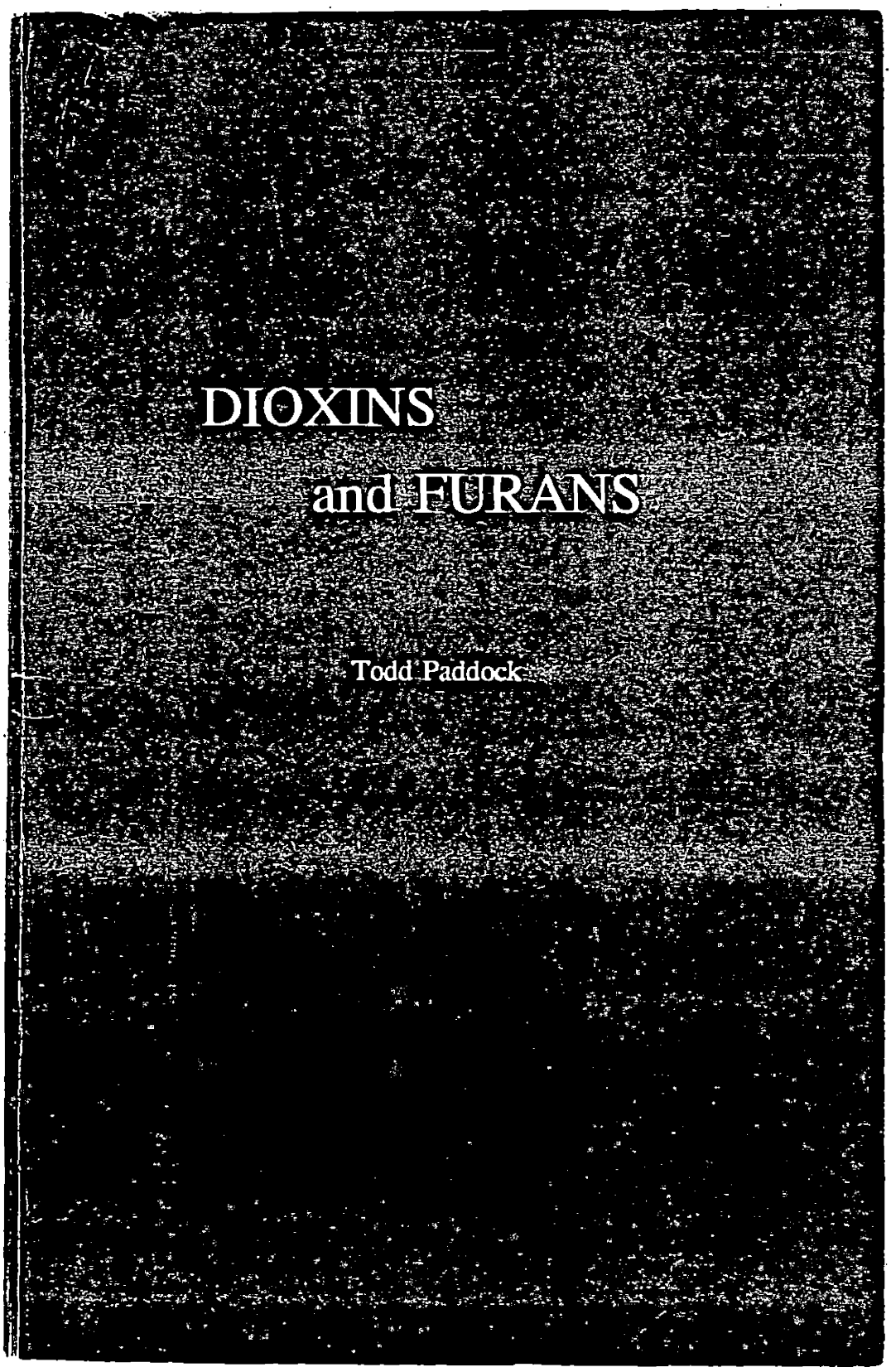
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Dioxins and Furans:
Questions and Answers

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Executive Summary

In the U.S., dioxin is infamous because it was the substance in Agent Orange blamed for a host of diseases in Vietnam veterans, and the substance that contaminated Times Beach, Missouri, prompting the federal government to purchase the entire town. Since then, the discovery of dioxins in incinerator emissions has caused further concern.

There are actually many kinds of dioxins. The chlorinated dioxins have caused the most concern. A related family of compounds called chlorinated furans are very similar to dioxins in their distribution, toxicity, and behavior, although not as much is known about them. The chlorinated dioxins and furans are the subject of this report.

One particular dioxin has received more attention than any other, because it was found in relatively large amounts in some widely used chemical products, and appears to be the most toxic. This dioxin is 2,3,7,8-TCDD. The word dioxin is commonly used to mean 2,3,7,8-TCDD. A great deal more research has been done on 2,3,7,8-TCDD than on the other dioxins and furans, and this report is based largely on studies of 2,3,7,8-TCDD.

How toxic are dioxins and furans?

Based on laboratory and field studies, 20 or so of the 210 chlorinated dioxins and furans are considered to be extremely toxic to animals. They accumulate in the tissues of organisms, and are often the only types found in organisms from the wild. Most dioxins and furans appear to be almost nontoxic, and do not accumulate.

What factors affect the toxicity of dioxins and furans?

The toxic effect of a dioxin or furan on an animal depends on more than the type of dioxin or furan. It also depends on the species of animal, the status of the animal, the route of exposure, and the substance in which the dioxin or furan is present. Some animal species are thousands of times more sensitive than others to the toxic effects of the same dioxin or furan.

How do dioxins and furans reach humans?

The major routes of exposure are generally considered to be eating contaminated food and breathing contaminated particles. Particles in air, especially near urban areas or large sources of combustion products, can be contaminated with dioxins and furans. Trace levels of

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dioxins and furans have been found in a variety of foods in Japan, Canada, and Europe, and the same is probably true of foods in the U.S. Dioxins have also been found in fish from certain areas in the U.S.

Are dioxins and furans found in humans?

Dioxins and furans have been found in samples of blood and fatty tissue from persons in numerous countries, including the U.S., Canada, Japan, Vietnam, and several countries in Europe. Based on these findings, it is generally agreed that the population at large in many parts of the world has been exposed to low levels of dioxins and furans.

What are the health effects of dioxins and furans?

The long-term health effects of these compounds have been intensely investigated, and they are now among the most-studied substances with respect to their effect on animal and human health. Dioxins and furans cause mortality and/or cancer in many animals, but there is no conclusive evidence that they cause cancer or any other life-threatening health problem in humans. No documented human death has occurred because of an exposure to dioxins or furans.

People have been exposed to relatively large amounts of dioxins and furans (1 microgram or more) during industrial accidents, and other incidents of accidental contamination. The health effects were serious, but subsided with time. With the exception of severe acne, most researchers have found no serious, long-term health problems associated with exposure to dioxins, even at the highest reported levels, and after ten to thirty years have elapsed.

How are dioxins and furans created?

Dioxins and furans have no useful purpose and have never been manufactured deliberately, except in small amounts for research purposes. They are known to be created in two major ways: (1) they are created by unwanted side reactions, which take place during the chemical processes used to manufacture useful products such as biocides, paper, disinfectants, and preservatives; (2) they are created when a substance containing chlorine is burned, or when a substance is burned in the presence of chlorine.

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Dioxins and/or furans have been found as contaminants in the following manufactured products:

Product	Contaminants
PCBs	Furans
hexachlorophene	Dioxins*
2,4,5-T	Dioxins*
2,4-D	Dioxins*
chlorophenols with 3 or more chlorines	Dioxins and Furans
polychlorinated benzenes	" "
diphenyl ether herbicides	" "
hexachlorocyclohexane	" "
paper products	" "

* I could find no analyses of these products for furans.

Other products derived from chlorophenols have been identified as possibly contaminated and are under investigation by the U.S. Environmental Protection Agency (USEPA) and others.

What products can produce dioxins and furans when they are burned?

The following chlorinated products or substances can produce dioxins and/or furans when burned:

Product	Contaminants
PCBs	Furans
polybrominated biphenyls (PBBs)	Dioxins and Furans
polybrominated diphenyl ethers (PBDEs)	" "
polychlorinated diphenyl ethers (PCDEs)	" "
chlorophenols	" "
tetrachloroethylene	" "
polychlorinated benzenes	" "
polyvinyl chloride (PVC)	" "
Leaded gasolines	" "
paper, wood, peat, and coal	" "
dioxins and furans	" "

Note: When a material is burned in the laboratory to determine whether it is capable of producing dioxins and furans, it is typically burned under those conditions most likely to produce dioxins and furans, or that produce the largest quantity of dioxins and furans. Therefore, the same substance may produce much smaller amounts of dioxins and furans under the uncontrolled conditions of a fire, or in the highly controlled conditions of an incinerator.

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incinerators and other large combustion sources, sewage plants, waste streams, and landfills are also sources of dioxins and furans.

There are many sources of dioxins and furans. The combustion sources are widespread and numerous. Highly contaminated manufacturing and waste sites are fewer in number and usually local problems. The relative importance of the two major sources of dioxins and furans (chlorophenols vs. combustion) is not yet known. It is generally agreed that modern industrial activity, not the burning of wood, is the primary source of dioxins and furans to the environment.

Where are dioxins and furans found?

Dioxins and furans are present at trace levels in many places, and at relatively high levels at a small number of contaminated sites. The presence of dioxins and furans is usually associated with the production, storage, use, or disposal of chlorophenol-based compounds. In addition, they have been detected at low levels in urban areas, where they are believed to be the product of numerous combustion processes. As our ability to detect dioxins and furans improves, it is likely that they will be found in more places.

Dioxins and furans have been found in organisms from contaminated areas, and from fish in many areas of the U.S. Plants typically contain these compounds at levels much below the surrounding environment, and only when they are grown in highly contaminated soil. Fruits do not appear to contain dioxins, although root crops may sometimes contain them.

How long do dioxins and furans last in the environment?

2,3,7,8-TCDD can be broken down in a matter of days in the environment, if it is mixed with the proper organic solvent and exposed to ultraviolet light. On the surface of the soil, without a solvent, 2,3,7,8-TCDD has an environmental half-life of one year or less, due to slow photodegradation and evaporation. Underground and in sediments, this very stable compound degrades much more slowly, with a half-life of about 10 years. Very little is known about the half-lives of other dioxins and furans. In general, dioxins and furans with fewer than four chlorines are broken down more quickly than 2,3,7,8-TCDD. The dioxin or furan with eight chlorines is more resistant to breakdown.

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To what degree do dioxins and furans spread when they are introduced into the environment?

Dioxins and furans bind very strongly to the organic carbon present in particles of soil, in sediments, and to the particles found in the air. These particles can be lifted and carried by wind or moving water. Dioxins and furans can be transported great distances in air, and smaller distances in water. In soil and sediments, dioxins and furans appear to migrate very little or not at all. Areas of severe contamination have typically remained local problems, and most are not expected to spread the contamination to nearby areas.

What has been done about dioxins and furans?

Because they are very potent toxins and carcinogens in laboratory animals, regulatory agencies from various countries have issued guidelines for human exposure to dioxins and furans. These guidelines vary by more than a thousandfold, due to different interpretations of the same animal laboratory studies.

As dioxins and furans have been identified as contaminants in products, the manufacturers have lowered the levels of contamination. The USEPA has cancelled or restricted the manufacture, use, and disposal of many of the products containing hazardous levels of dioxins and furans, and the disposal of wastes from the manufacture of many products. Many contaminated sites have been cleaned up, and many more are on the Superfund list.

Conclusions and Recommendations

Based on the many studies already published, dioxins and furans do not appear to be a hazard to our health when we are exposed to the low levels typically present in the environment. Several groups of people need special attention because they may be exposed to greater than normal amounts of dioxins and furans. These include breast-fed babies (because dioxins and furans are usually present in breast milk), and workers in industries that use contaminated products.

We should continue to monitor the health of groups of people who were exposed to large amounts of these compounds, or who were exposed to smaller amounts for a long time. This will enable us to identify any long-term health effects not already recognized. Now that

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we have the ability to determine the extent of an individual's past exposure to dioxins and furans through a blood test, the identification of exposed persons is much easier and more accurate, making such studies more powerful.

We also need to determine how dioxins and furans cause the effects that they do, and why their toxicity varies so greatly between species. Such an understanding would not only have preventive or therapeutic value, but is also critical for accurate assessments of the risks these compounds pose to humans and other organisms.

Because of their demonstrated toxicity to laboratory animals, plus their distribution and persistence, some dioxins and furans are a potential hazard to organisms in the environment. There is a great need for more study in this area. We need to know what are unacceptably harmful levels for the various organisms in the environment, and the nature and amount of dioxins and furans from the various sources.

What appears to be the largest amount of toxic dioxins and furans is buried in landfills or stored at past or present sites of manufacture or use. We need to develop safe, yet practical ways to contain, or better yet destroy, this contaminated waste.

We may need to reduce the amounts of dioxins and furans occurring as unwanted contaminants of some products. Great reductions have been made in certain products in the past, and further reduction may be necessary. Unfortunately, such reduction can result in very highly contaminated waste streams, and proper disposal is difficult and expensive. Therefore, the emphasis needs to be on preventing their creation during the manufacturing process.

We also need to reduce the unwanted creation of dioxins and furans during combustion. For example, incinerators can be operated under conditions that reduce the creation of dioxins and furans.

More than one billion dollars has been spent in the U.S. alone for the research of dioxins and furans, and this research has greatly expanded our knowledge. With this knowledge, and with improved technologies for both reducing future sources of these compounds and cleaning up presently contaminated areas, dioxins and furans should be regarded as a manageable problem.

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Dioxins and Furans: Introduction and a Brief History

When most people use the term dioxin, they mean a single chemical compound, often referred to as "the most toxic substance ever made by man". In the U.S., dioxin is infamous because it was the substance in Agent Orange blamed for a host of diseases in Vietnam veterans, and the substance that contaminated Times Beach, Missouri, prompting the Federal Government to purchase the entire town. However, the dioxins (short for chlorinated dibenzo-p-dioxins) are a large family of compounds whose toxicity varies or, for many types, is unknown. A closely related and very similar group is the furans (chlorinated dibenzofurans).

Dioxins and furans have been discovered in unexpected places. They are in fish from lakes and rivers, in common paper products, in auto exhaust, and in the ash from municipal and industrial incinerators. Dioxins and furans will continue to be found in new places. In part, this is because they can be measured in extremely small amounts, and have been looked for in many places. Also, many dioxins and furans are very stable, can remain in the environment for years, and can accumulate in organisms.

One particular dioxin has received more attention than any other, because it was found in relatively large amounts in some widely used chemical products, and appears to be the most toxic[1]. This dioxin is 2,3,7,8-tetrachlorodibenzo-p-dioxin, also known as 2,3,7,8-TCDD, or simply TCDD. The word dioxin is commonly used to mean 2,3,7,8-TCDD.

Much of the general public's knowledge of dioxins and furans was gained as a result of a few widely-publicized incidents or controversies. A brief history of dioxins and furans, with summaries of a few of the most publicized incidents, can serve to remind us of how these compounds became so well known.

[1] Here and in the rest of the report, "toxic" means that the substance is associated with adverse health effects, but not necessarily effects that are serious, or permanent.

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What is the history of dioxins and furans?

Dioxins and furans were synthesized by organic chemists in the 1930s and were patented in 1939 as constituents in a product used for electric insulation (cited in Huff and Wassom, 1973). Different dioxins and furans were tested and in some cases patented as agents against bacteria, insects, and fungi, as intermediates in chemical processes, and as flame retardants, from the 1930s to at least 1970 (cited in Huff and Wassom, 1973).

Discovering their toxic effects on humans and animals

The toxic effects of dioxins and furans in humans were more or less unrecognized until 1957, when Dr. Karl Schultz, a dermatologist at the University of Hamburg in West Germany, tried to determine why workers at a nearby chemical plant were developing a severe form of acne, called chloracne (Gough, 1986: pp 29-33). After some careful tests, Schultz concluded that a contaminant in one of the plant's products (2,4,5-trichlorophenol) was responsible for the chloracne, and identified the contaminant as 2,3,7,8-TCDD. We now know that 2,4,5-trichlorophenol is always contaminated with 2,3,7,8-TCDD, and therefore trace levels of 2,3,7,8-TCDD are also present in the products made from 2,4,5-trichlorophenol.

Schultz and colleagues published several papers in 1957, showing that several dioxins and furans induced acne when applied to rabbit ears (the standard and most sensitive test at that time), and that small doses of 2,3,7,8-TCDD caused liver damage and death in rabbits. The plant chemists found a way to reduce the amount of the contaminants formed during the production of their 2,4,5-trichlorophenol, and the chloracne problem in their workers subsided.

Chick edema disease

Also in 1957, scientists in the U.S. Food and Drug Administration (USFDA) were trying to determine the cause of an outbreak of millions of deaths of chickens in the U.S. (Firestone, 1973). They determined in 1958 that the cause of the disease (chick edema) was toxic contaminants in commercial fatty acids that were part of the chicken feed. Symptoms included excessive fluid in the heart sac and abdominal cavity, and liver damage. Although the contaminant was not yet identified, the USFDA issued a regulation in 1960 that required fatty

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acids to be proven free of the contaminants through a 3-week chick-feeding bioassay. Advances in detection techniques made it possible to further identify the contaminants, and in 1966 it was determined that 1,2,3,7,8,9-hexachlorodibenzo-p-dioxin was one of the contaminants and that a synthetic hexachlorodibenzo-p-dioxin produced the disease in chickens (Firestone, 1973).

It had been shown as early as 1936 (cited in Huff and Wassom, 1973) that dioxins could be produced by heating chlorophenols, and the USFDA scientists proposed that chlorophenols were the source of the dioxins in the toxic fats being fed to chickens. Chlorophenols, including trichlorophenol and pentachlorophenol, were widely used as agents against slimes, bacteria, termites, and as herbicides. The USFDA scientists were able to produce a variety of dioxins by heating various commercial chlorophenols, and found these same dioxins in the toxic fat. They tested these dioxins on chickens, found that they produced chick edema, and found that the most toxic dioxin was 2,3,7,8-TCDD. Finally, it was determined in 1972 that the source of chick edema disease was fat derived from hides (from cattle, hogs, sheep, and other animals) that had been treated with commercial pentachlorophenol (Metcalfe, 1972, cited in Firestone, 1973).

Concern about 2,4,5-T and related herbicides

In 1970, at hearings before the U.S. Congress, evidence was presented to show that the widely-used herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was capable of causing birth defects in rats and mice (cited in Huff and Wassom, 1973). During that same year, a study showing similar results was published (Courtney et al., 1970). It was proposed that the relatively high levels (approximately 30 parts per million [ppm]) of 2,3,7,8-TCDD contamination in 2,4,5-T were probably responsible for the birth defects (cleft palate, cystic kydney) in mice and rats, and a study on 2,3,7,8-TCDD found that it affected reproduction in rats (Sparschu et al., 1970).

Based on these findings and the earlier findings of the toxicity of 2,3,7,8-TCDD to humans and animals, the U.S. Department of Agriculture (USDA) decided in 1970 to cancel registrations for the use of 2,4,5-T in certain areas, for example its use on human food crops, near bodies of water, and around homes (Gough, 1986: p 138). They did not cancel registration

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for other uses of 2,4,5-T if the levels of 2,3,7,8-TCDD were below 1 ppm (cited in Huff and Wassom, 1973). However, the USDA and U.S. Department of Health, Education, and Welfare issued a joint statement that 2,4,5-T and 2,3,7,8-TCDD may cause birth defects in mice and rats (cited in Huff and Wassom, 1973). Also in 1970, the U.S. Department of Defense stopped using the herbicide Agent Orange in Vietnam. Agent Orange was a half-and-half mixture of 2,4,5-T and 2,4-dichlorophenoxyacetic acid (2,4-D), and was contaminated with 2,3,7,8-TCDD.

In 1971, a Science Advisory Council from the U.S. National Academy of Sciences recommended to the U.S. Environmental Protection Agency (USEPA) that the registration of 2,4,5-T be restored for use on forests, rangeland, and rice fields, with certain restrictions on its use (Gough, 1986: p 138). For example, they required that present stocks of 2,4,5-T have less than 0.5 ppm 2,3,7,8-TCDD and that newly manufactured 2,4,5-T have less than 0.1 ppm (cited in Huff and Wassom, 1973). In 1973, the Science Advisory Council recommended that no more than 0.1 ppm 2,4,5-T be allowed in water or on edible food products, if they were for human consumption (cited in Huff and Wassom, 1973).

In 1979, the USEPA issued an emergency suspension of most remaining uses of 2,4,5-T, based on accumulating evidence that 2,3,7,8-TCDD caused cancer and birth defects in laboratory animals, and because of a 1979 study that showed elevated levels of spontaneous abortions among women near Alsea, Oregon, where 2,4,5-T was sprayed (Gough, 1986: p 138-145). A panel of scientists subsequently reviewed this 1979 study and concluded that because of a flawed design, the study was incapable of showing either the presence or absence of effects of exposure to 2,4,5-T (Coulston and Olajos, 1980, cited in USEPA, 1985: p 9-25). From 1979 until 1984, 2,4,5-T was used on rice and sugarcane fields, pending settlement of a suit between the USEPA and manufacturers of 2,4,5-T. All companies withdrew from the suit by 1984, and the USEPA then cancelled all uses of 2,4,5-T and the closely related herbicide Silvex.

Times Beach and other areas in Missouri

(Much of the following discussion is based on a chapter in Gough, 1986)

Hexachlorophene, a germicidal product used as a skin disinfectant in hospitals, in germicidal soaps, and in some veterinary products, is produced from 2,4,5-trichlorophenol, the

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same chemical used to manufacture 2,4,5-T and related herbicides. When manufacturers of 2,4,5-trichlorophenol-based products became aware of the toxic properties of 2,3,7,8-TCDD, they found ways to greatly lower the concentrations in their final products. However, this purification process can create wastes with very high concentrations of 2,3,7,8-TCDD. If this waste is not disposed of properly, it can be a hazard to humans and other organisms.

Improper disposal of such wastes was the cause of the contamination of Times Beach, Missouri. A chemical plant in Verona, Missouri, produced hexachlorophene from 1970 to 1972. The purification of their product (by distillation) resulted in a thick, oily, 2,3,7,8-TCDD-contaminated waste called still bottoms. The plant's still bottoms were hauled away by a separate company, and sold in turn to a waste oil buyer. The waste oil buyer then used the still bottoms in a secondary business, in which he sprayed oil to keep dust down in horse arenas, private roads, and parking lots. Horses, birds, and other animals became sick and died following the spraying of one of the horse arenas in 1971, and a child who played in the horse arena was hospitalized with bladder inflammation and bleeding.

The U.S. Centers for Disease Control (CDC) began an investigation of the poisoning episode at that stable in August, 1971, three months after the spraying. They and local doctors diagnosed the episode as chemically related, but could not identify the disease or the contaminant. In the meantime, the owners of the first horse arena and another arena had suspected the cause of the illness was the sprayed oil, and had removed the top layer of soil from their arenas and placed it in a nearby landfill. The CDC investigation continued, and in 1973 they had identified 2,3,7,8-TCDD in samples of dirt from the arenas. They notified the Missouri Department of Health in 1974, and the CDC and state began a joint investigation of the episode.

The origins of the waste oil were quickly determined by the investigators, but identifying the many areas that had been sprayed with the oil was more difficult. Fortunately, the owners of the first arena had secretly followed and kept records of the spraying done by the waste oil buyer, and these records were used to locate other contaminated areas. Arenas and trailer parks had been contaminated by spraying, and soil from the arenas was removed and

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placed in landfills, on farms, and used as fill for building projects. Also, the waste oil buyer had sprayed 23 miles of unpaved streets in the town of Times Beach, Missouri, from 1972 to 1976.

The contamination of Times Beach was not confirmed by the federal government until December 1982, when their soil tests showed 5-300 parts per billion (ppb) 2,3,7,8-TCDD in the soil from the streets of Times Beach. Based on the results, the CDC recommended that the town of Times Beach was unsafe for its inhabitants, and in February 1983 the federal government announced it would buy the homes, properties, and businesses of Times Beach. The many other contaminated sites in Missouri (51 in all, according to USEPA, 1987a: pp 2.3-2.35) are being dealt with in various ways. The USEPA and CDC have removed soil, decontaminated the soil, restricted access, and advised minimal contact with soil, depending on the level of contamination and possibility of exposure (USEPA, 1987a: pp 2.18-19).

Based on studies of residents of the most highly contaminated areas in Missouri, it appears that the people exposed to 2,3,7,8-TCDD have suffered few if any long-term effects, although several commonly used areas were contaminated with extremely high levels of 2,3,7,8-TCDD (as much as 31 ppm in the soil of one horse arena). The people who were exposed to high levels of 2,3,7,8-TCDD in the arena soil suffered from a variety of effects, including nausea, headaches, diarrhea, nosebleeds, chloracne, and severe bladder inflammation. Their health subsequently returned to normal (reviewed in Reggiani, 1980).

A study of the residents of a trailer park that had 39-1100 ppb of 2,3,7,8-TCDD in the soil found abnormal levels of some blood constituents, that suggested long-term effects on the liver and immune system (Stehr-Green et al., 1987). However, no liver changes could be directly detected, and no excess of illness was reported. The authors noted, "The effects we observed may be merely part of the normal, adaptive response to a toxicologic challenge, and well within the normal reserve capacity of the affected organ systems (i.e., they are markers of exposure and not signs of disease)."

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Agent Orange

Herbicides were used by the U.S. in the Vietnam War from 1962 to 1970. Agent Orange, a half-and-half mixture of the herbicides 2,4,5-T and 2,4-D, was sprayed over South Vietnam from 1965 to 1970, accounting for more than half of all herbicides used there. Earlier mixtures were called Agents Purple, Pink, and Green. Based on analyses of Agent Orange manufactured before 1970, the mixture sprayed on South Vietnam was contaminated with from 0.02-54 ppm 2,3,7,8-TCDD (Esposito et al, 1980: p 98). The U.S. Air Force (USAF) estimates that Agent Orange had a mean concentration of 2 ppm 2,3,7,8-TCDD, Agent Purple had a mean concentration of 33 ppm, and Agents Pink and Green were estimated to have 66 ppm (Albanese, 1988: p 3).

The large-scale use of herbicides in Vietnam ended in 1970 because of concern about the effects on the environment and inhabitants of South Vietnam. However, that concern has been almost entirely eclipsed by concern about the possible effects on the health of soldiers from the U.S. and other countries who were exposed to large amounts of the herbicides. Because of this concern, several large studies by the CDC and USAF have been conducted or are underway. The USAF study investigated the general health and rates of cancer and death among Vietnam veterans who were exposed to herbicides, as well as birth defects among children of the veterans. The CDC study investigated the health of Vietnam veterans in general, most of whom were not exposed to Agent orange, and therefore is not relevant to this discussion.

The USAF Health Study is investigating the health of USAF personnel who participated in the aerial spraying of herbicides over Vietnam. The spraying program was called Operation Ranch Hand; Ranch Hands include the men who flew the planes, and all men who worked with the herbicide and spray equipment used for the aerial spraying. The health of Ranch Hands is being compared to a carefully matched group of USAF personnel who flew in and out of Southeast Asia (but not Vietnam) during the same period. The most recent report from this study focused on eleven health effects that studies of animals and humans have associated with exposure to 2,3,7,8-TCDD (Albanese, 1988). They reported that for 6 of the 11 effects,

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differences were detected between the two groups, and for 5 of the effects the difference was in the direction that would be expected from current knowledge of the effects of 2,3,7,8-TCDD.

The six effects were increases in the rate of cancer, increases in the number of birth defects reported in children of the veterans, an increase of psychological changes, an increase of liver changes, an increase of cardiovascular changes, and an increase in changes in the endocrine system. However, no differences were detected in the sperm count or proportion of normal sperm, the rate of heart disease and heart attack, the immune system, overall mortality, and other health effects that studies of animals and humans have associated with exposure to 2,3,7,8-TCDD. Follow up studies, including an investigation of the birth certificates and health records of all children of the veterans, are now under way.

Albanese (1988) concluded that while "At this time one cannot ascribe the observed group differences to an effect of dioxin", the study also "...does not exonerate dioxin as a causative agent of these differences." He noted that the study did not find that the health effects increased in severity with a greater exposure to Agent Orange, and that some of the characteristic effects of exposure to 2,3,7,8-TCDD were not observed. He also noted that five of the six group differences were in the direction of 2,3,7,8-TCDD effects, and that the calculation of exposure was only an estimate. Finally, he noted that the sample sizes used in the study made it possible to detect common diseases and death, but almost impossible to detect rare diseases.

An important part of the controversy over the possible health effects of Agent Orange is the question of determining exposure: what veterans were exposed and what was the level of exposure? Recent studies indicate that 2,3,7,8-TCDD persists for decades in the fatty tissues of humans (Schecter and Ryan, 1988), and levels of 2,3,7,8-TCDD in the blood can be correlated with levels in the fatty tissues (Patterson et al., 1988). Measurements of 2,3,7,8-TCDD in the blood can therefore be used to determine a person's level of exposure to 2,3,7,8-TCDD, even if the exposure occurred many years before.

The CDC recently reported the levels of 2,3,7,8-TCDD in the blood of approximately 700 veterans who were part of the Vietnam Experience Study or the Ranch Hand Study. Ranch

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Hand veterans had significantly higher levels of 2,3,7,8-TCDD in their blood (mean of 49 parts per trillion [ppt]) than did other Vietnam-era Air Force veterans (mean of 5 ppt). Seventy-nine percent of Ranch Hands had blood levels above 10 ppt, and many had levels of 100-300 ppt (CDC, 1988). However, other Vietnam veterans did not have higher levels of 2,3,7,8-TCDD in their blood (mean of 4.8 ppt) than did non-Vietnam veterans (mean of 4.9 ppt) (CDC, 1987). It appears that Ranch Hands, but not other Vietnam veterans, were exposed to significant amounts of Agent Orange.

To determine past exposure, the CDC used an estimated half-life[1] of about seven years (CDC, 1988), and therefore assumed that two to four half-lives have passed since Vietnam veterans were exposed to 2,3,7,8-TCDD in Agent Orange. Recent work by Kissel and Robarge (1988) suggests that the half-life of 2,3,7,8-TCDD is inversely related to the concentration of 2,3,7,8-TCDD in the body, and that the half-life is significantly shorter when concentrations are relatively high (e.g. immediately after exposure). If so, the CDC may be underestimating Vietnam veterans' past exposure to 2,3,7,8-TCDD in Agent Orange.

The Seveso accident

In 1976, a cloud of 2,4,5-trichlorophenol and caustic lime, contaminated with 2,3,7,8-TCDD, was released over the town of Seveso, Italy and nearby towns, when a chemical plant's reactor vessel went out of control. Approximately 700 acres inhabited by 38,000 people were contaminated with 2,3,7,8-TCDD (Mastroiacovo et al., 1988). The level of 2,3,7,8-TCDD ranged from greater than 150 ppt in the soil of the most contaminated area to less than 50 ppt in the soil of the least contaminated area (Wipf and Schmid, 1983). Vegetation (including garden vegetables and orchard fruits) was contaminated with from more than 1000 ppb 2,3,7,8-TCDD to less than 1 ppb, which was the limit of detection at that time (Wipf and Schmid, 1983).

The accident resulted in the deaths of about 3000 domestic animals (almost all rabbits and chickens); some of these animals died almost immediately after the accident, and their

[1] The half-life is the amount of time during which the 2,3,7,8-TCDD present in the body is reduced by one-half.

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deaths were attributed not to 2,3,7,8-TCDD, but to the other chemicals in the cloud or to deliberate slaughter. (All animals given a lethal dose of 2,3,7,8-TCDD take at least two to three weeks to die after their exposure.) One hundred and eighty-three people, mostly children, were confirmed as having contracted chloracne, indicating that they were exposed to significant amounts of 2,3,7,8-TCDD. It was presumed that children were exposed to greater amounts because they played in the vegetation, and may also be more sensitive. All cases of chloracne cleared up within 5 years, although the 15 most severe cases resulted in permanent scars.

Aside from chloracne, no other health effects were confirmed by an investigation of the accident (Wipf and Schmid, 1983). However, there was concern that the effects of the exposure might include birth defects. The Italian health authorities advised the inhabitants of the area to avoid becoming pregnant, and many women requested abortions after the accident. A panel set up by the Italian government investigated, from the beginning of 1977 to the end of 1984, all suspected birth defects in children born to women who were inhabitants of the area at the time of contamination (Mastroiacovo et al., 1988). This study did not find an increased rate of birth defects in children born to the exposed women. The authors noted that rare defects could not have been detected because of the small number of births, and that it was possible that spontaneous abortions decreased the number of children born with defects. They cited one study that found an increase in spontaneous abortions after the accident (Santi et al., 1983), and one study that did not (Bianco et al., 1986).

Conclusion

The contamination of Times Beach and Seveso, and the health effects attributed to use of the herbicide 2,4,5-T, especially from Agent Orange, have made dioxin a household word with in infamous connotation. Much attention has been paid to the potential danger of dioxins and furans and their wide presence in very small quantities. Yet investigators of the health of the people exposed to relatively large quantities have found little evidence to justify the enormous reaction to these incidents. Nevertheless, dioxins and furans are present in our environment, in organisms, and in us, and can be extremely toxic, at least to other organisms. A great deal of money and other resources have been spent on the research of dioxins and

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furans, and will continue to be spent. The question then is, what do we know about dioxins and furans?

Detecting Trace Amounts of Dioxins and Furans

One of the difficulties of discussing dioxins and furans is the almost unimaginably small quantities we can detect with modern techniques: as little as one trillionth, or in some cases even a few quadrillionths of a gram. The following examples illustrate how little these quantities are. If we assume a drop of water to be equal to 0.05 milliliters (20 drops to a milliliter), then one ppb is equivalent to one drop of water in 13,200 gallons, or a pool 20-feet square and approximately 4.5-feet deep. One ppt is equivalent to one drop of water in 13,200,000 (thirteen million two hundred thousand) gallons, or a pool of water as long and wide as a football field, and approximately 28-feet deep. One part per quadrillion (ppq) is equivalent to one drop of water in 13,200,000,000 (thirteen billion, two hundred million) gallons, or a 6-square-mile lake, approximately 10.5-feet deep.

Dioxins and furans were among the very first compounds chemists could measure in one billionth of a gram (in 1970) and one trillionth of a gram (in 1976), and they are now approaching the detection of one quadrillionth of a gram. As the new techniques for detection were developed, dioxins and furans were used to test the techniques because they appeared to be toxic at extremely low levels. Before the detection techniques were developed, researchers used animals such as young chickens to test the toxicity of dioxins and furans (pers. comm., David Firestone, USFDA).

Dioxins lend themselves to the techniques used to detect amounts this small. They react with very few other chemicals, many last a long time, and they dissolve readily in organic solvents. Also, dioxins and furans are relatively simple molecules. The molecules of many other toxins are very complex (pers. comm., David Firestone, USFDA).

In order to detect amounts this small, it is first necessary to isolate the substance you want to measure from all the other substances that are present in a typical sample. This process

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is called analytical clean-up; the chemist puts the sample through a complicated and delicate preparation process, involving extractions, washings, and separations. Once the sample has been cleaned up, the separated materials need to be detected and their amounts measured. This can be done using several techniques, including an Electron Capure Detector, Flame Ionization Detector, or Mass Spectrometer, along with a Gas Chromatograph (pers. comm., Annette Guiseppi-Elie, Drexel University).

In addition to the difficulty of detecting these very small amounts, the toxicity of these compounds necessitates great care in their handling. Therefore, detecting the presence of dioxins and furans at levels approaching 1 ppt is a very time-consuming, expensive, and difficult undertaking. An experienced analytical chemist needs several months in order to prepare the laboratory for detecting a particular chemical at these extremely low concentrations. Once everything is in place, the analysis of just one sample costs about $2000 in materials, requires the attention of an experienced analytical chemist, and typically consumes a half-day of work (pers. comm., Robert Baughman, Harvard University).

A much cheaper and quicker method has been developed for determining the level of toxicity of a mixture of dioxins and furans in a sample. It has been established that dioxins and furans induce a specific enzyme and bind to a specific protein in animals and humans (Poland et al, 1979). Rather than using analytical chemistry to determine the amounts of various toxic congeners[3] of dioxins and furans in a sample, cell cultures are exposed to the sample and the above biochemical responses are measured. The accuracy of these methods have been verified by comparing their results with the results of chronic toxicity tests using laboratory animals (Safe, 1987; Sawyer et al, 1983).

[3] A congener is a single, particular dioxin or furan, e.g. 2,3,7,8-TCDD.

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Dioxins and Furans: What They Are, Where They Are Found, and How They Behave

What is dioxin?

The dibenzo-para-dioxin structure (see Figure 1), which provides the framework for tens of thousands of compounds, is made up of two benzene rings joined by two oxygen atoms (hence dibenzo dioxin). The "para" means the benzene rings are attached to opposite sides of the dioxin molecule, and it is usually designated as simply "p". Benzene is a ring of six carbon atoms (one at each corner) and six hydrogen atoms (one bonded to the carbon atom at each corner).

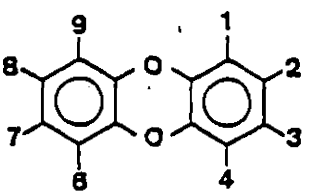


Figure 1. Dibenzo-p-dioxin

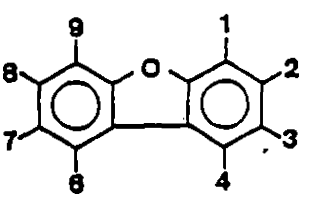


Figure 2. Dibenzofuran

In addition to dibenzo-p-dioxin, there is the related structure called a dibenzofuran (see Figure 2). A dibenzofuran, like a dibenzo-p-dioxin, is composed of two benzene rings. However, the benzene rings are joined by one oxygen atom, instead of two. The single oxygen that joins the two benzene rings forms the furan part of the molecule. A furan is a ring like benzene, but is made up of four carbon atoms plus one other atom (in this case, that other atom is the oxygen atom). Both dioxins and furans have eight corners (four on each benzene ring), free to react with other atoms. These other atoms can be, for example, hydrogen, chlorine, bromine, fluorine, iodine, or nitrogen. The corners can also react with groups made up of these atoms. The number of possible combinations is enormous.

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There are 75 polychlorinated dibenzo-p-dioxins (PCDDs) and 135 polychlorinated dibenzofurans (PCDFs). PCDFs are similar to PCDDs in their distribution, toxic properties, and behavior, although not as much is known about them. While those dibenzo-p-dioxins and dibenzofurans with atoms other than chlorine on their corners (bromine, nitrogen, and so on) may have similar toxic potential (tests indicate this to be true for some types), they are not as common or numerous, and are not considered to be as important as chlorinated dibenzo-p-dioxins and dibenzofurans. However, there has been some concern about the polybrominated dibenzo-p-dioxins and dibenzofurans, because polybrominated compounds are used as gasoline additives, and have been widely used as flame retardants in plastics, carpets, textiles, and so on (Buser, 1987). It has been shown that when these compounds are burned, polybrominated dioxins and furans can form (Buser, 1986; Haglund et al., 1988). Almost nothing is known, however, about the extent of their presence in the environment. For the rest of this report, only the chlorinated dibenzo-p-dioxins and dibenzofurans will be discussed.

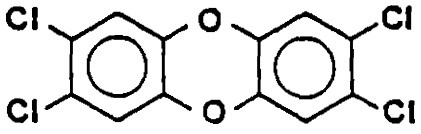


Figure 3, 2,3,7,8-TCDD

As illustrated in Figure 3, the numbers 2,3,7,8 refer to the positions on the benzene rings where the four (thus tetra) chlorine atoms are attached. 2,3,7,8-TCDD is the most toxic of the dioxins or furans, and can be formed in significant amounts during the production of trichlorophenol-based chemicals. Also, 2,3,7,8-TCDD is accumulated preferentially by animals and humans, compared to the other types of dioxins and furans. A great deal more research has been done on 2,3,7,8-TCDD than on the other dioxins and furans, and the following discussion is based largely on studies of 2,3,7,8-TCDD.

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Are all dioxins and furans dangerous?

Only 20 or so of the 210 chlorinated dioxins and furans are considered to be extremely toxic. These have 4 to 7 chlorines, with a chlorine in each of the 2,3,7, and 8 positions (2,3,7,8-substituted dioxins and furans). Table 1 lists these very toxic types of dioxins and furans, and all other dioxins and furans, along with their toxicity relative to the most toxic dioxin or furan, 2,3,7,8-TCDD.

Table 1. Relative toxicity of dioxins and furans

Compared to the most toxic, 2,3,7,8-TCDD.
From the USEPA National Dioxin Study (USEPA, 1987a: p 1.7)

Type of dioxin or furan		Relative toxicity
2,3,7,8-tetra-CDD	(4 chlorines)	1 (Most Toxic)
1,2,3,7,8-penta-CDD	(5 chlorines)	1/2 as toxic
2,3,7,8-tetra-CDF	(4 chlorines)	1/10th
1,2,3,7,8-penta-CDF	(5 chlorines)	"
2,3,4,7,8-penta-CDF	" "	"
1,2,3,4,7,8-hexa-CDD	(6 chlorines)	1/25th
1,2,3,6,7,8-hexa-CDD	" "	"
1,2,3,7,8,9-hexa-CDD	" "	"
Other tetra-CDDs	(4 chlorines)	1/100th
1,2,3,4,7,8-hexa-CDF	(6 chlorines)	"
1,2,3,6,7,8-hexa-CDF	" "	"
1,2,3,7,8,9-hexa-CDF	" "	"
2,3,4,6,7,8-hexa-CDF	" "	"
Other penta-CDDs	(5 chlorines)	1/200th
1,2,3,4,6,7,8-hepta-CDD	(7 chlorines)	1/1000th
Other tetra-CDFs	(4 chlorines)	"
1,2,3,4,6,7,8-hepta-CDF	(7 chlorines)	"
1,2,3,4,7,8,9-hepta-CDF	" "	"
Other penta-CDFs	(5 chlorines)	"
Other hexa-CDDs	(6 chlorines)	1/2500th
Other hexa-CDFs	" "	1/10,000th
Other hepta-CDDs/CDFs	(7 chlorines)	1/100,000th
Other CDDs/CDFs	(1-3, 8 chlorines)	Not toxic

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Dioxins and furans usually occur in a mixture of several or many types, and the mixture varies with the source of the dioxins and furans. In order to arrive at a number that represents the total toxicity of the mixture, relative to 2,3,7,8-TCDD, the appropriate factor is applied to the amount of each type of dioxin or furan in the mixture and the result then added, for all the types present. The total amount is then expressed in "2,3,7,8-TCDD equivalents". This allows the direct comparison of the toxicity of different mixtures of dioxins and furans, from different sources.

For example, let us say we have analyzed samples of soil from two contaminated waste sites, and found the following:

Site One	Site Two
3 ppb 2,3,7,8-TCDD	1 ppb 2,3,7,8-TCDD
6 ppb 2,3,7,8-TCDF	4 ppb 1,2,3,7,8-penta-CDD
200 ppb other tetra-CDDs	100 ppb 1,2,3,7,8-penta-CDF

How can we compare the toxicity of these two samples? The toxicity equivalent method is one way. The toxicity of these two samples would be computed as follows:

Site One	Factor	Result
3 ppb 2,3,7,8-TCDD	X 1	3 ppb
6 ppb 2,3,7,8-TCDF	X 0.1	0.6 ppb
200 ppb other tetra-CDDs	X 0.01	2 ppb
		5.6 ppb

Site Two	Factor	Result
1 ppb 2,3,7,8-TCDD	X 1	1 ppb
4 ppb 1,2,3,7,8-penta-CDD	X 0.5	2 ppb
100 ppb 1,2,3,7,8-penta-CDF	X 0.1	10 ppb
		13 ppb

Therefore, the toxicity of the soil in Site One is equal to 5.6 ppb 2,3,7,8-TCDD equivalents and in Site Two is equal to 13 ppb 2,3,7,8-TCDD equivalents.

The USEPA used data from several kinds of studies to determine these factors (Barnes et al., 1986). Of first importance were data concerning cancer and reproductive effects in animals, based on long-term studies, but this information is available for only a few types of

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dioxins and furans. Therefore, data from studies of the biochemical effects of these compounds on cell cultures were also used. These studies, called enzyme induction and receptor binding studies, measure certain biochemical responses that are related to the toxic effects of dioxins and furans. The USEPA chose not to use data from short-term animal toxicity tests, stating that such information is not useful for predicting the long-term health effects of dioxins and furans.

These factors are the result of interpretation, and agencies from other countries have released somewhat different guidelines (Barnes et al., 1986). This is an area of ongoing investigation, and the USEPA will probably revise this table. For example, a recent study indicates that octa-CDD/CDF are toxic, not non-toxic as this table shows (Couture et al., 1988). Other types of dioxins and furans have also been investigated and found to have toxicities higher or lower than the present factors indicate (Pleuss et al, 1988a; 1988b). The use of such factors to determine the toxicity of a mixture is somewhat controversial. The CDC, for example, feels that this method is not scientifically valid, given our limited knowledge of the health effects of the many dioxins and furans, especially in a mixture. However, Pleuss et al (1988b) investigated the toxicity of a mixture of dioxins and furans in rats, and found the USEPA's factors to be adequate for estimating the total toxicity.

What factors affect the toxicity of dioxins and furans?

The toxic effect of a dioxin or furan on an animal depends on more than the basic toxicity as expressed in the table above. It also depends upon the kind of animal being exposed, the condition of the animal, and the medium that the compound is mixed with. Some animals are thousands of times more sensitive than others to the toxic effects of the same dioxin or furan. For example, guinea pigs (the most sensitive animal tested so far) are approximately 3000-5000 times more sensitive to the lethal effects of 2,3,7,8-TCDD than are hamsters (the most resistant animal so far tested). Also, because they are very lipid-soluble (soluble in fats, oils, and solvents such as acetone), dioxins and furans have had a greater toxic effect on an

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animal when they were administered in, for example, corn oil, than when they were administered in dry food.

Different kinds of dioxins and furans have different behaviors within animals; some are excreted rather quickly, while others are stored in the tissues for long periods of time. This may affect their toxicity to the animal. Laboratory studies showed that rats and carp fed a mixture of dioxins and furans accumulated higher amounts of 2,3,7,8-substituted types (these are the most toxic types of dioxins and furans) than other types (van den Berg et al., 1983; Kuehl et al., 1987a; 1987b). They also found that carp retained 2,3,7,8-TCDD and its counterpart furan (2,3,7,8-TCDF) for longer periods than dioxins or furans with fewer or more chlorines.

Researchers who have made congener-specific analyses of tissues from organisms in the wild have also found that the 2,3,7,8-substituted congeners of dioxins and furans are the only types found (Rappe et al., 1987a; Heida et al., 1986). Crustaceans appear to be an exception to this general rule; Rappe et al. (1987a) detected dioxin and furans congeners in crabs and lobsters that were not 2,3,7,8-substituted.

Soils from different 2,3,7,8-TCDD-contaminated sites show greatly varying toxicities. Researchers from the National Institute of Environmental Health Sciences and Rutgers Medical School fed guinea pigs 2,3,7,8-TCDD-contaminated soils from two different sites (McConnell et al., 1984; Umbreit et al., 1986). The animals showed greatly different reactions, although the amount of 2,3,7,8-TCDD given to them was similar. They concluded that the nature of the soil affected the toxicity of the 2,3,7,8-TCDD, presumably by determining how much of the 2,3,7,8-TCDD was absorbed by the animal (this is known as bioavailability). The researchers suggested that the difference between the toxicity of the soils was caused by the difference in the amount of carbon in the soil. It is known that dioxins bind strongly to carbon and are thus less likely to be absorbed during digestion.

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Where are dioxins and furans found?

In soil, water, and air

There are areas contaminated with relatively high levels (1 ppb or more) of dioxins and furans; most are sites where 2,4,5-trichlorophenol and 2,4,5-trichlorophenol-based biocides were manufactured or processed, and/or their associated waste sites. Wood-preserving facilities that have used pentachlorophenol, and sawmills that have used chlorophenolic solutions to prevent staining, can be contaminated with dioxins and furans and in some cases have contaminated the local environment. In addition, there are many contaminated areas in Missouri where 2,3,7,8-TCDD-laden oil was sprayed on roads and horse arenas, and several military bases where Agent Orange was stored or heavily sprayed.

Apart from these recognized sites of contamination, 2,3,7,8-TCDD has not been found commonly in soil. As part of their National Dioxin Study, the USEPA randomly selected 221 urban sites and 142 rural sites where no previously known sources of dioxins or furans had been recorded. Seventeen of two hundred and twenty-one urban sites and one of one hundred and thirty-eight rural sites had detectable amounts of 2,3,7,8-TCDD in the soil (USEPA, 1987a: pp 3.26-32). The limit of detection was about 1 ppt, and levels ranged from 1-11 ppt.

Low levels of 2,3,7,8-TCDD have been found in areas that were sprayed with the herbicide 2,4,5-T. During their nationwide survey, the USEPA found 2,3,7,8-TCDD at 15 of 26 sites where 2,4,5,-T was sprayed commercially; these sites included sugarcane and rice fields, rangeland, and forests (USEPA, 1987a: pp 3.12-21). The highest levels (1-6 ppb in soils or sediment) were generally found where spraying equipment was loaded, or where the herbicide accumulated. Where the herbicide was simply sprayed, levels were very low or below the limit of detection (1-5 ppt).

Dioxins and furans have also been found in sediments in the Great Lakes and some associated rivers, and in some large river systems near urban centers. They are ubiquitous in low ppb levels in the sediments of Lakes Erie, Ontario, Huron, and Michigan (Czuczwa and Hites, 1986). They have also been found in sediments of the Niagara River (Hallet and

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Brooksbank, 1986). In many areas, samples of fish or other organisms have been used to detect the presence of dioxins and furans in the water ecosystem, rather than direct sampling of the sediments. (See the following section on dioxins and furans in animals and plants.)

Dioxins and furans have not been detected in treated drinking water in the U.S. However, furans have been found in tap water in Japan (Shiraishi et al., 1985). In the U.S., dioxins have been found in water of the Niagara River adjacent to the severely contaminated Love Canal area, and in groundwater near the canal (Hallet and Brooksbank, 1986). Groundwater and surface waters in the vicinity of wood-preserving facilities and sawmills, that use chlorophenol-based solutions to treat wood, have been contaminated with dioxins and furans (USEPA, 1988a: pp 53323-4). Both groundwater and surface waters can be sources for municipal drinking water, although the water is treated first. Those people who live near contaminated sites and use untreated well water have been notified.

Dioxins and furans have been detected in air in urban areas of the U.S. (Czuczwa and Hites, 1986), West Germany (Rappe and Kjeller, 1987; Rappe et al, 1988), and Japan (Nakano et al., 1987), in air near contaminated sites in the U.S. (Fairless et al., 1987), and in the air emissions from incinerators. It is suspected that incinerators and automobiles burning leaded gasoline are important sources of dioxins and furans in urban areas.

In conclusion, dioxins and furans are present in very low levels in many places, and at high levels at a small number of contaminated sites. Their presence is usually associated with the production, storage, use, or disposal of chlorophenol-based compounds. In addition, they have been detected in low levels in urban areas, where they are believed to be the product of numerous combustion processes. As our ability to detect dioxins and furans improves, it is likely that they will be found in more places.

Are dioxins and furans found in animals and plants?

Dioxins and furans are much more soluble in fats than in water and therefore tend to accumulate in the fatty tissues and organs of animals exposed to them. Levels of 1-200 ppt

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2,3,7,8-TCDD have been detected in fish and other aquatic organisms in some parts of the U.S., and fish from the Great Lakes and associated rivers generally contain higher amounts than elsewhere in the country (USEPA, 1987a: p 3.29-32). The USEPA detected 2,3,7,8-TCDD in 17 of 90 samples of fish from randomly selected national monitoring sites, at levels of 1-19 ppt. Samples were also taken from 305 areas of general interest, chosen because they were near population centers, were used for commercial or recreational fishing, or because other water quality information was already available. Of these samples, 95 of 305 from regionally selected sites had 1-85 ppt 2,3,7,8-TCDD. Of fish samples from the Great Lakes, 23 of 29 had 1-41 ppt 2,3,7,8-TCDD.

The USEPA also found as much as 85 ppt 2,3,7,8-TCDD in fish, from rivers that were receiving effluents from some pulp and paper mills (USEPA, 1987a: p 3.31). Since then, dioxins and furans have been found in the sludge, wastewater, and products of pulp and paper mills that use a chlorine bleaching process (Amendola et al., 1987).

A study of dioxins and furans in fish and Herring Gulls from the Great Lakes (Stalling et al., 1983) found a wide range of total levels of dioxins (undetectable to 223 ppt) and furans (15-290 ppt) in fish from various locations in the Great Lakes watershed. 2,3,7,8-substituted congeners were responsible for the bulk of the dioxins and furans present, and 2,3,7,8-TCDD was the predominant congener. The two Herring Gulls analyzed had 27 and 26 ppt total furans, and 196 and 110 ppt total dioxins, including 165 and 75 ppt 2,3,7,8-TCDD. 2,3,7,8-TCDD has also been detected in Herring Gull eggs from the Great Lakes, at levels ranging from 9-90 ppt (Norstrom et al., 1982).

Because 2,3,7,8-TCDD is a contaminant of the herbicide 2,4,5-T, there was concern about 2,3,7,8-TCDD being spread through the use of this herbicide (2,4,5-T is no longer produced or used in the U.S.) Therefore, a joint study by the USEPA and the University of Nebraska was undertaken to determine if animals showed detectable amounts of 2,3,7,8-TCDD after normal application of 2,4,5-T to Oregon forests (Gross, 1980, cited in Weerasinghe and Gross, 1985). Animal tissue and whole animals (mice, shrews, birds, and newts) were sampled

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and, although 3 ppt were found in several samples by one laboratory, this could not be confirmed by the second laboratory.

However, some studies have detected low levels of 2,3,7,8-TCDD in organisms after normal applications of 2,4,5-T. In a 1984 study by the USEPA, deer were placed in a plot that was then sprayed with 2,4,5-T (Harless et al., 1983). They found 1-27 ppt 2,3,7,8-TCDD in the fat, muscle, and liver tissue of the deer, and the frequency of detection increased during the four-week sampling period. No 2,3,7,8-TCDD was found in the bone marrow of the deer, or in a deer placed in a separate area. Also, 2,3,7,8-TCDD in levels as high as 1 ppb have been detected in whole-animal samples of fish and shellfish from areas in South Vietnam (Baughman and Meselson, 1973) that were heavily sprayed with Agent Orange during the Vietnam War.

At exceptionally contaminated sites, such as an improperly managed waste site (Heida et al., 1986), a military base where Agent Orange was repeatedly handled and sprayed (Young and Cockerham, 1985), or where 2,3,7,8-TCDD was released during an industrial accident (Fanelli et al., 1980), 2,3,7,8-TCDD has been found in many different organisms, such as rodents, insects, lizards, earthworms, and birds. Researchers in Italy found that plants grown in soil contaminated with 2,3,7,8-TCDD accumulated the compound in their roots, and to a much lesser extent, in the above-ground portion of the plants (Facchetti et al., 1986). However, in Seveso, one year after the accident, no traces of 2,3,7,8-TCDD were found in the flesh of fruits or in corn kernels and cobs, from trees and plants grown in soil contaminated with approximately 10 ppb total dioxins. Dioxins were found in the peels of fruits; the researchers concluded that they were contaminated externally by dust, and not by dioxins in the soil (Wipf et al., 1982).

Dioxins and furans have been found in organisms from contaminated areas, and from fish in many areas of the U.S. Most animals do not appear to accumulate these compounds at levels greater than in their surrounding environment, although "bioconcentration" can occur. Plants typically contain these compounds at levels much below the surrounding environment, and only when they are grown in highly contaminated soil. The fruits do not appear to contain dioxins; however, root crops may be more susceptible to contamination.

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How long do dioxins and furans last in the environment?

Many factors influence the persistence of dioxins and furans in the environment: what kind of dioxin or furan it is, whether it is present as a solid or gas, what it is mixed with, and what kind of environment it is exposed to, among other things. Photodegradation (breakdown by light) is believed to be the most important environmental process for the breakdown of dioxins and furans. Crosby et al. (1971) and Crosby and Wong (1977) have demonstrated that ultraviolet (UV) light is responsible for the photodegradation of 2,3,7,8-TCDD. They also found that the rate of breakdown varied with the material the dioxins were mixed with, and the surface to which the mixture was applied.

They reported that 2,3,7,8-TCDD applied to wet and dry soil, or dissolved in water, showed no signs of breakdown after as much as four days of exposure to ultraviolet light. However, when it was mixed with the herbicide Agent Orange, the commercial herbicide Esteron, or a solvent such as methanol (wood alcohol), and applied to soil, glass, or plant leaves, the 2,3,7,8-TCDD was partially or totally degraded in less than eight hours. The authors noted that these conditions would often have been met during the application of 2,3,7,8-TCDD-containing herbicides such as 2,4,5-T, and may explain why the normal application of 2,3,7,8-TCDD-containing herbicides typically does not leave persistent, detectable amounts of 2,3,7,8-TCDD on soil or foliage.

In addition to photodegradation, there are other processes that can break down dioxins and furans. For example, some breakdown of these compounds in soil appears to be the result of digestion by microorganisms (Matsumura and Benezet, 1973). However, this breakdown occurs much more slowly than the photodegradation process mentioned above. Efforts to isolate a microorganism that can digest 2,3,7,8-TCDD have met with little success: only a small percentage of the substance is broken down, even after months of incubation. It also appears that dioxins and furans bound to particles and dissolved in water are very slowly released to the atmosphere as a gas (Palausky et al., 1986; Nash and Beall, 1980). The significance of this

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process is not yet agreed upon. Once released to the air by this process, it is expected that dioxins and furans are broken down by sunlight.

In sum, 2,3,7,8-TCDD can be broken down in a matter of days in the environment, if it is mixed with the proper organic solvent (not water, but acetone or even corn or olive oil) and exposed to ultraviolet light (Wipf et al., 1978). On the surface of the soil, without a solvent, 2,3,7,8-TCDD has an environmental half-life of one year or less, due to slow photodegradation and volatilization (Crosby and Wong, 1977). Underground and in sediments, this very stable compound has broken down much more slowly, with a half-life of about 10 years (DiDomenico et al., 1980a; 1980b). Very little is known about the half-lives of other dioxins and furans. In general, dioxins and furans with fewer than four chlorines are broken down more quickly than 2,3,7,8-TCDD (Crosby et al., 1971). The dioxin or furan with eight chlorines is more resistant to breakdown.

To what degree do dioxins and furans spread when they are introduced into the environment?

The most important manner of transport of dioxins and furans appears to be the physical movement of particles to which they are bound. Dioxins and furans have a great affinity for organic carbon; thus they bind very strongly to the organic carbon present in particles of soil, in sediments, and to the particles found in the air. These particles can be lifted and carried by wind or by water.

In the air, dioxins and furans can be transported considerable distances. For instance, they have been found in the sediment of a lake on Isle Royale, an island in Lake Superior (Czuczwa et al., 1984). It is believed that dioxins and furans could reach this lake only by transport in air. Also, dioxins and furans have been found in Arctic seals taken in the Arctic Circle, many thousands of kilometers from any known sources of the compounds (Oehme et al, 1988).

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There is also evidence of the transport of dioxins and furans in water. 2,3,7,8-TCDD has been detected in fish downstream from known sources, and in groundwater and river water near landfills in which large amounts of 2,3,7,8-TCDD-contaminated wastes were disposed (Hallet and Brooksbank, 1986). Dioxins and furans have also migrated from wood-preserving facilities and sawmills through surface waters and groundwater (USEPA, 1988a: pp 53323-4).

Measurements of the movement of 2,3,7,8-TCDD through soil indicate that the process is generally a very slow one, from one to ten centimeters per year. Palausky et al. (1986) reported that the initial depth to which the 2,3,7,8-TCDD contaminates the soil greatly influences the resulting movement by vapor phase. This depth of contamination is related to the type of solvent in which the 2,3,7,8-TCDD is carried: the volatility (at what temperature it evaporates) of the solvent, its viscosity (how easily it flows), and how it interacts with soil organic matter.

Because they are so insoluble in water, only very small amounts of dioxins and furans are washed from soil particles or released from sediments to the water above it (Isensee and Jones, 1975). Their extremely low solubility in water makes it very hard to measure just how much dissolves; consequently, there is a wide range of reported values. The recently accepted value for 2,3,7,8-TCDD is about 20 ppt in pure water (Marple et al., 1986).

Dioxins and furans can be transported as a gas, but Palausky et al (1986) detected this only at temperatures above 30oC (87°F); it was therefore concluded that this vapor phase transport of 2,3,7,8-TCDD takes place only in upper layers of soil, during hot weather.

In conclusion, dioxins and furans attached to particles can migrate considerable distances in the air, and to a lesser extent in water. But because these compounds are so insoluble in water, and because they bind so strongly to particles in the soil and water, they appear to migrate very little once they reach sediments and soil. Areas of severe contamination have typically remained local problems, and contamination appears to be limited to nearby areas.

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Are dioxins and furans found in humans?

Dioxins and furans have been found in samples of human blood and fat tissues from numerous countries, including the U.S., Canada, Japan, Vietnam, and several countries in Europe. Based on these findings, it is generally agreed that the population at large in many parts of the world has been exposed to low levels of dioxins and furans.

In North America, for example, 2,3,7,8-TCDD is typically present in human fat, some other tissues, and breast milk, at the level of approximately 7 ppt (Ryan, 1986). In addition to 2,3,7,8-TCDD, penta-CDD is typically found at 10 ppt, penta-CDF at 15 ppt, and the hepta- and octa-CDDs/CDFs at levels approaching 1 ppb. In humans as in other animals, the most toxic dioxins and furans, with chlorines in the 2,3,7 and 8 positions, have been found in greater amounts than the other dioxins and furans with the same number of chlorines.

How do dioxins and furans reach humans?

Although we can be exposed to enough of these compounds to detect them in our bodies, the mechanisms of this process are still under investigation. Dioxins and furans are believed to reach humans in the following ways: when we inhale contaminated dust or vapors; when we contact or ingest contaminated water, sediment, or soil; when we eat contaminated vegetable, meat, or dairy products (Mukerjee et al., 1986). At the present time, the relative importance of these routes of exposure is not understood. However, drinking and contacting contaminated water is generally considered a minor route because dioxins and furans have not been detected in treated drinking water in the U.S., and our contact with untreated water and sediments is normally minimal (e.g. swimming, other watersports). Ingestion of and contact with soil is also considered a minor route, except for small children.

The major routes of exposure, then, are generally considered to be breathing contaminated particles and eating contaminated food. Particles in air, especially near urban areas or large sources of combustion products, can contain low levels of dioxins and furans, and

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the accumulated lifetime exposure from inhaling these particles is considered a potentially important source (Marklund et al., 1986). Studies by Rappe et al. (1986a) and Travis and Hattemer-Frey (1987) compared breathing to food consumption, and estimated that breathing is far less important, even near a large source of combustion. However, very little is known about the actual amounts contributed by breathing air particles.

Dioxins and furans have been found in a variety of foods in Japan and Canada, in dairy products in Sweden, and in selected foods in the U.S. They have been found in fish from the Great Lakes and associated rivers, fish from many major rivers near urban centers, and fish from rivers below some pulp and paper mills. As a result, advisories for limiting consumption of fish in some of these areas have been issued (see Appendix).

In 1984, the USEPA found ppt levels of 2,3,7,8-TCDD in 3 of 85 samples of beef fat from cattle that had grazed on land sprayed with 2,4,5-T (USEPA, 1985: p 4.30). Dioxins and furans, but not 2,3,7,8-TCDD, have also been found in chicken livers and chicken eggs, and in gelatin from supermarkets (USEPA, 1985: p 4-31) In both cases, the sources of the contamination were hides (from cattle, hogs, sheep, and other animals), treated with pentachlorophenol, a product which is usually contaminated with dioxins and furans. Fat from the treated hides was used in the manufacture of the chicken feed and the gelatin (Firestone, 1973).

Investigators in Sweden found dioxins and furans (but not 2,3,7,8-TCDD or 2,3,7,8-TCDF) in levels below 1 ppb in cow fat, milk, cream, and liver (Nygren et al., 1986), and investigators in Japan (Ono et al., 1987) found similar amounts of dioxins and furans in a variety of meat and dairy products. A researcher in Canada also found ppt levels of dioxins and furans in fruits and vegetables (Davies, 1988).

2,3,7,8-TCDD has not commonly been found in food in the U.S.; its presence in selected foods has been related to specific sources, as above. However, it is probable that trace amounts of other dioxins and furans are present in common foods, as has been found in other countries. Similar tests have not yet been reported for the U.S.

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Breast milk, like fatty human tissues, has low levels of 2,3,7,8-substituted dioxins and furans, and they can be transferred to a baby through the mother's milk (reviewed in Lindstrom, 1988: p 34). Because a baby is tiny, undergoing rapid development, and may have breast milk as its sole source of nourishment, a breast-fed baby may be subject to a greater than normal risk (Tarkowski and Yrjanheikki, 1986; Schecter and Gasiewicz, 1987). Breast-fed babies may receive levels of dioxins and furans that exceed safe guidelines (Nygren et al., 1986). However, a study by the World Health Organization (WHO) Regional office in Europe determined that breast-fed babies receive dioxins and furans in amounts far below what are capable of causing adverse health effects (WHO, 1988, cited in Lindstrom, 1988).

Young children ingest more soil than older children and adults, and are probably subject to greater contact exposure as well (Houk, 1986). The Center for Disease Control took this greater exposure into account when they determined that 1 ppb or more of 2,3,7,8-TCDD in the residential soil of Times Beach suggested the need for corrective action.

Workers in several occupations are or have been subject to a greater potential exposure (Choudhary, 1983). These include: mills where wood is treated with pentachlorophenols or where treated wood is sawed; leather and tanning industries, which have used chlorophenols as preservatives; facilities that manufacture, ship, or formulate chlorophenols; pulp and paper mills that use chlorophenol-based fungicides; health-related facilities, where hexachlorophene is used as a bactericide; occupations where workers apply chlorophenol-based biocides.

In sum, it is generally believed that eating contaminated food and breathing contaminated particles are the most important sources of exposure. We need to know more about the routes of human exposure to dioxins and furans. Also, breast-fed babies, small children, and some workers in the industries that use contaminated products, are subject to greater than normal potential exposure. These cases merit special attention.

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Sources of Dioxins and Furans

How are dioxins and furans created?

Dioxins and furans have no useful purpose and have never been manufactured deliberately, except in small amounts for research purposes. They are known to be created in two major ways: (1) they are created in trace quantities by unwanted side reactions, which take place during the chemical processes used to manufacture useful products such as biocides, paper, disinfectants, and preservatives; (2) they are created when a substance containing chlorine is burned, or when a substance is burned in the presence of chlorine (chlorine is a constituent element of many substances). In addition to the dioxins and furans from these two major sources, there is evidence that dioxins and furans can be created when certain mixtures of substances are exposed to sunlight. The importance of this process is not yet known.

Determining the relative or absolute importance of the various sources of dioxins and furans is complicated. Simply, the question is: What kind of hazard does the source represent to humans and other organisms? In order to estimate this, at least the following information should be considered:

1) What types of dioxins and furans are produced by the source?

2) What amounts of the different types are produced?

3) How will humans and other organisms be exposed?

- For how long?
- To what levels?

4) How many humans or other organisms will be exposed?

Because this information is unavailable for most of the sources of the dioxins and furans, it is impossible to make more than a rudimentary qualitative estimate of the importance of the various sources, relative to each other. For some sources, even this is unwarranted because of lack of information. Therefore, while a discussion of the sources is worthwhile, it should be noted that the various known sources may prove to be much more or less important

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than our present understanding makes them appear. Also, new sources of dioxins and furans will continue to be identified.

What products can be contaminated with dioxins and furans?

Dioxins are formed as unwanted byproducts during the manufacture of some chlorophenols (especially those with three or more chlorines), and during the manufacture of chemical products that are derived from these chlorophenols: phenoxy herbicides such as 2,4,5-T, and biocides and preservatives such as pentachlorophenol. Manufacturers have greatly reduced the levels of dioxins and furans in their chemical products since they were identified as contaminants in the early 1970s. In some cases this resulted in much higher concentrations of dioxins and furans in the waste from these processes. The safe disposal of such waste is difficult, and inadequate disposal methods have resulted in environmental contamination.

Dioxins and/or furans have been found as contaminants in the following products:

Table 2. Products that can be contaminated with dioxins and/or furans

Product	Contaminants
PCBs	Furans
hexachlorophene	Dioxins*
2,4,5-T	Dioxins*
2,4-D	Dioxins*
chlorophenols with 3 or more chlorines	Dioxins and Furans
polychlorinated benzenes	• •
diphenyl ether herbicides	• •
hexachlorocyclohexane	• •
paper products	• •

* I could find no analyses of these products for furans.

Note: The above list is based on information available in journals or reports. Under the auspices of the Toxic Substances Control Act (TSCA), the USEPA published a larger list of chemical products that may be contaminated with chlorinated or brominated dioxins and furans (USEPA, 1987b). For more information, see under "What has been done about dioxins and furans?", page 69.

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PCBs

PCBs and PCB-containing mixtures can be a source of furans and to a lesser extent a source of dioxins. They were widely used prior to 1977, primarily as dielectric fluids in electrical equipment such as transformers and capacitors. PCBs have also been used as plasticizers, hydraulic lubricants, and in a variety of other applications. PCBs are no longer produced in the U.S. or installed in new equipment, and their use and disposal is restricted (see under "What Has Been Done About Dioxins and Furans?", page 69).

Levels of furan contamination in PCBs vary with the manufacturer and the type of PCB (Bowes et al., 1975; Rappe and Buser, 1980). A recent and particularly sensitive study of both U.S. and Japanese PCBs found up to 60 different kinds of furans, including most of the 2,3,7,8-substituted types, in low ppb levels (Wakimoto et al., 1988). No dioxins were detected above the limit of detection of 2 ppb.

Used PCBs can have higher levels of furans. Apparently, the heat that they may be subjected to during use can promote the further formation of furans (pers. comm., P.E. des Rosiers, USEPA). However, several studies have shown that simple overheating or arcing in electrical equipment does not generate furans (des Rosiers, 1987). While dioxins generally have not been detected in PCBs, a recent study found relatively high levels of dioxins in used oil (R. Adams, et al., 1986). For many applications, such as dielectric fluids, PCBs are combined with other compounds such as chlorinated benzenes. These other compounds are believed to be responsible for the presence of dioxins (see discussion of chlorinated benzenes, below).

Approximately 1.4 billion pounds of PCBs were purchased by U.S. industries prior to 1977. Based on these sales, an estimated 84-94 kilograms (kg) of furans were present in the PCBs produced during this period, including 8 kg of 2,3,7,8-TCDF and 14 kg of 2,3,4,7,8-penta-CDF (Hutzinger et al., 1985). The authors noted that these were underestimates, because of missing sales information for some types of PCBs.

Most PCBs have been incinerated by licensed hazardous waste facilities or buried in designated landfills. However, a January 1988 U.S. General Accounting Office report estimates that 312 million pounds of PCBs still remain in millions of pieces of electrical equipment in the

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U.S., and that as much as 150 million pounds of PCBs have been released to the environment (pers. comm., USEPA).

Low levels of PCBs are now ubiquitous in the environment, and it may be presumed that they are contaminated with trace levels of furans. In areas that are heavily contaminated with PCBs, it may be presumed that furans are present as well. Based on a comparison of the furan congeners in the tissues of aquatic animals (a snapping turtle, seal, and killer whale), to the furan congeners present in PCBs, several authors have concluded that PCBs were the source of the furans in the tissues (Rappe et al., 1981; Wakimoto et al., 1988). The release of PCBs is now highly restricted, and they are probably no longer a significant source of new releases of furans (the uncontrolled burning of PCB-containing fluids can be an exception; see "PCBs" on page 39).

Hexachlorophene

Hexachlorophene is a bactericide used in special soaps, veterinary medicine, and in restricted fashion as a surface disinfectant. It was once widely used in hospitals, in the cosmetics industry, and in some consumer products such as baby powder and skin cleansers. The USEPA no longer allows its use in non-prescription products. Very few measurements of dioxins in hexachlorophene have been made, but levels of from 0.2-0.5 ppb 2,3,7,8-TCDD were found in three samples (Baughman, 1974, cited in Rappe, 1984). Present levels have been reported as equal to or less than 30 ppb (USEPA, 1985: p. 4.12). Hexachlorophene is currently not produced in the U.S. (USEPA, 1986: p 3.16).

2,4,5-trichlorophenoxyacetic acid (2,4,5-T)

The herbicide 2,4,5-T was used widely in the U.S. before 1980, and in a restricted fashion until 1983. It is no longer produced or used in the U.S. Dioxin concentrations as high as 100 ppm were found in 2,4,5-T produced in the 1950s and 1960s, with an estimated average of about 1 ppm (Rappe et al., 1982: p 496). 2,3,7,8-TCDD was present in greater concentration than other isomers[4] (Rappe, 1984). When this contamination was verified in the early 1970s,

[4] An isomer is a single member of a homologue; a homologue is that group of dioxins or furans with the same number of chlorines.

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manufacturers lowered the level of contamination considerably, and in 1984 producers of 2,4,5-T reported that the levels of 2,3,7,8-TCDD were below 0.1 ppm (Rappe, 1984).

The authors of one study estimated the maximum amount of 2,3,7,8-TCDD that could have been present in 2,4,5-T produced from 1960-1970 (Hutzinger et al., 1985). Based on total 1960-1970 production of 48.2 million kg, and a maximum concentration of 100 ppm 2,3,7,8-TCDD, they estimated a maximum total of 4800 kg 2,3,7,8-TCDD present in 2,4,5-T. The authors added that after 1970, both the level of contamination and level of production dropped sharply.

The defoliant Agent Orange, which was sprayed heavily over most of South Vietnam during the Vietnam war, was composed of a 50:50 mixture of 2,4,5-T and 2,4-D. Levels of 2,3,7,8-TCDD in Agent Orange were found to vary between 0.02 and 54 ppm, with an average of 2 ppm (reviewed in Esposito et al., 1980: p 98). The very heavy spraying of South Vietnam resulted in significant contamination of the environment with 2,3,7,8-TCDD (Baughman and Meselson, 1973).

In the U.S., normal spraying with 2,4,5-T appears to have contributed only trace levels of 2,3,7,8-TCDD to the environment, presumably because the 2,3,7,8-TCDD is present in a solvent (2,4,5-T) and therefore can be quickly broken down by sunlight. However, it is possible that the use of 2,4,5-T contributed to the low background levels of 2,3,7,8-TCDD in the fat of persons in the U.S.

The herbicide 2,4,5-T is no longer produced or used in the U.S. and is therefore no longer a source of dioxins here. However, a large amount of contaminated waste was created during the production of 2,4,5-T, resulting in many contaminated manufacturing and waste disposal sites (USEPA, 1987a). Some of these sites are still contributing dioxins to the environment, predominantly the local area (see "What about dioxins and furans from sewage plants, waste streams, and landfills?", page 45).

2,4-dichlorophenoxyacetic acid (2,4-D)

The widely used herbicide 2,4-D is made from dichlorophenol. Dichlorophenol has only rarely been found to be contaminated with dioxins and furans, and 2,3,7,8-TCDD is not

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formed in the manufacture of 2,4-D. However, other, much less toxic dioxin congeners have been found in 2,4-D at levels of from less than 1000 ppm to less than 10 ppm total dioxins (Cochrane et al., 1982). The authors of this study analyzed 2,4-D as acid, ester, and amine formulations. The ester formulations contained the highest levels of dioxins. The majority of the dioxin congeners identified in 2,4-D by this study and others are considerably less toxic than 2,3,7,8-TCDD (e.g. 1,3,6,8-TCDD), or not toxic (dioxins with 1-3 chlorines).

While the dichlorophenol from which 2,4-D is derived is not normally contaminated with 2,3,7,8-TCDD, isolated cases of 2,3,7,8-TCDD contamination of 2,4-D may have occurred when equipment formerly used to process 2,4,5-T was used to produce 2,4-D (USEPA, 1986: pp 3.21-3.23). The contaminated equipment would not be expected to contribute more than very low levels of 2,3,7,8-TCDD in the first few batches of 2,4-D.

In Canada, an annual production of 8 million kg of 2,4-D was estimated to be the source of 9 kg of dioxins with from 2-4 chlorines (Tosine, 1983). In the U.S., 7.1 million kg of amine formulations of 2,4-D were produced in 1986, and 6 million kg sold (U.S. Department of Commerce, 1987). No ester formulations were produced in the U.S. in 1986, but 3.7 million kg were sold. Because the level of production is similar to that in Canada, I assume it to be the source of a similar amount of dioxins. As in 2,4,5-T, dioxins in 2,4-D are present in a solvent (the herbicide) and therefore may be broken down by sunlight soon after their application. This possibility, along with proper disposal of the wastes from the production of 2,4-D, make it probable that the production of 2,4-D is a relatively unimportant source of dioxins in the environment.

Chlorophenols

Chlorophenols are a principal ingredient in the processes used to manufacture many of the above chemicals. They or their derivatives are used to preserve wood and drilling muds, and as biocides for process and cooling waters in some industries. Some of these uses may be phased out in the near future. In the past, chlorophenols and their derivatives have been used to preserve hides, textiles, paints, glues, and other materials, as disinfectants, and as biocides in fluids that aid in the cutting of metals.

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Chlorophenols with one or two chlorines (chlorophenol and dichlorophenol) have rarely been found to be contaminated with dioxins or furans. Chlorophenols with three or more chlorines have been found to be contaminated with many congeners of dioxins and furans, and 2,4,5-trichlorophenol is always contaminated with 2,3,7,8-TCDD (pers. comm., Rolf Hartung, University of Michigan). Chlorophenols with four or fewer chlorines are no longer produced in the U.S. (pers. comm., John Wilkinson, Vulcan Chemicals). There is only one current manufacturer of pentachlorophenol in the U.S., and pentachlorophenol and a product made from pentachlorophenol, sodium pentachlorophenate, are used almost entirely to preserve wood or prevent wood staining (USEPA, 1986: pp 3.17-21; USEPA, 1988a: pp 53286-7).

Pentachlorophenol has been commercially produced by two basic methods: the hydrolysis of hexachlorobenzene or the direct chlorination of phenol (Esposito et al, 1980: pp 78-88). Although both methods result in the formation of dioxins and furans, the hydrolysis of hexachlorobenzene can result in higher levels of dioxins and furans that are of most concern. In the U.S., pentachlorophenol has always been produced by the direct chlorination of phenol (Esposito et al, 1980: pp 78-88).

The USEPA has reported that solutions of U.S.-produced pentachlorophenol in use at wood-preserving facilities average approximately 300 ppb total dioxins and furans, in 2,3,7,8-TCDD equivalents (USEPA, 1988a: p 53301). 2,3,7,8-substituted hexa-CDDs are responsible for most of this calculated toxicity, and in the past were thought to be responsible for pentachlorophenol's demonstrated toxicity. However, the results of a recent bioassay (NTP, 1988) showed that pentachlorophenol alone is a carcinogen in mice, and the presence of 10 ppm hexa-CDDs in the pentachlorophenol made little or no difference. 2,3,7,8-substituted dioxins and furans with fewer than six chlorines are present in pentachlorophenol only at very low detectable levels or below the limits of detection. Tetra-dioxins, including 2,3,7,8-TCDD, have not been detected in numerous analyses of U.S.-produced pentachlorophenol (pers. comm., P.E. des Rosiers, USEPA).

A recent and particularly sensitive analysis of 10-year old pentachlorophenols and sodium pentachlorophenates, manufactured in Europe by the hydrolysis of hexachlorobenzene,

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found very low ppb and ppt levels of 2,3,7,8-substituted tetra- and penta- dioxins and furans, in addition to 2,3,7,8-substituted dioxins and furans with six or more chlorines (Hagenmaier and Brunner, 1987). However, the low levels would have little impact on the estimated overall toxicity of the products; it would not differ significantly from that of U.S.-produced pentachlorophenols.

Hagenmaier and Brunner (1987) postulated that, in West Germany, the production and use of pentachlorophenol and sodium pentachlorophenate may be, or have been in the past, the predominant source of dioxins and furans in their environment. The USEPA considers the wastes from wood-treatment facilities and sawmills to be sources of significant environmental contamination, and for that reason has regulated or proposed to regulate such wastes as hazardous (see "What Has Been Done About Dioxins and Furans?", page 69). It should be pointed out that alternative wood preservatives (creosotes and inorganic arsenicals) are considered human carcinogens (USEPA, 1988a), and a study by Carnegie-Mellon University concluded that pentachlorophenols are the safest wood preservatives available (Carnegie-Mellon University, 1982).

Chlorophenols, especially 2,4,5-trichlorophenol, were probably sources of large quantities of 2,3,7,8-substituted dioxins and furans in the past (Tosine, 1983; Hutzinger, 1985), and many contaminated sites remain from this era. However, levels of chlorophenol production and contamination have been greatly reduced. Based on a total annual production of pentachlorophenol in North America of 11 million kg (USEPA, 1988a), and an average 2,3,7,8-TCDD- equivalent content of 300 ppb, this production would be the source of 3.3 kg of 2,3,7,8-TCDD equivalents annually in North America. Most of this amount is present in used pentachlorophenol, and is incinerated as a hazardous waste.

Polychlorinated benzenes

Polychlorinated benzenes have been used as biocides, solvents, and in electrical and chemical industries. They were often mixed with PCBs in dielectric fluids. Dioxins and furans have been found in some chlorobenzenes at ppb and ppm levels, although no information is available on the specific types (reviewed in Heindl and Hutzinger, 1986).

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Diphenyl ether herbicides

Diphenyl ether herbicides can be derived from trichlorophenol and a variety of dioxins and furans were found in three types of diphenyl ether herbicides in one study (Yamagishi et al., 1981, cited in Rappe, 1984: p 83a). Total concentration of dioxins and furans ranged from less than 2 ppm to about 50 ppm. No 2,3,7,8-TCDD was found and the few individual congeners that were identified are not considered very toxic.

Hexachlorocyclohexane

Hexachlorocyclohexane is used primarily to produce the insecticide Lindane. Dioxins and furans are apparently formed by this process, and the waste has been found to be contaminated with ppm levels of dioxins and furans, including ppb levels of 2,3,7,8-substituted congeners (Scholz and Engler, 1987). The significance of this source is not known.

Paper mills and products

Pulp and paper mills that use a chlorine bleaching process have been identified as sources of dioxins and furans. Dioxins and furans are believed to be formed when naturally occurring phenolic compounds in wood called lignins react with chlorine during the bleaching operations in the manufacture of pulp (Beck et al., 1988). A preliminary study by the USEPA and the pulp and paper industry of five pulp and paper mills that use a chlorine bleaching process, found that the wastewater effluent from these mills had concentrations of less than 1 ppt total dioxins and furans, and the sludge had concentrations of less than 1 ppb total dioxins and furans (Amendola et al., 1987).

Because of the large volume of effluents from pulp and paper mills and the potential for the accumulation of dioxins and furans in organisms, even these low levels may pose significant risks to organisms exposed to the effluents. As noted earlier in this report, low levels of 2,3,7,8-TCDD were found in fish downstream from some pulp and paper mills and these findings are of concern to people who eat fish from these waters. The USEPA and the pulp and paper industry have a more extensive study underway.

Researchers in Sweden determined that the isomeric pattern of dioxins and furans from pulp plants is easily distinguishable from that of dioxins and furans from incinerators (Swanson

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et al, 1988). They found that crabs and sediments near the pulp mill had dioxins and furans with an isomeric pattern similar to that in pulp, which then changed to the incinerator pattern as they moved away from the mill. The authors estimated that the pulp industry in Sweden was a source of 5-15 grams of 2,3,7,8-TCDD equivalents per year, and reported that the Swedish EPA estimates 1 kg of 2,3,7,8-TCDD equivalents per year for all sources of dioxins and furans in Sweden.

A study of West German paper products such as newsprint, coffee filters, and recycled paper found that they can contain a variety of forms of dioxins and furans. Total concentrations of dioxins and furans were below 1 ppb with concentrations of the more toxic congeners at levels below 50 ppt (Beck et al., 1988). A study sponsored by the National Council of the Paper industry for Air and Stream Improvement (NCASI) determined that contact with paper products poses an insignificant health risk to humans (NCASI, 1987a). However, studies by researchers in Canada suggest that cardboard cartons made of bleached pulp can transmit dioxins and furans to certain food products, such as milk (Ryan et al, 1988). Any such assessments include a large number of uncertainties, and further research is necessary.

What products can produce dioxins and furans when they are burned?

Dioxins and furans can be formed when many materials containing some form of chlorine are burned, or when certain materials are burned in the presence of chlorine. The amount and kind of dioxins and furans produced varies greatly with the source and conditions.

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The following chlorinated products can all produce dioxins and/or furans when burned.

Table 3. Chlorinated products that can produce dioxins and/or furans when burned

Product	Contaminants
PCBs	Furans
polybrominated biphenyls (PBBs)	Dioxins and Furans
polybrominated diphenyl ethers (PBDEs)	• •
polychlorinated diphenyl ethers (PCDEs)	• •
chlorophenols	• •
tetrachloroethylene	• •
polychlorinated benzenes	• •
polyvinyl chloride (PVC)	• •
Leaded gasolines	• •

Note: When a material is burned in the laboratory to determine whether it is capable of producing dioxins and furans, it is typically burned under those conditions most likely to produce dioxins and furans, or that produce the largest quantity of dioxins and furans. Therefore, the same substance may produce much smaller amounts of dioxins and furans under the uncontrolled conditions of a fire, or in the highly controlled conditions of an incinerator.

PCBs

PCBs are fire-resistant compounds and do not readily support combustion. However, if they are incompletely burned in fires fueled by other materials, furans may be formed. More than 1 ppm of each of the most toxic congeners of furans (4-7 chlorines and 2,3,7,8 positions filled) have been produced by burning PCBs in the laboratory (Erickson et al., 1984; Swanson et al., 1985). Fires involving electrical equipment have contaminated buildings and other places with soot containing as much as 5000 parts per million total furans, including low ppm levels of 2,3,7,8-TCDF (Erickson et al., 1984; Rappe et al., 1983: p 121). In the U.S., PCBs are no longer produced or used in new equipment, and PCB-containing equipment in buildings is subject to strict regulation. However, large numbers of transformers and capacitors with PCB-contaminated dielectric fluids are still in use, and these contaminated dielectric fluids can produce furans when they are burned (Narang et al, 1988).

Polybrominated biphenyls (PBBs) and polybrominated diphenyl ethers (PBDPEs)

Polybrominated biphenyls (PBBs) and polybrominated diphenyl ethers (PBDPEs) have been widely used as flame retardants in textiles, carpets, and plastics. Although the addition of these substances makes products such as clothing and carpets less susceptible to igniting, the materials can burn in a building fire or incinerator. When burned in the laboratory, they have

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produced brominated dioxins and furans, and smaller amounts of chlorinated dioxins and furans if they are burned in the presence of some form of chlorine (Buser, 1986; 1987; Thoma et al., 1987).

Polychlorinated diphenyl ethers (PCDEs)

Polychlorinated diphenyl ethers (PCDEs) are used as herbicides and have been found as low-level contaminants in chlorophenols (Soikkeli et al., 1986). When burned in the laboratory, they can produce significant amounts of dioxins and furans (Rappe, 1984: p 83a).

Chlorophenols

When burned, chlorophenols can produce significant amounts of dioxins and furans, especially those with from 4-6 chlorines, and including the toxic 2,3,7,8 congeners (Rappe et al, 1978; Rappe et al., 1983: p 108). An unknown amount of chlorophenol-treated wood is eventually burned, and may be a widespread source of significant amounts of dioxins and furans. Current USEPA registration for pentachlorophenol used in wood treatment specifies that the wood is not to be burned, although wood-treatment facilities may burn wood scrap in high-temperature boilers.

One study found that levels of octa-CDD were doubled when PCP-treated plywood was burned, and dioxins with six and seven chlorines were also detected (Crosby et al., 1973). Wood-treatment facilities often collect waste pentachlorophenol in ponds, and in the past they periodically set fire to the ponds to reduce their volume. This practice generated large amounts of dioxins and furans (P.E. des Rosiers, USEPA, cited in USEPA, 1986c p 3-18).

Tetrachloroethylene and polychlorinated benzenes

Tetrachlorethylene and polychlorinated benzenes are used as cooling fluids in electrical equipment, often in combination with PCBs. When burned in the laboratory, they can produce furans and lesser amounts of dioxins, including the most toxic congeners (Erickson et al., 1984). Polychlorinated benzenes produced less than 1/10th the amount of furans produced by PCBs, and tetrachloroethylene produced less than 1/1000th. However, these are significant amounts nonetheless. For example, polychlorinated benzenes are believed to be responsible for the dioxin contamination of an office building in Binghamton, New York, after a transformer fire.

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The soot produced by this fire had 20 ppb total dioxins, including 0.6 ppb 2,3,7,8-TCDD (Rappe et al., 1983: p 121).

Polyvinyl chloride (PVC)

Polyvinyl chloride (PVC) is a common plastic. When burned in the laboratory, it can produce low ppb levels of dioxins and furans (Marklund et al., 1986: p 90). Large amounts of PVC plastic are present in municipal waste, and are suspected of contributing to the dioxins and furans emitted from incinerators.

Leaded gasoline

Low levels of dioxins and furans have been detected in motor oil and in the exhaust of cars that use leaded gasoline (Marklund et al., 1987), but not cars that use unleaded gasoline. The authors noted that dioxins and furans may be destroyed in the catalytic converters of the cars burning unleaded gasoline. Dioxins have been found in mufflers from diesel trucks (Bumb et al., 1980), and dioxins and furans have been found in motor oils that are recycled from used motor and other oils (Rotard et al., 1987). Dichloro and dibromo ethane (DCE and DBE) are used as additives in leaded gasolines, and are thought to be the precursors to dioxins and furans.

Leaded gasoline is a very widely used product, and therefore even the low levels formed are potentially important. It was estimated that autos burning leaded gasoline in Sweden produce emissions with a total of 10-100 grams of 2,3,7,8-TCDD equivalents per year (Marklund et al., 1987). Using their emission values (30-540 picograms of 2,3,7,8-TCDD equivalents/kilometer) and assuming their test autos averaged 20 miles (32 kilometers) per gallon of gasoline (the models were a Saab 900, a VW Golf, an Opel Kadett, and a Volvo 245), total emissions from U.S. autos can be estimated. Based on an average figure of approximately 57 million gallons of leaded gas used per day in 1988 in the U.S. (pers. comm., American Petroleum Institute), and the values above, autos burning leaded gasoline in the U.S. may produce emissions with from (57 million gallons/day x 365 days x 32 kilometers/gallon x 30 picograms/kilometer) to (57 million gallons/day x 365 days x 32 kilometer/gallon x 540 picograms/kilometer), or 20 to 360 grams of 2,3,7,8-TCDD equivalents per year. The use of

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leaded gasoline is steadily decreasing in the U.S. and much of Europe, thus reducing the contribution from this source.

The burning of dioxins and furans

The incineration of dioxins and furans in the laboratory has produced other dioxins and furans with fewer chlorines. For instance, incineration of octa-CDD or octa-CDF can produce dioxins or furans with from four to seven chlorines (Marklund et al., 1986; Swanson et al., 1986). This is a potentially significant process because the toxicity of dioxins and furans generally increases as the number of chlorines decreases from eight to four. Also, octa-CDD and octa-CDF are often the most numerous types of dioxin or furan in contaminated products or waste.

Does the burning of paper, wood, peat, or coal produce dioxins and furans?

Several studies have shown that wood, peat, and coal can produce dioxins and furans when they are burned. Apparently, naturally occurring phenolic compounds in these substances (lignins) can react with naturally present chlorine to form chlorinated phenols, which then form dioxins and furans (Beck et al., 1988). Measurements of dioxin and furan emissions from coal- and peat-fired power plants indicate that they emit only very small amounts of dioxins and furans, perhaps in part because the combustion conditions are carefully controlled for high efficiency (Kimble and Gross, 1980; Marklund et al., 1986; USEPA, 1987a: pp 4.1-26). Likewise, the combustion of paper has been found to produce only very small amounts of dioxins and furans (Olie et al., 1982; USEPA, 1987a: pp 4.1-26).

The combustion of wood, on the other hand, is a potentially significant source of dioxins and furans. Dioxins and furans, including the most toxic congeners, have been detected in small amounts in emissions from wood stoves and wood boilers (Nestrick and Lamparski, 1983; USEPA, 1987a: pp 4.1-26; Thoma, 1988). Because of the large number of wood combustion sources in the U.S., this could be a significant source. However, analyses of lake sediments from an island in Lake Superior did not find dioxins and furans in sediment layers

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from before 1940, when wood combustion was prevalent in North America (Czuczwa and Hites, 1986).

Wood that has been treated with pentachlorophenol or sodium pentachlorophenate would be especially likely to emit dioxins and furans when burned. However, the major use of pentachlorophenol is for the treatment of utility poles, and few if any are burned in wood stoves.

Do municipal incinerators produce dioxins and furans?

Municipal solid-waste incinerators are recognized as important sources of dioxins and furans. They are found on the dust and in the gas emitted through the stack; on the fly ash and bottom ash that is collected and placed in landfills; and in the scrubber water that is part of the emission control system (Marklund et al., 1986; USEPA, 1987a: pp 4.1-26; Hiroaka et al., 1987). While municipal incinerators have been found to produce all types of dioxins and furans (Karasek and Hutzinger, 1986), the amounts and distribution of the different dioxins and furans produced by municipal incinerators vary widely and therefore so do estimates of the amounts of dioxins and furans produced in total by municipal solid-waste incineration. Incinerators operated under the proper conditions and equipped with the most modern emission control devices have greatly reduced dioxin and furan emissions (Hay et al., 1987; USEPA, 1988b).

Rappe et al. (1987a: p 1604) estimates that a normal-sized municipal solid waste incinerator (which burns 50,000-200,000 tons of garbage per year), operating under normal conditions, emits through the stack 1-100 grams of 2,3,7,8-TCDD equivalents per year (based on a range of 1-100 nanograms of 2,3,7,8-TCDD equivalents per cubic meter of stack effluent). This figure can thus be interpreted as 5 millionths (1/200000) to 2 thousandths (100/50000) of a gram of 2,3,7,8-TCDD equivalent emitted through the stack per ton of garbage burned. According to a recent USEPA report (USEPA, 1988b), 16.5 million tons of garbage are incinerated in the U.S. each year. Using Rappe's range as given above, municipal incinerators in the U.S. may be the source of from (5 x 10-6 grams/ton x 16.5 x 10^6 tons) to (2 x 10^3

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grams/ton x 16.5 x 10^6 tons), or from 0.0825 to 33 kg of 2,3,7,8-TCDD equivalents per year, distributed in their stack effluents. This estimate does not take into account the dioxins and furans in the bottom ash, fly ash, and scrubber water, the majority of which are placed in landfills. In Canada, dioxin and furan emissions from municipal incinerators has been estimated to be 6.3 kg/year TCDD equivalent in the fly ash, and 6.1 kg/year TCDD equivalents in the stack emissions (Tosine, 1983).

A study in Switzerland showed that levels of dioxins and furans in cow milk were higher in samples collected near incinerators (Rappe et al., 1987b). However, in another study (Rappe et al., 1987c), the authors mention that while Sweden has 25-30 incinerators and Yugoslavia has none, similar levels of dioxins and furans are found in human milk from these countries. Apparently, incinerators are only one of many sources that contribute dioxins and furans to the environment in these countries.

Do other large combustion sources produce dioxins and furans?

There are other potentially large combustion sources of dioxins and furans. Hazardous and hospital waste incinerators, sewage sludge incinerators, and smelters and mills that recycle metals can all produce significant amounts of dioxins and furans, including the more toxic types (USEPA, 1987a: pp 4.1-26; Clement et al., 1987; Marklund et al., 1986). The dioxin and furan emissions from other large combustion sources have not been as well studied as those from municipal incinerators. The available data indicate that the emissions from a hazardous waste or sewage sludge incinerator are of the same magnitude as a municipal incinerator (USEPA, 1987a: pp 4.1-4.25; Tsuji et al., 1987; Clement et al., 1987). Because there are many more large industrial combustion sources than municipal incinerators, their total emissions of dioxins and furans could be greater than from municipal incinerators (Rappe, 1987).

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What about dioxins and furans from sewage plants, waste streams, and landfills?

Dioxins and furans have been found in sewage sludge from municipal waste-water treatment plants in Germany, in levels below 100 ppb (Hagenmaier et al., 1986). The toxic varieties of dioxins and furans were present at levels below 1 ppb. Based on an analysis of the ratios of the various congeners of dioxins and furans present, the authors proposed that pentachlorophenol and its derivatives are the source of most of the dioxins and furans in the sludge.

In addition to the products themselves, the waste generated by the production of chlorophenol-based products is a potentially important source of dioxins and furans. Ironically, the contamination of the wastes is thought to have increased significantly as a result of efforts to reduce the contamination of the products: the dioxins and furans were concentrated further in the wastes. Landfills that contain the wastes from the manufacture of chlorophenols and chlorophenol-based products, that contain PCBs, or that contain fly ash, are suspected sources of dioxins and furans. The Love Canal and associated chemical landfills in New York, for example, have contaminated the adjacent Niagara River with dioxins, and the Vertac manufacturing and waste-disposal site in Jacksonville, Arkansas, has contaminated the nearby Bayou Meto and Arkansas River. Also, sites where chlorophenols and chlorophenol-based products were manufactured or processed are now considered sources of dioxins and furans (USEPA, 1987a: pp 3.1-26). As a result of these incidents, wastes containing dioxins and furans have been disposed of much more carefully.

Are there other processes that create dioxins and furans?

It has been shown that dioxins and furans can be broken down by sunlight (this is called photolysis) to other types of dioxins and furans with fewer chlorines. For example, the octachlorodioxin (OCDD), when exposed to UV light, can be broken down to potentially more toxic dioxins with fewer numbers of chlorines (Crosby et al., 1971). Combustion processes

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often produce greater amounts of octa-CDD than all other dioxins combined, and it is often the most common dioxin in chlorophenols. However, the photolysis would then be expected to continue, eventually breaking down any toxic varieties into much less toxic forms.

Photolysis can also form dioxins and furans from other compounds. It has been shown that a mixture of polychlorinated benzenes and phenol, when exposed to UV light, can produce furans (Choudhary et al., 1983). Both polychlorinated benzenes and phenols are common environmental contaminants. Also, the photolysis of chlorophenols has been shown to produce octa-CDD (USEPA, 1985: p 4.17). Thus, wood and other materials preserved with chlorophenols and exposed to sunlight may result in the formation of dioxins. The significance of these photochemical processes is not yet known.

Conclusions on sources of dioxins and furans

There are many sources of dioxins and furans. Some of them, such as sources of combustion, are widespread and numerous. Others, such as contaminated manufacturing and waste sites, are fewer in number and usually local problems. The significance of the photochemical processes that can convert less toxic compounds to dioxins and furans is not yet known. Also, the relative importance of what appears to be the two major sources of dioxins and furans (chlorophenols and chlorophenol-based products, and sources of combustion) is not yet known.

It is generally agreed that dioxins and furans in our environment come primarily from recent human activities (chemical production and use, incineration, automobiles). Czuczwa and Hites (1985) analyzed sediment cores from the Great Lakes and a Lake in Switzerland. Dioxins and furans in these sediments were at or below the limit of detection until they reached the layers deposited after approximately 1940, a time that they say corresponds to the beginning of large-scale manufacture and disposal (often by incineration) of chlorinated aromatic compounds. Also, levels of dioxins and furans in human fatty tissue and breast milk from North Vietnam are the lowest yet measured while levels in South Vietnam are similar to the U.S., Japan, and

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Europe (Schecter et al., 1986; Rappe et al., 1987c: p 233). This indicates that modern industrial activity, not the burning of wood, is the primary source of dioxins and furans to the environment.

There is some evidence that the amount of dioxins and furans being released into the environment reached a peak in the mid-1970s and has declined since. The sediment cores analyzed by Czuczwa and Hites (1986) showed such a trend. An analysis of breast milk in Sweden also appeared to show this decline: the levels of dioxins and furans in combined samples of breast milk have dropped since the study was initiated in 1972 (Noren, 1988). However, studies of dioxins and furans in breast milk in Yugoslavia (1981 to 1987) and Japan (1978 to 1984) do not show changes over time (reviewed in Lindstrom, 1988: p 36).

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The Health Effects of Dioxins and Furans, in Animals and Humans

What are the health effects on animals?

Some general features

Dioxins and furans have some very unusual features with respect to their toxicity to animals. Animals given fatal doses of dioxins and furans do not die immediately; several weeks elapse between the administration of the fatal dose and the death of the animal. Also, many of the acute (short-term) effects of less than fatal doses are temporary. When the animal stops receiving the substance, it recovers from most of the effects. The rate of recovery varies, and some animals have required more than six months to return to normal (USEPA, 1985: p 8.48).

The toxicity of dioxins and furans is highly variable. They produce a wide spectrum of effects, involving many different organs and body systems, and the affected areas usually vary with the species of animal. The toxicity of different dioxins and furans varies widely for a given species of animal, and the toxicity of a given dioxin or furan varies widely between different species of animals. See Tables 4 and 5 for acutely lethal (causes death quickly) doses of 2,3,7,8-TCDD for a variety of species. Finally, some individuals of a sensitive species (rats or mice, for example) do not die even when given doses as much as 100 times greater than the amount that is fatal to the other individuals in the same study.

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Table 4. Acutely lethal single doses of 2,3,7,8-TCDD (LD50)
(from USEPA, 1985, Table 8.1)

Species	Gender	Dose micrograms per kg of body weight (ug/kg)
Guinea Pig	Male	0.6-2.1
Guinea Pig	Female	2.5-19
Rat	Male	22
Rat	Female	45
Monkey	Female	<70
Rabbit	Male	115
Rabbit	Female	115
Mouse	Male	114-285
Mouse	Female	>450
Hamster	Male	1157-5051

Note: The lethal values above represent doses that caused the death of 50 percent of the test animals. Such a value is known as an LD50, and is a standard endpoint in a toxicity test. For aquatic animals, different concentrations of the substance in water are used, instead of a dose. The concentration that causes death of 50 percent of the test animals is called the LC50 (see table below). There are also studies that use different endpoints, such as LD95 or LC95, where 95 percent of the test animals die.

Table 5. Lethal exposures to 2,3,7,8-TCDD in water (LC50)
(reviewed in Kenaga and Norris, 1983)

Species	Concentration in Water (ug/liter)	Duration (days)
Channel Catfish	0.0042 (4.2 ppt)	15
Coho Salmon	0.0056 (5.6 ppt)	1-3
Guppy	0.1-10 (ppb)	5

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Generally speaking, all toxic members of the dioxin and furan families produce similar effects in a given species of animal, and several effects are common to all mammals that receive a lethal dose of these compounds. Although most studies of the effects of dioxins and furans on laboratory animals have been done using 2,3,7,8-TCDD, these studies can be used to predict the toxic effects of other dioxins and furans. The chief difference is that a greater dose of other dioxins or furans is necessary to produce the same effects. Many dioxins and furans, are so much less toxic than 2,3,7,8-TCDD that an experiment would require an impracticably large dose to produce lethal effects.

Doses that produce no observable adverse health effects

Long-term studies of mice and rats have shown that there are doses of 2,3,7,8-TCDD that produce no observed effects on the animals, even after receiving the substance for two years. The USEPA estimates the No Observed Adverse Effects Level (NOAEL) for toxic effects other than cancer to be approximately 0.001 ug/kg/day for both mice and rats. They also cited a need for long-term studies using lower doses (USEPA, 1985: p 14.10); some researchers feel that this dose can produce adverse reproductive effects (USEPA, 1985: p 14.10), and is therefore a Lowest Observed Adverse Effect Level (LOAEL). See Tables 6, 7, and 8 for NOAELs and LOAELs for other species. Also, the toxicity of 2,3,7,8-TCDD is at least partially cumulative; a smaller dose than the acutely lethal dose can cause lethal effects if the smaller dose is given for a longer period of time (McConnell, 1980: pp 110-111).

Table 6. Lowest dose with an observed adverse effect (LOAEL)
(from USEPA, 1985, Tables 8-4, 8-5)

Species	Dose	Duration	Effects
Rat	0.01 ug/kg/day	2 years	Liver damage
Mouse	0.001 ug/kg/day	1 year	Several
Guinea Pig	0.006 ug/kg/day	8 weeks	Several

(For the studies above, the animals received 2,3,7,8-TCDD in their food or were force-fed 2,3,7,8-TCDD once per week in a medium such as corn oil.)

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Table 7. Lowest concentration with observed adverse effect (LOAEL)

Species	Concentration in Water ug/liter (ppb)	Duration days	Effect
Northern Pike (eggs)*	0.0001 (0.1 ppt)	4	Lowered Survival
Rainbow Trout*	0.0001 (0.1 ppt)	4	Lowered Survival
Fathead Minnows**	0.0017 (1.7 ppt)	28	Lowered Survival

* (Helder, 1982)
** (W. Adams, et al., 1986)

Table 8. Highest concentration with no observed adverse effect (NOAEL)
(reviewed in Kenaga and Norris, 1983)

Species	Concentration in Water ug/liter (ppb)	Duration days
Coho Salmon	0.00056 (0.56 ppt)	4
Rainbow Trout	0.0001 (0.1 ppt)	4
Mosquito larvae (Aedes aegypti)	0.2	17
Daphnia (Daphnia magna)	1.33	32
Alga (Oedogonium cardiacum)	1.33	32

The short-term health effects on animals

All mammals given an acutely lethal dose of a dioxin or furan suffer from wasting (losing weight), and it is sometimes the only effect leading to death. The animals simply waste away. This occurs primarily because the animals do not eat enough food to maintain their weight. Apparently, the animals' internal weight "setting" is lowered below normal, and so the animals do not eat enough food. However, there is evidence that this reduction of nourishment is not entirely responsible for the weight loss. Researchers who force-fed the animals could prevent some of the weight loss, but not all, and the animals died anyway (Gasiewicz et al., 1980).

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The thymus is affected in all mammals exposed to a toxic dose, and the liver is affected in most mammals. The thymus, and to a lesser extent the spleen and lymph nodes, typically suffer damage and a reduction in size, while the liver typically suffers damage and an increase in size. In rodents, liver damage is suspected to be a principle cause of death. Skin disorders have occurred in rabbits, monkeys, cattle, and hairless mice. The effects include severe acne, thickening of the eyelid, and abnormal growth or loss of hair, fingernails, toenails, or hooves. Dioxins and furans have also affected the cardiovascular system, gastrointestinal and urinary tracts, spleen, bone marrow, and gallbladder of animals.

For reviews of the above health effects, see Gupta et al., 1973; Poland and Knutson, 1982; USEPA, 1985.

Cancers

2,3,7,8-TCDD is a carcinogen in mice and rats, when fed to the animal or applied to the skin. Two-year studies at Dow Chemical Corporation (Kociba et al., 1978) and at the National Cancer Institute (NTP, 1980a; 1980b) found increased cancers of several types, resulting from chronic (long-term) doses as low as 0.01 ug/kg/day. Rats fed this dose for two years had, at the end of the Dow study, 1700 ppt 2,3,7,8-TCDD in their fat. The rats fed this dose also suffered increased mortality, decreased weight gain, and increased excretion of porphyrins (a pigment produced by the liver which indicates liver damage). Rats fed 0.001 ug/kg/day suffered no health effects of consequence, and had 540 ppt 2,3,7,8-TCDD in their body fat at the end of the study.

Long-term studies have also been conducted using a mixture of two hexa-CDDs, both containing chlorine atoms in the 2,3,7,and 8 positions (NTP, 1980c; 1980d. The mixture caused liver tumors in both mice and rats when administered orally in doses of 2.5-5.0 ug/kg/week (for female rats), 5.0 ug/kg/week (for male mice and rats), and 10.0 ug/kg/week (for female mice), but not at lower doses. The mixture was not carcinogenic when 0.1 ug was applied to the skin three times per week.

Many researchers have concluded that 2,3,7,8-TCDD is a promoting, rather than an initiating, carcinogen. A promoter provides a favorable environment for tumor growth, while an

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initiator produces a change in DNA that causes tumorous growth. Tumor promotion is reversible and is not inherited, while tumor initiation is not reversible and is passed on through cell division (Paustenbach et al, 1986). Pitot et al (1980) used diethylnitrosamine to initiate tumors in mice, and found that 2,3,7,8-TCDD promoted the growth of the initiated tumors. Also, Kouri et al (1978) used 3-methyl-cholanthrene to initiate subcutaneous tumors in mice, and found that 2,3,7,8-TCDD promoted the growth of these tumors.

Reproductive effects

Dioxins and furans appear to be especially toxic to embryos and fetuses, and the reproductive system of adult animals appears to be especially sensitive as well. Mice and rats have produced litters with abnormally high rates of birth defects such as cleft palates, extra ribs, and deformed livers and kidneys, after receiving doses of 2,3,7,8-TCDD as low as 0.01 ug/kg/day, or a single dose of 1 ug/kg (reviewed in USEPA, 1985: pp 9.1-23). Reproductive effects such as smaller litters, lower birth weights, and premature abortions have been reported in monkeys and ferrets, as well as in rats and mice, that were administered 2,3,7,8-TCDD. When 2,3,7,8-TCDD was fed continuously to three generations of laboratory rats, reproduction was impaired by a dose of 0.01 ug/kg/day, but not 0.001 ug/kg/day (reviewed in USEPA, 1985: p D.1). Female Rhesus monkeys suffered reduced fertility after being fed 0.0015 ug/kg/day 2,3,7,8-TCDD (reviewed in USEPA, 1985: p D.1).

These effects have only been observed when the embryo or mother was exposed to 2,3,7,8-TCDD, and appear to be the result of direct exposure or transfer of the toxin from mother to embryo across the placenta. Birth defects have not been associated with exposure only to the father, nor has reproduction been reduced when only the father was exposed to 2,3,7,8-TCDD (reviewed in Kamrin and Matsumura, 1985; reviewed in Silbergeld and Mattison, 1987: p 137). However, 2,3,7,8-TCDD can reduce the weight of testes, lower levels of testosterone, and impair the development of sperm, in rodents, monkeys, and chickens (reviewed in Poland and Knutson, 1982: p 524).

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Mutagenic effects

An important question about any toxic substance is whether it causes changes in the DNA of exposed organisms, because such changes can then cause cellular mutations. Such a substance is called a mutagen. A small number of early studies found 2,3,7,8-TCDD to be a weak mutagen, while many subsequent studies have detected no evidence of mutagenicity. Most researchers have concluded that 2,3,7,8-TCDD is not a mutagen (reviewed in Hay, 1984; reviewed in Poland and Knutson, 1982: p 525).

Immune effects

2,3,7,8-TCDD has been shown to inhibit the immune system of mice, rats, and guinea pigs, at doses much below the fatal level. The thymus, spleen, and bone marrow have all been shown to suffer loss of tissue, and the enzyme immune system and cell immune system which normally react to a potentially toxic substance are inhibited (reviewed in Poland and Knutson, 1982: p 522; reviewed in USEPA, 1985: pp 8.85-86). Similar responses have been observed with 2,3,7,8-TCDF, at doses thirty times higher than for 2,3,7,8-TCDD. The effects of the 2,3,7,8-TCDF disappeared six weeks after dosing ended (Vecchi et al., 1983).

Effects on blood constituents

2,3,7,8-TCDD and 2,3,7,8-TCDF have been shown to affect a variety of constituents in the blood of test animals. 2,3,7,8-TCDD has been shown to reduce the number of red and white blood cells (Kociba et al., 1978), change the level of blood lipids such as triglycerides and esters, and change the levels of thyroid hormones such as thyroxine and thyrotropin (reviewed in Poland and Knutson, 1982: pp 526-529). Blood levels of progesterone and estrogen decreased in Rhesus monkeys fed 2,3,7,8-TCDD, and blood levels of testosterone decreased in rats (reviewed in Peterson et al., 1984). 2,3,7,8-TCDF has been shown to increase the level of serum globulin in mice, and decrease serum cholesterol and serum albumin in monkeys (Moore et al., 1979).

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Are dioxins and furans hazardous to organisms in the environment?

Very little is known about the impact of dioxins and furans on organisms in the environment, especially at low levels. Many animals died after being exposed to relatively high levels of dioxins and furans, as a result of the accidental contamination of areas surrounding Seveso, Italy, and Times Beach, Missouri (reviewed in Reggiani, 1980: pp 318, 325). It was reported that six months after the Seveso accident, normal populations of wild and domestic animals were present in the contaminated area, and appeared to be in good health (Homberger et al., 1983, cited in OME, 1985: p 3.92). Fourteen rabbits from the zone of highest contamination were collected, sacrificed, and examined, three months after the accident. All had more than 300 ppb 2,3,7,8-TCDD in their livers, and 5 showed evidence of liver damage. Blood constituents were normal in all 14 rabbits, and no other health problems were observed (Abbruzzi et al., 1977, cited in OME, 1985: p 3.92).

2,3,7,8-TCDD is also suspected of causing reproductive failure in fish-eating birds of Lake Erie and Lake Ontario (Gilbertson and Fox, 1983). The reproductive failure was primarily the result of unusually high embryo and chick mortality. A study of several gull colonies found that the embryos and chicks were suffering from chick edema disease and hepatic porphyria, which are both indicators of poisoning by dioxins, furans, and related compounds such as PCBs. Relatively high levels of 2,3,7,8-TCDD were later detected in gulls from Lakes Ontario and Erie (Norstrom et al., 1982). Other contaminants such as PCBs, DDT, and hexachlorobenzene were also detected in relatively high levels in these birds, and may have been responsible for these health effects.

Several environmental studies of organisms exposed to 2,3,7,8-TCDD and other dioxins and furans have found little or no health effects. A 15-year study of beachmice at a highly contaminated site in Florida (Young et al., 1987) found that the 1 ppb levels of 2,3,7,8-TCDD in the soil had no observable effect on the birthrate of the mice, and enlarged livers was the only observed effect. Also, NCASI sponsored a five-month study of plants and animals exposed to dioxins and furans from paper sludge spread on a pine plantation (NCASI, 1987b). The 10

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ppt 2,3,7,8-TCDD and 100 ppt 2,3,7,8-TCDF in soil had no observable effects on the health and reproduction of the organisms studied. More studies of the environmental effects of dioxins and furans are needed.

What are the pharmacokinetics of dioxins and furans?

In order to understand why many dioxins and furans accumulate and persist in animals and humans exposed to them, why the processes vary with the type of dioxin or furan, and how these characteristics relate to the varying toxicity of dioxins and furans, it is helpful to know something about the pharmacokinetics of dioxins and furans (their absorption, distribution, metabolism, accumulation, and elimination, in animals and humans).

Dioxins and furans are intermediate in their affinity for fat (this is known as lipid-solubility). They are soluble enough to be readily absorbed through the walls of the digestive tract, but not so lipid-soluble that, once absorbed, they are found only in the adipose (fatty) tissues of the body. They are also found in the liver and in other organs. Adipose tissue is the body's storage depot, and substances there are "in limbo". They are not interacting with the mechanisms that cause effects (toxic or otherwise), nor with the mechanisms that metabolize the substances. Therefore, the intermediate lipid-solubility of dioxins and furans makes it possible for them to persist in the body and be available to the mechanisms that cause toxic effects (Matthews and Birnbaum, 1983).

Studies with rodents show that 2,3,7,8-TCDD is absorbed primarily through the gastrointestinal tract, and to a much lesser extent through the skin and lungs. When 2,3,7,8-TCDD was administered orally in food or in corn oil, from 50-90% of the amount was then absorbed through the gastrointestinal tract (reviewed in USEPA, 1985: p 7.2). When applied to the skin in a solvent such as methanol, from 1-10% was absorbed. The absorbed 2,3,7,8-TCDD is distributed rapidly to many parts of the body, but the major sites of storage are almost entirely the liver and the fatty tissues (reviewed in Gasiewicz et al., 1983). The sites of storage vary somewhat with the species. 2,3,7,8-TCDD is stored in its original form, not as a changed

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product of metabolism. Apparently, metabolites are excreted once they are formed. It appears that the absorption of dioxins and furans decreases as the number of chlorines increases (reviewed in Matthews and Birnbaum, 1983).

The metabolism of 2,3,7,8-TCDD has been studied primarily in rodents. Metabolism is affected by the number and position of chlorine atoms (reviewed in Matthews and Birnbaum, 1983). The critical factor for metabolism is the presence of two adjacent unsubstituted carbon atoms (not attached to a chlorine or other atom). When chlorines fill the 2,3,7, and 8 positions, no adjacent unsubstituted carbon atoms are left on the dioxin or furan molecule, and very little metabolism takes place. A year after people in Japan ingested a number of different types of furans that were accidentally present in rice oil (known as the "Yusho" incident), no furans with adjacent unsubstituted carbon atoms remained in their tissues. Apparently, these types had been metabolized and excreted (Rappe et al., 1979, cited in Matthews and Birnbaum, 1983). Furans with the 2,3,7, and 8 positions filled were still present in the tissues. In the general population, the only dioxins and furans found in people are those that are 2,3,7,8-substituted (Ryan, 1986).

The accumulation of dioxins and furans is the product of absorption and metabolism, and varies with the type of dioxin or furan. For instance, studies with carp and rodents have shown that they preferentially absorb those types with the 2,3,7,and 8 positions filled, and they absorb a greater proportion of the available dioxin or furan as the number of chlorines decreases (Kuehl et al., 1986; 1987a; 1987b; Opperhuizen et al., 1986; van den Berg et al., 1986). Although relatively small amounts of hepta- and octa-CDDs/CDFs are absorbed, these smaller amounts accumulate and persist in the body for longer periods of time.

2,3,7,8-TCDD has been eliminated slowly from the bodies of exposed animals; the half-life varies from 10 days to approximately one year, depending on the species. In humans, 2,3,7,8-TCDD appears to be eliminated quite slowly. Based on the rate of disappearance of one billionth of a gram of self-ingested 2,3,7,8-TCDD, Poiger and Schlatter (1986) estimate a half-life of about six years. They also found that the dioxin was distributed quickly in the blood and stored almost entirely in the fatty tissues. A half-life of about seven years was

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estimated by researchers from the CDC, based on measurements of 2,3,7,8-TCDD in the blood of Ranch Hand personnel (Pirkle et al., cited in CDC, 1988).

Recent work with pharmacokinetic models suggests that the half-life of 2,3,7,8-TCDD is inversely related to its concentration in human fatty tissue (Kissel and Robarge, 1988). They predict that the half-life is 4.4 years when 2,3,7,8-TCDD is present in fatty tissue at 100 ppt, but increases to 20 years as the tissue concentration approaches 10 ppt (a typical level in a person from an industrial country).

The rate of elimination of dioxins and furans increases as the number of chlorines decreases (reviewed in Matthews and Birnbaum, 1983; Kuehl et al., 1987b), and studies with rodents and fish have shown that furans are eliminated more quickly than their counterpart dioxins (van den Berg et al., 1983; Kuehl et al., 1987b). Apparently, 2,3,7,8-TCDD must be metabolized to different compounds in order to be excreted in the urine and bile (reviewed in Olson et al., 1983: p 97). However, unmetabolized 2,3,7,8-TCDD can be excreted in the feces (USEPA, 1985: p 7.20) and in breast milk (Moore et al., 1976; van den Berg et al., 1986). Studies of people exposed to furans in the Yusho incident showed that the furans were also stored in the fat tissue and in the liver, and were metabolized at a slightly faster rate than that found for 2,3,7,8-TCDD, above (reviewed in Reggiani, 1983: pp 59-61). It has also been shown that nursing women excrete both dioxins and furans in their breast milk, and there is some evidence that nursing can reduce the amounts of these compounds in the body (Noren, 1988; Ogaki et al., 1987, reviewed in Lindstrom, 1988: p 34).

In some cases, the toxicity of a dioxin or furan has been related to the rate of metabolism. For instance, the toxicity of 2,3,7,8-TCDF to different species was found to be inversely proportional to the rate of metabolism (reviewed in Matthews and Birnbaum, 1983). Also, a study of 2,8-di-CDD in goldfish found that, when the metabolism of this dioxin was prevented, it was 1/60th as toxic as 2,3,7,8-TCDD (Sijm and Opperhuizen, 1988). Normally, 2,8-di-CDD is metabolized too quickly to accumulate, and is almost nontoxic. Metabolism alone cannot explain the varying toxicities of dioxins and furans, however. For instance, rats metabolize 2,3,7,8-TCDD about three times faster than do guinea pigs, yet the LD50 for the rat

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is 3000 to 5000 times greater than that for the guinea pig (reviewed in Poland and Knutson, 1982: p 521). Also, octa-CDD is metabolized very slowly (Birnbaum and Couture, 1988), yet is very much less toxic than other dioxins and furans with chlorines in the 2,3,7,8 positions.

What are the effects of dioxins and furans on human health?

Our understanding of the effects of dioxins and furans on humans is not based on controlled studies such as were done with animals. Instead, it is based on studies of people who were inadvertently exposed to these substances, as a result of an accident, their occupation, or other circumstances. This presents two major difficulties: 1) we cannot control and often do not know the level of exposure (the dose); 2) people have almost never been exposed to these substances alone; the dioxins and furans have been mixed with other substances (PCBs, PBBs, chlorophenols, chlorophenol-based herbicides, and so on).

These other substances have been shown to cause some of the same health effects as dioxins and furans, but only when people have been exposed to much larger amounts. An important difference between the toxicity of dioxins and furans and these other substances is the comparatively small amounts of dioxins and furans that can cause serious health effects. In most cases, the level of exposure to the other substances has been too low to cause such effects.

The health effects of toxic substances can be divided into two major groups: 1) acute (short-term) effects, which occur soon after exposure and quickly subside; 2) chronic (long-term) effects, which can occur anytime during the life of the person and can be persistent. Our knowledge of the acute effects of dioxins and furans is based on studies of people who were exposed to relatively large amounts, usually as a result of an industrial accident. Our knowledge of the chronic effects of dioxins and furans is based on long-term studies of these same groups of people, or studies of people exposed to relatively small amounts for a long period of time. With these long-term studies, it is more difficult to remove the confounding effects of exposure to organic chemicals other than dioxins.

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The short-term health effects on humans

Dioxins and furans have caused a host of reported acute effects on people who were exposed to relatively high levels. The reported clinical symptoms (directly observable or felt by the patient) include nausea and vomiting, headaches, irritation of the eyes, skin, and gastrointestinal tract, and a general feeling of not being well. Pain, especially in the limbs, has been reported as persisting for months, as has an enlarged and tender liver, and irritability and nervousness. There is no documented case of a person dying because of exposure to dioxins and furans.

In addition to the clinical symptoms, there are reported effects detectable by laboratory tests or study. They include liver damage, elevated levels of constituents of the blood such as triglycerides and lipids, and damage to the nerve fibers. Although most people recover quickly from the acute effects of dioxins and furans, there are reports that some of these effects have lasted for months or years. For reviews of the acute human health effects of dioxins and furans see USEPA (1985: pp 8.60-8.65); Hay (1982); Reggiani (1982).

The long-term health effects on humans

The most common chronic effect of exposure to dioxins and furans is severe and persistent acne, called chloracne, which can occur in many parts of the body. Excessive body hair and pigmentation, and elevated levels of blood constituents such as triglycerides, cholesterol, liver enzymes, and porphyrins have also been reported several years after the high exposure. Nerve disorders have been reported, including problems with or loss of some vision, hearing, taste, and smell. Also, general weakness and a loss of sexual drive has been reported to occur for some years after high exposure.

The reported symptoms and effects of exposure to dioxins and furans have been inconsistent, with the exception of chloracne. However, in most cases an enlarged and/or damaged liver and neuromuscular symptoms have also been reported. For reviews of the chronic human health effects of dioxins and furans, see USEPA (1985: pp 8.65-8.69); Hay (1982); Reggiani (1982).

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Cancers

A few epidemiologic studies have found an association between cancers and occupational exposure to dioxins and furans. The studies that are generally considered to offer the best evidence were carried out in Sweden. Abnormally high levels of a rare type of cancer, called soft-tissue sarcoma, were found in men who were exposed to chlorophenols and chlorophenol-based herbicides (reviewed in Hardell, 1983). The Swedish studies have been criticized for unreliable diagnosis of soft-tissue sarcoma (Hajdu, 1984), and for relying on the workers' memory of their exposure to the substances (Cook, 1983). In general, the diagnosis and classification of this rare type of cancer is difficult, and relying on memory for an appraisal of a subject's health is an admitted weakness.

Similar studies have since been conducted in several countries, and no such association has been found (reviewed in USEPA, 1985: 11.64-91). Several of these studies are not considered sensitive enough to detect the small numbers of a cancer as rare as soft-tissue sarcoma (reviewed in USEPA, 1985: 11.64-91).

After the Swedish results were published, a review of the medical history of men in the U.S. who were occupationally exposed to chlorophenol-based chemical products showed an abnormally high number (seven cases) of soft-tissue sarcomas (Honchar and Halperin, 1981). The authors of the U.S. review subsequently helped to reexamine the data, and they determined that only two of the seven workers were both 1) exposed to dioxin- and furan-contaminated products and 2) correctly diagnosed as having soft-tissue sarcoma (Fingerhut et al., 1984). Nevertheless, two cases still represents a significantly higher number of soft-tissue sarcomas for this group (USEPA, 1985: p 11.91). A similar study in Sweden found no association between rates of soft-tissue sarcoma and exposure to phenoxy-acid herbicides, among 350,000 Swedish agricultural or foresury workers, when compared to 1.7 million Swedish workers in other industries (Wiklund and Holm, 1986, cited in Fishbein, 1987).

In their 1985 Health Assessment for dioxins, the USEPA stated that the Swedish studies offer "limited" evidence for the carcinogenicity of dioxin-contaminated chlorophenols and

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chlorinated phenoxyacetic herbicides (such as 2,4,5-T), but "inadequate" evidence for the carcinogenicity of 2,3,7,8-TCDD alone (USEPA, 1985: p 11.138-140).

In addition to the association between exposure to dioxin- and furan-contaminated products and soft-tissue sarcomas, a few researchers have reported finding such an association with nasal cancer (reviewed in Hardell, 1983), stomach cancer (reviewed in USEPA, 1985: pp 98-107), and cancer of circulating cells (reviewed in USEPA, 1985: pp 92-98; reviewed in Hardell, 1983). Similar studies of workers exposed to dioxin- and furan-contaminated products have not found elevated levels of cancers (reviewed in Reggiani, 1983; p 483). The USEPA Health Assessment considered the available evidence for these other cancers to be inadequate, and this is an opinion shared by a majority of researchers.

The great majority of researchers in the field of toxicology or epidemiology feel that the numerous studies of the human carcinogenicity of dioxins and furans have provided inadequate evidence that they cause cancer in humans. Based on the epidemiological evidence and the knowledge that some dioxins and furans are proven carcinogens in animals, dioxins and furans are suspected human carcinogens.

Abortions, birth defects

A small number of researchers have found an association between exposure to 2,3,7,8-TCDD-contaminated products and abnormally high levels of abortions or birth defects (reviewed in USEPA, 1985: pp 9.23-36; reviewed in Hatch, 1984; Albanese, 1988). Children of mothers who consumed rice oil contaminated with PCBs and furans (the Yusho incident) were born with dark pigmentation and deformed fingernails (reviewed in Kuratsune, 1980: p 299). Some of these babies also suffered from slowed growth, and chloracne. It has generally been concluded that furans were responsible for these effects (Kunita et al, 1984). Other researchers conducting similar studies have found no such association (reviewed in USEPA, 1985: pp 9.23-36; reviewed in Hatch, 1984; Mastroiacovo et al., 1988). Many of these studies have been largely discounted because of weaknesses in the methodology (USEPA, 1985: pp 9.23-36).

There is thus far inconclusive evidence of an association between dioxins and furans and abortions, and an association with birth defects was detected only in the rare circumstance

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where the mother was exposed to extremely large amounts while pregnant (the Yusho incident). If these substances are capable of causing such effects on humans, then either the level of exposure must be extremely high, or they cause rare types of birth defects that researchers have not been able to detect in significant numbers. Again, this is an area where further investigation is needed.

Immune effects

A few researchers have reported an association between long- term exposure to 2,3,7,8-TCDD and an impaired immune system (reviewed in Hay, 1982). Other long-term studies have found no effects on the immune system (Albanese, 1988; Fara et al., 1982). A study of people exposed to 2,3,7,8-TCDD in Missouri found evidence of an abnormal cellular immune system (Stehr-Green et al., 1987). However, the effects did not result in increased illness. Also, the authors noted that these effects may be part of a normal response to a toxin in the body, i.e., they could be considered indicators of exposure and not signs of disease.

Conclusions on health effects

The long-term health effects of these compounds have been intensely investigated, making them among the most-studied substances with respect to their effect on animal and human health. Dioxins and furans cause mortality and/or cancer in many animals, but there is no conclusive evidence that they cause cancer or any other life-threatening health problem in humans. No documented human death has occurred because of an exposure to dioxins or furans.

People have been exposed to relatively large amounts of dioxins and furans (1 ug or more) during industrial accidents, and other incidents of accidental contamination (Yusho, Times Beach). The symptoms of these high-level exposures include dizziness, headaches, liver damage, leg pains, and reduced sex drive. These symptoms, though serious, have typically subsided with time. An exposure to these amounts can also cause severe acne, called chloracne, which in some cases has persisted for decades. With the exception of chloracne, most

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researchers have found no serious, long-term health problems associated with exposure to dioxins, even at the highest reported levels.

However, some studies have found an association between long-term diseases and exposure to dioxins and furans. Several Swedish studies have shown an association between a person's exposure to 2,3,7,8-TCDD-containing herbicides and the incidence of a rare form of cancer. Other and subsequent studies have not confirmed this association, and the Swedish studies have been criticized on several grounds. Nevertheless, because they are very potent toxins and carcinogens in laboratory animals, the U.S. Environmental Protection Agency, Centers for Disease Control, Food and Drug Administration, and other groups, have issued guidelines for human exposure to dioxins and furans, based on the results of animal studies in the laboratory (see "How do government agencies arrive at acceptable human exposure to dioxins and furans?", page 65.

How do dioxins and furans cause the health effects that they do?

We don't know yet why dioxins and furans cause the health effects that they do. But because these substances affect so many systems of the body, it is expected that the mechanism of toxicity involves metabolism at the cellular level. For example, it has been proposed that many of the toxic effects of dioxins and furans are similar to vitamin A deficiency (Hakansson et al, 1988).

Some of the toxic effects of dioxins and furans (and other halogenated aryl hydrocarbons such as PCBs and PBBs) have been linked with the cellular machinery that makes and releases certain enzymes (Poland et al., 1979). Also, 2,3,7,8-TCDD has been linked with changes in the outer membrane of the cell (reviewed in Matsumura, 1985). It is not likely, however, that these changes in the cell are directly responsible for the toxic effects. Instead, it appears that the toxic effects are somehow mediated by these enzyme systems and the cell membrane.

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Regulating Dioxins and Furans

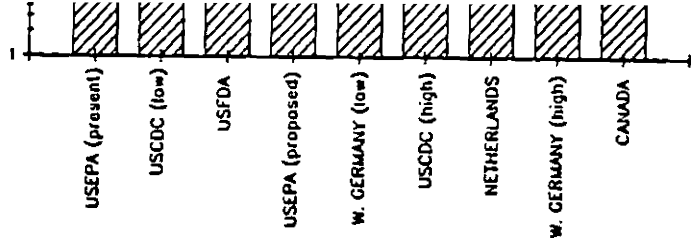
How do government agencies arrive at acceptable human exposure to dioxins and furans?

Government agencies from many different countries have determined maximum acceptable doses of 2,3,7,8-TCDD for humans, which are then used to regulate practices that expose people to dioxins and furans. These acceptable doses (also called tolerable, allowable, and virtually safe) vary from country to country, and can vary from agency to agency within a country (see Figure 4, following page). This variation is primarily the result of differing interpretation of the same experimental animal data, and to a lesser extent the choice of different data. In order to understand how this comes about, a brief summary of how an agency determines an acceptable dose is in order.

First, suitable data must be chosen. The results of animal laboratory studies are relied upon most heavily and are often the only data considered. This is because human studies (epidemiological studies) are after-the-fact investigations of uncontrolled exposure, making it extremely difficult to confidently correlate a given dose of the substance with the observed health effects. Animal laboratory studies allow several controlled doses, including lethal doses, to be used, and allow the researchers to examine the health effects over most or all of the lifetime of the animal.

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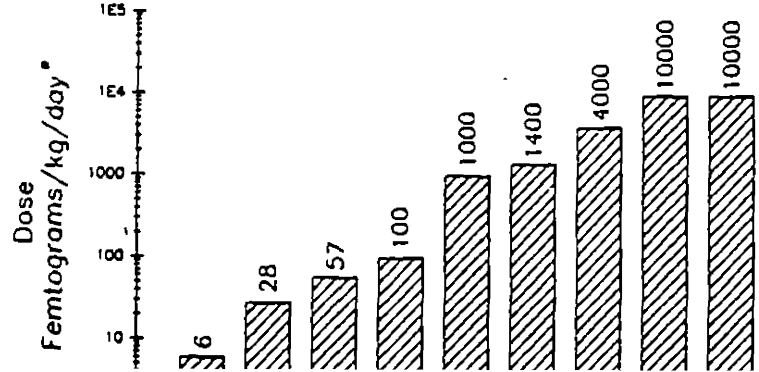
[a] A femtogram is one quadrillionth of a gram (1×10^{-15} grams)

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Figure 4. Virtually safe lifetime daily doses of 2,3,7,8-TCDD from various agencies and countries.

from Figure 1 in USEPA, 1988c: p 4.



The effects of different doses on long-term health aspects such as cancer, birth defects, and changes in the immune system are typically used by regulators, because they have very serious consequences. In the United States and Canada, for example, data from long-term studies of cancer in rats have been used to estimate an acceptable dose of 2,3,7,8-TCDD. Rats were chosen as subjects because they are physiologically similar to humans, reliable strains of rats have been developed for use in such studies, and large numbers of the animals can be used for the experiment.

Regulators must then extrapolate an acceptable dose for humans from the chosen data. It is this extrapolation that accounts for most of the variation in acceptable doses. The extrapolation can be done using two major methods: 1) applying safety factors to the largest dose that could be correlated with no observed adverse effect (the NOAEL); 2) using the various doses and their correlated adverse effects to extrapolate a dose that would be theoretically associated with an acceptably small risk of causing the effect. For example, the USEPA considers a risk of one tumor in one million people during an average lifetime of 70 years to be an acceptably small risk.

A safety factor (method 1) is used when the regulators decide that there is a threshold dose below which there is only a remote risk that the health effect will occur. The safety factor is then applied to allow for individuals that are more sensitive to the toxic effects than the average, and to allow for the possible difference between the test species and humans. Safety factors of from 10 to 1000 have been applied, depending on the confidence the regulators have in the available data.

The dose extrapolation (method 2) is used when the regulators decide that any dose, no matter how small, increases the risk of the health effect. Because the aim of this method is to quantify the risk and then choose an acceptable level of risk, such an approach is called quantitative risk assessment. There are many different mathematical models that can be used to extrapolate quantitative risks from a range of doses, and the extrapolated degrees of risk can vary considerably, depending on the model chosen.

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The choices outlined above account for the range of acceptable daily lifetime doses of 2,3,7,8-TCDD as presented in Figure 4. The lowest (USEPA) and highest (Ontario Ministry of the Environment) doses in that table were derived from the same long-term studies of rats by Kociba et al (1978). The USEPA also used a similar study by the National Cancer Institute (NTP, 1980a); the Ontario Ministry of the Environment also used a similar study by Murray et al., (1979).

Because the USEPA feels that there is no threshold below which 2,3,7,8-TCDD does not cause an increased risk of cancer, they used a dose-extrapolation model to calculate the acceptable daily dose. The model they used to arrive at the present virtually safe dose is the most conservative of the five models they tested; it extrapolates a greater risk than do the other models at low doses.

The Ontario regulators recognize 2,3,7,8-TCDD as a promoting rather than an initiating carcinogen, and therefore feel that there is a threshold dose below which 2,3,7,8-TCDD probably does not increase the risk of cancer. They applied a safety factor of 100 to the dose that they felt represented the NOAEL in these studies, to calculate an acceptable daily dose. Thus, we have two very different interpretations of essentially the same data.

The USEPA's recently proposed virtually safe dose is greater because it reflects the median virtually safe dose from the different models, rather than the most conservative. As has been pointed out by Lave (1983), when the mechanism of action of a toxin is unknown, all applicable models are equally valid and should be used to derive a range of risk estimates. No single model should be chosen, because undue emphasis is then placed on one estimate in what is a large range of equally relevant estimates. At the same time, regulators should make conservative assumptions. Then, as their knowledge about the toxin increases and they replace their conservative assumptions with more accurate ones, the estimated risks should decrease. This provides an incentive for industry to conduct research on toxic substances.

Because of lack of information about dioxins and furans, the USEPA and other regulatory agencies have made very conservative assumptions when estimating the health risks of these compounds. Many researchers have argued that the assumptions are overly

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conservative, and these arguments have grown more persuasive as our knowledge of dioxins and furans has increased. For example, Paustenbach et al (1986) critically examine the assumptions used by the CDC and USEPA to determine the risk posed by 2,3,7,8-TCDD in soil. Their analysis indicates that the CDC and USEPA significantly overestimated the risk of 2,3,7,8-TCDD in soil. Also, although the CDC made it clear that their risk assessment was 1) specific to the residential area in Times Beach, Missouri and 2) not applicable for industrial sites, the USEPA subsequently adopted their guideline of 1 ppb 2,3,7,8-TCDD in soil to determine the need for cleanup at industrial sites, where the level of exposure would be much lower.

What has been done about dioxins and furans?

The manufacture and use of 2,4,5-T and the related herbicide Silvex in the U.S. was restricted in 1970, and prohibited in 1984. The manufacture and use of chlorophenols has been greatly reduced. No chlorophenols with four or fewer chlorines are being produced in the U.S. (pers. comm., John Robinson, Vulcan Chemicals), and hexachlorophene is not being produced because of the lack of 2,4,5-trichlorophenol (USEPA, 1986: p 3-16).

The USEPA requires that pentachlorophenol produced in the U.S. must now average no more than 2 ppm hexa-CDDs, and cannot have more than 4 ppm hexa-CDDs (Vulcan Chemicals, 1988). Approximately half of the hexa-CDDs present are 2,3,7,8-substituted (pers. comm., John Wilkinson, Vulcan Chemicals). The USEPA intends to cancel most non-wood uses of pentachlorophenol (USEPA, 1987b: p. 21413).

Wastes from the manufacture of tri-, tetra-, and pentachlorophenols, and from the manufacture of tetra-, penta-, and hexachlorobenzene under alkaline conditions, were designated as acutely hazardous by the USEPA in 1984. The regulations also apply to discarded, unused chlorophenols or products containing them, to products made with equipment previously used to manufacture the substances above (except pentachlorophenols), and to soil that has been contaminated by these substances. The manufacturers or owners of such wastes are required to notify the USEPA. These wastes cannot be placed in a landfill if they contain more than 1 ppb

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of certain dioxin and furans congeners (USEPA, 1986: p 2.5), and if incinerated, 99.9999% of the contamination must be removed (USEPA, 1986:p 2.3).

The USEPA has proposed that wastewaters and drippings, from wood-preserving facilities and sawmills that use chlorophenolic solutions, or that use equipment formerly used for such solutions, be regulated as hazardous wastes (USEPA, 1988a). Also, 2,3,7,8-TCDD is now listed under the Clean Water Act as a compound which the USEPA must control in industrial effluents (pers. comm., USEPA).

PCBs are no longer produced in the U.S., and any new use of them is strictly controlled and must be extremely well justified. A gradual phase-out of PCB-containing equipment is taking place. Transformers that contain fluid with more than 500 ppm PCBs are now prohibited where they can contaminate food or feed, must be registered and inspected, and leaks must be repaired within 48 hours. Capacitors with more than 500 ppm PCBs are also prohibited from areas where they might contaminate food or feed, and large capacitors had to be placed in enclosures by November 1988.

The disposal of PCBs is also highly restricted. Fluid contaminated with more than 500 ppm PCBs must be incinerated with a removal efficiency of 99.9999%, and fluid contaminated with more than 50 ppm cannot be placed in a landfill. It is a violation to store PCBs for more than one year before disposal (pers. comm., USEPA).

The USEPA has published lists of chemical products that may be contaminated with chlorinated or brominated dioxins and furans (USEPA, 1987b). Manufacturers or importers of the chemical products on these lists must test for and report to the USEPA on the presence of dioxins and furans in these products. The USEPA also published a list of precursor products, which are not contaminated themselves but may produce dioxins or furans if they are used to manufacture other products. Manufacturers or importers of products made with these precursors must notify USEPA of that fact (USEPA, 1987b).

The USEPA conducted a nationwide survey in order to determine the extent of contamination by 2,3,7,8-TCDD. The most severely contaminated sites were assigned to the Superfund List (most were already on the list because of the presence of other toxic substances).

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Where the USEPA found relatively high levels of 2,3,7,8-TCDD in fish, advisories were issued to limit consumption. Also, a program was put in place for cleaning up the many contaminated sites in Missouri, and many of the sites have since been cleaned up.

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Conclusions and Recommendations

We need to know more about the toxicity, distribution, and behavior of chlorinated dioxins and furans other than 2,3,7,8-TCDD, especially those that are found in relatively large amounts in the environment. For instance, current research indicates that the common octa-CDD is biologically active, not non-toxic as has been believed (Couture et al, 1988).

Based on the many studies already published, dioxins and furans do not appear to be a hazard to our health when we are exposed to the low levels typically present in the environment. The epidemiological evidence indicates that dioxins and furans do not cause cancer, immune effects, or other serious, long-term health effects on humans, even at very high doses. Some groups of people, such as workers who are exposed occupationally to greater amounts over a long time, and breast-fed babies, need special attention. We should continue to monitor the health of those persons who have been exposed to large amounts of these compounds, or who are exposed to smaller amounts for a long time. This will enable us to identify any long-term health effects not already recognized. We also need to determine the mechanism of action. Such an understanding would not only have preventive or therapeutic value, but is also critical for accurate assessments of the risks these compounds pose.

Because of their demonstrated toxicity to laboratory animals, plus their distribution and persistence, some dioxins and furans are a potential hazard to organisms in the environment. There is a great need for more study in this area. We need to know what are unacceptably harmful levels for the various organisms in the environment, and the nature and amount of dioxins and furans from the various sources.

If we are to reduce the amounts of toxic dioxins and furans in our environment, we need to deal both with the large amounts already created, and the processes that continue to create them. What appears to be the largest amount of toxic dioxins and furans is buried in landfills or stored at former manufacturing sites. We need to develop safe yet practical ways to destroy this waste or ensure that it is contained. We may also need to reduce the amounts of dioxins and furans occurring as unwanted contaminants of some products. Great reductions

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have been made in certain products in the past, and further reduction may be necessary. The emphasis needs to be on preventing their creation during the manufacturing process. Finally, we need to reduce the unwanted creation of dioxins and furans during combustion. For example, incinerators can be operated under conditions (high temperatures, complete burning) that reduce the creation of dioxins and furans (USEPA, 1988b).

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Appendix

The Virtually Safe Lifetime Daily Dose (VSD) of 2,3,7,8-TCDD is that quantity of 2,3,7,8-TCDD estimated to cause a maximum of one tumor in one million people who are exposed for 70 years. In other words, one person faces a maximum one in one million risk of a tumor due to 2,3,7,8-TCDD during their lifetime, if they are exposed to the VSD of 2,3,7,8-TCDD for their lifetime.

Virtually Safe Lifetime Daily Dose of 2,3,7,8-TCDD, from the U.S. Environmental Protection Agency

6 femtograms/kg/day
A femtogram is 1×10^{-15} grams or 1 quadrillionth of a gram. **Note:** The USEPA has proposed to increase this daily dose to 100 femtograms/kg/day.

Virtually Safe Levels of 2,3,7,8-TCDD in Soil and Water, from the USEPA

Soil: Below 1 ppb requires no immediate action (from the U.S. Center for Disease Control's recommendation for Times Beach, Missouri).

Water: 13 parts per quadrillion (1.3×10^{-14} grams/liter).

The Advisory for Fish Contaminated with 2,3,7,8-TCDD, from the U.S. Food and Drug Administration

Fish with levels of 2,3,7,8-TCDD above 50 ppt should not be eaten.

Fish with levels from 25 to 50 ppt can be eaten twice a month.

Fish with levels below 25 ppt can be eaten with no resuriction.

(These levels can also be used for 2,3,7,8-TCDD in other foods).

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Recommended Reading

For a succinct, informative pamphlet that summarizes the health risks of 2,3,7,8-TCDD in an easy-to-read style:

"Dioxin in the Environment: Its Effect on Human Health" 1986.
Available from the American Council on Science and Health,
47 Maple St., Summit, NJ 07901. Telephone (201) 277-0024.
Price is $2 per copy.

For a well-written synopsis of the issues surrounding 2,3,7,8-TCDD and the relevant scientific information:

"Dioxin" by F. H. Tschirley, in Scientific American,
February, 1986. Volume 254, Number 2, pages 29-35.
Available from Scientific American, 415 Madison Ave.
New York, NY 10017, Attention Rosa Davis. Cost of issue is $4.

For a book that discusses in detail the issues surrounding 2,3,7,8-TCDD, especially the incidents that received the most publicity in the U.S., and includes much of the relevant scientific information on 2,3,7,8-TCDD:

Dioxin, Agent Orange by Michael Gough, 1986.
Plenum Press, New York.

For a summary of the current research findings on dioxins and furans, consult the proceedings of the annual International Symposium on Chlorinated Dioxins and Related Compounds, published annually in the journal Chemosphere (Pergamon Press).

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Glossary and List of Abbreviations

2,3,7,8-TCDD	2,3,7,8-tetrachlorodibenzo-para-dioxin
2,4-D	2,4-dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
CDC	United States Centers for Disease Control
ADI	acceptable daily intake
CDD	chlorinated dibenzo-p-dioxin
CDF	chlorinated dibenzo furan
congener	a single, particular member of a chemical family
DNA	deoxyribonucleic acid
dioxins	chlorinated dibenzo-p-dioxins
furans	chlorinated dibenzo furans
homologue	a group of dioxins or furans with the same number of chlorine atoms in their structure
isomer	a single member of a homologue
kg	kilogram
LC50	lethal concentration for 50% of test organisms
LD50	lethal dose for 50% of test organisms
LOAEL	lowest-observed-adverse-effects level
milligram	1×10^{-3} grams
microgram	1×10^{-6} grams
nanogram	1×10^{-9} grams
picogram	1×10^{-12} grams
femtogram	1×10^{-15} grams
NCASI	National Council of the Paper Industry for Air and Stream Improvement
NOAEL	no-observed-adverse-effects level
NOEL	no-observed-effect level
NTP	National Toxicology Program
PBBs	polybrominated biphenyls
PCBs	polychlorinated biphenyls
ppm	parts per million
ppb	parts per billion
ppt	parts per trillion
ppq	parts per quadrillion
ug	microgram
USAF	United States Air Force
USDA	United States Department of Agriculture
USEPA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration
WHO	World Health Organization

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SENSITIVITY ANALYSIS FOR DIOXIN RISK ASSESSMENT AND IMPLICATIONS FOR DETERMINING ACCEPTABLE LEVELS OF DAILY EXPOSURE

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SENSITIVITY ANALYSIS FOR DIOXIN RISK ASSESSMENT AND IMPLICATIONS FOR DETERMINING ACCEPTABLE LEVELS OF DAILY EXPOSURE

EXECUTIVE SUMMARY

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is one of the most studied environmental chemicals. The great interest in TCDD shown by scientists and laymen alike is due partly to its high toxicity in bioassays involving laboratory animals. The fact that TCDD-contaminated Agent Orange was used in the Vietnam War as a defoliant and herbicide has sparked additional public concern and debate far in excess of the interest surrounding any other environmental chemical.

Considerable research has been carried out to identify the long-term adverse health effects associated with exposure to TCDD. Studies of the most highly exposed human populations have failed to associate increased cancer rates with exposure to dioxin or dioxin-contaminated chemicals (Armstrong, 1983; Bond et al., 1983, 1989; Cook et al., 1985; Eriksson et al., 1984; Filipini et al., 1981; Lathrop et al., 1983, 1984; Lipson, 1983; Mastroiacovo et al., 1988; Minister of Veterans' Affairs, 1983; Moses et al., 1984; Nelson et al., 1979; Pocchiari et al., 1979; Reggiani et al., 1978, 1980; Smith et al., 1982; Suskind and Hertzberg, 1984; Zack and Gaffery, 1983). In addition, the total weight of evidence currently available does not support a conclusion that any of the phenoxy herbicides, presumably contaminated with TCDD, presents a carcinogenic hazard to humans (Bond et al., 1989).

Agencies throughout the world have regulated TCDD because of its presumed human carcinogenic effects. In the absence of adequate dose-response data from epidemiologic studies, extrapolations from animal toxicity bioassays have been made in an attempt to characterize the relationship between TCDD uptake and an anticipated carcinogenic response in humans. Clearly, the assumptions used in extrapolating the dose-response curve generated from these experiments can have a significant impact on estimates of human risk associated with exposure to TCDD (Paustenbach et al., 1986; Sielken, 1987).

A number of regulatory agencies in Western Europe and North America have estimated human exposure limits for TCDD based on the application of classical toxicological safety factors to either the no-observable-adverse-effect level (NOAEL) or to the lowest-observable-adverse-effect level (LOAEL) of exposure in rodents. The use of the classical safety factor approach is based on the weight of scientific evidence that an exposure threshold exists below which adverse effects will not occur (Shu et al., 1987; CanTox, 1989). Using this methodology, a number of countries have developed lifetime allowable daily intakes (ADIs) ranging between 1 and 10 picograms per kilogram of body weight per day (pg/kg-day).

The U.S. Environmental Protection Agency (EPA) and certain other federal policy and regulatory groups, however, employ a very different extrapolation procedure to the same animal bioassay data (EPA, 1985). Through the use of the linearized multistage (LMS) model, human exposure levels at a selected

probability of increased cancer risk are extrapolated from rodent bioassay data. The use of this model is based on an assumption that there is no threshold for carcinogenesis; i.e., any dose, regardless of the quantity, poses some level of risk. At a risk level of 1 in 100,000 (1×10^{-5}), the use of the multistage model and the EPA assumptions results in a risk-specific dose (RsD) of 0.064 pg/kg-day[1].

Acceptable daily intakes and risk-specific doses developed for TCDD by various U.S. agencies and other countries are shown in Figure 1 for comparative purposes. It is clear that the application of the two different extrapolation procedures to the same animal cancer bioassay data results in markedly different exposure limits for TCDD.

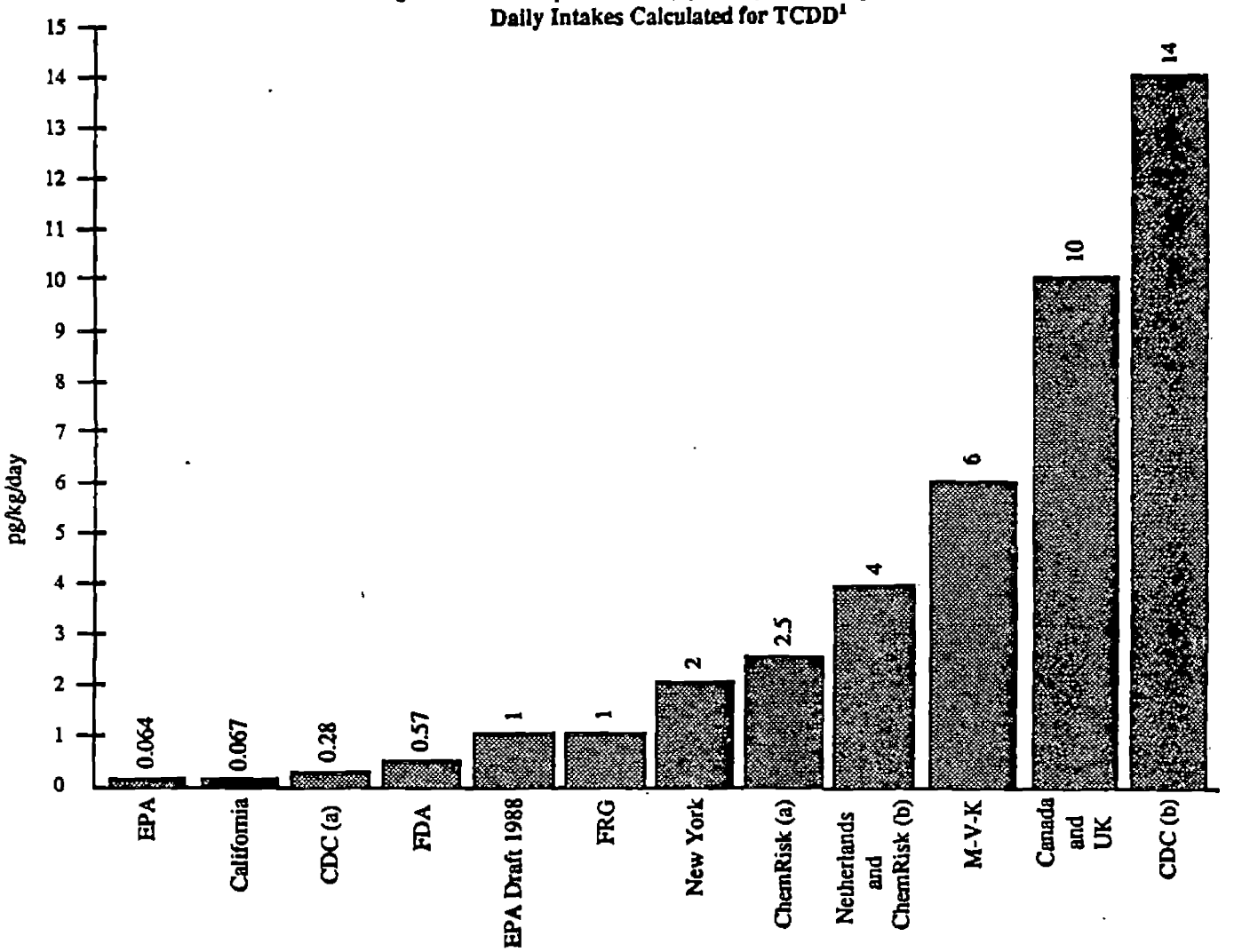
Shu et al. (1987) have critically reviewed the TCDD bacterial, animal, and human data on mutagenesis, carcinogenesis, and tumor promotion and have concluded that the scientific evidence does not support risk estimates for TCDD which are based on linear low-dose extrapolation models and that alternative means for evaluating risk should be investigated. CanTox (1989), in their critical review of the biological data, concluded that the most appropriate method for the estimation of permissible limits for human exposure would be the application of a safety factor to the NOAEL observed in the two-year chronic toxicity and oncogenicity study of TCDD in Sprague-Dawley rats conducted by Kociba et al. (1978). This resulted in a recommended ADI for carcinogenic effects of 10 pg/kg-day, in agreement with that of certain Western, industrialized nations.

This report by ChemRisk™ presents a sensitivity analysis of the EPA (1985) dose-response assessment for TCDD and a reinterpretation of the biological data from the Kociba et al. (1978) bioassay prior to using the LMS model for estimating scientifically defensible RsDs for TCDD. The quantitative impacts of several choices involved in the quantitative risk assessment of TCDD were reviewed, including the definition of the carcinogenic response of concern within the experimental dataset, the pathological evaluations of the original researchers and the EPA, methods of making the fitted multistage model responsive to the data at the lower experimental doses, the choice of scaling factor between rats and humans, and the method of estimating the maximum acceptable dose. Each of these choices affects the range of plausible risk estimates predicted through the use of the LMS model.

In this analysis, RsDs were determined by ChemRisk at a lifetime incremental risk level of 1 in 100,000 (1×10^{-5}), since this level of risk is generally considered to be *de minimis* by regulatory agencies. In fact, examination of numerous regulatory decisions has shown that for effects on small populations, regulatory action was never taken for individual risk levels below one in ten

[1] U.S. agencies, including the EPA, have calculated and presented RsDs at various levels of risk. For the sake of consistency and to enhance the clarity of this discussion, the risk level of 1 in 100,000 has been used in this document when presenting RsD estimates.

Figure 1. Risk-specific Doses (at 10^{-5}) and Acceptable Daily Intakes Calculated for TCDD[1]



EPA	U.S. Environmental Protection Agency - RsD
California	State of California - RsD
CDC	Centers for Disease Control, U.S. Public Health Service - RsD (a) Most conservative figure of range (b) Least conservative figure of range
FDA	U.S. Food and Drug Administration - RsD
M-V-K	Mangarian, Vouzon, Knudsen - RsD
FRG	Federal Republic of Germany - ADI (ADI shown is most conservative figure of a range calculated by FRG)
New York	State of New York - ADI
Netherlands	- ADI
Canada	Ministry of Ontario Health and Welfare Canada - ADI
UK	United Kingdom - guideline level
ChemRisk	- RsD (a) Conservative plausible alternative using 95% LCL (b) conservative plausible alternative using MLE

1. U.S. agencies, including the EPA, have calculated and presented RsDs at various levels of risk. For the sake of consistency and to enhance the clarity of this document, the risk level of 1 in 100,000 has been used in the presentation of RsD estimates.

thousand (1×10^{-4}) (Travis et al., 1987). Furthermore, it is appropriate to consider the variance associated with background risk in the human population when determining an appropriate level of *de minimis* incremental risk (Farber, 1989) His analysis has illustrated that incremental risks associated with chemical exposures do not alter the probability of an individual contracting a disease if the incremental risk estimate is smaller than the variance estimate for the background rate of disease incidence. In the case of exposure to TCDD, Farber (1989) has concluded that an incremental risk level of 1 in 100,000 is indeed insignificant and *de minimis* on both mathematical and biological grounds.

The low-dose risk estimate derived by the EPA through the application of the LMS model to the Kociba et al. (1978) and the Squire (EPA, 1985) datasets is very sensitive to the choice of modeling assumptions. The principal conclusions of the ChemRisk sensitivity analysis are summarized below.

(1) Hepatocellular carcinomas are the only indicators of a carcinogenic response in the Kociba et al. (1978) bioassay having any conceivable use in a dose-response extrapolation via the LMS model. Considering only hepatocellular carcinomas, no statistically significant differences between controls and the lowest (1,000 pg/kg-day) or intermediate (10,000 pg/kg-day) dose groups were observed. Therefore, the dose-response curve is determined by only a single data point and the underlying assumptions of the model which force a fit to the origin in order to preserve the notion of "linearity". Furthermore, the high dose level administered to the rats (100,000 pg/kg-day) clearly exceeded the Maximum Tolerated Dose (MTD), evident from Kociba's observations of severe liver toxicity, diminished weight gain, and increased mortality.

(2) The statistically significant (95% confidence limit) increased incidence of hyperplastic nodules observed by Kociba et al. (1978) in the intermediate and highest (100,000 pg/kg-day) TCDD treatment groups should not be modeled via a nonthreshold approach because: (a) There is no evidence in this study to indicate that a hyperplastic nodule is likely to progress to a hepatocellular adenoma and then to a hepatocellular carcinoma. (b) Kociba et al. (1978) identified and reported the occurrence of adenomas in other organs and tissues, while no adenomas were reported in the liver. This clearly indicates that the observed lesions were considered to be a less serious form of hepatocellular proliferative lesion, i.e. hyperplasia. Furthermore, Kociba et al. (1978) reported that there was no evidence of metastasis of any of these hyperplastic nodules. (c) "Neoplastic nodules", a term used by Squire (EPA, 1985) in his re-evaluation of the Kociba analysis, represents terminology from a different morphological classification system developed by Squire and another researcher. Its use by Squire (EPA, 1985) does not provide convincing evidence that the hyperplasia was actually a more serious condition. The

classification system of Squire and Levitt (1975) failed to define the exact position and significance of the "neoplastic nodule", which included both hyperplasia and more serious lesions (EPA, 1986b). Today, the National Toxicology Program (NTP) no longer uses the classification scheme of Squire and Levitt and neoplastic nodules are no longer identified during histopathological examinations by most toxicological laboratories, including the NTP. "Neoplastic nodule" has been replaced with two terms: hyperplasia and hepatocellular adenoma (EPA, 1986b; Maronpot et al., 1986; McConnell et al., 1988). (d) Schulte-Hermann et al. (1983) demonstrated that a significant incidence of hyperplastic liver nodules are found in the livers of untreated rats. The incidence increased to 100% over the course of the animals' two-year average lifetime and lesion size increased with age. In addition, these hyperplastic nodules showed similar characteristics to those observed in rats treated with known liver carcinogens. Schulte-Hermann et al. (1983) have concluded that the high incidence of hyperplastic liver nodules in untreated aged rats precludes the distinction between chemical promoters and initiating agents during long-term carcinogenicity bioassays in laboratory rodents, particularly when liver tumor incidence data are the primary criteria.

(3) The statistically significant increased incidence of hyperplastic nodules observed by Kociba et al. (1978) in the intermediate (10,000 pg/kg-day) and highest (100,000 pg/kg-day) TCDD treatment groups should be appropriately extrapolated to human exposure only through the use of a classical safety factor approach: the LOAEL divided by a safety factor of 1,000. In this case, the LOAEL for hyperplastic nodules in the rat liver (10,000 pg/kg-day) divided by 1,000 would yield an ADI of 10 pg/kg-day. This approach is identical to that taken by the United Kingdom (1989) when they derived a guideline level of 10 pg/kg-day, "which, when exceeded, should trigger investigation and appropriate measures to reduce environmental levels."

(4) The frequencies of tumors in other organ systems, specifically in the lung, tongue and palate, should not be considered in the dose-response assessment of TCDD. The Office of Science and Technology Policy (1985) does not recommend combining tumor incidences in different organs for the purpose of estimating a cancer potency. Furthermore, Kociba (1984) has suggested that the tumor incidence observed in the lung and oropharynx is likely the result of periodically high localized concentrations of TCDD associated with surface contact of TCDD from the animal's diet, rather than an effect of systemic uptake.

(5) For TCDD, the use of a body weight correction factor in the rat-to-human extrapolation procedure is biologically more relevant than the use of a surface area correction factor. The use of a surface

area scaling factor, based on the premise that the magnitude of the adverse effect of the chemical is dependent on the basal metabolic rate of the species of concern (O'Flaherty, 1988; Clayson, 1988), is inappropriate for poorly metabolized chemicals such as TCDD. In the case of TCDD, basic pharmacokinetics suggest that the parent compound is the biologically active moiety (Shu et al., 1987; Leung et al., 1988, 1989). Despite this evidence, the EPA used a surface area correction factor of 5.4 when extrapolating doses between Sprague-Dawley rats in the Kociba et al. (1978) study and humans.

(6) Based on this analysis and on the other scientific evidence summarized in this report, it is clear that the use of the linearized multistage model in conjunction with the Kociba et al. (1978) bioassay to describe the carcinogenic dose-response of TCDD can result in a wide range of plausible risk estimates (Table 1). The choice of maximum likelihood estimate (MLE) for the RsD in preference to the 95% lower confidence limit (LCL)[2] estimate, body weight instead of surface area scaling factor, and a better definition or biological interpretation of the reported liver lesions will result in RsDs much different than the estimates developed by the EPA (1985, 1988) and the CDC (Kimbrough, 1984).

(7) ChemRisk further concludes that the weight of scientific evidence indicates that TCDD should be regulated as if it exhibited a threshold of carcinogenic action. The scientific evidence does not support risk estimates for TCDD which are based on linear low-dose extrapolation models. The most appropriate method for estimating permissable limits for human exposure, given the present state of knowledge in this interim period before a biologically-based pharmacokinetic model is validated experimentally, is the application of a safety factor to the NOAEL for carcinogenicity observed in the Kociba et al. (1978) study.

(8) Although the use of the safety factor approach may be most appropriate for the dose-response assessment of materials such as TCDD that lack genotoxic potential, it is recognized that others may wish to use the linearized multistage model or some other nonthreshold model of low-dose extrapolation in conjunction with the Kociba et al. (1978) dataset. Since this is currently the case with U.S. regulatory agencies, then the carcinogenic behavior of TCDD in female Sprague-Dawley rats should be extrapolated to humans only on the basis of the incidence of hepatocellular carcinomas. A body weight scaling factor should be used in preference to the surface area assumptions. If the 95% LCL of the human RsD is adopted, then an RsD at a 1 x 10^{-5} risk level of 2.5

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[2]The 95% LCL is that dose for which there is a 95% statistical confidence that the true RsD is no lower than this value.

pg/kg-day of TCDD is estimated. If the MLE is adopted, then the appropriate RsD at a 1 x 10^{-5} risk level is 4.0 pg/kg-day of TCDD (Table 1). Both of these RsDs are more conservative than the ADI of 10 pg/kg-day, estimated to be protective of hepatic hyperplasia, derived via a classical safety factor approach.

Table 1. Plausible Alternative Risk-specific Doses (RsDs) for 2,3,7,8-TCDD

Plausible Choices[a]	Risk-specific Dose at 1 x 10^{-5} Incremental Carcinogenic Risk (pg/kg-day)
1. Body Weight + 95% LCL[b] + Hepato. carcinomas	2.5
2. Body Weight + MLE[c] + Hepato. carcinomas	4.0

a. These assumptions are considered by ChemRisk™ (1989) to represent the most plausible basis for an alternative human RsD for 2,3,7,8-TCDD.
b. The 95% LCL is that dose for which there is a 95% statistical confidence that the true RsD is no lower than this value.
c. Maximum likelihood estimate.

1.0 INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is one of the most studied environmental chemicals. The great interest in TCDD shown by scientists and laymen alike is due partly to its high toxicity in bioassays involving laboratory animals. The fact that TCDD-contaminated Agent Orange was used in the Vietnam War as a defoliant and herbicide has sparked additional public concern and debate far in excess of the interest surrounding any other environmental chemical.

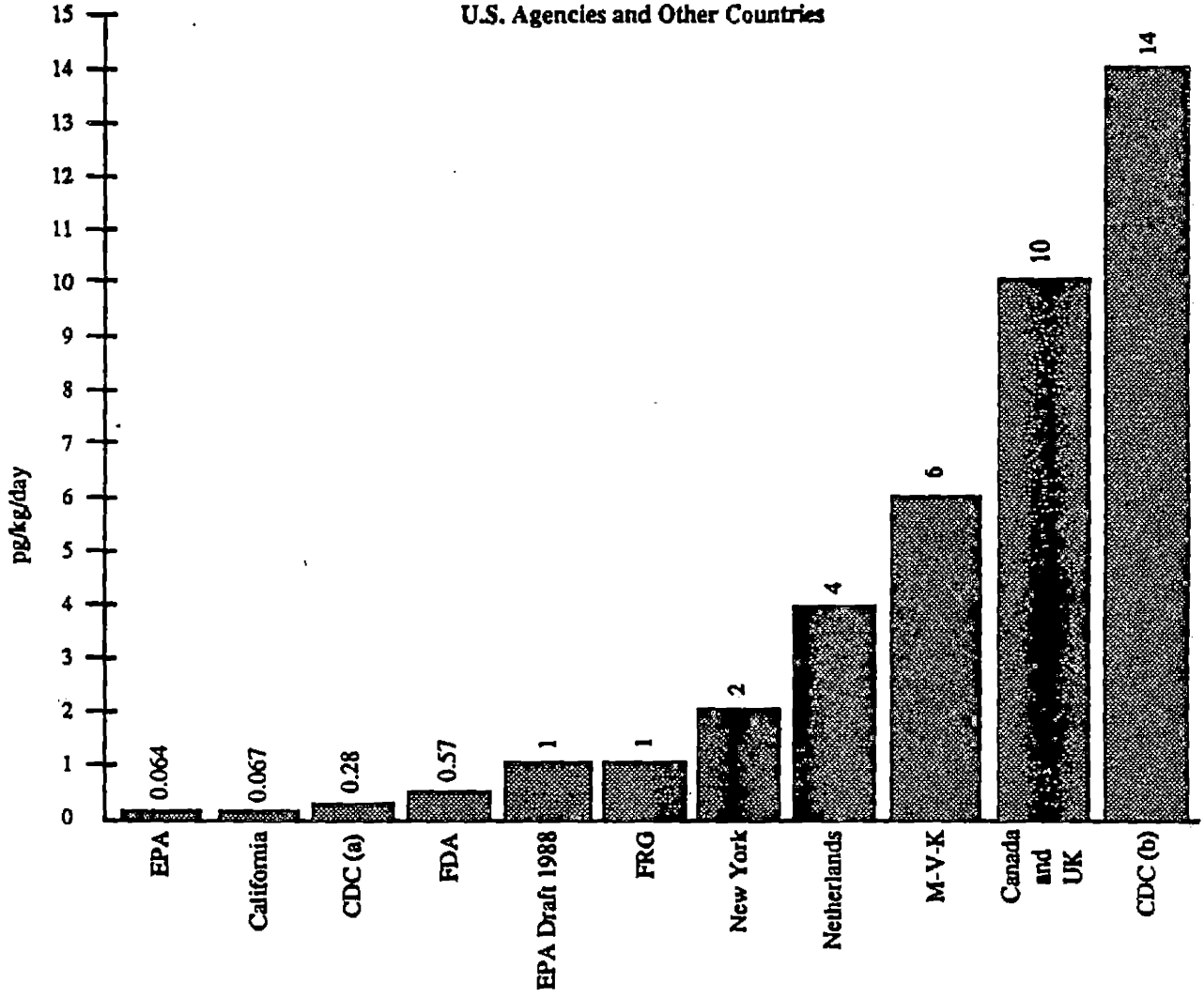
Considerable research has been carried out to identify the long-term adverse health effects associated with exposure to TCDD. Studies of the most highly exposed human populations have failed to associate increased cancer rates with exposure to dioxin or dioxin-contaminated chemicals (Armstrong, 1983; Bond et al., 1983, 1989; Cook et al., 1985; Eriksson et al., 1984; Filipini et al., 1981; Lathrop et al., 1983, 1984; Lipson, 1983; Mastroiacovo et al., 1988; Minister of Veterans' Affairs, 1983; Moses et al., 1984; Nelson et al., 1979; Pocchiari et al., 1979; Reggiani et al., 1978, 1980; Smith et al., 1982; Suskind and Hertzberg, 1984; Zack and Gaffery, 1983). In addition, the total weight of evidence currently available does not support a conclusion that any of the phenoxy herbicides, presumably contaminated with TCDD, presents a carcinogenic hazard to humans (Bond et al., 1989).

Agencies throughout the world have regulated TCDD because of its presumed human carcinogenic effects. In the absence of adequate dose-response data from epidemiologic studies, extrapolations from animal toxicity bioassays have been made in an attempt to characterize the relationship between TCDD uptake and an anticipated carcinogenic response in humans. Clearly, the assumptions used in extrapolating the dose-response curve generated from these experiments can have a significant impact on estimates of human risk associated with exposure to TCDD (Paustenbach et al., 1986; Sielken, 1987).

A number of regulatory agencies in Western Europe and North America have estimated human exposure limits for TCDD based on the application of classical toxicological safety factors to either the no-observable-adverse-effect level (NOAEL) or to the lowest-observable-adverse-effect level (LOAEL) of exposure in rodents (Table 2). The use of the classical safety factor approach is based on the weight of scientific evidence that an exposure threshold exists below which adverse effects will not occur (Shu et al., 1987; CanTox, 1989). Using this methodology, a number of countries have developed lifetime allowable daily intakes (ADIs) ranging between 1 and 10 picograms per kilogram of body weight per day (pg/kg-day) (Figure 2).

The U.S. Environmental Protection Agency (EPA) and certain other federal policy and regulatory groups, however, employ a very different extrapolation procedure to the same animal bioassay data (EPA, 1985). Through the use of the linearized multistage (LMS) model, human exposure levels at a selected probability of increased cancer risk are extrapolated from rodent bioassay data. The use of this model is based on an assumption that there is no threshold for carcinogenesis; i.e., any dose, regardless of the quantity,

Figure 2. Risk-specific Doses (at 10^{-5}) and Acceptable Daily Intakes Calculated for TCDD by Various U.S. Agencies and Other Countries



EPA	U.S. Environmental Protection Agency - RsD
California	State of California - RsD
CDC	Centers for Disease Control, U.S. Public Health Service - RsD (a) Most conservative figure of range (b) Least conservative figure of range
FDA	U.S. Food and Drug Administration - RsD
M-V-K	Mrugovitar, Vesnina, Kmoleun - RsD
FRG	Federal Republic of Germany - ADI (ADI shown is most conservative figure of a range calculated by FRG)
New York	State of New York - ADI
Netherlands	- ADI
Canada	Ministry of Ontario Health and Welfare Canada - ADI
UK	United Kingdom - guideline level

1. U.S. agencies, including the EPA, have calculated and presented RsDs at various levels of risk. For the sake of consistency and to enhance the clarity of this document, the risk level of 1 in 100,000 has been used in the presentation of RsD estimates.

Table 2. Toxicological Criteria (pg/kg-day) for TCDD

Health Effect	Toxicological Criteria	Extrapolation Factor	ADI
Carcinogenicity (Kociba et al., 1978)	10,000 (NOAEL)	+ 1000	10
Hyperplastic nodules (Kociba et al., 1978)	10,000 (LOAEL)	+ 1000	10
	1,000 (NOAEL)	+ 100	10
Reproductive effects (Murray et al., 1979)	1,000 (NOAEL)	+ 100	10

poses some level of risk. At a risk level of 1 in 100,000 (1×10^{-5}), the use of the multistage model and the EPA assumptions results in a risk-specific dose (RsD) of 0.064 pg/kg-day[1]. Acceptable daily intakes and risk-specific doses developed for TCDD by various U.S. agencies and other countries are shown in Figure 2 for comparative purposes.

Shu et al. (1987) have critically reviewed the TCDD bacterial, animal, and human data on mutagenesis, carcinogenesis, and tumor promotion and have concluded that the scientific evidence does not support risk estimates for TCDD which are based on linear low-dose extrapolation models and that alternative means for evaluating risk should be investigated. CanTox (1989), in their critical review of the biological data, concluded that the most appropriate method for the estimation of permissible limits for human exposure would be the application of a safety factor to the NOAEL observed in the two-year chronic toxicity and oncogenicity study of TCDD in Sprague-Dawley rats conducted by Kociba et al. (1978). This resulted in a recommended ADI of 10 pg/kg-day, in agreement with that of certain Western, industrialized nations.

It is clear that the application of the two different extrapolation procedures to the same animal cancer bioassay data results in markedly different exposure limits for TCDD. It is certainly true that a large component of this difference between the two approaches is based upon whether one believes that the dose-response curve for TCDD is best described by either a threshold or by a nonthreshold model. However, there is also reason to suspect that other, secondary assumptions made in the dose-response assessment may be contributing to the widely varying estimates of acceptable levels of daily exposure obtained through the use of the two procedures. We hypothesized that each of these secondary choices might possibly have a substantial impact upon the range of plausible risk estimates predicted through the use of the LMS model.

In this report, ChemRisk™ presents a sensitivity analysis of the EPA (1985) dose-response assessment for TCDD and a reinterpretation of the biological data from the Kociba et al. (1978) bioassay prior to using the LMS model for estimating scientifically defensible RsDs for TCDD. These alternative RsDs were then compared to the estimates of acceptable daily intake, obtained via a classical safety factor approach to human extrapolation of rat dose-response data. The quantitative impacts of several choices involved in the quantitative risk assessment of TCDD were reviewed, including the definition of the carcinogenic response of concern within the experimental dataset, the pathological evaluations of the original researchers and the EPA, methods of making the fitted multistage model responsive to the data at the lower experimental doses, the choice of scaling factor between rats and humans, and the method of estimating the maximum acceptable dose.

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[1] U.S. agencies, including the EPA, have calculated and presented RSDs at various levels of risk. For the sake of consistency, the risk level of 1 in 100,000 has been used in this document when presenting RsD estimates.

2.0 CURRENT UNDERSTANDINGS OF THE TOXICOLOGICAL BEHAVIOR OF TCDD

2.1 Acute and Chronic Human Toxicity

Numerous epidemiologic studies have examined the potential association between TCDD exposure and a number of disease or mortality endpoints in humans (NCASI, 1987). The epidemiologic database for TCDD has been reviewed by AMA (1984), EPA (1985), Fishbein (1987), NCASI (1987), UAREP (1988), and Bond et al. (1989). Available data on human exposure is found primarily in studies involving industrial workers, herbicide sprayers, the exposed population in the Seveso, Italy accident, residents of Times Beach, Missouri and U.S. Air Force personnel involved in the use of Agent Orange in Operation Ranch Hand. In these studies, the concentrations of TCDD to which people were exposed was much greater than would typically be encountered in the environment. The applicability of these studies to environmental exposures, therefore, is unclear. The populations evaluated in many of these studies were exposed to multiple chemicals, thereby complicating the assessment of disease or mortality endpoints potentially attributable to TCDD exposure.

Chloracne is the only consistently demonstrated long-term adverse health effect associated with human exposure to TCDD (Suskind, 1985). This characteristic persistent dermatosis has been observed in cases of both acute and chronic exposure to significant concentrations of TCDD and can be induced following systemic uptake or dermal exposure (Kociba and Schwetz, 1982; Suskind, 1985; Kimbrough and Houk, 1987). Due to the seven year half-life of TCDD in humans, chloracne can persist for several years following high-level occupational exposures. While chloracne is associated with exposure to a number of other chlorinated aromatic hydrocarbons (Kimbrough et al., 1984), Suskind (1985) identifies TCDD as the most potent chloracnegen.

Other health effects that have been reported in individuals exposed to substances contaminated with TCDD include porphyria cutanea tarda, hyperpigmentation, hirsutism, and altered liver function, (Bleiberg et al., 1964; Pazderova-Vejlupkova et al., 1981; Singer et al., 1982; Moses et al., 1984). Most of these effects are derived from case histories or clinical surveys that lacked an assessment of these effects in control groups and also failed to assess concomitant exposure to other chemicals. Certain Swedish studies have suggested a positive association between phenoxy herbicide or chlorophenol (presumably contaminated with TCDD) exposure and soft tissue sarcoma and malignant lymphoma (Hardell and Sandstrom, 1979; Eriksson et al., 1981; Hardell et al., 1981). However, other epidemiologic studies involving herbicide exposure have not confirmed the positive associations in these studies (Smith et al., 1982, 1983; Wiklund and Holm, 1986; Smith and Pearce, 1986; Pearce et al., 1986).

The epidemiologic database on TCDD supports the conclusion that chloracne is the only consistently demonstrated long-term health effect associated with exposure to TCDD. Studies of the most highly exposed human populations have failed to associate increased cancer rates with exposure to dioxins or dioxin-

contaminated chemicals (Armstrong, 1983; Bond et al., 1983, 1989; Cook et al., 1985; Eriksson et al., 1984; Filipini et al., 1981; Lathrop et al., 1983, 1984; Lipson, 1983; Mastroiacovo et al., 1988; Minister of Veteran's Affairs, 1983; Moses et al., 1984; Nelson et al., 1979; Pocchiari et al., 1979; Reggiani, 1978, 1980; Smith et al., 1982; Suskind and Hertzberg, 1984; Zack and Gaffery, 1983). The total weight of evidence, reviewed in the recent paper by Bond et al. (1989), shows that TCDD, as a contaminant of phenoxy herbicides, is unlikely to be a human carcinogen at low doses. Therefore, based on the available epidemiologic data, it is not appropriate to conclude that environmental exposures to TCDD are likely to produce cancer in humans.

2.2 Average Daily Intake of PCDDs and PCDFs

Human exposure to TCDD is widespread. A number of studies have reported uptake of TCDD by persons with no known exposure to dioxins. In these individuals, adipose tissue levels of TCDD ranged from 3 to 10 ppt (Ryan et al., 1985; Graham et al., 1985; Patterson et al., 1986). Several studies have been conducted to estimate average daily intakes of PCDDs and PCDFs (Table 3). These studies propose that food intake is the primary source of PCDD and PCDF exposure for humans (Travis and Hattemer-Frey, 1987; Ontario, 1988; Beck et al., 1988). It has been estimated that approximately 98% of the daily TCDD intake is from food (Travis and Hattemer-Frey, 1987). Estimates of average adult daily intakes of TCDD equivalents range from 0.54 to 2.33 pg/kg-body weight (Table 3). Potential routes of dioxin exposure through the food chain include fish, beef, and dairy products (Fishbein, 1987).

2.3 Acute and Chronic Animal Toxicity

The noncarcinogenic toxicity of TCDD in animals has been studied in a number of acute, subchronic and chronic studies. This compound has been shown to be extremely toxic to certain rodent species that have been tested. For example, the acute LD_{50} (the dose which is lethal to 50% of the animals tested) for guinea pigs is reported to be 0.6 µg/kg body weight. The sensitivity to TCDD toxicity is extremely variable among laboratory animal species. Kociba and Cabey (1985) have shown the LD_{50} of hamsters to be as high as 5,051 µg/kg; i.e., TCDD is over 8,400 times less toxic to the hamster. Clinical signs of acute toxicity in laboratory animals are severe weight loss, hepatotoxicity, chloracne, thymic atrophy, and death.

Chronic exposure to TCDD has been shown to induce reproductive effects in laboratory animals, including several strains of mice (Smith et al., 1976) and rats (EPA, 1985). The no-observed-adverse-effect-level (NOAEL) for rats was found to be 1,000 pg/kg-day (Murray et al., 1979), while in the mouse, the NOAEL was 100,000 pg/kg-day (Smith et al., 1976). In general, 1,000 pg/kg-day is considered the NOAEL for noncarcinogenic effects. Several agencies and authorities have developed acceptable daily intakes (ADIs, also known as reference doses) to protect against noncarcinogenic effects in humans using

Table 3. Average Daily Intake of TCDD (pg/kg Body Weight)

	Gilman, personal communication	Birmingham, et al. 1989	Travis and Hattemer-Frey, 1987
•Air	0.04	0.07	0.014
•Soil	0.01	0.02	
•Water	<0.01 - 0.05	0.002	.0065
•Consumer Products	<0.01	0.005	
•Food	0.49-2.0	2.32	0.657
TOTAL	0.54-2.1	2.33	0.678

reproductive effects in animals as the critical effect (EPA, 1984; NCASI, 1987; UK, 1989). These ADIs have ranged from 1 to 10 pg/kg-day.

A number of regulatory agencies throughout the world have regulated TCDD because of its presumed human carcinogenic effects. Although the epidemiologic database does not support this presumption, extrapolations from animal toxicity bioassays have been made in an attempt to characterize the relationship between TCDD uptake and an anticipated carcinogenic response in humans.

Chronic high-level TCDD exposure has been shown to be carcinogenic in rats and mice (Kociba, 1984). A number of different tumor types are elevated, but the liver is the primary and most important target tissue (Kociba et al., 1978). However, carcinogenicity was evident only at doses that elicited a severe non-carcinogenic toxic response in the study animals. This was evidenced by the Kociba et al. (1978) observations of severe liver toxicity, diminished weight gain, and increased mortality.

Carcinogens may be generally classified as initiators or promoters. An initiator compound must bind to cellular DNA (Williams and Weisburger, 1986) and alter it in order to initiate carcinogenesis. The initiated cell incorporates the mutated (damaged) DNA into its replicating genome, which may be locked in the cell for as long as the cell line continues to reproduce. A promoter acts by increasing the tumorigenic response of a cell to an initiator when applied after the initiator (Williams and Weisburger, 1986). Promoters require prolonged and repeated exposure or persistence in the body before tumor formation occurs in animals, whereas for tumor initiators, short-term exposure may cause tumors (Shu et al., 1987). Tumor initiation is considered an irreversible event, while tumor promotion may be reversible upon removal of the promoter (Shu et al., 1987).

TCDD binding to DNA, a necessary event in mutagenesis, does not occur to any significant extent (Poland and Glover, 1979) and mutagenicity tests generally indicate that TCDD is nonmutagenic (Shu et al., 1987). These data support the conclusion that TCDD is not genotoxic. Studies by Pitot et al. (1980) and Poland and Knutson (1982) have shown that TCDD is a potent promoter. Many promoters, including TCDD, affect cellular growth and differentiation and alter a number of cell membrane properties (Weinstein, 1984). Unlike genotoxic (initiator) carcinogens, tumor promoters may exhibit a threshold in their dose response (Williams and Weisburger, 1986).

Shu et al. (1987) have critically reviewed the TCDD bacterial, animal, and human data on mutagenesis, carcinogenesis, and tumor promotion and have concluded that the scientific evidence does not support risk estimates which are based on TCDD as a tumor initiator. The authors noted that risk assessments which incorporate tumor promotion activity, more accurately reflect the scientific understanding of the mechanism of action of TCDD than do those assuming a nonthreshold (initiation) mechanism.

A EPA special advisory committee (Dioxin Update Committee, 1986) noted that TCDD acts as a potent promoting agent in at least two different tissues in two species, but there is no evidence for initiation activity in any species. Regarding risk assessment, the EPA Committee concluded that mechanistic models should be used for quantitative risk estimation for TCDD and related compounds. Models should consider epidemiological data, sex-species susceptibility, the promoting action of TCDD, and its pharmacokinetic properties in predicting risks for exposed populations. Current models used by U.S. agencies do not, however, consider these important factors in deriving risk estimates.

The wide range in the sensitivity of different laboratory animals to TCDD exemplifies the problem inherent in the use of laboratory animal models to develop dose-response estimates for humans. Substantial bias can be introduced easily into potency estimates by the improper selection of an animal model. The example of the guinea pig and hamster demonstrates this point clearly. There is in excess of 3 orders of magnitude difference in sensitivity of these species to the acute toxicity of TCDD. Such disparity will have obvious implications in the regulatory process. Issues such as similarities or differences between laboratory animals and humans in such critical aspects of metabolism as biological half-life of TCDD, systems biochemistry, and physiology should drive the process of selecting study animals. All too frequently, the rat or mouse is selected as the animal of choice for no more scientifically-based a reason than convenience.

3.0 DETERMINING ACCEPTABLE LEVELS OF EXPOSURE TO TCDD

Critical to the development of meaningful acceptable levels of exposure to TCDD is the determination of appropriate carcinogenic risk estimates for this compound. As discussed in Section 2.0 of this report, studies of the most highly exposed human populations have failed to associate increased cancer rates with exposure to dioxin or dioxin-containing chemicals. In the absence of adequate dose-response data from epidemiologic studies, extrapolations from animal toxicity bioassays have been made in an attempt to characterize the relationship between TCDD uptake and an anticipated carcinogenic response in humans. Clearly, the assumptions used in extrapolating the dose-response curve generated from these experiments can have a significant impact on estimates of human risk associated with exposure to TCDD (Paustenbach et al., 1986; Sielken, 1987).

3.1 Various Approaches to the Carcinogenic Dose-Response Assessment of TCDD

A number of regulatory agencies in Western Europe and North America have estimated human exposure limits for TCDD based on the application of classical toxicological safety factors to either the no-observable-adverse-effect level (NOAEL) or to the lowest-observable-adverse-effect level (LOAEL) of exposure in rodents. The use of the classical safety factor approach is based on the weight of scientific evidence that an exposure threshold exists below which adverse effects will not occur (Shu et al., 1987; CanTox, 1989). Using this methodology, a number of countries have developed lifetime ADIs ranging between 1 and 10 picograms per kilogram of body weight per day (pg/kg-day) (Table 4).

The Ontario Ministry of the Environment (Ontario, 1985) calculated a maximum acceptable daily intake for humans of 10 pg/kg-day based on a NOEL of 1,000 pg/kg-day and a safety factor of 100. The State Institute of National Health (SINH) in the Netherlands derived a maximum ADI of 4 pg/kg-day based on a NOEL of 1,000 pg/kg-day and a safety factor of 250 (van der Heijden et al., 1982). A range of ADIs of 1 to 10 pg/kg-day was developed by the Federal Republic of Germany based on a NOEL of 1,000 pg/kg-day and safety factors of 1,000 or 100 (NCASI, 1987). Recently, the United Kingdom derived a guideline value of 10 pg/kg-day for TCDD and its toxic equivalents as "a level which, when exceeded, should trigger investigation and appropriate measures to reduce environmental levels" (UK, 1989).

The FDA, prior to their adoption of a linear interpolation model (FDA, 1983), had originally used a safety factor approach to support advisory levels for TCDD in fish. The agency (Cordle, 1983) calculated a TCDD exposure level of 13 pg/kg-day from consuming fish containing 25 ppt TCDD at the 99th percentile of U.S. fish consumption. The FDA noted that this exposure level was less than 1/70th of the animal NOEL of 1 ng/kg-day. This approach was used to support 25 ppt TCDD as a "safe" level in Great Lakes' fish.

Table 4. Maximum Acceptable Daily Intake (ADI) of TCDD

Jurisdiction	Safety Factor	Max ADI (pg/kg-day)	Reference
Canada	100	10	Ontario, 1988
Denmark	200	5	NCASI, 1987
Federal Republic of Germany	100-1000	1-10	NCASI, 1987
Netherlands	250	4	van der Heijden et al., 1982
United Kingdom			UK, 1989
Reproductive toxicity	100	1	
Carcinogenicity	1000	10	
Immunotoxicity	100	60	

A model developed by Moolgavkar, Venzon, and Knudson (M-V-K) (Moolgavkar and Venzon, 1979; Moolgavkar and Knudson, 1981) has recently been applied to TCDD to estimate human cancer risk based on a tumor promotion mechanism (Thorslund, 1987). The M-V-K model is a two-stage biological model for carcinogenesis. Thorslund assumed that TCDD exerts it carcinogenic effect by increasing the net growth rate of preneoplastic, initiated cells. Two forms of the M-V-K model, negative exponential and log-logistic, were used to extrapolate from observed animal liver tumor rates to expected human response rates. The results using the negative exponential model can be used to calculate a risk-specific dose of 6.0 pg/kg-day at a 1×10^{-5} risk level (originally reported as 0.6 pg/kg-day at a 1×10^{-6} risk level) (EPA, 1988).

Acceptable daily intakes and risk-specific doses developed for TCDD by various U.S. agencies and other countries are shown in Figure 3 for comparison purposes. A risk-specific dose developed using the M-V-K model is also included in Figure 3. It is clear that the application of the two different extrapolation procedures to the same animal cancer bioassay data results in markedly different exposure limits for TCDD. The approach taken by EPA is at the most extreme end of the range of acceptable intake levels proposed by the regulatory agencies of Western industrialized nations.

3.2 Traditional U.S. Agency Dose-Response Analyses

The EPA and certain other federal policy and regulatory groups, however, employ a very different extrapolation procedure to the same animal bioassay data (EPA, 1985). Through the use of the LMS model, human exposure levels at a selected probability of increased cancer risk are extrapolated from rodent bioassay data. The use of this model is based on an assumption that there is no threshold for carcinogenesis; i.e., any dose, regardless of the quantity, poses some level of risk.

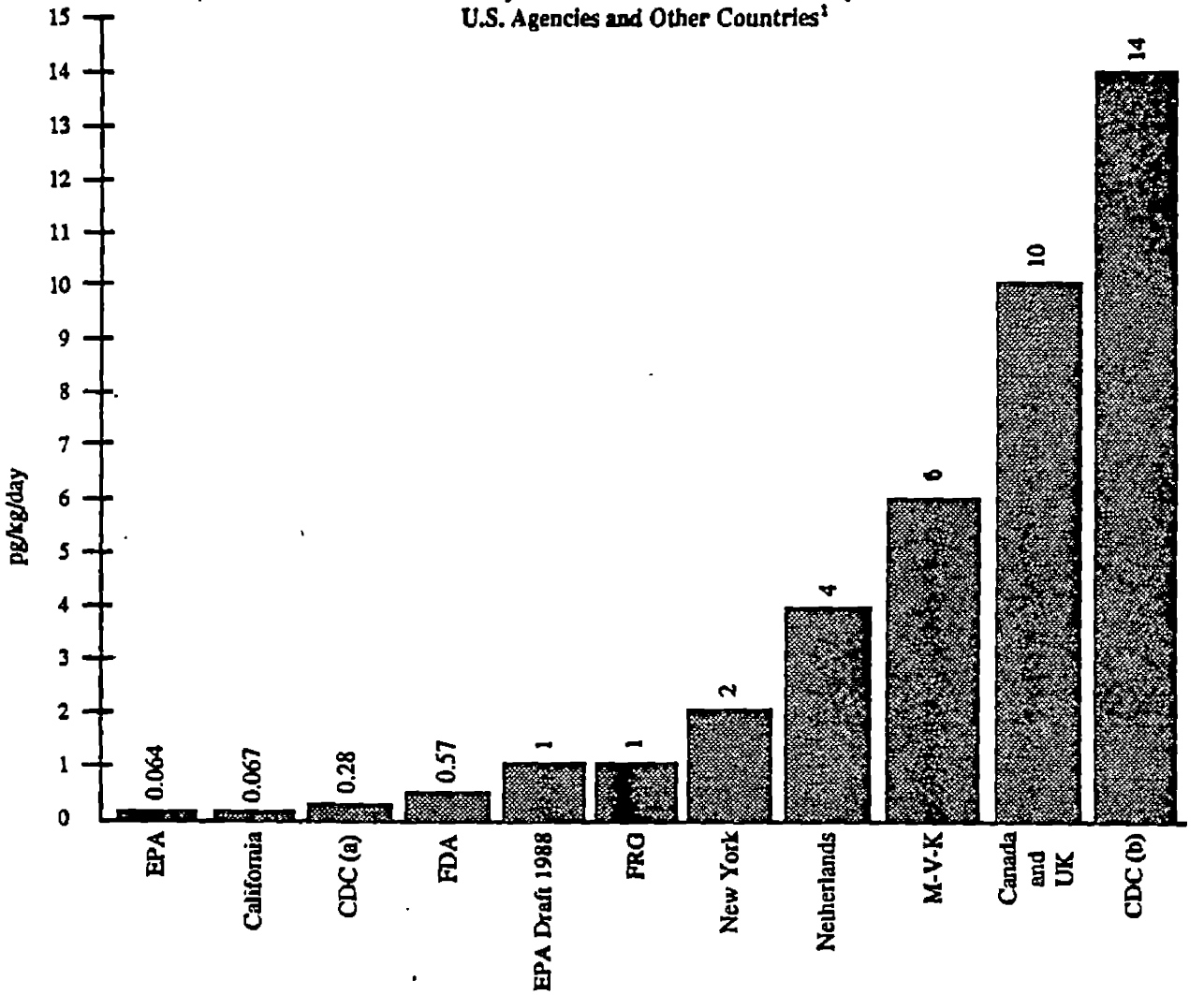
The Office of Science and Technology Policy (OSTP) prepared a framework for U.S. regulatory agencies involved in the process of assessing human cancer risks from chemical exposures (OSTP, 1985). OSTP proposed that

> "...a single long-term (animal) study may be utilized to obtain data on chronic toxicity, on carcinogenic potential, and for carcinogenic risk assessment."

The EPA risk assessment policies are consistent with the OSTP proposal; chronic bioassay data, most commonly from rodents, are used to calculate upper-bound estimates of the human carcinogenic risk associated with chemical exposure.

The EPA (1985), the Centers for Disease Control (CDC) (Kimbrough et al., 1984), and the Food and Drug Administration (FDA, 1983) have developed estimates of cancer potency for TCDD and corresponding estimates of the risk-specific dose (RsD). By definition, an RsD is defined as that dose of a

Figure 3. Risk-specific Doses (at 10^{-5}) and Acceptable Daily Intakes Calculated for TCDD by Various U.S. Agencies and Other Countries[1]



EPA	U.S. Environmental Protection Agency - RsD
California	State of California - RsD
CDC	Centers for Disease Control, U.S. Public Health Service - RsD (a) Most conservative figure of range (b) Least conservative figure of range
FDA	U.S. Food and Drug Administration - RsD
M-V-K	Mongerian, Vermin, Kandean - RsD
FRG	Federal Republic of Germany - ADI (ADI shown is most conservative figure of a range calculated by FRG)
New York	State of New York - ADI
Netherlands	- ADI
Canada	Ministry of Country Health and Welfare Canada - ADI
UK	United Kingdom - guideline level

1. U.S. agencies, including the EPA, have calculated and presented RsDs at various levels of risk. For the sake of consistency and to enhance the clarity of this document, the risk level of 1 in 100,000 has been used in the presentation of RsD estimates.

compound which is associated with a given probability of a toxic effect in humans. This relationship is described by the following equation:

$$RsD = \frac{\text{Level of Risk}}{\text{Cancer Potency Factor}}$$

The EPA and CDC have used the linearized multistage model (Howe and Crump, 1982) to estimate the dose-response relationship for low-level human exposures based on data obtained from rodents exposed to high doses. A linear interpolation model (Gaylor and Kodell, 1980) has been employed by the FDA (1983).

The current EPA cancer potency estimate for TCDD is 1.56×10^5 $(mg/kg\text{-}day)^{-1}$, based on the combined incidence of a number of tumor types (including, the liver, lung, and oropharynx) observed in female Sprague-Dawley rats from the Kociba et al. (1978) two-year bioassay. Using the upper confidence limit (UCL) of the cancer potency estimate derived from the multistage model, an RsD associated with a risk of 1 in 100,000 can be calculated at 0.064 pg/kg/day. This estimate is the most conservative among the RsDs calculated by the EPA (1985), the CDC (Kimbrough et al., 1984), and the FDA (1983).

When the CDC evaluated the bioassay data from NTP (1982) and Kociba et al. (1978), they presented virtually safe doses (VSDs) involving a number of plausible scenarios. Rather than simply rely on one set of assumptions about the behavior of TCDD in rodents, the CDC adopted a range of assumptions from the most sensitive species/strain/sex/tumor type combination to the least sensitive species/strain/sex/tumor type combination (Kimbrough et al., 1984). The 95% lower confidence bounds of the VSDs that would correspond to a 1×10^{-5} risk level range from 0.28 to 14.28 pg/kg/day. [Note: the term VSD is synonymous with the term RsD which is currently used by the EPA.] The calculated cancer potency estimates corresponding to the VSDs reported by the CDC range from 7.0×10^2 to 3.6×10^4 $(mg/kg\text{-}day)^{-1}$.

The FDA (1983) has suggested a cancer potency estimate of 1.75×10^4 $(mg/kg\text{-}day)^{-1}$, based on the incidence of hepatic tumors in female rats in the Kociba et al. (1978) study. Their estimate corresponds to an RsD associated with a risk of 1×10^{-5} of 0.57 pg/kg/day (FDA, 1983). The FDA analysis adopted a linear interpolation model (Gaylor and Kodell, 1980), which assumed a linear dose-response relationship between the origin and the upper 95% confidence level on the doses.

Cancer potency figures and corresponding risk-specific doses for TCDD are summarized in Table 5. Table 5 also includes the revised cancer potency estimate recently proposed by the EPA. Risk-specific doses are shown corresponding to incremental cancer risks of 1 in 100,000 (1×10^{-5}). It is evident that the three agencies' cancer potency estimates differ considerably. The EPA (1985) potency estimate exceeds the most conservative CDC estimate by a factor of 4 and exceeds the FDA estimate by a factor of 9. Differences among the three agencies' cancer potency estimates are due to differences in

Table 5. U.S. Agency Risk-specific Doses for 2,3,7,8-TCDD[a]

	Cancer Potency $(mg/kg\text{-}day)^{-1}$	Risk-specific Dose (pg/kg-day) 1×10^{-5}	Reference
EPA	1.56×10^{5}	0.064	EPA, 1985
EPA[b]	1.0×10^{4}	1	EPA, 1988
CDC	3.6×10^{4} to 7×10^{2}	.28-14.28	Kimbrough et al., 1984
FDA	1.75×10^{4}	.57	FDA, 1983

a. U.S. agencies, including the EPA, have calculated and presented RsDs at various levels of risk. For the sake of consistency and to enhance the clarity of this document, the risk level of 1 in 100,000 has been used in the presentation of RsD estimates.
b. Recommended in EPA (1988) draft document as revised RsD for TCDD.

data interpretation and modeling analysis. Due to the size difference between humans and rats, an adjustment of the animal dose is necessary to properly predict the possible human response. It should be pointed out that the regulatory agencies do not agree on the appropriate method for making this correction.

Sielken (1987, 1988) has challenged the use of the LMS model for predicting human cancer risk and, in particular, has challenged the appropriateness of adopting evidence from the Kociba rodent bioassay. Sielken concluded that when the multistage model is fitted to the animal data, trade-offs inherent in curve fitting may lead to questionable fits in the low-dose region. Compared to the response rates observed in the bioassay, the tumor response rates estimated by the LMS model are too large at the lowest nonzero experimental dose level and too small at the intermediate dose level. Sielken's analysis clearly demonstrated how the sensitivity of the low-dose extrapolation from rats to humans is dependent on the assumptions employed in the multistage model. His analysis indicated that the extrapolation approaches which have been used by various agencies may considerably overestimate the actual cancer potency of TCDD.

The statistical uncertainties of the LMS model described by Sielken (1987, 1988) have been disputed by Crump (1988). Crump did not agree that the chi-square goodness-of-fit test is an inadequate statistical measure of the multistage model. According to the author, the chi-square method is a universally accepted and widely used statistical test. Crump also did not agree with Sielken (1987, 1988) that the LMS model cannot handle saturation-type phenomenon at high dose levels and non-linear behavior at low doses. When the high TCDD dose group is omitted from the model, as Sielken (1987, 1988) demonstrates, Crump believed that the remaining data offer little assurance that the true dose-response relationship is non-linear; the gap between the lowest and middle doses is too large to discern the true shape of the dose-response curve for TCDD.

Recently, a draft report prepared by a EPA Workgroup reexamined the hazard identification and dose-response assessment regarding the potential carcinogenicity of TCDD (EPA, 1988). The Workgroup recommended on the basis of the weight of evidence that a revised RsD of 0.1 pg/kg-day (100 fg/kg-day) be adopted as the dose most likely to be associated with an increased lifetime cancer risk of 1×10^{-6}. This corresponds to a cancer potency estimate of 1×10^{4} $(mg/kg\text{-}day)^{-1}$. This potency estimate is 16 times lower than the cancer potency adopted by the EPA in 1985 (Table 5). The EPA's Science Advisory Board recently has reviewed the draft report. However, a formal statement of their conclusions has not been released to the public (Inside EPA, 1988).

The Workgroup assessed several potential approaches for estimating the cancer potency of TCDD and concluded that none of the available models, including the multistage model, adequately describe the carcinogenic behavior of TCDD at low doses. The EPA Workgroup encouraged the types of analyses generated by Sielken and the use of the M-V-K model for TCDD. However, the Workgroup did not consider these approaches sufficiently refined or accepted in the scientific community to warrant their consideration as the basis for

recommending changes in the current cancer potency or RsD estimates for TCDD. They stated that while arguments could be made that a threshold for the carcinogenicity of TCDD may exist, the evidence for such a conclusion is not compelling.

The EPA Workgroup noted, however, that the EPA (1985) analysis based on the linearized multistage model is likely to have led to an overestimation of the carcinogenic risk of TCDD. The weight of evidence indicated that a more appropriate upper-bound estimate would result in an unspecified reduction in the cancer potency of TCDD. The Workgroup noted that all of the cancer potency and corresponding risk-specific dose estimates made by various federal and state agencies, including the EPA, FDA, CDC, and the State of California, are arguably of equal scientific merit at the present time (EPA, 1988).

There appears to be little, if any, scientific basis for using a linear nonthreshold model for low-dose risk extrapolation for TCDD. According to the EPA Guidelines for Carcinogen Risk Assessment (EPA, 1986a),

> ". . . when pharmacokinetics or metabolism data are available, or when other substantial evidence on the mechanistic aspects of the carcinogenesis process exists, a low-dose extrapolation model other than the linearized multistage procedure might be considered more appropriate on biological grounds."

3.3 Determining an Appropriate Level of Risk

It is a common misperception within risk assessment that all occupational and environmental regulations have as their goal a theoretical maximum cancer risk of one in one million (i.e., 1 in 1,000,000 or 1 x 10^{-6}). In the U.S., regulatory agencies frequently adopt the cancer risk guideline of one in one million as a negligible or *de minimis* risk level when very large populations are likely to be exposed to a suspect carcinogen. Travis et al. (1987) demonstrated that this view is not compatible with past regulatory practice. In a retrospective examination of the level of risk which triggered regulatory action in 132 federal decisions, the authors considered three measures of risk: individual risk (an upper-limit estimate of the probability that the most highly exposed individual in a population will develop cancer as a result of a lifetime exposure), the size of the population exposed, and population risk (an upper-limit estimate of the number of additional cases of cancer in the exposed population).

Travis et al. (1987) found that for exposures resulting in a small-population risk, the *de manifestis* level was approximately 4 in 1,000 (4 x 10^{-3}), i.e. the level of risk above which agencies almost always acted to reduce risk. For large-population risks (the entire U.S. population) the *de manifestis* level dropped to about 3 in 10,000 (3 x 10^{-4}). Risks that are so low that agencies almost never act to reduce them are termed *de minimis* risks. For effects on small populations, regulatory action was never taken for individual risk levels below 1 in 10,000 (1 x 10^{-4}). For large-population effects, the

de minimis risk level dropped to 1 in 1,000,000 (1×10^{-6}). The review by Travis et al. (1987) shows that regulatory agencies have found risks far in excess of 1 in 1,000,000 (1×10^{-6}) to be insignificant and of no concern if the size of the population is small.

Farber (1989) proposes that the decisions regarding the selection of the appropriate level of incremental risk should be driven by the existing level of "background" risk. He defines background risk as that level of risk which is present regardless of whether the source of the "incremental" risk is present or not. In a real population , this "background" risk translates into incidences of disease or death which, when monitored in a population over time, are variable. The inherent variance associated with background risk estimates needs to be considered when selecting an appropriate level of incremental risk. Farber mathematically modeled the relationship between background and incremental population risk. His analysis indicates that incremental risks which are smaller than the variance estimate for the background risk do not alter the probability of an individual contracting a disease or increase case incidence. He further suggests that when the ratio of background risk/incremental risk increases beyond 100, the additional risk no longer affects the case incidence and may be considered a "negligible" risk.

Farber (1989) concluded that the use of a risk level smaller than 1×10^{-5} for deriving acceptable levels of daily exposure is almost always mathematically meaningless and unsound. The use of such a small risk level would require the estimation of small differences between two large numbers, neither of which is known with the required degree of precision. "Background" risk alone and the "background" risk plus "incremental" risk do not exist as precise quantities; thus, any discussion of incremental or excess risk is highly tenuous at risk levels below 1×10^{-4} or 1×10^{-5}.

State regulatory agencies have not adopted uniformly a one in one million (1×10^{-6}) risk criterion in making environmental and occupational decisions. The State of Maine Department of Human Services (DHS) uses a lifetime risk of one in one hundred thousand .(1×10^{-5}) as a reference for nonthreshold (carcinogenic) effects in its risk management decisions regarding exposures to environmental contaminants (Maine DHS, 1988). Similarly, a lifetime incremental cancer risk of one in one hundred thousand is used by the Commonwealth of Massachusetts as a cancer risk limit for exposures to substances in more than one medium at hazardous waste disposal sites (Mass DEQE, 1988). This risk limit represents the total cancer risk at the site associated with exposure to multiple chemicals in all contaminated media. The State of California has also established a level of risk of one in one hundred thousand (1×10^{-5}) for use in determining levels of chemicals and exposures that pose no significant risks of cancer under the Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65) (California, 1986). Additionally, the states of Maryland, Michigan, Minnesota, Ohio, Virginia, and Wisconsin have employed the 1×10^{-5} level of risk in their risk management decisions (individual state offices of environmental quality, personal communication).

Workplace air standards developed by the Occupational Safety and Health Administration (OSHA) typically reflect theoretical risks of one in one thousand (1×10^{-3}) or greater (Rodricks et al., 1987).

When considering these limits it is interesting to note that many common human activities entail annual risks greatly in excess of one in one million (Tables 6 and 7). These have been discussed by Grover Wrenn (1986), former director of Federal compliance and State Programs at OSHA, as follows:

> "Examination of the risks of common human activities demonstrates . . . a lifetime risk of 1 in 100,000 or more is within the realm of, or orders of magnitude below, everyday risks that generally do not cause undue concern. These are risks that people, while they are aware of them and may have some concern or fear over them, do not in general alter their behavior to avoid. The risks from many activities greatly exceed the level of one in 100,000. In comparison to these background risks of "everyday activities," a lifetime risk of 1 in 100,000 is relatively small. Accordingly, regulatory action will not generally be justifiable unless risks are substantially higher than this 1 in 100,000 "benchmark"."

Ultimately, the selection of an acceptable and *de minimis* risk level is a policy decision in which both costs and benefits of anticipated courses of action should be thoroughly evaluated. However, actuarial data and risk estimates of common human activities, regulatory precedence, and the relationship between the magnitude and variance of background and incremental risk estimates all provide compelling support for the adoption of the *de minimis* risk level of 1×10^{-5} for regulatory purposes.

Table 6. Activities Associated with 1×10^{-6} Increased Risk of Death in Any Year

Activity	Cause
Smoking 1.4 cigarettes	Cancer, heart disease
Drinking 0.5 L wine	Cirrhosis
Eating 40 tablespoons of peanut butter	Liber cancer due to aflatoxin
Eating 100 charcoal-broiled steaks	Cancer from benzopyrene
Bicycling 10 miles	Accident
Flying (jet) 1000 miles	Accident
One chest x-ray	Cancer due to irradiation

Adapted from Allman, 1985

Table 7. Comparison of Risks from Selected Activities on a Per Capita Basis

Activity	Average Annual per Capita Risk of Mortality
Smoking	
cancer only	1×10^{-3}
all effects	3×10^{-3}
Scuba Diving	4×10^{-4}
Motor Vehicle Accident	2×10^{-4}
Boating	5×10^{-5}
Hunting	3×10^{-5}
Swimming	2×10^{-5}
Lightning	5×10^{-7}

Adapted from Crouch and Wilson (1982)

4.0 SENSITIVITY ANALYSIS FOR DIOXIN RISK ASSESSMENT

4.1 Overview

In the absence of adequate human epidemiological evidence, laboratory animals typically serve as models for the study of the toxicological effects of chemicals in humans, based on the assumption that the extrapolation of biological data from animals to humans is valid, at least for some physiological parameters. A critical component of the quantitative risk assessment process, interspecies extrapolation, assumes that a reasonably stable relationship exists between the potency of a chemical in laboratory animals and in humans. Moreover, it assumes that the parameters of this relationship have been adequately estimated and characterized.

In the case of TCDD, low-dose risk estimates derived by the EPA (1985) through the application of the LMS model to the incidence of hepatic lesions observed in the Kociba et al. (1978) bioassay are very sensitive to the modeling assumptions chosen by the EPA to extrapolate cancer risks from Sprague-Dawley rats to humans. Each of these assumptions contributes to the uncertainties expressed by a number of researchers (Portier et al., 1984; Kimbrough, 1984; Sielken 1987, 1988; Paustenbach et al., 1986; Shu et al., 1987; CanTox, 1989; Farber, 1989; Bonvalot et al., 1989) concerning the EPA's reliance on the LMS model to simulate the dose-response behavior of TCDD in laboratory animals and/or the appropriateness of an RsD of 0.064 pg/kg-day at a 1×10^{-5} risk level.

In order to understand these uncertainties, a sensitivity analysis of the dose-response behavior of TCDD observed in the Kociba et al. (1978) study was conducted. It was hypothesized that each of the following considerations in the dose-response assessment of TCDD might have a substantial impact upon the range of plausible risk estimates predicted through the use of the LMS model:

- The histopathological interpretation of the observed tumor incidence among female Sprague-Dawley rats exposed to various doses of TCDD.
- Use of the term "neoplastic nodules" in histopathological examinations of laboratory animals to describe tissue anomalies.
- The effect of high spontaneous rates of hepatocellular lesions in rodents on the determination of chemically-induced carcinogenic responses in the livers of Sprague-Dawley rats.
- Consideration of hepatocellular carcinomas as the most appropriate primary carcinogenic response in laboratory rodent bioassays.
- Low-dose risk estimates based on the incidence of hepatocellular carcinomas observed during the Kociba et al. (1978) bioassay.
- The use of Maximum Likelihood Estimates (MLE) versus the 95% Lower Confidence Limit (LCL) of the human RsD.

- The use of a body weight correction factor in the rat-to-human extrapolation procedure rather than the use of a surface area correction factor.

4.2 Histopathological Interpretation of the Kociba et al. (1978) Bioassay

Among the several animal bioassays described by the EPA in their 1985 document entitled "*Health Assessment Document for Polychlorinated Dibenzo-p-dioxins*", the two-year study conducted by Kociba et al. (1978) has been cited as the primary evidence supporting the carcinogenicity of TCDD in laboratory animals. The EPA (1985) also cited a two-year bioassay conducted by the National Toxicology Program in 1982 as additional evidence supporting the findings of the Kociba et al. (1978) study.

In the Kociba et al. (1978) study, groups of 50 Sprague-Dawley rats, Spartan substrain, of each sex were maintained for up to 24 months on diets containing 1,000, 10,000, and 100,000 pg/kg-day of 2,3,7,8-TCDD. A population of 86 animals of each sex was maintained as study controls. Gross and histopathological examinations were performed on all animals. Tissues considered as possible target organs were examined in most animals from each treatment group and included liver, lungs, kidneys, urinary bladder, tongue, brain, testes/ovaries, and prostate/uterus.

High early mortality was observed in all treatment groups in the Kociba et al. (1978) study. A substantial proportion of the animals in the study, including those in the control groups, died before the end of the two-year period (78% to 92% among males and 68% to 92% among females) (Kimbrough et al., 1984; Portier et al., 1984). However, early mortality was statistically significant compared to controls only among female animals in the highest dose-group (100,000 pg/kg-day) (Kociba et al., 1978; EPA, 1985). Progressive mortality among female animals in the high-dose group began as early as the 12th month and reached 50% mortality by the 21st month of the study (EPA, 1985). The mortality of only the group of males given the lowest dose (1,000 pg/kg-day) was significantly different from control animals (Kociba et al., 1978). Forty-four percent of the male animals in that group died within the first 18 months of the study (EPA, 1985).

The EPA (1985) noted that the principal impact of the high early mortality rate observed in the Kociba et al. (1978) study was an overall reduction in the sensitivity of the bioassay because of a decrease in the number of animals at risk during the time of expected tumor formation.

Farber (1989) and CanTox (1989) regarded the high mortality rate, coupled with depressed hematologic parameters, increased urinary levels of porphyrins, decreased weight gain, and increased activity of serum enzymes, as indicative of severe liver toxicity in rats in the highest dose-group (100,000 pg/kg-day). Similar effects were also observed in the NTP (1982) study (CanTox,

1989). These researchers have suggested that hepatocellular toxicity would result in marked increases in cell proliferation, which could significantly enhance the susceptibility of hepatocytes to both tumor initiators and promotors.

A summary of the principal histopathological findings of the Kociba et al. (1978) study are presented in Table 8. The lesions that are listed are those which were statistically different from control levels for at least one treatment dose and in one sex.

The incidences of lesions observed among exposed animals that were found to be statistically depressed below those of control animals are listed in Table 9. In general, the lesions reduced by TCDD treatment were those that typically occur at high spontaneous rates in the particular strain of Sprague-Dawley rat employed in the bioassay (Kociba, 1984). These included neoplasms of the pancreas, uterus, mammary glands, and pituitary glands. Kociba (1984) found it difficult to ascertain whether the reduction in these incidences was associated with exposure to TCDD or related to nontreatment factors (e.g., diet, age, cage conditions). Several researchers believe that the reduction in the incidence of various tissue lesions is associated with hormonal alterations or with the mediating role of one or more thyroid hormones (Potter et al., 1983; Rozman et al., 1984; Holder and Menzel, 1989; CanTox, 1989).

The lesions observed among animals exposed to 2,3,7,8-TCDD that were found to be statistically increased above control levels are listed in Table 10. In the highest dose groups, 2,3,7,8-TCDD induced statistically significant increases in stratified squamous cell carcinomas of the hard palate and/or nasal turbinates in both males and females, squamous cell carcinomas of the tongue in males, and keratinizing squamous cell carcinomas of the lungs, hepatocellular carcinomas and hyperplastic nodules in females.

Kociba (1984) has argued that lesions in tissues other than the liver (lung, hard palate, and nasal turbinates) were not systemic and probably resulted from the incidental inhalation of some of the TCDD-treated food or with prolonged direct contact of the TCDD-treated food with the mouth and respiratory tract. Furthermore, the pathological data presented in Kociba et al. (1978) indicate that hepatic lesions were observed in all animals that had one or more tumors at these other locations.

The incidences of the principal lesions reported in female Sprague-Dawley rats by Kociba et al. (1978) that are considered by the EPA (1985) as the quantitative basis for TCDD risk extrapolations are presented in Table 11. The description of hyperplastic nodules used by Kociba et al. in 1978 to characterize this type of lesion was referred to by the EPA in 1985 as neoplastic nodules (see section 4.3). The total combined incidences of lesions in the liver, lung, hard palate, or nasal turbinate were revised by EPA (1985) to adjust for high early mortality in the first year of the study. Since hepatic lesions were observed in all animals that had one or more lesions of the lung, hard palate, or nasal turbinates, the total combined incidence of lesions presented in Table 11 represent the response frequencies

Table 8. Summary of Neoplastic Lesions Produced by 2,3,7,8-TCDD in Sprague Dawley Rats, Spartan Substrain that are Statistically Significant in at Least One Sex

	Males				Females			
Treatment Dose (µg/kg/day)	0	0.001	0.01	0.1	0	0.001	0.01	0.1
Number of Animals Tested	85	50	50	50	86	50	50	49
Hepatocellular Hyperplastic nodules	6	0	3	2	8	3	18*	23*
Hepatocellular carcinoma	2	0	0	1	1	0	2	11*
Stratified squamous cell carcinoma of hard palate or nasal turbinate	0	0	0	4*	0	0	1	4*
Keratinizing squamous cell carcinoma of lung	0	0	0	1	0	0	0	7*
Benign tumor of uterus	–	–	–	–	28	12	11	7*
Subcutaneous fibroma/ fibroadenoma/lipoma	10	1*	5	6	1	1	0	0
Benign mammary gland neoplasm	0	0	0	1	73	35	36	24*
Mammary gland carcinoma	0	0	0	0	8	4	4	0*
Stratified squamous cell carcinoma of tongue	0	1	1	3*	1	0	0	2
Pituitary adenoma	26	6	11	13	43	18	13	12*
Acinar adenoma of pancreas	14	7	5	2*	0	1	0	1
Adenoma of adrenal cortex	0	0	2	5*	9	6	2	5
Pheochromocytoma of adrenal	28	6	10	4*	7	2	1	3

Source: Kociba et al., 1978.
* Denotes a statistically significant difference ($p \leq 0.05$) from control by the Fisher Exact Test.

Table 9. Tissue Lesions Observed by Kociba et al. (1978) Among Sprague-Dawley Rats Exposed to 2,3,7,8-TCDD that Were Statistically Depressed Below Controls

Lesion	Sex	Treatment Group (µg/kg/day)
Benign tumor of uterus	Female	0.1
Benign mammary gland neoplasms	Female	0.1
Mammary gland carcinomas	Female	0.1
Pituitary adenomas	Female	0.1
Subcutaneous fibroadenomas, fibromas, and lipomas	Male	0.001
Acinar adenomas of the pancreas	Male	0.1
Pheochromocytomas	Male	0.1

Source: EPA (1984).

Table 10. Tissue Lesions Observed by Kociba et al. (1978) Among Sprague-Dawley Rats Exposed to 2,3,7,8-TCDD that Were Statistically Increased Above Controls

Lesion	Sex	Treatment Group (µg/kg/day)
Hepatocellular hyperplastic nodules	Female	0.01, 0.1
Hepatocellular carcinoma	Female	0.1
Keratinizing squamous cell carcinoma of lung	Female	0.1
Stratified squamous cell carcinoma of palate or nasal turbinate	Male & Female	0.1
Stratified squamous cell carcinoma of tongue	Male	0.1
Adenoma of adrenal cortex	Male	0.1

Source: EPA (1984).

Table 11. Principal Lesions in Female Sprague-Dawley Rats Exposed to 2,3,7,8-TCDD Reported by Kociba et al. (1978)

Treatment Dose (µg/kg/day)	0	0.001	0.01	0.1
Hepatocellular hyperplastic nodules	8/86 (9%)[a]	3/50 (6%)	18/50 (36%)*	23/49 (48%)*
Hepatocellular carcinoma	1/86 (1%)	0/50 (0%)	2/50 (4%)	11/49 (22%)*
Keratinizing squamous cell carcinoma of lung	0/86 (0%)	0/50 (0%)	0/49 (0%)	7/49 (14%)*
Stratified squamous cell carcinoma of hard-palate or nasal turbinates (revised diagnosis in EPA, 1985)	1/54 (2%)	0/30 (0%)	1/27 (4%)	5/24 (21%)*
Total combined incidence[b] (liver, lung, hard palate or nasal turbinate)	9/85 (11%)	3/48 (6%)	18/48 (37%)*	34/40 (85%)*

Source: Kociba et al. (1978) and EPA (1985).

* Denotes a statistically significant difference ($p<0.05$) from control by the Fischer Exact Test.

a. Number of responses/number of animals examined (percent response).

b. The Kociba et al. (1978) dataset was adjusted by the EPA (1985) to eliminate the first year's data to account for high early mortality in the high-dose group. The EPA (1985) replaced the term "hyperplastic nodules" from Kociba et al. (1978) with "neoplastic nodules". These data were employed by EPA (1985) in an LMS analysis of the Kociba et al. (1978) bioassay.

adopted by the EPA in 1985 for their dose-response assessment of TCDD using an LMS model.

The histopathology of the Kociba et al. (1978) study was reviewed by Dr. Robert Squire, pathologist at the Johns Hopkins University Medical School and consultant to EPA's Carcinogen Assessment Group (CAG). The results of Dr. Squire's interpretation of the Kociba dataset were reported in EPA (1985) and are reproduced in Table 12. Similar to the EPA's (1985) interpretation, the total combined incidences of lesions in the liver, lung, hard palate, or nasal turbinates also were derived from an elimination of the first year of data to adjust for the high early mortality of female rats in the highest dose group.

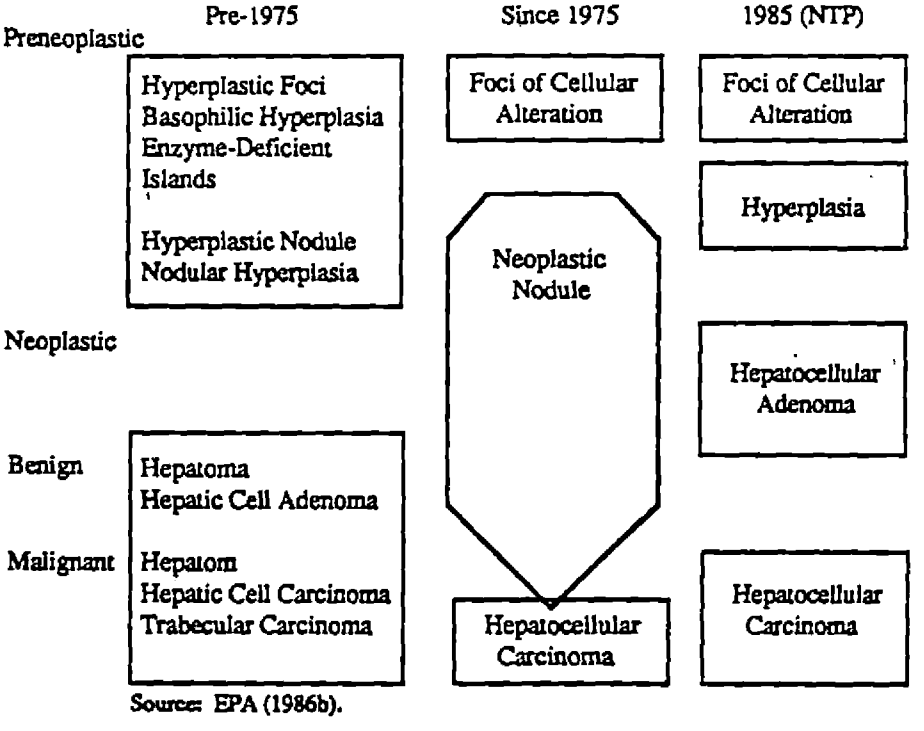
In the NTP (1982) carcinogenicity bioassay in rats and mice, considered by the EPA (1984, 1985) as significant support for the findings in Kociba et al. (1978), the liver also appears to be the predominant target organ of TCDD carcinogenicity. A summary of hepatic lesions observed in Osborne-Mendel rats and B6C3F1 mice exposed to 2,3,7,8-TCDD in the NTP (1982) study is presented in Table 13. Fifty rats of each sex and 50 male mice were exposed by gavage to 0, 0.01, 0.05, and 0.5 µg/kg-week of TCDD for 104 weeks. Animals were dosed twice each week during the study. Fifty female mice were similarly exposed to doses of 0, 0.04, 0.2, and 2.0 µg/kg-week of TCDD. A significant increase in follicular cell adenomas or carcinomas of the thyroid were elevated in male rats, and both neoplastic nodules and hepatocellular carcinomas were elevated in female rats in the highest dose groups. Mice of both sexes had statistically significant elevated levels of hepatocellular carcinomas at the highest treatment doses.

4.3 Interpretation of Hyperplastic Nodules and Neoplastic Nodules

During chemical hepatocarcinogenesis in experimental animals, various cellular changes also are observed either in conjunction with carcinomas or at lower doses than those at which carcinomas occur. Since approximately 1975, two lesions of hepatocytes have been generally related to cancer development: altered foci and neoplastic nodules. The characterization of these lesions among pathologists has involved a range of adjectives, namely "degenerative", "regenerative", "hyperplastic", "preneoplastic", and "neoplastic" (Bannasch et al., 1982). The changes in the terminology of rat liver lesions over time (Figure 4) reflects the difficulties in the understanding of the biological behavior of these lesions and their significance in hepatocellular carcinogenesis.

According to the EPA (1986b), liver foci, or areas of altered hepatocytes, are generally considered to be the first indications of abnormalities in the rat liver, followed by the appearance of neoplastic nodules, and finally carcinomas. Over the last decade, this sequence of events in hepatocarcinogenesis suggested that foci were a precursor stage in the development of neoplastic nodules, which in turn were precursors of carcinomas. However, under certain circumstances, foci and nodules may regress to a condition that is morphologically indistinguishable from the

Figure 4. Terminology of Rat Liver Lesions



Source: EPA (1986b).

Table 12. Histopathological Interpretation by Squire (EPA, 1985) of Principal Lesions in Female Sprague-Dawley Rats Exposed to 2,3,7,8-TCDD in the Kociba et al. (1978) Bioassay

Treatment Dose (µg/kg/day)	0	0.001	0.01	0.1
Keratinizing squamous cell carcinoma of lung	0/86 (0%)[a]	0/50 (0%)	0/49 (0%)	8/47 (17%)*
Stratified squamous cell carcinoma of hard palate or nasal turbinates	0/54 (0%)	0/30 (0%)	1/27 (4%)	5/22 (23%)*
Liver neoplastic nodules[b] and hepatocellular carcinoma	16/86 (19%)	8/50 (16%)	27/50 (54%)*	33/47 (70%)*
Total combined incidence (liver, lung, hard palate or nasal turbinates)[c]	16/85 (19%)	8/48 (17%)	27/48 (57%)*	34/40 (85%)*

Source: EPA (1985).

* Denotes a statistically significant difference ($P<0.05$) from control by the Fischer Exact Test.

a. Number of responses/number of animals examined (percent response).

b. The term "neoplastic nodules" was adopted by Dr. Squire and the EPA (1985) and was not used by Kociba et al. (1978). These data were not reported separately in EPA (1985).

c. These data were employed by EPA, 1985 in an LMS analysis of Squire's interpretation of Kociba et al. (1978).

Table 13. Summary of Hepatic Lesions Observed in Osborne-Mendel Rats and B6C3F1 Mice Exposed to 2,3,7,8-TCDD in the NTP (1982) Bioassay

	Osborne-Mendel Rats							
	Males				Females			
Treatment Dose (µg/kg/week)	0	0.01	0.05	0.5	0	0.01	0.05	0.5
Number of Animals Tested	74	50	50	50	75	49	50	49
Neoplastic nodule	0 (0%)	0 (0%)	0 (0%)	3 (6%)	5 (7%)	1 (2%)	3 (6%)	12 (24%)*
Hepatocellular carcinoma	0 (0%)	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0 (0%)	0 (0%)	3 (6%)
Toxic Hepatitis[a]	0 (0%)	1 (2%)	0 (0%)	14 (28%)	0 (0%)	0 (0%)	1 (2%)	32 (65%)

	B6C3F1 Mice							
	Males				Females			
Treatment Dose (µg/kg/week)	0	0.01	0.05	0.5	0	0.04	0.2	2.0
Number of Animals Tested	73	49	49	50	73	50	48	47
Hepatocellular carcinoma	8 (11%)	9 (18%)	8 (16%)	17 (34%)*	1 (1%)	2 (4%)	2 (4%)	6 (13%)*
Hepatocellular adenoma	7 (10%)	3 (6%)	5 (10%)	10 (20%)	2 (3%)	4 (8%)	4 (8%)	5 (11%)
Toxic Hepatitis	1 (1%)	5 (10%)	3 (6%)	44 (88%)	0 (0%)	1 (2%)	2 (4%)	34 (73%)

Source: NTP (1982).
* Denotes a statistically significant difference ($p<0.05$) from control by the Fisher Exact Test.
a. Identified by NTP (1982) to describe liver toxicity.

surrounding hepatic tissue. Thus, an alternative interpretation is that foci and so-called "neoplastic nodules" may be independent of the development of hepatocellular carcinomas.

Based on a review of a number of hepatocarcinogenic studies in laboratory rodents, the EPA (1986b) and the NTP (McConnell et al., 1988) observed that under certain experimental conditions, early proliferative lesions (i.e., foci and nodules) that have been induced by hepatocarcinogens may regress following removal of the carcinogenic agent. The EPA concluded that, to the extent that proliferative lesions regress, there may be some modulation of the potential hazard for cancer development. Nonetheless, there is no certain scientific evidence or consensus among pathologists regarding the biological significance of these liver nodules or their interpretation in rodent carcinogenesis studies.

There was no evidence in the Kociba et al. (1978) study to indicate that a hyperplastic nodule would likely progress to a hepatocellular adenoma and then to a hepatocellular carcinoma. Kociba et al. (1978) identified and reported the occurrence of adenomas in other organs and tissues, while no adenomas were reported in the liver, clearly indicating that the observed lesions were considered to be a less serious form of hepatocellular proliferative lesion, i.e. hyperplasia. Furthermore, Kociba et al. (1978) reported that there was no evidence of metastasis of any of these hyperplastic nodules.

"Neoplastic nodules", the term used by both the USEPA (1985) and Squire (EPA, 1985) in their histopathological interpretations of the Kociba et al. (1978) study, represents terminology from a different morphological classification system developed by Squire and another researcher. Its use by Squire (EPA, 1985) does not provide convincing evidence that the hyperplasia was actually a more serious condition. The classification system of Squire and Levitt (1975) failed to define the exact position and significance of the "neoplastic nodule", which included both hyperplasia and more serious lesions (EPA, 1986b).

The National Toxicology Program (NTP) instituted a change in the classification of rat liver proliferative lesions in 1986 and no longer uses the scheme of Squire and Leavitt (1975) in histopathological evaluations (Maronpot et al., 1986; McConnell et al., 1988). The NTP retained the terms foci and hepatocellular carcinoma but replaced neoplastic nodule with two terms: hyperplasia and hepatocellular adenoma (Figure 4). Hyperplasia is characterized by a mild compression of surrounding hepatic tissue that is not observed in the focus of cellular alteration. Hyperplasia is further viewed as the result of cell injury and regeneration. In contrast, hepatocellular adenoma is indicated by clear differentiation of cells from the surrounding normal hepatic tissue, including tissue compression and the loss of the normal lobular architecture of the liver.

Some researchers have reported spontaneous incidences of hepatocellular proliferative lesions in various strains of rats, including Fischer-344, Sprague-Dawley, and Osborne-Mendel strains (Hollander and Burek, 1978; Ogawa

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et al., 1981; Schulte-Hermann and Parzefall, 1981; Bannasch et al., 1982; Schulte-Hermann et al., 1983; EPA, 1986b). These studies indicate that the high incidence of hyperplastic liver nodules in untreated, ageing rats may often preclude the distinction between chemical treatment and nontreatment effects in the liver.

For example, Schulte-Hermann et al. (1983) demonstrated that a significant incidence of hyperplastic liver nodules are found in the livers of untreated rats. The incidence increased to 100% over the course of the animals' two-year average lifetime. The size of these lesions also increased with age. In addition, these hyperplastic nodules showed similar characteristics to those observed in rats treated with known liver carcinogens. Schulte-Hermann et al. (1983) have concluded that the high incidence of hyperplastic liver nodules in untreated aged rats precludes the distinction between chemical promoters and initiating agents during long-term carcinogenicity bioassays in laboratory rodents, particularly when liver tumor incidence data are the primary criteria.

The use of rodents as a model for liver cancer in humans and, in particular, the consideration of the incidence of hepatocellular neoplastic nodules, may considerably overestimate the potential risks associated with exposure to TCDD (CanTox, 1989; Sielken, 1987; Shu et al., 1987). The combined incidence of hepatocellular neoplastic nodules or carcinomas in aged rats is commonly as high as 18 to 20% (Sielken, 1987) but is relatively rare in the U.S. human population (less than 5 per 100,000, or 0.005%; ACS, 1989).

In a 1986 review of proliferative hepatocellular lesions in rats and their use in risk assessment, the EPA acknowledged that the exact contribution of neoplastic nodules to the overall incidence of hepatocellular tumors in the rat remains unclear. Accordingly, the EPA adopted the following position:

> "Determination of carcinogenic hazard will be based upon consideration of the incidence of hepatocellular carcinoma alone, neoplastic nodule alone, and a combination of carcinoma and nodule. The range of responses could vary from cases where there are very significant increases in the incidence of carcinomas backed up by increases of nodules, through situations where only the combined frequency of animals with carcinomas or nodules is significant, to cases where increases are limited to nodules alone.
>
> (i) At one end, responses showing an overwhelming increase in carcinomas alone will be interpreted as providing sufficient evidence of animal carcinogenicity when other criteria for the sufficient category are met. In the absence of human evidence and supporting information, such evidence will generally be given a weight-of-evidence designation of Probable Human Carcinogen (Group B2), and a quantitative risk assessment is appropriate.

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> (ii) At the other end, where increases in lesions and statistical significance are restricted to neoplastic nodules alone, such data will be interpreted as only limited evidence of animal carcinogenicity. When neither human evidence nor supporting information accompany the increase in nodules, the weight-of-evidence designation is Possible Human Carcinogen (Group C), but quantitative assessment is not warranted.
>
> (iii) Situations within the two extremes will be evaluated on a case-by-case basis....In cases where quantitation is performed, methods of expressing the possible uncertainty in the estimated risks should be explored (e.g., expressing risk as a range between that determined from extrapolation of carcinoma incidence alone and that from a combination of carcinomas and nodules)."

At a minimum, the EPA in their 1988 draft document (EPA, 1988) should have reported a range of risk for TCDD in order to convey the uncertainties associated with the quantification of the carcinogenic response in female Sprague-Dawley rats by Kociba et al. (1978). In addition to presenting an RsD based on an analysis of carcinomas and nodules combined, the EPA should have presented an alternative RsD based on the incidence of hepatocellular carcinoma alone.

4.4 Principal Carcinogenic Response in Female Sprague-Dawley Rats

The EPA combined the incidences of lesions observed by Kociba et al. (1978) in the liver, lung, hard palate, or nasal turbinates of female Sprague-Dawley rats for the purposes of estimating a TCDD cancer potency factor. As stated in the Office of Science and Technology Policy's (1985) document on chemical carcinogenesis, this procedure is not recommended :

> "Generally, most experts agree that the incidence of total tumors at all organ sites is not a very useful expression of cancer incidence, nor is the calculation of the incidence of total benign or total malignant tumors. Most useful appears to be the number of histologically unique tumors at specific organ sites."

The combining of the incidences of hyperplastic nodules (referred to as neoplastic nodules in EPA, 1985) and hepatocellular carcinomas observed by Kociba et al. (1978) also is not warranted. As discussed in Section 4.3, there was no evidence in the Kociba et al. (1978) study to indicate that a hyperplastic nodule would likely progress to a hepatocellular adenoma and then to a hepatocellular carcinoma. Kociba et al. (1978) identified and reported the occurrence of adenomas in other organs and tissues, while no adenomas were reported in the liver, clearly indicating that the observed lesions were considered to be a less serious form of hepatocellular proliferative lesion,

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i.e. hyperplasia. Furthermore, Kociba et al. (1978) reported that there was no evidence of metastasis of any of these hyperplastic nodules.

Hepatocellular carcinomas are the only indicators of a carcinogenic response in the Kociba et al. (1978) bioassay having any conceivable use in a dose-response extrapolation via the LMS model. Considering only hepatocellular carcinomas, no statistically significant differences between controls and the lowest (1,000 pg/kg-day) or intermediate (10,000 pg/kg-day) dose groups were observed (Table 10).

The statistically significant increased incidence of hyperplastic nodules observed by Kociba et al. (1978) in the intermediate (10,000 pg/kg-day) and highest (100,000 pg/kg-day) TCDD treatment groups should be appropriately extrapolated to human exposure only through the use of a classical safety factor approach: the LOAEL divided by a safety factor of 1,000. In this case, the LOAEL for hyperplastic nodules in the rat liver (10,000 pg/kg-day) divided by 1,000 would yield an ADI of 10 pg/kg-day. This approach is identical to that taken by the United Kingdom (1989) when they derived a guideline level of 10 pg/kg-day as protective for potential carcinogenic effects, "which, when exceeded, should trigger investigation and appropriate measures to reduce environmental levels."

4.5 Reassessment of Carcinogenic Risk Estimates Based on Hepatocellular Carcinomas

Using the combined incidences of lesions in the liver, lung, hard palate, or nasal turbinate observed in female Sprague-Dawley rats from Kociba et al. (1978), the EPA in 1985 developed a cancer potency estimate for TCDD of 1.56×10^5 $(mg/kg/day)^{-1}$ with an associated 95% LCL on the RsD at a 1×10^{-5} risk level of 0.064 pg/kg-day (Table 14). These estimates were derived from the geometric mean of risk estimates developed from the EPA's interpretation of Kociba et al. (1978) (Table 11) and from the interpretation of the histopathology of the Kociba et al. (1978) dataset by Squire (EPA, 1985) (Table 12). Both interpretations were revised in the EPA analysis to eliminate the first year's data to account for the high early mortality observed by Kociba et al. (1978) in the high-dose groups. Risk estimates derived from the pooled incidences of lesions in the liver, lung, and oropharynx by EPA (1985) and Squire (EPA, 1985) are shown in Table 14.

The EPA's position in this instance appears to represent a desire to reach a statistical compromise between different histopathological interpretations of the same carcinogenic bioassay. While the differences in the interpretations of the histopathology contribute some degree of uncertainty to the calculated potency of TCDD, this approach does not represent a major source of variation.

To calculate revised risk estimates for TCDD based on the incidences of hepatocellular carcinomas observed in female Sprague-Dawley rats exposed to TCDD, computer modeling was conducted using the results of the Kociba et al. (1978) bioassay and the Global'86 Linearized Multistage (LMS) Model (Howe et

Table 14. Risk Estimates[a] Derived from the Incidence of Hepatocellular Carcinoma and From the Pooled Incidence of Various Tumor Types Reported in Kociba et al. (1978)

Interpretation:	ChemRisk™	EPA, 1985	Squire, 1985	EPA, 1985
Lesions Evaluated:	Hepatocellular[b] carcinoma	Pooled Tumors (Liver, Lung, and Oropharynx)	Pooled Tumors (Liver, Lung, and Oropharynx)	Geometric Mean[c] EPA & Squire
Animal q1* $(mg/kg/day)^{-1}$	3.93×10^{-3}	2.82×10^{-4}	2.99×10^{-4}	2.88×10^{-4}
Human q1*[d] $(mg/kg/day)^{-1}$	2.12×10^{-4}	1.52×10^{-5}	1.62×10^{-5}	1.56×10^{-5}
95% LCL on the Human RsD at 1×10^{-5} Risk Level[e] (pg/kg-day)	0.47	0.066	0.062	0.064
Maximum Likelihood Estimate (MLE) on Animal RsD at 1×10^{-5} Risk Level[f] (pg/kg-day)	4.0	0.48	0.47	0.48

a. U.S. agencies, including the EPA, have calculated and presented RsDs at various levels of risk. For the sake of consistency, the risk level of 1 in 100,000 has been used when presenting RsD estimates.
b. Risk estimates were calculated by ChemRisk™ using the Global '86 (Howe, Crump, & Landingham, 1986) LMS model and the incidence of hepatocellular carcinomas observed by Kociba et al. (1978).
c. The geometric mean of risk estimates by EPA, 1985 and by Squire (EPA, 1985) from the Kociba et al. (1978) study was adopted by the EPA as the appropriate estimate of risk to humans from exposure to TCDD.
d. The human q1* was calculated by multiplying the animal q1* by a surface area correction factor of 5.4.
e. The 95% LCL on the human RsD was calculated from the equation: risk = potency x dose; where risk = 1×10^{-5} and potency = human q1*.
f. The MLE was calculated by Global '86 (Howe, Crump, & Landingham, 1986).

al., 1986). The Global'86 LMS model is the latest revision to an earlier version of the model, Global'82 (Howe and Crump, 1982). Both Global'86 and Global'82 are currently employed interchangeably by the EPA Carcinogen Assessment Group (CAG) to evaluate potential human carcinogens (CAG, 1989).

Data were fitted to a conventional 3-stage Global'86 LMS model (one minus the number of dose groups in the bioassay). Estimates were derived for the animal aq1* and the human q1* (i.e., cancer potencies), the 95% lower confidence limit (LCL) on the human RsD, and the MLE of the animal RsD.

The human q1* was calculated as the product of the cancer potency in animals (aq1*) and a rat-to-human surface area correction factor of 5.4. The aq1* is estimated by Global'86 at the 95% confidence limit. The rat-to-human surface area correction factor of 5.4 is employed by the EPA in their dose-response assessment process.

The MLE of the RsD for the rat was estimated by Global'86 from the fitted 3-stage model. The 95% LCL on the human RsD at a 1×10^{-5} risk level was calculated as the ratio between a 1×10^{-5} risk level and the human q1* from the equation:

$$RsD = \frac{\text{Risk Level}}{\text{Cancer Potency}}$$

On the basis of the incidence of hepatocellular carcinomas reported by Kociba et al. (1978), a human q1* was calculated to be 2.12×10^{4} $(mg/kg\text{-}day)^{-1}$ with an associated 95% LCL on the RsD at a 1×10^{-5} risk level of 0.47 pg/kg-day (Table 14). The estimate of the human q1* and the 95% LCL on the RsD are approximately seven times lower and higher, respectively, than the estimates derived by the EPA (1985). The MLE at a 1×10^{-5} risk level was calculated to be 4.0 pg/kg-day (Table 14).

4.6 Use of the MLE versus the 95% LCL of the Human RsD

Empirical studies have shown that the 95% lower confidence limit (LCL) of the RsD is never smaller than the best estimate of the RsD (or Maximum Likelihood Estimate, MLE) when the underlying dose-response relationship is convex or upward curving, as it is for TCDD (Sielken, 1987). Therefore, if it is believed that the true dose-response relationship for TCDD follows one of the curves of the multistage model family, then the fitted model value, or MLE, is the more appropriate basis for a risk estimate.

Comparisons between the MLE and the 95% LCL of the human RsD have been conducted by Sielken (1987) and Kimbrough et al. (1984). Large differences between the MLE and the 95% LCL of the human RsD were observed by Sielken (1987) when the hepatic tumor incidence in the high TCDD dose group (100,000 pg/kg-day) from the Kociba et al. (1978) bioassay was not included in the LMS model. The resulting fitted curve resembled an upwardly sloping line for the control and two lowest dose groups (1,000 and 10,000 pg/kg-day). The analysis of hepatic tumors in female rats by Kimbrough et al. (1984) indicates that

when the high TCDD dose group was not dropped from the analysis, little difference was observed between the MLE and the 95% LCL of the human RsD. The reason for these disparate results appears to lie in the mathematical function that describes the LMS model:

$$P(d) = 1 - \exp\left[(q_1^d + q_2^{d2} + \ldots + q_k^{dk})\right]$$

where P(d) is the lifetime risk (probability) of cancer at dose d. In the analysis by Kimbrough et al. (1984), when the linear term (q_1) of the multistage model defines the dose-response curve from the experimental data, as in the case of the incidence of hepatic tumors in female rats from Kociba et al. (1978), there is little difference (a factor of about 1.3) between the MLE and the 95% LCL of the human RsD. In Sielken's (1987) analysis, when the high dose group was not included in the multistage model, the linear term was not adequate to describe the dose-response curve for TCDD. The upwardly sloping fitted curve of the multistage model that was observed by Sielken (1987) suggests that the quadratic term (q_2) best describes the dose-response curve for the tumor incidence observed in the control and two lowest TCDD dose groups. In this case, differences between the MLE and the 95% LCL of the human RsD can be very large.

Risk estimates developed in this analysis using a 3-stage Global'86 LMS model and the incidence of hepatocellular carcinoma reported in Kociba et al. (1978) were compared to estimates developed by EPA (1985) from Kociba et al. (1978) and Squire (EPA, 1985) and to estimates calculated by Kimbrough et al. (1984) from NTP (1982).

Estimates of the MLE and 95% LCL on the human RsD at a 1×10^{-5} risk level calculated by the EPA (1985) from Kociba et al. (1978) and from Squire (EPA, 1985) are shown in Table 14. The 95% LCL of the human RsD was calculated, in part, on the basis of surface area differences between rats and humans. In order to compare the MLE to the 95% LCL of the human RsD at a 1×10^{-5} risk level, the surface area correction factor of 5.4 was also applied to the MLE. A comparison of these estimates developed by EPA (1985) from Kociba et al. (1978) and Squire (EPA, 1985) is presented in Table 15.

The comparisons in Table 15 demonstrate that the corrected MLE is consistently higher than the 95% LCL of the human RsD at a 1×10^{-5} risk level. The increase, however, is not substantial. In all cases, the MLE, corrected for surface area differences between rats and humans, is approximately 1.3 times larger than the 95% LCL of the human RsD. The geometric mean of the MLE, corrected for surface area differences, is slightly higher, 0.088 pg/kg-day, than the 95% LCL of the human RsD at a 1×10^{-5} risk level, 0.064 pg/kg-day.

Comparisons between estimates calculated by the EPA (1985) of the MLE and the 95% LCL of the human RsD were also conducted on the basis of a body weight correction factor (Table 16). Since a body weight correction factor assumes a 1:1 relationship between the weight of a rat and a human, the estimate of the human cancer potency is assumed to be equal to the animal q1* (aq1*) predicted

Table 15. Comparison at a 1×10^{-5} Risk Level Between EPA (1985) Estimates of the 95% Lower Confidence Limits (LCL) on the Human RsD and the Maximum Likelihood Estimates (MLE) of the Animal RsD Based on Surface Area Correction

	95% LCL on Human RsD[a] (pg/kg-day)	MLE on Animal RsD Corrected for Surface Area[b] (pg/kg-day)
EPA, 1985 Pooled Tumors (liver, lung, oropharynx)	0.066	0.089
Squire (EPA, 1985) Pooled Tumors (liver, lung, oropharynx)	0.062	0.087
Geometric Mean EPA & Squire Interpretations of Kociba et al. (1978)	0.064	0.088

a. The 95% LCL on the human RsD was calculated from the equation: risk = potency x dose; where risk = 1×10^{-5} and potency = $q1^*$ from Table 14.

b. The MLE was calculated by Global '86 (Howe, Crump, & Landingham, 1986); The MLE dose in the rat was corrected for surface area differences between rats and humans by dividing the MLE by a surface area correction factor of 5.4.

Table 16. Comparison at a 1×10^{-5} Risk Level Between EPA (1985) Estimates of the 95% Lower Confidence Limits (LCL) on the Human RsD and the Maximum Likelihood Estimates (MLE) of the Animal RsD Based on Body Weight Correction

	95% LCL on Human RsD Corrected for Body Weight[a] (pg/kg-day)	MLE on Animal RsD[b] (pg/kg-day)
EPA, 1985 Pooled Tumors (liver, lung, oropharynx)	0.35	0.48
Squire (EPA, 1985) Pooled Tumors (liver, lung, oropharynx)	0.33	0.47
Geometric Mean EPA & Squire Interpretations of Kociba et al. (1978)	0.34	0.48

a. The 95% LCL on the human RsD was corrected for body weight differences between rats and humans from the equation: risk = potency x dose; where risk = 1×10^{-5} and potency = $aq1^*$ from Table 14.
b. The MLE was calculated by Global '86 (Howe, Crump & Landingham, 1986).

by the Global'86 LMS model. This approach is identical to removing the surface area correction factor from the estimates of the 95% LCL of the human RsD in Table 14.

Similar to Table 15, the comparisons in Table 16 demonstrate that the MLE is approximately 1.3 times higher than the corrected 95% LCL of the human RsD at a 1×10^{-5} risk level. The geometric mean of the MLE, 0.48 pg/kg-day, is slightly higher than the 95% LCL of the human RsD, corrected for surface area differences, at a 1×10^{-5} risk level, 0.34 pg/kg-day.

The effect of employing a body weight versus a surface area correction factor also was considered for potency estimates based on the incidence of hepatocellular carcinomas observed in the Kociba et al. (1978) bioassay. This tumor type should be considered as the most appropriate carcinogenic response observed during the Kociba et al. (1978) bioassay. Regardless of whether an adjustment is made on the basis of surface area or body weight, the MLE was approximately 1.6 times higher than the 95% LCL of the human RsD at a 1×10^{-5} risk level (Table 17).

In Table 17, when risk estimates were based on body weight and a 1×10^{-5} risk level, the MLE was 4.0 pg/kg-day and the 95% LCL of the human RsD was 2.5 pg/kg-day. When these estimates were based on a correction for surface area differences between rats and humans at the same risk level, the MLE was 0.74 pg/kg-day and the 95% LCL of the human RsD was 0.47 pg/kg-day.

Risk estimates developed in this analysis from the incidence of hepatocellular carcinomas reported in Kociba et al. (1978) were also compared to the incidences of similar lesions reported in Osborne-Mendel rats and B6C3F1 mice in NTP (1982). Comparisons between the MLE and the 95% LCL on the human RsD calculated from Kociba et al. (1978) and NTP (1982) were conducted on the basis of a correction for body weight differences between rodents and humans. These comparisons are shown in Table 18.

Based on the incidence of hepatocellular carcinomas in the liver, it appears that B6C3F1 male mice may be the most sensitive species/strain/sex to exposure to TCDD (Table 18). Differences in the route of administration of TCDD between animals in the NTP (1982) study and Kociba et al. (1978), however, must be carefully considered before reaching a more definitive conclusion. The MLE and 95% LCL on the human RsD at a 1×10^{-5} risk level were calculated to be 2.6 and 1.5, respectively, in male B6C3F1 mice. In contrast, the MLE and 95% LCL on the human RsD at a 1×10^{-5} risk level were estimated in this analysis to be 4.0 and 2.5, respectively, in female Sprague-Dawley rats.

4.7 Biological Basis for Extrapolating Across Species (Interspecies Scaling Factors)

In attempting to account for the various factors that contribute to interspecies variation in the toxicological response to a chemical, including both carcinogenic and noncarcinogenic compounds, scientists have long sought to establish a common biological baseline for extrapolating across species.

Table 17. Comparison at a 1 x 10^{-5} Risk Level Between Estimates of the 95% Lower Confidence Limits (LCL) on the Human RsD and the Maximum Likelihood Estimates (MLE) of the Animal RsD Based on the Incidence of Hepatocellular Carcinoma Reported by Kociba et al. (1978)

	RsD at 1 x 10^{-5} Risk Level (pg/kg-day)
95% LCL on Human RsD Corrected for Body Weight[a]	2.5
Maximum Likelihood Estimate on Animal RsD	4.0
95% LCL on Human RsD	0.47
Maximum Likelihood Estimate Corrected for Surface Area[b]	0.74

a. The 95% LCL on the human RsD was corrected for body weight differences between rats and humans from the equation: risk = potency x dose; where risk = 1 x 10^{-5} and potency = $aq1^*$ from Table 14.

b. The MLE was calculated by Global '86 (Howe, Crump, & Landingham, 1986). The MLE dose in the rat was corrected for surface area differences between rats and humans by dividing the MLE by a surface area correction factor of 5.4.

Table 18. Comparison Between Estimates[a] of the 95% Lower Confidence Limits (LCL) on the Human RsD and the Maximum Likelihood Estimate (MLE) of the Animal RsD Based on the Incidence of Hepatocellular Carcinomas Reported by Kociba et al (1978) and NTP (1982).

	Maximum Likelihood Estimate on RsD at 1 x 10^{-5} Risk Level (pg/kg-day)	95% LCL on Human RsD at 1 x 10^{-5} Risk Level, Corrected for Body Weight[b] (pg/kg-day)
ChemRisk™ Female Sprague-Dawley Rats	4.0	2.5
NTP Female Osborne-Mendel Rats	18,000	6.2
NTP Male Osborne-Mendel Rats	26,000	11.0
NTP Female B6C3F1 Mice	25.0	12.0
NTP Male B6C3F1 Mice	2.6	1.5

a. Risk estimates were calculated by ChemRisk™ using the Global '86 (Howe, Crump, & Landingham, 1986) LMS model.

b. The 95% LCL on the human RsD was corrected for body weight differences between rats and humans from the equation: risk = potency x dose; where risk = 1 x 10^{-5} and potency = aq1* from Table .

In the absence of detailed pharmacokinetic information on interspecies differences, the two extrapolation procedures most often considered to address physiological and toxicological differences between mammalian species are adjustments by body weight scaling or by surface area scaling.

Adolph (1949) was among the first researchers to correlate a number of anatomical, biochemical, and physiological properties of mammals with their body weights using the relationship:

$$\text{Property} = a(\text{body weight})^k$$

His most significant finding was the empirical demonstration that a large number of physiological characteristics could be extrapolated across a wide range of mammalian species if they were first standardized in terms of some power of body weight. Estimates for the value of *k* tended to be less than 1.0 for relationships depicting physiological functions, indicating that as body weight increased, the physiologic function per unit of body weight increased. Fractional powers of body weight between 0.6 and 0.8 are very close to the fractional power of 0.67, which relates the surface area of cylindrical objects to their volume. Since the density of most mammalian bodies is about the same, mass or body weight can be used instead of volume in the above relationship. This is the origin of the term *surface area* or $(\text{body weight})^{0.67}$ scaling factor.

Adjustment based on differences in body surface area is based on the premise that the magnitude of the adverse effect of the chemical is dependent on the basal metabolic rate (BMR) of the species of concern (O'Flaherty, 1988; Clayson, 1988).

Standardization of risk estimates on the basis of body surface area is not broadly accepted as the best approach to the estimation of cancer risk. For example, Krasovskii (1976) observed that expressing dosage in mg/m^2 in order to adjust for species surface area differences in extrapolation only seemed to be appropriate in about half of the cases considered. Furthermore, the appropriateness of body weight as a scaling factor has been substantiated by an interspecies comparison of carcinogenic potency for approximately 70 chemicals which gave reliable interspecies correlations between potencies (Crouch and Wilson, 1979).

The TCDD dose extrapolation procedure used most often by the EPA (1985) is based on the assumption that dose per unit body surface area is equivalent between species. In contrast, the CDC and the FDA use dose per unit body weight when the active carcinogen is thought to be the administered compound, and only use dose per unit surface area when the active carcinogen is a metabolite of the administered compound (Bayard, 1988). Both the CDC and the FDA have chosen to use the body weight scaling factor in the case of TCDD, based on their opinion that strict dose per unit body weight considerations ought to apply since the administered compound does not have to be metabolized in order to be carcinogenic in rodents (Bayard, 1988).

To estimate the dose-response relationship for low-level human exposure to TCDD based on the data obtained from high doses used in the Kociba et al. (1978) bioassay, the use of a body weight scaling factor is biologically more relevant to the pharmacokinetic behavior of TCDD than the surface area correction factor employed by the EPA.

The use of a scaling factor based on surface area is inappropriate as a surrogate for relative rates of metabolism between species for poorly metabolized chemicals such as TCDD. Basic pharmacokinetics suggest that the parent compound is the biologically active moiety (Shu, et al. 1987; Leung et al., 1988, 1989). In addition, the scientific literature reports markedly different tissue distributions of TCDD between species (EPA, 1985). Thus if the liver is the organ of primary toxicological concern, a human would have to be given anywhere from 10 to 50 times the dose on a mg/kg-body weight basis to have the same liver concentrations as the rat (Bayard, 1988). Even if one were to consider the longer relative half-life of TCDD in humans than in rats, it is the opinion of ChemRisk that the body weight scaling factor is more appropriate than surface area for this extrapolation between rats and humans.

With the further development of biologically-based pharmacokinetic models for TCDD, the application of basic pharmacokinetic principles will provide the most accurate means available to extrapolate doses between species (Mordenti, 1985, 1986; Lutz and Dedrick, 1987; Anderson et al., 1987; Rietz et al., 1988; Travis, 1989).

5.0 CONCLUSIONS

ChemRisk™ has conducted a sensitivity analysis of the EPA (1985) dose-response assessment for TCDD, and has reevaluated the biological data from the Kociba et al. (1978) bioassay, prior to using the LMS model for estimating scientifically defensible RsDs for TCDD. The quantitative impacts of several choices involved in the risk assessment of TCDD were reviewed, including the definition of the carcinogenic response of concern within the experimental dataset, the evaluation of liver pathology by the original researchers and the EPA, the methods used to make the fitted LMS model responsive to the data at the lower experimental doses, the choice of rat-to-human scaling factor, and the method of maximum acceptable dose estimation. Each choice affects the range of plausible risk estimates predicted using the LMS model.

5.1 Conclusions of the Sensitivity Analysis

The low-dose risk estimate derived by the EPA through the application of the LMS model to the Kociba et al. (1978) and the Squire (EPA, 1985) datasets is very sensitive to the choice of modeling assumptions. The principal conclusions of the ChemRisk sensitivity analysis are summarized below.

- Hepatocellular carcinomas are the only indicators of a carcinogenic response in the Kociba et al. (1978) bioassay having any conceivable use in a dose-response extrapolation via the LMS model. Considering only hepatocellular carcinomas, no statistically significant differences between controls and the lowest (1,000 pg/kg-day) or intermediate (10,000 pg/kg-day) dose groups were observed. Therefore, the dose-response curve is determined by only a single data point and the underlying assumptions of the model which force a fit to the origin in order to preserve the notion of "linearity". Furthermore, the high dose level administered to the rats (100,000 pg/kg-day) clearly exceeded the Maximum Tolerated Dose (MTD), as evident from Kociba's observations of severe liver toxicity, diminished weight gain, and increased mortality.

- The statistically significant (95% confidence limit) increased incidence of hyperplastic nodules observed by Kociba et al. (1978) in the intermediate (10,000 pg/kg-day) and highest (100,000 pg/kg-day) TCDD treatment groups should not be modeled via a nonthreshold approach because:

  (a) There is no evidence in this study to indicate that a hyperplastic nodule is likely to progress to a hepatocellular adenoma and then to a hepatocellular carcinoma.

  (b) Kociba et al. (1978) identified and reported the occurrence of adenomas in other organs and tissues, while no adenomas were reported in the liver, clearly indicating that the observed lesions were considered to be a less serious form of hepatocellular proliferative lesion, i.e. hyperplasia.

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Furthermore, Kociba et al. (1978) reported that there was no evidence of metastasis of any of these hyperplastic nodules.

(c) "Neoplastic nodules", a term used by Squire (EPA, 1985) in his re-evaluation of the Kociba analysis, represents terminology from a different morphological classification system developed by Squire and another researcher. Its use by Squire (EPA, 1985) does not provide convincing evidence that the hyperplasia was actually a more serious condition. The classification system of Squire and Levitt (1975) failed to define the exact position and significance of the "neoplastic nodule", which included both hyperplasia and more serious lesions (EPA, 1986b). Today, the National Toxicology Program (NTP) no longer uses the classification scheme of Squire and Levitt, and neoplastic nodules are no longer identified during histopathological examinations by most toxicological laboratories, including the NTP (Maronpot et al., 1986; McConnell et al., 1988). "Neoplastic nodule" has been replaced with two terms: hyperplasia and hepatocellular adenoma (EPA, 1986b; Maronpot et al., 1986; McConnell et al., 1988).

(d) Schulte-Hermann et al. (1983) demonstrated that a significant incidence of hyperplastic liver nodules are found in the livers of untreated rats. The incidence increased to 100% over the course of the animals' two-year average lifetime and lesion size increased with age. In addition, these hyperplastic nodules showed similar characteristics to those observed in rats treated with known liver carcinogens. Schulte-Hermann et al. (1983) have concluded that the high incidence of hyperplastic liver nodules in untreated aged rats precludes the distinction between chemical promoters and initiating agents during long-term carcinogenicity bioassays in laboratory rodents, particularly when liver tumor incidence data are the primary criteria.

- The statistically significant increased incidence of hyperplastic nodules observed by Kociba et al. (1978) in the intermediate (10,000 pg/kg-day) and highest (100,000 pg/kg-day) TCDD treatment groups should be appropriately extrapolated to human exposure only through the use of a classical safety factor approach: the LOAEL divided by a safety factor of 1,000. In this case, the LOAEL for hyperplastic nodules in the rat liver (10,000 pg/kg-day) divided by 1,000 would yield an ADI of 10 pg/kg-day. This approach is identical to that taken by the United Kingdom (1989) when they derived a guideline level of 10 pg/kg-day, "which, when exceeded, should trigger investigation and appropriate measures to reduce environmental levels."

- The frequencies of tumors in other organ systems, specifically in the lung, tongue and palate, should not be considered in the dose-response assessment of TCDD. The Office of Science and Technology Policy (1985) does not recommend combining tumor incidences in different organs for the purposes of estimating a cancer potency. Furthermore, Kociba (1984)

has suggested that the tumor incidence observed in the lung and oropharynx is likely the result of periodically high localized concentrations of TCDD associated with surface contact of TCDD from the animal's diet, rather than an effect of systemic uptake.

- The use of a body weight correction factor in the rat-to-human extrapolation procedure is biologically more relevant than the use of a surface area correction factor for TCDD. In the case of TCDD, basic pharmacokinetics suggest that the parent compound is the biologically active moiety (Leung et al., 1988, 1989). Despite this evidence, the EPA used a surface area correction factor of 5.4 when extrapolating doses between Sprague-Dawley rats in the Kociba et al. (1978) study and humans.

5.2 Implications for Determining Acceptable Levels of Daily Exposure

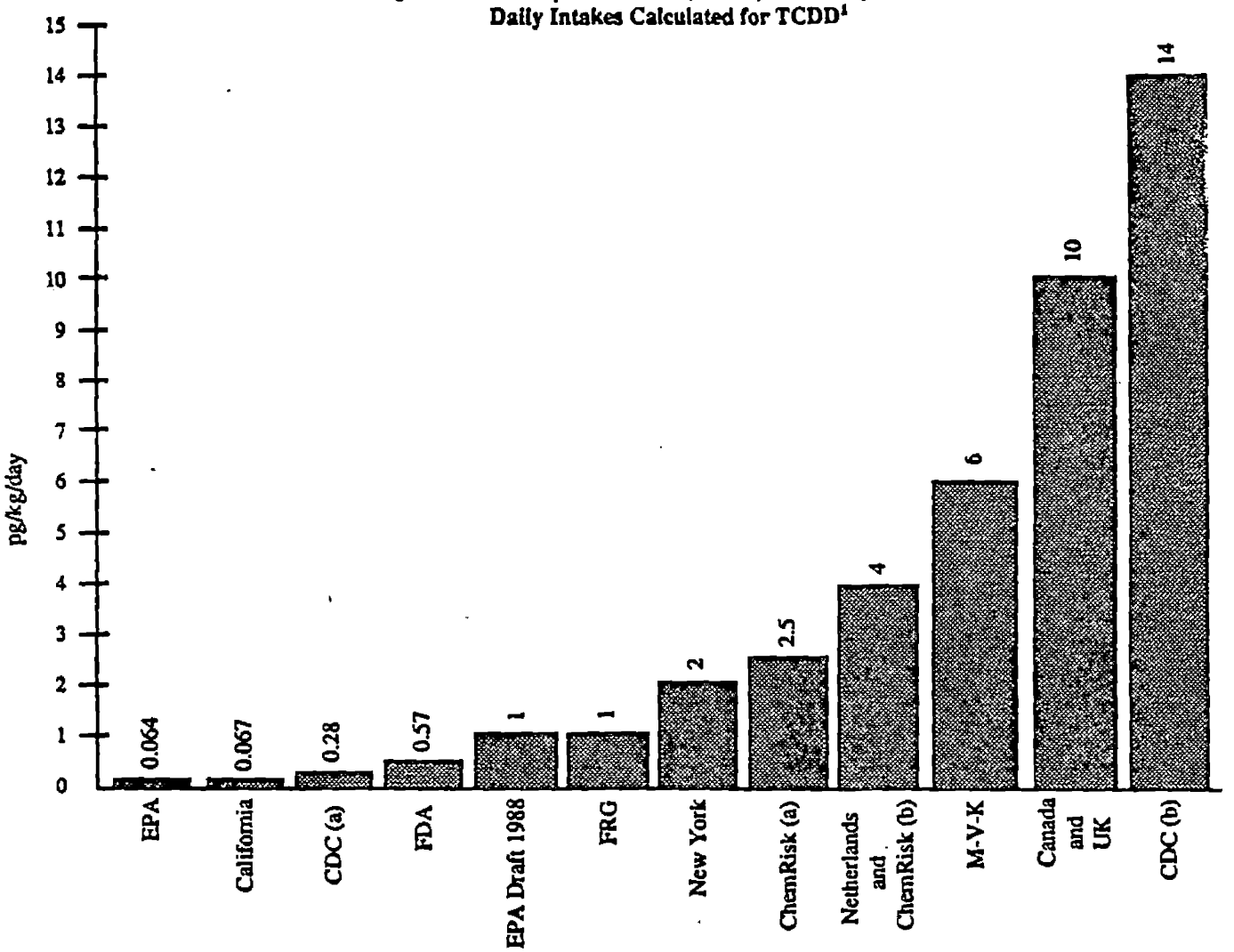
Based on this analysis and on the other scientific evidence summarized in this report, it is clear that the use of the LMS model in conjunction with the Kociba et al. (1978) bioassay to describe the carcinogenic dose-response of TCDD can result in a wide range of plausible risk estimates (Table 19). The choice of maximum likelihood estimate (MLE) for the RsD in preference to the 95% lower confidence limit (LCL) estimate, body weight instead of surface area scaling factor, and a better definition or biological interpretation of the reported liver lesions will result in RsDs much different than the estimates adopted by the EPA (1985, 1988) and the CDC (Kimbrough, 1984).

Acceptable daily intakes and RsDs have been developed by various U.S. agencies and other countries on the basis of some of these considerations. It is evident from Figure 5 that the application of different extrapolation procedures to the same animal cancer bioassay data has resulted in markedly different exposure limits for TCDD. Risk estimates by various regulatory agencies have ranged from 0.064 pg/kg-day (EPA, 1985) to 14 pg/kg-day (Kimbrough, 1984) when expressed at the 1×10^{-5} level of incremental risk.

ChemRisk further concludes that the weight of scientific evidence indicates that TCDD should be regulated as if it exhibited a threshold of carcinogenic action. The scientific evidence does not support risk estimates for TCDD which are based on linear low-dose extrapolation models. The most appropriate method for estimating permissible limits for human exposure, given the present state of knowledge in this interim period before a biologically-based pharmacokinetic model is validated experimentally, is the application of a safety factor to the NOAEL for carcinogenicity observed in the Kociba et al. (1978) study.

Although the use of the safety factor approach may be most appropriate for the dose-response assessment of materials such as TCDD that lack genotoxic potential, it is recognized that others may wish to use the linearized multistage model or some other nonthreshold model of low-dose extrapolation in

Figure 5. Risk-specific Doses (at 10^{-5}) and Acceptable Daily Intakes Calculated for TCDD[1]



EPA	U.S. Environmental Protection Agency - RsD
California	State of California - RsD
CDC	Centers for Disease Control, U.S. Public Health Service - RsD (a) Most conservative figure of range (b) Least conservative figure of range
FDA	U.S. Food and Drug Administration - RsD
M-V-K	Mesgaritar, Verizon, Kanalson - RsD
FRG	Federal Republic of Germany - ADI (ADI shown is most conservative figure of a range calculated by FRG)
New York	State of New York - ADI
Netherlands	- ADI
Canada	Ministry of Ontario Health and Welfare Canada - ADI
UK	United Kingdom - guideline level
ChemRisk	-RsD (a) Conservative plausible alternative using 95% LCL (b) conservative plausible alternative using MLE

1. U.S. agencies, including the EPA, have calculated and presented RsDs at various levels of risk. For the sake of consistency and to enhance the clarity of this document, the risk level of 1 in 100,000 has been used in the presentation of RsD estimates.

Table 19. Range of Plausible Risk-specific Doses (RsDs) for 2,3,7,8-TCDD Based on Considerations of Extrapolation Between Rats and Humans, the 95% LCL versus the MLE, and the Principal Carcinogenic Response Observed in Kociba et al. (1978)

Set of Assumptions[a]	Risk-specific Dose at 1×10^{-5} Incremental Carcinogenic Risk (pg/kg-day)
1. Surface Area + 95% LCL[b] + Total Hepatic Tumors[c]	0.064
2. Surface Area + MLE + Total Hepatic Tumors	0.088
3. Body Weight + 95% LCL + Total Hepatic Tumors	0.34
4. Surface Area + 95% LCL + Hepato. carcinomas	0.47
5. Body Weight + MLE + Total Hepatic Tumors	0.48
6. Surface Area + MLE + Hepat. carcinomas	0.74
7. Body Weight + 95% LCL + Hepato. carcinomas[d]	2.5
8. Body Weight + MLE + Hepato. carcinomas[d]	4.0

a. Each set of assumptions were developed from the Kociba et al. (1978) bioassay in female Sprague-Dawley rats, Spartan substrain, on the basis of three choices:

1) extrapolation from rats to humans using a surface area versus a body weight correction factor;
2) adoption of the 95% Lower Confidence Limit (LCL) of the human RsD versus the Maximum Likelihood Estimate (MLE) of the potency in the rat; and,
3) the consideration of hepatocellular hyperplastic nodules and carcinomas (total hepatic tumors) versus hepatocellular carcinomas as the principal response observed in the Kociba et al. (1978) bioassay.

b. The 95% LCL is that dose for which there is a 95% statistical confidence that the true RsD is no lower than this value.

c. These assumptions represent the basis for the EPA's current estimate of the human RsD for 2,3,7,8-TCDD.

d. These assumptions are considered by ChemRisk to represent the most plausible basis for an alternative human RsD for 2,3,7,8-TCDD.

conjunction with the Kociba et al. (1978) dataset. Since this is currently the case with U.S. regulatory agencies, then the carcinogenic behavior of TCDD in female Sprague-Dawley rats should be extrapolated to humans only on the basis of the incidence of hepatocellular carcinomas. A body weight scaling factor should be used in preference to the surface area assumptions. If the 95% LCL of the human RsD is adopted, then the RsD at a 1×10^{-5} risk level is 2.5 pg/kg-day of TCDD. If the MLE is adopted, then the appropriate RsD at a 1×10^{-5} risk level is 4.0 pg/kg-day of TCDD (Table 20). Both of these RsDs are more conservative than the ADI of 10 pg/kg-day, estimated by ChemRisk to be protective of hepatic hyperplasia, derived via a classical safety factor approach.

Table 20. Plausible Alternative Risk-specific Doses (RsDs) for 2,3,7,8-TCDD

Plausible Choices[a]	Risk-specific Dose at 1 x 10^{-5} Incremental Carcinogenic Risk (pg/kg-day)
1. Body Weight + 95% LCL[b] + Hepato. carcinomas	2.5
2. Body Weight + MLE[c] + Hepato. carcinomas	4.0

a. These assumptions are considered by ChemRisk™ (1989) to represent the most plausible basis for an alternative human RsD for 2,3,7,8-TCDD.
b. The 95% LCL is that dose for which there is a 95% statistical confidence that the true RsD is no lower than this value.
c. Maximum likelihood estimate.

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7.0 GLOSSARY OF TERMS

Acceptable Daily Intake (ADI) The amount of a chemical to which a person can be exposed on a daily basis over an extended period of time (usually a lifetime) without suffering a deleterious effect.

Adenoma A benign epithelial tumor in which the cells form recognizable glandular structures or in which the cells are clearly derived from glandular epithelium.

B2 carcinogen Probable human carcinogen; EPA weight-of-evidence category for an agent which has sufficient evidence of carcinogenicity in animals, but inadequate evidence of carcinogenicity in humans.

Cancer potency The incremental excess cancer risk per unit of exposure (usually in mg/kg-day) derived from the upper-bound estimate of the low-dose slope of the dose-response curve.

Carcinogenic Possessing the capacity to initiate or promote cancer; producing carcinoma.

Carcinoma A malignant new growth made up of epithelial cells, tending to infiltrate the surrounding tissues and give rise to metastases.

Chloracne An acneiform skin condition caused by exposure to chlorinated hydrocarbons.

***De manifestis* risk** Term used to identify those risks which are of obvious or evident concern.

***De minimis* risk** Term used to characterize risks which are insignificant, negligible or of no concern, such that regulatory action is unwarranted.

Dose The quantifiable amount of a material introduced into an animal either through a route of administration or as a result of exposure, e.g., injection, ingestion, inhalation, or dermal contact.

Dose-response curve A mathematical function describing the relationship between the dose (i.e., the quantity) of the chemical administered to the organisms and the percentage response of the test population. The curve is plotted as response versus dose.

Epidemiology The study of the distribution and determinants of diseases and injuries in human populations.

Exposure Contact of a receptor organism with a chemical, biological, or physical agent which can be quantified as the amount of the agent available at the exchange boundaries of the organism (e.g., skin) and available for absorption.

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Foci (hepatic) Small lesions of less than one liver lobule in size; do not show any distruptions of the normal hepatic architecture; may be part of the spectrum of lesions capable of progressing to hyperplastic nodules. The first observable morphological hepatocellular lesion following initiation.

Genome The complete set of hereditary factors, as contained in the haploid assortment of chromosomes.

Genotoxic DNA-reactive; induces an adverse effect on the genetic material (DNA) of living cells that may be expressed as a mutagenic or carcinogenic event.

Hirsutism Abnormal, excessive growth of hair.

Hepatocyte A liver cell.

Hyperpigmentation Increased pigmentation.

Hyperplastic Nodule A small mass of tissue, either normal or pathological that has an increase in the number of normal cells.

Hyperplasia The multiplication or increase in the number of normal cells in normal arrangement in a tissue.

Initiator A carcinogen which, if not already electrophilic, undergoes metabolic transformation to an electrophile and reacts covalently with DNA such that the affected cell incorporates the DNA damage into its replicating genome.

LCL (95% Lower Confidence Limit) Dose for which there is a 95% statistical confidence that the true RsD is no lower than that value.

LD_{50} (median lethal dose) The statistically derived single dosage of a substance that can be expected to cause death in 50 percent of the animals tested.

Lipophilic Having an affinity for fats or oils.

LOAEL (Lowest-Observed-Adverse-Effect-Level) The minimum dose level, determined from chronic toxicity studies in laboratory animals, at which the first indications of adverse (toxic) effects are observed in the species tested.

Metaplasia The change in the type of differentiated cells in a tissue to a form which is not normal for that tissue.

Metastasis The transfer of disease from one organ or part to another not directly connected with it. The capacity to metastasize is a characteristic of all malignant tumors.

MLE (Maximum Likelihood Estimate) Derived from the linearized multistage model; the model's best estimate of the RsD.

Mutagenic Inducing genetic mutation or change in genetic material.

Mutation A permanent transmissible change in the genetic material, usually in a single gene; the change may be in the form of a loss (deletion), gain (translocation), or exchange (transduction) of genetic material.

Neoplasia The formation of a neoplasm, i.e., the progressive multiplication of cells under conditions that would not elicit, or would cause cessation of, multiplication of normal cells.

NOAEL (No-Observed-Adverse-Effect-Level) The highest dosage administered to laboratory animals that does not produce toxic effects.

Nodule A small mass of tissue in the form of a swelling, knot, or protuberance; either normal or pathological.

Pharmacokinetic model Quantitative predictive approach used to describe and predict events occurring during the process of drug or chemical disposition throughout the body, thus yielding tissue levels of the drug or chemical.

Pharmacokinetics The study of the action of a chemical in the body over a period of time, including the process of absorption, distribution, localization in tissues, biotransformation, and excretion.

Physiologically-Based Pharmacokinetic (PB-PK) Model Quantitative predictive approach which utilizes physiological parameters of the experimental animals to describe the pharmacokinetic process; allows for prediction of the relationship between administered concentrations of a drug and the resulting concentration found in target tissues.

Porphyria cutanea tarda Disorder characterized by disturbances of the metabolism of porphyrins and chronic skin lesions ranging from slight skin fragility to severe scarring.

Promoter A substance which, after repeated or prolonged exposure, acts to increase the tumorigenic response of a cell exposed to an initiator. Promoters are thought to affect cellular growth and differentiation and may alter a number of cell membrane properties. Promoters exhibit a threshold in their dose response and tumor promotion may be reversible upon removal of the promoter.

Reference Dose (RfD) An estimate of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. The EPA RfD is derived from the LOAEL or NOAEL by application of uncertainty factors or safety factors.

Risk A statistical concept defined as the expected frequency or probability of undesirable effects resulting from a specified exposure to known or potential environmental concentrations of a substance. A substance is considered safe if the risks associated with its exposure are judged to be *de minimis*. Estimates of risk may be expressed in absolute or relative terms. Absolute risk is the excess risk due to exposure. Relative risk is the ratio of the risk in the exposed population to the risk in the unexposed population.

Risk-specific Dose (RsD) The dose estimated to result in an upper-bound estimate of incremental lifetime cancer risk, such as 1 in 100,000.

Statistical significance An inference that the probability is low that the observed difference in quantities being measured could be due to variability in the data rather than an actual difference in the quantities themselves. The inference that an observed difference is statistically significant is typically based on a test to reject one hypothesis and accept another.

Threshold concentration A concentration above which some effect (or response) may be produced and below which it will not.

Toxicant An agent or material capable of producing an adverse response (effect) in a biological system, seriously injuring structure or function or producing death.

Toxic Equivalency Factor (TEF) Conversion factor used to quantify toxicity of measured dioxin and furan congeners relative to 2,3,7,8-TCDD.

Toxicity The inherent potential or capacity of a material to cause adverse effects in a living organism.

Virtually Safe Dose (VSD) See Risk-specific Dose. Term used by CDC which is synonymous with the term RsD which is currently used by the EPA.

Occupational exposure.
R. Kimbrough and P. Grandjean, 1989.

Purpose: A review of occupational exposure to PCBs and other compounds, focusing on exposure potential, retention levels in human tissues and body fluids, and systemic effects.

Conclusion: *Exposure scenarios have been documented since World War I, of which chlorance is the most characteristic clinical effect caused by PCB compounds. Several systemic effects have been linked to PCBs, including liver dysfunction and respiratory irritation. Occupational exposure recommendations range from 0.5 to 1.0 mg/m^3 in other countries, while in Sweden and Japan have limits of 0.01 and 0.1 mg/m^3, respectively.*

Exposure History: In World War I, chlorinated naphthalenes were used for production of gas masks and material for detonators. Chlorinated biphenyls were used for insulating material for cables and condensers. These compounds were used extensively in the shipbuilding industry during World War II. PCBs were introduced in the early 1930s for dielectric fluids for capacitors and transformers. Repair and maintenance exposure levels for PCBs ranged up to 60 μg/m^3, while surface contamination ranged from 4-60 μg/m^2 and chronic leaking was 33 μg/m^2. Skin exposure from capacitor production was 5 μg/cm^2. PCB levels in blood from chronic exposure have been found to range from 6-3500 ng/ml.

Exposure Effects: Exposure to chlorinated compounds was revealed by the appearance of chloracne, which is the most characteristic clinical effect caused by these agents. The onset of chloracne occurred within a few days of exposure, as well as, being delayed for several weeks. Lesions formed on the face, neck, skin area behind the ears, back, arms, and legs. In males, the genitals were also involved.. Chloracne remained active for many years, with deep-pitted scars remaining as a residual effect. Treatment has included vitamin A acid, UV light, X-ray, lancing and expressing of pustules. Temporary remissions were achieved with accutane, but possible teratogenic effects are a side effect. Chloracne was thought to be due to external contact and not the result of systemic exposure to the acnegenic agents. Accidental ingestion of chloracnegenic agents resulted in a variety of adverse effects, including chloracne. The identity of all agents which may be chloracnegenic in humans has not been established. Most reported cases of PCB-related chloracne involved exposure to vapors which developed when PCBs or mixtures of chlorinated terphenyls were heated. It is extremely difficult to relate particular effects to individual compounds, due to the occurrence of several compounds in occupational exposure.

Occupational exposure.
(continued)

Systemic Effects:

Specific effects attributed to PCBs are difficult to describe. Chloracne was present in all 17 workers engaged in PCB production. Other symptoms included loss of appetite and libido and lassitude. One study reported complaints of burning eyes, nose and throat, dry throat, nausea, and dizziness. Three fatalities from jaundice prompted a study of systemic

effects of PCBs. Recent studies include PCB-related changes in hepatocytemorphology. Liver dysfunction and chloracne occurred in workers exposed to PCB vapors for 5-14 months. Capacitor production workers complained of face and skin burns, persistent body odor, and eczematous rashes. In other studies, liver injury was found. Acute PCB exposure has caused elevated serum triglycerides, total cholesterol and phospholipids. Experimental animal studies indicated that lipid metabolism in the liver may be affected by halogenated biphenyls. Higher serum lipid levels may be directly responsible for increased serum PCB levels, since PCBs are lipid-soluble. Thus, one study found correlations between serum PCB and serum lipids to disappear when the PCB concentration was expressed on a lipid basis. The induction of mixed-function oxidases is another effect of PCBs on the liver. Exposed workers had a plasma antipyrine half-life significantly lower than controls. Upper respiratory irritation was reported by 48% of 326 workers in a capacitor plant, with a restrictive pattern of impairment demonstrated from spirometric studies. Immunotoxicity may provide a mechanism for some adverse effects, although the evidence available is very limited.

Cancer:

Research has been delineated into two main areas. First, cancer incidence rates have been determined in cohorts of workers with documented exposures to chlorinated compounds. Second, case-referent studies have mainly linked soft-tissue sarcomas to usage of herbicides with possible dioxin contaminants. Workers exposed to a PCB mixture in a refinery plant had significantly higher than expected rates for pancreatic cancer. A cohort study of 2567 workers from a capacitor plant followed for 15 yrs, with PCB exposure of 2 yrs or less, resulted in increased deaths due to liver cancer, liver cirrhosis and rectal cancer. In Italy, increased cancer mortality occurred in capacitor plant workers.

Prevention:

In 1973, OECD requested member countries not to use PCBs except for enclosed systems in transformers, large capacitors, heat-exchangers and hydraulic systems in mines. A subsequent directive from the CEC prohibited use of products containing more than 0.1% PCBs and PCTs. OSHA has determined a PEL of 1mg/m^3 for PCBs containing 42%

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Occupational exposure.
(continued)

chlorine and 0.5 mg/m^3 for PCBs with 54% chlorine. NIOSH has recommended that exposure to PCBs in the workplace be limited to or below the minimum reliable detectable concentration of 1 μg/m^3. According to the ILO and WHO, occupational exposure to PCBs in other countries ranged from 0.5 mg/m^3 to 1.0 mg/m^3. In Sweden, the exposure limit for all PCBs is 0.01 mg/m^3, with a short term limit of 0.03 mg/m^3; while in Japan the limit is 0.1 mg/m^3.

Specific PCB congener distribution in adipose tissue of Canadians.
Jos Mes et al. 1990.

Purpose: Human adipose tissue was analyzed for 34 selected PCB congeners of which 24 were confirmed.

Conclusion: *Several congeners were found to be prevalent in adipose tissue of Canadians; while other congeners were found to have a higher percentage in females and different age groups.*

Remarks: Selected congeners represented greater than 85% of total PCBs in human milk fat. The possible effects of age, sex, and geographical area of sampling on PCB congener distribution were explored. Samples were taken from the abdominal and kidney region of 81 males and 27 females, with an average age of 42 yrs (range 3-85 yrs).

Results: Congeners 138, 153 and 180 made up 52% of all congeners found in adipose tissue of Canadians, therefore being the major PCB contributors to body burden. Lower total congener levels were found in the western region of Canada than the other regions. Since there were no outstanding differences among the regions, the authors assumed a common source of contamination. The percent distribution of congeners 52, 99, and 157 was significantly higher in females than in males. Also the percentage of many tri- to hexachlorobiphenyls were significantly higher in breast milk than in adipose tissue, while many hepta- to nonachlorobiphenyls were significantly lower. Residue levels of PCB congeners 156, 180, 189, 194, 201, 203, 206 and 209 were significantly lower in the 0-25 age group than in the >51 year age group. Previous observations have shown a positive correlation between PCB residue levels and age, based on total PCBs, not individual congeners.

GENP 011476

Organochlorine pesticides and PCBs in tissues from Dutch citizens (1968 -1986). P.A. Greve and P. Van Zoonen. 1990.

Purpose: To present results of monitoring data of occurrence of organochlorine pesticides and PCBs in humans (adipose tissue, milk, blood) from 1968-1986 in Dutch citizens to compare with results from other countries.

Conclusion: *Only a limited number of organocholrine compounds can be monitored satisfactorily in blood due to low concentrations. The values from this study can serve as 'reference' or normal values, even though variations in blood levels are higher than those in adipose tissue. The time trends appear to be a good instrument for monitoring the effect of certain measures against the use of certain persistent fat-soluble chemicals.*

Remarks: Earlier old methods were compared with new analytical methods to ensure comparable results over a 20 year period. Very carefully detailed and checked for accuracy of methods. Adipose tissue was collected from older adults post-mortem. Milk samples were collected from mothers at 2 maternity centers and blood samples were taken from 19 year old males. Samples are not random and some groups are overrepresented.

Results: One of the objectives of the study was to establish possible time trends for the compounds investigated. p.p'-DDT had a downward trend reflecting the ban of this compound in many countries. The metabolite p.p'-DDE does not show a tendency to decrease in concentration due to more resistance to degradation and excretion than p.p'-DDT. HCB had an increase in the early 1970s associated with the increase in products of animal origin. Recently, measures taken to avoid contamination have lowered HCB concentration in tissue. No trends were found for dieldrin although use of dieldrin and precursor aldrin were strongly reduced in last 15 years. Little, if any, downward trend seen for B-HCH which was surprising due to prohibition of BHC mixtures in many parts of the world on edible crops and animals. The persistance of B-HCH in fatty tissues is high. No significant downward trend was found for PCBs, although their use has been reduced over the course of the study and the effect of these prohibitory measures remains invisible from the data. Other organochlorine levels were low and levels found in milk samples had a more profound downward trend.

The highest influence of age was found for p.p'-DDE and PCBs followed by HCB and B-HCB. The levels in blood follow the same trend as the ones for adipose tissue and milk. Significant increases of the levels in milk were observed during lactation for HCB, B-HCB and PCBs; while a significant decrease resulted for p.p'-DDE. Several other influences were

found to have no significant effect including: sex, origin (rural or urban), eating habits, or medication.

GENP 011478

Regional differences of PCB and PCQ concentrations in the blood and subcutaneous fat tissue of residents of Nagasaki.
T. Ohgami et al. 1989.

Purpose: To analyze PCB and PCQ concentrations in the subcutaneous fat tissue of residents of Nagasaki Prefecture and compare these levels between blood and subcutaneous fat tissue of people living in different regions.

Conclusion: *Regional differences were found for PCB blood and tissue levels, with fishery area samples having higher values. It was assumed that dietary exposure was greater for fishery regions than for agricultural or urban regions.*

Remarks: Samples were obtained from seventy-one people surveyed with no history of PCB contamination living in urban, agricultural, fishery and combined fishery and agriculture areas.

Results: Regional mean PCB concentration levels ranged from 1.39 to 6.0 ppb in blood and 320 to 1942 ppb in fat tissue and were higher for fishery areas than the urban or agricultural areas. PCB concentrations in subcutaneous fat tissue were 100 times higher than that in blood samples. PCQ concentrations detected in almost all subcutaneous fat tissue but with no regional differences. PCQ concentrations in blood were non-detectable.

Discussion: The authors concluded that it is a necessity to clarify occupations, diet, and living environment in controls due to regional differences.

GENP 011479

Coplaner PCBs in Swedish human milk.
K. Noren et al. 1990.

Purpose: To find the occurrence of PCB congeners (tetrachloro, pentachloro, hexachlorobiphenyl) in the Swedish population and investigate the long term trends of these concentrations.

Conclusion: *Non-ortho coplaner PCBs (77, 126, 169) were excreted in human milk. The concentrations of non-coplaner PCBs were higher than those of the most toxic PCDDs and PCDFs. The most toxic PCB, 3,3',4,4',5-PeCB (126) was the most abundant non-ortho coplaner PCB in Swedish human milk. The concentrations of the PCB congeners decreased during the time period studies and only minor changes in distribution of congeners occurred from 1976 to 1989.*

Remarks: Milk was taken from two mothers (25 and 36 years old) in 1989 during nursing of their first infant. The pooled samples were collected at the Mothers Milk Center in Stockholm in 1972, 1976, 1980 and 1988/89 with 60, 204, 305 and 120 samples, respectively.

Results: PCB levels were higher in milk from the older mother at 0.97 ug/g fat compared to 0.76. Total PCBs and DDT were also higher in the older mother. The distribution of congeners were similar despite the different total levels and the age of the mothers. The average levels of non-coplaner PCBs in milk from the Mothers Milk Center decreased from 1972 to 1989. PCB 126 was the most abundant among the congeners and is considered the most toxic with a response and potency similar to TCDD. Levels of PCB 126 are 50 times higher than those of TCDD, which were 1-6 pg/g fat.

GENP 011480

The relation of polychlorinated biphenyls to birth weight and gestational age in the offspring of occupationally exposed mothers.
P.E. Taylor et al. 1989.

Purpose: The authors studied the relation of PCBs to birth weight and gestational age among the live offspring of women occupationally exposed during manufacture of capacitors. Interviews were conducted in 1982 with 200 women having direct exposure jobs and 205 women who never held a direct-exposure related job in order to ascertain information concerning reproductive outcome.

Conclusion: *The authors concluded that the data indicate a significant relation between increased estimated serum PCB level and decreased birth weight and gestational age, and that the decrease in birth weight is at least partially related to shortened gestational age. The magnitude of these effects is likely to be negligible except among already low birth weight or short gestation limits.*

Remarks: Exposure was assessed as high-homolog PCB (Aroclor 1254), a continuous exposure variable estimated from an independently derived prediction model. Direct exposure jobs were defined as those in which direct contact with PCBs occurred during the manufacturing process. Indirect-exposure jobs including those performed in office and manufacturing areas where PCBs not directly used.

Results: Direct and indirect exposure groups had 172 and 184 births analyzed, respectively. A significant effect of high-homolog exposure was seen for birth weight. For gestational age a small but significant decrease was observed with an increase in estimated exposure.

Trends in the levels of some chlorinated hydrocarbon residues in adipose tissue of Canadians.
Jos Mes, 1990.

Purpose: Presentation of results of nationwide surveys of chlorinated hydrocarbon residues from 1969, 1972, 1976, and 1985 in Canadians and possible effects of age and sex.

Conclusion: *In general, the adipose tissue burden of reported residues in Canadians increased with age. A loss of fatty deposits during advanced age may have been reflected to some extent in the diminishing number of residues which are significantly different between the 26-50 and >51 age groups. Age did not appear to affect the depletion of residues from body over the years. Although rates of elimination may differ, the same general downward trend was observed regardless of age group.*

Remarks: Data was categorized in three age groups, 0-25 yrs, 26-51 yrs, and >51 yrs.

Results: Statistical significant differences were observed for all residues between the years of 1972 and 1985. Residues of dieldrin and DDT decreased steadily and was significant from 1969 to 1985 in both females and males. PCBs significantly decreased in males between 1976 and 1985. DDE residue levels in males decreased significantly until 1976. PCBs and OCl in adipose tissue of males did not change significantly from 1972 to 1976. There were few significant differences in residue levels between males and females. In 1972, significantly higher PCB levels were recorded for males than females. In general it was found that residue levels tend to increase with age. No definite trend was observed for PCBs. A downward trend was observed in each region between 1969 and 1985.

Discussion: PCB use was restricted in 1977, in Canada, with prior surveys showing no decrease in residue levels, but by 1985 adipose tissue burden had halved. There were no consistent and significant difference in residue levels between the sexes.

PCBs and organochlorine pesticides in milk of Norwegian women during lactation.
J.U. Skaare and A. Polder. 1990.

Purpose: To investigate the variation of organochlorine contamination levels in human milk during lactation and to assess the influence of parity, dietary and smoking habits.

Conclusions: *The general tendency seemed to be a decrease in organochlorines in milk fat during lactation periods. Also findings of a statistically significant decline during lactation of PCB and HCB; and a decline in DDE demonstrated that mothers decrease their body burden of these compounds by milk excretion.*

Remarks: There were a limited number of subjects (n=14) as the data were pooled, using data only from first child lactation period. Eight of the women were Norwegian natives while six others were from Europe with at least 7 years of residency in Norway.

Results: HCB (hexachlorobenzene) and PCB levels were significantly reduced during the period of observation. DDE levels were also reduced but not significantly. There were significant declines in the first child only group for HCB, DDE, PCB but not for the second child group. The mean levels of PCB, HCB, and DDE in the first child group were higher than corresponding levels in milk for the second or third child groups. PCB, DDE, and HCB levels reduced 80% from first to second child lactation periods starting 3.5 years later. The highest HCB levels were found in milk from non-smoking mothers born outside of Norway.

Discussion: Overall levels of HCB, PCB and DDE were lower than levels found earlier in Norway in 1972, 1978 and 1981. Mean levels of organochlorines in human milk are low compared to corresponding concentrations found in other European countries. Higher HCB levels in mothers born outside of Norway are assumed to reflect previous exposure, since they correspond to milk fat levels reported in their home countries. Also, HCB in Norway is an industrial waste product and not directly used in agriculture. The findings of higher levels of PCB, DDE, and HCB in first lactation agreed with earlier studies. No pronounced changes in levels during the second child lactation were attributed to short accumulation time from first child and a corresponding low contamination of these compounds resulting from a ban on DDT (1971) and restriction (1971) and ban (1980) of PCB use.

Yu-Cheng
W.J. Rogan, 1989.

Purpose: A review of the literature concerning the oil disease epidemic known as Yu-Cheng, occurring in Taiwan in 1979.

Conclusion: *Comparisons to the Yusho outbreak in Japan showed that total PCBs were about 900 ppm to 30-90 ppm for Yu-Cheng rice oil. Although Yu-Cheng patients consumed ten times the amount of contaminated oil, the same amount of PCBs and PCDFs were found for both outbreak patients. Some evidence has been reported for a decline in PCB blood level concentrations for some congeners, while hexachlorinated biphenyls remained persistent.*

Background: Yu-Cheng refers to a syndrome of chloracne, hyperpigmentation, dilatation and hypersecretion of conjunctival glands caused by the ingestion of contaminated rice oil. Blood from victims as well as oil from both the purchaser and seller contained PCBs resembling Kanechlor 500, a PCB mixture of Japanese manufacture. As of 1983, 2061 cases had been identified with 5-10% of the cases severe. The cooking oil was speculated as being contaminated from pipe heating to remove odors and off-colors in the final processing stage. Repeated heating of the PCB mixture would lead to formation of PCTs, PCQs and PCDFs.

Clinical Features: Eye discharge and/or disturbance of vision was the most common complaint, while general malaise, numbness of limbs and headaches were also persistent. High rates of mucocutaneous pigmentation, acne and deformed nails also occurred. A limited number of patients had neurological testing which showed some sensory and motor nerve slowing, reduced sensory nerve conduction and mildly abnormal EEGs. Examination of offspring born to affected women showed that exposed children consistent delay in achievement of developmental milestones. PCBs and similar chemicals induce enzymes of the mixed-function oxidase class. Placentae from four women showed large increases in ary hydrocarbon hydroxylase levels. The persistence of this phenomenon was concluded to be due to the persistence of the chemicals, since the placental tissue was not present at time of exposure. Outbreak patients suffered more frequent severe skin and respiratory infections and a conviction that they had lowered resistance to illness.

Results: Most samples contained about 30-90 ppm total PCBs, compared to 900 ppm in Yusho rice oil. The Taiwan oil had between 0.1 and 1.68 ppm total PCDFs with these amounts strictly correlated with the amount of total PCBs, with a ratio of about 0.1-0.3%. Patients consumption of oil was estimated at a rate of 1.4 kg/month for 2.7 months before they became symptomatic, and then for another six months before the oil was

withdrawn. PCB concentrations ranged from 67-99 ppm and PCDFs from 0.21-0.40 ppm. Thus, patients consumed about 1 g of PCBs and 3.8 mg of PCDFs total. Taiwanese patients consumed roughly ten times as much contaminated oil as the Yusho patients, but got about the same amount of PCBs and PCDFs. PCB levels in blood, as of 1985, had a mean of 53.5 ppb. Samples drawn after only two years of exposure found a range of total PCBs from 10-720 ppb, with a mean and median of 38 and 28 ppb, respectively. The values were lower for the Japanese Yusho patients, probably due to a longer post-exposure period. Total PCB concentrations of 15 ppb were found in samples drawn in 1985, resulting in selective elimination of certain congeners. PCDFs in blood drawn within 6 months of exposure had a median of 0.09 ppb and a range from <0.005 to 0.27 ppb. As of 1983, 24 deaths occurred among exposed patients and 8 deaths among 39 babies born to affected mothers. Liver disease and hepatoma were the primary reasons for death, although in Taiwan there is an extraordinary prevalence of hepatitis B, and that cirrhosis and liver cancer are common.

Discussion: The majority of information consists of observations without controls on groups who are not well defined. Nevertheless, the evidence from Taiwan coupled with that from Japan leads to a reasonably consistent picture of the illness produced by exposure to thermally degraded PCBs.

Levels of dioxins, dibenzofurans and other chlorinated xenobiotics in human milk from the Soviet Union.
A. Schecter et al. 1990.

Purpose: To determine differences in levels between various geographical areas of PCDDs and PCDFs. To characterize the existence of chlorinated dioxins, dibenzofurans, PCBs and other chlorinated chemicals in the Soviet Union in human tissue, food, wildlife and the environment, with results to be compared with WHO studies on dioxins in human milk.

Conclusion: *The study documents for the first time the existence of dioxins, dibenzofurans, and other chlorinated chemicals in the USSR in human tissue and milk. The findings suggest levels and a pattern of dioxin and dibenzofuran congeners which permit "fingerprinting" of Soviet tissues from humans in comparison to other countries. Regional differences were noted within the Soviet Union.*

Remarks: All samples were pooled before analyses with the following breakdown by country: Soviet Union (24), USA (40+), W. Germany (140+). No statistical analyses were reported.

Results: The OCDD mean value was markedly lower for Soviet Union (30-80 ppt) in samples on a fat basis, than for W. Germany (185 ppt) and USA (163-303 ppt). The average heptachlorinated dioxin value was also much lower in the Soviet Union (5-16 ppt) than for W. Germany (34 ppt) and USA (50 ppt). Three hexachlorinated dioxins held the same pattern with lower values reflecting less pollution in the Soviet Union. PnCDD values were similar for all three countries with Siberian samples reflecting a more recent industrialization there with less accumulated pollution due to lower values. TCDD, the most toxic of the dioxins and dibenzofurans, values are similar.

For OCDF, the Soviet samples are all below the levels of the USA and W. Germany. Soviet HpCDF mean levels (0.6-2.6 ppt) were less than those from USA (2.4-5.7 ppt) and W. Germany (9.9 ppt). Values were similar for 1,2,3,4,7,8-HxCDF, 1,2,3,6,7,8-HxCDF and 2,3,4,6,7,8-HxCDF in all three countries. For 1,2,3,7,8-PnCDF, the Soviet samples are higher than the USA or W. Germany, possibly due to sampling or different pollution patterns. The highly toxic 2,3,4,7,8-PnCDF was found in W. Germany to exceed the values for the USA and Soviet Union, while TCDF values were similar in all three countries.

Mean levels of dioxins (total PCDD) in the Soviet Union are lower than those in the USA and W. Germany. PCDFs showed little difference among the countries with W. Germany having the highest values. Total PCDD/Fs in human milk are lower in the Soviet Union.

Adipose tissue/serum partitioning of chlorinated hydrocarbon pesticides in humans. L.L. Needham et al. 1990.

Purpose: To measure the levels of chlorinated hydrocarbon pesticides in both adipose tissue and serum to investigate how these compounds partition between these two compartments.

Remarks: More measurements are taken from serum blood than tissue. Partitioning is expressed as a ratio of adipose tissue (lipid weight) to serum concentration (lipid weight and albumin content).

Results: Data by gender found no significant differences in the concentration ratios for men and women. Concentration ratios were given for adipose tissue and serum blood samples in the following measurements:

- lipid weight to whole weight
- lipid weight to lipid weight
- lipid weight to albumin weight

Distributions of chlorinated hydrocarbons analytes in adipose tissue and serum are presented with concentrations being very close to the detection limits.

Discussion: Mean and median concentration ratios are greater than 1 for all analytes. PCB concentration ratios should be approximately 1.0 when calculated on a per unit of lipid. Concentration ratios for various compounds are influenced by how they are transported in blood. In an earlier study, the authors reported that nearly 100% of in vitro spiked 2,3,7,8-TCDD was found in the plasma portion of whole blood.

Comments: Concentration ratios were never explained as to their importance or relevance to a discussion on chemical exposure in humans.

GENP 011487

Neurobehavioral dysfunction in firemen exposed to PCBs: possible improvement after detoxification. K.H. Kilburn et al. 1989.

Purpose: A comparison of neurobehavioral functions of PCB exposed firemen before and after a detoxification regimen and to a comparable non-exposed group.

Conclusion: *Changes across the detoxification interval were selective. There were minimal changes in affective status between the testing sessions (exposed group) which suggests that subjects perception of distress did not improve. It was concluded that affective disorders were not causing neurobehavioral dysfunction due to only one test being significant correlated to Profile of Mood States (POMS). The effectiveness of the detoxification program was not answered conclusively, because memory and cognitive function may be influenced by prior testing. The non-exposed group had other exposures over a 2 to 25 year period, discounting their validity as a referent group. This study suggests that subjects with neurobehavioral dysfunction may be aided by diet, exercise, and sauna-induced excretion of PCBs.*

Remarks: Fourteen firemen were exposed to fumes, smoke and gases from transformers containing PCBs. Inhalation time was 15 to 30 minutes without a breathing apparatus, while dermal contact occurred with skin and soaked hands with and without gloves. Symptoms began 2 days to 3 months after the fire and included: fatigue, headaches, muscle weakness, aching joints, memory loss, hypertension, insomnia, irritability and loss of balance. Four months after the fire neurobehavioral, medical and biochemical studies were performed. Afterwards a 2-3 week detoxification program was implemented including a regulated diet, exercise twice daily, and a daily sauna (heat stress). Neurobehavioral tests were repeated for the exposed group after completion of the detoxification program. A matched comparison group consisting of fourteen non-exposed firemen from the area served as the control.

Results: Firemen exposed to PCBs had poorer neurobehavioral functions than the unexposed group. There were significant differences for most of the tests performed. Serum PCB and body content of PCBs were not significantly correlated nor were they correlated with any neurobehavioral test. After the detoxification program some of the test scores improved significantly, while only one test worsened significantly.

Discussion: There is a concern that memory and cognitive function may improve on repeat testing because of familiarity, practice, motivation and learning (although considered unlikely in this study). Another concern is whether affective disorders adversely affect neurobehavioral test scores as does post-traumatic stress.

Polychlorinated Biphenyls (PCBs):
Mutagenicity and Carcinogenicity
S. Safe (1989)

Purpose: To examine the mutagenetic and carcinogenetic properties of polychlorinated biphenyls (PCBs) in laboratory animals and humans.

Conclusions: *Overall, highly chlorinated PCBs (i.e.: Aroclor 1254) are not mutagenic in assays done with Salmonella typhimurium and E. coli. In rodents, highly chlorinated PCBs have been shown to cause hepatocellular carcinomas, adenocarcinomas, and neoplastic nodules. The carcinogenicity of the lower chlorinated biphenyls has not yet been determined. PCBs can also act as cocarcinogens and anticarcinogens which can enhance or inhibit the tumorigenic activity of other carcinogens. In humans it appears that overall mortalities and cancer mortalities resulting from exposure to PCBs is lower than expected, however, there was a significant increase in the number of deaths due to cancers of the liver, gall bladder, and biliary tract combined. There may be a connection between the degree of chlorination of the PCB and exposure.*

Remarks: This paper is based on reviews of studies done by other researchers and authors.

Results: Mutagenicity: As reported by Wyndham et al. (1976), the 4-chlorobiphenyl and the lower chlorinated PCB mixture, Aroclor 1221, were mutagenic to *Salmonella typhimurium* strain TA1538 in the presence of an external source of metabolic activation. The more highly chlorinated biphenyls (Aroclor 1254 and 2,2',5,5'-tetrachlorobiphenyl) were not mutagenic. However, studies done in the same laboratory pater on failed to reproduce the original observations. Others have also reported a lack of mutagenicity in bacteria of Arochlor 1254, 4-chlorobiphenyl, 3,3',4,4'-, 2,2',4,4'-tetrachlorobiphenyl and 2,2',4,4',6,6'-hexachlorobiphenyl (Schoeny et al. 1979).

In another study, Heddle and Bruce (1977) reported that Aroclor 1254 did not cause cytogenic effects in mice and Aroclors 1242 and 1254 did not cause cytogenic effects in the bone marrow of rats. It was also determined that Clophen A30 and A60 did not show clastogenic effects in *Drosophila melanogaster* (Nilsson and Ramel 1974). In ring dove embryos, chromosomal aberrations were observed when tested with Aroclor 1254 (Peakall et al. 1972).

Carcinogenicity - Laboratory Animals: In a chronic feeding studies done with Donryu rats, Kimura and Baba (1973) demonstrated that adenomatous nodules formed in the liver of female rats but not male rats. However, it was evident that the dose levels used were toxic. Kimbrough et al. (1975) used Arochlor 1260 (at 100 ppm for 21 months) in Sherman strain female rats and

found that 26/184 (14%) developed hepatocellular carcinomas; 144/184 (78%) developed neoplastic nodules and 182/184 showed foci or areas of cytoplasmic alteration. This is compared to 1/173, o/173 and 28/173 controls respectively for each type of lesion. In Sprague-Dawley rats (Norback and Weltmen 1984) Aroclor 1260 preparations caused hepatocellular trabecular carcinoma (23%), adenocarcinoma (26%), and neoplastic nodules (8%). Female rats were more susceptible than males to the hepatocarcinogenic effects of this PCB preparation.

The National Cancer Institute investigated the effects of Aroclor 1254 in male and female F344 rats in 1978. The results showed that at dose levels of 0,2,5,50, and 100 ppm in the diet for a period of 105 weeks, there was a dose-dependent increase of hepatocellular carcinomas and hyperplastic nodules in both sexes of rats. There was also a significant increase in intestinal metaplasia (male & female combined) at the 100 ppm dose level (Ward 1985). This is in contrast to the previously mentioned study where female S.-D. rats were affected by Aroclor 1260 more than male S.-D. rats; in this study the distribution of carcinogenic effects was equal between the F344 rats.

A study was done using Clophen A60 and A30 in the diet (100 ppm for up to 832 days, Schaeffer et al. 1984) on male Wistar rats. The Clophens both caused neoplastic nodules to form but only Clophen A60 caused hepatocellular carcinomas in 61% of the rats which was significantly different ($p < 0.05$) from controls (2%). Also significantly different was the formation of neoplastic nodules (40%(Clophen A60) vs 4%(Controls)), adenofibrosis (2% vs 23%), thymoma (0% vs 40%) and Wistar nephritis (0 vs 40%). The differences between Clophen A30 and A60 lies in the markedly increased hepatocarcinogenic potency of the higher chlorinated Clophen A60 (60% by weight of Cl).

The feeding of Kanechlor 500, 400, and 300 to male dd mice for 32 weeks at dietary levels of 500, 250, and 100 ppm showed that at the 500 ppm levels, Kanechlor 500 caused hepatocellular carcinomas (41.7%, Nagasaki et al. 1972). In BALB/CJ mice fed Aroclor 1254 (300 ppm) for 11 months, hepatomas increased by 40% where this did not occur in the control animals (Kimbrough and Linder 1974).

PCBs can also act as cocarcinogens or anticarcinogens which enhance or inhibit the tumorigenic activity of other carcinogens. However it should be noted that the more highly chlorinated commercial PCBs (>50% by weight) were reported as promotors of hepatocarcinogenesis in rodents and the lower chlorinated PCBs have not been determined. For example, Kanechlor 500 decreased the incidence of liver tumors in rats treated with the heaptocarcinogens 3'-methyl-4-dimethyl-aminoaxobenzene, 2-acetylaminofluorene, and diethylnitrosamine in the diet. Some anitcarcinogenic activities of Aroclor 1254 and PCB congeners have been looked at in mouse

skin models using PAHs as initiators. Results of one study by DiGiovanni et al. (1979) showed that Aroclor 1254 (100 μg/mouse) given 18 hours before the initiator 7,12-dimethylbenz[a]anthracene (DBMA), significantly decreased the incidence of papilloma formation in female Charles River CD-1 mice. 3,3',4,4'-tetrachlorobiphenyl was even more active as an anticarcinogen than Aroclor 1254. TCDD and the later mentioned PCB both significantly reduced the number of papillomas/mouse.

Human Studies: Analytical studies have shown that most individuals carry significant body burdens of PCBs in adipose tissues, ranging from 0.1 to 1.0 ppm. The residues found in these individuals come from many environmental pathways and their potential carcinogenic or anticarcinogenic effects have not been determined. The exception to this is the two major groups which have experienced very high levels of exposure to PCBs through the consumption of contaminated rice oil in Japan and Taiwan and through occupational exposure.

The most complete and comprehensive study was done by Brown and Jones (1981) and Brown (1987) on 2588 workers employed at two capacitor plants. The overall mortality and cancer mortalities were lower than expected, 295 and 62 respectively. In examining the cancer mortality data, the authors noted that the only category where there was a significant increase ($p > 0.05$) in deaths was associated with liver, gall bladder, and biliary tract cancers combined. Females were more susceptible to these cancers than males and the length of employment in plant number two was also important because more highly chlorinated PCBs were used there initially.

Discussion: Several conclusions can be drawn from looking at the cases mentioned in the results section:

1) PCBs can covalently adduct DNA *in vivo* and *in vitro* with the more highly chlorinated PCBs being poorly metabolized and therefore binding less frequently with DNA.

2) PCB mixtures and individual compounds exhibit very little mutagenic activity in most assay tests.

3) The more highly chlorinated PCB mixtures (>50% by weight) are hepatocarcinogens in rodents whereas data on lower chlorinated PCBs appear to not be carcinogenic.

4) In some model systems, higher chlorinated PCB mixtures act as promotors of preneoplastic lesions and hepatocellular carcinomas in rodents treated with a variety of initiators.

5) Occupational studies show that exposure to PCBs may cause an excess of cancers at some sites but the Brown study suggests that there are no significant increases in the overall cancer rate among workers.

Since PCBs are not mutagenic and do not easily form covalent adducts with cellular DNA it is unlikely that the higher chlorinated biphenyls are genotoxic and act as promotors of carcinogenesis in rodents. Remember that the incidence of hepatocellular carcinomas was much lower in the Aroclor 1254-treated animals than Aroclor 1260 which suggests a possible difference in the carcinogenic potencies of the two mixtures related to the differences in their composition.

Carcinogenicity of Polyhalogenated Biphenyls: PCBs and PBBs
E.M. Silberhorn, H.P. Glauert, and L.W. Robertson (1990)

Purpose: To report on the findings of the role that PCBs and PBBs play in the carcinogenic process and how they might influence tumor production by other agents.

Conclusions: *Polychlorinated biphenyls are capable of inducing preneoplastic lesions, neoplastic nodules, and hepatocellular carcinomas in rats and mice. They are suspected of being human carcinogens as well, but current evidence is inconclusive. PCBs do not appear to be mutagenic or genotoxic in the assays done to date. However, recently there has been some indication that they might be. PCBs are also active tumor promoters and are antitumorigenic under certain conditions.*

Remarks: This review will focus on the polychlorinated biphenyls (PCBs) only.

Results: Carcinogenicity: After reviewing many studies that involve PCB mixtures such as Aroclor 1254, Kanechlor 500, and Clophen A30 and others for example, evidence indicates that these mixtures induce preneoplastic lesions and hepatocellular carcinoma in animals when given at appropriate doses for long periods of time. PCB mixtures with a high chlorine content (ie: Aroclor 1254, Clophen A60, and Kanechlor 500) are more likely to induce neoplastic nodules and hepatocellular carcinomas than less chlorinated ones. Some studies have shown that female animals to be more sensitive to the tumorigenic effects of PCBs than males. For example, in a study done by Norback and Weltman (1985) male and female Sprague-Dawley rats were fed Aroclor 1260 (100 ppm for 16 months and 50 ppm for an additional 8 months) for two years followed by a control diet for five months. The results of the study showed that 96% of the female rats had hepatocellular neoplasms where in the males it was only 15%. Malignant tumors were found in 91.5% of the females and in 4.3% of the male rats.

There have been studies done on the modulation of carcinogenesis in rainbow trout. Results of these studies show that PCBs are not carcinogenic in trout or other fish species nor do they contain any promoting activity in the trout model. PCBs may modulate hepatocarcinogenesis in trout but this depends on a number of factors like choice and dose of the initiating carcinogen and the modulation (PCB) and the dosing regimen of the modulator relative to carcinogen exposure. When Aflatoxin B_1 (AFB_1) was coadministered with PCBs, or if PCBs were fed prior to AFB_1, it inhibited carcinogenesis in trout (Hendricks 1977; Shelton 1983, 1984) and the amount of inhibition was dose-related to Aroclor 1254. A null effect on tumor incidence has been observed when trout embryos were treated with AFB_1 then fed Aroclor 1254-containing diets after hatching (Hendricks 1977).

Mutagenicity & Genotoxicity: A number of PCB mixtures and congeners have been tested for mutagenicity using the *Salmonella* Ames Test. The majority of the studies done have found that PCBs are not mutagenic in this bacterial system. Schoeny et al (1979) tested Aroclor 1254 using this test and found it inactive. Others have found similar results in other strains of *Salmonella typhimurium*. Arochlor 1268, Kanechlor 300 and 500 have also shown negative results for mutagenicity. However, several PCB congeners have been tested for mutagenicity and have been mutagenic to strains TA98 and TA100 either with or without an exogenous activating system (S9). These congeners were 4-chlorobiphenyl, 2,2',4,4'-tetrachlorobiphenyl, 3,3',4,4'-tetrachlorobiphenyl, and 2,2',4,4',6,6'-hexachlorobiphenyl. Several bacterial PCB biodegradation products were also tested and found to be nonmutagenic in the *Salmonella* assay (Sayler et al. 1982).

In some *in vivo* and *in vitro* test systems PCBs have produced negative results. Mixtures of Aroclor 1242 and 1254 did not cause chromosomal abnormalities in bone marrow and spermatogonial cells or induce dominant lethality of rats (Dikshith et al. 1975; Garthoff et al. 1977; Green et al. 1973, 1975). Aroclor 1254 also did not caused unscheduled DNA synthesis in primary rat hepatocyte cultures (Probst et al. 1981). When V79 Chinese hamster cells were treated with up to 150 μg/ml of Aroclor 1242 or Clophen A60, neither produced mutagenic effects.

Despite overwhelming negative results concerning PCBs and mutagenicity, there have been some studies with positive results. The Japanese Ministry of Health and Welfare sponsored a study that found that Kanechlor 300 produced chromosomal abnormalities in mammalian cells treated *in vitro* and induced repair activity in certain bacterial strains. Kanechlor 500 was found to cause chromosomal aberrations in mouse bone marrow after *in vivo* PCB administration. Sargent et al. (1989) reported that Aroclor 1254 at 0.011 to 1.1 μg/ml caused chromosome breakage, rearrangements and mitotic delay in human lymphocytes cultured *in vitro*. There have also been studies done with PCB congeners and binary mixtures but they will not be discussed in this review.

Promotion of Tumors: Hirose and associates (1981) studied the effects of PCBs on hepatic and renal carcinogenesis induced by N-ethyl-N-hydroxyethylnitrosamine (EHEN) in male F344 rats. They found that rats fed 0.05% PCBs in the diet for 32 weeks after the EHEN treatment showed a statistically significant increase ($p < 0.001$) in the incidence of hepatocellular carcinoma when compared to controls given EHEN alone. However, there were no significant effects on renal carcinogenesis.

Anderson et al. (1986) were the first to show that tumor promotion outside the liver occurred in the lung. A single dose of Aroclor 1254 (500 mg/kg) given after initiation by dimethylnitrosamine (DMNA) in young male Swiss mice

caused almost twice the number of tumors in the lung than were found in the DMNA only controls. Mice given 50 and 250 mg/kg PCBs also had increased numbers of tumors but it was not significantly different from DMNA controls.

The promotion of putative preneoplastic lesions has been studied using a few two-stage liver carcinogenesis models. A single dose of Aroclor 1254 (500 mg/kg) promoted enzyme-altered foci (GGT+) in rat livers after partial hepatectomy and initiation by DENA (diethylnitrosamine) (Pereira et al. 1982). Oesterle and Deml (1981) showed that both Clophen A50 and A30 promoted enzyme-altered foci initiated by DENA in rat livers. Doses of 0.2 Nm/kg body weight per week the Clophen's enhanced the number of foci by 9 to 11-fold and 13 to 17-fold respectively. These investigators also found a no-effect-dose (2 mg/kg) for the promotion by Clophen A50 in weanling rats but not adult rats, which may suggest evidence for a threshold dose. In another study done by Oesterle and Deml (1982) it was discovered that there were sex-dependent-promoting effects of PCBs on altered hepatic foci induced by DENA in the rat liver.

Antitumor Activity: Hayes and associates (1987) transplanted liver nodules generated by DENA in the resistant hepatocyte model into the spleen of syngeneic rats previously given DENA. Subsequent exposure to Aroclor 1254 reduced transplant survival to an average of 8 versus 21% in controls.

Two studies by Kerkvliet and Kimeldorf (1977) have demonstrated that PCBs (Aroclor 1254) have antitumor activity and inhibit tumor growth in Sprague-Dawley rats inoculated with a transplantable tumor (Walker 256 carcinosarcoma). The authors speculate that PCBs may inhibit tumor growth by altering the immune response of the host either by increasing cellular immunity or by decreasing production of blocking factor.

Cocarcinogenesis and Modulation of Carcinogenesis: Makiura et al. (1974) found that Kanechlor 500 in the diet of rats inhibited tumorigenesis when treated concurrently with a known hepatocarcinogen (3'-methyl-4-dimethylaminoazobenzene). When Aroclor 1254 was given to pregnant Swiss CD-1 mice on day 19 of gestation and then the newborns were given DENA postnatally on day four or 14, two affects of pretreatment were noted. One, it reduced lung and liver tumor incidence at some times in mice given the DENA on day 14 but, two, it also increased the numbers of mice whose livers were completely tumorous at 18 months when DENA was given on day four. The researchers suggest that certain lung tumors with long latency may be selectively responsive to the protective effect of Aroclor 1254.

Intercellular Communication: Tumor promoters have been shown to affect gap junction structure and/or function and to inhibit cell communication. Inhibition of communication may disrupt normal cellular growth control thus allowing preneoplastic cells to progress towards neoplasia. This will not be

explored any further in this paper.

Human Epidemiologic Studies: Brown and Jones (1981) looked at workers employed by two U.S. electrical plants where PCBs were used by manufacturers. The PCBs used included Aroclors 1254, 1242, and 1210. Study results showed that overall total cancer mortality was lower than expected (39 observed vs. 43.8 expected) while there was excess liver cancer (3 observed vs. 1.07 expected) which was not significant. But Brown and Jones suggest that there is an association between both liver cancer and cirrhosis of the liver and occupational exposure to PCBs. There was no increase in risk associated with a longer length of employment Brown later discovered.

Bertazzi et al. (1987) reported on cancer mortality capacitor manufacturing workers (2100) exposed to PCBs (Aroclor 1254, Pyralene 1476 (54% Cl), and Pyralene 3010 and 3011 (42% Cl)) used between 1946 and 1982. The study revealed a statistically significant excess number of cancer deaths for both sexes compared to local population rates. Malignant tumors accounted for 14 deaths with only 7.6 expected in male workers while in female workers there were 12 cancer deaths as opposed to 5.3 expected. Bertazzi was reluctant to make conclusions about the study because of the small number of deaths and other study limitations.

Zack and Musch (1979) found a statistically significant increase in deaths from circulatory diseases in white males employed at a U.S. PCB production facility.

Gustavsson et al. (1986) examined Swedish capacitor workers exposed to PCBs. The number of cancer deaths was slightly elevated (7 observed vs. 5.39 expected) but the overall risk was well within the expected values. The only supporting evidence for chemically induced effects was the discovery of two relatively rare tumors in one person who had intermediate exposure for five years. Gustavsson concluded that his study did not indicate any excess mortality or cancer incidence at this factor to date.

Davidorf and Knupp (1979) looked at the epidemiology of ocular melanoma in the state of Ohio. They were unable to correlate the incidence of ocular melanoma with exposure to PCBs.

Chase et al. (1989) have looked at cohort mortality studies in terms of criteria for the association between PCB exposure and the development of cancer and he concludes that "There is insufficient evidence to show a causal relationship between PCB exposure and the subsequent development of any form of cancer."

Estimation of Carcinogenic Risk to Humans: The U.S. Environmental Protection Agency (EPA), Food and Drug Administration (FDA), and the National Institute for Occupational Safety and Health (NIOSH) have proposed or adopted standards or criteria for regulating exposure to PCBs. These guidelines have been used for the most part to quantify risk assessments for carcinogenesis by these compounds. The federal standards and criteria for PCB exposure do not make a distinction between PCB mixtures and individual congeners - they are all considered to be equally toxic or carcinogenic. Based on consumption of 2 L of drinking water and 6.5 g of fish and shellfish per day, water concentrations of PCBs of 0.79, 0.079, and 0.0079 ng/L were estimated to produce incremental lifetime cancer risks of 10^{-5}, 10^{-6}, and 10^{-7} respectively (Kimbrough et al. 1975). More recently, (Federal Register, Vol. 54, No. 97, May 22, 1989)the EPA has proposed a maximum contaminant level goal (MCLG) and a maximum contaminant level (MCL) for PCBs in drinking water. EPA has realized that an MCLG level of zero is unrealistic so they have proposed a MCL of 0.0005 mg/L for PCBs. This MCL level corresponds to an excess lifetime cancer risk of slightly less than 10^{-4}.

The American Conference of Governmental Industrial Hygienists (ACGIH) in 1980 set a threshold limit value (TLV) for an 8-hour time weighted average (TWA) concentration of 0.5 and 1.0 mg/M^3 for Arochlor 1254 and 1242 respectively. Short-term exposure limits (STEL) were set at 1.0 mg/M^3 for Aroclor 1254 and 1242. NIOSH has also recommended that workers not be exposed to a PCB concentration above 1.0 μg/M^3. This was a TWA concentration for a 10-hour workday and was the most reliable detectable limit at that time, 1977.

Deml and Oesterle (1987) evaluated the promoting activity of PCBs using a rat liver foci bioassay and found the lowest effective dose level to be 1 mg/kg body weight per day (430 μg/kg/day). They estimate the risk of exposure to PCBs to be very low since the LOEL was more than 1000-fold greater than the average daily intake for an individual as estimated by Cordle et al. (1978) to be 5 to 10 μg or 0.07 to 0.14 μg/kg/day for a 70 kg person.

Discussion: Polychlorinated biphenyls induce preneoplastic lesions, neoplastic nodules, and hepatocellular carcinomas in rats and mice when given at appropriate doses for extended periods of time. PCB mixtures that are high in their chlorine content (i.e., Aroclor 1260, Clophen A60, Kanechlor 500) are more potent than the lesser chlorinated PCBs in inducing neoplastic nodules and hepatocarinomas. It has also been noted that female species are somewhat more sensitive to the tumorigenic effects of PCBs than males of the same species. Experimental evidence appears to show that PCBs are not mutagenic or genotoxic although there have been some recent studies that suggest otherwise. The Ames bacterial mutagenicity assays, as well as the genotoxic assays are not optimized for the slow rate of metabolism of individual halogenated biphenyls.

Therefore it is possible that one or more PCB congeners may be metabolized to a reactive species with highly genotoxic potential.

PCB mixtures and individuals congeners are active tumor promoters in both rats and mice when given for extended periods of time after an initiating agent. The promoting effects of PCBs have been demonstrated in the liver, as well as in the lung and skin. Generally PCBs are immunosuppressive but it has been found that PCBs contain antitumor activity when they are administered either before or after tumor transplantation. PCBs also can act as cocarcinogens or modulate carcinogenesis. The timing of the PCB dosing relative to the administration of the initiator can enhance, inhibit, or have no effect on carcinogenesis.

Human epidemiological evidence is little and for the most part incomplete. However, some studies, like those relating to Yusho or Yu-Cheng, suggest that exposure to PCBs may increase the risk of hepatocellular carcinoma. Because of the results obtained through animal studies on carcinogenicity, it may be reasonable to assume that PCBs have the potential to be a human carcinogen as well.

Effects of Exposure to PCBs and Related Compounds on Growth and Activity in Children
J.L. Jacobson, S.W. Jacobson, and H.B. Humphrey (1990)

Purpose: The authors examined 236 children from two cohorts that were at risk for exposure to PCBs and related compounds at the age of four years. They explored growth patterns and activity levels of these children based on composite ratings provided by the mothers and independent examiners.

Conclusions: *Prenatal exposure to PCBs has effects on the growth in height and weight as well as activity in children. Both weight and activity are negatively correlated in a dose-dependent fashion to exposure to PCBs. Depressed activity levels in children can also be attributed to postnatal exposure from maternal milk when children are breast fed for at least 12 months. Prenatal exposure may cause more problems than postnatal exposure because of the sensitivity of migratory cells and cells undergoing mitosis to toxic insult, incomplete development of the blood brain barrier and/or the absence of important drug-metabolizing capacities that are found postnatally.*

Remarks: Cord serum PCB levels and mothers reported contaminated fish intake (over a six year period) from Lake Michigan were associated with reduced birth weight and shorter gestation.

Cohorts are represented by 236 children chosen from 8482 women who delivered infants in four Michigan hospitals in 1980-81 and from children who were exposed to PCBs through contaminated farm products (milk and meat) from farms whose animals had consumed silage with PCBs in it.

75% of the children assessed as infants were assessed at age four and four years and three months. Tests administered were the McCarthy Scales of Children's Abilities and reaction time tests.

Mothers were asked to complete the Peabody Picture Vocabulary Test-Revised (PPVT-R) and the Buss and Plomin Emotionality Activity Sociability (EAS) Temperament Survey for Children.

Results: Cohort Comparisons: The farm exposure families were more likely to live in rural areas, have more children, and less likely to be divorced or separated. Overall socioeconomic status (SES) was about the same for each cohort. There were no cohort differences in other perinatal risk factors including duration of prenatal care, gravidity, birth weight, gestational age, and delivery complications. Farm exposure serum PCB levels were about the same as fish consumers due to exposure from non-fish sources. Data was pooled because of these similarities except for cord serum and maternal milk levels which were not available for the farm cohort.

Cord serum PCB levels ranged from 0 to 12.3 ng/ml (mean = 2.5, s.d. = 2.0). Four-year serum levels ranged from 0 to 23.3 ng/ml (mean = 2.1, s.d. = 3.3). Mothers providing milk samples breast fed for an average of 29.6 weeks and PCB levels (fat basis) in milk ranged from 185.7 to 2600.0 mg/ml (mean = 835.9, s.d. = 388.4).

Prenatal exposure was associated with lower weight at age four in a dose-dependent fashion. Children that had a cord serum level of 5.0 ng/ml or greater tended to weigh 1.8 kg less on the average than lower exposed children. This was significant for girls but not for boys. None of the measures of PCB exposure were related to height or head circumference at four years of age.

The affects of PCBs on composite activity levels was negatively correlated to four-year PCB level in a dose-dependent manner. Thirty-one percent of the children with levels of 9 ng/ml or more fell in the bottom tenth percentile for activity. In the highest exposed group (9 ng/ml) 15.4% and 14.3% were rated "usually quiet and inactive" on the Child Behavior Record at the first and second testing sessions as compared to 5.4% and 7.8% for the lowest exposed group on visits one and two.

Activity level was also negatively related to maternal milk PCB level. In mothers with higher than average PCB levels and who breast fed for at least 12 months, the effect on the children's activity was the strongest.

Discussion: The data seems to indicate that the effects of *in vitro* exposure to PCBs and related compounds extend beyond the fetal and infant stages. There are affects on growth in height and weight as well as affects on activity in children. The four-year weight deficit is associated with prenatal but not postnatal exposure, a pattern which held up to five months postpartum. Although larger quantities of these compounds are transferred in breast feeding, most of the effects of low-dose exposure in humans have been linked to the prenatal period. This may be due to the sensitivity of migratory cells and cells undergoing mitosis to toxic insult, incomplete development of the blood brain barrier, and/or the absence of important drug-metabolizing capacities that are found post-natally.

The effects on activity due to prenatal exposure to PCBs and related compounds are contradictory in some cases. In some laboratory animal studies, increased levels of activity has been observed as well as in the children involved in the Yu-Cheng episode. However, decreased activity was reported in those children exposed to PCBs in the Yusho incident and in U.S. newborns. Postnatal exposure on the other hand seems to be more consistent. Reduced activity was found in two laboratory studies in rhesus monkey juveniles exposed both prenatally and via lactation and in two studies done with rats. In this study this was also observed. These depressed activity levels may

be associated with the duration of breast feeding; ie: they may be negligible . unless the infant is breast fed for at least one year.

Future studies may wish to explore more thoroughly the effects of exposure on behavior, based on a more direct behavioral coding of activity and/or automated recording of the child's movements. This is because the effects seen here was relatively subtle and its clinical significance is uncertain.

Polychlorinated Biphenyls and the Developing Nervous System: Cross Species Comparisons
H.A. Tilson, J.L. Jacobson, and W.J. Rogan (1990)

Purpose: The study looked at the effects that polychlorinated biphenyls have on the developing nervous system in various species and then to compare those effects.

Conclusions: Developmental exposure to polychlorinated biphenyls results in neurobehavioral alterations in monkeys, rats, mice, humans, and other species. Prenatal exposure results in hyperactivity, decreased motor activity, poor visual recognition memory, lower birth weights and shorter gestation periods in many of the species observed. Overall, the tests of higher cortical function in animals are affected, while in humans it is the delays in the development of motor skills. The data from these studies can be evaluated for the development of NOAELs, LOAELs, and RfDs for each of the species studied (see attached table).

Remarks: Comparisons of effects were made between Rhesus monkeys, quail, rats, mice, and humans.

This study was based on a series of laboratory studies done by other scientists to measure behavioral effects in various species.

Results: Rhesus monkeys: Bowman et al. (1978) fed 2,5 ppm Aroclor 1248 to nine adult female monkeys for 86-89 weeks (0.084 mg PCB/kg/day). Out of eight conceptions there were five live births, three of which were tested. Birth weights of the three-PCB-treated monkeys were 21% lower than controls and by the age of two months had developed characteristic PCB toxicity. The animals with the highest peak levels were more hyperactive at six and 12 months of age. The monkeys exposed to PCBs were also tested for PCB-induced alterations in learning and memory and changes in performance using the Wisconsin General Test Apparatus (WGTA). This test was given beginning at seven months and lasting until 24 months of age. The PCB-treated monkeys showed deficits on the first two discrimination reversal tasks (spatial & color). Monkeys were also slow in learning progressive probability shifts and the object alternation tasks. A peak PCB body burden at age four months corresponded significantly with increased errors in five of the nine learning tasks conducted from eight to 24 months of age. In a second study, Bowman took eight surviving breeding monkeys from the first study (cohort 1) that had been off the PCB diet for 22 to 84 weeks before the conception of a second set of infants. Five infants (cohort 2) survived for testing and the average PCB content in subcutaneous fat tissue for the mothers was 0.8 $\pm$ 0.9 ppm (SD) was only slightly higher than controls. The level of PCBs in two stillborn infants was 2-2.5 μg/g tissue. In four months of nursing, the infants experienced hyperpigmentation around the hairline, and showed increasing

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levels of PCBs in skin biopsies from birth (virtually not detectable) to three months (3.31 μg/g tissue). However, Bowman reported that there was very little PCB detected in subcutaneous fat of the offspring at eight and 12 months of age. These offspring showed hyperactivity in 24 daily 90-minute motor activity sessions at age 12 months. This is similar to the first cohort. In a third cohort (new breeding mothers), monkeys were fed Aroclor 1248 three times a week at 0.5 and 1.0 ppm respectively for 65 to 102 weeks. When motor activity was tested at 12 months and compared to the activity levels of control offspring in cohort 2, PCB exposure resulted in significant dose-dependent increases in motor activity.

Controls and treated animals from mothers removed from the 2.5 ppm PCB diet (cohort 2) and the offspring from mothers who received 0.5 ppm during gestation and nursing (cohort 3) were used in subsequent testing of schedule-controlled operant responding. Monkeys were tested under a series of fixed-interval schedules of food reinforcement. There was a slight but significant lowering of the index of curvature in the PCB-treated monkeys.

Quail: Japanese quail chicks were exposed to Aroclor 1254 (200 ppm) in feed beginning at seven days of age (Kreitzer and Heinz 1974). Exposure continued for eight days followed by six days of no exposure. After 24 hours of initial dose, chicks were tested for avoidance response to a moving silhouette. This response was suppressed in exposed animals even after untreated food was restored.

Rats: Behavioral testing began at 12 weeks of age after mothers were dosed at 20 or 100 mg/kg of Kanechlor 500 on days 8-14 or 15-21 of gestation. Open field trials and multiple T-maze trials (with escape from water as an endpoint) were conducted. Exposure to Kanechlor had no significant effect on total number of implants, number of resorptions, average litter size, or number of externally malformed fetuses. No pups from the 100 mg/kg group survived. Males exposed to 20 mg/kg on days 15-21 of gestation had increased numbers of errors in the water maze.

Koja et al. (1978) administered Kanechlor 400 (100 mg/kg orally) for six days to rats three and 20 days old. In a five minute test period there was decreased spontaneous motor activity in both groups. When three day old rats were dosed for 12 days at 100 mg/kg of Kanechlor 400 and were tested at three and four weeks there was decreased spontaneous motor activity and impaired performance on an inclined screen.

Overmann et al. (1987) exposed female Wistar rats via diet to 0.02, 2.5, 26, or 269 ppm of Aroclor 1254 from mating to weaning of their pups. The highest doses of PCBs decreased the number of litters and pup birth weight; most pups delivered to those mothers died within a week of birth. Lower concentrations of PCBs had no effect on pregnancy success, pup birth weight,

dam body weight, and food intake. Body weights were reduced and ontogeny of negative geotaxis, auditory startle and air righting reflexes in pups from mothers exposed to 26 ppm were altered prior to weaning.

Pantaloni et al. (1988) subjected female rats to Fenchlor 42 for five days, two weeks prior to mating, during gestation or during lactation. Exposure to PCBs did not affect maternal or pup weight up to 21 days of age. Preconception or in utero exposure had no effect on cliff avoidance, while in postnatally exposed groups this measure was suppressed. Development of swimming behavior was also affected in all three groups of exposed rats. Preconception exposure depressed activity in an open field at 14 and 21 days of age while in postnatally exposed groups, rats were suppressed at day 14.

Mice: Chou et al. (1979) studied the effects of 3,4,3',4'-tetrachlorobiphenyl (4-CB) on mice. 32 mg/kg of 4-CB were administered by gavage on days 10 to 16 of gestation. About one-half of the litters displayed a neurological syndrome which included increased locomotor activity at day 15. Animals showed jerking or rotational movement of the head and episodes of constant circling around the home cage. All the 4-CB treated mice had heterotopic motor neurons and astroglia in ventral spinal fibers and cylindrical CNS peninsulas that projected into the ventral cranial and spinal nerve roots.

Tilson et al. also studied 4-CB in mice. CD-1 mice whose mothers were exposed to 4-CB during gestation displayed at 35 and 65 days of age a neurobehavioral syndrome consisting of intermittent stereotypic circling and hyperactivity. In the dark phase of the diurnal cycle, 4-CB-affected mice were markedly hyperactive and had impaired grip strength and balance. The mice were also impaired in the acquisition of a one-way, shock-motivated avoidance response. In another study, Tilson found that at one year of age, mice exposed in utero to 4-CB had elevated levels of motor activity that were associated with decreased levels of dopamine and dopamine receptor binding sites in the caudate nucleus.

Human: Two cohorts of children have been followed in Michigan and North Carolina. Of the 313 newborns in MI, 242 of the mothers had consumed Lake Michigan fish presumed to be contaminated with PCBs. Higher cord serum PCB levels and maternal consumption of contaminated fish was thought to be related to smaller birth size and shorter gestation. This size deficit persisted beyond the newborn stage. The administration of the Brazelton Neonatal Behavioral Assessment Scale in MI to 287 newborns, tested motor coordination, muscle tone, orientation, and state changes (arousal & self-quieting). Infants whose mothers consumed large quantities of contaminated fish exhibited abnormally weaker reflexes and were less responsive to the stimulation of the Brazelton examination, and showed more jerky, unbalanced movement and more startles.

In NC, among 802 infants followed from a previous study, prenatal PCB exposure was associated with poorer performance on the Psychomotor Index from the Bayley Scales of Infant Development at six and 12 months of age. In addition, more highly exposed infants were hypotonic and exhibited a greater number of abnormally weak reflexes.

Again in MI, a test of visual recognition memory was administered to the infants. Cord serum PCB level and maternal consumption of contaminated fish were associated with poorer visual recognition memory performance at seven months among 123 children (a subgroup of those tested at birth). Postnatal exposure through nursing was not related to any of the scales used in MI or NC. These cohorts are still being studied.

Comparisons: Qualitative: "Developmental exposure to PCB results in persistent neurobehavioral alterations in monkeys and non-primates, similar neurological or behavioral effects are observed across species...[and] can be observed in the absence of reduced body weights or gross signs of PCB intoxification during development." The behavioral effects seen in rodents and avians exposed developmentally to PCBs were qualitively similar to those seen in Rhesus monkeys.

The most common finding in the animal studies was that developmental exposure to PCBs resulted in behavioral hyperactivity. This was observed in mice, male rats, and monkeys in which exposure was pre- or perinatal. Developmental exposure also decreased motor activity in short time periods of testing (five minutes or less). Higher cognitive processes or learning was influenced by exposure to PCBs during development in rats, mice, and Rhesus monkeys. Learning effects were observed using more complex discrimination tasks and schedule-controlled performance in monkeys as well as unconditioned and conditioned responses in non-primates.

In humans, lower birthweight in children with greater exposures was seen in MI but not in NC. The only other toxicity seen in either group was neurodevelopmental. Primarily motor effects were detected in both studies within the Brazelton Assessment. The other tests, the Bayley Psychomotor Index, was impaired by prenatal exposure in NC at six and 12 months and in MI it appeared to affect a subset of fine motor tasks within the Bayley. There is consistent evidence which points to prenatal exposure to PCBs at levels encountered in the U.S. which produce noticeable effects on motor maturation and some evidence of impaired infant learning. The authors of this paper conclude that these effects in humans are not due to DDE or social and demographic characteristics of the families.

Overall, the tests of higher cortical function in animals are affected, while in children it is the delays in the development of motor skills. The evidence of poor performance on visual recognition memory, a well-validated assessment

of infant cortical function is consistent with animal data. Tests on motor function in animals are relatively unaffected.

Quantitative: The available data were subjected to the risk assessment model proposed by the USEPA for developmental toxicity (1989). A NOAEL or "no-observed-adverse-effect level and LOAEL or a "lowest-observed-adverse-effect level were determined from the data and from these reference doses or RfDs were developed for each species of animal (see copy of attached table). In looking at the table, we can see that monkeys respond to the lowest doses and for rhesus monkeys, fetotoxicity, chloracne, and decreased postnatal body weights have reference doses that are an order of magnitude higher than for those for motor activity and cognitive tests. Dose information is not as readily available for the women who participated in these studies so a few references must be made about the estimated amount of PCB present in the fat of breast milk at term. If they assume that a woman weighs 60 kg, and the percentage of that weight which is fat, 25%, then a body burden for PCBs can be determined. If the theoretical level of PCBs in breast milk was 3.4 ppm, then the body burden of PCBs would be 51 mg of PCBs which the mother got from a daily dose of 5.6 μg/day, or 0.093 μg/kg/day. The safety factor would be 10 for susceptibility variation among exposed humans which would yield a reference dose of 9.3×10^{-3} μg/kg/day. For the specific and limited purpose of setting a reference dose, the rhesus monkey data yield an estimate of the reference dose one order of magnitude higher than the estimate calculated from the human data (Table 4); and the rodent data gives an RfD that is three orders of magnitude higher. The comparisons were made using that effect which results in the lowest RfD among the endpoints considered.

TABLE 4

REFERENCE DOSE COMPARISONS

	Type of Effect	Dose (mg/kg/day) LOAEL Range	NOAEL Range	Reference Dose*
Rodents:	Fetotoxicity	32–269	20–26	3.2×10^{-2}
	Postnatal Body Weights	—	2–10	2.0×10^{-2}
	Reproduction	32–269	4–26	3.2×10^{-2}
	Motor Activity	1–32	0.2–2	2.0×10^{-4}
	Learning/Memory	2–32	1–5	1.0×10^{-2}
Rhesus Monkeys: Aroclor 1248	Fetotoxicity and Chloracne	0.084	0.014	1.4×10^{-4}
	Postnatal Body Weights	0.084	0.014	1.4×10^{-4}
	Motor Activity	0.014	—	1.4×10^{-3}
	Learning/Memory	0.014	—	1.4×10^{-3}
	Performance	0.014	—	1.4×10^{-3}
Humans:	Hypotonicity on the Brazelton Scale		9.3×10^{-5}	9.3×10^{-6}
	Psychomotor Scale Decrement on 12-Month Bayley		9.3×10^{-5}	9.3×10^{-6}
	Impaired Visual Recognition Memory		2.7×10^{-5}	2.7×10^{-6}
	Carcinogenicity, Risk = 1×10^{-6} (EPA quantitative estimate, lifetime exposure) [(1), p. 95]		1.3×10^{-7}	
	Current EPA Reference Dose (based on low birthweight in Rhesus monkeys) [(1), p. 94]		1.0×10^{-4}	

*For animal experiments, intra- and interspecies variability were considered. For human data, only intraspecies variability was considered.

GENP 011507

Background Levels In Humans. ***In Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Products*****, R.D. Kimbrough and A.A. Jensen, 1989**

Purpose: To look at background levels of polychlorinated biphenyls (PCBs), polychlorinated terphenyls (PCTs), polychlorinated quaterphenyls (PCQs), polybrominated biphenyls (PBBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) in human blood, milk, adipose tissue, and other tissues.

Conclusions: *There are many factors that can affect the levels of PCBs present in the human body such as where they live, what they eat, how old they are, and what their past exposure has been. Levels of PCBs range from 0.5 to 4 ppm in milk, 0.5 to 10 ppm in adipose tissue, and 2 to 5 ppb in blood serum. However PCBs are not limited to just these mediums; they can also be found in the liver and the lungs. Levels of PCBs tend to be higher in the urban and industrialized areas of contries like the United States, Norway, and Sweden as well as higher in men than women.*

Remarks: Although this paper focuses on all of the above mentioned chemicals, this review will focus only on polychlorinated biphenyls (PCBs).

Results: Milk: There is a wide distribution of PCBs in milk ranging from 0.5 to 4 ppm (mg/kg) in the industrialized nations. This average background concentration varies with donors, sampling time and the analytical methods used. More than 60% of the total PCB content in human milk is made up of the following seven congeners: 2,4,4'-trichlorobiphenyl, 2,4,4',5-tetrachlorobiphenyl, 2,3',4,4',5-pentachlorobiphenyl, 2,2',3,4,4',5-, 2,2',4,4',5,5'-hexachlorobiphenyl, 2,2',3,3',4,4',5'- and 2,2',3,4,4',5,5'-heptachlorobiphenyl (Yakushiji et al. 1978; Schulte and Malisch 1984; Safe et al. 1985).

It should be noted that average background levels of PCBs in human milk do not vary much between countries when similar quantitation procedures are used. Levels of PCBs can vary depending on the lactation period, mothers' place of residence (PCB levels tend to be higher in urban and industrialized areas) and the degree of air pollution both indoors and outdoors. PCB levels tend to decrease with maternal age, parity, and during the lactation period.

Diet may also play a role in determining PCB levels in milk. For example, dairy and meat products mat be contaminated through the food chain or from feedstuffs. In some parts of the world (Michigan, Sweden, and Japan) PCB-contaminated fish are a key source of PCBs in milk.

Blood: In the blood, PCBs are bound to lipoproteins and are mainly in the plasma/serum fraction. The average background level in blood plasma or serum is estimated to be less than 5 ppb (Wasserman et al. 1979), and it may

be close to 2 ppb. The concentration of blood serum PCB levels tends to be higher in the United States but variations in analytical techniques and sampling methods may affect this.

The most abundant PCB congeners in blood from Upstate New York were 2,2',3,4,4',5'-hexachlorobiphenyl > 2,2',4,4',5,5'-hexachlorobiphneyl > 2,2',3,3',5,6,6'-heptachlorobiphenyl > 2,2',3,3',4,4'-hexachlorobiphenyl (Bush et al. 1984). Similar levels were found in Yusho/Yucheng patients.

During pregnancy, PCB levels in maternal blood were raised to approximately two times the levels before pregnancy, and decreased back to lower levels five months after delivery (Kodama and Ota 1980). PCB levels in cord blood are much lower than maternal blood at delivery (Masuda et al. 1978; Kodama and Ota 1980; Schwartz et al. 1983; Bush et al. 1984).
Some studies have shown a strong positive correlation between PCB levels in blood and age (Finklea et al. 1972; Kreiss et al. 1981, 1982; Schwartz et al. 1983; Stark et al. 1986; Humphrey 1983). PCB blood levels have also been positively related to alcohol intake and serum cholesterol (Kreiss et al. 1981; Stark et al. 1986) but negatively linked with obesity and enzyme-inducing medication (Kreiss et al. 1981).

Adipose Tissue: Most data concerning PCB levels in adipose tissue is obtained from autopsy samples not biopsy samples and are usually from people much older than those who provide blood and milk samples. Background levels of PCB in adipose tissue are reported to be 0.5 to 10 ppm (Wasserman et al. 1979), which is within the same range as milk levels.

Data from various countries are not comparable primarily because of the different analytical methods used. Donor materials also differ especially in males where levels often are much higher than females (Solly and Shanks 1974; Kraul and Karlog 1976; Grant et al. 1976; Mes et al. 1977, 1982; Mori et al. 1983; Wolff et al. 1982; Williams et al. 1984; Anasari et al. 1986), and increase with age.

In regard to background levels of PCBs in adipose tissue, the order of the different congeners is 2,2',4,4',5,5'-hexachlorobiphenyl > 2,2',3,4,4',5'-hexachlorobiphenyl, 2,2',3',4,4',5,5'-heptachlorobiphenyl > 2',3,4,4',5'-pentachlorobiphenyl (Jensen and Sundstrom 1974). In Italy it is these four congeners plus 2,2',3,3',4,4',5-hexachlorobiphenyl that constitute 60% of the total PCB content in adipose tissue.

Other Human Tissues: PCBs have been found in adult human liver and lungs from the general population in Japan. Average levels were between 10 and 100 ppb (Wantanabe et al. 1980); Masuda and Yoshimura 1984; Masuda et al. 1985). In Norway, Finland, and Denmark PCBs were also found in liver tissue with levels ranging from 1.87 ppm, 2.50 ppm, and 3.2 ppm lipid

basis respectively (Bjorseth et al. 1977; Hattula et al. 1976; Kraul and Karlog 1976). PCB levels in the brain were lower and averaged 0.76 ppm and 1.19 ppm in Denmark and Finland (Kraul and Karlog 1976; Hattula et al. 1976).

Discussion: It is known that PCBs accumulate in human tissues such as the liver as well as in blood and milk. This occurs most noticeably in the industrialized nations, for example, Norway, Sweden, France, and the United States. It appears that the background levels of PCBs in industrialized nations are about the same magnitude and at present time no significant downward trend seems to be occurring.

Genetic Toxicity. ***In Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins, and Related Products*****, eds. R.D. Kimbrough and A.A. Jensen, 1989**

Purpose: To look at the genetic toxicity of polychlorinated biphenyls, polybrominated biphenyls, polychlorinated dibenzodioxins, polychlorinated naphthalenes, and polychlorinated dibenzofurans.

Conclusions: Overall, the authors feel that polychlorinated biphenyls are not genotoxic. Test performed on Salmonella, E. coli, cultured mammalian cells, rats, mice, and other species show that exposure to Aroclor 1242, and 1254, Clophen A 30, 40, and 50, and Kanechlor 500 did not result in significant genotoxic endpoints.

Remarks: This paper looked at effects in bacteria and yeast, cultured mammalian cells, mammals, humans, and other categories of organisms.

Although this paper looks at all the chemicals mentioned in the purpose section, this review will focus only on polychlorinated biphenyls.

Results: Bacteria & Yeast: The majority of studies done have been with Aroclor or Kanechlor. Studies done with bacteria have shown with near unaminity in the literature that PCBs are nonmutagenic in *Salmonella typhimurium* (Aroclor 1254 being the test agent). Aroclor 1254 was also nonmutagenic in *E. coli*. Wyndham et al. (1976) reported that Aroclor 1254 was weakly mutagenic in *Salmonella* strain TA1538 in the presence on rabbit liver S-9; the negative results mentioned above used rat, mouse and hamster liver S-9s. Wyndham et al. (1976) also states that mutagenic activity increased as the degree of chlorination increased. Aroclor 1254 did not induce error-prone DNA repair in *E. coli* (Quillardet et al. 1985). Also in *E. coli*, Kanechlor 500 was positive in a test that measured induction of the *umu* operon in *Salmonella* (Oda et al. 1985).

Cultured Mammalian Cells: Tests in cultured mammalian cells showed neither gene mutations nor chromosome damage in cultured rodent cells treated with Aroclor 1254, 1242 or Clophen A40 (Amacher et al. 1979; Hattula 1985). Aroclor 1254 did not transform primary Syrian hamster embryo cells or induce chromatid breaks or mitotic inhibition when incubated with dividing human lymphocytes in culture (Hoopingarner et al. 1972). Unscheduled DNA synthesis in primary rat hepatocytes and single-strand breaks were produced when cells were treated with Aroclor 1254. This also happened in a mammalian cell line when treated with 2,2',5,5'-tetrachlorobiphenyl and its epoxide and derivatives (Stadnicki et al. 1979).

Mammals: In mammals, chromosome aberrations were not induced in the bone marrow of rats fed Aroclor 1254 for five weeks (Garthoff et al. 1977) or

when given Aroclor 1242 or 1254 by gavage in single or multiple doses. In mice, there was no induction of micronuclei in bone marrow when injected with Aroclor 1254 or Kanechlor 500 in ethanol. However there was a slight increase when Kanechlor 500 in corn oil administered by gavage.

Arochlor 1242 and 1254 did not produce dominant lethal effects in rats when given by gavage or by feed, just Aroclor 1254. However, some dominant lethal effects were noted in male rats weaned on female rats given Aroclor 1254 throughout lactation. These two Aroclors did not induce aberrations in rat spermatogonia as well (Dikshith et al. 1975; Garthoff et al. 1977; Green et al. 1975b).

Other Species: Other species tested for genetic toxicity were *Drosophila* larvae or adults fed the PCB mixtures, Clophen 30 or 50, silkworms fed Kanechlor 300 or 500, and three carp species injected with Aroclor 1254. Neither sex chromosome nondisjunction or breakage was induced in Drosophila (Nilsson and Ramel 1974), nor were mutations induced in silkworms (Kawachi et al. 1980). There were some dose-related chromosome breaks and fragments seen in the kidneys of the carp (Al-Sabti 1985).

Discussion: This group of chemicals is with few exceptions not genotoxic. The positive results that do appear have rarely been duplicated in a second laboratory. The majority of the studies have been done with PCB mixtures so it is difficult to ascribe positive results with these biphenyl mixtures to individual isomers.

PCB Reduction and Clinical Improvement by Detoxification: An Unexploited Approach?
Z. Tretjak, M. Shields, and S.L. Beckman (1990)

Purpose: To study a patient who has been occupationally exposed to PCBs and had elevated levels of these chemicals in serum, adipose tissue, and nipple discharge. Then to look at the outcome of a detoxifying method used to remove the chemicals from the patient's body.

Conclusions: *The detoxification program presented here and administered to the patient appears to have favorable results in reducing PCB levels in blood serum, adipose tissue, skin lipids, and nipple discharge. Other symptoms present before the treatment began were also alleviated. This method of treatment works well in detoxifying a patient of PCB residue build-up.*

Remarks: Patient worked in a capacitor plant inspecting for the leakage of PCBs for a period of nine months. She otherwise did not work in direct contact with PCBs but was working in an unprotected area where PCBs were being used.

The sum total of PCBs detected by GC-MS was 17 congeners.

Other symptoms observed included abdominal pain, bloating, general fatigue, muscle pain, chloracne eruptions, sun sensitivity, hyperpigmentation of the lower eyelids, joint pains and swelling of the limbs, as well as menstrual irregularities.

Results: After a complete physical and chemical examination, everything was normal except for some abnormalities noticed in a liver biopsy. A chromosome study showed chromatid and isochromatid breaks, gaps, and minutes in 20% of the 200 analyzed lymphocytes. The patients history of exposure to chemicals in the work place was supported by the elevated levels of PCBs in the samples taken from the liver and analyzed microscopically. The highest amounts of congeners detected were congeners 31/28, 74, 66, and 60. Major contributors to the total PCB concentration were the congeners nominally substituted at the 4; 2,4; 2,5; or 2,4,5 positions.

Treatment was administered to the patient to mobilize and enhance the removal of stored lipophilic xenobiotics, including PCBs. The treatment consisted of the following:

•Daily aerobic exercise followed by periods of low-heat (60-80°) sauna.

•Niacin and polyunsaturated oil were given to sustain mobilization and elimination.

•Vitamins and minerals were supplemented and daily liquid losses substituted.

•Body weight was maintained throughout the program

Initial PCB levels were high in adipose tissue (102 mg/kg), serum (512 μg/L), skin lipids (66.3 mg/kg), and in nipple discharge (712 μg/L). The detoxification treatment lasted 23 days and resulted in reduced adipose tissue deposits of PCBs and lower blood serum levels of PCBs. Detoxification treatment reduced PCB levels to 37.4 mg/kg in adipose tissue, and 261 μg/L in serum; a 63% and 49% reduction, respectively. Skin lipids were also reduced to a level of 44.3 mg/kg (33.2%) and nipple discharge disappeared. Other symptoms were reduced or disappeared completely. Excretion of intact PCBs in sebum was good before treatment but was helped by up to five-fold with the detoxification treatment. Unchanged was hyperpigmentation of the eyelids and the loss of the sense of touch. A post-treatment chromosome study showed a decrease in the number of isochromatid and chromatid breaks and gaps to 10% in 200 examined lymphocytes. The pre- and post-treatment levels of PCB congeners in tissue samples were considered to be statistically significant ($p < 0.05$). No adverse side-effects to the treatment were noted. At the end of six months after treatment was terminated, the patient was reported to be faring well.

Discussion: Clinical problems of the patient were alliviated during treatment. Given the excess body burden of PCBs, the remaining amounts may produce further clinical consequences that require repetitive treatment. Overall, though, the attained reduction in PCB levels in adipose tissue, serum, and the nipple discharge appears to validate the treatments effectiveness.

The authors state that prior studies incorporating their method of detoxification is safe and effective, with results acquired within an acceptable time frame. They feel that their results from this case involving very high PCB levels and a history of exposure to other lipophilic xenobiotics support others findings.

Polychlorinated Biphenyl Isomers in the Blood and Biopsy Fat Specimens of a Selected Population of British Columbia (Canada)
J. Mes, L. Marchand, and K. Karpinski (1989)

Purpose: To report on a number of selected PCB isomers in blood and biopsy fat of residents from the town of Golden and surrounding vicinity in British Columbia.

Conclusions: *None of the PCB congeners were related to the incidence of Down's Syndrome. There were low levels of PCB residues in blood (0.09%) but rather high levels in adipose tissue (76.4%).*

Remarks: This area was chosen by the British Columbia health authorities because of this area's high incidence of Down's Syndrome in order to rule out possible effects of environmental chemicals such as PCBs.

25 blood and biopsy samples were collected from donors with and without a Down's Syndrome child and were analyzed for 29 PCB isomers

Fat samples were taken most often from the abdominal region; also one in the buttocks and two from the breast.

Results: Mean lipid contents of the blood and adipose tissue were 0.09 and 76.4% respectively. Mean recovery of selected PCB isomers in blood ranged from 57-100% with a mean of 79%. Mean PCB isomer recovery in adipose tissue was 83% with a range of 78-86% for all selected isomers. The coefficient of variation (CV) for triplicate determinations of all detectable PCB isomers in blood was <20% and was also <20% for adipose tissue except for 2,4,4'-tri, 2,3,2',5-, 2,4,2',5', and 2,3,4,4'-tetrachlorobiphenyls (CV range of 25-55%). The higher CV's of these four congeners may be due to the relatively low residue levels encountered and/or small interferences from coeluting isomers or non-PCB-related compounds.

The small differences in residue levels observed for some PCB isomers between donors with and without a Down's Syndrome child were not statistically significant. None of the maximum PCB isomer levels in blood and adipose tissue was associated with Down's Syndrome related donors. Median residue levels of PCBs that were below GC detection were: 37, 44, 49, 52, 60, 87, 90, 105, 110, 180, 201, and 209. Levels of 118,138, and 153 were about the same as reported previously by Mes in a study of blood samples of children from Toronto. Most of the PCB isomers detected in adipose tissue were also observed in blood and vice versa. The only exception to this was 3,4,4'-trichlorobiphenyl isomer which was sometimes observed in blood but never in adipose tissue.

Discussion: The ratio profiles indicate that higher chlorinated PCB congeners have lower and more constant blood/adipose ratios than the lower chlorinated congeners reflecting their more stable metabolic nature and a possible equilibrium between two tissues. It also appears that exposure to PCBs has no relationship with the development of Down's Syndrome. In both blood and adipose tissue there appears to be a tendency for mean PCB isomer levels to be higher in males than in females but this was not statistically significant.

Polychlorinated Biphenyl Congener Residues in Human Adipose Tissue Samples From Five Ontario Municipalities
D.T. Williams and G.L. LeBel (1990)

Purpose: To extend previous investigations on total PCB levels in human adipose tissue by determining and comparing levels of specific PCB congeners in tissue samples from municipalities in the Great Lakes Basin.

Conclusions: *The 16 PCB congener levels appear to be almost equivalent in tissues from the various municipalities studied with the exception of congeners 170, 180, 187, 194, and 201. There appeared to be no difference between males and females levels of these congeners. Therefore, PCB exposure may be the same in these municipalities.*

Remarks: Human tissue samples obtained from unembalmed cadavers during autopsies in five Canadian municipalities; Cornwall, London, St. Catharines, Welland, and Windsor.

Tissue samples taken from the greater omentum area.

16 specific congeners of polychlorinated biphenyls were analyzed by gas chromatography-mass spectrometry (GC-MS).

Results: The sum of the congeners 118 to 209 would represent approximately 60% of the total amount of PCBs previously measured in these municipalities. Mean residue levels for males were not significantly different ($p<0.05$) from those for females for any of the PCB congeners. Total (males & females) mean residue value showed no significant differences ($p<0.05$) between municipalities for 11 of the 16 PCB congeners. However, two congeners, 170 (180 ng/g for ♂, 130 ng/g for ♀) and 180 (260 ng/g for ♂, 280 ng/g for ♀) were significantly higher in those tissues obtained from St. Catharines than in tissues from other municipalities. Congeners 187, 194, and 201 were also significantly higher ($p<0.05$) in tissues from St. Catharines compared to Welland tissues but tissue levels for either of these municipalities were not significantly different from tissue levels of these congeners for the other three municipalities. There is no explanation of these differences except for the fact that it might be due to non-uniformity in donor backgrounds and lifetime exposures, variations in individual metabolism, and the small number of samples.

Discussion: There is limited data available on levels of PCB congeners in human adipose tissue and the same congeners have not always been analyzed. However, these results are in general agreement with those from studies done in Sweden, the United States, and Italy. PCB exposures for the general population appear to be similar worldwide and these PCB tissue levels are not likely to cause adverse health effects.

Kidney Cancer in Utility Workers Exposed to Polychlorinated Biphenyls (PCBs)
S.L. Shalat, L.D. True, L.E. Fleming, P.E. Pace (1990)

Purpose: To report on a few cases of kidney cancer among utility workers who were responsible for maintaining electrical transmission equipment including power transformers.

Conclusions: *After examining utility workers for evidence of kidney cancer, the authors found that there was conclusive evidence showing that exposure to PCBs can result in carcinogenic growths in the kidney. The authors agree that more research needs to be done in relating PCB exposure to cancer.*

Remarks: The three subjects of various ages in this report worked as linesmen and maintenance workers for a public utility company and were engaged in servicing and repairing electrical power transformers. These subjects reported being exposed to organic solvents, herbicides, electromagnetic fields, and PCBs.

Results: All tumors were unifocal, located within the center portion of the kidney, and with a maximum dimension of 6-14 cm. None of the tumors showed capsular or vascular invasion or nodal metastases. All three cases showed the following characteristics. Case one showed a majority of solid sheets of cells having an eosinophilic granular cytoplasm and a minority component consisting of cuboidal cells forming irregular granular cytoplasm. The tumor in case two exhibited solid sheets of cells with predominantly clear cytoplasm and associated necrosis and had a prominent vascular component. The tumor in case three exhibited predominantly clear cells that in some areas grew in sheets and in others formed tubular and alveolar structures associated with broad fibrous septae.

Serum concentrations for PCBs were not elevated in cases one and two but traces of pesticides, herbicides, and their breakdown products were found.

Discussion: The authors state that there needs to be more work done on relating kidney cancer with occupational exposure. They suggest the need for an epidemiological investigation of renal adenocarcinoma and exposures that occur in the electrical utility industry.

GENP 011518

Decrease Over a Six Year Period of Dioxin and Dibenzofuran Tissue Levels in a Single Patient Following Exposure
A. Schecter, J.J. Ryan, and P.J. Kostyniak (1990)

Purpose: This paper is a follow-up on a patient that was monitored over three years (extended over a six year time period) beginning two years after exposure to a PCB transformer fire. The study monitored levels of 2,3,4,7,8-PnCDF, 1,2,3,4,7,8-HxCDF, 1,2,3,6,7,8-HxCDF, 1,2,3,6,7,8-HxCDD, and 1,2,3,4,6,7,8-HpCDF, and PCB blood levels as well.

Conclusions: *The study showed that over a period of six years, blood and adipose tissue levels of dioxin, PCDFs, and PCDDs decreased. There was also a decrease in the toxic equivalent levels for dioxin. The authors feel that data from this study will help others determine body burdens of these compounds over time or a lack of build up over time.*

Remarks: Surgical biopsy for adipose tissue was performed on the exposed worker - a white male whose age ranged from the late fifties to mid-sixties during the course of the study.

Results: There was a decrease over six years in the blood and fat levels of one PnCDF, two HxCDFs, one HpCDF, and one HxCDD to a level they now think to be a plateau. The study also showed a decrease in "dioxin toxic equivalent" (TEQ) levels over time from 113 ppt to 64 ppt, lipid basis between 1983 and 1987-89 where the TEQ also reached a plateau. Overall, total PCDD/F levels remained similar over the years due to the usual, but high, OCDD levels found in this patient and the general population. PCB blood levels were mentioned but no values were given in the report.

Discussion: The authors feel that data from this paper will "permit determination of increased body burden (or lack of increase over time) and estimates of target organ dose, after potential occupational or environmental exposure." With information like this it may be possible to determine how long after exposure these measurements may be useful. The decrease in toxic equivalents appears to have reached a plateau, six years after exposure, by 1987. General population tissue levels of PCDD/Fs are believed to be derived from food as well as general ambient OCDD levels in the environment, and this may account for the slight increase in recently sampled OCDD levels and a general lack of decline in total PCDD/F levels. However, the congeners to which the patient was exposed, did decrease over time which accounts for the decline in dioxin toxic equivalents from 113 to 65 ppt, 2 years and 8 years after exposure respectively.

Dermatological Findings in Children Exposed Transplacentally to Heat-Degraded Polychlorinated Biphenyls in Taiwan
B.C. Gladen, J.S. Taylor, Y-C. Wu, N.B. Ragan, W.J. Rogan, and C-C. Hsu (1990)

Purpose: To study 128 children who were transplacentally exposed to polychlorinated biphenyls and dibenzofurans in Taiwan; also their parents and siblings who were directly exposed and 115 control children from the same neighborhoods.

Conclusions: The major symptoms that manifest themselves in children as a result of transplacental exposure to PCBs are: growth deficiencies, developmental delays, dental abnormalities, and neuro-ectodermal dysplasia. Other symptoms occur as well such as finger and toe-nail deformities, chloracne, hyperpigmentation, and acneiform lesions. The conclusion is that transplacental exposure to polychlorinated biphenyls results in more serious and extreme effects than direct exposure does. This suggests that the route of exposure plays a key role in what symptoms appear at a later stage.

Remarks: Acute direct exposure ceased after the rice-bran cooking oil was recalled in the autumn on 1979.

Any children born in or after June 1978 were considered to have been at risk of transplacental exposure. There were 132 such children born to 74 mothers ranging in age from a few months to just under seven years old.

Results: At birth, exposed children had increased rates of hyperpigmentation, eyelid swellings and discharge, deformed nails, acne, natal teeth and swollen gums compared to controls. The exposed children also had an increased rate of dystrophic finger-nails and pigmented or dystrophic toe-nails than controls. In addition, exposed children had more generalized itching, localized skin infections and hair loss. These findings differ from those subjects who were exposed directly rather than transplacentally. Of the 57 hyperpigmented children, 46 (all transplacentally exposed) were reportedly pigmented over their entire body. The most common sites of pigmentation were the forehead (8), trunk (8), and cheeks (7). Some children had only one of the above symptoms (75), others had multiple symptoms (50). Those children born shortly after direct exposure ceased were neither more or less likely to have these problems than children born much later ($p > 0.10$).

A physical examination was performed on the transplacentally exposed children and the controls. Hyperpigmentation was persistent and occurred with a high prevalence in transplacentally exposed children especially in the genitalia ($p=0.0178$) and feet ($p=0.0701$). Noticeable facial hair was three times more frequent in exposed children. Rates of gingival hyperpigmentation were unaffected by transplacental exposure ($p > 0.10$) but older siblings and exposed mothers showed high rates.

Rates of dystrophic finger-nails, dystrophic toe-nails, and pigmented toe-nails were all significantly higher in the transplacental group than the control ($p<0.001$). The affected digits were often the first and fifth ones; 71% of all the children with abnormal finger-nails and 61% with abnormal toe-nails were affected in this manner. The most common deformities seen in the finger-nails were grooves and ridges and other unspecified dystrophy (10), transverse overcurvature (pincer and tile-shaped nails), and longitudinal overcurvature (forward-curving and humped) also affecting 10 children. In the toe-nails the noticeable effects were koilonychia (spooning, 37), ridging, onychauxis (thickening, 26), onychoschizia, splitting/scaling (20), plication (flattening, 17), and transverse overcurvature.

Acneiform lesions and/or scars at the time of examination were twice as common in transplacentally exposed children as in controls ($p>0.10$). They were also seen in almost half of the older siblings. Seven out of eleven exposed had follicular keratoses. The transplacentally exposed children had a higher frequency of scaly or keratotic disorders than controls, not significant at $p>0.10$, and their older siblings had an even higher rate. Exposed children also had higher rates of sweat gland disorders although rates were low and not significant ($p>0.10$).

Discussion: In this outbreak the major target organ was the skin. The transplacentally exposed children appear to have acquired neuro-ectodermal dysplasia with dental abnormalities, a growth deficit, developmental delay, and a behavior disorder. In these children whose mode of exposure was different and who were exposed at a critical point of development, the dermatological findings are somewhat different from those seen in adults. The observation that one-half of the older siblings with potential direct exposure had acneiform lesions and/or scars, compared to only 17% of the transplacentally exposed children. This observation calls into question the concept of chloracne as the most sensitive biological indicator of significant exposure to this class of compounds. The nail changes were very significant and remarkable. The pigmentation of nails appears to involve both the nail plate and bed. This pigmentation has been reported in PCB-exposed workers.

In examining lifetime dermatological findings as reported by the parents of the children in the study, the authors found that they could easily compare the transplacentally exposed children to the controls but not to their older siblings and mothers because they had more time to develop those conditions mentioned in the results section as well as abscesses or boils, warts, blistering in the sun, dark red urine, hair loss, numbness of hands and feet, and localized thickening of palms and soles. There were some inconsistencies in the reported numbers of neonatal acne, deformed nails (both finger and toe) where the numbers reported in the lifetime findings were lower than the neonatal findings. The reason for this is not known but may be a result of inaccurate reporting by the

mothers. Many reported lifetime findings in transplacentally exposed children were more prevalent in older children than younger. The higher prevalence of reported lifetime findings in older children may be due to being born soon after the poisoning (more severely affected) as opposed to later or because older children have had more time for the finding to occur.

There is little doubt that chemical exposure during gestation produced the abnormalities and that PCBs were the major contaminants of the cooking oil ingested by these children's mothers. The PCBs themselves are mixtures of over 200 distinct congeners. The PCBs themselves were heat-degraded during use and thus were contaminated by the PCDFs and by polychlorinated quaterphenyls. Workers and offspring of workers exposed to relatively contaminant-free PCBs are not so severely affected. The authors suspect that mixtures are toxic but that the PCDFs play a key role in their toxicity.

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Correlation between acute toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and total body fat content in mammals

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Summary

Single oral 30-day LD_{50}s of 2,3,7,8-tetra[illegible]izo-*p*-dioxin (TCDD) were correlated with total body fat (TBF) content in various species and st[illegible] of laboratory mammals. LD_{50} values and TBF contents were either obtained from the literature or determined by experiments. A log (LD_{50}) vs. log (TBF) plot yielded a highly significant linear regression equation ($r^2 = 0.834$, $P < 0.001$, $n = 20$). It is suggested that this correlation exists for at least two reasons: (1) increasing TBF content in organisms represents an enhanced capacity to remove TCDD from the systemic circulation and (2) different TBF content reflects a differential role and regulation of fat metabolism for various organisms. Extrapolation of this correlation to man suggests that adult humans are among the less sensitive species to the acute toxicity of TCDD.

Key words: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; Acute toxicity; Species differences; Total body fat content

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is not produced for commercial purposes and has no reported use other than as a research tool. It is formed as a contaminant in the process of chemical synthesis of chlorinated phenols, especially of 2,4,5-trichlorophenol and in smaller quantities during the combustion of municipal and industrial wastes. TCDD is a very lipophilic and persistent chemi-

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cal. It accumulates in fat of aquatic as well as of terrestrial organisms including man [1—3].

TCDD is an extremely toxic chemical to some animal species. However, there are marked differences in the sensitivity of various species to the toxic effects of TCDD. The LD_{50} of TCDD in different species varies by nearly 4 orders of magnitude. Male guinea pigs with an LD_{50} of about 1 μg/kg and male golden Syrian hamsters with an LD_{50} of about 5 mg/kg represent the two extremes (Table I). Like guinea pigs, American dark minks [4] and rabbits are also very sensitive, whereas adult Beagle dogs and a mouse strain (DBA/2 J) are relatively resistant to acute TCDD intoxication. Interstrain differences have also been reported for rats [5] and mice [6].

In spite of extensive investigations in recent years, neither the cause of death nor the marked species differences in the acute toxicity of TCDD can be explained. Rozman [7] and Rozman et al. [8,9] suggested that species differences in TCDD toxicity may be due to different amounts of brown adipose tissue (BAT) in various species. BAT occurs in relatively high amounts in hibernators such as hamsters, while non-hibernators like adult guinea pigs contain little, if any, identifiable amounts of this tissue.

Because the main toxic effect of TCDD in all animals studied is a loss of body weight, which is largely a result of body fat loss, we have extended the hypothesis of Rozman et al. [7—9] to investigate if a quantitative relationship exists between the acute toxicity (LD_{50}) of TCDD and total body fat content (brown adipose tissue plus white adipose tissue) of various mammals.

Materials and methods

Acute toxicity data of TCDD

The acute oral toxicity data (LD_{50}) of TCDD in different species and strains of laboratory mammals were taken from the original literature and are compiled with references in Table I. LD_{50} values are influenced by many factors such as animal species, strain, body weight, age, sex, health, diet, food deprivation, observation period, etc. The route of dosing (oral, intraperitoneal, dermal, subcutaneous, etc.) has also a great impact on LD_{50} values of TCDD [10]. Therefore, in Table I, only single oral LD_{50} data are given with an observation period of 30 days after dosing. In most instances the vehicle was oil, in a few cases oil/acetone (95:5 v/v). When important information regarding body weight, age, etc. of the animals was not reported in the original publications, such data were obtained directly from the authors.

Determination of body fat

Total body fat (TBF) content for the various species was either obtained from the literature or determined experimentally (Table I). Animals used for TBF determination were of the same strain, similar weight and/or age as those for which LD_{50} values but no TBF contents were available in the literature. Rats, mice and hamsters were bred in the Zentralinstitut für Versuchstierzucht, Hannover, F.R.G. Male Hartley guinea pigs were obtained from Savo, Kisslegg,

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F.R.G. All animals were kept under standard laboratory conditions (12 h light/dark cycle, 55% humidity) and had access to species-specific feed (Altromin standard diet, Lage, F.R.G.) and water ad libitum. For each determination of TBF content three animals were used.

Animals were weighed individually, sacrificed and dried to a constant weight in an oven at 110°C (time: 2 days for mice, 3 days for hamsters, 4 days for rats and guinea pigs). The dried carcass was ground in a mortar, mixed with perchloroethylene and allowed to stand for 24 h at room temperature. TBF was then extracted by the FOSS-LET method according to the operating instructions of the manufacturer Foss Electric (Skandinavien), A/S, 1 Huginsvej, DK-3400 Hilleroad, Denmark. TBF content on a % live weight basis was calculated using a calibration curve (specific gravity as a function of fat content).

TBF content of those species or strains obtained from the literature — except the rhesus monkey — was determined chemically by extraction with organic solvents (petrolether, diethylether etc.). In the rhesus monkeys TBF content was estimated via the tritiated water method (Bowman, R.E., pers. commun.).

The FOSS-LET method for TBF determination is in good agreement with the other extraction methods taken from the literature [Rapp, K., pers. commun., Herberg, L., pers. commun., 11]. These data and our own measurements are compiled with references in Table I.

Statistics

Statistical analyses were carried out using a STATGRAPHICS (version 2.6) computer program of STSC, Inc., USA. The relationship between LD_{50} (30 day) of TCDD in different species and strains and TBF content was analyzed by linear least-squares regression analysis.

Results

The single oral median lethal doses (30-day LD_{50}) of TCDD in different species and strains were correlated with their TBF content. Using a two variable linear regression [log (LD_{50}) vs. log (TBF)] the following regression equation [1] was obtained:

$$\log(LD_{50}) = 5.30 \log (TBF) - 3.22 \quad (1)$$

The antilog of equation (1) is

$$LD_{50} = 6.03 \times 10^{-4} (TBF)^{5.30} \quad (2)$$

Correlation coefficient $r^2 = 0.834$; level of significance $P < 0.0001$; standard error of the estimate (square root of the variance of estimate) of log data $S_{y.x} = 0.460$; standard error of slope of equation (1) is $\pm$ 0.559; standard error of the intercept is $\pm$ 0.565 and the number of data points used in the calculation of the regression equation $n = 20$. Data are plotted and equation (1) is presented in Fig. 1 graphically.

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TABLE I

BODY WEIGHTS AND SINGLE ORAL 30-DAY LD_{50}s OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN (TCDD) IN DIFFERENT MAMMALS AND TOTAL BODY FAT CONTENT (TBF) OF THE SAME SPECIES AND STRAINS WITH SIMILAR BODY WEIGHT

Species or strain	Sex	Initial body wt[a] (g) and/or age (weeks)	LD_{50}[b] (μg/kg)	Refs.	Body wt[a] (g)	TBF[c] (% body wt)	Refs.
Guinea pigs (Pirbright-white, Dunkin-Hartley)	M	200 (ca. 3w)	0.7[e] (0.6—0.8)[d]	12	195.8 ± 3.5	4.5 ± 0.8	Own determination
Guinea pigs (Hartley)	M	213.5 ± 8.2 173—238 (2.5 w)	1.75 (1.26—2.4)[d]	13, McConnell, E.E., pers. comm.	Same data used as above,		Own determination
Guinea pigs (Hartley)	M	200—250 (3—4 w)	2.0	14	Same data used as above,		Own determination
Rabbits harecolored	M	ca. 3000	10	15, Schulz, K.H., pers. comm.	2560	7.5	24
Rabbits (New Zealand White)	M + F	ca. 2000—3000	115	16, Oehring, P.J. and Betso, J., pers. comm.	2580	10.1	24
Rhesus monkeys *Macaca mulatta*	F	2100—2600 (juvenile)	50	13, McConnell, E.E., pers. comm.	2340	10.3	Bowman, R.E., pers. comm.
Rats (Fischer F 344)	F	100—130 (ca. 4—5 w)	40	17	113.3 ± 2.2	10.8 ± 2.3	Own determination
Rats (Fischer F 344)	M	130—150	47	18, McConnell, E.E., pers. comm.	143.5 ± 5.3	10.1 ± 1.4	Own determination
Rats (Sprague—Dawley)	M	200 (6—7 w)	50	19	187.7 ± 5.3	8.9 ± 0.14	Own determination

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Rats, outbred (CD, Sprague—Dawley)	F	168.8 ± 4.9 (6—8 w)	100
Rats, outbred (Charles River, CR/CD Sprague—Dawley)	M	352 ± 5 (10—11 w)	297 (240—360)[r]
Rats (Frederick, Fischer, F/F 344N)	M	240 ± 4 (11—12 w)	303 (250—360)[r]
Mice (C57 BL/6J)	M	25.8—26.3 (10—12 w)	182 (161—201)[r]
Mice (C57 BL/6fh)	M	21—25 (9 w)	283.7
Mice (B6 D2F$_1$)[s]	M	22—32 (10—12 w)	296 (268—324)[r]
Mice (DBA/2J)	M	27.6—28.9 (10—12 w)	2570 (2206—2912)[r]
Dogs (Beagle)	M	7000—13 000	1000[t] (300—3000)[t]
Hamsters (golden Syrian) *Mesocricetus auratus*	M	49.3 44—54 (ca. 3—4 w)	1157 (829—1583)[r]

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20	166.1 ± 2.8	11.4 ± 0.45	Own determination
21	344.5 ± 3.6	9.4 ± 0.3	Own determination
21	250.0 ± 7.1	10.5 ± 0.2	Own determination
6	25.1 ± 1.4	7.9 ± 1.5	23
14	23.6 ± 2.7	9.8 ± 0.2	Own determination
6	27.7 ± 2.8	14.3 ± 1.9	Own determination
6	27.23 ± 0.4	20.0 ± 2.6	Own determination
16, Gehring, P.J. and Betso, J., pers. comm.	9220 ± 2190 (7180—13 070)	13.8 ± 4.8	25
10, Olson, J.R., pers. comm.	46.8 ± 0.3	9.8 ± 0.8	Own determination

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TABLE I

Hamsters, outbred (golden Syrian) *Mesocricetus auratus*	M	101.8 ± 10.3 70—120 (ca. 8 w)	5051 (3876—18487)[f]	22, Betso, J., pers. comm. Rao, K.S., pers. comm.	96.2 ± 0.35	17.3 ± 0.4	Own determination
Hamsters, outbred (golden Syrian) *Mesocricetus auratus*	M	Same data used as above		22, Betso, J., pers. comm. Rao, K.S., pers. comm.	127.4 ± 6.4	20.2 ± 4.3	26

[a]Mean ± S.E. and/or range.
[b]Amount required to kill 50% of the animals within 30 days post-exposure.
[c]Calculated by the authors.
[d]The number in parentheses indicate the range of lethal doses (0 and 100% mortality).
[e]95% confidence.
[f]Pirbright-white, Dunkin-Hartley strain.
[g](C57 BL/6J female × DBA/2J male) F_1.

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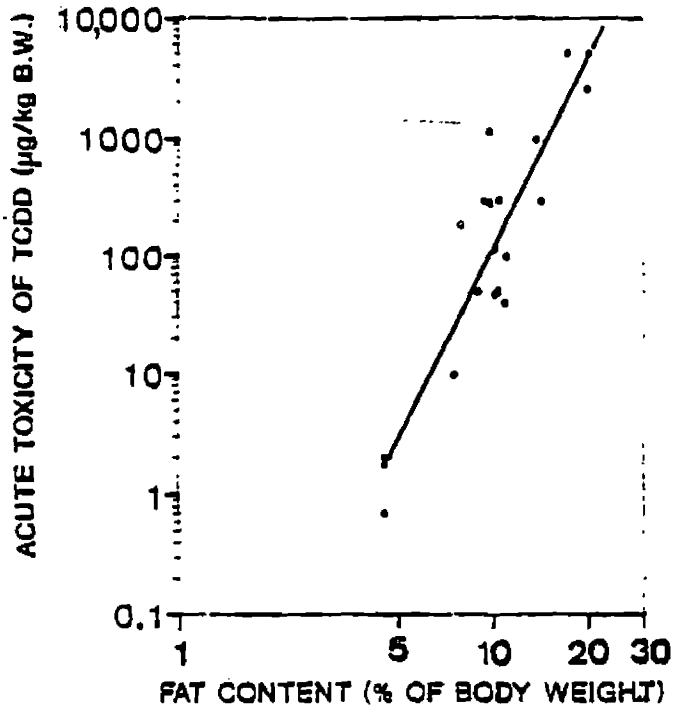


Fig. 1. Relationship between acute oral toxicity (log LD_{50}) of TCDD and total body fat content (log TBF) in different mammals.

Discussion

The above data demonstrate a positive linear relationship between the logarithm of single oral acute doses of TCDD and the logarithm of TBF content in the species and strains examined. The correlation is highly significant (correlation coefficient $r^2 = 0.834$, significance level $P < 0.001$) and therefore suggests that TBF content is associated with the acute toxicity of TCDD.

Adipose tissues (white and brown adipose tissue) contain large amounts of fat which is the predominant form of stored energy in an organism. Due to this large fat content, adipocytes are important storage sites for lipophilic compounds such as polychlorinated biphenyls, hexachlorobenzene and TCDD. Storage of chemicals in fat results in their removal from the systemic circulation. This in turn reduces the concentration of such substances at potential target sites of toxicity. Therefore, one might suspect that the correlation between acute toxicity of TCDD and TBF is a result of increased storage of TCDD. Despite the attractiveness of such a straight forward explanation, it appears to be an oversimplification. First, the correlation between LD_{50} and TBF exists on a logarithmic scale, but storage of TCDD in fat does not increase logarithmically. Second, the oral LD_{50} for both the Sprague—Dawley rat and the rhesus monkey is 50 µg/kg (Table I), but the main storage site of TCDD for the Sprague—Dawley rat is the

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liver and fat is only second in importance [27], whereas for the rhesus monkey fat represents the major site of TCDD storage [28]. Therefore, the depot function of fat stores alone is probably not sufficient to explain the herein reported strong correlation between acute toxicity of TCDD and TBF content.

Recently, disturbances of major pathways of intermediary metabolism (glucose, fat and protein metabolism) and their endocrine control have been reported in the rat after TCDD exposure [29—37]. It should be noted that intermediary metabolism is differently regulated and the various substrates play differential roles in energy metabolism of various species. For example, the guinea pig is a herbivore with fermentation of cellulose to short chain fatty acids taking place in the cecum. These short chain fatty acids are the major source of energy for this species. In addition, guinea pigs and other herbivores have frequent bouts of feeding which reduces their need for large energy stores. Not surprisingly this species has the lowest TBF (about 5% of body weight in young guinea pigs). Rats and rhesus monkeys are omnivors with carbohydrate being their major form of energy intake. These two species have less frequent bouts of feeding (about two daily) and convert a substantial proportion of their energy intake into stored fat. Correspondingly larger fat depots are present in these species (about 10% body wt). The hamster is a hibernator with a seasonal cycle superimposed over the daily feeding pattern and a nearly complete switch from glucose to fat metabolism during hibernation. The need for large fat depot in such species is self evident (about 20% of body weight in the adult hamster). These brief physiological/teleological considerations of species differences indicate the differential contribution and role of fat metabolism and fat depots for intermediary metabolism in various species.

It should be noted that both the acute toxicity of TCDD and TBF seem to be age dependent (Table I). Unfortunately no systematic investigations exist on age dependence of TCDD toxicity or TBF content across the herein examined species. Therefore, it is not possible to make a strong case for predicting the acute toxicity of TCDD for children based on this correlation. However, the fact that this strong correlation holds for a variety of species and strains of different ages suggests that an extrapolation for children may also be warranted. According to Geigy Scientific Tables [38] the TBF content of 4—36-month-old children is 26.3—18.3%, whereas that of newborns is about 13.6% of body weight. The correlation thus predicts that young children would be in the same range or slightly less sensitive whereas newborns would be about 10 times more sensitive to an acute dose of TCDD (DL_{50}: 614 μg/kg) than the adult 'Reference Western Man'.

We suggest that the cause of a strong correlation between acute toxicity of TCDD and TBF may be a combination of storage capacity (toxicokinetics) and of the differential role and regulation of intermediary metabolism among species and strains. Assuming that this correlation is valid for most and perhaps all mammals, including man, the 30 day oral LD_{50} in the adult 'Reference Western Man' of 70 kg body weight and 21% total body fat (ICRP, 39) would be 6230 μg/kg of TCDD. This prediction supports suggestions of Ayres et al. [40], Tschirley [41] and Poiger (Poiger, H., pers. commun.) that adult humans are on the less sensitive side of the TCDD toxicity spectrum.

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We realize that the selection of studies for this evaluation may be subject to criticism. For example, the inclusion of 3 different guinea pig studies with the same TBF content and one hamster study with 2 different TBF contents may unduly influence the correlation coefficient and the slope of the regression line. However, omission of 2 guinea pig and one hamster studies would not change the conclusions of this paper. In that case linear regression analysis yields the equation

$$LD_{50} = 1.55 \times 10^{-3} (TBF)^{4.92}$$

with $r^2 = 0.7$ and $P < 0.001$ predicting an LD_{50} of 4962 μg TCDD/kg for adult 'Reference Western Man'. In fact Table I allows for interested scientists to select any combination of these studies based on their own exclusion criteria and perform linear regression analysis.

In conclusion, there is a good correlation between log (TBF) and log (LD_{50}) of TCDD across many species and strains. It appears that both toxicokinetic and toxicodynamic factors contribute to the existence of this relationship. Exceptions to this correlation may occur and reveal additional factors important in the acute toxicity of TCDD.

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ENVIRON

July 5, 1990

Prof. Dr. med. Helmut Greim
Gesellschaft für Strahlen- und
Umweltforschung mbH
Institute für Toxicologie
Ingolstädter Landstraße 1
D-8042 Neuherberg
West Germany

Dear Dr. Greim:

About two and a half years ago you graciously responded to a letter I wrote to you asking two questions about a publication in *Toxicology and Applied Pharmacology* (Vol.75, p. 278-288, 1984) written by you, and Drs Scheaffer and Goessner. PCBs continue to be of interest to us and our clients. The reason I am writing to you now is related to some work that has been conducted recently in this country in which slides from a 1978 cancer study of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in rats have been re-evaluated in light of more recent histopathological criteria for diagnosis of proliferative liver lesions in rats. This work has been conducted by Dr. Robert Squire of Johns Hopkins University and some of his colleagues. Dr. Squire found that many of the lesions that had originally been classified as "neoplastic nodule," or "hepatocellular adenoma" would, under current criteria[1] be diagnosed as foci rather than as neoplasms.

Because of the similarities between PCBs and TCDD, one of our clients has expressed an interest in what would be the result of re-evaluating the existing cancer studies of PCBs using the new diagnostic criteria used by Squire. Dr. Gene McConnell, formerly of the National Toxicology Program, who is working with us on this project, has contacted the authors of two other PCB studies[2] in rats to enlist their aid in performing such a re-analysis. I am writing to you to ask if you would be willing to permit Dr. McConnell to come to your lab

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[1] Squire, R.A., Evaluation and Grading of Rat Liver Foci in Carcinogenicity Tests. Presented at National Toxicology Program Symposium "The Significance of Foci of Cellular Alteration in the Rat Liver," May 1989.

[2] Kimbrough, R.D. et al. 1975. Induction of liver tumors in Sherman strain female rats by polychlorinated biphenyl Aroclor 1260. J. Natl. Cancer Inst. 55:1453-1459;
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and review the slides from your study to determine whether the tumor incidences would change under Dr. Squire's diagnostic criteria.

Sincerely,

Duncan Turnbull

Duncan Turnbull, D.Phil.
Senior Science Advisor

SCIENCE AND THE CITIZEN

"A Great Poison"

Dioxin helps elucidate the function of genes



Boss Tweed as hacker, honeybee dancing, liquid-crystal cosmos, Dead Sea Scrolls dispute

One man's poison can be another man's passion. Even dioxin—the infamous contaminant in Agent Orange—is loved by some. This small and relatively silent band of followers has been probing the molecular mechanisms of the toxic compound for some 20 years, trying to stay out of the political and scientific jungle that envelops the chemical's toxicity.

These researchers are revealing the intricate details of dioxin's activity in cells, where it now appears that the chemical mimics a hormone. Their discoveries could elucidate dioxin's diverse biological effects to support or contest conflicting epidemiological studies and could also provide valuable insights into gene expression and target genes in general. "The next five years are going to reveal what genes dioxin affects and how they relate to important toxic effects such as cancer and immune suppression," predicts Ellen K. Silbergeld, a toxicologist with the Environmental Defense Fund. "At last we have a molecular handle on what's going on."

There are 75 kinds of dioxins, but the most potent, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, also called TCDD or, simply, dioxin, has been the principal object of scrutiny. This compound is created during combustion and as a contaminant in some chemical manufacturing processes. Trace amounts were present in the defoliant Agent Orange, which was widely used during the Vietnam War. An accident at a chemical plant in Seveso, Italy, led to widespread local contamination in 1976.

Claims that the herbicide had injured Vietnam veterans and chemical plant workers touched off numerous investigations. Since then, animal data have shown TCDD to be lethal at some doses and to cause a host of different effects—including cancer, thymus and liver damage, birth defects and immune-system depression—that vary by species. Epidemiological studies finding increased occurrence of soft-tissue sarcoma and non-Hodgkin's lymphoma in people exposed to dioxin have been hotly contested; studies finding no such associations have also been widely criticized. One uncontested long-term human effect is chloracne—a sometimes disfiguring skin condition.

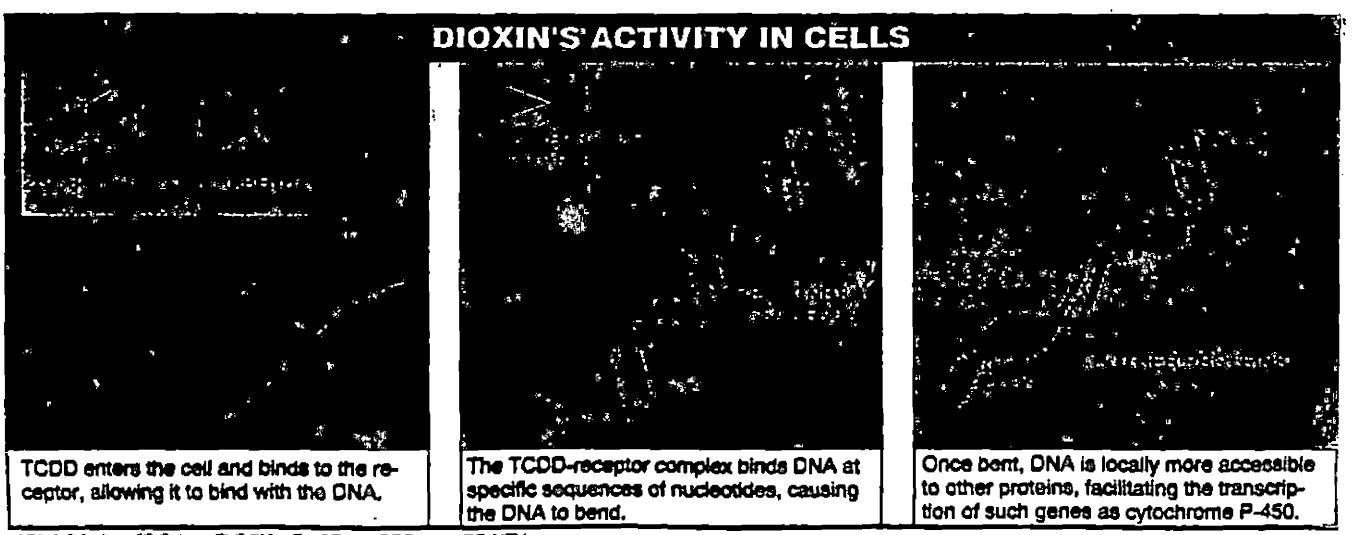
Understanding dioxin's biological activity entails finding a mechanism that explains why different species respond in different ways. Why, for example, are hamsters unaffected by a dose that can kill a guinea pig? Why do female rats develop liver cancer after doses of TCDD, and male rats do not?

Researchers now know that dioxin works by reversibly binding to an intracellular receptor, which also binds with similar compounds such as polychlorinated biphenyls (PCBs). The receptor is soluble, that is, it is not bound to the cell membrane. "All [of TCDD's] toxicity is mediated through this receptor," says Alan P. Poland, a professor of oncology at McArdle Laboratory for Cancer Research at the University of Wisconsin at Madison, who isolated the dioxin receptor in 1976. The potency of dioxins or of PCBs is a direct reflection of their ability to bind with the receptor—and TCDD binds most avidly, explains James P. Whitlock, Jr., a pharmacologist at Stanford University.

The receptor-dioxin complex binds with DNA at what may be one of several dioxin recognition sites. This site is a regulatory region located upstream from a gene that encodes an enzyme from the cytochrome P-450 family. Although dioxin induces the expression of other genes as well, this response is the most thoroughly documented.

The cytochrome P-450 protein works to detoxify cells. This "garbage disposal enzyme," as Poland describes it, helps break down fats. Increasing levels of this enzyme, however, can lead to the formation of potentially dangerous compounds. One researcher describes P-450 as a double-edged sword. The effect of dioxin on P-450 levels and the consequence of these levels remain to be explored.

Despite dioxin's ability to initiate the metabolism of many other compounds, TCDD itself is not broken down,



SOURCE: JAMES P. WHITLOCK, JR., STANFORD UNIVERSITY

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"which is one of the reasons to be concerned about it," Whitlock says. TCDD persists in the body and has an estimated half-life of five years.

Dioxin may also regulate other target genes. Silbergeld and her colleagues at the University of Maryland, where she is a visiting professor, have identified six genes, aside from that for P-450, whose expression is affected by dioxin. William F. Greenlee, a toxicologist at the Chemical Industry Institute of Toxicology in Research Triangle Park, North Carolina, has found evidence of two more.

The dioxin receptor has been observed in many kinds of cells from many species, including humans and sharks—suggesting some evolutionary significance. Although there is some variation in the receptor between species, investigators say these differences alone do not account for the varying toxicity observed between animals. One explanation is that other factors, such as the environment or genetic makeup, could modulate TCDD's effect. The region on the DNA is "like an electrical switch with a dimmer: TCDD may turn it on, but something else turns it up or down," Whitlock says.

Dioxin does not appear to damage DNA. That, along with other laboratory evidence, has led researchers to postulate that it could be a cancer promoter rather than inducer—meaning that other factors could initiate a cancer but that TCDD would help it along. Indeed, animal experiments have shown that dioxin promotes the formation of tumors once a carcinogen has been introduced into the cell.

In the laboratory, TCDD has also been shown to affect the normal growth and differentiation of human skin cells and other tissues, including rat liver. George W. Lucier, a biochemist at the National Institute for Environmental Health Sciences, has found that TCDD will cause cancer in the rat liver only in the presence of estrogen. Female rats develop cancer after doses of dioxin, but ovariectomized rats and male rats do not—even after "huge" doses. "Dioxin's carcinogenic effects are probably related to cell proliferation," Lucier says. In turn, "these pathways are related to estrogen."

Although its role in cancer remains unclear, TCDD "is unique in that few carcinogens bind to a specific intracellular receptor," Greenlee says. Tracking it may reveal how chemical carcinogens alter normal growth processes.

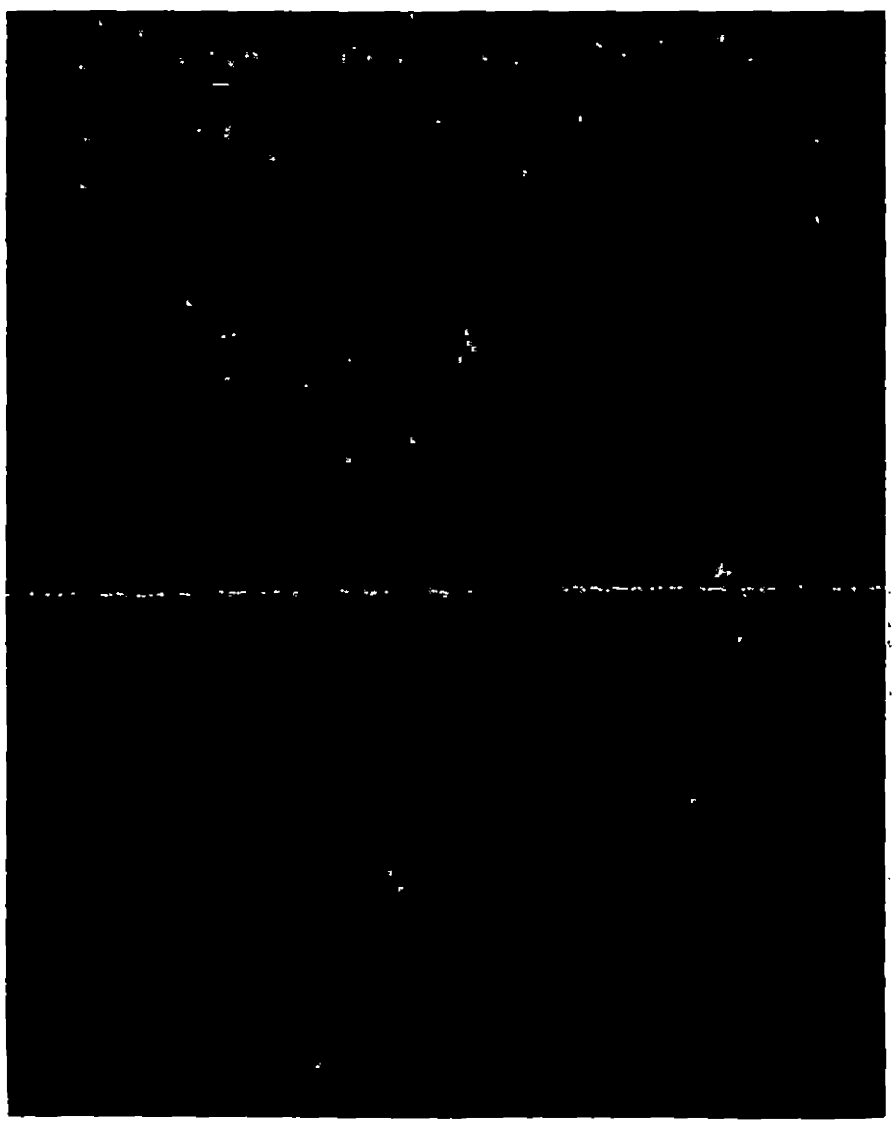
Recently Whitlock discovered that TCDD may bend DNA—a potential stage in gene transcription. "In the test tube, the binding of the receptor to the DNA bends the DNA, distorting it," Whitlock says. Usually DNA is packed into the nucleus and hard to get to. When bent, DNA is exposed and may become more accessible to proteins involved in gene expression. "It is as if that region has been opened up in some way," he observes.

Whitlock and others have also determined the seven-nucleotide sequence of the site where the dioxin-receptor complex binds to the DNA (nucleotides are the component molecules of DNA). This pattern recurs four or five times in the same region—all within 400 nucleotides of one another. Such repetition is "not a random occurrence," Whitlock says, and supports the idea that the site served an evolutionarily beneficial purpose.

The existence of the receptor and the binding sites has suggested to researchers that TCDD may be analogous to a hormone. The "true" fit to the receptor could be an as of yet undiscovered compound. "The natural compound must be very important," Silbergeld says, who notes that dioxin is hormonelike in that it reversibly binds to a specific protein receptor.

The receptor, however, may not have any physiological counterpart. "Maybe the toxicological response is a vestigal one," Poland says—meaning that the receptor bound with something external that may no longer exist, leaving P-450 as a response to the environment. But, Poland asks, why should immune suppression or cell proliferation be triggered by environmental contaminants?

Whatever the outcome, though, the new TCDD data are more than supporting evidence in an epidemiological debate. Just as morphine led to the discovery of endorphins, dioxin is leading to an understanding of gene expression. "This was a secret wired part of the body. Dioxin pushed the button, and it lit up," Poland says. "Dioxin is a great poison, and poisons elucidate physiology." —*Marguerite Holloway*



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AMERICAN INDUSTRIAL HEALTH COUNCIL

SCIENCE COMMENTARY

Vol. 2, Issue 2 December 1990

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1. AIHC Comments Strengthen EPA's Interest in Developing a Biologically Based Model for Expressing the Carcinogenic Risk of Dioxin.

Background

In June 1988, (see *Science Commentary*, Vol. 1, Issue 1, Item 9), an Environmental Protection Agency (EPA) inter-office dioxin working group issued two draft reports, "A Cancer Risk-Specific Dose for 2,3,7,8-TCDD" and "Estimating Exposure to 2,3,7,8-TCDD." These reports described the EPA working group's decision to recommend a revised standard for exposure to dioxin. In the latter draft, the working group recommended changing the existing RsD (Risk-specific Dose) value for dioxin by a factor of 16, on the basis that the available scientific information did not support continued reliance on the default assumption requiring use of the linearized multi-stage (LMS)

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procedure, and on the working group's judgement that the weight-of-the-evidence supported a less stringent standard. It was the EPA working group's contention that new scientific information supported a change in the dioxin exposure standard set in 1985. Shortly after the release of the working group's draft reports, the Agency requested that they be reviewed by EPA's Science Advisory Board (SAB). An *ad hoc* panel was appointed by the SAB to conduct such a review.

The SAB *ad hoc* dioxin panel generally agreed with the EPA working group's interpretation of the science, but stated in their draft report that the available evidence did not compel a change in existing reliance on the LMS model. The SAB *ad hoc* panel noted that although "there are promising alternative models which may be expected to more accurately reflect the biological basis of 2,3,7,8-TCDD carcinogenesis, such newer models need to be further developed and validated." In effect, the *ad hoc* panel recommended continuing the LMS procedure as the default option of choice; however, the panel strongly urged EPA to build upon the working group's excellent review and move "to develop and validate a new risk model capable of more accurately estimating the risk of human cancer caused by dioxins and related compounds."

AIHC Actions

In a letter of February 23, 1989, to the chairman of the SAB *ad hoc* dioxin panel, AIHC supported the EPA working group's position for a revised dioxin standard and expanded on what the Council perceived as the nature of the EPA working group report, and the weight-of-the-evidence process the group used to reach their recommendation. AIHC disagreed with an SAB panel statement that the change in the dioxin RsD, as recommended by the EPA working group, was a "science policy" choice. AIHC suggested that the working group's document was a "hazard characterization" that supported reconsideration of the exposure standard for dioxin. It justly questioned the applicability of two default assumptions: the linearized multi-stage (LSM) procedure, and the use of the standard surface area scaling factor for interspecies conversion (body weight to the 2/3 power).

In verbal and written communications presented to the SAB Executive Committee on October 24, 1989, AIHC again supported the EPA dioxin working group's recommendations and questioned the reluctance of the

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SAB *ad hoc* panel to recommend against the scientifically inappropriate LMS default option. Further, AIHC stated that the SAB *ad hoc* panel's recommendation seemed to imply that the Agency should not permit its scientific staff to exercise expert judgement to choose from other scientifically plausible options an alternative to the obviously inappropriate LMS default option. AIHC noted that the deliberative process used by the EPA working group was consistent with the Agency's own Guidelines for Carcinogenic Risk Assessment and with the Office of Science and Technology Policy (OSTP) principles. AIHC observed that by using this process the working group had rightly concluded that use of the LMS default option was not supported by the science and had chosen instead, based on the working group's scientific judgement, one value for the RsD from among a spectrum of alternative values generated by other models and procedures.

Present Status

On November 28, 1989, the SAB *ad hoc* panel on dioxin issued their report to the chairman of the SAB. Subsequently, the SAB Executive Committee sent a summary of that report to the EPA Administrator. Pertinent excerpts are as follows:

"It should be noted that the "Dioxin" Panel was not asked, nor did it choose to address directly or in, detail, the adequacy of the Agency's 1985 cancer risk assessment for 2,3,7,8-TCDD. However, in the course of the current review, the Panel generally agreed with the EPA Working Group's criticism of the linear multi-stage model as applied to the specific case of 2,3,7,8-TCDD in 1985."

"This criticism reflects, in part, the existence of a series of important and innovative mechanistically oriented studies which have provided new insight into the toxicological effects of 2,3,7,8-TCDD and related compounds. The EC (SAB Executive Committee) joins the Panel in encouraging the Agency to support research which will incorporate this new information into risk assessment approaches currently under development, where appropriate."

"The EC concludes that the existing, LMS-based risk assessment for 2,3,7,8-TCDD lacks a firm scientific foundation. However, until the new approaches are fully developed and peer-reviewed, estimates based on other models are equally questionable. Unfortunately, the direction and extent of any change from the LMS-based risk estimate that might result from the application of more appropriate models cannot be determined at this time."

EPA's review of the 1985 dioxin standard, and the SAB *ad hoc* panel's recommendations, have provided a significant new opportunity for the Agency to look at biologically based, less ultra-conservative risk assessment methodology. As a result of the SAB's recommendation, EPA published a notice (55 FR 30513), "Development of a Biologically Based Model for Dioxin", which announced "a working meeting to be held by EPA's Office of Health and Environmental Assessment (OHEA) and the Institute for Evaluating Health Risks to discuss the development of a preferred model for quantitatively expressing the carcinogenic risk of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin)." The topic of this meeting, which was held August 2,1990, was also discussed at the October 21-24,1990, Banbury Conference on the "Biological Basis for Risk Assessment of Dioxins and Related Compounds."

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2. AIHC Sends Letter to Congressman Dingell on Scientific Issues Surrounding the Residual Risk Provisions in H.R. 3030, a Clean Air Act Reauthorization Bill.

Background

In late 1989, the Health and Environment Subcommittee of the House Energy and Commerce Committee reported out H.R. 3030, an amended version of the Bush Administration's Clean Air proposal. This bill deleted the "bright line" approach of Senate bill S. 1630, which required use of a numerical standard to establish what constitutes risk to a toxic air pollutant for the purpose of regulation, such as 10^{-4} or 10^{-6}. Under H.R. 3030 the "residual risks" of toxic air pollutants (those risks remaining after "maximum achievable control technology" (MACT) is installed) would be evaluated after initial technology controls are in place and a determination made by EPA as to whether further risk reduction measures are necessary. The final version of the House bill, as reported by the full Energy and Commerce Committee on April 5, 1990, did not contain the Senate's "bright line" approach.

AIHC Action

AIHC was concerned that amendments to H.R. 3030 might be introduced during deliberations of the full Energy and Commerce Committee, which would attempt to establish a numerical "bright-line" standard of residual risk for a maximally exposed individual (MEI) of one in a million. AIHC's Scientific Committee stated their opposition to this approach in a December 22, 1989, letter to Congressman John D. Dingell, Chairman of the Energy and Commerce Committee. In their letter, the Scientific Committee supported the "residual risk" provisions of H.R. 3030, as reported by the Health and Environment Subcommittee, as providing a sound framework for risk decisions. AIHC noted that H.R.3030 was "consistent with scientific principles underlying risk assessment and would permit regulatory consideration of anticipated advances in scientific information." AIHC cautioned that "any 'bright' line approach removes EPA's discretion to address uncertainty and to use the best available science as a basis for regulatory action."

On February 8, 1990, Congressman Dingell forwarded AIHC's letter to EPA Administrator William Reilly for review and comment. The House bill, as passed on May 23, 1990, did not adopt the "bright line" standard.

3. Senate Clean Air Act Bill and Residual Risk Discussed at Toxicology Forum

On February 19, 1990, AIHC Board Member Emeritus Robert Barnard, Esq., addressed the 1990 Annual Winter Toxicology Forum on the scientific issues raised by the residual risk provisions in the Clean Air Act bills under consideration. He focused his analysis on the Senate Bill and misuse of the population-based statistical 10^{-4} and 10^{-6} "bright line" risk estimates to predict the risk of any one person in -

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line" standard had been criticized because their report had stated "the science for assessing these risks is yet too new to be locked into absolute limits." He said the Committee report had also noted that critics urged discretion and the use of other risk dimensions and data in setting standards for cancer incidence, potency, and human exposure. Mr. Barnard stated that although the Senate report agreed that these concerns are legitimate, the Senate committee had rejected them because they felt that EPA had not followed "discretion" logic in fixing recent standards. He noted the Senate report further reasoned that "the 'bright line' boundaries on acceptable risk contained in the Senate legislation are necessary to bring health considerations in the standard-setting process at the beginning." In closing, Mr. Barnard suggested that the scientific community might want to become educators of Congress on science issues in order to prevent passage of legislation with a "bright line" approach, or any other legislation that may be similarly scientifically flawed.

4. AIHC Issues Concept Paper on Combining the Residual Risk Provisions of the House and Senate Versions for an Amended Clean Air Act.

Background on Proposed Residual Risk Amendments to the Clean Air Act

Senate Action

On April 3, 1990, the Senate passed S. 1630, its version of legislation to amend and reauthorize the Clean Air Act. The Senate bill contained the "bright line" approach and required industrial sources of 191 hazardous air pollutants to meet emission limits based on "maximum achievable control technology" (MACT) requirements to be developed by EPA and installed by industry within two to ten years. According to the Senate bill, before residual emissions could be required to be controlled further, a series of studies and reports must be completed concerning the "residual risks" from carcinogens that may remain after applying MACT.

Of particular concern to AIHC were provisions in the Senate bill freezing the numerical standard "bright line" approach, and the current risk assessment methodology, unless Congress agreed to change the law within five years. The bill did contain the following provisions which had potential to bring about changes in existing risk assessment methodology:

- Within three months after passage, the Administration must request the National Academy of Sciences (NAS) to undertake a study and make recommendations for changes in the current risk assessment methodology. A report on the NAS study would be due two years after passage of the bill.

- Eighteen months after passage of the bill, a ten-member Risk Assessment and Risk Management Commission must have been appointed: three members appointed by the President, three by the House, three by the Senate, and one by NAS.

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• Following the receipt of the NAS report, EPA must issue new risk assessment guidelines accepting or rejecting the NAS recommendations.

• The Risk Assessment and Risk Management Commission was charged to review the NAS report, the EPA guidelines, and undertake any necessary additional study. A report by the Commission, with legislative recommendations, was to be made to Congress and the President not later than 42 months after passage of the bill.

• Congress would consider the Commission's recommendations under an accelerated schedule.

House Action

On April 5, 1990, the House Energy and Commerce Committee passed H.R. 3030 and on May 23, 1990, this legislation passed the full House. The House version adopted language similar to that in the Senate bill on air toxics. Both bills required that any source emitting one or more of 189 to 191 listed air pollutants to install MACT within ten years. However, the two bills differed on residual risk -- the risk remaining after MACT is applied. The House bill did not adopt the Senate's "bright line" standards and deferred the issue of residual risk for ten years after enactment, or eight years after MACT is installed, whichever was later. At that time Congress must act or not act based on recommendations developed from a study conducted by EPA and the Surgeon General. If Congress did not legislate stricter standards, EPA could lower the emission standard to provide an "ample margin of safety," in accordance with the language of Section 112 of the 1977 law.

AIHC Action

AIHC has always taken the position that it is not appropriate to legislate regulatory criteria in the form of hypothetical statistical values, i.e., one-in-a-million risk (10^{-6}) to the most exposed individual. This value, calculated on the unrealistic assumption of 70 years residence at constant exposure, relates to a random representative of the exposed population and was never intended to serve as an estimate of risk for any actual person. Based on these and other scientific reasons, AIHC has strongly opposed the establishment of any numerical "bright line" standard for regulating risks.

Both the House and Senate proposals contained provisions for the evaluation, and, as necessary, regulation of residual risks remaining after mandated technological controls are in place. These provisions were to become operative within five to eight years after enactment. Both bills provided for Congressional review and had "default" provisions if Congress failed to act.

To express AIHC's views on the "bright line" approach, and to suggest a framework for the evaluation and regulation of residual risks, AIHC developed a concept paper, "Residual Risk: A Conceptual Framework for a Conference Proposal." It was hoped that AIHC's approach would aid the House-Senate conference committee in their deliberations for a compromise on the air toxics provisions in the House- and Senate-passed versions.

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The AIHC paper addressed ways to assure that the evaluation and regulation of residual risk would be based on the latest and best scientific information. AIHC suggested that the compromise bill would be enhanced and strengthened by a combination of:

- the House provisions and criteria on residual risk,
- the Senate provision for an in-depth study of risk assessment by the National Academy of Sciences,
- the Senate provision for an EPA revision of risk assessment guidelines in response to the Academy study report, and
- the Senate provision for establishment of the Bipartisan Commission on Risk Assessment and Risk Management.

Present Status

Conferees on the House-Senate conference committee reached a compromise on the legislation during the closing days of the last session of Congress. Both the House and the Senate ratified the Clean Air Act Amendments of 1990 on October 26, 1990. The President signed the measure into law on November 15, 1990.

There is evidence that several of AIHC's recommendations were considered in the air toxics provisions of the legislation. An analysis and study of the act (750 plus pages) is still underway; however, the following summarizes the residual risk provisions which have been of direct concern to AIHC:

- National Academy of Sciences Study: Within three months of enactment the EPA Administrator must enter into arrangements with NAS to conduct a study of risk assessment methodology. Within thirty months of enactment the NAS must submit a report to the Senate Committee on Environment and Public Works, the House Committee on Energy and Commerce, the Risk Assessment and Management Commission, and the EPA Administrator.
- EPA and Surgeon General Report: Not later than six years after enactment, the EPA in consultation with the Surgeon General, shall report their recommendations to Congress as to needed legislation regarding residual risk.
- Default Provision: If Congress does not act on the several recommendations within eight years, EPA shall promulgate emission standards which provide "an ample margin of safety to protect public health." If the technological standards (MACT) for those pollutants classified as a known, probable or possible human carcinogen do not reduce lifetime excess cancer risk to the individual most exposed to less than one-in-one million (10^{-6}), then further regulation of residual risk will be considered. (Note the 10^{-6} standard is a trigger, not a "bright line"; thus EPA is not prevented from looking at all dimensions of risk, as it did in the 1989 benzene rule.)

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5. AIHC's Reproductive and Developmental Effects Subcommittee Provides Technical Input on Draft Reproductive Toxicity Guidelines Proposed by California's Department of Health Services (DHS).

Background

On December 29, 1989, the Reproductive and Cancer Hazard Assessment Section, California Department of Health Services (DHS) released for review "Draft Guidelines for Identification and Hazard Assessment of Agents Causing Developmental and/or Reproductive Toxicity." The guidelines, prepared to satisfy the mandates of Proposition 65, were announced as providing DHS, and the "scientific community of California," with guidance "for the analysis and interpretation of information relating to the potential of an agent to cause reproductive or developmental harm."

The draft guidelines addressed the first two stages of the risk assessment process, Hazard Identification and Dose-Response Assessment/Reference Dose Determination (RfD). The draft identified three types of reproductive toxicity - male, female, and developmental toxicity. It also provided rules for assessing epidemiological studies and for the proper use of these studies as well as the draft proposed rules for animal studies. DHS suggested that the draft guidelines be implemented in conjunction with pertinent EPA and National Research Council (NRC) guidance documents. The later stages of the risk assessment process, Exposure Assessment and Risk Characterization, are not covered in the draft. Comments on the draft guidelines closed May 1, 1990. After DHS review, the guidelines may be amended and re-released for public comment.

AIHC Action

On April 26, 1990, AIHC's Reproductive and Developmental Effects Subcommittee supplied comments on California's draft Reproductive Toxicity Guidelines through the Technical Committee of the Environmental Working Group, a California industry-sponsored organization.

The AIHC comments commended DHS for recommending that a "weight-of-the-evidence" approach be used to evaluate reproductive and developmental toxicity data, and supported the California agency's position to incorporate all epidemiological data from both positive and negative studies. However, AIHC cautioned that it was unclear just how the weight-of-the-evidence process would be used by DHS and whether certain "levels of evidence" would be subsequently proposed for incorporating reproductive or developmental toxicants into Proposition 65 lists. AIHC also noted that it was often unclear as to whether certain DHS positions expressed in the guidelines were based on science or on DHS policy.

In their comments AIHC pointed to the implications of Karnofsky's Law, which states that any compound can be positive in a developmental toxicity assay under some combination of experimental conditions. The AIHC subcommittee suggested that "any categorization of compounds needs to be qualified by the circumstances under which the compound is positive or negative." They stated that the DHS guideline "appears to equate lack of data with hazard" and suggested that "it is not scientifically appropriate to assume a hazard exists in the absence of data."

The proposed DHS guidelines were largely based on the EPA "Guidelines for the Health Assessment of Suspect Developmental Toxicants", as reviewed by the EPA/SAB Environmental Health Committee on October 27, 1989, and currently under revision. (See below Item 6 and *Science Commentary*, Vol. 1, Issue 2, Item 1, October 1989.) AIHC recommended that the DHS document not be published in final form until the EPA guidelines have been amended to reflect the Environmental Health Committee's recommendations.

6. EPA's Environmental Health Committee Review of "Proposed Amendments to the Guidelines for the Health Assessment of Suspect Developmental Toxicants."

Present Status

As reported in the *Science Commentary* (Vol. 2, Issue 1, Item 6, June 1990), the EPA Science Advisory Board's (SAB) Environmental Health Committee met on October 27, 1989, to review EPA's document "Proposed Amendments to the Guidelines for the Health Assessment of Suspect Developmental Toxicants." AIHC's Reproductive and Developmental Effects Subcommittee addressed the SAB Committee at the time of their October 27 deliberations, principally on three areas of interest: the proposed weight-of-the-evidence classification scheme, the quantitative evaluation/extrapolation of data, and maternal toxicity.

On April 23, 1990, the SAB and the Environmental Health Committee issued a report on the Committee's review of the proposed amended guidelines. In general the Committee found the amended document "to be adequately founded in toxicological and teratological science, and to reflect the current status in these fields." The Committee did suggest that the Agency rethink their proposed weight-of-the-evidence classification scheme "in order to avoid confusion with more commonly applied uses of such classifications, and to develop a more powerful conceptual approach." Further, the Committee recommended that "the Agency should begin to move away from the current use of the No Observed Adverse Effects/Lowest Observed Adverse Effects Level (NOAEL/LOAEL) basis for calculating the Reference Dose, to a benchmark dose/confidence limit approach, tied to empirical models of dose-response relationships."

Several of AIHC's recommendations were given consideration in the SAB report. In particular, the Committee was in close agreement with AIHC's concern for the proposed weight-of-the-evidence classification scheme.

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7. AIHC Dialogue With the Department of Health and Human Services (DHHS) on Need to Communicate Proper Interpretation and Utilization of the National Toxicology Program's Annual Report on Carcinogens.

Background

Public Law 95-622, signed into law on November 8, 1978, requires the Secretary, DHHS, to publish an annual report containing "..a list of all substances (i) which either are known to be carcinogens or which may reasonably be anticipated to be carcinogens and (ii) to which a significant number of persons residing in the United States are exposed...." In addition, the annual reports are intended to provide information "on the nature and degree of exposures to such carcinogens and the extent to which Federal regulations are effective in reducing the risk to the public health from exposures to these substances." Responsibility for preparation of the annual report was delegated by the Secretary DHHS to the Director of the National Toxicology Program (NTP) in 1979. To date five annual reports have been issued, the first in July, 1980, and the last during the fall of 1989. Some 162 substances have now been selected by NTP for inclusion on these lists.

Within the past few years Federal regulators, and state legislators and regulators, when drafting new laws and regulatory initiatives, have, directly or by reference, included for regulatory restrictions, without further study, the list of "chemical carcinogens" as published in the Annual Report on Carcinogens (ARC). The best-known examples of regulatory programs affected to date include the Occupational Safety and Health Administration (OSHA) Hazard Communication Standards, the Environmental Protection Agency's community right-to-know program under Title III of the Superfund Amendments and Reauthorization Act (SARA) of 1986, and the listing of carcinogens under California's Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65). The ARC is also a basis for listing carcinogenic or hazardous substances under right-to-know laws in Alabama, Delaware, Florida, Illinois, Maine, Massachusetts, Michigan, Pennsylvania, and Vermont. Other states indirectly adopt the ARC list by incorporating the OSHA Hazard Communication Standard into their laws or regulations by reference.

This unintended, but almost automatic incorporation of the ARC lists into other laws and regulations, continues to occur even though DHHS and NTP have recently emphasized, both in a "Note to the Reader" and in the "Introduction" of the Fifth Annual Report on Carcinogens (1989), that the ARCs "are informational documents which serve as meaningful compilations of data on the carcinogenicity of the listed substances in humans and/or animals.... The reports represent the first step in hazard identification of the substances selected for inclusion....The evaluation of the degree of human risk from substances included in the Annual Reports requires a wider analysis than has been made in preparing the Reports. That is properly the purview of the Federal, State, and local health regulatory and research agencies authorized to implement the laws relating to carcinogens."

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A review of past events shows that NTP had previously stated, but had not widely publicized, that the ARC is based only on the first step in hazard identification. It was, and is, NTP's position that any evaluations beyond the first step is "the province of the regulatory agencies, not NTP in its preparation of the Annual Reports...." In a July, 1989 declaration, NTP defined the second step in hazard identification (as part of the risk assessment process to be performed by the regulatory agencies) as "the consideration of other data that are pertinent to risk assessment, i.e., assessing the estimated level of human carcinogenic risk." NTP goes on to explain that "the 'weight-of-evidence' approach is used in such an assessment, which includes a review of not only the epidemiological and experimental animal carcinogenicity data, but also, if available, pharmacokinetics and mechanisms of action data. Mechanisms of action studies are performed in an effort to ascertain the mechanism at a cellular and subcellular level by which a chemical produces a toxic effect, e.g., cancer. It is the province of the regulatory agencies, not NTP in its preparation of the Annual Reports, to perform this second step in hazard identification as part of the risk assessment process." In spite of these statements, the NTP position was not generally known in the regulatory community, and certainly not by the general public.

Due to concerns for the misuse of the ARC list of carcinogens, and public misunderstanding regarding the purpose of the lists, DHHS, in December 1987, requested that the inter-agency Committee to Coordinate Environmental Health and Related Programs (CCEHRP) review how the ARCs are developed. As a result of the CCEHRP review, which considered recommendations from several groups including AIHC, language as discussed above was added to the "Introduction" of the ARC, and in the "Note to the Reader," which does help to clarify the purpose and intended use of the annual reports. Regardless of these steps toward clarification, there remains a pressing need to inform users of the report that the purpose of the ARC list is to convey the results of the first step of hazard identification, and that the ARC is not meant to report the results of a complete hazard identification, which can then be used as the primary basis for regulatory restrictions of listed substances.

AIHC Action

Over the years, AIHC has interacted with DHHS on many different scientific and policy issues. Since 1986 the Council has maintained dialogue with officials in DHHS, NTP, and with CCEHRP concerning the importance of establishing the best available scientific basis for the development of the Annual Reports on Carcinogens, and the need to ensure that the ARCs are neither misinterpreted nor misused for regulatory and/or legislative purposes.

In December, 1986, comments to NTP on the draft Fifth Annual Report, AIHC focused their discussion on those substances that had been included on the ARC list based on experimental evidence from animal tests. To more accurately describe the basis NTP used for listing such chemicals, AIHC recommended that language be added in the ARC "Introduction" that would stress the need for an analysis of the data beyond carcinogenitic activity in animal tests. Specifically, AIHC suggested that the wider analysis should include "information and data not considered in the original

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review and data generated subsequently"—and should consider "the physiological, pharmacological, and toxicological differences between test animals and humans."

AIHC's Scientific Committee reaffirmed these views in testimony before an NTP sponsored meeting on the ARC in April, 1987. AIHC urged that the ARC state unequivocally that no evaluation had been made concerning use of the report for regulatory or corrective action, and that the ARC also state that no decision concerning regulatory use of the lists should be made without further scientific evaluation. As alternatives, AIHC proposed that NTP either perform a full weight-of-the-evidence hazard evaluation, including an assessment of relevance to humans, or simply continue to review positive data without a full review, but provide a prominent notice of the limited nature of the scientific review involved and place qualifications on possible regulatory or legislative use of the ARC lists. (This latter course was adopted in part by NTP stating certain limitations in the "Note to Reader" and "Introduction" of the Fifth Annual Report, as released in the fall of 1989).

AIHC's views, as outlined above, were expanded in July, 1988 testimony before the CCEHRP subcommittee convened to review the criteria and process used by NTP in preparing the ARCs. In particular, AIHC suggested that the Secretary, DHHS, should assume responsibility for educating regulators and the public as to the strengths and weaknesses of the ARC, and how the ARCs should or should not be used.

In spite of the progress made by NTP to clarify and explain the meaning and purpose of the ARC, AIHC believes that there remains a growing need for DHHS to initiate a more aggressive communications program directed to the users of the ARC, particularly the regulatory community and legislators. To discuss this situation, AIHC met with the DHHS Assistant Secretary for Health, Dr. James Mason, and his staff, on October 17, 1989. This meeting has resulted in an exchange of correspondence in which AIHC has again outlined concerns, and presented further examples of where the ARC continues to be a primary basis for regulatory restrictions. Also discussed at the October 17th meeting were the problems of incorporating new scientific information into the risk assessment process, and the application of negligible risk standards under the Federal Food, Drug and Cosmetic Act.

Science Commentary

Edited by: M. J. Sloan, Ph.D., Consultant
Designer/Production Coordinator: Tracey J. Carl

This newsletter summarizes important Federal regulatory and policy proposals relating to human chronic health matters. AIHC welcomes and encourages the involvement of academic scientists, whose input is essential to the development and incorporation of scientific advances in regulatory policy.

"The mission of AIHC is to promote the sound use of scientific principles and procedures in the assessment and regulation of risks of chronic human health effects and directly related public policy issues."

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Neurological Effects

Q. Is there epidemiological evidence to suggest that PCBs cause neurological problems in children?

A. There are two studies in the U.S. (Michigan and North Carolina) that suggest that environmental exposure to organic contaminants may cause minor defects in learning abilities in children. These defects have not been clearly linked to PCBs. Other contaminants, which might be the cause, have not been measured. In the Michigan study, there was no correlation between the deficits and PCB levels.

Q. What are ortho substituted congeners?

A. These are PCB molecules that have chlorines in either of the four positions around the bond connecting the two rings in biphenyl. These congeners may have chlorines in some of the meta and para positions as well. Most PCBs are ortho-substituted.

Q. What is the significance of the NYS finding that certain "ortho" congeners affect dopamine levels in rats and monkeys and tend to concentrate in brain tissue?

A. There are numerous questions that need to be answered before the significance can be determined:

1. Exposure levels in the NYS tests were very high (500 and 1000 ppm 1254 in one study and up to 3.1 mg/kg-day 1016 in another.) These levels are much higher than humans exposed either occupationally or environmentally (e.g., eating fish). The NYS papers do not allow one to determine the response at various doses — therefore the possible relevance of these data to human exposure is not clear.

2. The significance of dopamine level depressions of the magnitude found is unknown. Dopamine levels are depressed by other chemicals, such as alcohol, reversibly. The nature of the PCB effect may be simply anaesthetic and also reversible.

3. The *in-vitro* tests on rat brain cells do not necessarily represent what happens in living mammalian systems. For example, 2,2' dichloro-biphenyl is a significant depressant for dopamine in cell studies, but is metabolized so rapidly *in vivo* that it will not accumulate in the brain.

Q. Is there any evidence for an association of PCB exposure and Parkinson's disease?

A. None of the occupational mortality or living worker studies have suggested that Parkinson's disease might be related to PCBs. If the disease does not show up with highly exposed workers, it is not likely to result from lower, environmental exposures.

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NIOSH (Sinks) Study of Westinghouse Capacitor Workers (Bloomington, Indiana)

Q. What were the major findings of the NIOSH study?

A. 8 deaths due to malignant melanomas vs. 2 expected
5 brain cancers vs. 2.8 expected
54 deaths due to all cancers vs. 63 expected

Q. Were any of the results related to PCB exposure?

A. No. Attempts to relate exposure of people who died of melanoma and brain cancer did not identify a relationship.

Q. Was there a relationship of latency period and exposure to PCBs?

A. No.

Q. Is there a biological rationale for a relationship of melanoma and brain cancer with PCB exposure?

A. No. No rationale was presented in the paper.

Q. Do the major findings from the study concur with those from other PCB mortality studies?

A. The absence of a relationship of cancer deaths to PCB exposures is consistent with other studies. The excesses of melanoma and brain cancer are not consistent with the results from other PCB mortality studies. For example, the study of GE workers by Taylor (NCI), which is most closely related to the NIOSH study, found no excess of melanoma and a reduction in brain cancers. Like the NIOSH study, the Taylor study found a reduction in deaths due to all cancers, combined.

Dioxin-like PCB Congeners - TEFs

Q. What are "dioxin-like PCB congeners"?

A. PCB structures having both para positions chlorinated, two or more meta positions chlorinated, and no more than one ortho position chlorinated — also known as coplanar PCBs.

Q. What are TEFs?

A. An approach to relating the toxicities of dioxins other than 2,3,7,8-TCDD, dibenzofurans and coplanar PCBs to 2,3,7,8-TCDD, which is considered to be the most toxic of these compounds.

Q. What is the relationship of the coplanar PCBs to 2,3,7,8-TCDD?

A. The coplanar PCBs exhibit the same type of acute toxicity as TCDD — thus include such end points as lethal dose, immunosupression, enzyme induction and skin effects. However, the relationship does not hold with respect to carcinogenicity. While TCDD is a powerful carcinogen, the most carcinogenic PCB mixture, Aroclor 1260, contains a small amount of coplanar PCBs. Aroclor 1254, which is negative as a carcinogen, contains a relatively large amount of coplanar PCBs.

Q. How does this impact on the concept of regulating PCBs on the basis of TEFs?

A. Since both TCDD and PCBs are regulated as carcinogens, and since carcinogenicity of PCBs appears to be related to highest level of chlorination, not TEFs, the application of TEFs to PCB risk assessment does not appear feasible.

Q. What are the levels of coplanar PCBs in Hudson River sediments?

A. The anaerobic dechlorination taking place in the upper river "hot spots" preferentially destroys coplanar PCBs. This finding has been determined both analytically and in biological test systems.

Q. What is the cancer risk related to PCBs in upper Hudson River sediments?

A. In animal tests, only 60% Cl PCB mixtures are carcinogenic. The PCBs in the sediments are derived from 1242 (42% Cl) but have been dechlorinated to a mixture that will not accumulate in humans. Workers exposed occupationally to very high levels of 1242 did not have an increased risk of cancer. The PCBs in the sediment should not represent a risk of cancer to humans.

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Neurological Effects

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THE WALL STREET JOURNAL TUESDAY, AUGUST 6, 1

The Dioxin Un-Scare—Where's the Press?

By Reed Irvine

Dioxin has been described as "the most nt carcinogen ever tested." An unted contaminant in some chemicals industrial processes, it has been the et of studies funded by the government ie tune of $400 million over the past de. Claims lodged by individuals who redly suffered or even feared serious age to their health from exposure to n have cost businesses and the govlent additional hundreds of millions of rs. Now a high government official us that an inordinate fear of dioxin just a scientific misunderstanding.

1982, the government ordered the uation of the 2,232 residents of Times h, Mo., because traces of dioxin were in the soil. At that time, the Centers isease Control believed that ingesting ing containing as much as one part illion of dioxin posed a significant risk man health. The Environmental Pro- ly spent $33 million to buy up declared it to be a dangerous e. Motorists passing through gway 44 were greeted with signs ng them to keep their windows closed ot to stop and leave their vehicles.

e government pinned the responsibil- the contamination of Times Beach other Missouri sites on Syntex Corp. f its subsidiaries had bought a plant ld once supplied dioxin-tainted waste a contractor who had sprayed it on eets of Times Beach and the other After years of costly litigation, Syned a consent decree a year ago ng to clean up the sites and incinerne 100,000 cubic yards of contamisoil. Cost estimates run as much as illion over the next decade.

l after the demolition of buildings in Beach began this spring, Dr. Vernon Houk, the CDC official who had recommended the evacuation in 1982, dropped a bombshell. At an environmental conference in Missouri, he said that he would not be concerned about the levels of dioxin at Times Beach because scientific studies have shown that low doses of dioxin pose minimal health risks.

Dr. Houk, who is director of the Center for Environmental Health and Injury Control at the CDC, told reporters that he now believes that the evacuation of Times Beach was unnecessary. Asked what he would tell the former residents of the town, who underwent the trauma of being torn from their homes, Dr. Houk said: "We should have been more upfront with Times Beach people and told them, 'We're doing our best with the estimates of the risk, but we may be wrong.' I think we never added 'but we may be wrong.' "

In debunking the claim that dioxin is a potent human carcinogen, Dr. Houk attacked the scientific theory and methodology that had led him and others to what he believes was a false conclusion. Dr. Houk said the method used to assess the risk was based on an assumption that violates a fundamental rule in toxicology: The dose makes the poison. It was assumed that feeding laboratory animals the maximum dose they could tolerate would enable scientists to determine whether trace amounts of a chemical would cause cancer in humans. One obvious problem with this is that different animals have different responses to the same chemical. What can be highly toxic to guinea pigs may have no effect on rats, and what may cause cancer in mice will not necessarily have the same effect on humans.

But Dr. Houk's attack went beyond this. He added his voice to that of Bruce Ames, head of the biochemistry department of the University of California at Berkeley, a leading critic of the methods used to assess the cancer risk from chemicals. Mr. Ames contends that the animal tests are fundamentally flawed because the maximum tolerated doses of the chemicals being tested kill cells due to the sheer size of the dosage. Mr. Ames argues that this can cause rapid cell division among the surviving cells, leading to cancer-causing mutations. This suggests that risks calculated from animal tests involving maximum tolerated doses are greatly exaggerated.

Dr. Houk says that most scientists now agree with this. He cites the dioxin case as "a good example of why we must use both animal and human data when evaluating the potential health effects of chemical exposure for humans." The epidemiologic evidence, he says, shows that "if dioxin is a human carcinogen, it is a rather weak one in the population exposed to high doses . . . and is not a carcinogen in the population exposed to lower doses." He adds that there are no convincing data that show that exposure to dioxin causes birth defects, chronic diseases of the liver or of the immune, cardiovascular, or neurologic systems.

Skeptics have long noted the glaring inconsistency of the risk assigned to dioxin based on animal tests and actual human experience. What has brought scientists such as Dr. Houk around is the mounting empirical evidence and the growing support for a theory that explains why humans are far less sensitive to dioxin than are guinea pigs.

The theory is that for dioxin to have a toxic effect it must first bind to receptors. There appears to be a dose, which varies by species, below which the receptors don't function; therefore there is no risk unless that dose is reached or exceeded. No one knows just what the level is for hum but it is apparently far higher than maximum acceptable intake level se the EPA of 0.006 trillionths of a gram kilogram of body weight per day. Car and some European countries set acc able levels 170 to 1,700 times that.

The EPA has yet to recognize that oxin's dangers have been greatly exag ated, but Dr. Houk predicts it will eve ally come around. It plans to begin stu ing the matter soon, but acceptance of Houk's analysis won't come easily. chael Gough of the Office of Technol Assessment says that if the EPA backs on dioxin, it will open the door to dema for reassessment of many other chemic "That," he says, "is a door they will rel tantly open."

In the meantime, the cleanup of Tim Beach proceeds. Dr. Houk says there little choice but to go ahead with it. " cause we've got the public so riled up The media that got people riled up w scare stories about dioxin-tainted Age Orange. Times Beach and paper-mill eff ent have done little to "unrile" them. I Louis Post-Dispatch under a front-pa banner headline, but it got little attenti in the East. ABC News reported it; CI and NBC did not. The newspapers that i fluence those in Washington who cou bring the costly Times Beach boondogg to a screeching halt buried a small A story deep on their inside pages.

Mr. Irvine is chairman of Accuracy i Media Inc., a media watchdog group.

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PCDF + DP's
in Elect.
Eq'n

GENP 011556

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#10

Measurement of PCDF and PCDD in Utility Equiment

Sydney H. Gordon, Michael Miller
IIT Research Institute, Chicago, Illinois

Fred L. DeRoos, Marcus Cooke
Battelle Memorial Institute, Columbus, Ohio

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Electric Power Research Institute, Palo Alto, California

Introduction

There is increasing interest in the potential for formation of polychlorinated dibenzofurans (PCDF) and polychlorinated dibenzo-p-dioxins (PCDD) from uncontrolled fires involving polychlorinated biphenyl (PCB)-containing dielectric fluids. At the temperatures that prevail in transformer fires, PCB may react to form PCDF and other toxic oxidation products. Information is sparse, however, on the distribution of PCDF and PCDD in the PCB-containing insulating fluids used by the electric utility industry. Even less is known about the effect of service time on the contaminant concentration in PCB-filled electrical equipment. There is also the possibility that abnormal operation (arcing, overheating) may create conditions that lead to the formation of PCDF and PCDD. In order to address this issue, the Electric Power Research Institute (EPRI) recently initiated a program to evaluate and develop compound-specific analytical procedures for characterizing PCDF and PCDD in PCB-containing insulating fluids.

Although the toxicological effects of PCDF and PCDD on humans is not well understood, tests using laboratory animals suggest that these compounds are much more toxic than PCB. Moreover, they generally occur at very low ambient concentrations, and toxicities can vary over a range of five orders of magnitude depending on the specific compound (i.e., degree of chlorination and location of chlorine atoms). Consequently, in order to measure specific compounds at pg/g (parts-per-trillion) levels, the EPRI-sponsored research has three basic goals:

- Improve the chemical analytical methods for individual compounds of PCDF and PCDD

GENP 011557

- Determine the concentration of PCDF and PCDD in utility equipment that has seen varied use
- Develop a screening test that determines PCDF and PCDD concentrations rapidly in order to reduce costs and time delays before undertaking cleanup activities after PCB fires.

To this end, the program has been divided into the following tasks:

- Round-robin method evaluation
- Analysis of spiked sample matrices and in-service dielectric fluids
- Development of a rapid MS/MS screening technique.

This paper summarizes the chemical analytical methods used and some of the early results of these studies. Results of the MS/MS investigation will be reported elsewhere.

Analytical Approach

The isomer-specific analysis of trace concentrations of PCDF and PCDD in PCB-containing fluids is a challenging problem. The analytical difficulty is often compounded by the presence of interfering substances such as PCB or polychlorinated diphenyl ethers. Combined capillary column gas chromatography/mass spectrometry (GC/MS) is the method of choice for the measurement of PCDF and PCDD in trace amounts. The selectivity of high resolution GC together with the sensitivity of mass spectrometry yields detection limits of 0.5 to 2 ng/g (parts-per-billion) for these compounds.

Since GC columns cannot handle PCB-contaminated fluids (sample matrices) directly, the samples must be extracted first with a suitable solvent to separate the analytes of interest (i.e., PCB, PCDF, PCDD) from them. This is followed by an enrichment step to remove co-extracted interferences and to concentrate the PCDF and PCDD in the sample extract. Finally, GC/MS analysis of the sample extracts provides the identification and quantitative measure of the PCDF and PCDD of interest, based on the premise that GC separation of each chlorinated compound class can be achieved so that a unique set of mass spectral indicator ions can be monitored for each of the appropriate GC retention time windows. The use of high resolution GC/high resolution MS (HRGC/HRMS) improves the isolation of PCDF and PCDD from impurities and reduces the need for extensive sample cleanup.

GENP011558

To determine the recovery efficiency and improve quantitation, appropriate ^{13}C-labeled isotopic analogs of the "dioxins" and "furans" are added to the samples. (^{13}C-containing compounds are not present in significant quantities in naturally occurring materials and are clearly distinguishable from native compounds by mass spectrometry. Spiking samples with labeled compounds thus provides unique internal standards for accurate analysis.)

Round-Robin Method Evaluation

The round-robin method evaluation is designed to validate the analytical procedure and to determine the reliability with which specific compounds can be identified and quantified. It includes the synthesis of native and isotopically labeled standards and the analysis of spiked sample matrices using the best available in-house procedure. Five laboratories are involved in this effort: Battelle Memorial Institute, IIT Research Institute (IITRI), New York State Department of Health (NYSDH), Radian Corporation, and University of Umea. Radian synthesized the standards and prepared the spiked matrix samples. These have been analyzed by the five laboratories.

The laboratories use a variety of extraction-cleanup procedures and analytical GC/MS techniques, the main elements of which are summarized in Table 1. Five baseline samples (Aroclor 1016, Aroclor 1242, Aroclor 1260, tri- and tetrachlorobenzenes, and aged mineral oil) were spiked with the isotopically labeled internal standards ^{13}C-2,3,7,8-TCDF, ^{13}C-2,3,7,8-TCDD, ^{13}C-1,2,3,7,8-PnCDF, and ^{13}C-OCDD at concentrations of 100 ng/g each. In addition, the three Aroclors and the tri- and tetrachlorobenzene samples were spiked with the native isomers 1,2,3,4,7,8-HxCDF and 1,2,3,4,6,7,8-HpCDF at concentrations of 100 ng/g each. The aged mineral oil sample contained several additional unlabeled isomers as well as 510 ug/g Aroclor 1260 and 530 ug/g tri- and tetrachlorobenzenes. A summary of the measured average total congener class concentrations obtained by the reporting laboratories is presented in Table 2.

In general, agreement between the values obtained by the various groups was found to be within a factor of two. In the case of the tri- and tetrachlorobenzene sample, fairly good agreement was also obtained between the expected and measured amounts for the isomers spiked into the matrix, and virtually no additional PCDF and/or PCDD was detected. By contrast, the Aroclor 1260 sample contained significant amounts of a large number of PCDF compounds. Lower, but still significant, amounts were measured for the remaining Aroclors, while the aged mineral oil sample had concentrations close to that of the spiked values.

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Analysis of In-Service Dielectric Fluids

A preliminary round-robin study was undertaken by three of the participating laboratories using four samples of dielectric fluids taken from in-service transformer and capacitor units. The average total congener class concentrations found for PCDF are shown in Table 3. Owing to differences in analytical procedures and variabilities in sample matrices, estimates of detection limit have not been included.

Sample ISL1O was a mineral oil contaminated with 100 µg/g PCB from a transformer that had failed in service by arcing. Sample ISL2A was an Askarel containing Aroclor 1242, which was removed from a capacitor that had bulged, but not ruptured, in service. Both samples had PCDF at concentration less than or equal to the limits of detection. Samples ISL3A and ISL4A both contained 70% Aroclor 1260 and 30% trichlorobenzene. Sample ISL3A was taken from an Askarel transformer after 20 years of service and contained 0.5 to 2.0 µg/g of PCDF. Sample ISL4A was taken from a transformer of a different manufacturer after 31 years of service. It had substantially higher concentrations of these partial oxidation products for all congener classes except total TCDF.

Work to date has developed improved measurement methods for compound specific PCDF and PCDD in PCB-contaminated insulating fluids. Thus far, the results do not suggest a preferred analytical technique. Analysis of the four in-service fluids indicate measurable quantities of specific PCDF compounds in 20- to 30-year old insulating fluids, however, there is insufficient information for generalization to all utility equipment.

Future work will focus on replicate analyses of another three baseline samples as well as 10 in-service samples.

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Table 1. Summary of Analytical Protocols for PCDF and PCDD Measurements

Laboratory	Cleanup		Analysis	
	Solvent Extraction	Column Chromatography	Capillary Gas Chromatography*	Mass Spectrometry**
Battelle	None	$SiO_2/Al_2O_3^{\ominus}/Al_2O_3^{\ominus}$	DB-5, CPSil-88	HRMS, EI
IITRI	None	Gel perm/$Al_2O_3^{\ominus}$/ $Al_2O_3^{\oplus}$	SP-2330/CPSil-88	LRMS, EI
NYSDH	None	$Al_2O_3^{\ominus}/C/Al_2O_3$	SP-2330	HRMS, EI
Radian	CH_3CN/ hexane	$SiO_2/Al_2O_3^{\ominus}/Al_2O_3^{\oplus}$	DB-5, SP-2340	LRMS, EI
Umea	None	C-fiber/rev elut/ Florisil	SP-2330	LRMS, NCI

*Fused-silica capillary columns

**LRMS = low resolution MS; HRMS = high resolution MS; EI = electron impact; NCI = negative ion chemical ionization (methane). (All use selected ion monitoring mode MS).

Table 2. Average Total Congener Class Concentrations of PCDF and PCDD in Spiked Baseline Samples

Congener Class	Sample, µg/g				
	Aroclor 1016	Aroclor 1242	Aroclor 1260	Tri- and tetra-chlorobenzenes	Aged Mineral Oil
ΣTCDD	ND	ND	ND	ND	0.03±0.02
ΣTCDF	0.01±0.01	0.94±0.78	0.55±0.39	ND	0.10±0.02
ΣPnCDF	0.01±0.02	0.33±0.24	1.15±0.68	ND	0.21±0.04
ΣHxCDF	0.18±0.12	0.10±0.03	2.73±2.08	0.34±0.44	0.44±0.44
ΣHpCDF	0.08±0.01	0.12±0.06	1.83±0.71	0.11±0.04	0.07±0.05
OCDF	0.01±0.01	0.63±1.12	2.81±1.22	ND	0.22±0.15

ND = not detected

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Table 3. Average Total Congener Class Concentrations of PCDF in Four In-Service Dielectric Fluids

Congener Class	In-service Sample, µg/g ISL4A	ISL3A	ISL2A	ISLIO
ΣTCDF	0.1	0.5	ND	ND
ΣPnCDF	2.6	1.6	0.01	ND
ΣHxCDF	7.6	1.6	0.01	ND
ΣHpCDF	16.3	2.0	ND	ND
OCDF	9.8	1.0	ND	ND
Total PCDF	36.4	6.7	0.02	ND

ND = not detected

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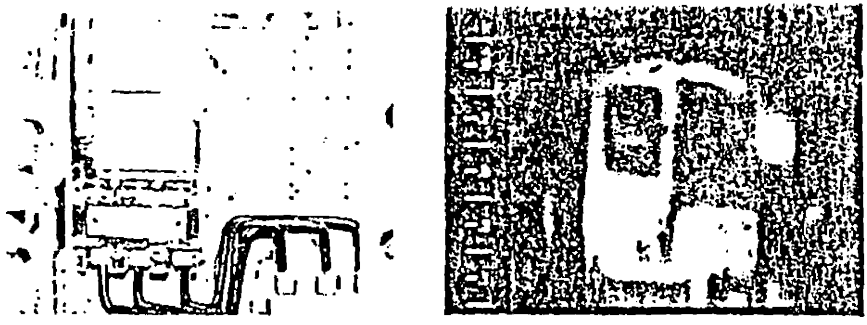
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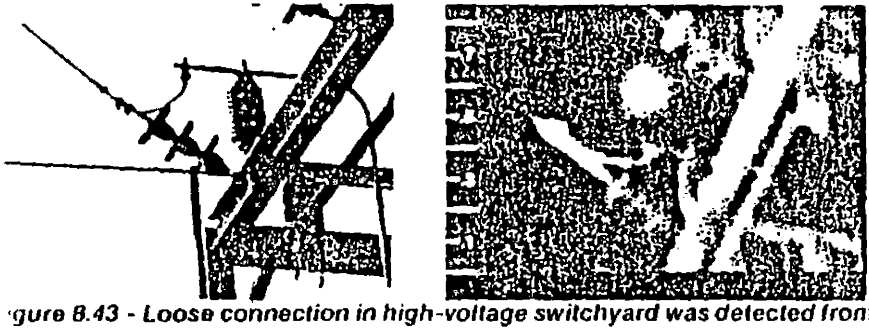
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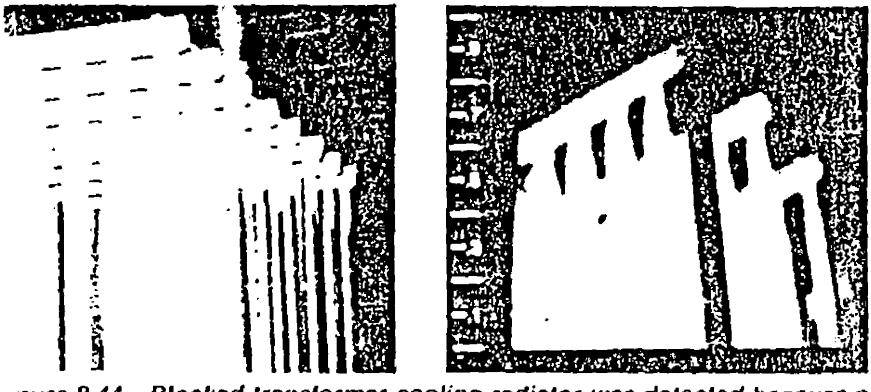
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gure 8.42 - Thermogram clearly pinpoints wiring with bad breaker/wire con-ections. (Courtesy of Thermotest Infra-Red Surveys).



gure 8.43 - Loose connection in high-voltage switchyard was detected from round level. (Courtesy of Plant Engineering® and AGA Corporation).



gure 8.44 - Blocked transformer cooling radiator was detected because of ck of heating in the radiator. In another similar case history IR thermograms abled a utility to prove certain radiators on a new transformer were either ocked or improperly designed. (Courtesy of Plant Engineering®).

PCB FILE

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What To Do About Askarel (PCB) Transformers (Past, Present, and Future)

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GENP 011564

Part 1—Straight Talk On Askarels

Introduction

Fluids containing polychlorinated biphenyl (PCB), known generically as "askarel," have been used for nearly 50 years for heat transfer and insulating electrical equipment in applications requiring a highly fire resistant liquid. Thus, transformers could be used indoors close to the load center.

PCB is exceptionally stable and resistant to oxidation from acids, bases, and other chemical agents. This same stability has caused the liquid to persist and so in the late 1970's the U.S. Environmental Protection Agency (EPA) established regulations governing PCB use, maintenance, handling, storage, and disposal (Figure 9.1). These regulations are not voluntary, but mandatory, and therefore an understanding of these rules must precede our primary focus—maintenance of service-aged transformers, in this case, askarel-filled equipment.

In addition, the EPA banned the manufacture of the fluid, and thus the production of *new* askarel-insulated transformers has ceased.

However, current U.S. government regulations do not require earlier than normal removal of PCBs from service in totally enclosed industrial and utility locations ("railroad transformers"—only 1000 units—are required to be retrofilled to alternative fluids.)* It is anticipated that many of the estimated 140,000 in-service askarel-filled transformers and 35 million oil-filled service-aged units (assumed by the EPA to be PCB-contaminated) may be around for many years to come.

**Pending legislation may ban continued use of PCBs in the food and feed industries.*

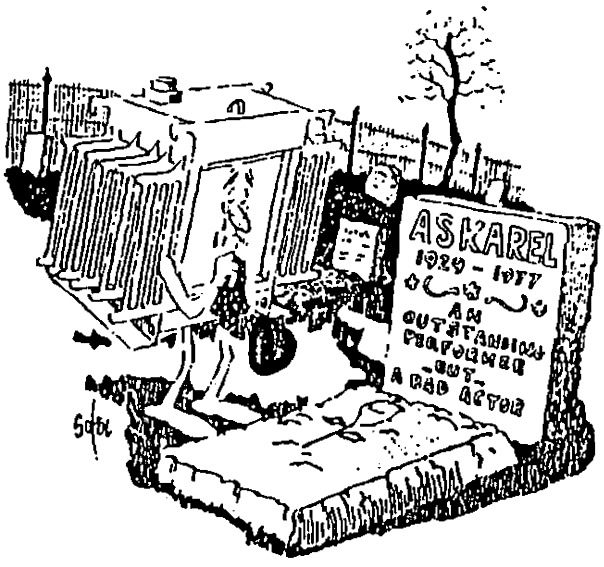


Figure 9.1 - PCBs: On their way out. (Courtesy of Plant Engineering®).

PCB History

Invention and Development (Primary Uses)

Askarel was first used in power capacitors (Figures 9.2 and 9.3) and then in power transformers (Figure 9.4). PCB was invented in 1929 as a fire-resistant dielectric fluid for capacitors by the Swann Chemical Company. The company was later bought out by Monsanto Chemical, St. Louis, Missouri. The late Dr. Frank M. Clark patented its application for use as a transformer insulating fluid for the General Electric Company in 1931. The first transformers were put in-service in 1933.

As summarized by Underwriters Laboratories (UL) Incorporated (Table 9.1):

> Transformer askarels are considered non-flammable at ordinary temperatures. Under practical conditions, formation of combustible or explosive mixtures is regarded extremely unlikely. The fire hazard is very small.

As noted in Table 9.2, certain applications such as the pulp and paper industry have particularly relied upon askarel-filled equipment.

TABLE 9.1

FLAMMABILITY RATING UNDERWRITER'S LABORATORY (UL)*	
Water	0
Transformer Askarel	2-3
Silicone	4-5
Transformer Oil	10-20
Ether	100

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GENP 011565

TABLE 9.2

ESTIMATED ASKAREL TRANSFORMER POPULATION	
Classification	PCB Units Per Total Units
All Units Power Units Pulp and Paper Industry	1 of 250 (140,000 of 35,000,000) 1 of 36 (140,000 of 5,000,000) 2 of 5[1]
[1]Non-random sampling of ten clients	

*See Chapter 2, Part 1, Table 2.1.

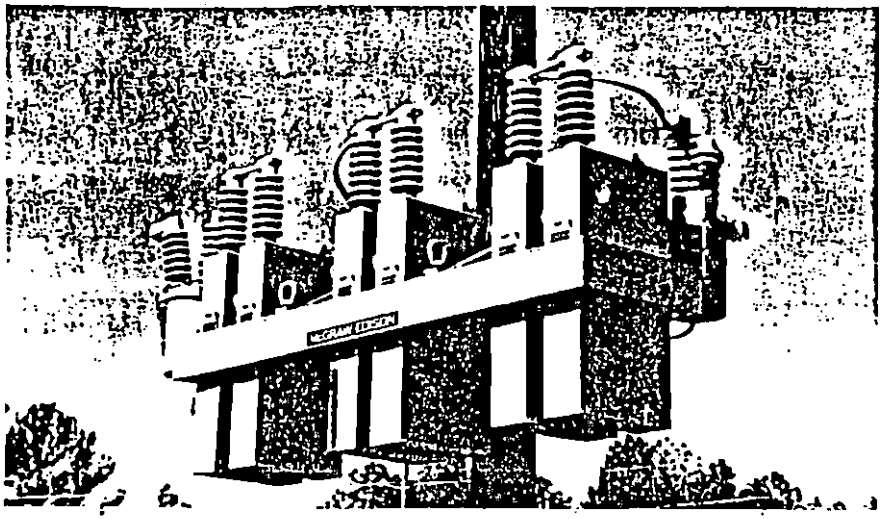


Figure 9.2 - A typical power capacitor bank. (Courtesy of McGraw-Edison Company).

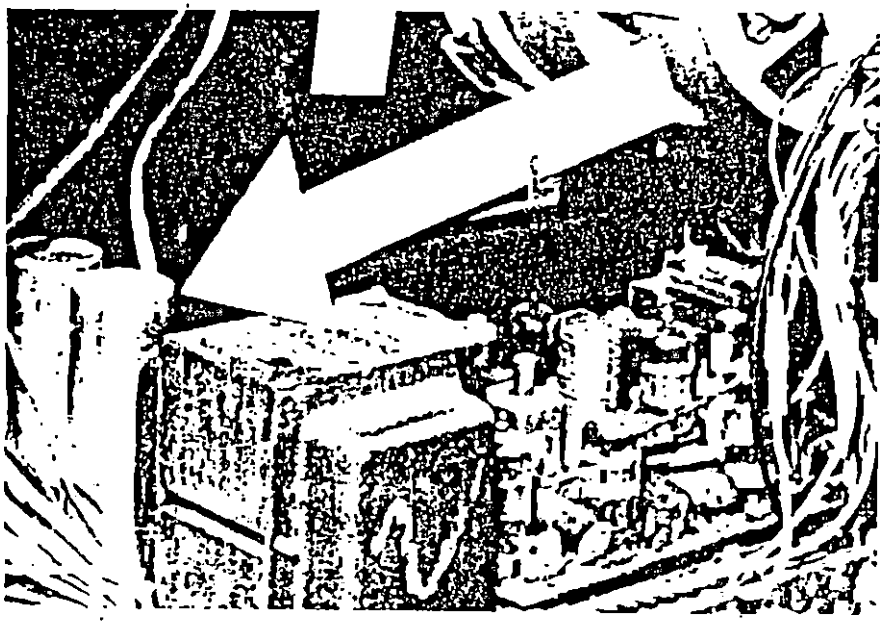


Figure 9.3 - A typical capacitor as used in electronic circuits.

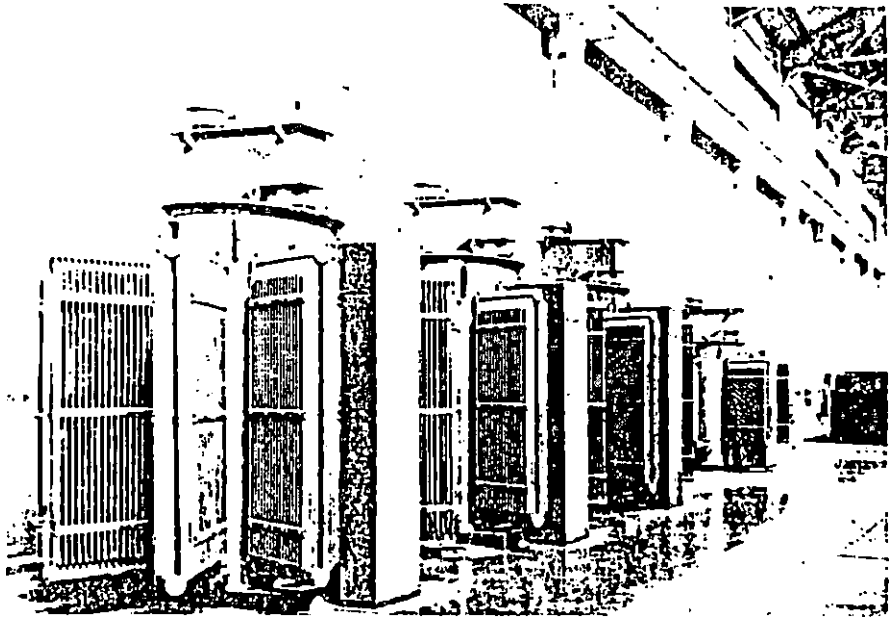


Figure 9.4 - A typical askarel transformer bank located inside a factory.

Secondary Uses

Unique fire resistant capabilities for PCB use extended far beyond its initial purpose, encompassing the following diverse applications (listed in order of predominant uses):

- **Capacitors,** including fluorescent light ballasts
- Plasticizers in synthethic resins and in rubbers
- Carbonless carbon paper (NCR)
- **Transformer liquids**
- Hydraulic fluids
- Lubricants, cutting oils
- Heat-transfer liquids
- Paint pigments
- Sealants
- Adhesives
- Printing inks
- Floor waxes
- Caulking compounds
- Dedusting agents
- Frying pans (heat transfer)
- Mounting medium for microscopic slides

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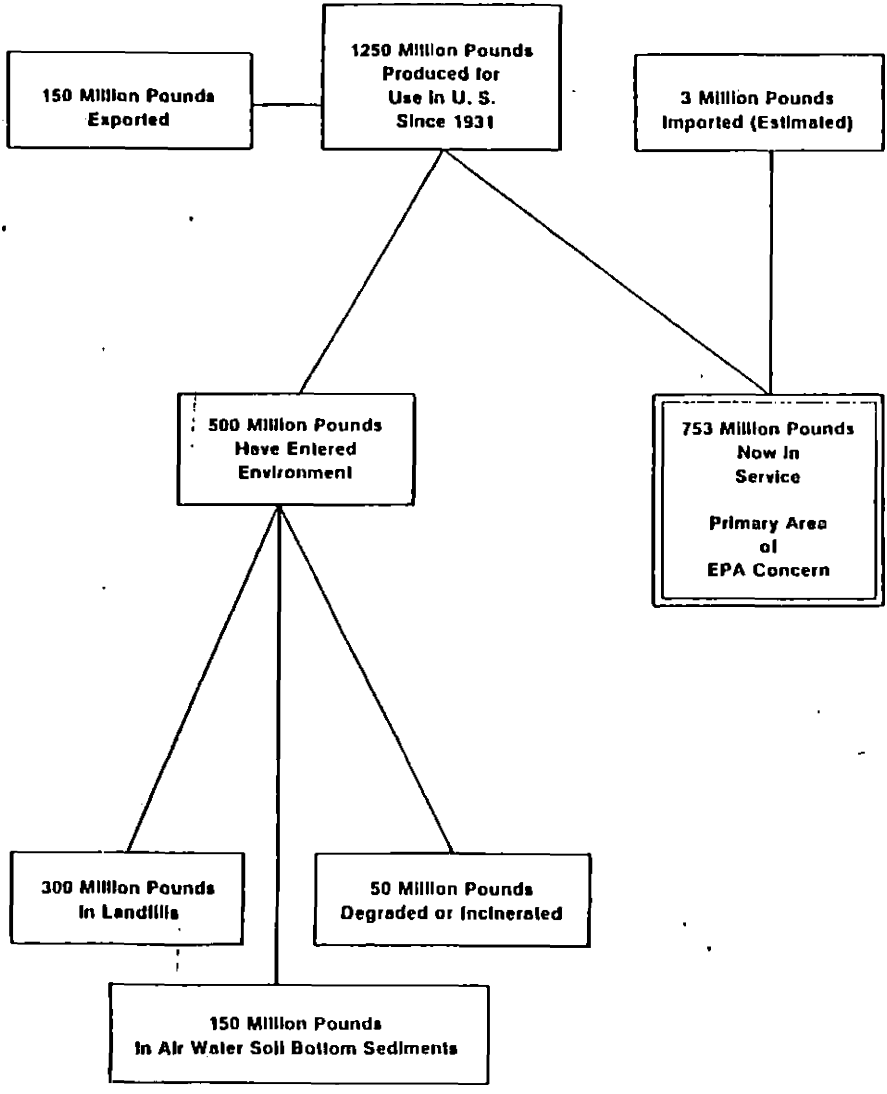
GENP 011566

Table 9.3 shows that over a billion pounds of PCB were produced between 1931 and 1977. Virtually all PCBs in existence today were

produced synthetically. About 40 percent of this synthetic fluid has entered the environment (Table 9.3).

TABLE 9.3

PCB HISTORY IN THE UNITED STATES



How to Identify PCBs

Throughout its history, PCB has been manufactured in the United States by Monsanto, under the trade name Aroclor®. It was then commonly wholesaled to second parties who would designate the generic "askarel" by a private label trademark for resale to end-users. Table 9.4 gives the more common registered trademarks used in the U.S. and overseas.

Most authorities state that some European PCBs are more toxic than U.S. Aroclors. In any event, designations are most likely to be used to identify the coolant in a high fire point transformer. The designations "askarel" and "PCB" are rarely found on a unit's nameplate.

PCB Characteristics

Molecular Structure

The PCB molecule consists of two phenyl molecules joined together, with two or more hydrogen atoms replaced by chlorine atoms (Figure 9.5). Two hundred and nine chlorine-substituted biphenyls can be created by the replacement of chlorine atoms at the various corners of the carbon rings. However, only three types of PCB are normally used in electrical transformers—1242, 1254, and 1260.* The first two digits, (12), designate the number of carbon atoms in the molecule and 42, 54, and 60 stand for the weight percent of chlorine respectively in each type. Other grades are used in capacitors.

Chemical Composition

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Commonly used transformer askarels are essentially a blend—mixtures of chlorinated biphenyl and chlorinated benzene (Table 9.5). The PCBs are mixed to give particular viscosity characteristics. Nevertheless, transformer askarels are generally interchangeable.

In Table 9.5 reference is made to additive(s). The scavenger acts as a "getter" for free hydrogen chloride gas should any be present as a result of spurious arcs.

An earlier scavenger, tin tetraphenyl (1944), had a tendency to precipitate out on top of askarel as a milky-white crystalline material.

ASTM D-2283 lists seven types of askarel that have been used in transformers over the years by various manufacturers.

GENP 011567

TABLE 9.4

ASKARELS CONTAINING PCBs IN U.S.A.	
Trademark	**Manufacturer**
Aroclor	Monsanto
Asbestol	American Corporation
Askarel	See Note 1
Chlorextol	Allis Chalmers
Diaclor	Sangamo Electric
Dykanol	Cornell Dubilier
Elemex	McGraw Edison
Hyvol	Aerovox
Inerteen	Westinghouse Electric
No-Flamol	Wagner Electric
Pyranol	General Electric
Saf-T-Kuhl	Kuhlman Electric

[1]Generic name used for insulating liquids in capacitors and transformers; may contain PCBs.*

**According to ANSI/IEEE/ASTM, "askarel" is "a generic term for a group of synthetic, fire resistant, chlorinated aromatic hydrocarbons used as electrical insulating liquids. They have a property under arcing conditions such that any gases produced will consist predominantly of noncombustible hydrogen chloride with lesser amounts of combustible gases." (IEEE Standard 591-1977; ANSI C57.12.80; ASTM D-2864).*

OVERSEAS	
Trademark	**Manufacturer**
Pyroclor	Monsanto (England)
Clophen	Bayer (Germany)
DK	Caffaro (Italy)
Fenclor	Caffaro (Italy)
Kennechlor	Mitsubishi (Japan)
Phenoclor	Prodelec (France)
Pyralene	Prodelec (France)
Santotherm	Mitsubishi (Japan)
Kanechlor	Kangegafuchi (Japan)
?	Sovol (U.S.S.R.)
?	Chemko (Czechoslovakia)

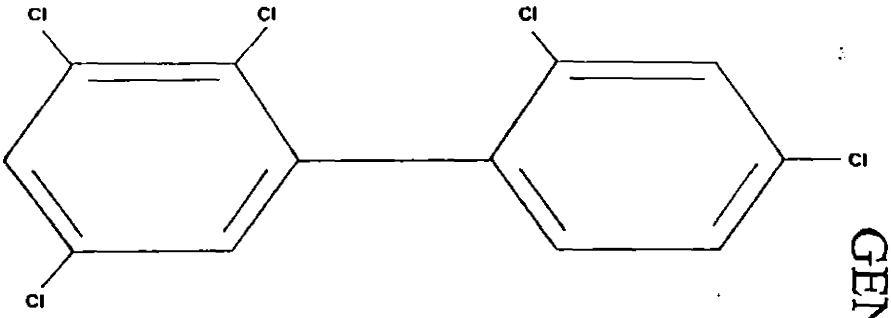


Figure 9.5 - PCB chemical structure. 784379

GENP 011568

TABLE 9.5

THE COMPOSITION OF TYPICAL TRANSFORMER ASKARELS[1]

Method ASTM D-2283

Trade Names	Type D Inerteen® 70-30	Type E Inerteen® 100-42	Type G Pyranol® A13B3B3
Ingredients (% by Weight)	–	–	–
Aroclor® 1254, Chlorinated Biphenyl (54% Chlorine by Weight)	70	–	60
Aroclor® 1242, Chlorinated Biphenyl (42% Chlorine by Weight)	–	100	–
Trichlorobenzene	30	–	40
Phenoxypropene Oxide Scavenger	0.18 to 0.22	0.18 to 0.22	–
Diepoxide Scavenger	–	–	0.115 to 0.135

[1]Monsanto Chemical Co.

This cloudy condition should be not interpreted as the presence of moisture in the early askarels.

Monsanto has not found evidence of reaction between the different scavengers when various askarels are mixed in any proportion.

Electrical Capability

Askarel, though one of the best insulating fluids developed by science, has been limited in usage. It has never been used on large transformers (beyond 69 KV, 15 MVA). In fact, during the early 1950's, transformer authorities recognized the practical askarel design limit as 34.5 KV (7.5 MVA). Most PCB units, therefore, fall in the 750-7500 KVA range.

Whereas mineral oil has an excellent impulse strength characteristic, askarel is limited in application because of its lightning strike capability (Figure 9.6).

On the other hand, oil must *never* be mixed with askarel. "Over two percent of oil by volume in askarel begins to lower its fire resistance."

Relationship to DDT

In some of the early analytical instrument studies PCB so resembled DDT (Dichlorodiphenyltrichloroethane) that part of the past impact of DDT in some situations may have been ascribed to PCBs. While similarities exist, the biggest difference is crucial; whereas DDT is a known acute toxic substance (physical effect apparent in a matter of hours), PCB by contrast is thought to be toxic because it is *bioaccumulative* (that is, its effect is *slow* accumulation in tissues of living matter over a period of years).

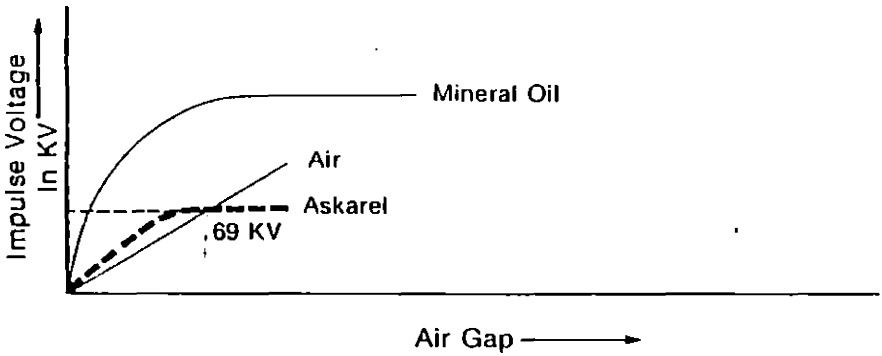


Figure 9.6 - Comparing lightning strike capability of insulating media.

The PCB Dilemma

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U.S. Hazardous Classification

Only after many years of use did the PCB ecological problem become apparent. Table 9.6 contrasts insulating fluid criteria between 1968 and 1976. Obviously, sacrificial trade-offs have taken place.

PCB has been the most publicized item in U.S. environmental regulations; it has received the most press because of its assumed toxicity and as one of the most abundant pollutants in the world. Unfortunately, however, because of its abundance, the mass media has wrongly called PCB a "deadly poison"; they have even gone as far as to classify PCB (along with dioxin) as one of the two most dangerous contaminants on the planet earth!

What is not sufficiently known is that the EPA alone has at least six different classes of hazardous substances ("X", "A", "B", "C", "D", and "Priority Pollutant"); the Department of Transportation (DOT) has at least four ("Class A" and "Class B Poisons", and "ORM-A and -E Substances"); and the Occupational Safety and Health Administration (OSHA) has at least one more grouping. One substance may also receive multiple classifications.

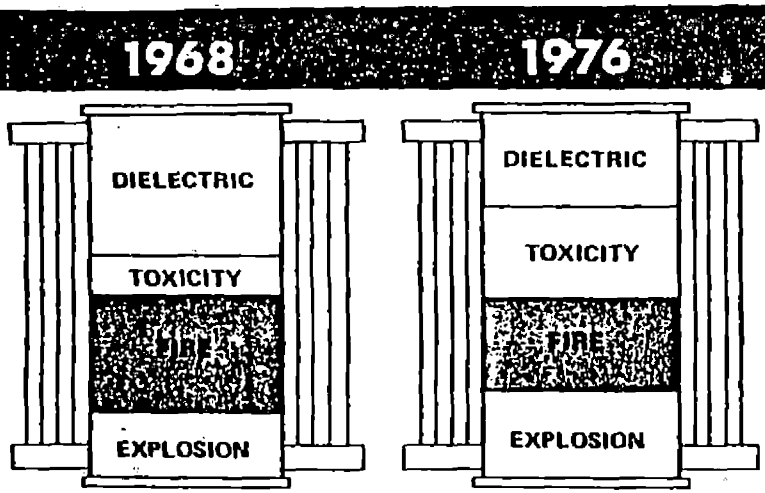
EPA lists PCB both as a "Category A" hazardous substance (a legal spill, 10 pounds or more), and as "a priority pollutant" but not as a "poison". For comparison's sake, the "worst possible case" would be a hazardous substance, listed as "Category X" (legal spill, one pound or more) and "Class A poison" under the DOT criteria. Furthermore, PCB is nowhere listed in these various classifications as an "extremely hazardous" substance and has yet to be proven as "toxic" to public health.

DOT lists PCB as an ORM-E Substance (other regulated material), a material that is not included in any other hazard class. DOT only considers PCB as a hazardous substance when its reportable quantity (RQ) is equal to or greater than 10 pounds. When such a quantity is shipped, the container must be marked with a label designating "polychlorinated biphenyls," its DOT classification (ORM-E), its DOT identification number (UN 2315) and the letters RQ (reportable quantity) 49 CFR 172.316 (a)(7), 172.324 (b). The 10 pound reportable quantity follows the EPA's definition of a reportable spill near a waterway (*Federal Register*, Vol. 44, No. 169, August 29, 1979, p. 50777).

GENP 011569

TABLE 9.6

CHANGING TRANSFORMER FLUID PRIORITIES[1]



[1]*Courtesy of Dow Corning Corporation.*

Recent studies by the National Research Council conducted for the Environmental Protection Agency (Office of Research and Development) included these remarks:

> Based on anticipated (PCB) use patterns, the expected human and environmental exposure to the chemical would be primarily from protective coatings (lacquers and varnishes). Some risk would be anticipated in occupational situations, but these exposures could be carefully controlled. If the anticipated (PCB) use had been limited to heat exchangers and closed electrical equipment (capacitors and sealed transformers), the testing data might not have suggested potential hazard to human health or the environment. In such a case, PCBs might have "passed" the toxic substance evaluation.

Cost-Benefit Evaluation

A dilemma becomes especially apparent when benefits are compared with social costs (Tables 9.7 and 9.8). As one example of the en-

vironmental dilemma, food packaging materials made from *recycled* paper containing carbonless NCR copy paper and printing inks, are even sources of some contamination.

Effect on the Ecological Food Chain

784381

The same characteristic of non-biodegradeability that has made PCBs useful for many industrial purposes has made them harmful in the food chain. This ecological process proceeds from the plankton (the simplest plant or animal), to fish, birds, and so on through to man himself (Figure 9.7). In fact, PCBs may biomagnify in the food chain—that is, as they move up the food chain towards man, their concentration may increase.

PCBs were first discovered in the flesh of Baltic fish in 1966. In humans the appearance of symptons of alleged PCB poisoning was observed in Japan in 1968 after inadvertent consumption of approximately 0.5 grams (2000 ppm) PCB in rice oil (from leaking heat transfer piping).

Several authorities now believe that the real culprit in this "Yusho" incident was not "normal" PCB at all, but one by-product of heated PCB (600°F)—polychlorinated *dibenzofurans*, which may be 500 times more toxic than PCBs!* This by-product in effect caused certain acute or immediate physical effects.

TABLE 9.7

COST-BENEFIT EVALUATION
BENEFITS OF PCBs

Transformer Safety
Transformer Reliability
Reduction In:
- Property loss
- Loss of life
- Medical costs
- Employment loss

Reduction in air pollution (PCB used in electrostatic precipitators used to control particulate matter)
Reduction in cost of controlling air pollution

GENP 011570

**This by-product may also result from insufficient incineration and may substantiate the closing of all PCB incineration sites as of April 18, 1978.*

COST-BENEFIT EVALUATION SOCIAL COSTS OF PCBs (ENVIRONMENTAL DAMAGE COSTS)	
Direct:	
	Commercial fisheries
	Recreational industry
	Contaminated food
	Non-recycling of paper
	Worker lost time & medical (?)
Indirect:	
	Recreational fishing & hunting
	Loss of birds
	Human loss of physical & mental well-being (?)

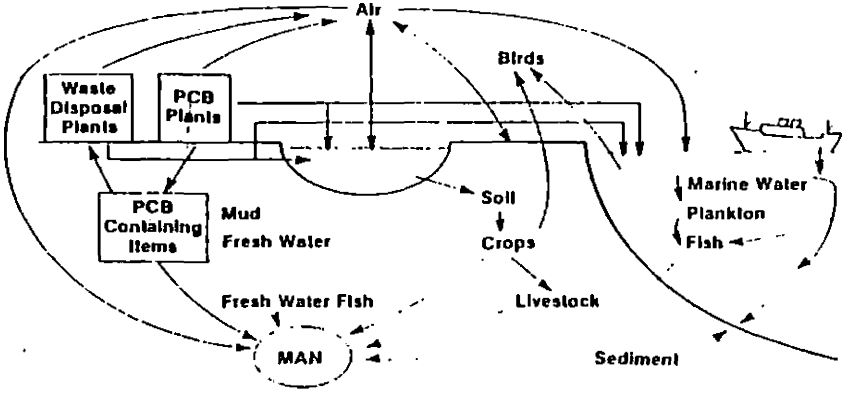


Figure 9.7 - Fate of PCBs in the environment. (K. Higuchi, PCB Poisoning and Pollution, Academic Press, 1976).

In the past, the EPA has stated that "...it is probably inappropriate to use the data from the Yusho episode to make quantitive estimates of the toxic hazard posed by PCBs to humans."* It would be like comparing apples with oranges! Keep in mind also the real possibility that non-U.S. PCB may be more "toxic" than American types.

**Federal Register, Vol. 42, No. 22 (Feb. 2, 1977), p. 6537.*

The [illegible] conclusion is that [illegible]
quired before any definite conclusions can be made. It is fair to say that the long term effect of PCB on the food chain may not be known for 20 to 30 years. Therefore, during this time of uncertainty, the best practice would be to minimize PCB exposure to the environment.

ASSESSMENT OF PCB HAZARD

An analysis of PCB data compiled following the criteria of FIFRA and TSCA* proposed guidelines, leads to the conclusion that PCBs are persistent, and are likely to accumulate. PCBs do not appear particularly toxic for short-term exposure, but results are subject to interpretation...

FIFRA Testing Results
The conclusion reached regarding the potential hazard posed to the environment by PCBs based on FIFRA requirements is **questionable.†** Acute toxicity is observed *only* for some aquatic invertebrates, but these species are not required test animals in the FIFRA guidelines.

**FIFRA - Federal Insecticide, Fungicide and Rodenticide Act. (1972)*
TSCA - Toxic Substances Control Act. (1976)

† - *TMI emphasis*

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Precaution in Handling PCB in the Field

Because PCB remains an excellent dielectric fluid, such equipment should be around for many years to come (or in retrofilled PCB units). Personnel working with PCB should take precautions to protect the environment.

Limit exposure to PCB! No chemical is safe all the time; for example, the sulfuric acid in a car battery, if not handled properly, will burn the body and if dumped into a waterway, it will kill fish.

Human PCB exposure normally consists of sampling fluids and testing and repairing transformers. Thus, when working with PCB or solvent rinses used in PCB retrofill (many of these are also on EPA or DOT hazardous material lists) please note:

- Avoid direct skin contact
- Avoid direct eye contact by use of goggles
- Avoid breathing of vapors for extended periods of time
- Avoid vapors from a severely arced askarel transformer

Seeking Our Way Out of the PCB Woods

Confronted with this dilemma, America began its efforts "to seek a way out of the PCB woods" (Figure 9.8).

When the harmful effects of PCBs became known, five events took place. In 1972:

1. Standards were set by U.S. Food and Drug Administration for food contamination.
2. Standards were proposed by the U.S. EPA for water contamination.
3. Monsanto voluntarily withdrew PCB from all markets except totally enclosed systems—that is, sealed transformers and capacitors.

In 1977:

4. Westinghouse and General Electric ceased production of PCB transformers.
5. Monsanto voluntarily discontinued manufacture of askarel, after acceptable substitutes had been developed.

The transition to using non-PCB high fire point transformer and capacitor dielectric liquids, took place in the mid-1970's. All transformers that were manufactured before the mid-1970's—and that meet the requirements of a high fire point transformer as defined in Section 450-23 of the 1981 *National Electrical Code (NEC)*—invariably contain PCBs. With some rare, special-application exceptions, capacitors that contain more than three gallons of coolant, and that qualify for indoor use without vaults under Section 460-2 (a) of the *NEC* will contain PCBs if they were manufactured before the mid-1970's. Such equipment manufactured in the latter half of the 1970's may or may not contain PCBs; however, no PCB-insulated electrical equipment is manufactured today.

Even with these several steps, voluntary regulation proved ineffective. Therefore, U.S. governmental regulations have been published.



Figure 9.8 - A PCB transformer in a residential neighborhood. (Courtesy of Chemical Week).

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Transformer Maintenance

By
S. D. Myers
J. J. Kelly
R. H. Parrish

TMI®

Transformer Maintenance Institute,
Division, S. D. Myers, Inc.
Akron, Ohio

DIVISION OF S D Myers TRANSFORMER MAINTENANCE INSTITUTE

Acts 4:12

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'rspective on

CHLORINATED DIBENZODIOXINS

And

DIBENZOFURANS

Chlorinated dibenzodioxins or dibenzofurans have been found as contaminants in fungicides (pentachlorophenol), herbicides (2,4,5-T), polychlorinated biphenyls (PCBs), and products from processes in which polychlorinated phenols are used (animal fats and tallows). Chloracne in chemical workers also seemed due, to some degree, to chemical processes in which trace chlorinated dioxin levels were present. Marked variation in toxicity seemed to be associated with the number and location of chlorines, with the 2,3,7,8-tetrachlorodioxin being one of the most toxic chemicals known.

Much of the data about these compounds is fragmentary, in obscure sources, or unpublished. A conference sponsored by the NIEHS was organized in order to provide a forum where the current knowledge of these compounds could be presented and discussed by the government, industry and university community. It hopefully also served as an indicator of where to focus future efforts. The conference was held April 2–3, 1973 at the Governors Inn, Research Triangle Park, North Carolina. Presentations included methods of detection, chemical processes involved in formation, bioaccumulation, and degradation, toxicity, pharmacologic effects, as well as distribution and fate in man's environment.

The papers presented at that meeting appear substantially as presented. The paper by Piper, Rose, and Gehring as well as the Bibliography prepared by Huff were not part of the Conference but are included for completeness of the subject. Your comments on the issue are certainly welcome.

JOHN A. MOORE
Conference Chairman

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Etiology of Chick Edema Disease

by David Firestone*

Chick edema disease first came to the attention of the Food and Drug Administration in December, 1957, when it was learned that millions of broilers died in the eastern and midwestern parts of the United States. Several groups in industry and government quickly determined that the disease was due to toxic components in certain feed fats (*1–3*), and that toxicity was associated with the unsaponifiable portion of the fat.

Characteristic symptoms included the presence of excessive fluid in the heart sac and in the abdominal cavity of chicks fed toxic fats (*2, 4, 5*). These and other symptoms such as subcutaneous edema and liver necrosis were accompanied by high mortality beginning approximately in the third week. Allen (*6, 7*) suggested several years later that the accumulation of large quantities of extravascular fluid in chickens might be due to altered permeability of the vascular bed as well as cardiac decompensation and liver necrosis.

Developments Prior to Chemical Identification of the Toxic Factors

Initial outbreaks of the disease in 1957 occurred at a time of increased demands by feed manufacturers for low-cost fats to raise the caloric level of diets for food animals. Investigations by the Food and Drug Administration in 1958 soon demonstrated that chick edema disease was caused by toxic material in by-product fatty acids obtained from production of oleic and stearic acids added to certain lots of feed-grade fats. Further investigation showed that toxic material was also present in various distillates and still residues obtained from several fatty acid producers who prepared commercial oleic and stearic acids from inedible tallow (*8*). The most toxic samples were batch still distillates obtained after repeated distillation of tallow fatty acids. The general scheme used for production of commercial fatty acids is shown in Figure 1. Fatty acids

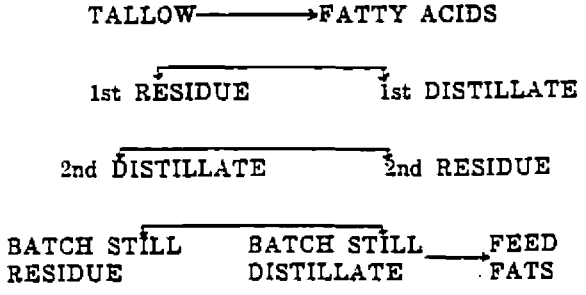


FIGURE 1. Scheme for commercial production of fatty acids and by-product (batch still) distillate from tallow.

obtained from hydrolysis of tallow were distilled in continuous stills to produce first and second (continuous still) distillates which were subjected to further processing to produce oleic and stearic acids. The second (continuous still) residue was then distilled in a batch still to yield by-product (batch still distillate) fatty acids which were sold as feed fats. Occasional vegetable oil fatty acid samples were also found to exhibit some chick edema activity (*8*), but it could not be determined whether these samples

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were inherently toxic or were made toxic by cross contamination in the plant with toxic tallows or toxic tallow acids.

Now that it was demonstrated that chick edema disease was caused by toxic substances present in the feed, the possibility existed that edible chicken flesh might contain these toxic materials. The unsaponifiables isolated from the carcasses of chickens fed toxic fat were fed to chicks at various levels (0.025–1.0%) in a test ration (*2*). Symptoms of edema disease were observed at all levels fed. The unsaponifiables from normal control chickens, fed to chicks at 0.5% of the diet produced no abnormal symptoms in test checks. Similarly, the presence of toxic factor was demonstrated in the meat of hogs that had been fed toxic fat (*9*).

The presence of toxic factor in commercial still distillates and residues prompted the examination of oleic acids and stearic acids collected in 1959 from food manufacturing plants. Oleic acids from several plants showed varying degrees of chick edema toxicity (*8*). In addition, oleic acid derivatives such as triolein and glyceryl monooleate were found to be toxic. Ames et al. (*10*) also found chick edema factor in a number of oleic acids and glyceryl monooleates. No toxicity was found in commercial stearic acids. At this stage, the identity of the toxic factors was unknown, but evidence clearly indicated that they were chlorinated aromatic compounds (*11*).

Since chick edema factor was found in food grade oleic acid and derivatives, the Food and Drug Administration issued a Food Additive Regulation in 1960 for fatty acids (*12*) specifying that they be free of chick edema factor in accordance with a 3-week chick feeding bioassay. The need for a rapid screening test was met by development of a microcoulometric gas chromatographic method (*13*) involving cleanup of isolated unsaponifiables by adsorption chromatography on activated alumina prior to gas chromatographic analysis. Portions of a reference toxic fat obtained in 1958 were made available as positive standards.

The availability of methodology assisted in bringing the chick edema disease under control after another outbreak in the Southeast in 1960. Although the structures of the substances causing the disease were yet to be determined, the typical pattern of gas chromatographic peaks with long retention times versus aldrin (Fig. 2) was found useful for detecting toxic fats.

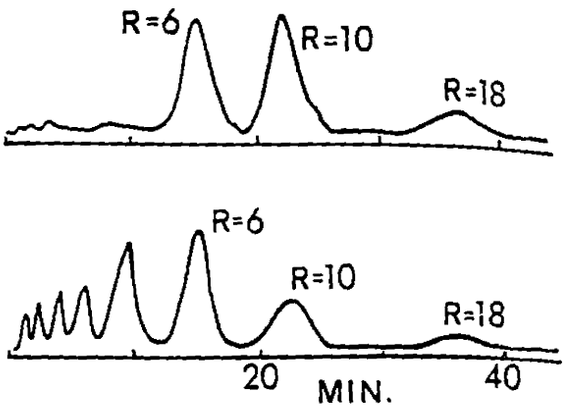
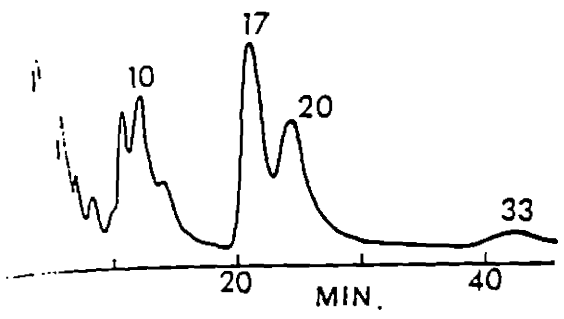


FIGURE 2. Microcoulometric gas chromatograms of material isolated from two toxic by-product fatty acids obtained from commercial fatty acid manufacturers. GLC column temperature 248°C; *R* denotes retention time versus aldrin.

Subsequently, electron capture–gas chromatographic procedures were developed (*14–17*) which were approximately 2000 times more sensitive than the microcoulometric methods and could detect less than 10 ppb of chick edema factors in lipid samples. Electron-capture gas chromatography also provided increased resolution so that additional peaks with characteristic retention times could be observed in extracts from toxic fats (see Fig. 3). In addition, a bioassay test with increased sensitivity was developed (*18,19*) using fertile chick eggs. Injection of chick edema factors resulted in decreased hatch and development of embryonic deformities and edema (*19*). Embryos which failed to hatch exhibited malformed beaks, eye defects, leg deformities, and lack of development of the right mesencephalon. Embryos which hatched exhibited

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. Electron-capture gas chromatogram of
.t from a toxic commercial glyceryl mono-
. GLC column temperature, 210° C; numbers
peaks are retention times versus aldrin.

and defective feathering and growth
tion.

ity of the Chick Edema Factors

omatographic behavior and ultraviolet
rption spectra of toxic fractions indi-
initially (*2*) that the toxic factors
substituted aromatic compounds, per-
substituted naphthalenes or phenan-
es or aromatic steroids. A major break-
gh on the arduous road to identifying
structure of chick edema factors oc-
ed in 1960 when Harmon et al. (*11*, *20*)
ted crystalline material from a toxic fat
d determined that it was an aromatic sub-
nce containing 47% chlorine (M. Tishler,
erck and Co., private communication). A
nilar crystalline material which was chick
ma-active at 0.1 ppm in the diet was
ated from a sample of commercial trio-
n found to be toxic to Cebus monkeys
2). The monkeys fed the toxic triolein
hibited fatty liver, liver necrosis, pan-
reatic atrophy and fibrosis, bile duct proli-
eration, hemosiderosis, gross hemorrhage in
he gastro-intestinal tract, and erythrocyto-
hagocytosis. Subsequently, Wootton and
rchene (*21*) characterized two com-

pounds isolated from a toxic feed fat as hexachlorohexahydrophenanthrenes on the basis of mass and other spectral data.

Finally, in 1966, Cantrell et al. (*22*) showed by x-ray crystallography that one of the active crystalline materials isolated earlier from toxic fat was 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin. Wootton immediately demonstrated that a synthetic hexachlorinated dibenzo-*p*-dioxin displayed properties similar to the material isolated from the toxic fat and, in addition, produced the chick edema disease in chickens (J.C. Wootton, Procter and Gamble Co., private communication, 1966).

Formation and Occurrence of Chlorodibenzo-*p*-dioxins (Dioxins)

Tomita et al. (*23*) had shown that chlorophenols and their salts can condense to form chloro derivatives of dibenzo-*p*-dioxin. Thus, chlorophenols appeared to be the source of dioxins. Accordingly, FDA scientists (*24*) pyrolyzed a number of commercially available chlorophenols, including pentachlorophenol, and obtained chlorodioxin mixtures with GLC peaks having retention times identical to those found in toxic fats. The most toxic pyrolysis product, identified as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, was obtained from 2,4,5-trichlorophenol. Hexa-, hepta-, and octachlorodioxins were obtained from pentachlorophenol. Individual components were isolated by preparative GLC from a reference toxic fat. Dioxins with 3, 4, 6, and 7 chlorine atoms were toxic to chick embryos. The presence of 2,3,7-trichloro- and 2,3,7,8-tetrachlorodioxin as well as hexa-, hepta-, and octachlorodioxins in the reference toxic fat indicated that the tallow from which the fat was derived was contaminated with 2,4,5-trichlorophenol as well as pentachlorophenol. Kimmig and Schulz (*25*) demonstrated in 1957 that 2,3,7,8-tetrachlorodioxin was highly toxic and chloracnegenic and was formed in the industrial production of 2,4,5-trichlorophenol by alkaline hydrolysis of 1,2,4,5-tetrachlorobenzene. Toxicological studies of individual chlorodioxins with chicks (*26*) and chick

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embryos (M. J. Verrett, private communication, 1970) again showed that the 2,3,7,8-tetrachlorodioxin was the most toxic. Hexachlorodioxins were about one fifth as toxic and 2,7-di- and octachlorodioxins were the least toxic.

Pentachlorophenol and other chlorophenols have been widely used as bactericides, slimicides, defoliants, and termite control agents in industry and agriculture. They are also used in the manufacture of food-packaging materials (Subpart E, Title 21 of the Code of Federal Regulations (21 CFR), Part 121 § 121.2001) and as components of articles that contact food (Subpart F, 21 CFR §121.2500). A list of food additive uses of 2,4,5-trichloro- and pentachlorophenol is given in Table 1. Because of their widespread use, a variety of commercial chlorophenols were examined for the presence of individual dioxins and related compounds (27) Nonacidic material isolated from the chlorophenols was chromatographed on alumina, and fractions were examined by combined gas chromatography–mass spectrometry.

Table 1. Food additive uses for 2,4,5-trichloro- and pentachlorophenol.[a]

Regulation No.	Specific compound[b]	Use
Subpart E:		
121.2001	TCP–Na; TCP–K; PCP–K	Slime control in manufacture of paper and paperboard
Subpart F:		
121.2505	TCP–Na; PCP–Na	Slimicides in manufacture of paper and paperboard
121.2514, (b) (3) (xxxi)	PCP–Na	Preservation in can end cements
121.2519 (d) (3)	PCP–Na; TCP–Na	Defoaming agents in manufacture of paper and paperboard
121.2526 (5)	PCP–Na	Paper in contact with aqueous and fatty foods
121.2534 (d) (3)	PCP–Na	Animal glue for articles holding food
121.2550 (b) (5)	PCP–Na; PCP–K	Closures with sealing gaskets for food containers
121.2556 (b)	PCP; PCP–Na	Wood preservative
121.2557 (d) (3)	TCP–Na; PCP–Na	Preservative for defoaming agents used in coatings
121.2562 (c) (4) (iii)	PCP–Na	Antioxidant in rubber articles used in producing, processing, or holding food
121.2596	PCP–Na	Preservative for ammonium alginate used in manufacture of poly(vinyl chloride) polymers that contact food

[a] Title 21, Code of Federal Regulations.
[b] TCP–Na=sodium trichlorophenate; TCP–K=potassium trichlorophenate; PCP=pentachlorophenol; PCP–Na=sodium pentachlorophenate; PCP–K=potassium pentachlorophenate.

A variety of polychlorodibenzofurans (furans) and polychlorodiphenyl ethers (ethers) was found in the chlorophenols in addition to dioxins. Up to 6.2 ppm 2,3,7,8-tetrachlorodioxin was found in six samples of 2,4,5-trichlorophenol, and up to 39 ppm hexachlorodioxins was found in eight samples of pentachlorophenol. In addition, chloromethoxyfurans and chloromethoxyethers were found in 2,4,5-trichlorophenol, and chlorohydroxybiphenyls were found in pentachlorophenol.

Chlorodioxins, furans, and ethers found in commercial pentachlorophenols are indicated in Table 2. Combined gas chromatography–mass spectrometry (GC–MS) was used to detect mixtures of these compounds which were unresolved by GLC. Characteristic mass spectral peaks for a dioxin, a furan, and an ether are shown in Table 3. The

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Dioxins, furans, and ethers in commercial pentachlorophenols.

	Detected by combined GC–MS		
	Dioxins [a]	Furans [b]	Ethers [c]
		+	+
		+	+
	+	+	+
	+	+	+
	+	+	+
			+
			+

[a] ...rodibenzo-*p*-dioxins.
[b] ...rodibenzofurans.
[c] ...rodiphenyl ethers.

...r weight and fragmentation pat... the dioxins, furans, and ethers were ...ntly different so that unresolved mix... were readily characterized by com... GC–MS. The results of analysis of the ...ercial chlorophenols indicated that the ...eaks of chick edema disease could have ...ated from tallows contaminated with ...henols containing preformed dioxins.

Characteristic mass spectral fragments of dioxins, furans, and ethers.

	Relative intensity, % [a]		
Fragment	Cl.–Dioxin [b]	Cl.–Furan [c]	Cl.–Ether [c]
M	78	72	22
M–Cl	7	10	2
M–2Cl	3	15	54
M– (CO + Cl)	35	50	—
M–2(CO + Cl)	25	—	—
M– (CO + 2 Cl)	—	15	—
M– (CO + 3 Cl)	—	75	8

[a] Based on ^{35}Cl; 1% or greater relative intensity.
[b] 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin.
[c] Positions of chlorine substitution unknown.

...obable Source of Chick Edema Factors

Reports in early 1960's indicated that pen...achlorophenol was widely used as a preser...ative in hide stripping operations, and might be present in the by-product tallows (fleshing greases) recovered from hides. A number of papers in leather trades periodicals (*28–30*) described the use of trichloro... pentachlorophenol as preservatives for hide curing operations. A major domestic manufacturer of chlorophenols recommends the use of the sodium salts of 2,4,5-trichlorophenol or pentachlorophenol for a variety of hide preservation and hide treatment operations as well as for general plant sanitizing procedures (*31*). Pretanning operations include trimming, brining (or salt curing) of the hides, followed by soaking, liming, bating (neutralization with buffering salts and treatment with a proteolytic enzyme), and pickling (*32*). The sodium salt of pentachlorophenol is recommended for use in salt curing, brining, soaking and pickling operations, and the sodium salt of 2,4,5-trichlorophenol is recommended for use in soaking operations (*31*). By-product fats are obtained after trimming, soaking and liming treatments (*32*) so that the use of chlorophenols as preservatives during processing can result in contamination of the hide greases with chlorophenols.

Contaminated hide greases may have been the source of the chick edema outbreaks of 1957 and 1960. Accordingly, three commercial oleic acids examined earlier for chick edema factor were reexamined for the presence of chlorophenols (*33*). Two of the samples that were previously found to be positive for chick edema factor were also found to be contaminated with 2,3,4,6-tetrachlorophenol and pentachlorophenol. The third sample contained a trace of pentachlorophenol.

Finally, Metcalfe (*34*) presented concrete evidence that the source of chick edema factor was fleshing grease from hides that had been treated with commercial pentachlorophenol. He examined samples of fleshing grease from pentachlorophenol-treated hides as well as industrial tallows containing glue emulsion with added pentachlorophenol. Chlorophenol-containing glue emulsions have been used in dry rendering operations. A fleshing grease sample was found to be extremely toxic when tested for chick edema factor (chick feeding bioassay) at the 16% level (*34*). A gas chromatogram of chlorodioxins isolated from this fleshing grease is shown in Figure 4. (The numbers above the

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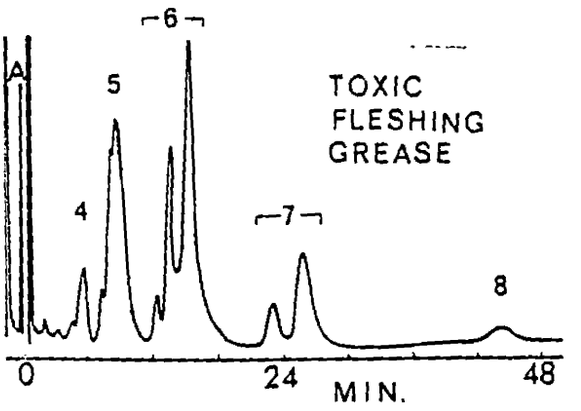


FIGURE 4. Electron-capture gas chromatogram (*34*) of dioxins extracted from a toxic fleshing grease. The numbers above the peaks refer to the number of halogen atoms in the individual dioxin molecules.

GLC peaks refer to the number of halogen atoms in the individual dioxins.) The Division of Chemistry and Physics, Food and Drug Administration, assisted with analysis of the sample. The presence of tetra-, penta-, hexa-, hepta-, and octachlorodioxins was confirmed by combined GC–MS. Penta- and hexachlorodioxins comprised 70% of the total dioxins found. The widespread use of chlorophenols as hide preservatives prior to the middle 1960's is the probable cause of the earlier outbreaks of chick edema disease. The use of chlorophenol-containing glue emulsions for dry rendering is another possible source of toxic tallows.

In 1970, a survey was made of a selected number of tallow processors and fatty acid producers. Ten of 45 samples of tallow and oleic acid were found to contain up to 77 ppb of dioxins (hexa-, hepta-, and octachlorodioxins). Four non-food-grade oleic acids contained 11 to 77 ppb of dioxins, and one food-grade oleic acid contained 59 ppb of dioxins. Fleshing grease from two hide processors contained low levels (5 and 11 ppb of dioxins), indicating that the use of chlorophenols for hide processing has not been entirely discontinued.

Origin of 1969 Outbreak of Chick Edema Disease

In early 1969, an outbreak of chick edema disease in North Carolina resulted in the death or destruction of some 300,000 chickens. An additional million birds were involved in further outbreaks in the next several weeks. The cause was traced to the use of contaminated vegetable oil by-product fatty acids in the feed.

Investigation by the Food and Drug Administration disclosed that the feed fat became contaminated at a vegetable oil refinery which also formulated antimicrobial water treatment products containing various chlorophenols. An underground pipe line was found leading from the "pesticide" product plant to traps used to collect by-product fatty acids (acidified soapstock) from the vegetable oil refinery. This pipe line unintentionally transferred "pesticide" plant wash water to the traps holding acidified soapstock, resulting in contamination of the acidified soapstock intended for sale as feed fats. The possibility of further contamination was eliminated by removal of the "pesticide" operation from the refinery site.

Prevention of Dioxin Contamination of Foods and Feeds

Since commercial chlorophenols are widely used as termite control and antimicrobial agents, there are numerous opportunities for direct and indirect contamination of food and food fats. Dioxin contamination of fats and fatty acids destined for use in foods or feeds can be minimized by control of sources of direct contamination with chlorophenols. These sources include the use of tri- and pentachlorophenol for hide preservation and other pretanning operations and in glue emulsions for dry rendering of fats. Care must also be taken in the use of chlorophenols industrially (as antimicrobials) or on the farm (as wood preservatives for fences, barns, etc.).

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Survey of the Embryotoxic Effects of TCDD in Mammalian Species

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Introduction

Only limited data are presently available on the embryotoxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). This is true for the number of animals used per experimental group, especially in the higher dose range, as well as for the number of different species of experimental animals tested. There has been an understandable reluctance to investigate these highly toxic compounds, because of the hazards of contaminating animal quarters and the risk to personnel. Nevertheless, we feel that the data available allow a rough estimate of the dangers that may be encountered with exposure to such dioxins during pregnancy.

The possibility that TCDD might have certain types of embryotoxic effects was realized for the first time when Courtney et al. (*1*) reported their data on the teratogenic effect of 2,4,5-T. Substances like TCDD have been known for some time (*2–4*) to be contaminants of chlorinated phenols and derivatives of such compounds and they were thought to be responsible for an intoxication called chloracne (*5*). Some commercial samples of 2,4,5-T apparently were rather highly contaminated with these extremely toxic substances.

In this paper we evaluate exclusively data which have been obtained using pure TCDD. No attempt is made to analyze the rather extensive literature on results obtained with 2,4,5-T samples contaminated to various degrees with TCDD or similar compounds.

Some Special Aspects of Embryotoxic Effects Induced by TCDD

Some General Aspects of Embryotoxic Action

Considerable confusion has been caused by many previous investigators with the nomenclature used in the field of prenatal toxicology. Unfortunately, the term used to specify certain experimental data in this field may suggest a special mode of action (*6*) and, more important, may trigger conclusions on a possible hazard to be expected during human embryonic development.

We, therefore, wish to suggest a system of nomenclature used by our group which is in accordance with the terms used in toxicological research and which at the same time takes into account the special situation of prenatal development.

The most comprehensive term is embryotoxic (fetotoxic) effect. A special situation of an embryotoxic effect may be characterized, e.g., a teratogenic effect, an embryolethal (fetolethal) effect, or a retardation (retarded growth) etc.

We feel that the following definitions prove to be convenient. By embryotoxic (fetotoxic) effects we denote all transient or

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permanent toxic effects induced in an embryo or fetus, regardless of the mechanism of action. By embryolethal (fetolethal) effect or embryomortality (or fetomortality) we denote prenatal mortality at any stage of embryonic or fetal development (may be referred to as $LD_5 - LD_{95}$). This term is certainly not identical with embryotoxic but refers to a special event that may occur in the course of an embryotoxic action.

We define a teratogenic effect as an abnormality originating from an impairment of an event typical for embryonic or fetal development (induction process, differentiation). The abnormality should be largely irreversible. It may be obvious by macroscopic appearance or "hidden" (micromorphological defect or inborn error of metabolism) or result in a mental abnormality.

Some examples of embryotoxic, not teratogenic effects are: general retardation of embryonic (or fetal) growth, retarded occurrence of certain ossification centers (reversible), fetal intestinal hemorrhages (without secondary effects). Representing a teratogenic effect would be an irreversible involution of lymphatic tissues (with consequences in postnatal life) or a severe mental defect resulting from an impairment of prenatal brain development.

Some Special Aspects Connected with the Action of TCDD

A brief analysis of the effects of TCDD on prenatal development may suggest that TCDD is a surprisingly specific teratogen, interfering only with a few special developmental processes. An increased frequency of cleft palate and kidney abnormalities of a special type are the only teratogenic effects which have been observed. Interestingly enough, it has not been possible so far to induce limb or head abnormalities with this agent, despite the fact that the drug was given at the "critical period" for the induction of such malformations. Larger experimental series over a sufficient dose range are necessary to permit conclusions on the specificity of the teratogenic action of TCDD, however.

TCDD is certainly not a teratogenic ag exclusively, since an increased fetomorta results if the drug is given at a high enou dose over a long enough time interval, though with such a dose schedule no obvic symptoms of a maternal toxicity beco evident. Apparently the higher doses lead a more general toxic effect on the embryor cells, but an effect on the placenta or mate nal tissues cannot be excluded at the momer

Some of the toxic signs which can demonstrated in the fetal tissue should n be referred to as teratogenic. This includ the intestinal hemorrhages (without pe manent secondary changes), a fatty infiltr tion (Fig. 1) of fetal livers (*7*) (to ou knowledge not reported before with any em bryotoxic agent), as well as subcutaneou edema and delayed ossification. Most of thes symptoms are reversible and they are cer tainly not the result of a typical interferenc with developmental processes. It is interesting that most of these symptoms can alsc be demonstrated in tissues of adult organisms under the toxic action of TCDD.

In adult mice a pronounced involution of some lymphatic tissues can be demonstrated as an early toxic sign, together with a loss of weight (*8*), predominantly in a drastic reduction of the size of the thymus, the spleen, and lymph nodes (*9*). A similar interference has been observed in our group with the development of these systems during fetal development in the presence of TCDD (*10*). This impaired development of lymphatic organs results in a typical postnatal insufficiency and in a pronounced reduction of the chances of postnatal survival.

Although these symptoms very much resemble changes produced in the adult organism by TCDD (*9*), we would consider these embryotoxic effects as teratogenic, since a typical developmental process is altered and the defect can be demonstrated long enough to handicap the newborn.

From all our studies we have seen no indication that the general growth of the fetal mice showing a teratogenic effect was affected to a significant degree.

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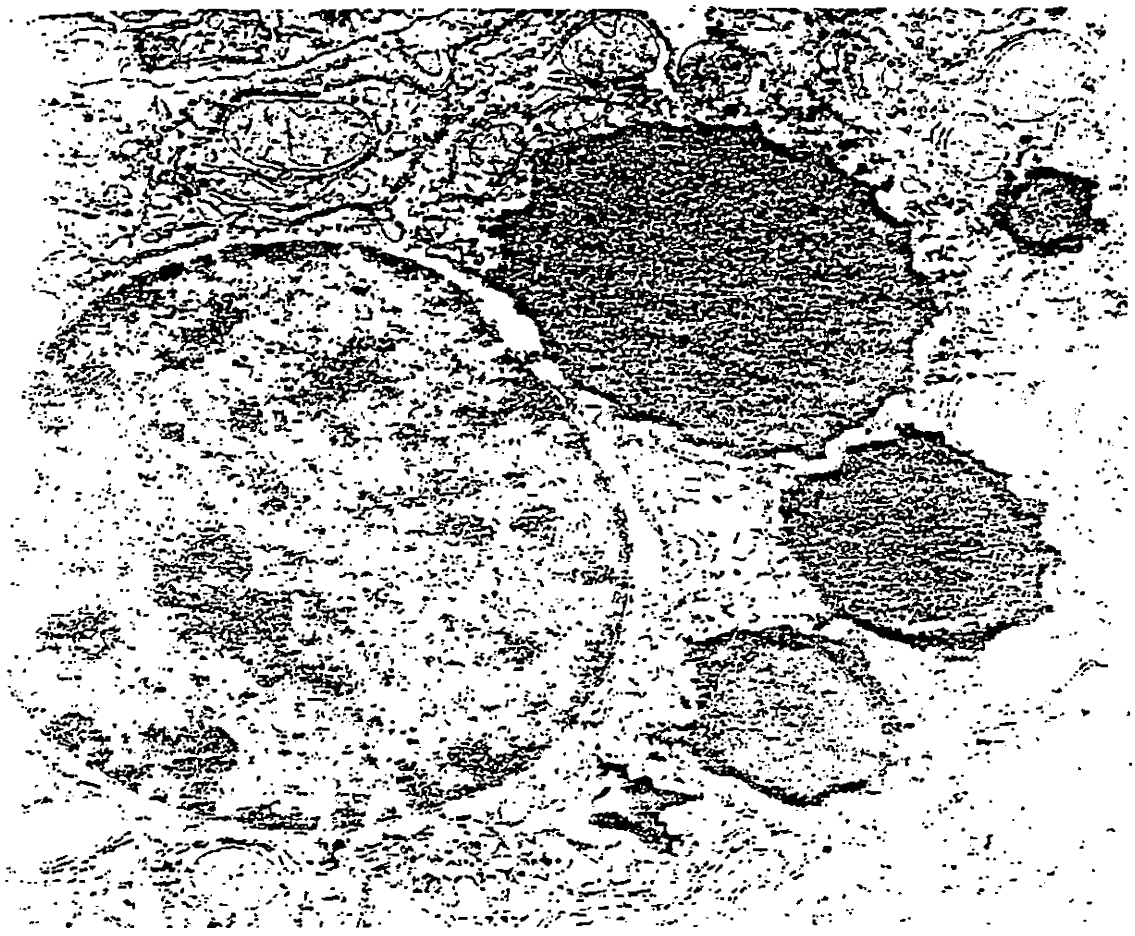


FIGURE 1. Example of an electron microscopic examination of fetal rat liver (day 18 of gestation). TCDD (15 μg/kg) was given once on day 17 of pregnancy. Using TCDD it has been possible to produce a fatty infiltration of the developing fetal liver as early as on day 14 of gestation.

We feel that the sort of embryotoxic effects induced by TCDD are very interesting from a theoretical point of view and to some extent unique. Therefore, they justify a further, closer analysis.

Results and Discussion

First indications of embryotoxic effects of TCDD were reported in 1970 by Sparschu et al. (*11*, *12*) from experiments with rats. Further data on embryotoxic effects then became available from reports of Courtney and Moore (*13*) and from our group (*14*) (see Table 1).

Three major effects were noted in these studies: intestinal hemorrhages in rat fetuses and an increased frequency of cleft palate and kidney abnormalities in mouse fetuses. Teratological studies with rats have apparently only been performed using repeated doses of TCDD while with mice effects produced by single as well as by repeated doses of this drug have been reported.

We shall try to evaluate the data from teratological studies available and to supplement them with unpublished data obtained in our laboratory. The following aspects will be discussed: (1) dose–response relationships after repeated doses of TCDD given to rats and mice; (2) dose–response relationships after single doses of TCDD given to mice and

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Table 1. Evaluation of the published teratogenic (embryotoxic) effects induced by TCDD in rats and mice.[a]

Species	Strain	Teratogenic effect (system)	Dose μg/kg: Minimal tested	Dose μg/kg: ≈ED_{50}[b]	Time TCDD given, days	Route	Reference
Rat		Intestinal hemorrhage	0.125	= 0.5 ?		Oral	Sparschu et al. (*12*)
	CD	Kidney abnormality	0.5	> 1	6–15	SC	Courtney and Moore (*13*)
Mouse	CD-I	CP	1 ? 3	> 3	6–15	SC	"
		Kidney abnormality	1	1–3	6–15	SC	"
	DBA/2J	CP	3	> 3	6–15	SC	"
		Kidney abnormality	3	> 3	6–15	SC	"
	C57Bl/6J	CP	3	> 3	6–15	SC	"
		Kidney abnormality	3	< 3	6–15	SC	"
	NMRI	CP	3	6.5	6–15	Oral	Neubert and Dillmann (*14*)
			9	< 9	9–13	Oral	"
			15	40	13	Oral	Neubert et al. (this paper)
			5	15	11	Oral	" "

[a] The smallest dose with which a significant teratogenic effect has been produced is indicated. Since sometimes only one dose level was tested this does not necessarily give the smallest dose from which a teratogenic effect could result. Routes were both oral and subcutaneous (SC). An attempt was also made to estimate the ED_{50} from the few data available.

[b] ED_{50}: dose required to produce effect in 50% of animals.

evaluation of phase specificity; (3) embryotoxic effects observed after application of TCDD together with other teratogens.

Dose–Response Relationship of Teratogenic Effects and Fetomortality After Repeated Doses of TCDD to Rats or Mice

The first striking result which is immediately apparent is the extremely low dose of TCDD which is able to induce teratogenic and fetolethal effects in mice and rats. Although these species must be considered to be comparatively insensitive during the adult status towards the toxic action of this compound, repeated or even single doses of as little as 1–10 μg/kg are capable of reproducibly triggering malformations of certain types. This is by far the smallest effective dose of any teratogen known today.

Interestingly, of the series of chlorinated dibenzodioxins, the tetrachloro derivative apparently is the most active one. The hexachloro derivative also seems to show some embryotoxic activity, while the dichloro and the octachloro derivatives have been reported (*15*) to be nonembryotoxic. The few data available suggest that embryotoxic activity is a property of those compounds having a pronounced chloracne potency (*15*).

Teratogenic effects induced by TCDD—Two major types of malformation have been reported so far in fetuses of rats or mice treated with TCDD during pregnancy: cleft palate—so far reported only in mice—and kidney abnormalities of a certain type, observed in both species after single or repeated doses of TCDD (Table 2).

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Embryotoxic effects induced by TCDD.[a,b]

Abnormality	Dose producing ~50% effect, μg/kg [c]	
Intestinal hemorrhage	0.5	?
Kidney abnormalities	1–2	?
Lethal	1–2	?
Cleft palate	6	
Kidney abnormalities	1–3	
Lethal	7	

[a] TCDD was given on days 6–15 of pregnancy.
[b] Literature data (*11*, *13*, *14*).
[c] ? = no exact data available from the literature. Doses assumed to be in the range indicated.

hermore, an involution of fetal thymus pleen and other lymphatic organs can bserved (*10*) which affects the survival r birth. Intestinal hemorrhage and fatty tration of the fetal liver do not represent atogenic effects, as discussed above. With- he adult organism similar symptoms can riced.

are convinced that looking more closely tuses affected by TCDD will reveal more malities; this has been the case with drug effect evaluated carefully enough th the modern biochemical and micro- rphological methods available.

Other than the reports on teratogenic and mbryotoxic effects produced by TCDD in ce and rats with documented experimental a we found only a few references which gest that embryotoxic effects may also ur in other animal species after a treat- ent with TCDD. Although we did not find erifiable data, TCDD apparently can also roduce embryotoxic effects in hamsters. Eye bnormalities and reduction of mean fetal weight—neither of which is typical of symp- ms seen in rats or mice after treatment with CDD—as well as gastrointestinal hemorr- ages and increased prenatal mortality are mentioned after doses of TCDD in the μg/ kg range for 5 days (*16*).

Unfortunately, we have no knowledge of teratological experiments performed with the highly TCDD-sensitive species guinea pig and rabbit. It would be important to know whether the comparatively high toxicity n in adult animals of these species is also paralleled by a high degree of sensitivity of the embryos or fetuses when compared with that of rats and mice.

Fetomortality induced by TCDD—Although the teratogenic effect induced by TCDD seems to be rather specific because the development of only a limited number of special organ systems is found to be impaired, TCDD when given in multiple doses does not represent an exclusively teratogenic agent. The occurrence of developmental abnormalities in chronic experiments is almost paralleled in a dose-response curve by the fetomortality (Fig. 2). However, as in many teratological experiments, there is no obligatory connection of these two parameters. In mice a high cleft palate frequency can be obtained without any apparent fetomortality (Fig. 3), just by reducing the time of treatment from 10 days (day 6–15) to 5 days (day 9–13 of gestation).

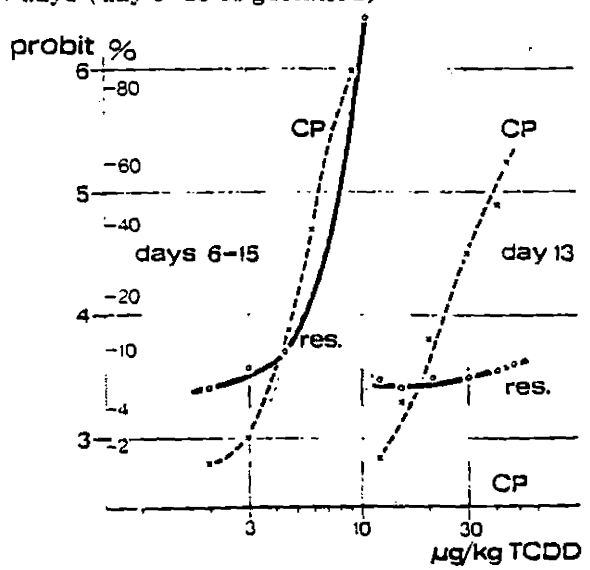


FIGURE 2. Comparison of the teratogenic effect and the degree of fetomortality induced by TCDD. The effect of a single dose given on day 13 of pregnancy is compared with that resulting after repeated doses given during days 6–15 of pregnancy. The points are derived from the evaluation of the total fetuses per treated group of mice (at least 12 litters per dose). TCDD was given in rape-seed oil by stomach tube once a day (1 P.M.). Cleft palate frequency is evaluated in this experiment as percent of the viable fetuses. Effect (probit scale) is plotted against dose (log scale). The control values represent 2000 fetuses from mice treated with rape-seed oil for 10 days.

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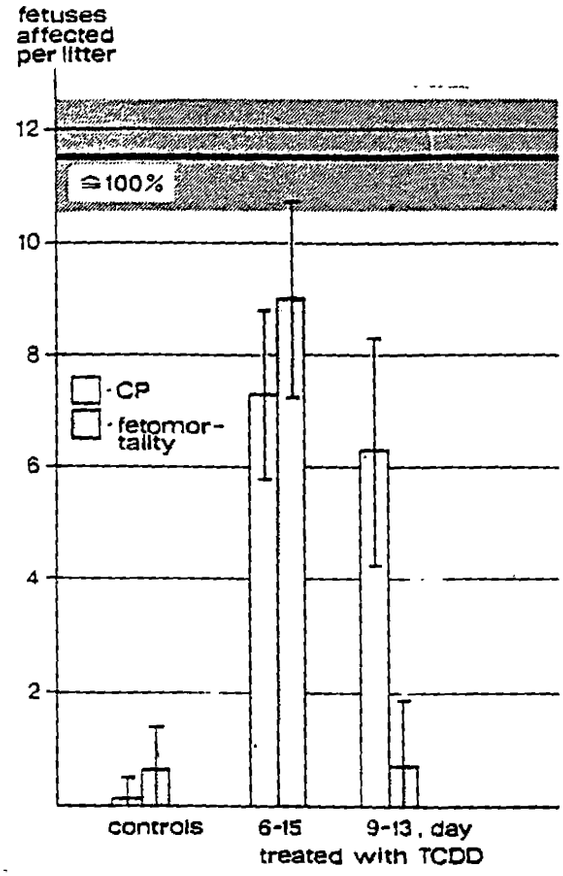


FIGURE 3. Comparison of teratogenic effect and fetomortality produced by TCDD. Ten pregnant mice each were treated with 9 μg/kg TCDD orally during the time interval indicated. The numbers of fetuses affected are given ($\bar{M} \pm$ S.D.) per litter. Average number of implantation sites per litter was 11.5 (= 100%). Controls received rapeseed oil only. CP denotes cleft palate frequency.

Differences among strains in the degree of teratogenic and fetolethal effects induced by TCDD in mice—Information on differences in the susceptibility towards TCDD of different strains of experimental animals are so far available only for mice, mainly through the data of Courtney and Moore (*13*). Tables 3 and 4 summarize these data, supplemented by information obtained with NMRI mice in our laboratory (*14*). For a better comparison we have recalculated some of the data. Since for half of the strains data are only available at one dose, the comparison is made for the effect produced by 3 μg/kg TCDD given during days 6–15 of gestation.

As can be seen from Table 3, with this scheme of treatment a noticible but small fetomortality occurs only with the CD-1 mice. With the same scheme of treatment a significant increase in the cleft palate frequency over that of the controls can be observed with all of the four strains tested. While the incidence of this type of teratogenic effect is roughly the same with three of the four strains (3–4%), the C57Bl mice are clearly more susceptible to the teratogen. Further data of Courtney and Moore (*13*) lead to the conclusion that the special sensitivity of the C57Bl mice also holds for the induction of kidney abnormalities. At the moment no clue is available for the cause of this higher sensitivity. An increased susceptibility of the target tissue or alternatively a special rate of drug metabolism in this strain, leading to a higher concentration of the effective drug in the fetus, may be responsible for this special sensitivity. Further data are needed to distinguish between these possibilities.

Dose–Response Relationship of Teratogenic Effects and Fetomortality in Mice after a Single Dose of TCDD (Phase Specificity)

We have started some systematic studies to clarify the question whether the teratogenic effects may be induced by single doses of TCDD—and by what doses—and what appears to be the most sensitive interval of fetal development. The induction of cleft palates was chosen as a criterion in these studies, but other types of malformation (kidney abnormalities) may also be evaluated from these experimental series.

The highest degree of malformations can be produced (*14*) when the drug is given to the mice on day 11 of pregnancy. Although a significant increase in the cleft palate frequency over that of the controls can also be induced by giving the teratogen on days 10 or 12 of gestation, the effect obtained on these days is only about half of that produced on day 11 for a given strain.

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Fetomortality induced by TCDD in different strains of mice.[a]

...se strain	Avg. fetomortality, %/litter[b]	
	TCDD	Controls
	12.4 (144)	8.6
	5.3 (110)	4.8
	27.0 (103)	26.1
...Bl[e]	5.3 (49)	10.8

[a] TCDD, 3 μg/kg, was given daily on days 6–15 of pregnancy.
[b] Values in parentheses denote percentage when compared with the corresponding controls receiving the vehicle only. In none of the strains tested was a pronounced fetomortality observed.
[c] TCDD given subcutaneously (SC) in DMSO; data of Courtney and Moore (*13*).
[d] TCDD given orally in oil; data of Neubert and Dillmann (*14*).

When TCDD is given on day 13 of gestation, an effect of only about one third of that seen on day 11 is produced. It should be mentioned that in all of these experiments where one time per day (at noon) was checked since it was not intended to pinpoint exactly the time of the highest sensitivity. The maximum of the effect may, therefore, be somewhat higher.

In order to compare the cleft palate-inducing effect of TCDD with that seen after treatment of pregnant mice with other teratogens we have performed most of the following experiments by giving TCDD on day 13 of gestation, even though the effect with TCDD obtained on this day is not maximal. Interestingly enough, cleft palates can be produced by giving different teratogens with a phase optimum which varies considerably, indicating quite different modes of action of the various teratogens (Fig. 4).

Because of our interest in the dose–response relationship of the teratogenic effect of TCDD we compared the dose–response curves (cleft palate frequency) obtained with five cleft palate-inducing drugs (Fig. 5). The steepness of the dose–response curves varies considerably when the different drugs are

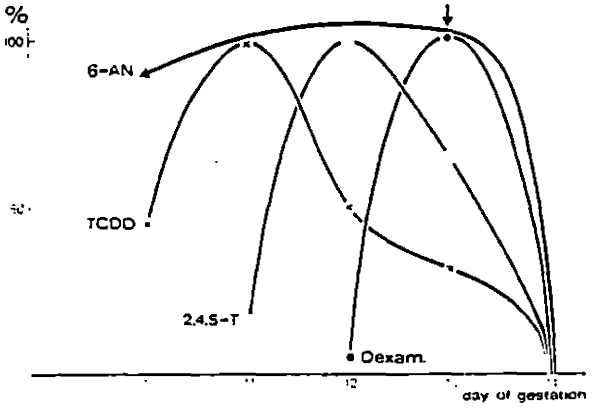


FIGURE 4. Phase specificity of cleft palate induction by various drugs. A rough survey is given on the optimal effect produced by different teratogens. The points are taken from large experimental series performed in our laboratory with NMRI mice. 100% indicates the maximum effect, not a cleft palate frequency of 100%. The arrow indicates the time at which most of our combination experiments have been performed. Not all teratogens exhibit their maximal effect at this stage of development.

Table 4. Cleft palate frequency induced by TCDD in different strains of mice.[a]

Mouse strain	TCDD			Controls	
	Fetuses evaluated	CP, %[b]	Affected litters (%)	CP, %[b]	Affected litters (%)
CD-1[c]	104	3	3/10 (30)	< 0.3	0/29 (< 3)
DBA[c]	55	4	2/9 (22)	< 1	0/23 (< 4)
NMRI[d]	271	3	7/24 (29)	0.7	10/160 (6)
C57Bl[c]	58	22	5/7 (71)	< 1	0/23 (< 4)

[a] TCDD, 3 μg kg, was given daily during days 6–15 of pregnancy (same experimental conditions as in Table 3). Strain C57Bl appears to be the most susceptible strain. All the other strains tested show about the same degree of sensitivity.
[b] % CP = percentage of cleft palates of the total fetuses examined in this group.
[c] TCDD given SC in DMSO; data of Courtney and Moore (*13*).
[d] TCDD given orally in rape seed oil, data of Neubert and Dillmann (*14*).

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compared. Table 5 gives, in addition, the tan α calculated from these curves as well as the dose range required for increasing the teratogenic effect from 2% (just significantly over the controls) to 50% (probit 3 to 5).

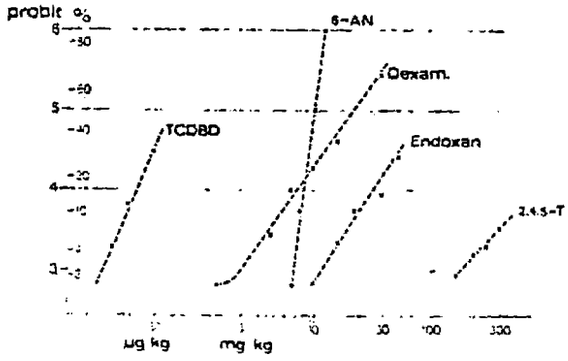


FIGURE 5. Dose-response relationship observed after single doses of different cleft palate-inducing drugs. All drugs were given on day 13 of pregnancy. TCDD and 2,4,5-T were given per stomach tube in rape-seed oil; 6-AN, dexamethasone, and cyclophosphamide were injected subcutaneously. Each point represents the frequency observed in the fetuses from at least 12 litters, at the lower dose range of at least 20 and generally about 30 litters. The data are given as percentage of the total fetuses evaluated at the dose specified. Tan α values of the dose–response curves are given in Table 5; threshold doses derived from these curves are given in Table 6.

Of the drugs tested, 6-aminonicotinamide (6-AN), a teratogen extensively studied biochemically as well as micromorphologically in our laboratory (*17–20*) shows the largest increase in cleft palate frequency with increasing doses (log dose), while 2,4,5-T shows the smallest. A dose–response curve of intermediate steepness is obtained when the effect of TCDD or of dexamethasone or cyclophosphamide (Endoxan) is plotted against the dose given. The dose required for producing a 10% effect with the various teratogens differs by more than four orders of magnitude!

Embryotoxic Effects Observed after Application of TCDD Together with Other Teratogens

Special emphasis was given to experiments

Table 5. Dose–response relationship with five palate-inducing drugs.[a]

Drug	tan α of dose–response curve	Increase in dose necessary to increase effect from probit 3 to 5 (2% to 50% effect)	ED_{50} (cleft palate) mg/kg
6-AN	7.6	1.3×	10
TCDD	2.3	3×	0.040
Endoxan	1.5	5×	60[b]
Dexamethasone	1.4	6×	20
2,4,5-T	0.8	11×	2000?[b]

[a] All drugs were given to the mice as a single dose on day 13 of pregnancy, (evaluation of the data shown in Fig. 5).
[b] No 50% effect obtained; value extrapolated.

in which TCDD was combined with other teratogens. We believe that such combination studies may serve several purposes. First, very little is presently known regarding the teratogenic effect of drug combinations, despite the fact that single compounds very rarely act on an organism, either under ambient environmental conditions or during treatment. Therefore, information on the chance of potentiating effects and the possible health hazards of combined drug actions on embryonic development is urgently needed. Also, almost no information is available today on the possibility of synergistic action of two or more drugs in producing an impairment of embryonic development which cannot be induced, even by high doses, by either substance alone. Information available from studies with carcinogens suggests that the likelihood that a certain drug will induce special teratogenic effects may be greatly increased by varying nutritional factors (e.g. vitamins, protein uptake, heavy metal concentrations) or by blocking certain metabolic pathways.

When designing experiments in which the teratogenic effect of combined drug actions is to be evaluated, several possibilities have to be considered. (1) Both (or all) drugs may be given simultaneously over an extended period of time. This has the advantage that all substances act on the target tissue

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...—probably different—optimal phase of ...elopment. The disadvantage of such an ...perimental setup may lie in the secondary ... interesting effects of the drugs pro... outside of the critical period (e.g., ... lethal effects) which could mask ... atogenic effect to be evaluated. (2) ... or all) drugs are given only once, ... the same time. This gives more favor... conditions for evaluating the mechanism ... action. However not all of the drugs may ... the chance to act at the most sensitive ...se. (3) Both (or all) drugs are given ... nce, but in sequence, each at the optimal ... of its effect. The advantages and dis... tages of this experimental design are ...us.

...ach of these experimental designs has ...vantages when studying special problems. ...ch one has practical as well as theoretical ...plications. We feel that in the long run ... the three models have to be tested in ...er to allow an exact elucidation of tera... nic effects of drug combinations. In the ...ent paper we present the results of some ...eriments performed with TCDD combi... ...ions by use of experimental designs 1 and ...

Cleft palate frequency induced by a combination of TCDD and 2,4,5-T given during days 6 and 15 of gestation—Studies with combinations of TCDD and 2,4,5-T were performed first, since an effect of this drug combination was of special interest. Large experimental series performed in various laboratories with 2,4,5-T preparations contaminated with dioxins to a very different extent led to such variable results that studies with a clear-cut combination of compounds were warranted.

Figure 6 shows the results of experiments in which a teratogenically active dose of 2,4,5-T (60 or 100 mg/kg) was combined with a threshold dose of TCDD or even much smaller doses.

Although we are completely aware of the fact that there is no "threshold dose" we use this term for convenience to indicate that dose (derived from a dose–response curve) which gives just no significant increase of cleft palate frequency over that of the controls, when 300–500 fetuses from treated mice and 2000 fetuses from controls are evaluated (1.5% cleft palate frequency, 0.7% seen with controls; under our experimental conditions, $\chi^2 > 3.5$).

A clear-cut effect is seen on combining 60 mg/kg 2,4,5-T with the "threshold dose" of TCDD (2 μg/kg), and a detectable potentiation is produced even with 1/10 of this

Table 6. Just nonteratogenic doses of the drugs used in our combination experiments (threshold doses).[a]

	TCDD, μg/kg		2,4,5-T mg/kg		6-AN, mg/kg	Dexamethasone, mg/kg	Endoxan, mg/kg
	Day 13	Day 6–15	Day 13	Day 6–15	Day 13	Day 13	Day 13
"Just teratogenic" dose[b]	15	3	250	40	7.5	4	15
"just nonteratogenic" dose	12	2	150	30	7	2	10
"Threshold dose" as fraction of ED_{50}[c]	1/3		1/13[d]		7/10	1/10	1/6[d]

[a] All doses were derived from dose–response curves, with at least 300 fetuses evaluated at the low dose levels. The data refer to single doses given on day 13 of pregnancy.

[b] The "just teratogenic" dose gives the lowest dose found to produce a significant effect ($\chi^2 > 3.5$) under our experimental conditions, 300–500 fetuses being evaluated per dose.

[c] Although we are aware of the fact that a "threshold dose" cannot be determined accurately, we use this term for the convenience of planning our combination experiments. In this paper this term is defined as the lowest dose found under our experimental conditions (300–500 fetuses) not to be able to produce an effect significantly different from the controls (2000 fetuses evaluated). For a more accurate definition this dose is also characterized as a fraction of the ED_{50}.

[d] Fetal ED_{50} extrapolated since this dose is toxic to maternal organism or fetolethal.

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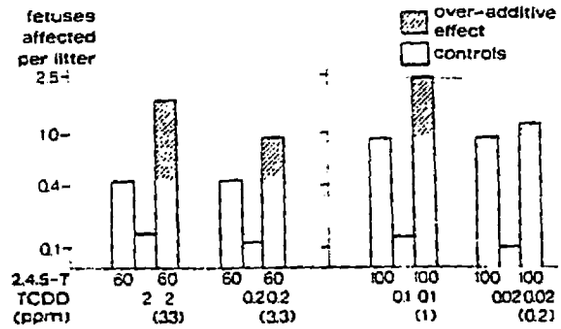


FIGURE 6. Cleft palate frequency in mice produced by a combination of TCDD and 2,4,5-T. Both drugs were given in the doses indicated (mg/kg for 2,4,5-T and μg/kg for TCDD) during the days 6–15 of pregnancy once daily by stomach tube. In each group 20 litters were evaluated. The data are given as number of fetuses per litter. (*M*). The effect produced by 60 mg/kg 2,4,5-T + 2 μg/kg TCDD is highly significant, that produced by 100 mg/kg 2,4,5-T + 0.1 μg/kg TCDD just significant ($P = 0.01$).

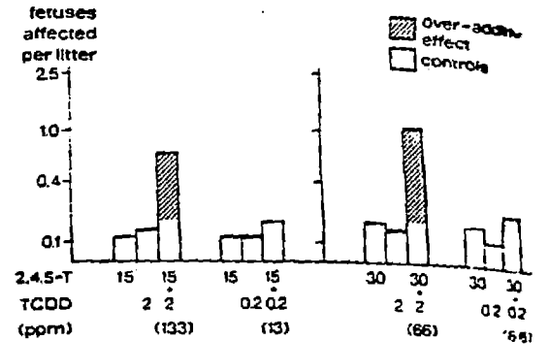


FIGURE 7. Cleft palate frequency in mice produced by a combination of TCDD and 2,4,5-T. Experimental conditions as in Fig. 6. The effects observed with both 15 mg/kg 2,4,5-T + 2 μg/kg TCDD and with 30 mg/kg 2,4,5-T + 2 μg/kg TCDD are highly significant ($P < 0.0027$).

dose of TCDD. This combination would correspond to a "contamination" of 3.3 ppm. When the dose of 2,4,5-T—and thereby the effect produced by this compound alone—is increased, even smaller doses of TCDD (0.1 μg/kg) lead to a detectable increase in the frequency of malformations. This combination would correspond to about 1 ppm. No significant potentiation can be observed when the dose of TCDD is lowered by another factor of 5 (to 0.02 μg/kg), corresponding to 0.2 ppm.

Although in such an experimental setup an effect by as little as 50 ng/kg TCDD can be detected, these data clearly show that a potentiating effect in this system cannot be obtained—even if highly teratogenic doses of 2,4,5-T are used—with a combination containing less than 0.5 ppm TCDD.

Further experiments were performed to get some information on what doses of TCDD are required to induce a detectable effect when just "nonteratogenic" doses of 2,4,5-T are used.

Under the conditions specified, 30 mg/kg was about a threshold dose. When this dose of 2,4,5-T is combined with 2 μg/kg TCDD (also a threshold dose), 66 ppm, a clear-cut potentiation can be observed (Fig. 7). The same dose of TCDD (2 μg/kg) increases the cleft palate frequency over the background also when half the 2,4,5-T dose is used (15 mg/kg), corresponding to 133 ppm. But 1/10 of the TCDD dose (0.2 μg/kg) does not induce any detectable effect with either of the doses 2,4,5-T used in this experimental series. This suggests that when just nonteratogenic doses of 2,4,5-T are used, there is no effedt with the system used if less than 10–20 ppm TCDD is present.

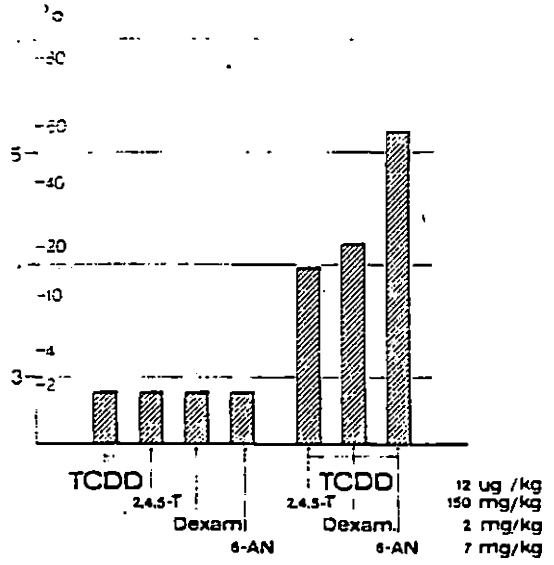
The results obtained with our experimental setup may give too high values since we have used neither the most sensitive strain of mice nor the malformation seen with the lowest dose in this strain (kidney abnormalities). It is furthermore interesting that with none of the combinations mentioned an increased fetomortality could be observed.

Cleft palate frequency induced by giving a combination of TCDD and other teratogens simultaneously on day 13 of gestation.—In these studies all the teratogens were given only once. Day 13 was chosen as the most convenient time of gestation (Fig. 4). In these studies, therefore, two of the teratogens were not given at the most sensitive phase of development.

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...ce 2,4,5-T cannot be considered a very ...rful teratogen we have studied the pos... of an occurrence of a potentiation ... TCDD is combined with other cleft ...ducing teratogens. Special empha... placed on the differences in dose ...se characteristics for these agents. Re... of such experimental series are com... in Figure 8. In this study the cleft ... frequency was measured when just ... ratogenic doses (threshold doses) of the ...gens were combined with a threshold ... TCDD (12 μg/kg).



...RE 8. Cleft palate frequency induced by drug ...mbinations. All drugs were given once on day ... of pregnancy (TCDD and 2,4,5-T in rape-seed ...il by stomach tube, all other drugs subcutan...ously) at the doses indicated, which represent ...he just nonteratogenic (or threshold) doses as ...xplained in Table 6. At least 12 litters were ...valuated in each group. The data are given as ...ercentage of the total fetuses evaluated per ...roup (probit scale).

It can be seen that a potentiation occurs ...fter a treatment with all the teratogens ...tudied. The degree of potentiation varied ...omewhat; a combination of TCDD with ...4,5-T resulted in about 15% cleft palate and ...out the same frequency was observed with ...mbination of TCDD with dexamethasone. With a combination of TCDD and 6-AN almost 60% of the mouse fetuses showed this teratogenic effect. It is interesting that the dose-response curve obtained with 6-AN is very steep when compared with those for 2,4,5-T or dexamethasone.

These data as well as data obtained with other combination (*21*) suggest that an especially pronounced potentiation is to be expected when two drugs showing both a rather steep dose–response curve are combined, while less potentiation is to be expected from the combined action of two drugs revealing a flat dose–response relationship. Of course, this prediction only holds as long as each drug does not act by interfering with the metabolism of the other. Accordingly, the TCDD–2,4,5-T pair should be expected to give a moderate degree of potentiation in the assay system used here.

Cleft palate frequency after treatment with a single dose of more than two teratogens.—We have, furthermore. performed studies to elucidate the possibility of TCDD potentiating the teratogenic action of other drugs when several such teratogens are given simultaneously in "subthreshold" doses. For these experiments all the drugs were given in doses of about half of the just nonteratogenic dose.

Figure 9 shows that neither a combination of 6-AN and dexamethasone nor the combination of the three drugs 6-AN, dexamethasone, and 2,4,5-T at this dose leads to an effect significantly different from that observed in controls. When TCDD is added to this drug combination as a fourth drug, however, at about half of the just nonteratogenic dose (6 μg/kg), a pronounced effect can be demonstrated. The cleft palate frequency is about 30%.

From these combination experiments and with regard to the model studied we wish to draw the following conclusions.

(1) When doses of TCDD much lower than the threshold dose are combined with teratogenic doses of drugs that are able to produce the same teratogenic effect, a potentiation may be expected, even with doses of TCDD as low as 1/20 to 1/50 of the threshold

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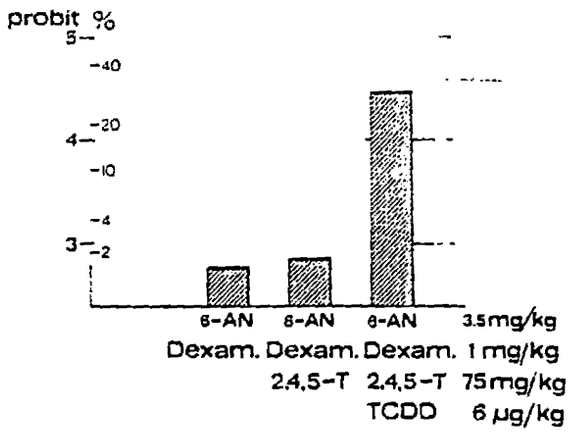


FIGURE 9. Cleft palate frequency induced by drug combinations. Experimental conditions as in Figure 8, except that more than two drugs were given simultaneously in some of the experiments. The doses given represent about half the threshold dose, as defined in Table 6.

dose (2 µg/kg).

(2) When just nonteratogenic doses of TCDD are combined with just nonteratogenic (threshold) doses of drugs which are able to produce the same teratogenic effect, a potentiation may be expected, the degree of which depends on tan α of the dose–response curve of the teratogens used.

(3) When two teratogenic drugs are combined at half the level of the threshold doses or lower, a significant effect is not to be expected unless both the teratogens exhibit very steep dose–response curves. In the case of TCDD a just significant effect is obtained with 6-AN but not in combination with 2,4,5-T, dexamethasone, or Endoxan.

(4) When TCDD at half the level of the threshold dose is combined simultaneously with three other teratogens, all at this dose, a clearcut potentiation is observed.

(5) Our results do not give any indication that in the system used TCDD in a dose of about 1/10 of the threshold dose would be able to induce a teratogenic effect, even in combination with up to three other teratogens simultaneously present in doses of half the threshold dose or lower.

All the foregoing conclusions are only valid as long as the drug metabolism of any of the teratogens used is not drastically altered during the combination studies.

Materials and Methods

For all experiments performed in this laboratory mice of the strain NMRI (purchased from Schwenke & Co., Bad Nauheim, Germany) weighing 29 ± 3 g were used. The animals were mated (20 females with 10 males per cage) for 2 hr and subsequently checked for vaginal plugs. The 24-hr period following the mating (8 A.M.) was called day 0 of pregnancy. Altromin R and tap water were given *ad libitum*.

The animals were sacrificed on day 18 of pregnancy and the number of viable fetuses was counted. Resorbed fetuses were regarded as dead. Litters with less than five implantation sites were not included in the evaluation. The average litter size was 11.1 ± 1.3 with this strain.

The results presented in this paper are based on the evaluation of about 1000 treated pregnant mice (ca. 11000 fetuses) and about 250 controls (ca. 2800 fetuses).

Embryotoxic effects have been evaluated in this paper as frequency per single litter, or alternatively as percentage of the total of fetuses examined. Although the litter should be considered the experimental unit for statistical evaluations in teratological studies we feel that a calculation of the embryotoxic effects based on the total number of fetuses examined is justified, since a closer analysis of our data always has revealed that the occurrence of embryotoxic effects is distributed randomly (*14*). In all general calculations of our data litters showing more than 75% resorptions have not been included but were listed separately.

Embryolethal and teratogenic effects were statistically evaluated from the total percentages or from the frequencies occurring in each litter (M ± S.D.) by using a t-test (*22*) or a χ^2-test.

Pure TCDD (Lot No. 851:142-26) was kindly provided by the Dow Chemical Co., Midland, Michigan, U.S. This extremely toxic substance was handled with utmost care. The purity (gas chromatographic analysis) in-

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was 98.6%. The drug was dissolved etone or chloroform and vigorously with rape-seed oil. Doses of 0.1 ml/10 se were given by stomach tube.

2,4,5-T (Lot SHG) was kindly pro- by C.H. Boehringer, Ingelheim, Ger- The dioxin content indicated was < ppm. Since this preparation was syn- ized by a special procedure it may be sidered dioxin-free. The drug was dis- ved in rape-seed oil by gently warming to C and given to the mice by stomach tube ml/10 g mouse). When 2,4,5-T and D were given together, stock solutions mixed before giving the solution (oil) e experimental animals at a dose of l 10 g.

-AN was purchased from Calbiochem (U.S.)

Dexamethasone (Fortecortin) was a gift E. Merck, Darmstadt, Germany. It was en to the mice in aqueous solution by utaneous injection.

ure cyclophosphamide (Endoxan) was ft of Asta-Werke, Brackwede, Germany. drug was given to the mice in aqueous ion by subcutaneous injection.

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Postnatal Effects of Maternal Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

by J.A. Moore,* B.N.Gupta,* J.G. Zinkl,* and J.G. Vos*

Previous studies reported that subcutaneous administration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) at a dose level of 3 μg/kg, in mice on days 6 through 15 of gestation produced pups with cleft palates and kidney anomalies (*1*). The C57B1/6 mouse was the most sensitive of the three strains tested to the TCDD-induced kidney effects, in that almost 100% of the fetuses developed kidney anomalies. The purpose of this paper is to report subsequent studies which attempt to characterize the teratogenic response as seen in the C57B1/6 mouse with specific emphasis on the nature and significance of the kidney anomaly. To distinguish between delayed kidney development, which if transient is of diminished significance, and irreversible effects, postnatal studies were also conducted. Reversible delays in metanephric kidney maturation in the rat have been described (*2*).

Materials and Methods

Inbred mice of the C57B1/6 strain were obtained from either the Jackson Laboratory, Bar Harbor, Maine, or the AR Schmidt Company, Madison, Wisconsin, and mated at the institute. Detection of a vaginal plug indicated day 0 of pregnancy. All experimental mice were singly housed in plastic cages and allowed free access to food and water.

The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (>99% purity, Dow Chemical Company, Midland, Michigan) was dissolved in acetone and subsequently diluted with at least 9 parts of corn oil. All mice were weighed prior to dosing, and the oral dose administered computed on the mean weight of the mice being treated. Control mice received an equivalent amount of 0.1 ml of an acetone–corn oil preparation.

Fetuses were removed from their mother on gestation day 18 and necropsied after fixation in Bouins solution. Mice necropsied in the postnatal studies were processed according to standard necropsy procedures. Tissues for histologic examination were fixed in either Bouins or 10% neutral buffered formalin, paraffin embedded and stained with hematoxylin and eosin. The incidence of an abnormality is given as mean average percent which is derived by determining its percent incidence in a litter and subsequently computing the mean of these percents.

Results

The effects of maternal treatment with TCDD on fetal palate closure and kidney development are shown in Table 1. TCDD at 3 μg/kg, administered on gestation days 10 through 13, produced cleft palate in pups from 12 of 14 litters with a mean average incidence of 55.4%. The mean average kidney and bilateral kidney incidence was 95.1% and 83.1%, respectively, with all lit-

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ters affected. When the dose of TCDD administered on gestation days 10 through 13 was reduced to 1 μg/kg, cleft palate incidence decreased to a mean of 1.9%. The litter incidence of kidney anomalies persisted at high levels; unilateral mean average pup incidence decreased to 58.9% and the corresponding figure for bilateral effect decreased to 36.3%. When TCDD administration at 1 μg/kg was a single dose administered on gestation day 10, no cleft palates were produced. The average mean incidence of unilateral kidney effects was 34.3% with anomalies occurring in 16 of 18 litters. Bilateral kidney effects at this dose occurred in seven litters with a mean incidence of 8.8%. No cleft palates or kidney anomalies occurred in controls.

The fetal kidney anomaly is best described as a renal papilla which is markedly reduced in size, or nonexistent in a few cases, resulting in an enlarged renal pelvis (Fig. 1). Given the stage of kidney development this may reflect a retardation or absence of papillae development rather than loss of an already formed structure. Nephron development appears similar to that occurring in control mice of the same age. The appearance of the affected kidneys resemble an early stage of hydronephrosis. It was further noted that when the renal anomalies were unilateral, the right kidney was affected 70.5% of the time. This preponderance of right kidney involvement had a high statistical significance ($P<0.01$).



FIGURE 1. Transverse section of a hydronephrotic kidney from an 18-day-old C57B1/6 fetus whose mother received 3 μg/kg TCDD on gestation days 10–13.

To assess the impact of this kidney change on the ability of a pup to survive in an extrauterine environment, postnatal studies were conducted. Pregnant C57B1/6 mice received 1 μg/kg TCDD on gestation day 10 and were allowed to litter. In one study litters from TCDD-treated mothers were fos-

Table 1. Incidence of cleft palate and kidney anomalies in C57B1/6 fetuses from TCDD-treated mothers.*

Treatment days	TCDD dose, μg/kg	No. litters	Cleft palate		Kidney anomalies		Bilateral kidney anomalies	
			No. affected litters	Mean average %	No. affected litters	Mean average %	No. affected litters	Mean average %
10–13	3	14	12	55.4	14	95.1	14	83.1
10–13	1	16	3	1.9	15	58.9	13	36.3
10	1	18	0	0	16	34.3	7	8.8
10, 10–13	0	27	0	0	0	0	0	0

* No significant differences in the number of live fetuses, resorptions, fetal weight and maternal weight was found at these dose levels when compared to controls.

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n control mothers; litters from con-
l mice nursed TCDD treated mice. To
tinguish a prenatal effect from a post-
l effect or from a combined prenatal
postnatal effect, a reciprocal cross fos-
g study was conducted. The results of
e studies as they affected kidney are de-
ted in Table 2. It was found that only one
p from a TCDD-treated mother, who
rsed an untreated mother, had kidney
sions when necropsied on postnatal day 14.
total of six pups, in four of the 14 litters,
nursed TCDD mothers but were born
ntreated mothers had hydronephrotic
eys. In the four-way cross foster study,
ost all kidney lesions occurred in pups
ose mother received 1 μg/kg TCDD and
bsequently nursed a TCDD treated
other. Five of 7 litters contained pups with
dronephrotic kidneys with a mean aver-
e pup incidence of 34%. Almost one half
the affected pups had both kidneys af-
ted. Three pups in two litters, from un-
ated mothers who nursed a TCDD-treated
, also had kidney anomalies; one was
ally involved. One pup from a TCCD-
eated female which nursed an untreated
other had an affected kidney, as did one of
e control pups.

To confirm that exposure to TCDD-treated
males during the nursing period was a
ajor factor in development of renal hy-
onephrosis in mouse pups, experiments
here mothers were treated on the day of
arturition were conducted. In these studies,
nice received 0, 1, 3 or 10 μg/kg at partur-
tion developed hydronephrosis. A mean average of 75% of the pups developed at least unilateral hydronephrosis; a mean average of 40% developed bilateral hydronephrosis. At the 3 μg/kg dose, a mean average of 71% of pups in three of three litters developed a right unilateral hydronephrosis. At the 1 μg/kg dose level, a mean average of 12% of pups in five of nine litters developed a unilateral hydronephrosis.

Gross and histological studies of affected mice reveal that the lesion produced is a progressive hydronephrosis. Essentially, total atrophy of the right kidney had occurred in several 55-day-old mice which had nursed a female that received 10 μg/kg TCDD at parturition (Fig. 2). There were

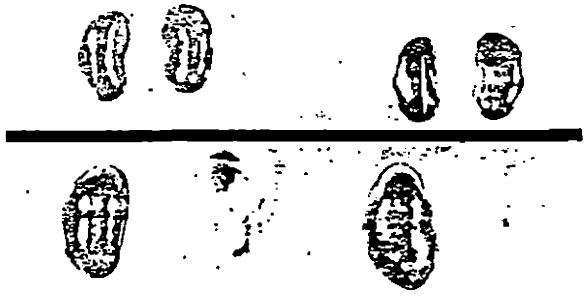


FIGURE 2. Gross photograph of kidneys from 55-day-old C57B1/6 mice: (top row) left and right kidneys from mice who nursed a control mother. (bottom row) kidneys from mice who nursed a mother treated with 10 μg/kg TCDD. Left kidney enlarged and fluid-filled. Right kidneys with marked hydronephrosis and complete atrophy of renal parenchyma (lower right).

Table 2. Incidence of hydronephrosis in C57B1/6 mouse pups following exposure to TCDD; mice treated at 1 μg/kg on day 10 of pregnancy.[a]

Period exposed to TCDD mother			Kidney hydronephrosis		Bilateral hydronephrosis	
In utero	Postnatal	No. litters	No. affected litters	Mean average %	No. affected litters	Mean average %
Yes	No	14	1	2.0	0	0
No	Yes	14	4	9.6	1	0.8
Yes	Yes	7	5	34.0	4	17.0
Yes	No	5	1	2.2	0	0
No	Yes	5	2	7.4	1	0.5
No	No	7	1	1.7	0	0

[a] significant differences in pup weight, maternal weight or litter mortality was observed.

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never any indications of a hydroureter accompanying the hydronephrosis. Serial sections of some renal pelves at the site of ureter entrance did not indicate a lack of patency. Serial sections at the site of ureter insertion into the bladder also indicated that there was no blockage. There is a weak impression that the ureter associated with a hydronephrotic kidney has a lumen of smaller diameter and that there may be a slight thickening or increase in the epithelial cells of the ureter mucosa. In those mice in which unilateral renal hydronephrosis occurred, the right kidney was affected over 90% of the time. The tendency for unilateral hydronephrosis to involve the right kidney suggests an anatomical variation which predisposes its involvement at low dose levels. The right kidney in mice is normally superior in position; the vena cava, due to its close proximity to the right kidney, provides less room for ureter insertion into the kidney hilus. Whether these anatomical characteristics contribute to this curious pattern of incidence remains a moot point.

Failure to mention possible thymus effects in a paper dealing with TCDD exposure would be misleading. Unfortunately, data are too meager and diverse in source to assemble specific figures for presentation; however, there appears to be a reduction in thymus weight at birth in some pups from females treated with TCDD during pregnancy. In postnatal studies, there was a dose-related thymus weight decrease when compared to controls in pups weaned from mothers who received 10 or 3 μg/kg TCDD at parturition. Definitive experiments to assess thymic effect and possible impairment of cellular immunity are planned.

Discussion

The results of these studies clearly indicate that exposing pregnant mice to TCDD during the period when progeny are undergoing metanephric kidney formation or maturation leads to the development of hydronephrosis. The incidence of this defect shows a dose–response relationship as scored by the litter, mean average pup, and unilateral or bilateral kidney incidence. Association with a treated mother during the nursing period accounted for the highest incidence of hydronephrosis in the studies described. Pups probably are being exposed to TCDD by its presence in milk.

The postnatal studies subsequent to prenatal exposure to TCDD at gestation day 10 would seem on first examination to discredit the significance of kidney anomalies recorded from fetuses examined on gestation day 18. However, a comparison of the salient features of the prenatal and postnatal kidney effects reveals striking similarities: (1) the lesion in both cases is best described as a hydronephrosis; (2) there is a similar dose–response relationship of kidney effects; (3) there is a tendency for right kidney hydronephrosis at low TCDD doses. The failure to demonstrate progressive hydronephrosis postnatally in pups exposed only *in utero* is likely a function of dose and

Table 3. Hydronephrosis in C57Bl/6 pups from mothers treated with TCDD at parturition.[a]

TCDD dose, μg/kg	No. litters	Average no. pups/litter	Kidney hydronephrosis		Bilateral hydronephrosis	
			No. affected litters	Mean average %	No. affected litters	Mean average %
0	4	6	0	0	0	0
10	5	6	5	75	3	40
0	4	6	0	0	0	0
3	3	5.3	3	71	0	0
0	8	8.0	0	0	0	0
1	9	7.7	5	12	0	0

[a] Pups nursing mothers treated with 10 μg/kg TCDD were reduced in size at weaning.

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of target organ exposure. This con- is supported by the findings that the greatest incidence of hydronephrosis oc- red in those litters which were exposure TCDD both *in utero* and during the post- period. It is hypothesized that the genesis of the hydronephrotic syn- e due to prenatal or postnatal maternal exposure is similar. Studies which test this hypothesis are in progress.

Summary

Hydronephrotic kidneys were produced in e pups that nursed a mother treated TCDD during pregnancy or at time of rition. Variations in kidney development, consistent with hydronephrosis, were observed in fetuses examined at gestation day 18. It was hypothesized that the prenatal and postnatal kidney anomaly are of the common etiology and that the incidence and degree of hydronephrosis is a function of dose and length of target organ exposure.

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GENP 011600

Toxicology of Chlorinated Dibenzo-p-dioxins

by B.A. Schwetz,* J.M. Norris,* G.L. Sparschu,* V.K. Rowe,* P.J. Gehring,* J.L. Emerson,† and C.G. Gerbig†

Severe toxicological responses have been associated with certain chlorodibenzodioxins. One of these responses is chloracne, a follicular dermatosis first associated with skin contamination by chlorohydrocarbons in 1899 (*1*). Serious outbreaks of chloracne-like lesions associated with runaway reactions in the production of 2,4,5-trichlorophenol occurred in Germany in the early 1950's (*2*). 2,4,5-Trichlorophenol itself does not cause acne [illegible], but the contaminants which may be formed in the uncontrolled production of 2,4,5-trichlorophenol are extremely potent acnegens (*2*). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and tri- and tetrachlorodibenzofuran were isolated from the contaminants formed in 2,4,5-trichlorophenol production and were demonstrated to be strongly positive acnegens when applied to rabbit ears (*3*). By using the rabbit ear test, the acnegenic potency of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) was confirmed in 1962 (*4*). In addition, 2,3,7,8-TCDD is extremely toxic in the chick embryo assay (*5*) and is highly embryotoxic in rats (*6*). Another chlorodibenzodioxin, hexachlorodibenzo-*p*-dioxin (HCDD), is known to be positive for the chick edema factor, a condition characterized by hydropericardium, ascites, and anasarca (*5*, *7*).

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Experimental

Materials

The chlorodibenzodioxin samples used in these studies are identified and described in Table 1. Studies were limited in some cases by availability of pure samples.

Acute Lethality

Samples of 2,7-dichlorodibenzo-*p*-dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, hexachlorodibenzo-*p*-dioxin, and octachlorodibenzo-*p*-dioxin were evaluated for acute oral lethality in several animals as summarized in Table 2.

Test materials were administered as suspensions in corn oil or as corn oil: acetone (9:1) solutions in single doses by gavage. The animals were deprived of feed for 16 hr before dosing. After dosing, they were observed for signs of toxicity including body weight changes for two to eight weeks.

Lethality of 2,3,7,8-TCDD via skin absorption was tested on rabbits of mixed sexes with doses of 31.6, 63, 126, 252, and 500 μg/kg body weight. The compound was applied as a 0.01% solution in acetone to the abdominal skin which had been shorn. After the acetone evaporated, the trunk of each rabbit was wrapped in cotton to prevent ingestion. The rabbits were housed in individual holding cages and were observed for signs of toxicity including body weight changes for three weeks.

Parenteral lethality was determined by injecting rabbits of mixed sexes intraperi-

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Table 1. Purity of samples used in the toxicology studies.

Sample no.	Sample identification	Source	Purity[a]	Tests[b]
	2,7-Dichlorodibenzo-*p*-dioxin (2,7-DCDD)			
1a	#104, shelf 142	Dow Chem. Co.	99.8%	1, 2, 3
1b	AR-570[c]	Dow Chem. Co.		3
1c	340-2-13A	Dow Chem. Co.	99.6%	1, 2, 3
1d	340-2-69A	Dow Chem. Co.	>99%	4
	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD)			
2a	Caustic insoluble isolate 1965	Dow Chem. Co.	96.4%	1
2b	851-142-24	Dow Chem. Co.	98%	1
2c	Skelly 11/11/64	Dow Chem. Co.	91%	1, 3, 5
2d	340-2-54B	Dow Chem. Co.	>99%	1, 2, 3, 5
	1,2,3,4-Tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TCDD)			
3a	FDA-F990	FDA	98.5%	3
	Hexachlorodibenzo-*p*-dioxin (HCDD)			
4a	252-44-12B-AL22	Dow Chem. Co.	65:35, 2 isomers	1, 3
4b	252-44-12B-AL11	Dow Chem. Co.	99%, 65:35, 2 isomers	3
4c	340-2-82A	Dow Chem. Co.	>99%, 89:11, 2 isomers	1, 2, 3, 4, 5
4d	FDA-F911	FDA	95.1%, 3 isomers	3
	Octachlorodibenzo-*p*-dioxin (OCDD)			
5a	251-1-142A	Dow Chem. Co.	98%	1, 2, 3
5b	340-2-29A	Dow Chem. Co.	94%	1, 3
5c	AR-570[d]	Dow Chem. Co.		3
5d	340-2-57A	Dow Chem. Co.	98.86%	1, 3, 4, 5

[a] Based on gas-liquid chromatographic (GLC) or GLC-mass spectrophotometric analysis.
[b] Test identifications: 1 = LD_{50}; 2 = eye irritation; 3 = chloracne; 4 = tetratogenicity; 5 = chick edema.
[c] Photolysis product of sample 1a.
[d] Photolysis product of sample 5a.

Table 2. Evaluation of acute oral lethality.

Test animal	Strain	Test material: 2,7-DCDD	2,3,7,8-TCDD	HCDD	OCDD
Rat	Sprague-Dawley	X		X	X
Rat	Sherman (Spartan)		X		
Mouse	Swiss Webster	X	X		X
Rabbit	New Zealand albino		X		
Guinea pig	Hartley		X		
Dog	Beagle		X		

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orally with 31.6, 63, 126, 252 and 500 mg/kg of 2,3,7,8-TCDD as a 0.01% corn oil suspension; control rabbits were injected with corn oil. The rabbits were housed in individual holding cages and were observed for signs of toxicity for four weeks. The LD50's were calculated by the Weil modification of the Thompson method (*8*, *9*) or by the Litchfield and Wilcoxon method (*10*). The acute lethality studies were terminated when it was evident that the survivors were not showing signs of toxicity.

Eye Irritation

Rabbit eyes were examined prior to experiments and found to be free from defects or irritation. Approximately 2 mg of 2,7-DCDD, 2,3,7,8-TCDD, HCDD, or OCDD were instilled in the conjunctival sac of one eye; the contralateral eye served as a control. The eyes were examined at various times after treatment for conjunctival redness and chemosis, iritis, and corneal injury. Responses were categorized according to intensity.

Rabbit Ear Bioassay For Acnegenic Activity

Acnegenic activity of 2,7-DCDD, 1,2,3,4-TCDD, 2,3,7,8-TCDD, HCDD, and OCDD was tested by applying 0.1 ml of either a solvent solution or the supernatant of a solvent suspension of each compound to the inner surface of the rabbit's ears five days a week for four weeks. The ears were examined weekly for signs of chloracne, inflammation and hyperkeratosis. The responses were divided into five categories: (1) none, (2) very slight, (3) slight, (4) moderate, and (5) severe.

Responses in the first three categories include no response to mild irritation, increased ear thickness, slight enlargement of the follicular aperture, slight exfoliation and slight crust formation. These responses alone are not considered indicative of chloracnegenic activity. Categories 4 and 5 are indicative of acnegenic response and are characterized by comedo formation, increased ear thickness and hyperkeratosis.

Teratology

Pregnant adult Sprague-Dawley (Spartan strain) female rats weighing approximately 250 g were used to study teratogenicity of the chlorinated dibenzo-*p*-dioxins. The day sperm were first present in a vaginal smear was considered day zero of pregnancy. The animals were housed individually in wire-bottom cages in a room controlled for temperature, humidity, light cycle and noise. Commercial laboratory rat chow and water were provided with choice.

Corn oil: acetone (9:1) solutions with varying amounts of test material were given in 2.5 ml/kg dosages by gavage. Dosages were calculated using daily body weights. Rats were treated with 100 mg of 2,7-DCDD/kg-day, 0.1, 1.0, 10, or 100 μg HCDD/kg-day and 100 or 500 mg OCDD/kg-day on days 6 through 15 of gestation. Control rats received 2.5 ml/kg of corn oil: acetone (9:1) orally. All rats were observed daily throughout pregnancy and were weighed on days 6, 13, and 21 of gestation. Pregnant females were sacrificed by carbon dioxide anesthesia on day 21 of gestation; the uterine horns were exteriorized through a midline incision in the abdominal wall, and the number and position of live, dead, and resorbed fetuses were noted. After being weighed and sexed, the fetuses were examined for external anomalies; the crown-rump length was measured with a vernier caliper. Half of each litter was preserved in Bouin's solution and later examined for soft tissue anomalies (*11*); the other half was preserved in alcohol, cleared and stained with Alizarin Red-S, and examined for skeletal abnormalities (*12*).

A 2 × 2 contingency table was used to evaluate the frequency of anomalies and resorptions within the fetal population and between litters. Body weight and body measurements were statistically analyzed by an analysis of variance and Tukey's test (*13*). In all cases, the level of significance was $P<0.05$.

Chick Bioassay for Chick Edema Factor

The bioassay for chick edema factor was

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conducted according to the Association of Official Agricultural Chemists method (*14*). Three-day-old white leghorn, single-comb cockerels were used. 2,3,7,8-TCDD, HCDD, and OCDD were the compounds studied. The diet used in the study was formulated specifically for conducting the chick edema bioassay (Nutritional Biochemicals, International Chemical and Nuclear Corp., Cleveland, Ohio). Body weights were recorded twice weekly for the oral intubation studies and at the start and termination of the dietary study. The chicks were observed daily for signs of toxicity, and food consumption was recorded weekly. After 20 or 21 days of treatment, all chickens were sacrificed by cervical dislocation and examined for gross lesions. The amount of pericardial and peritoneal fluid was measured, and all gross lesions were recorded. If the calculated t was greater than +1.3, the mean logarithm (100 × ml pericardial fluid) was greater than 1.1461 for the chicks receiving the test compound, and the mean logarithm of the negative control was less than 1.1460, the compound was considered positive for chick edema.*

Pathology

Toxicology studies were not designed to study the pathological changes associated with chlorodibenzodioxin administration, but in some cases, gross pathological and histopathological examinations were performed. For microscopic examination, tissues were fixed in 10% buffered formalin and were stained with hematoxylin and eosin. Sections of fetuses of control dams and dams treated with 100 mg 2,7-DCDD/kg-day were stained with hematoxylin and eosin, hematoxylin–phloxine–saffron, Masson's trichrome stain, and Mallory's phosphotungstic acid-hematoxylin stain.

*The calculated mean of logarithms of pericardial fluid voluemes of the test group and of concurrent negative control group x_t and x_c, respectively, is given by

$$t = (x_t - x_c)/[(s_t^2/n_t) + (s_c^2/n_c)]^{1/2}$$

where n_t and n_c are the number of chicks in the test and control groups, respectively, and s_t^2 and s_c^2 are variances of test and control groups, respectively (*14*).

Results

Acute Lethality

The lethality of 2,3,7,8-TCDD is presented in Table 3. The data reveal that the single oral LD50 ranges from 0.0006 mg/kg in male guinea pigs to 0.115 mg/kg in rabbits of mixed sex. Data on rats indicate that males are more sensitive than females; lethality is essentially the same following intraperitoneal, oral or skin administration for rabbits. Limited data show that dogs are less sensitive to 2,3,7,8-TCDD than rabbits. For female and male mice, single oral doses ranging from 0.001 to 0.130 mg/kg produced a few sporadic deaths without any definitive dose-response relationship; therefore the data are not presented in the table.

Limited lethality data are available for 2,7-DCDD, HCDD, and OCDD. HCDD (sample c) killed 1 of 2 and 0 of 2 male rats given oral doses of 100 and 10 mg/kg, respectively. No deaths occurred in four male mice given 2.0 g/kg of 2,7-DCDD (sample a or b) orally or in two female rats given 1 g/kg (sample a). For OCDD, oral doses of 1 g/kg (sample d) to five female rats did not cause death: in four male mice, doses of 4 g/kg also did not cause death. No signs of toxicity were observed in animals treated with either 2,7-DCDD or OCDD. The only sign of toxicity among animals treated with HCDD was loss of body weight.

While all species lost body weight following treatment with 2,3,7,8-TCDD, other signs of toxicity were species dependent. Ascites was seen in mice. Anorexia, dehydration, depression, emaciation, intestinal hemorrhage and alopecia were seen in dogs. Certain rabbits treated intraperitoneally with 2,3,7,8-TCDD developed skin lesions typical of those associated with acnegens.

Rabbit Eye Irritation

Instillation of the chlorodibenzodioxins into the conjunctival sac caused slight, trans-

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Table 3. Lethality of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [a]

...s and sex	Sample [b]	Route of administration	Time of death, days postadminis-tration	LD_{50}, mg/kg	Dose, mg/kg	Number deaths/ number treated
...le	c	Oral	9–27	0.022	0.008	0/5
					0.016	0/5
					0.032	10/10
					0.063	5/5
...t, female	c	Oral	13–43	0.045 (0.030–0.066)		
...nea pig, male	c	Oral	5–34	0.0006 (0.0004–0.0009)		
...nea pig, male	d	Oral	9–42	0.0021 (0.0015–0.0030)		
...bbit, mixed	c	Oral	6–39	0.115 (0.038–0.345)		
	c	Skin	12–22	0.275 (0.142–0.531)		
	c	Intraperitoneal	6–23	—	0.032	0/5
					0.063	2/5
					0.126	2/5
					0.252	2/5
					0.500	3/5
...s, male	c	Oral	9–15		0.30	0/2
					3.00	2/2
...s, female	c	Oral	—		0.03	0/2
					0.10	0/2

[a] Responses to individual doses are given in those cases in which an LD_{50} could not be calculated. The LD_{50} for oral administration to rabbits was calculated by using the method of Litchfield and Wilcoxon (9); the remaining values were calculated by using the Weil modification of the method of Thompson (15, 16).

[b] Letters refer to sample identification in Table 1.

...pain and conjunctival inflammation, ...itially. Treatment with 2,3,7,8-TCDD was ...sociated with delayed conjunctival chemo... 13-22 days later. By day 27, the chemosis ...d subsided, but the rim of the eyelid was ...ickened and encrusted. In rabbits treated ...h HCDD, the rim of the eyelid was en...sted 27 days after treatment. Neither ...rneal injury nor iritis was observed in any ...f the animals following instillation of the ...hlorodibenzodioxins in the conjunctival sac.

Acnegenic Response

Both 2,3,7,8-TCDD and HCDD produced ...ne in the rabbit ear bioassay as indicated ...y the formation of comedones. Solutions of ...3,7,8-TCDD (sample c) in benzene rang...ng in concentration from 0.04 to 400 μg/ml produced a positive response with severity increasing with concentration. A negative response was obtained with a solution of 0.004 μg/ml. In contrast, a chloroform solution of 1,2,3,4-TCDD, 50 μg/ml, did not produce a positive response. With HCDD (samples a, b, c, and d), a response was produced by solutions of 10 to 50 μg/ml in chloroform and dimethoxyethane. Chloroform extracts from 10% suspensions of 2,7-DCDD or OCDD were negative, indicating that these have a low order or possibly no acnegenic activity.

Teratogenicity

The effects of chlorodibenzodioxins on maternal and fetal body measurements, incidence of fetal resorptions and anomalies are given in Tables 4 and 5.

2,7-DCDD. Rats treated with 100 mg/kg-day on days 6 through 15 of gestation gained slightly more weight during pregnancy than controls but showed no toxicity. There was no effect on fetal body measurements, or incidence of resorptions, or gross, soft tissue or skeletal anomalies.

HCDD. Administration of 0.1–100 μg HCDD/kg-day was associated with a dose-related decrease in maternal weight-gain

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during gestation. Gross necropsy examination at the time of cesarean section revealed evidence of maternal toxicity only among dams receiving 100 μg/kg-day (pale, friable liver 3/20 dams; serous atrophy of fat, 1/20 dams).

Treatment with 10 or 100 μg HCDD/kg-day was highly lethal to fetuses during late gestation. While the incidence of early resorptions was not increased at any dose level of HCDD (5–7% in the treated versus 7% in the controls), there was a significant increase in late resorptions (0% at 0.1 μg/kg-day to 79% at 100 μg/kg-day). The weight and length of surviving fetuses were significantly decreased.

A significant increase in the incidence of fetal soft-tissue and skeletal anomalies was seen following treatment of pregnant rats with HCDD at the 100 μg/kg-day dose level. The incidence of cleft palate, subcutaneous edema, vertebrae with split or unfused centra, and split sternebrae was significantly greater than among control litters or the control fetal population. Among dams treated with 1 or 10 μg/kg-day, only subcutaneous edema occurred at a significantly greater incidence than in the control litters or fetal population. Treatment with 0.1 μg/kg-day of HCDD did not increase fetal anomalies among the litters or the fetal population. The incidence of delayed ossification of sternebrae was significantly increased among the fetal population but not among litters.

OCDD. Signs of maternal toxicity were not observed in rats given 100 or 500 mg/kg-day OCDD. Examination of the fetuses did not reveal changes in fetal body measurements, incidence of fetal resorptions, or incidence of any fetal anomaly among litters or the fetal population. At 500 mg/kg-day, the incidence of subcutaneous edema was significantly increased among the fetal population (23/100 compared with 8/156 in controls) but not among litters (9/18 compared with 6/28 in controls).

Chick Edema Bioassay

Chick edema was produced in groups of birds treated with 1 and 10 μg/kg-day of 2,3,7,8-TCDD and 10 and 100 μ/kg-day of HCDD (Table 6). The mean logarithm for pericardial fluid volume of the negative control groups was greater than 1.1460 and could negate the results if the guidelines for interpreting chick edema bioassay studies were rigidly followed. However, since the volume of pericardial fluid was markedly increased by the treatments indicated above, the treatments were considered to be positive for the production of chick edema. A positive response was not observed in chicks maintained on a diet containing 0.5% OCDD.

Severe dyspnea, subcutaneous edema, and distended abdomens were observed in some birds receiving 1 or 10 μg 2,3,7,8-TCDD/kg-day. Dyspnea and mucus accumulation in the mouth prior to death were observed in birds receiving 100 μg 2,3,7,8-TCDD/kg-day. No overt clinical signs were observed in birds receiving OCDD.

The gross lesions seen in chicks treated with chlorodibenzodioxins are summarized in Table 7. The most consistent gross lesions were increased pericardial and peritoneal fluid, subcutaneous and pulmonary edema, hepatomegaly and a mottled appearance of the liver.

Histopathologic examination of tissues of selected birds from the 2,3,7,8-TCDD (1 and 10 μg) and HCDD (10 and 100 μg) groups revealed similar lesions consisting of: atrophy of germinal centers of the spleen, a paucity of lymphocytes in the bursa of Fabricius, pulmonary edema, intersititial edema of the myocardium, fatty degeneration and coagulation necrosis of the liver. Many birds died as a result of pulmonary edema.

Pathology

Gross necropsy and histological examinations were conducted on relatively few mammals treated with the chlorinated dibenzo-*p*-dioxins. Therefore, the results reported here are incomplete and preliminary. The liver of animals treated with 2,3,7,8-TCDD and HCDD was most consistently affected.

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Table 4. Effect of treatment with chlorinated dibenzo-*p*-dioxin on maternal and fetal body measurements and the incidence of fetal resorption.

Test compound (sample) [a]	No. of litters	Maternal weight gain, g [b]			Fetal body weight, g [c]	Fetal crown–rump [c] length, mm	Fetal resorptions, %	
		Days 6–13	Days 13–21	Days 6–21			Population [d]	Litter [e]
Control	30	36 ± 2	101 ± 6	137 ± 8	5.68 ± 0.05	44.5 ± 0.1	7 (22/337)	47 (14/30)
2,7-Dichlorodibenzo-*p*-dioxin (d)								
100.0 mg/kg-day	7	31 ± 1	122 ± 4	152 ± 5	5.80 ± 0.09	44.2 ± 0.2	6 (5/ 86)	57 (4/ 7)
Hexachlorodibenzo-*p*-dioxin (c)								
0.1 μg/kg-day	19	28 ± 2	102 ± 5	130 ± 5	5.73 ± 0.04	43.8 ± 0.1	5 (10/217)	47 (9/19)
1.0 μg/kg-day	19	27 ± 3	99 ± 5	126 ± 6	5.93 ± 0.16	45.7 ± 0.5	9 (20/218)	74 (14/19)
10.0 μg/kg-day	18	22 ± 3 [f]	97 ± 5	119 ± 6	5.12 ± 0.05 [f]	42.6 ± 0.2 [f]	25 [f] (57/229)	94 [f] (17/18)
100.0 μg/kg-day	19	6 ± 2 [f]	13 ± 7 [f]	19 ± 9 [f]	3.65 ± 0.28 [f]	35.2 ± 0.7 [f]	85 [f] (194/227)	100 [f] (19/19)
Octachlorodibenzo-*p*-dioxin (d)								
100.0 mg/kg-day	12	32 ± 2	100 ± 8	131 ± 7	5.73 ± 0.09	43.6 ± 0.4	8 (11/131)	42 (5/12)
500.0 mg/kg-day	17	35 ± 3	115 ± 4	150 ± 5	5.69 ± 0.05	44.5 ± 0.2	5 (9/199)	41 (7/17)

[a] Sample identified in Table 1; administered on days 6–15 of gestation as a corn oil: acetone (9:1) solution.
[b] Mean ± S.E. for various gestation times.
[c] Mean of litter means ± S.E.
[d] % (number resorptions/number implantations).
[e] % (number litters with at least one resorption/number litters).
[f] Significantly different from control by an analysis of variance and Tukey's test (measurements) or the 2 × 2 contingency table (resorptions), $P < 0.05$.

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Table 5. Effect of treatment with hexachlorodibenzo-*p*-dioxin on the incidence of fetal anomalies.

		Incidence with treatment on days 6–15 of gestation							
		0		0.1 μg/kg-day		1.0 μg/kg-day		10 μg/kg-day	
Soft tissue anomalies									
Cleft palate	P[a]	0	(0/156)	1	(1/104)	0	(0/99)	0	(0/86)	47	(8/17)[c]
	L[b]	0	(0/ 28)	5	(1/ 19)	0	(0/19)	0	(0/18)	73	(8/11)[c]
Dilated renal pelvis	P	0.6	(1/156)	0	(0/104)	2	(2/99)	6	(5/86)[c]	12	(2/17)[c]
	L	4	(1/ 28)	0	(0/ 19)	5	(1/19)	17	(3/18)	18	(2/11)
Subcutaneous edema	P	5	(8/156)	6	(6/104)	55	(54/99)[c]	100	(86/86)[c]	100	(17/17)[c]
	L	21	(6/ 28)	32	(6/ 19)	100	(19/19)[c]	100	(18/18)[c]	100	(11/11)[c]
Skeletal anomalies									
Split vertebral centra	P	6	(9/158)	2	(2/103)	1	(1/99)	7	(6/86)	31	(5/16)[c]
	L	19	(5/ 27)	5	(1/ 19)	6	(1/18)	29	(5/17)	56	(5/ 9)[c]
Split sternebrae	P	0.6	(1/158)	1	(1/103)	2	(2/99)	2	(2/86)	31	(5/16)[c]
	L	4	(1/ 27)	5	(1/ 19)	11	(2/18)	12	(2/17)	56	(5/ 9)[c]
Delayed ossification of sternebrae	P	11	(18/158)	28	(29/103)[c]	12	(12/99)	34	(29/86)[c]	56	(9/16)[c]
	L	44	(12/ 27)	74	(14/ 19)	50	(9/18)	71	(12/17)	56	(5/ 9)

[a] Incidence among fetal population; % (number of affected fetuses/number fetuses examined).
[b] Incidence among litters; % (number of affected litters/number litters examined).
[c] Significantly different from control by 2 × 2 contingency table, $P < 0.05$.

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Table 6. Results of chick edema bioassay: body weight, food consumption, and pericardial fluid volume calculations of chicks treated with chlorodioxins.

Treatment (sample)[a]	n	Body weight, g[b] Day 0	Body weight, g[b] Day 21	Food consumption, g[c]	Pericardial fluid volume ml ± S.E.	Pericardial fluid volume Mean log (100 × ml)	Calculated t value	Positive for chick edema factor based on Calculations	Positive for chick edema factor based on Gross lesions
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (d)[d]									
0 µg/kg	10	46 ± 1	199 ± 5	17.4	0.16 ± 0.02	1.1717	—	—	—
0.01 µg/kg	16	44 ± 1	196 ± 7	16.7	0.14 ± 0.02	1.1181	−0.74	No	No
0.10 µg/kg	10	45 ± 1	203 ± 7	17.2	0.19 ± 0.01	1.2688	+1.69	No	No
1.0 µg/kg[e]	2	42 ± 1	196 ± 24	33.7	2.34 ± 0.08	2.3680	+21.7	No	Yes
10.0 µg/kg[f]	9	42 ± 1	No survivors	11.2	1.29 ± 0.62	1.5661	+1.47	No	Yes
Hexachlorodibenzo-*p*-dioxin (c)[d]									
0 µg/kg	9	38 ± 1	194 ± 5	17.4	0.15 ± 0.01	1.1771	—	—	—
0.1 µg/kg	10	42 ± 1	197 ± 4	17.8	0.11 ± 0.02	0.9978	−1.93	No	No
1.0 µg/kg	10	43 ± 1	196 ± 6	18.2	0.09 ± 0.01	0.9387	−4.57	No	No
10.0 µg/kg[g]	9	38 ± 1	187 ± 7	16.7	0.81 ± 0.01	1.7294	+3.82	No	Yes
100.0 µg/kg[h]	10	36 ± 1	No survivors	13.3	0.62 ± 0.24	1.5650	+2.72	No	Yes
Octachlorodibenzo-*p*-dioxin (d)[i]			(Day 20)						
0% of diet	12	45 ± 1	141 ± 8	13.3	0.08 ± 0.01	0.8053	—	—	—
0.1% of diet	11	45 ± 1	124 ± 5	9.5	0.06 ± 0.01	0.7889	−0.21	No	No
0.5% of diet	11	43 ± 1	196 ± 10	10.9	0.09 ± 0.01	0.9002	+0.91	No	No

[a] Sample identified in Table 1.
[b] Mean ± S.E.
[c] Grams/chick/day.
[d] Administered orally as a corn oil: acetone solution.
[e] Animals died on days 9, 11, 11, 14, 15, 17, 18, and 19 of treatment.
[f] Animals died on days 3, 4, 4, 4, 5, 8, 8, 9, 12 and 15 of treatment.
[g] One animal died on day 19 of treatment.
[h] Animals died on days 5, 5, 6, 7, 8, 10, 11, 11, 15 and 17 of treatment.
[i] Fed in the diet (0.1% ≅ 100 mg/kg, 0.5% ≅ 500 mg/kg).

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Table 7. Results of chick edema bioassay: summary of gross lesions observed in chicks treated with chlorodioxins.

Treatment (sample)[a]	Mortality	Pericardial fluid >0.2 ml	Peritoneal fluid	Subcutaneous edema	Pulmonary edema	Atrophy of spleen and/or bursa	Liver swollen and/or mottled	Gizzard erosions
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (d)[c]								
0 μg/kg	0/10[b]	2/10	0/10	0/10	0/10	0/10	0/10	0/10
0.01 μg/kg	0/10	1/10	0/10	0/10	0/10	0/10	0/10	0/10
0.10 μg/kg	0/10	2/10	0/10	0/10	0/10	0/10	0/10	0/10
1.0 μg/kg	3/10	10/10	9/10	9/10	5/10	2/10	7/10	1/10
10.0 μg/kg	10/10	5/10	9/10	9/10	5/10	0/10	6/10	0/10
Hexachlorodibenzo-*p*-dioxin (c)[c]								
0 μg/kg	1/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
0.1 μg/kg	0/10	1/10	0/10	0/10	0/10	0/10	0/10	0/10
1.0 μg/kg	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
10.0 μg/kg	1/10	9/10	3/10	1/10	1/10	1/10	3/10	0/10
100.0 μg/kg	10/10	6/10	3/10	8/10	8/10	0/10	4/10	0/10
Octachlorodibenzo-*p*-dioxin (d)[d]								
0% of diet	0/12	0/12	0/12	0/12	0/12	0/12	0/12	2/12
0.1% of diet	0/12	0/12	0/12	0/12	0/12	0/12	0/12	7/12
0.5% of diet	0/12	0/12	0/12	0/12	0/12	0/12	0/12	7/12

[a] Sample identified in Table 1.
[b] Number affected/total number in group.
[c] Administered orally as a corn oil: acetone solution.
[d] Fed in the diet (0.1% ≅ 100 mg/kg, 0.5% ≅ 500 mg/kg).

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...scopic examination of this organ revealed a highly variable pattern and degree ...epatic necrosis with various degrees of ...eration and regeneration of the hepato... depending upon the post-treatment in... Necrosis was observed both in the ...ilobular and periportal areas. The degree of necrosis of the liver was not sufficient ...conclude that it was responsible for death. Hepatic lesions were observed in rats, mice, rabbits, and dogs. In addition to hepatic involvement, other changes observed sporadically include fat necrosis, periarteritis, ser... ...trophy of fat, and ascites.

Discussion and Summary

The studies reported here confirmed the high toxicity of 2,3,7,8-TCDD. In addition, some perspective of the relative toxicities of 2,7-DCDD, HCDD, and OCDD has been obtained. 2,7-DCDD and OCDD failed to cause death in female rats given oral doses ... 1 g/kg; even larger doses were given to ...e without causing death. Limited data ...est that oral doses of approximately ... mg/kg of HCDD are needed to cause death in male rats. In the teratology study, no deaths occurred following administration of 100 μg/kg of HCDD to female rats for 10 consecutive days.

2,3,7,8-TCDD is much more toxic than the other chlorodibenzodioxins studied; the LD_{50} ranged from 0.6 μg/kg in male guinea pigs to 115 μg/kg in rabbits. Dogs appear to be less sensitive than rabbits. Others have reported 100% mortality in rabbits treated with 10 μg/kg (*15*) and chick embryos treated with 0.05 μg/egg (*5*).

Death following treatment with a lethal dose of 2,3,7,8-TCDD is often delayed for several weeks. Among the animals which died following treatment, approximately half the deaths occurred between 13 and 18 days after treatment, with one animal dying as late as 43 days after a single oral dose. In mice and rabbits, there is a marked individual difference in susceptibility to this compound which makes it difficult to conduct acute lethality studies.

...f the results of the rabbit eye irritation test can be extrapolated to man, accidental contact of these chlorodibenzodioxins with the eyes should not present a serious threat to vision. However, repeated contact with the skin of small amounts of either 2,3,7,8-TCDD or HCDD may be expected to produce chloracne. Sensitivity to 2,3,7,8-TCDD was recognized by industry years ago, and precautions have been taken to minimize its occurrence and prevent contamination of worker's skin. HCDD is apparently a less potent acnegen than 2,3,7,8-TCDD.

As previously reported, 2,3,7,8-TCDD is highly embryotoxic (*6*). The no-effect level for embryotoxicity was 0.03 μg/kg-day of 2,3,7,8-TCDD. In contrast to the high embryotoxicity of the symmetrical 2,3,7,8-TCDD, 1,2,3,4-TCDD was not embryotoxic at doses as high as 800 μg/kg-day (*16*).

By previously described definitions of teratogenicity and embryotoxicity (*17*), HCDD is teratogenic in the rat at a 100 μg/kg-day dose level, given orally on days 6 through 15 of gestation. Treatment of pregnant rats with HCDD caused embryotoxicity evidenced by a dose-related decrease in fetal body weight and crown–rump length and an increase in the incidence of fetal resorptions (Table 4). Likewise, the incidence of certain soft tissue and skeletal anomalies increased in a manner related to the dose level of HCDD (Table 5). A 0.1 μg/kg-day dosage of HCDD had no effect on embryonal or fetal development.

OCDD caused embryotoxicity but was not teratogenic at 500 mg/kg-day. OCDD and 2,7-DCDD caused neither teratogenicity nor embryotoxicity at 100 mg/kg-day. Khera and Ruddick (*16*) reported that the administration of 2 mg 2,7-DCDD/kg-day was associated with microscopic myocardial and pericardial lesions in rat fetuses. However, examination of sections of myocardium and pericardium from fetuses of dams treated with 100 mg doses in this study revealed no morphological differences from controls.

Both 2,3,7,8-TCDD and HCDD give positive results in chick edema bioassays (Table 6). This HCDD result is consistent with a previous report that the HCDD isolated

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from pentachlorophenol produced chick edema (*5*). These same authors reported that 2,3,7,8-TCDD was extremely toxic in the chick embryo assay but did not report that it produced chick edema.

Pathological changes observed in animals treated with chlorodibenzodioxins were inconsistent from animal to animal and species to species. Hepatic lesions were observed consistently, but the nature, degree, and distribution of the lesions were variable. Changes in organs other than the liver were sporadic and unpredictable. Gross and microscopic examination of tissues after chlorodibenzodioxin treatment did not reveal the cause of death. An in-depth evaluation of the toxicity associated with chronic exposure to the chlorobenzodioxins is needed.

Isomers of a chlorodibenzodioxin can produce different degrees of toxicity; 2,3,7,8-TCDD is highly embryotoxic and a potent acnegen, but 1,2,3,4-TCDD is neither embryotoxic nor acnegenic.

The toxicity of chlorodibenzodioxins other than those evaluated in this study has not been reported. Purified samples of trichloro-, pentachloro-, and heptachlorodibenzo-*p*-dioxin which are free of tetrachloro- and hexachlorodibenzo-*p*-dioxin need to be synthesized for study. However, heptachlorodibenzo-*p*-dioxin cannot be highly toxic, since studies on octachlorodibenzo-*p*-dioxin containing several per cent of heptachlorodibenzo-*p*-dioxin have tested the same as the pure product.

Studies on the chlorodibenzodioxins have led to the following conclusions: (1) 2,7-dichlorodibenzo-*p*-dioxin and octachlorodibenzo-*p*-dioxin have a low acute toxicity; (2) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin has an unusually high toxicity; (3) hexachlorodibenzo-*p*-dioxin is highly toxic but less toxic than 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; (4) all chlorodibenzodioxins are not alike in their toxicological properties. Isomers of the same dibenzo-*p*-dioxin vary in toxicological properties, making it important to identify them specifically.

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GENP 011613

General Biological Effects of TCDD in Laboratory Animals

by M.W. Harris,* J.A. Moore,* J.G. Vos,* and B.N. Gupta*

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is reported to be one of the most toxic chemicals known. There has been variation in such reported toxic parameters as single oral LD_{50} dose, range of time interval from dosing until death, and toxic manifestations which an animal exhibits (*1–4*). It seemed most appropriate, therefore, that the mean values and ranges of these and other general biological parameters be determined under the experimental condition to which animals are exposed at this Institute.

Materials and Methods

The TCDD utilized in the studies was graciously supplied by the Dow Chemical Company and was found by analyses to contain more than 99% TCDD. The majority of the studies reported by NIEHS scientists at this conference utilized a single stock solution prepared by dissolving 5 mg of chemical in 67.5 ml of reagent grade acetone. Once dissolved, this TCDD acetone solution was added to 432.5 ml of corn oil purchased at a local retail store. This solution was calculated to contain 10 μg TCDD/ml. Chemical analysis of an aliquot of this solution performed by the Dow Chemical Company yielded a value of 11 μg/ml ± 2 μg/ml. This compares favorably with the calculated value. By using appropriate dilutions of the stock solution with corn oil, the actual dosing solution was prepared in a volume appropriate for the requirements of the experiment.

All animals used, rats, guinea pigs, and mice, were housed in animal quarters maintained under a rigid sanitary regimen. Temperature was maintained at 70 ± 2°F and 50 ± 5% RH. Food and water were available at all times. All animals were administered the TCDD–acetone-corn oil solution via gastric intubation. The volume administered ranged from 0.2 ml to 1.6 ml in rats, from 0.17 to 0.22 ml in guinea pigs, and from 0.1 to 0.2 ml in mice. The volume administered in any one experiment was the same. Control animals received an equal amount of acetone–corn oil, the actual proportion of each used being equal that contained in the TCDD solution. All animals were weighed at least once weekly. Rat food consumption as measured by disappearance of the blocks from a suspended stainless steel feeder was determined at least twice each week. Rats and mice were dosed according to a mean average weight of the dose group. Guinea pigs were dosed according to the weight of each animal. In multiple-dose experiments, dose was recalculated each week based on the weights of the animals on the date of recalculation. All animals were assigned to a given dose group according to a table of random numbers.

Rats used were of both sexes, 6 to 8 weeks of age, specific pathogen-free, and had been

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acclimated to NIEHS conditions at least 2 weeks prior to use. All were random bred albino rats, CD stock, purchased from Charles River Breeding Laboratories, Wilmington, Massachusetts. Female albino guinea pigs, Hartley strain, were purchased from Carworth, Inc., New City, New York. Mice used were of two types, random bred albino CD-1 stock from Charles River or inbred C57B1/6Sch strain reared at the Institute.

The data obtained in the four experiments are presented as mean values and standard errors or deviations. Dunnett's multiple comparisons test (*8*) was used to make treatment control comparisons, usually two-sided, except for organ weights. In addition, a nonparametric test (Jonckheere's test) (*9*) was used to test for monotonic dose–response relationships.

Results

Live Animal Effects

In the course of various rat experiments the frequency of dose administration varied from a single administration to daily dosing for 30 consecutive days or weekly administration for a period of 6 weeks. The effects of a single dose of TCDD at 1, 5, 25, 50, or 100 μg/kg on body weight and survival in rats are illustrated in Figures 1–3; the actual body weights and statistical values are given in Tables 1–4. Male and female rats at the 1 or 5 μg/kg dose gained weight at the same rate as the controls. At the 25 μg/kg dose an actual weight loss was observed (for 1 week) in females (Fig. 1, Table 1). Subsequent to week 1 until the end of the experiment (week 9) animals at this dose gained weight at the same rate as controls. Male rats at the same dose had a significantly decreased weight gain 1 and 2 weeks subsequent to the TCDD dose (Fig. 2, Table 3). Their weight gain after 2 weeks was equivalent to control rats. In a later experiment (Fig. 3, Table 4), male rats showed a dose-related decrease in body weight gain in the first two weeks subsequent to a 25 or 50 μg/kg dose with the reduced weight gain observed at the higher

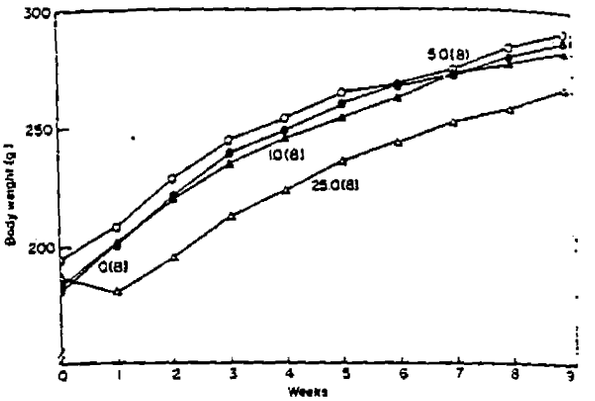


FIGURE 1. Body weights of female rats receiving a single dose of (●) 0; (△) 1.0 μg/kg; (○) 5.0 μg/kg; (△) 25.0 μg/kg. TCDD: There were 8 animals per dose group. All animals survived.

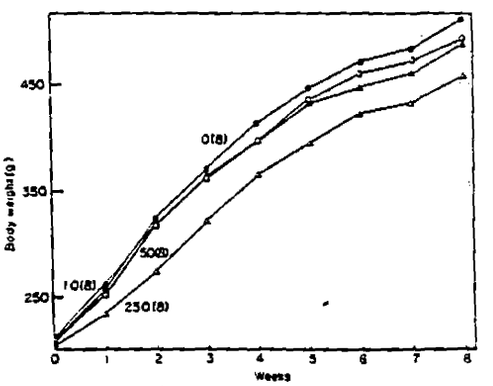


FIGURE 2. Body weights of male rats receiving a single dose of TCDD: (●) 0; (△) 1.0 μg/kg; (○) 5.0 μg/kg; (△) 25.0 μg/kg. There were 8 animals per dose group. All animals survived.

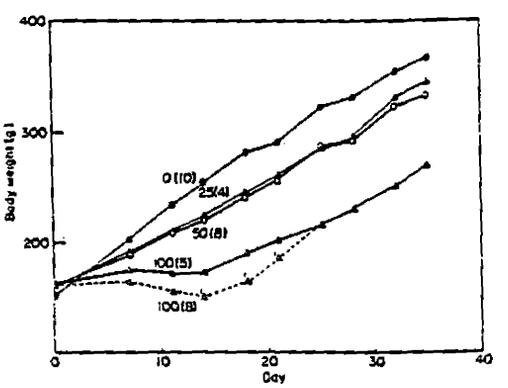


FIGURE 3. Body weights of male rats receiving a single dose of TCDD: (●) 0; (△) 25 μg/kg; (○) 50 μg/kg; (△) 100 μg/kg. The number of animals is shown in parentheses. In the 100 μg/kg group, the mean weight of the animal surviving the experimental period is also plotted ( ). The superscript 1 denotes days on which one animal died.

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Table 1. Weight gain of female rats receiving a single dose of TCDD.[a]

Single TCDD dose, μg/kg	Number of animals	Initial body weight, g	Weight gain, g	
			Week 0–1	Week 1–9
0	8	181.5 ± 7.2	20.3 ± 5.5	85.3 ± 16.8
1.0	8	183.8 ± 7.1	18.3 ± 2.3	81.3 ± 12.2
5.0	8	194.3 ± 6.9[b]	14.3 ± 5.2	82.8 ± 13.6
25	8	187.3 ± 5.7	−6.4 ± 11.4[b]	86.5 ± 20.6

[a] Mean values ± SD.
[b] $P < 0.01$.

Table 2. Weight gain of female rats receiving a single dose of TCDD.[a]

Single TCDD dose, μg/kg	Number of animals	Initial body weight, g	Weight gain, g	
			Week 0–1	Week 1–6
0	6	166.0 ± 1.8	22.5 ± 7.8	57.2 ± 12.3
50	6[b]	162.2 ± 8.3	−11.5 ± 14.8[d]	66.2 ± 11.4
100	6[c]	168.8 ± 4.9	−20.5 ± 12.8[d]	32.7 ± 31.8

[a] Mean values ± SD.
[b] One animal died on day 14.
[c] Three animals died between 18 and 21 days.
[d] $P < 0.01$.

Table 3. Weight gain of male rats receiving a single dose of TCDD.[a]

Single TCDD dose, μg/kg	Number of animals	Initial body weight, g	Weight gain, g	
			Week 0–2	Week 2–8
0	8	209.1 ± 7.7	115.3 ± 13.0	184.4 ± 30.8
1.0	8	211.0 ± 12.1	107.0 ± 13.6	169.5 ± 23.1
5.0	8	212.8 ± 9.7	107.6 ± 18.5	172.9 ± 29.3
25	8	204.0 ± 5.6	72.3 ± 23.6[b]	182.1 ± 36.0

[a] Mean values ± SD.
[b] $P < 0.01$.

Table 4. Weight gain of male rats receiving a single dose of TCDD.[a]

Single TCDD dose, μg/kg	Number of animals	Initial body weight, g	Weight gain, g	
			Week 0–2	Week 2–5
0	10	153.6 ± 9.8	100.7 ± 14.1	113.9 ± 22.6
25	4	158.8 ± 2.5	66.5 ± 39.5	122.5 ± 27.7
50	8	163.6 ± 9.2	58.0 ± 30.5[c]	112.8 ± 22.1
100	8[b]	161.0 ± 9.9	−9.9 ± 30.4[c]	97.6 ± 51.6

[a] Mean values ± SD.
[b] Three animals died between 14 and 21 days.
[c] $P < 0.01$.

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dose significant at the 1% level. At the 100 μg/kg dosage, 3 of 8 rats died between 14 and 21 days. The mean weight gain of the survivors at the high dose between weeks 2 and 5 was not significantly different from other TCDD dose levels or controls. Female rats which received 50 or 100 μg/kg exhibited a dose-related absolute decrease in body weight during the first week (Table 2). One of six and three of six rats died between 14 and 21 days at the 50 and 100 μg/kg dose, respectively. Weight gain between weeks 1 and 6 was not significantly different from that of controls; however, as the large standard deviation at the high dose suggests, appreciable variation among the survivors did exist.

The mean time interval until death was 18.3 days in the 6 of 14 male and female rats which died at the 100 μg/kg dose. Five of six deaths occurred between the 18th and 21st day; the female rat at the 50 μg/kg dose which died, survived 14 days.

Clinical symptoms of toxicity, which were seen at the 100 μg/kg dose, were ruffled hair coat, hunched posture, and inactivity (depression). Jaundice was observed for several days in those rats that died. Food consumption in male rats which received 25, 50, and 100 μg/kg was recorded and analyzed for the initial 2-week period subsequent to TCDD intubation (Table 5). Although the average amount of food consumed decreased in all treatment groups when compared to controls, the difference is significant only at the 100 μg/kg dose ($P<0.05$). If the food consumption of the three rats which subsequently died in the 100 μg/kg group is excluded and the mean recomputed, the value is no longer significantly different from controls. The standard deviation for food consumption in all TCDD treatment groups is relatively large which suggests marked variation within a dose group.

In the daily dose experiments, female rats received either 0, 0.1, 1, or 10 μg/kg TCDD for 31 consecutive days. The body weight changes which occurred in one of these experiments are given in Table 6. Although not included in the table because it was not part of this particular experiment, the effect on body weight in rats receiving daily TCDD at 0.1 μg/kg was negligible. Rats at the 10 μg/kg dose lost 21.8 g weight during the first 7 days of the experiment. Fifteen of 16 rats died or became moribund a mean of 21.8 days after the study commenced. Weight gain at the 1 μg/kg dose group was significantly less than that of controls for the 1–35 day time period. Weight gain during the 35–63 day time period exceeded that of controls by 14 g.

Table 5. Fourteen-day food consumption in male rats receiving a single dose of TCDD.

Single TCDD dose, μg/kg	Number of animals	Food consumption, g ± SD [a]
0	10	359.4 ± 19.3
25	4	313.2 ± 70.8
50	8	315.6 ± 56.4
100	8	257.5 [b] ± 87.2
100	5 [c]	275.0 ± 84.7

[a] Dose response test for food consumption significant at 0.01 level.
[b] $P<0.05$.
[c] Three rats in group which later died excluded.

Table 6. Weight gain of female rats receiving 31 daily doses of TCDD.

Daily TCDD dose, μg/kg	Initial no. of animals	Initial body weight, g ± SD	Weight gain, g ± SD		
			Days 1–7	Days [a] 1–35	Days [a] 35–63
0	16	183.3 ± 6.28	15.8 ± 6.6	65.8 ± 8.0	28.4 ± 9.7
1	12	185.6 ± 5.04	12.1 ± 6.4	34.8 ± 12.2 [b]	42.0 ± 11.9 [c]
10	16	184.0 ± 8.00	−21.4 ± 14.9 [b]	—	—

[a] Values based on 5 and 8 rats at 0 and 1 μg/kg respectively; 15 of 16 rats died or were killed when moribund at mean of 21.8 days.
[b] $P<0.01$.
[c] $P<0.06$.

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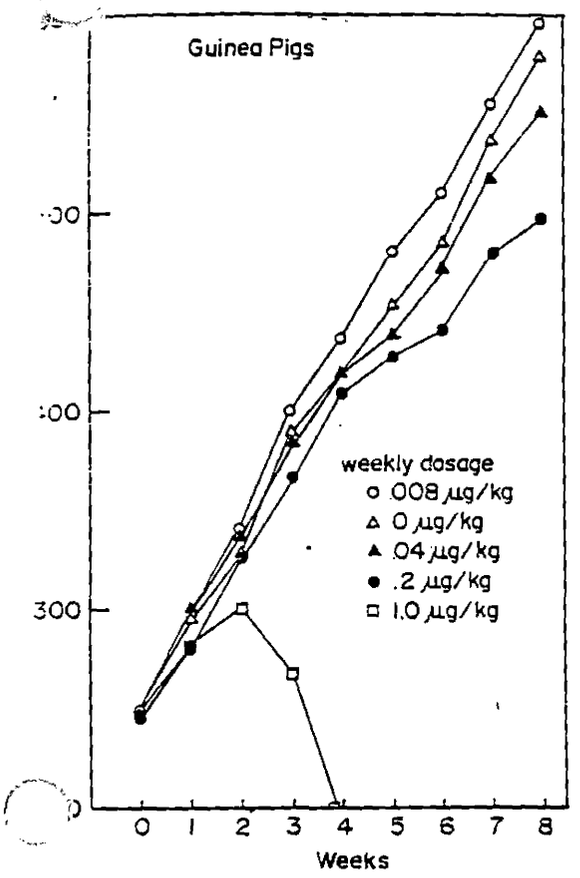


FIGURE 4. Body weight of guinea pigs receiving eight weekly doses of TCDD. There were 10 animals per dose group. All animals at the 1.0 μg/kg dose died.

Rats also received weekly doses of 0.02, 1.0, or 5.0 μg/kg for 6 weeks. During the dosing period, decreased body weight gain occurred in the 5.0 μg/kg dose group (7).

Guinea pigs were dosed weekly for 8 weeks with 0, 0.008, 0.04, 0.2, or 1.0 μg/kg. In other experiments, guinea pigs received a single 1.0 or 3.0 μg/kg dose of TCDD. Nine of ten animals died a mean 19.2 days after receiving the single 3 μg/kg dose. Severe weight loss preceded death. No guinea pigs died following the single 1.0 μg/kg dose, although a reversible decreased body weight gain was observed. All guinea pigs receiving the 1.0 μg/kg weekly dose 24–32 days after the study began died (mean survival time 28 days). Animals which received the weekly 0.2 μg/kg dose weighed significantly less at the end of the experiment (7). As Figure 4 illustrates, weight gain depression at this dose level primarily occurred during the fifth and sixth week of the study.

Adult CD-1 mice received a single oral dose of TCDD at 0, 1.0, 10, or 50 μg/kg. No effect on body weight was observed during the subsequent 5-week observation period. Adult C57B1/6 mice received weekly doses of 25, 5, 1, or 0.2 μg/kg for four weeks. Significant weight loss occurred at the 25 μg/kg dose, with one of seven mice dying on the day 25 of the study.

Organ Weights

In some experiments, rats were killed and organ weights recorded at predetermined intervals after a single 0, 5, or 25 μg/kg TCDD dose. These organ weights from randomly selected rats were obtained 1, 3, 8, 9, 16, and 28 days after TCDD intubation.

Table 7. Liver and thymus weights of male rats receiving a single dose of TCDD.

Time since dose, days	No. of animals	0 μg/kg		5 μg/kg		25 μg/kg	
		Liver, g ± SE	Thymus, mg ± SE[a]	Liver, g ± SE	Thymus, mg ± SE[a]	Liver, g ± SE	Thymus, mg ± SE[a]
1	5	9.02 ± 1.15	934 ± 53	10.34 ± 0.76	866 ± 89	8.65 ± 0.67	768 ± 29
3	5	11.06 ± 0.44	864 ± 62	11.94 ± 0.84	724 ± 108	11.14 ± 0.53	568 ± 36[b]
8	5	13.65 ± 0.88	826 ± 99	14.08 ± 0.62	644 ± 65	13.92 ± 0.59	334 ± 25[c]
9	5	14.96 ± 0.47	836 ± 107	14.28 ± 0.84	646 ± 62	13.38 ± 0.81	280 ± 30[c]
16	5	16.22 ± 0.70	790 ± 57	16.40 ± 1.27	570 ± 55[b]	14.28 ± 1.99	264 ± 45[c]
28	5	17.99 ± 0.95	764 ± 60	15.61 ± 0.93	664 ± 45	15.72 ± 1.60	494 ± 54[c]

[a] Dose-response tests for reduced thymus significant on each day at $P < 0.05$.
[b] $P < 0.05$.
[c] $P < 0.01$.

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Table 8. Liver and thymus weights of females rats receiving daily doses of TCDD.

No. of doses	No. of animals	0 μg/kg		0.1 μg/kg		1.0 μg/kg		10 μg/kg	
		Liver, g ± SE	Thymus, mg ± SE[a]	Liver, g ± SE	Thymus, mg ± SE[a]	Liver, g ± SE	Thymus, mg ± SE[a]	Liver, g ± SE	Thymus, mg ± SE[a]
3	4	7.80 ± 0.48	—	8.62 ± 0.41	—	9.32 ± 0.45	—	9.58 ± 0.43	—
6	4	8.41 ± 0.36	—	10.35 ± 0.45[b]	—	9.00 ± 0.14	—	10.84 ± 0.97	—
10	4	9.14 ± 0.21	—	10.78 ± 0.41[b]	—	11.53 ± 0.40[c]	—	10.53 ± 0.36[b]	—
13	4	7.90 ± 0.31	530 ± 48	10.17 ± 0.94[b]	492 ± 81	9.76 ± 0.62	390 ± 58	9.80 ± 1.08	142 ± 12[c]
17	4	9.26 ± 0.42	615 ± 59	10.91 ± 0.37[b]	468 ± 22	11.33 ± 0.29[c]	270 ± 11[c]	7.52 ± 1.37	110 ± 48[c]
24	4	9.45 ± 0.31	525 ± 43	11.40 ± 1.05	412 ± 28[b]	11.39 ± 0.98	372 ± 28[b]	4.67 ± 0.21[c]	32 ± 10[c]
31	4	9.70 ± 0.49	568 ± 87	11.78 ± 0.81	475 ± 49	12.84 ± 0.84[b]	260 ± 41[c]	—	—

[a] All dose response tests for thymus significant at $P < 0.01$.
[b] $P < 0.05$.
[c] $P < 0.01$.

Table 9. Liver and thymus weights of CD-1 mice treated with a single dose of TCDD.[a]

Time since dose, weeks	No. of animals	0 μg/kg		1.0 μg/kg		10 μg/kg		50 μg/kg	
		Liver, g ± SD	Thymus, mg ± SD	Liver, g ± SD	Thymus, mg ± SD	Liver, g ± SD	Thymus, mg ± SD	Liver, g ± SD	Thymus, mg ± SD
1	4	1.68 0.16	82.5 19.8	1.65 0.15	75.5 16.3	1.90 0.26	74.2 19.2	2.27[b] 0.16	60.8 17.7
3	3	1.89 0.38	81.7 8.1	2.05 0.26	83.3 3.2	1.94 0.41	60.3[c] 5.9	2.43 0.19	60.7[c] 14.2
5	3	1.77 0.17	51.0 18.0	1.61 0.13	47.0 8.5	1.63 0.10	54.7 6.7	1.83 0.04	38.7 22.0

[a] Dose-response test: $P < 0.01$ (liver) at week 1; $P < 0.05$ (thymus) at week 3.
[b] $P < 0.01$.
[c] $P < 0.05$.

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ney, heart, lung, spleen, adrenal, testes and thymus weights were recorded and evaluated. Significant differences in organ weights were seen with the thymus. Wet weights for this organ and liver are given in Table 7. There is a tendency for thymus weights to decrease with age. A significant reduction in thymus weight was first observed 3 days after rats received 25 μg/kg TCDD. Thymus weights at this dose continued to decrease with the lowest weights observed on day 16. The day 28 mean value was 220 mg higher than day 16 but still far below control values. The only significant ($P<0.05$) decrease in thymus weight in the 5 μg/kg group occurred at day 16. However, the average thymus weight reduction at this dose level for the entire 28-day period is significant ($P<0.01$). Liver weights were not significantly different from controls at either dose level. Reduced spleen weights were observed on days 3, 9, and 16 at the 25 μg/kg dose.

Organ weights were also collected at predetermined times in some rats being inoculated daily with 0, 0.1, 1.0, or 10 μg/kg. Liver and thymus weights for these doses are illustrated in Table 8. Mean liver weights were always greater at the 0.1 and 1 μg/kg dose; however, the statistical significance of these increases was inconsistent. At the daily 10 μg/kg dose livers were increased in size on the first four days organ weights were collected (days 3, 6, 10, and 13). There was a marked decrease in liver weight, falling below control mean values, on days 17 and 24. It should be remembered that body weight at this dose level markedly decreased and death occurred in 15 of 16 rats. Thymus weights were 20–25% of control values in the daily 10 μg/kg dose group when recorded on days 13 and 17. Weights were further reduced at death. At the 1 μg/kg level, thymus was first found significantly smaller than controls at day 17; it continued to remain so through the 31-day dosing period. Thymus weights at the 0.1 μg/kg dose were significantly reduced when evaluated for an overall dose response effect.

Weight changes were not seen in heart, lung, or kidney. Weight decreases occurred in spleen at the high dose group as they approached death.

Pathologic, hematologic, and clinical chemistry changes observed in rats, mice and guinea pigs used in these experiments are reported elsewhere (*5*, *6*). Experiments which measured humoral or cell-mediated immune capabilities are the subject of another report (*7*).

Table 9 lists the liver and thymus weights of CD-1 mice 1, 3, or 5 weeks after a single dose of TCDD. Liver weight was significantly increased after 1 week in mice which received 50 μg/kg TCDD. Thymus weight was decreased in the 10 and 50 μg/kg groups at week 3. The measurement of an overall dose response was significant for the liver increase at week 1 and thymus decrease at week 3. No weight effects on spleen or adrenal were observed.

Guinea pig liver, kidney, thyroid, and uterus weights collected after 8 weekly doses of 0.2, 0.04, or 0.008 μg/kg TCDD were not significantly lower than controls. Decreased thymus weight was observed at the 0.04 and 0.2 μg/kg dose level (Table 10). Marked thymic atrophy was also seen at time of necropsy in those guinea pigs which died in the weekly 1.0 μg/kg or single, 3.0 μg/kg dose groups.

Table 10. Liver and thymus weights of female guinea pigs receiving 8 weekly doses of TCDD and killed after 56 days.

Weekly dose of TCDD, μg/kg	No. of animals	Liver g ± SD	Thymus, mg ± SD [a]
0	10	29.4 ± 3.8	901 ± 246
0.008	10	29.5 ± 4.3	741 ± 163
0.04	10	26.9 ± 3.6	672 ± 161 [b]
0.2	7	26.9 ± 3.9	476 ± 70 [c]

[a] Thymus dose response $P<0.01$.
[b] $P<0.05$.
[c] $P<0.01$.

Discussion

Table 11 summarizes and contrasts some TCDD effects seen in the 3 species tested.

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Table 11. Summary of biological effects of TCDD.

	Rats			Guinea pigs			Mice
	Dose, μg/kg	MTD, days[a]	Mortality[b]	Dose, μg/kg	MTD, days[a]	Mortality[b]	Dose, μg/kg
Lethal dose							
Single	100	18	6/14	3	18	9/10	>50
Weekly	4 × 25	28	2/10	5 × 1	28	10/10	>4 × 25
Daily	10	22	15/16	—			—
Body weight							
Lowest dose effect							
Single	25			1			—
Weekly	6 × 5			8 × 0.2			4 × 25
Daily	30 × 1			—			—
No effect							
Single	5			—			50
Weekly	6 × 1			8 × 0.04			4 × 5
Daily	30 × 0.1			—			—
Thymus weight							
Lowest dose effect							
Single	5			—			10
Weekly	6 × 5			8 × 0.04			4 × 5
Daily	30 × 0.1			—			—

[a] Mean time to death after first exposure.
[b] Number of animals dying/number of animals treated.

Guinea pigs are most sensitive to the lethal effects of TCDD with 90% dying from a single 3 μg/kg dose. In contrast, a 100 μg/kg dose was lethal to 6 of 14 rats. In both species the time interval until death is similar and a large weight loss over a period of days preceded death. We did not observe a great range in the time interval from exposure until death which was observed by others (*1*).

Female rats appear to be more sensitive to TCDD than males. Although death incidence at 100 μg/kg was similar, one female did die at the 50 μg/kg dose. Additionally, at the 25 and 50 μg/kg dose females underwent an actual weight loss, whereas males exhibited only a 30–40% reduction in weight gain. Body weight effects of a single dose are primarily observed during the first two weeks after exposure. Subsequently, weight gain equates with controls, although treated rats never narrow the actual weight divergence which appears during the first 2 weeks.

There is an overall tendency for feed consumption to be reduced following TCDD exposure, but this depression is not sufficient magnitude to account for the body weight changes which occurred. Food consumption at the 100 μg/kg dose was significantly decreased primarily due to the greater anorectic state observed in rats for the several days preceding death.

Administration of daily or weekly sublethal doses does not seem to raise the threshold level of TCDD toxicity. For example decreased weight gain at the 1 μg/kg daily dose occurred in rats once the total dose administered exceeded approximately 20 μg/kg; decreased weight gain first occurred at the single 25 μg/kg level. A comparison of the body weight gains of 5 μg/kg weekly dose and the 1 μg/kg daily dose also reveals a similar weight pattern change. Parallel growth rates after 1 or 2 weeks in the single dose rat experiments and weight gains exceeding that of controls subsequent to 30-day exposure at 1 μg/kg/rat suggest that toxicity

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of TCDD, at least in some circumstances, are reversible. As Table 11 indicates, thymus appears to be a most sensitive indicator of TCDD exposure. Decreases in thymus weight consistently occurred in all species at a dose level below which body weight effects occurred.

Acknowledgement

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Hematologic and Clinical Chemistry Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Laboratory Animals

by J.G. Zinkl,* J.G. Vos,* J.A. Moore,* and B.N. Gupta*

Introduction

Chlorodibenzo-*p*-dioxins, especially 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are among the most toxic compounds known. These compounds are found as contaminants of technical chlorophenols and their derivatives. A variety of pathologic condition have been associated with the injestion of products containing chlorodibenzo-*p*-dioxins.

Toxic fat, the cause of chick edema disease (*1*, *2*) and a variety of pathologic manifestations in monkeys (*3*), has been found to contain chlorodibenzo-*p*-dioxins, among which is TCDD (*4*, *5*). TCDD has been implicated in a variety of other toxicoses, including outbreaks of chloracne in chemical workers (*6*), hepatonecrosis in rabbits (*6*), and hepatonecrosis and thymic atrophy in rats (*7*). However, only in the studies in which monkeys were fed toxic fat (*3*) and in which rats were given TCDD orally (*7*, *8*) were substantial clinical pathologic analyses performed.

Toxic fat caused in the monkeys, among other lesions, anemia, leukopenia and hypoproteinemia. The hypoproteinemia was primarily due to decreased serum albumin concentration. The blood urea nitrogen (BUN), serum bilirubin, cholesterol, sodium and potassium concentrations, and the prothrombin times were not altered (*3*).

Rats given 10 mg TCDD/kg body weight had, among the other lesions, increased activities of serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvate transaminase (SGPT), lactic dehydrogenase (LDH), and hydroxybuturate dehydrogenase as well as decreased arylesterase and cholinesterase activities. In addition, the rats had decreased serum glucose, sodium, and protein concentrations, and increased serum urea, lipid and bilirubin concentrations, hemoconcentration, and neutrophilia. Serum cholesterol, potassium and chloride concentrations and aldolase and alkaline phosphatase (AP) activities were not altered (*7*, *8*).

Because the clinical pathologic and pathologic changes found in these and other studies suggest that the most profound effect of chlorodibenzo-*p*-dioxins are on the liver and various hematopoietic organs, we decided to determine the sequential clinical pathologic changes which might occur in rats given different doses of TCDD over a period of time. These methods might also determine which functions are the most sensitive to TCDD. In addition, hematologic studies were conducted as an adjuvant to studies of immunologic effects of TCDD in

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guinea pigs (*9*) and in mice in which the effects of TCDD on oögenesis are being determined (J. McLachlan, personal communication).

Materials and Methods

Animals

Female CD rats weighing 150–175 g were given, orally, 0.1, 1.0, or 10.0 μg TCDD/kg daily for 30 days. Blood for the clinical pathologic studies was obtained from the heart 3, 6, 10, 13, 17, 24, and 31 days after the TCDD dosing commenced.

Blood was obtained from the retroorbital sinus for hematologic studies 1, 3, and 5 weeks after 8-week-old female CD-1 mice were given oral doses of TCDD of 1.0, 10.0, or 50 μg/kg.

Female Hartley strain guinea pigs weighing about 250 g received oral weekly doses of 0.008, 0.04, 0.2, or 1.0 μg TCDD/kg for 8 weeks. The 1.0 μg TCDD/kg group became moribund between 3 and 5 weeks. Half the animals were injected with tetanus toxoid (group A) and half with killed *Mycobacterium tuberculosis* (group B) in order to determine the effects of TCDD on humoral and cell-mediated immune responses. The details of these experimental procedures can be found elsewhere in this issue (*9*). The highest dose guinea pigs when moribund and the other animals at 8 weeks were bled from the heart for hematologic analysis.

Procedures

Routine methods were used for hematologic determinations. Platelet counts were determined in counting chambers (Unopette, Becton, Dickinson and Company, Rutherford, N. J.), fibrinogen by a heat-denaturation method (*10*); urea nitrogen was determined by a urease method (Boehringer-Mannhein Co., New York, N. Y.), cholesterol by a ferric chloride method (Hyland, Costa Mesa, California), serum proteins by the biuret technique (*11*), glucose by an *o*-toluidine method (Hyland, Costa Mesa, California), creatinine by an alkaline picrate method (*12*), bilirubin by a sulfanilic acid–caffeine–sodium benzoate method (Boehringer-Mannheim Co., New York, N. Y.), sodium and potassium by flame photometry, and chloride by coulometric titration. The serum enzymes, alkaline phosphatase (AP), lactic dehydrogenase (LDH), glutamic-oxaloacetic transaminase (SGOT), and glutamic pyruvate transaminase (SGPT) were determined by kinetic methods by use of commercially available kits (Sigma Chemical Co., St. Louis, Missouri) in a recording spectrophotometer.

Analysis of Data

Statistical analysis for differences between groups was done by Dunnett's test (*13*). Determination of a dose response was done by Jonckheere's method (*14*). The 5% level was selected as the value where variables would be considered significantly different.

Results

Rats

Enzymic alterations in the TCDD-treated rats consisted of increased SGPT activity in the high-dose (10.0μg TCDD/kg-day) rats at days 17, 24, and 31 (Fig. 1), and increased SGOT activity at days 13, 17, 24, and 31 in the high-dose rats as well as days 13 and 17 in the middle-dose (1.0 μg TCDD/kg-day) rats (Fig. 2). LDH was

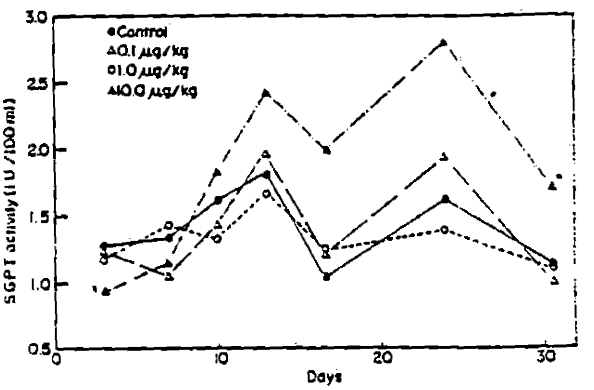


FIGURE 1. SGPT activity in rats treated with 0.1, 1.0, or 10.0 μg TCDD/kg-day. High-dose rats had increased SGPT activity at days 17 ($P < 0.01$) and 24 ($P < 0.05$). The asterisk (*) indicates a single observation. All other points are the means of three or four observations.

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ted only on day 24 in the high-dose rats, and AP was not altered at any time. Serum cholesterol was increased on days 17, 24, and 31 in the high-dose rats and 24 in the middle-dose animals. Significant dose–response elevations for cholesterol concentrations were also found on days 17 and 24 (Fig. 3). Blood glucose concentration decreased with time in all the treated groups. At day 10, glucose concentrations in all treated animals were significantly decreased and on days 24 and 31 in the high and middle dose animals it was decreased. Significant dose–response blood glucose decreases were found at days 10, 17, and 24 (Fig. 4). Total serum protein fluctuated with the treatment. On days 24 and 31, the high-dose rats had decreased protein

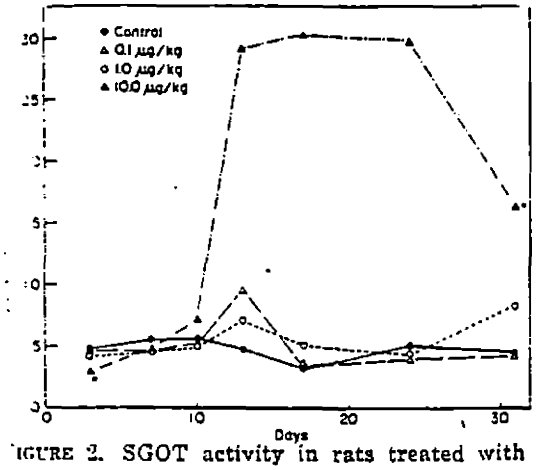


FIGURE 2. SGOT activity in rats treated with 0.1, 1.0, or 10.0 µg TCDD/kg-day. Significant increases were found in the high-dose rats on days 13, 17, and 24 ($P < 0.01$) and in the middle-dose rats on days 13 and 17 ($P < 0.05$). Significant dose responses were found on days 13 ($P < 0.01$), 17 ($P < 0.01$), and 31 ($P < 0.05$). The asterisk (*) indicates a single observation. All other values are means of three or four observations.

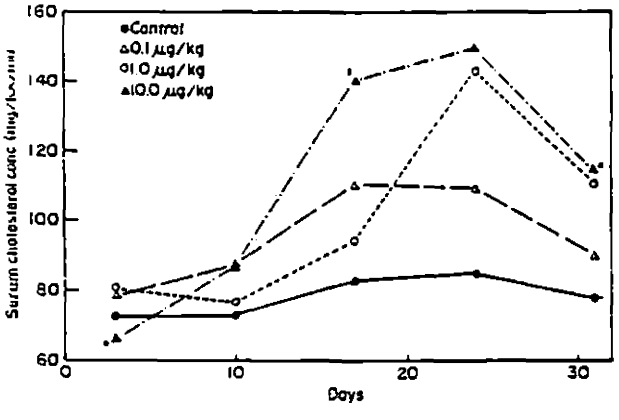


FIGURE 3. Serum cholesterol concentration in rats given 0.1, 1.0 or 10.0 µg TCDD/kg-day. Significant elevations occurred on days 10 ($P < 0.05$) and 24 ($P < 0.01$) in the high-dose rats and on day 24 ($P < 0.05$) in the middle-dose rats. Significant ($P < 0.05$) dose responses were found on days 17 and 24. The asterisk (*) indicates a single observations. All other values are the means of three or four observations.

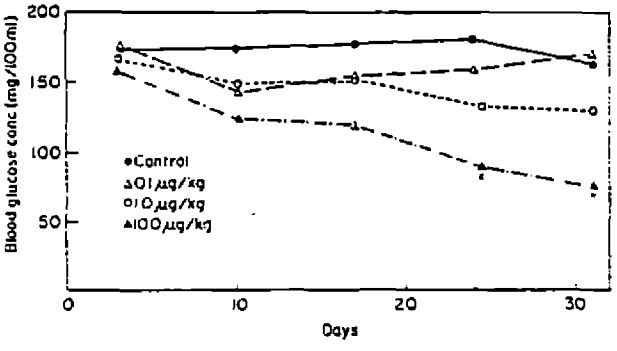


FIGURE 4. Blood glucose concentration in rats treated with 0.1, 1.0, or 10.0 µg TCDD/kg-day. Significant ($P < 0.05$) decreases occurred on day 10 in the low-dose rats and the middle-dose rats. Highly significant ($P < 0.01$) decreases occurred in the middle-dose rats on day 24 and in the high-dose rats on days 10 and 17. Significant dose responses occurred on days 10 ($P < 0.01$), 17 ($P < 0.05$ and 24 ($P < 0.01$). The asterisk (*) indicates a single observation. All other values are the means of three or four observations.

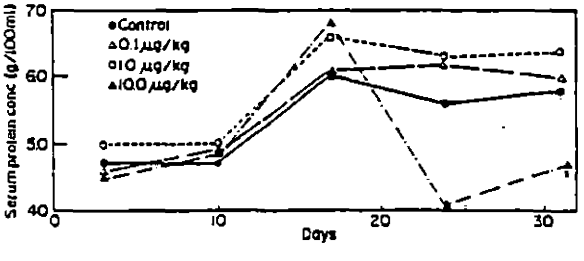


FIGURE 5. Serum protein concentration in rats treated with 0.1, 1.0, or 10.0 µg TCDD/kg-day. Significant ($P < 0.05$) increased concentrations occurred in the middle-dose rats on days 31 and decreased concentration in the high-dose rats on day 24. The asterisk (*) indicates a single observation. All other values are the means of three or four observations.

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concentration while at day 31 it was increased in the middle-dose rats (Fig. 5). In addition, serum bilirubin concentrations were increased in the high-dose animals on days 17, 24, and 31 (Table 1). No significant changes were found for serum creatinine, sodium, potassium, chloride, fibrinogen, and BUN concentrations.

Table 1. Bilirubin concentration in TCDD-treated rats.[a]

Time, days	Bilirubin concentration, mg/100 ml serum		
	Control	1.0 μg/kg-day	10.0 μg/kg-day
13	0.41 ± 0.10		0.83 ± 0.28 [b]
17	0.25 ± 0.08	0.22 ± 0.09	1.94 ± 0.42 [c]
24	0.64 ± 0.51	0.44 ± 0.15	5.62 ± 1.07 [c]
31	0.52 ± 0.15	0.40 ± 0.09	2.16 [d]

[a] Mean ± 1 S.D.
[b] $P < 0.05$.
[c] $P < 0.01$.
[d] Single value.

Hematologic changes were confined to hemoconcentration, as previously noted in rats (*7*), in the high-dose animals on days 17 and 24 (Figs. 6–8), and to striking thrombocytopenia in all the groups. After only 3 days treatment, the high and middle-dose animals had depressed platelet counts which remained depressed throughout the study. In the low-dose rats (0.1 μg TCDD/kg-day), platelets were decreased

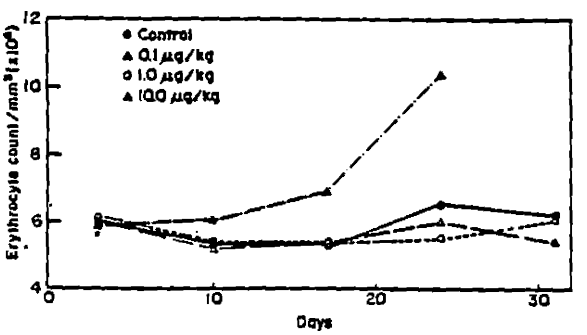


FIGURE 6. Erythrocyte count in rats given 0.1, 1.0, or 10.0 μg TCDD/kg-day. Significant increases occurred in the high-dose rats on days 17 and 24 ($P < 0.01$). All values are the means of three or four observations.

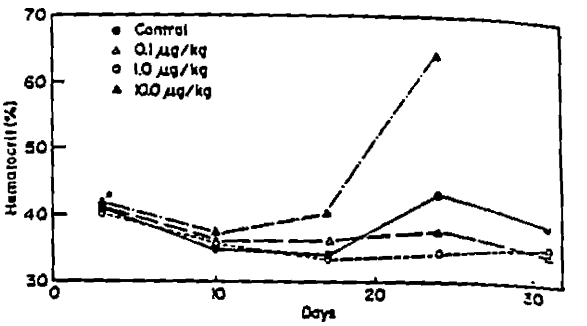


FIGURE 7. Hematocrit in rats given 0.1, 1.0, or 10.0 μg TCDD/kg-day. Highly significant ($P < 0.01$ increases occurred in the high-dose rats on days 17 and 24. All values are the means of three or four observations.

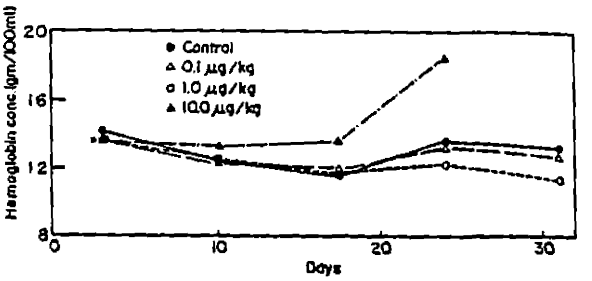


FIGURE 8. Hemoglobin concentration in rats given 0.1, 1.0, or 10.0 μg/kg-day. Highly significant ($P < 0.01$) increases occurred on days 17 and 24 in the high-dose rats. All values are the means of three or four observations.

significantly only on day 17. Significant dose response decreases in platelets occurred throughout the study (Fig. 9). No significant changes occurred in leukocyte counts or differentials (in particular, lymphocytes) in these rats.

Mice

Mice given a single oral dose of 1.0, 10.0, or 50.0 μg TCDD/kg had significantly decreased leukocyte (Table 2) and lymphocyte counts (Table 3) after 1 week. After 3 weeks, none of the treatment groups had significant differences from control mice; however, a significant dose-response lymphocyte depression remained. The leukocyte depression was on the borderline of being a

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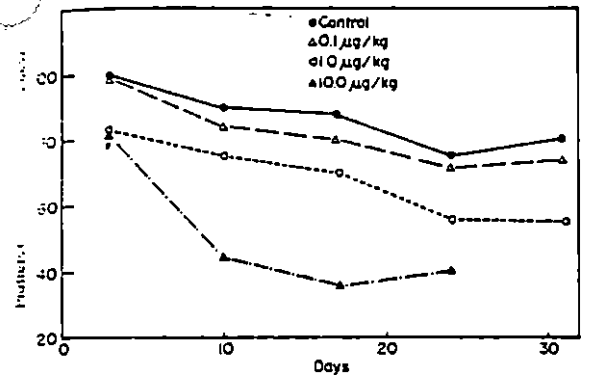


FIGURE 9. Relative platelet counts in rats treated with 0.1, 1.0, or 10.0 μg TCDD/kg-day. Significant ($P < 0.05$) decreases occurred in the low-dose rats on day 17, the middle-dose rats on days 3, 10, and 31 and in the high-dose rats on day 24. Highly significant ($P < 0.01$) decreases occurred in the middle-dose rats on day 24 and the high-dose rats on days 10 and 17. Significant dose-responses occurred on days 3 ($P < 0.05$), 10 ($P < 0.01$), 17 ($P < 0.01$), 24 ($P < 0.01$, and 31 ($P < 0.05$). The mean numbers of platelets at day 3 in the control rats were 1109 × 10^3/mm³ blood. All values are the means of three or four observations.

significant dose-response. After 5 weeks, no TCDD effects were detected except that the mice treated with 10 μg TCDD/kg had significantly elevated erythrocyte counts (Table 4). This difference is probably due to a lack of variability in the parameter rather than a real elevation and likely does not reflect an effect of TCDD.

Guinea Pigs

The TCDD-treated guinea pigs in the tetanus toxoid-injected group (group A) (*9*) had consistently lower leukocyte counts than the controls, but only for the middle-dose animals (0.04 μg TCDD/kg) was this decrease significant (Table 5). The lymphocyte counts were significantly decreased in all the TCDD-treated animals in this group. A significant dose response occurred for the leukocyte and lymphocyte decreases also, but these effects simply reflect the depression observed at all three TCDD treatment levels.

Table 2. Leukocyte counts in mice given a single oral dose of TCDD.

	Leukocyte count × 10^{-3} per mm³								
	Controls		1 μg/kg		10 μg/kg		50 μg/kg		Dose response [a]
Week	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	
1	12.05	2.58	8.35 [b]	1.72	8.78 [b]	0.74	6.08 [c]	0.90	$P_1 < 0.01$
3	8.07	2.50	6.53	0.65	5.43	0.91	6.10	2.29	$P_1 = 0.052$
5	8.70	3.67	8.23	3.82	7.30	1.49	7.27	3.95	$P_1 = 0.222$

[a] P_1 = one-side P value.
[b] P (one-sided) < 0.05.
[c] P (one-sided) < 0.01.

Table 3. Lymphocyte counts in mice given a single oral dose of TCDD.

	Lymphocyte count per mm³								
	Controls		1 μg/kg		10 μg/kg		50 μg/kg		Dose response [a]
Week	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	
1	9233.0	1551.7	6276.5 [b]	1405.8	6443.5 [b]	1112.9	3752.2 [b]	378.6	$P_1 < 0.01$
3	6170.7	1872.1	5363.0	422.2	3788.3	839.8	4414.3	1764.6	$P_1 = 0.037$
5	6353.3	3261.2	6587.7	3482.6	5024.3	405.7	4864.0	2679.8	$P_1 = 0.182$

[a] P_1 = one-sided P value.
[b] P (one-sided) < 0.01.

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Table 4. Erythrocyte counts × 10^6 in mice given a single oral dose of TCDD.

Week	Erythrocyte count × 10^{-6} per mm³								Dose response
	Controls		1 μg/kg		10 μg/kg		50 μg/kg		
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	
1	7.28	0.320	7.30	0.141	6.90	0.356	7.33	0.479	NS [a]
3	7.63	0.115	7.57	0.306	7.43	0.058	7.33	0.252	NS [a]
5	7.53	0.058	7.73	0.153	7.80 [b]	—	7.83	0.569	$P_2 = 0.045$

[a] NS = not significant.
[b] P_2 = two-sided P value.

Table 5. Leukocyte, lymphocyte, and neutrophil counts in group A guinea pigs treated with TCDD for 8 weeks.

Variable	Count per mm³								Dose response
	Control		0.008 μg/kg-wk		0.04 μg/kg-wk		0.2 μg/kg-wk		
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	
Leukocytes	6407.8	1878.0	5050.0	1161.7	4850.0 [a]	1237.6	4914.3	990.7	$P = 0.022$
Lymphocytes	4159.7	1465.3	2390.9 [b]	726.8	2872.6 [a]	1031.4	2591.7 [a]	459.3	$P = 0.021$
Neutrophils	1885.6	559.2	2286.5	1269.5	1668.7	466.6	2096.7	634.7	NS [c]

[a] $P < 0.05$.
[b] $P < 0.01$.

Table 6. Hematologic parameters for group B guinea pigs treated with TCDD for 8 weeks.

	Control		0.008 μg/kg-wk		0.04 μg/kg-wk		0.2 μg/kg-wk		Dose response
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	
Leukocytes per mm³	8840.0	4205.1	8230.0 [a]	2482.9	7260.0	1659.5	6140.0 [a]	2910.2	$P < 0.01$
Lymphocytes per mm³	4270.5	2293.3	4229.1	1312.0	3717.5	1169.1	3628.2	2371.7	$P = 0.024$
Neutropil per mm³	3517.6	2127.6	3405.9	1204.4	3104.3	654.8	2179.3 [a]	718.7	$P = 0.020$
Platelets × 10^{-3} per mm³	796.500	103.496	—	—	—	—	645.000 [b]	84.755	—
Erythrocytes × 10^{-6} per mm³	4.16	0.28	4.24	0.25	4.18	0.30	4.26	0.25	NS [c]
Hemoglobin, g/100 ml	10.74	0.57	11.00	0.40	11.29	0.57	10.59	0.66	NS
Hematocrit, %	41.70	1.84	42.25	1.77	41.90	2.34	40.45	2.22	NS

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] NS = not significant.

For Group B (*Mycobacterium tuberculosis* tuberculin-treated) (*9*) only the highest dose (0.2 μg TCDD/kg) caused significantly decreased leukocyte counts (Table 6). Neutrophil counts were also reduced in these guinea pigs, but there was no treatment versus control differences in lymphocyte counts. Significant dose-related decreases were found for leukocytes, neutrophils, and lymphocytes. In addition, the guinea pigs treated with 0.2 μg TCDD/kg also had lower platelet counts than the controls ($P < 0.01$).

The leukocyte and lymphocyte counts of the group A and group B control guinea pigs did not differ significantly (Table 7). However, the group B controls did have higher neutrophil counts than the group B controls ($P < 0.05$). When the group A control guinea pigs were compared with the moribund 1.0 μg TCDD/kg guinea pigs, decreased lymphocyte and increased neutro-

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Table 7. Leukocyte counts of tetanus toxoid-treated (group A), *Mycobacterium tuberculosis* tuberculin-treated (group B), and TCDD-treated guinea pigs.

Variable	Group A controls		Group B controls		1.0 μg TCDD/kg-wk	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
cyte count per mm³	6407.8	1878.0	8840.0	4205.1	6800.0	4018.7
nocyte count per mm³	4159.7	1465.3	4270.5	2293.3	1804.0 [a]	522.4
trophil count per mm³	1885.6	559.2	2517.6 [b]	2127.6	4686.4 [a]	3534.7

[a] $P<0.01$.
[b] $P<0.05$.

phil counts were found in the treated animals. However, this comparison may not be l because the TCDD-treated guinea pigs 3 to 5 weeks younger than the group ontrols and were in a moribund state in addition to not having been injected with tetanus toxoid. Hemoconcentration was also present in these dying animals, there being an average erythrocyte count of 5.1×10^6 per mm³ and a hematocrit of 48.9%. Platelet counts were decreased as well.

scussion

The clinical pathologic findings of this d other studies (*3*, *7*, *8*) suggest that the major sites of the toxic action of TCDD and the chlorodibenzo-*p*-dioxins of toxic fat are the hematopoietic system and the liver. Mice and guinea pigs given TCDD and monkeys fed toxic fat (*3*) were leukopenic, which in mice and guinea pigs is characterized by lymphopenia. In mice which received a single dose of TCDD the lymphopenia was reversed 5 weeks after TCDD exposure. Perhaps the lymphopenia seen in mice and guinea pigs is related to the decreased cell-mediated immune response observed in these two species after TCDD exposure (*9*). It is interesting that lymphopenia and cell-mediated immunosuppression were not found in rats treated with TCDD.

Rats and guinea pigs were thrombocytopenic. The cause of this was not determined. Examination of rat bone marrow in one experiment did not reveal any differences in numbers or morphology of megakaryocytes following TCDD treatment (*17*), but in another experiment in rats, spleen and ne marrow megakaryocytes were degenerated and appeared to be reduced in numbers (*15*). Therefore, further work is indicated to determine, perhaps by radioisotope tagging, whether the lowered platelet counts are due to decreased production or increased distruction of platelets. Nevertheless, thrombocytopenia or defects in the clotting mechanism (*17*) might have played a role in the production of hemorrhages that were occasionally seen in rats that died (*15*).

Anemia was not found in these studies, but it was seen in a previous study in monkeys fed toxic fat (*3*). That anemia was accompanied by atrophic bone marrow and normal serum bilirubin suggesting that the anemia was aplastic or depression type rather than hemolytic anemia.

We observed only terminal hemoconcentration in rats and guinea pigs, similar to that previously reported in rats given TCDD (*7*). This is probably an effect of terminal shock and dehydration rather than increased erythrocyte production. Dehydration is further evidenced by the increased serum protein concentrations found in the middle-dose rats. However, hypoproteinemia was observed in the high-dose rats probably as a result of liver damage, and may have contributed further to the hemoconcentration by decreasing the colloidal osmotic pressure of the blood and allowing fluid to accumulate in the tissues.

Liver damage in rats was evidenced by increased SGOT and SGPT activity. The increased SGOT activity might also be due to, in part, myocardial necrosis (*15*). Hyperbilirubinemia, hypercholesterolemia, and hypoproteinemia (in the high-dose rats) are

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probably other effects of the liver pathology. Since increased cholesterol concentrations have previously been seen in rats (*18*) and rabbits (*19*) which have sustained hepatocellular damage from polychlorinated biphenyls, perhaps metabolism of cholesterol is altered in liver damage caused by these chlorinated compounds. Hypoglycemia in the TCDD-treated rats might have been due to decreased food consumption (*16*), but there might also be an effect on the gluconeogenic ability of the damaged liver. Although clinical chemical parameters to assess liver damage were not determined in guinea pigs and mice, histopathologic evidence suggests that hepatocellular damage was minimal in these species (*15*).

It appears that hepatocellular necrosis is the main toxic action of TCDD in rats but with effects on platelets being important. The clinical pathologic changes are consistent with this hypothesis. Ultimately, hemoconcentration due to shock and dehydration occurs as a terminal event in rats as well as guinea pigs.

The lymphopenia observed in mice and guinea pigs are consistent with the immunosuppressive effects of TCDD in these species (*9*). Pathologic studies suggest that liver damage does not play a part in the death of guinea pigs given high doses of TCDD (*15*). However, in guinea pigs, atrophy of the adrenal zona glomerulosa (*9*, *15*) suggests that electrolyte imbalance might occur. Perhaps a study of serum electrolyte and aldosterone concentrations in guinea pigs given lethal doses of TCDD is warranted.

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Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin upon Hemostasis and Hematologic Function in the Rat

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Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) has been identified as a toxic contaminant of chlorophenols and related hydrocarbons that are widely used in agriculture and industry. It has been implicated in various diseases, including human cases of chloracne (*1*) and porphyria cutanea tarda (*2*) and in chick edema (*3*). Its teratogenicity has been documented in rats (*4*) and mice (*5*). Monkeys fed toxic fat, later found to contain TCDD and other chlorinated derivatives of dibenzo-*p*-dioxin, were observed to undergo a decrease in the cellular elements of the bone marrow and the peripheral blood (*6*). In contrast, increases in hematocrit and leukocyte count were observed in rats treated with TCDD (*7*). Depression of blood platelets in TCDD-treated rats and guinea pigs has been recently observed (*8*). The present studies were undertaken in order to characterize hematologic changes and in particular platelet function alterations in rats exposed to TCDD.

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Methods

Animals

Female CD rats, weighing 150–175 g, received daily oral doses of TCDD in an acetone–corn oil mixture at a level of 10 μg/kg. Control rats received an equivalent dose of acetone and corn oil. On days 10 and 14 of treatment animals were anesthetized with methoxyflurane, and blood was withdrawn by cardiac puncture.

Marrow Megakaryocyte Studies

Bone marrow preparations were made as described by Eurenius (*9*), and smears were prepared with a Wright-Leishman-Giemsa stain.

Platelet Function Studies

Bleeding times were determined on anesthetized animals. Lacerations were made on the ear with a blood lancet, and puncture sites were blotted with filter paper at 30-sec intervals until bleeding stopped. Clot retraction was determined as described in the literature (*10*). Platelet factor III activity was evaluated with the prothrombin consumption test (*11*) performed on blood that was allowed to clot with and without added Inositihin. Platelet aggregation was determined macroscopically in a mixture containing, in a final volume of 0.5 ml, 0.1 μmole ADP, 1.0 μmole calcium chloride,

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5.0 μmole Tris HCl, pH 7.35, and platelet-rich plasma with a final platelet concentration of 200,000/mm³.

Prothrombin times were performed as described in the literature (*12*). Factor X assays were performed by measuring clotting time in a mixture containing test serum, 0.1 ml, factor X-deficient plasma (Dade), 0.1 ml, and Simplastin (General Diagnostics), 0.2 ml.

Fibrinogen Degradation Products

Fibrinogen degradation products were assayed in the serum of control and treated rats by using the Wellcome FDP Kit (Burroughs-Wellcome), which employs a hemagglutination-inhibition system.

Other hematologic parameters were determined by routine methods.

Statistical analysis was performed by the Student's *t* test.

Results

Red cell parameters of control and treated rats are given in Table 1. Packed cell volumes were elevated in TCDD-treated rats at days 10 and 14 of treatment. Similarly, erythrocyte counts were increased in the treatment as compared to control groups at days 10 and 14. Reticulocyte counts differed significantly at day 14, presumably representing lowered values in the control animals. Mean corpuscular volume and mean corpuscular hemoglobin were significantly depressed in treatment groups at day 10 but not day 14. TCDD-treated rats did not differ significantly from controls with regard to either hemoglobin or mean corpuscular hemoglobin concentration.

White cell parameters of control and treated rats are given in Table 2. Total leukocyte counts were elevated, but not significantly, in TCDD-treated animals at 10 and 14 days. Neutrophil counts were significantly increased in both treatment groups when compared to controls. Lymphocyte and monocyte counts were elevated in rats treated 10 and 14 days, and eosinophil counts were slightly depressed. These differences, however, were not statistically significant.

Table 3 demonstrates that platelet counts were significantly lowered in treated rats at days 10 and 14. Bone marrow preparations did not show a corresponding decrease in megakaryocyte numbers of animals treated 14 days. Megakaryocyte size and numbers of nuclei per megakaryocyte were also evaluated, and these were observed to be equivalent in control and treatment groups.

Bleeding times were equivalent in treated and control rats at day 14. These results are presented in Table 4. Clot retraction was diminished in both 10-day and 14-day treatment groups compared to controls. The velocity of platelet aggregation was not significantly altered in TCDD-treated rats.

Table 1. Red cell parameters in rats administered daily oral doses of TCDD of 10 μg/kg.[a]

	10 Days		14 Days	
	Control	Treated	Control	Treated
Hemoglobin, g/100 ml	12.3 ± 0.7	13.5 ± 0.8	12.6 ± 1.2	14.8 ± 1.4
Packed cell volume, %	37.4 ± 2.3	43.4 ± 3.7*	36.6 ± 2.3	45.0 ± 4.0*
Erythrocyte count × 10^{-6}, per mm³	5.0 ± 0.5	6.7 ± 0.4**	5.2 ± 0.3	6.2 ± 0.7*
Reticulocyte count, %	4.2 ± 1.2	4.1 ± 1.0	1.5 ± 0.6	3.3 ± 1.2*
Mean corpuscular volume, μ³	75.8 ± 4.1	65.0 ± 7.0*	71.1 ± 1.8	73.0 ± 2.2
Mean corpuscular Hb concentration, %.	32.8 ± 1.4	31.2 ± 1.7	34.5 ± 1.3	32.9 ± 1.4
Mean corpuscular Hb, pg/cell	24.9 ± 1.2	20.2 ± 1.1**	24.5 ± 0.9	24.3 ± 1.6

[a] Values presented are means ± standard deviation. Four animals were tested in each group. Values marked with asterisks differ significantly from control values: *$P < 0.05$; **$P < 0.01$.

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Table 2. White cell parameters in rats administered daily oral doses of TCDD of 10 μg/kg.[a]

	10 Days		14 Days	
	Control	Treated	Control	Treated
·yte count × 10^{-3} per	4.6 ± 1.5	9.4 ± 4.2	5.1 ± 2.6	9.2 ± 2.1
ıphil count × 10^{-3} per	0.5 ± 0.1	0.9 ± 0.3*	0.6 ± 0.3	2.7 ± 1.5*
·mphocyte count × 10^{-3} per mm³	3.9 ± 1.4	8.1 ± 3.9	4.3 ± 2.4	6.1 ± 1.3
·ocyte count per mm³	137 ± 92	254 ± 182	89 ± 50	243 ± 135
·nophil count per mm³	61 ± 31	21 ± 36	77 ± 39	65 ± 55

[a] alues presented are means ± standard deviation. Four animals were tested in each group. Values rked with an asterisk differ significantly from control values at a level of $P < 0.05$.

Table 3. Effect of daily oral doses of TCDD of 10 μg/kg upon marrow megakaryocytes and blood platelets in rats.[a]

	10 Days		14 Days	
	Control	Treated	Control	Treated
·clet count × 10^{-3} er mm³	911 ± 100	427 ± 225*	688 ± 58	388 ± 124*
·nkaryocytes per 10^4 ·ucleated marrow cells	—	—	444 ± 75	615 ± 219

[a] Values presented are means ± standard deviation. Four animals were tested in each group. Values marked with an asterisk differ significantly from control values at a level of $P < 0.01$.

However, prothrombin consumption tests revealed markedly prolonged times, both with and without Inosithin, in rats from both treatment groups. This contrasted with equivalent prothrombin times observed in control and treated animals. No difference in factor X levels between control and treated rats was observed at 14 days.

Serum fibrinogen degradation products were not observed in either control or treatment groups at days 10 and 14. Four animals were tested in each group.

Discussion

The elevations in packed cell volumes and erythrocyte counts of TCDD-treated rats reported in the present study are consistent with dehydration and consequent hemoconcentration. These results are in agreement with earlier investigations of TCDD (*7*). Presumably, the previously reported depression of hematopoiesis in monkeys fed toxic fat (*6*) represents species variation or the effect of a different toxic contaminant.

The alterations in red cell indices and the leukocytosis with neutrophilia, lymphocytosis and eosinopenia that have been observed in this study are nonspecific hematologic changes consistent with widespread toxicity of TCDD. Of interest is the observed selective depression of blood platelets in treated rats. In view of the finding of normal marrow megakaryocytes, decreased production of platelets in treated rats is unlikely. It is possible that TCDD results in disseminated intravascular coagulation with thrombocytopenia due to the aggregation and incorporation of platelets into platelet-fibrin microthrombi. However, the absence of serum fibrinogen degradation products in treated animals argues against this possibility. Increased peripheral des-

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Table 4. Platelet function and procoagulant activity in rats administered daily oral doses of TCDD of 10 μg/kg.*

	10 Days		14 Days	
	Control	Treated	Control	Treated
Bleeding time, min	—	—	3.6 ± 0.9	4.1 ± 2.7
Clot retraction, %	73.6 ± 5.8 (7)	60.8 ± 4.3** (6)	81.2 ± 6.0	69.8 ± 4.3*
Velocity of platelet aggregation, sec	18.0 ± 1.4 (2)	26.0 ± 8.5 (2)	23.0 ± 6.4 (3)	51.2 ± 40.0
Prothrombin time, sec	11.8 ± 0.5	11.7 ± 1.9	11.7 ± 1.8	13.5 ± 3.1
Prothrombin consumption test, sec	22.8 ± 6.7	58.5 ± 1.7***	18.9 ± 3.6	53.0 ± 8.2***
Prothrombin consumption test with Inosithin, sec	26.2 ± 9.3	60.0 ± 0.0***	19.8 ± 6.8	51.9 ± 16.2*
Factor X, sec	—	—	19.0 ± 2.0 (2)	21.0 ± 0.0 (2)

* Values presented are means ± standard deviation. Numbers in parentheses indicate number of animals tested; in all other groups four animals were tested.
Values marked with asterisks differ significantly from control values: *P <0.02; **P <0.01; ***P <0.001.

truction of platelets due to an antibody response is a possible mechanism for the observed thrombocytopenia. Such a mechanism is proposed in human cases of hypersensitivity to quinidine and Sedormid (*13*).

The diminished clot retraction in TCDD-treated animals is consistent with the observed thrombocytopenia. Otherwise, platelet function, as assessed by bleeding time and velocity of aggregation, was not altered. Similarly, decreased platelet factor III activity, which would be reflected in a shortening of the prothrombin consumption time that was corrected by the addition of inosithin, was not observed in treated rats. On the contrary, prothrombin consumption times were consistently prolonged in the treatment groups. In view of the normal prothrombin times, this prolongation of prothrombin consumption times is not readily explainable. Factor X levels were normal in treated animals. Deficiency or inactivation of factor VII, which has been reported in human cases of liver and renal failure and after exposure to propylthiouracil, salicylates and indanedione drugs (*14*), is a possibility. Prolongation of the prothrombin consumption time in factor VII-deficient dogs has been reported (*15*), but this is accompanied by prolonged prothrombin times. Similarly, deficiency or inactivation of prothrombin might prolong the prothrombin consumption test, but again, an abnormal prothrombin time would be expected. It remains to perform factor VII assays and other coagulation studies in animals exposed to TCDD.

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Pathologic Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Laboratory Animals

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Introduction

The technical chlorophenols and products produced from them, such as the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) have been found to contain chlorodibenzo-*p*-dioxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TC-DD). TCDD and other chlorodibenzo-*p*-dioxins are among the most toxic compounds known, and have been implicated in outbreaks of chloracne among chemical workers (*1*). Hepatic and cardiac lesions and thymic involution were found in rats treated with TCDD (*2*). Generalized subcutaneous edema, ascites, hydrothorax, and hydropericardium were observed in monkeys given toxic fat (*3*) which purportedly contained TCDD. It also caused focal necrosis of parenchymal cells of liver and gastric ulcers in these monkeys. The purpose of this paper is to describe the sequence of pathologic changes which occurred following exposure to TCDD in rats, guinea pigs, and mice.

Materials and Methods

Young adult male and female random bred albino rats (CD stock, Charles River Breeding Laboratories, Wilmington, Massachusetts) were used. They were housed individually under conditions of controlled temperature (68–72°F) and 12 hr daily lighting. The diet consisted of commercially available sterilized diet. Water was provided *ad libitum*. Young, short-haired female albino guinea pigs (Hartley strain) and female mice (CD-1) were also used to study the toxic effect of TCDD. These animals were also housed under optimal controlled conditions.

TCDD (Dow Chemical Company, Midland, Michigan) was dissolved in acetone and diluted with corn oil. All animals (rats, guinea pigs and mice) were given TCDD by gastric intubation. Control animals were treated similarly with acetone and corn oil only. Dose range and frequency of treatments were as follows:

(a) rats treated daily with 10.0, 1.0, or 0.1 μg TCDD/kg body weight and examined after 3, 6, 10, 13, 17, 24, and 31 treatments; (b) rats given a single treatment with 100.0 or 50 μg TCDD/kg body weight and examined when the rats became moribund or dead; (c) rats given a single treatment with 25.0 or 5.0 μg TCDD/kg body weight and examined 1, 3, 9, 10, 17, and 29 days after treatment; (d) rats given 6 weekly treatments with 5.0, 1.0, or 0.2 μg TCDD/kg body weight and examined 10 days after the last treatment; (e) guinea pigs given 8 weekly

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treatments with 1.0, 0.2, 0.04, or 0.008 μg TCDD/kg body weight and examined 56 days after the initiation of the treatment; (f) guinea pigs given a single treatment with 3.0 μg TCDD/kg body weight and examined either after death or moribund condition; (g) mice given a single dose with 50.0, 10.0, or 1.0 μg TCDD/kg body weight and examined 7, 21, and 35 days after treatment. All animals received the scheduled treatment unless death intervened.

Necropsies were performed on euthanized animals and those which became moribund or died before the scheduled kill day. Tissue samples from liver, spleen, kidney, urinary bladder, heart, lung, thymus, trachea, brain, spinal cord, salivary glands, axillary and mesenteric lymph nodes, tongue, esophagus, stomach, small and large intestines, pancreas, striated muscle from hind legs and diaphragm, bone marrow from femur, tibia, sternum, and ribs, adrenal, thyroid, uterus, ovary or testes, and skin were collected from most of the animals and fixed in 10% buffered neutral formalin for histopathologic evaluation. All tissue specimens were paraffin-embedded, sectioned 6 μ thick and stained with Harris' hematoxylin and eosin Y (H & E). Liver, kidney, bone, and total body of dead or moribund rats and guinea pigs were grossly examined under the ultraviolet light (Wood's lamp) for the presence of red fluorescence as an indication of porphyrin accumulation.

Results

Gross Pathologic Findings

Most of the rats given TCDD at a level of 10.0 μg/kg became moribund or died between 17 and 31 daily treatments. Six of 14 rats given a single dose at a level of 100.0 μg/kg also died 18 to 21 days after treatment (4). These rats had ruffled hair and appeared depressed. They usually sat in a corner of the cage without much movement. Gross pathologic changes observed in dead or moribund rats given these doses were similar and will be described together (Table 1). The ears, subcutaneous tissues and visceral organs appeared icteric. Occasionally, there were subcutaneous hemorrhages in tail, paws, and under the nails; some had a loss of hair from the ventral surface of the body. There was a loss of subcutaneous and abdominal fat in TCDD-treated rats. The size of the uterus in these rats appeared to be smaller. The liver was friable and dark tan in color. The liver and spleen sizes were small. There were ulcerations and hemorrhages in the stomach which also contained blood clots (Fig. 1). Congestion of meningeal vessels and submeningeal hemorrhages were also observed (Fig. 1). All thymuses were markedly atrophied.

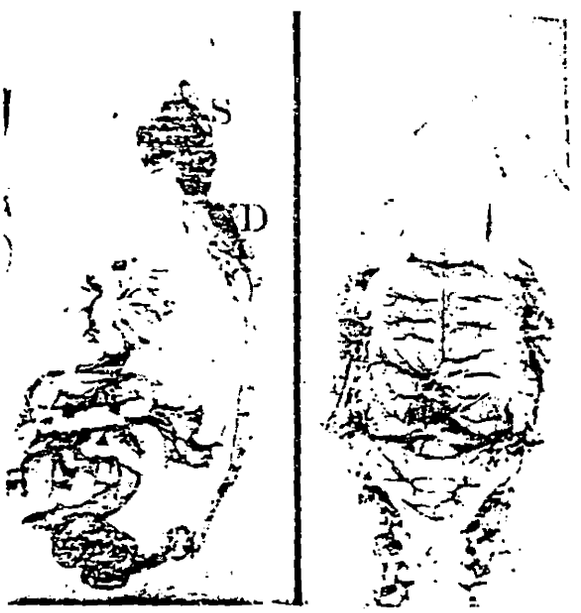


FIGURE 1. Gastrointestinal tract and brain of a rat given 26 daily treatments of 10.0 μg TCDD/kg body weight. Notice congestion and hemmorrhage in the brain (arrow), stomach (S) and duodenum (D).

The livers from rats killed after 10 to 17 daily intubations at 10.0 μg/kg were dull gray (cooked appearance), swollen, and enlarged. There was accentuation of lobular markings (Fig. 2). The small intestine and mesenteric lymph nodes appeared slightly congested and the intestinal contents were more mucinous than that of control rats. Thymic atrophy (Fig. 2), which was dose-dependent, occurred in all rats examined. Significant gross pathologic changes were

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FIGURE 2. Rats (left) given 10 daily treatments of 0.0 μg TCDD/kg body weight and (right) control. Notice the accentuation of lobular markings in the liver (L) and thymic atrophy (arrow).

not observed in other organs of rats given 25.0 or 5.0 μg TCDD/kg single dose, 1.0 or 0.1 μg/kg multiple daily doses, and 5.0, 1.0, or 0.2 μg/kg weekly doses.

All guinea pigs given weekly treatment at a level of 1.0 μg/kg became moribund or died between 24 and 32 days after the initiation of the treatment (Table 1). Nine out of ten guinea pigs given a single dose of 3.0 μg TCDD/kg body weight died between 15 and 28 days after treatment. There was a severe body weight loss, decreased amount of subcutaneous and visceral adipose tissues, and the guinea pigs appeared to be dehydrated. Hemorrhages were also observed in the adrenal gland, urinary bladder, gastrointestinal tract and mesenteric lymph nodes. There was marked atrophy of the thymus. Other than the relative thymic atrophy, remarkable gross tissue changes were not observed in guinea pigs given weekly doses of 0.2, 0.04 or 0.008 μg TCDD/kg.

Red fluorescence under the ultraviolet light indicating the presence of excess amounts of porphyrins was not observed grossly in any organ of the rats and guinea pigs examined.

Table 1. Summary of Pathologic changes in rats, guinea pigs, and mice given TCDD orally.

Species	Doses, μg TCDD/kg body weight (no. of treatments)	Mortality, %	Period of death, days	Pathologic changes		
				Thymic atrophy	Liver damage	Other major changes
Rat	100 (single)	43	18–21	Severe	Severe	Icterus, hemorrhage depletion of lymphoid organs
Rat	50 (single)	7	11	Severe	Severe	None
Rat	25 (single)	0	—	Slight/ moderate	None	None
Rat	5.0 (6 weekly)	0	—	Moderate	Slight	None
Rat	10.0 (16–31 daily)	94	15–31	Severe	Severe	Icterus, hemorrhage depletion of lymphoid organs
Rat	1.0 (31 daily)	0	—	Moderate	Slight/ moderate	Degenerative changes in kidney and thyroid glands
Guinea pig	3.0 (single)	90	15–28	Severe	Slight	Hemorrhage atrophy of adrenal zona glomerulosa, depletion of lymphoid organs
Guinea pig	1.0 (4–5 weekly)	100	24–32	Severe	Slight	Same as above, and hyperplasia of urinary bladder epithelium
Guinea pig	0.2 (8 weekly)	0	—	Slight/ moderate	None	None
Mouse	50.0 (single)	0	—	Slight	Slight	None

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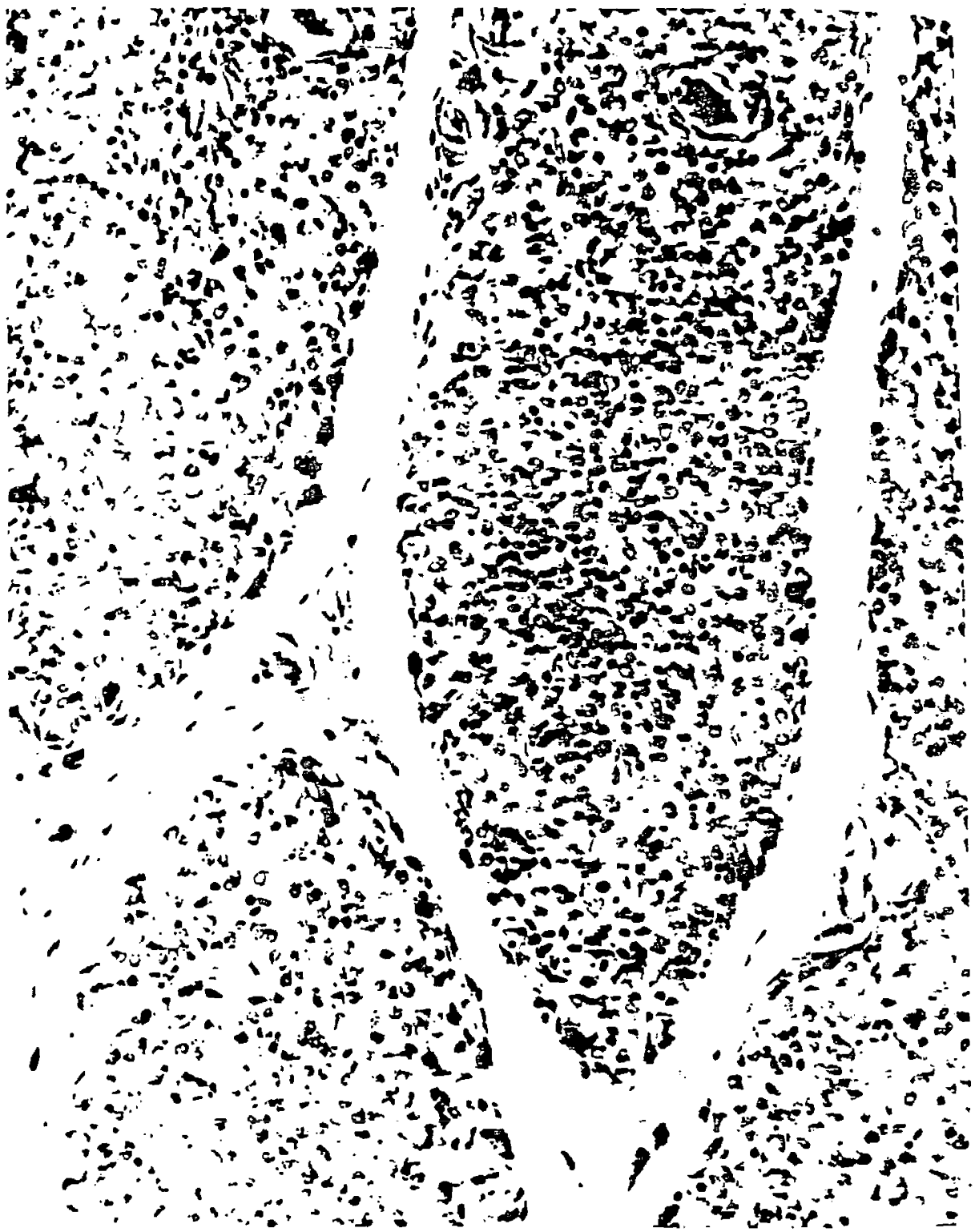


FIGURE 3. Thymus of a rat treated with 10.0 μg TCDD/kg daily for 16 days. Notice the marked decrease in the number of cortical thymocytes and loss of demarcation between the cortex and medulla. H&E stain; magnification 307×.

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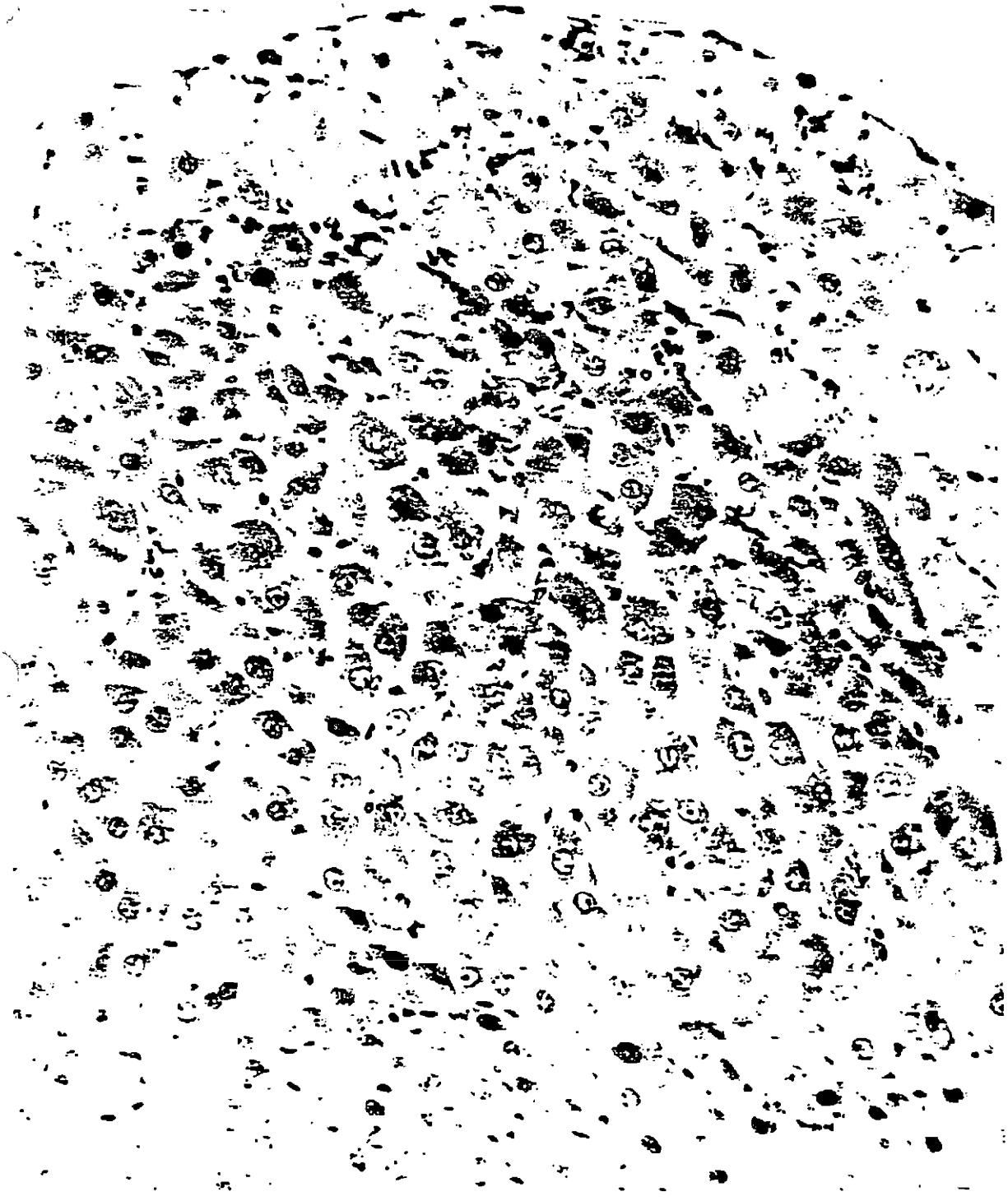


FIGURE 4. Liver of a rat given 31 daily treatments of 10.0 μg TCDD/kg body weight. Regenerated hepatocytes are disorganized with moderate to marked degenerative changes. H&E stain; magnification 320×.

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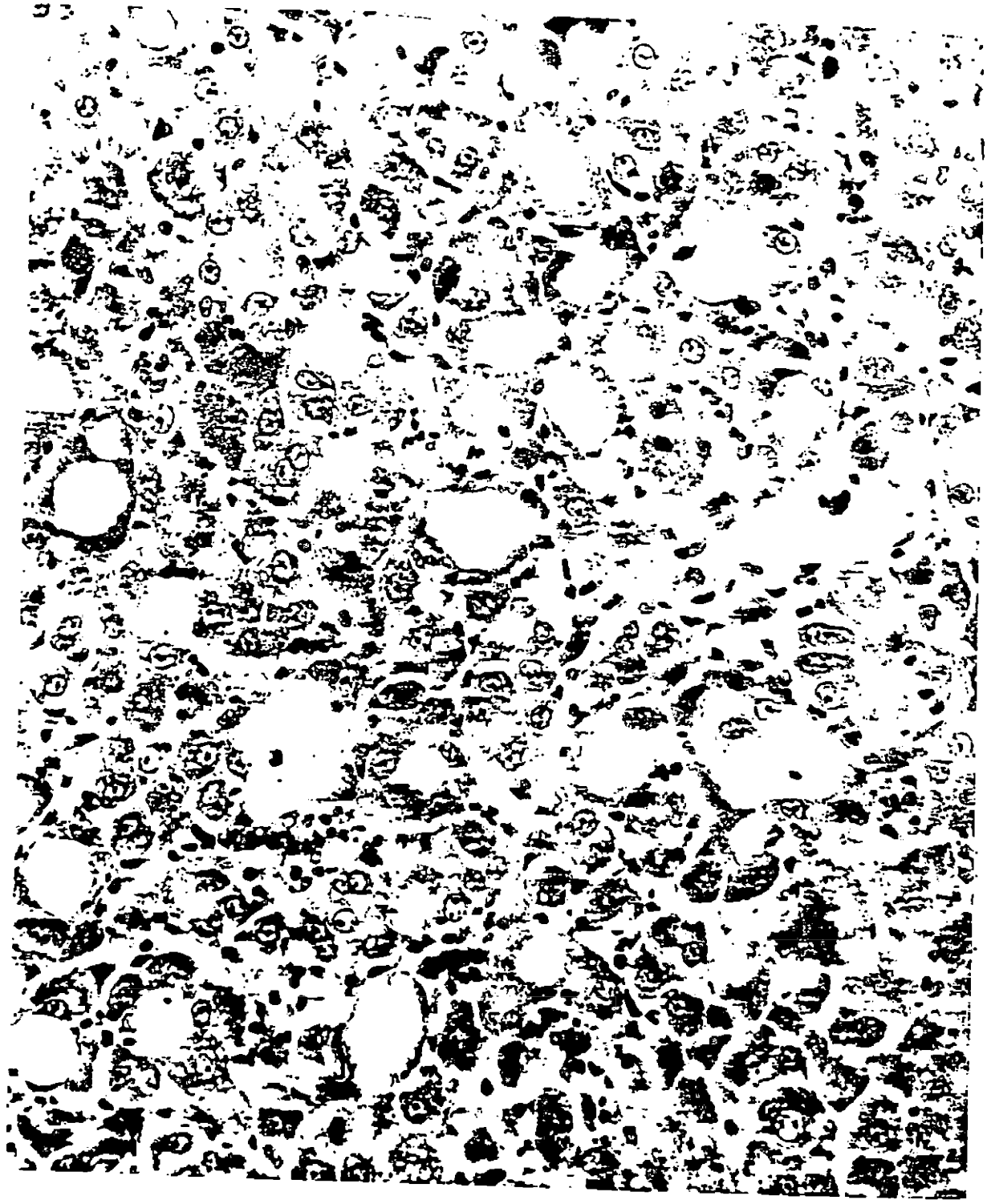


FIGURE 5. Liver of a rat given 31 daily treatments of 10.0 μg TCDD/kg body weight. Notice necrosis and tubule-like structures in the parenchymatous tissues of liver. H&E stain; magnification 320×.

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URE 6. Heart of a rat given 18 daily treatments of 10.0 μg TCDD/kg body weight, showing massive hemrhages (H) separating the myocardial fibers (arrows). H&E stain; magnification 320×.

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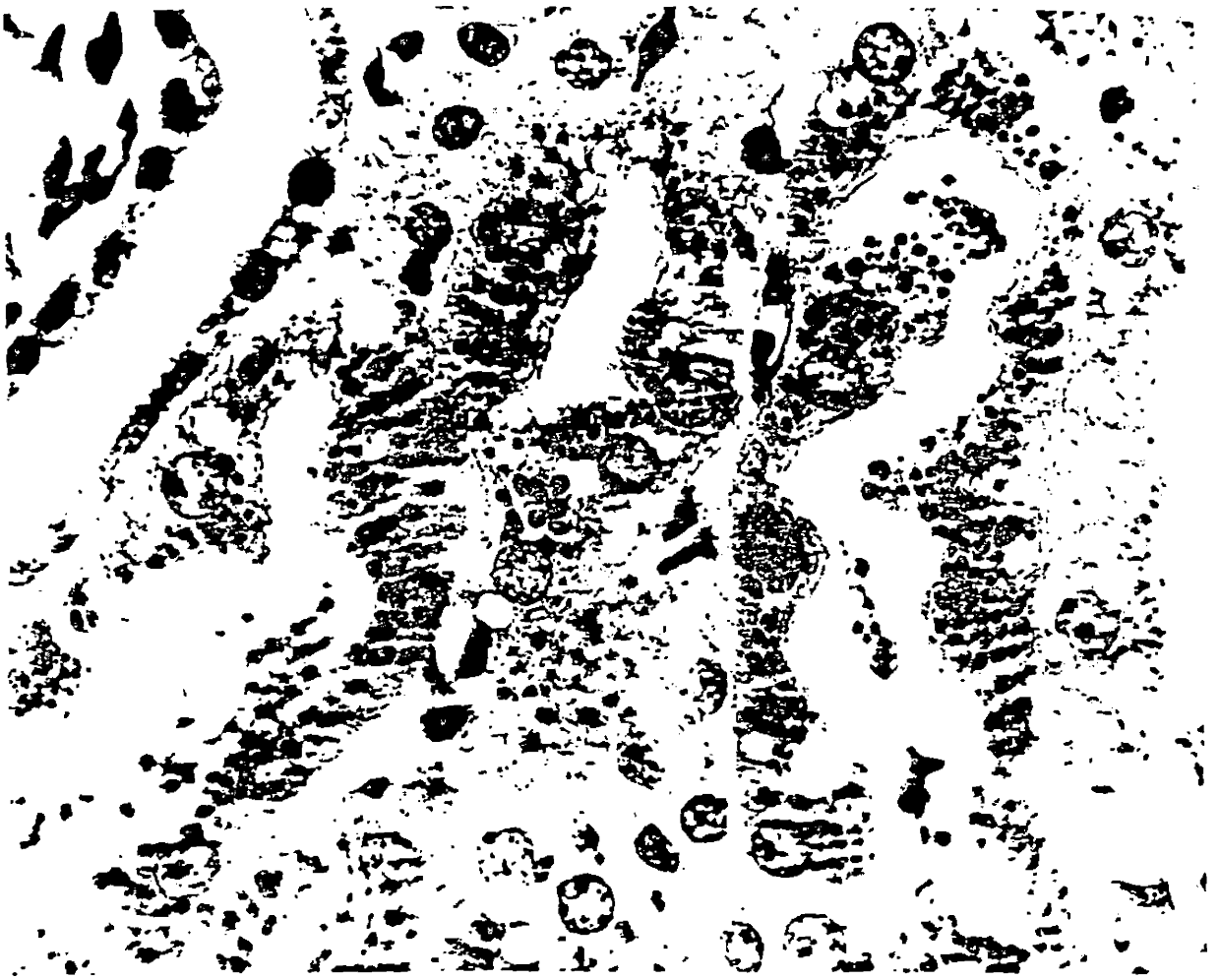


FIGURE 7. Kidney of a rat given 18 daily treatments of 10.0 μg TCDD/kg body weight. Notice hyaline droplets, and foamy and vacuolated cytoplasm in the renal tubules. H&E stain; 904×.

Histopathologic Findings

Rats—In those rats which became moribund or died, remarkable changes were consistently observed in the thymus, liver, spleen and lymph nodes (Table 1). Changes in the ovary, uterus, gastrointestinal tract, heart, and brain were frequently observed. Marked atrophy of thymus was denoted by decrease in the number of cortical thymocytes (Fig. 3). The thymic lobules were markedly smaller, and there was no demarcation between the cortex and medulla. There was also a relative depletion of lymphoid cells in the spleen and lymph nodes. Pyknosis of the nuclei and degenerative changes in the multinucleated megakaryocytic type giant cells of spleen and bone marrow were observed more frequently in treated rats. There was moderate to marked distortion of the architecture of liver parenchyma with marked necrosis of hepatocytes. The hepatocytes were round and large, and the hepatic cords were disorganized (Fig. 4). Some of the necrotic hepatocytes had tubulelike structures (Fig. 5). Rats which died or were killed when moribund had massive hemorrhages in the heart (Fig. 6), liver, brain, adrenal gland, and gastrointestinal tract. Occasionally, organized thrombi were observed in the heart.

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FIGURE 8. Liver of a rat given 6 weekly treatments of 5.0 μg TCDD/kg body weight. Notice multinucleated giant hepatocytes. H&E stain; magnification 500×.

brain, and lungs. There were necrosis and ulceration in the glandular part of the stomach. Atretic changes of the ovarian follicles were observed more frequently in treated than in control rats. There was also atrophy of mucosal folds and glandular structures of the uterus. The epithelial cells of renal tubules were foamy and vacuolated and contained numerous hyaline droplets (Fig. 7). This change was found in 3 out of 16 rats treated with 10.0 μg TCDD/kg body weight given daily (Table 1).

Rats given sublethal doses of TCDD had regeneration of hepatic parenchyma denoted by megalocytosis and multinucleated hepatocytes (Fig. 8). There were increased mitoses in the parencymatous tissue of liver. Some liver contained unusually large hepatocytes with seven to ten nuclei. Occasionally, a part of the cytoplasm of some hepatocytes appeared more eosinophilic and homogeneous while others contained eosinophilic hyaline bodies (Fig. 9). Swelling of hepatocytes, granular appearance of the cytoplasm, aggregation and condensation of cytoplasmic contents either around the nucleus or periphery of the cells, fatty infiltration, and vacuoles of different sizes and shapes were also observed in less affected livers.

Pyknosis and degenerative changes were observed in the connecting and collecting renal tubules of rats given multiple daily dosage during the early and middle of the study (Fig. 10). Moderate to marked degenerative changes were also observed in the epithelial cells of thyroid follicles. In addition, the epithelial lining was disorganized, exfoliated, or with papillary projections in the lumen of the follicle (Fig. 11). The follicular colloid was either absent or appeared foamy and thin. There were congestion and elongation of intestinal villi, and the goblet cells appeared to be increased only during the first half of the study.

Significant microscopic changes were not observed in different organs of rats given TCDD at levels of 0.1 μg/kg (multiple daily doses), 5.0 μg/kg (single dose), or 1.0 and 0.2 μg·kg (multiple weekly doses).

Guinea pigs—Microscopic changes in moribund or dead guinea pigs were characterized by a severe atrophy of the cortex of thymus with destruction of lymphocytes (Table 1). Hassall bodies were large and cystic and filled with polymorphonuclear leukocytes. There was lymphoid cell depletion in spleen and lymph nodes. Hemorrhage

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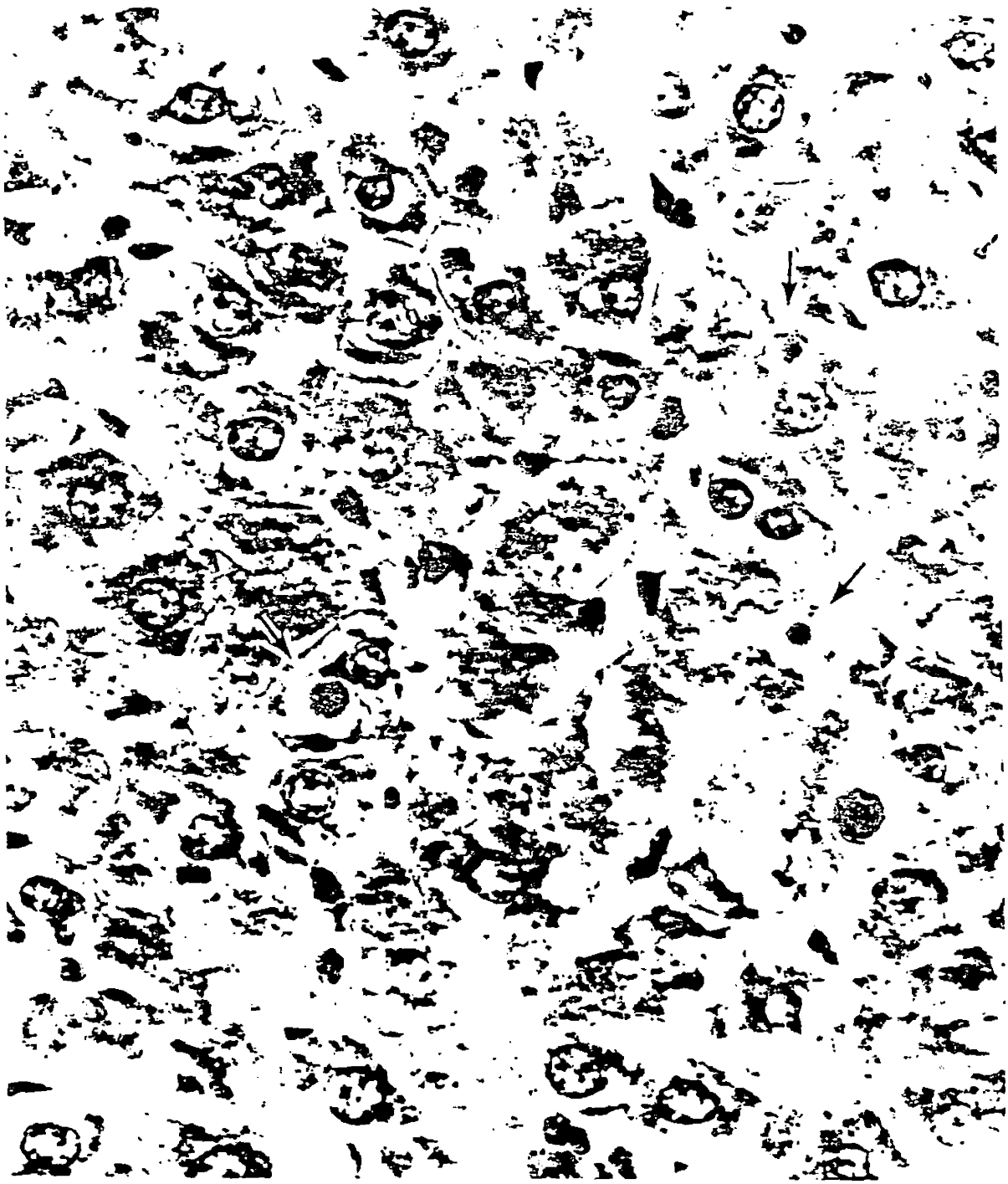


FIGURE 9. Liver of a rat given 10 daily treatments of 10.0 μg TCDD/ kg body weight. Notice the intracytoplasmic hyaline bodies (arrows). H&E stain; magnification 950×.

in the adrenal medulla extended into the zona reticularis and sometimes into the zona fasciculata. Atrophy of zona glomerulosa, and mitotic figures and loss of lipid vacuoles were observed in zona fasciculata (Fig. 12). Liver effects were confined to a diffuse single cell necrosis of hepatocytes, predominantly in the periportal area. There

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GURE 10. Degenerative changes and pyknosis of epithelial cells of renal tubules of a rat given 6 daily reatments of 10.0 μg TCDD/kg body weight. H&E stain; magnification 731×.

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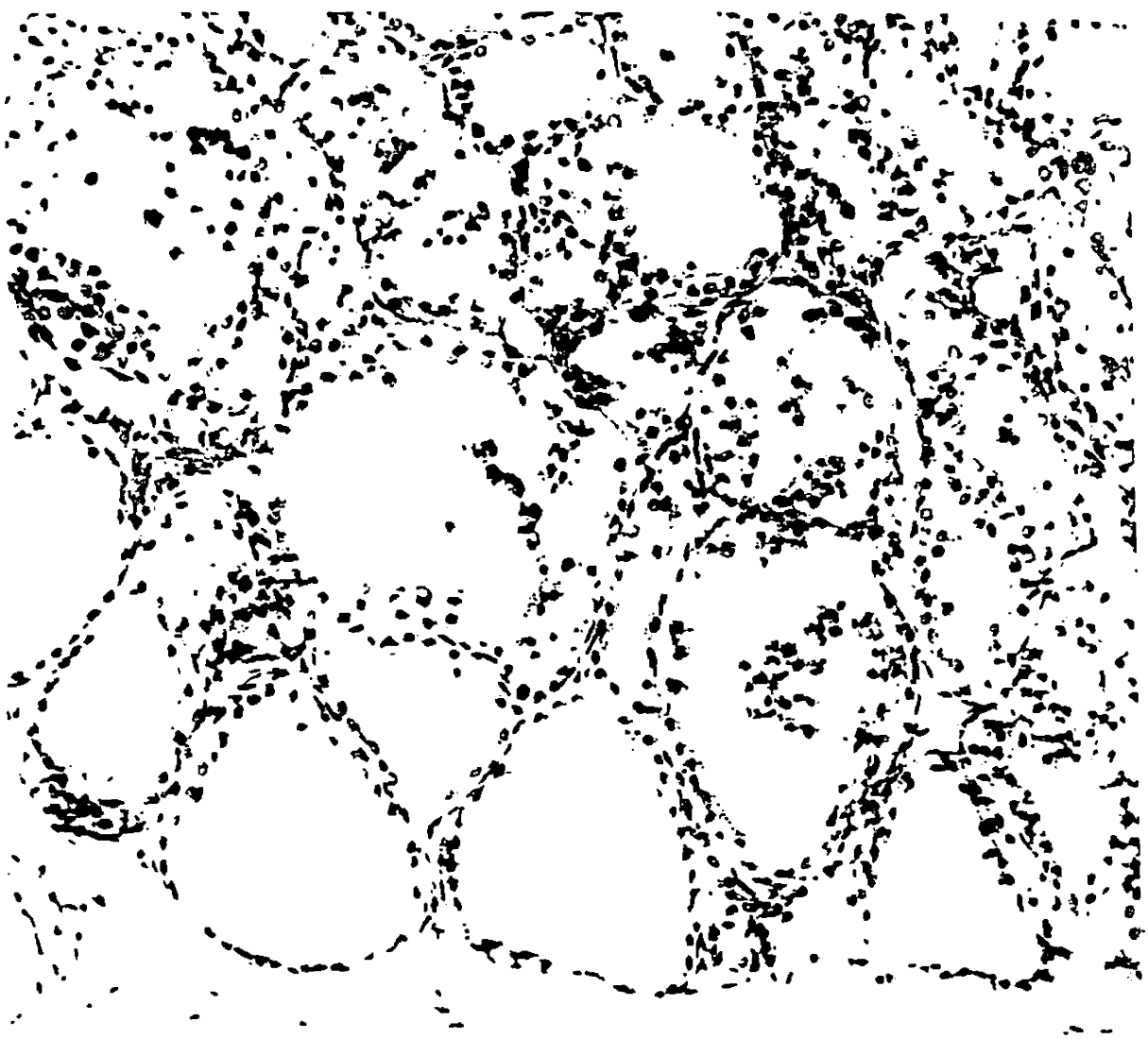


FIGURE 11. Pyknosis of nuclei, exfoliated epithelial cells and absence of colloid from the thyroid follicles of a TCDD-treated rat (10 daily treatments, 10.0 μg TCDD/kg body weight). H&E stain; magnification 243×.

were diffuse hemorrhages in the urinary bladder, and subserosal hemorrhages in the gastrointestinal tract. Blood pigment, probably hemosiderin, was observed in the medulla of cervical lymph nodes and lamina propria of the cecum. The transitional epithelium of urinary bladder appeared markedly hyperplastic, and the submucosal layer was edematous (Fig. 13). Occasionally, there were focal necrosis of smooth muscle cells and infiltration of inflammatory cells in the urinary bladder.

There was slight to moderate atrophy of thymic cortex of guinea pigs given weekly doses of TCDD of 0.2 μg/kg.

Mice—There was some hepatocellular swelling and a relative reduction of lymphoid cells in the thymus of mice 21 days after receiving a single 50 μg/kg dose of

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FIGURE 12. Atrophy of zona glomerulosa (G) and mitoses (arrows) in the zona fasciculata of adrenal gland of a guinea pig given 4 weekly doses of 1.0 μg TCDD/kg body weight. H&E stain; magnification 176×.

TCDD. However, microscopic changes were variable from one animal to another. No changes were observed in these tissues in mice necropsied 7 or 35 days after TCDD exposure. No effects were seen in mice given lower doses.

Discussion

The main target organs of TCDD appear to be the liver of rats and the thymus of rats, guinea pigs, and mice. TCDD caused thymic atrophy in all three species. Liver lesions severe enough to account for death were seen only in rats. The liver lesions seen in guinea pigs and mice were mild by comparison. Other lesions such as hemorrhages, degenerative changes in kidney, thyroid, megakaryocytes in spleen, ovarian follicles, lymphoid depletion of the spleen and lymph nodes in rats, and hemorrhages, hyperplasia of the urinary bladder mucosa, and atrophy of the adrenal zona glomerulosa in guinea pigs were also seen.

The liver lesions in rats dying after TCDD exposure were strikingly similar. This hepatotoxic reaction was characterized by such degenerative changes as swelling of hepatocytes, fatty metamorphosis and ultimately necrosis in rats which received 10 μg TCDD/kg-day for 10–13 days. At this time there was also an increase in serum transaminases activities (*5*). Thereafter, the hepatic lesions progressed and the weight of the liver decreased as more parenchymatous tissue was destroyed. Hyperbilirubinemia and hypoproteinemia (*5*) became inadequate and death soon followed in the icteric animals. Besides these degenerative lesions, large multinucleated giant hepatocytes were also seen in liver of TCDD treated rats. The presence of these cells, increased numbers of mitotic figures and pleomorphism of cord cells suggest that a long term study should be done to assess the possibility of the development of hyperplastic nodules and/or neoplasm. Increases in liver weight (*4*) can be explained by swelling of hepatocytes, fatty metamorphosis, proliferation of smooth and rough endoplasmic reticulum as shown by electron microscopy (*6*), concomitant with induction of hepatic microsomal enzymes (*7*).

Although a marked difference in the amount of TCDD needed to produce thymic atrophy occurred between species, the severity of the lesion was dose-dependent within a species (*4*). It was also the organ most sensitive to TCDD treatment (*4*). In both guinea pigs and mice, the cell-mediated immune response was depressed; lymphopenia also occurred. In contrast, cell-mediated immunity was not suppressed and lymphopenia

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FIGURE 13. Urinary bladder of a guinea pig treated with 4 weekly doses of 1.0 μg TCDD/kg body weight: (*A*) Notice marked hyperplasia of epithelial cells, congestion, hemorrhage, and edema; (*B*) infiltration of inflammatory cells into the submucosal and muscular layers. H&E stain; magnification 214×.

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l not occur in the rat (*8*).

The only other lesion seen with some consistency was hemorrhage of various organs in dead or dying rats and guinea pigs. A possible cause of these hemorrhages might be the thrombocytopenia seen in these two species (*5*). The thrombocytopenia might be due to the reduction in number of and necrosis of megakaryocytes seen in the spleen and bone marrow of rats. However, in another study, megakaryocyte numbers and morphology were not altered by TCDD treatment (*9*). The hemorrhages might be caused by platelet deficiency or abnormalities in the clotting mechanism (*9*).

Porphyria was not seen in rats and guinea pigs dying from TCDD which agreees with the finding that TCDD did not increase hepatic δ-aminolevulinic acid synthetase activities in rats (*10*). However, a marked increased activity of this enzyme was demonstrated in chick embryo liver (*11*). Therefore, it appears that species differences to the porphyrogenic action of TCDD exist.

Summary

Gross pathologic and histopathologic examinations were performed on rats, guinea pigs and mice treated with TCDD. In rats and guinea pigs, dose ranged from no effect to one which produced death; frequency of dose ranged from a single intubation to daily or weekly administration for several weeks. Lymphoid organs, primarily thymus, were consistently affected over a wide spectrum of dose ranges in all species examined. Atrophy of the thymus, as denoted by a dose-related decrease in weight and decrease in cortical thymocytes, was a very sensitive index of TCDD exposure. Relative depletion of lymphoid cells in spleen and lymph nodes was also observed.

The liver was the only other organ in which microscopic changes were observed consistently. The degree of hepatic involvement seemed dose-dependent, but severity of changes produced was quite variable between species. The most severe hepatic effects were seen in rats which received a lethal dose of TCDD. These rats, which were jaundiced, had diffuse degenerative and necrotic changes in the liver. At sublethal dose levels, transient degenerative changes in the liver were followed by megalocytosis, regeneration and unusual numbers of multinucleated giant hepatocytes. Other changes found inconsistently were hemorrhage in the gastrointestinal tract, heart, and brain as well as organized thrombi in various organs. Moderate to marked degenerative and necrotic changes were also observed in the epithelial cells of renal connecting and collecting tubules and thyroid follicles.

Although degenerative and necrotic changes in liver were also observed in guinea pigs and mice, the magnitude of these effects were markedly diminished. To illustrate this point, hepatic changes produced at lethal TCDD levels in the rat were severe enough to be a contributing cause of death, while hepatic changes in the guinea pig receiving a lethal dose were quite mild.

Acknowledgement

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Ultrastructural Changes in Rat Liver Cells Following a Single Oral Dose of TCDD

by Bruce A. Fowler,* George W. Lucier,* Hayes W. Brown,* and O.S. McDaniel*

Ultrastructural alterations of liver parenchymal cells involving the endoplasmic reticulum are known to occur in animals exposed to chlorinated diphenyl-*p*-dioxins in the diet (*1–4*). The observed changes are similar to those reported for other aromatic chlorinated hydrocarbons (*5–8*) and are usually associated with induction of hepatic microsomal enzyme systems (*7, 9, 10*).

The present study was undertaken to correlate ultrastructural changes in rat liver cells with biochemical studies on liver microsomes and mitochondria for a period of 28 days following a single oral dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

Materials and Methods

Ninety male Charles River rats were separated into three groups of 30 each. Animals in the first group received a single oral dose by gavage of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in 0.5 ml of acetone and corn oil at a dose of 5 μg/kg while the second group was given a dose of 25 μg/kg. The third group served as controls. Five animals from each group were killed by decapitation at 1, 3, 6, 9, 16, and 28 days after treatment.

Blocks of liver tissue were fixed in buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for light microscopy. Other blocks of liver were placed in a glutaraldehyde-formaldehyde fixative described previously (*11*), embedded in Epon, and sectioned with diamond knives. These sections were double-stained with lead citrate and uranyl acetate prior to examination in a Philips EM 300.

Results

Histologic sections of liver from all treated animals were indistinguishable from controls. Liver parenchymal cells of all animals killed on the first day after treatment exhibited ultrastructural architecture similar to controls (Fig. 1). By the third day, liver cells of treated animals contained increased amounts of smooth endoplasmic reticulum (SER) which were prominent around the periphery of cells particularly in areas adjacent to bile canaliculi (Fig. 2). More SER was observed in rats given the higher dose of TCDD. Some cells of treated animals also appeared to contain more rough endoplasmic reticulum (RER) than controls.

Large aggregates of SER and massive amounts of RER (Fig. 3) were observed in the liver cells of treated animals killed on days 6 and 9. This observation was most readily appreciated in rats given the

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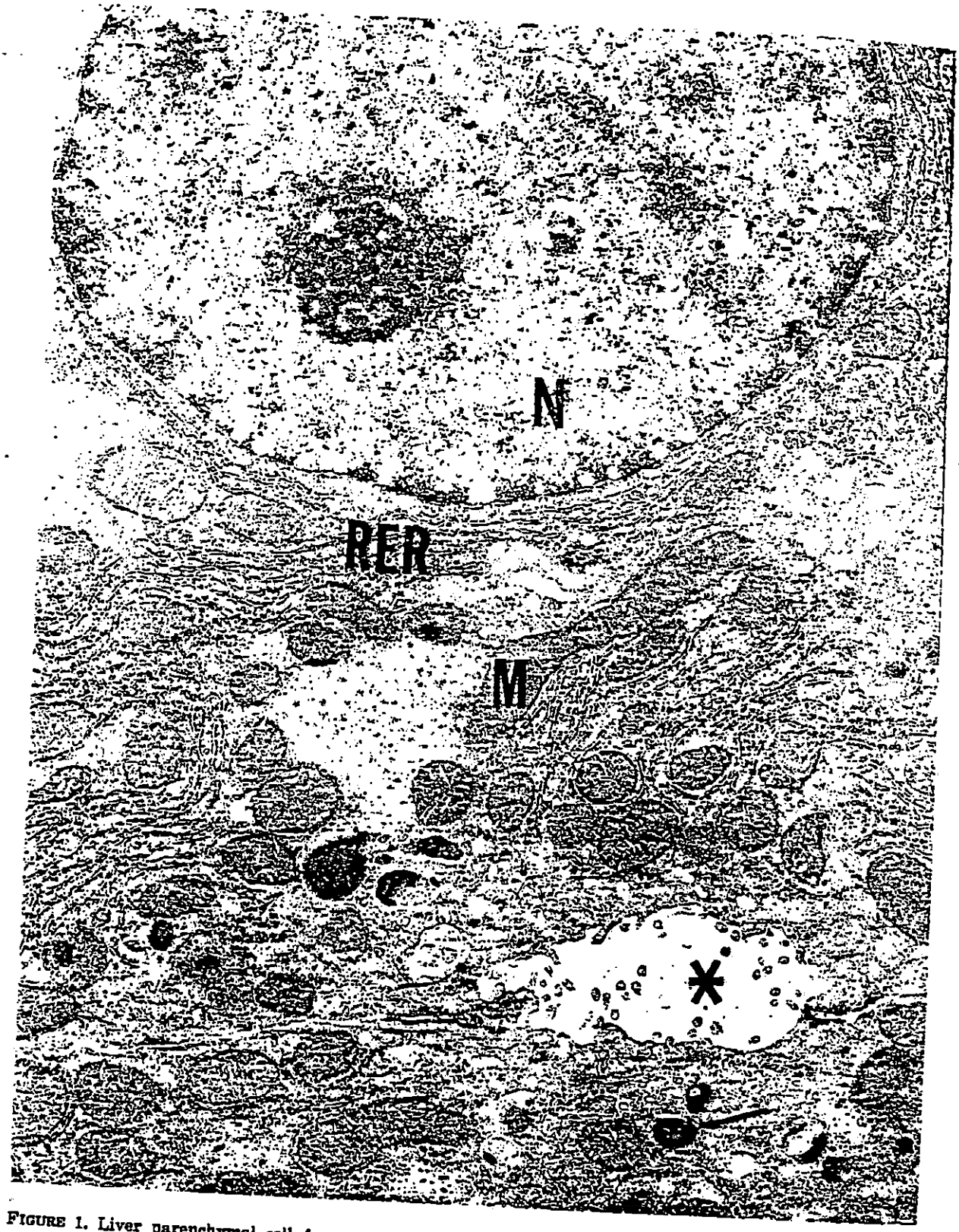


FIGURE 1. Liver parenchymal cell from an animal killed 1 day after dioxin treatment displaying usual architecture. Nucleus (N) mitochondria (M), rough endoplasmic reticulum (RER), cytosomes (arrow) and bile canaliculus (*) are present in the cytoplasm. 15,102×.

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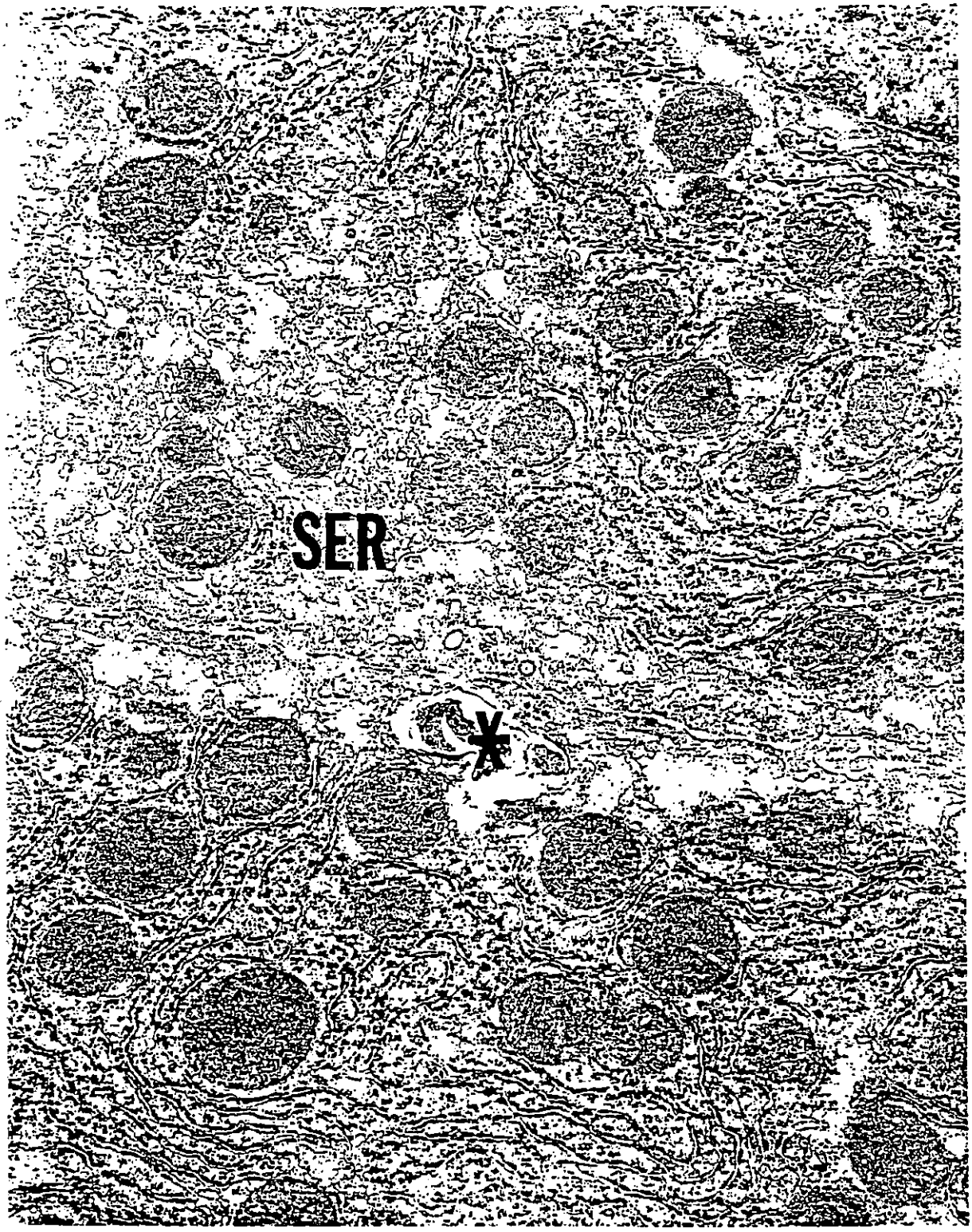


FIGURE 2. Liver cell from a rat 3 days after exposure to dioxin. Increased amounts of smooth endoplasmic reticulum (SER) are present around a bile canaliculus (*). The RER also seems to be more prominent. 26,520×.

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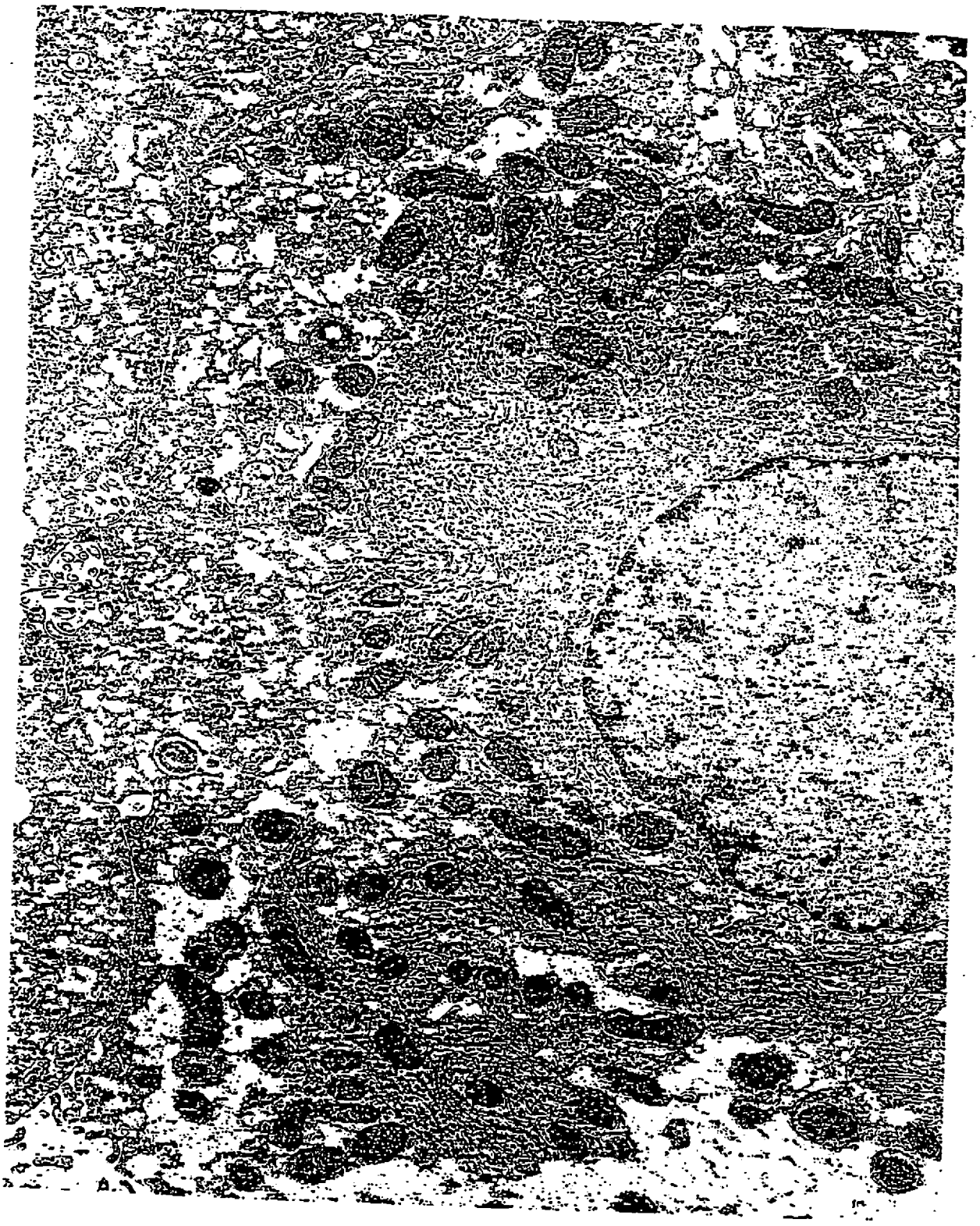


FIGURE 3. Cell from a rat killed 6 days after administration of dioxin. Large amounts of SER are seen around the cellular periphery and massive amounts of RER surround the nucleus. 11,080×.

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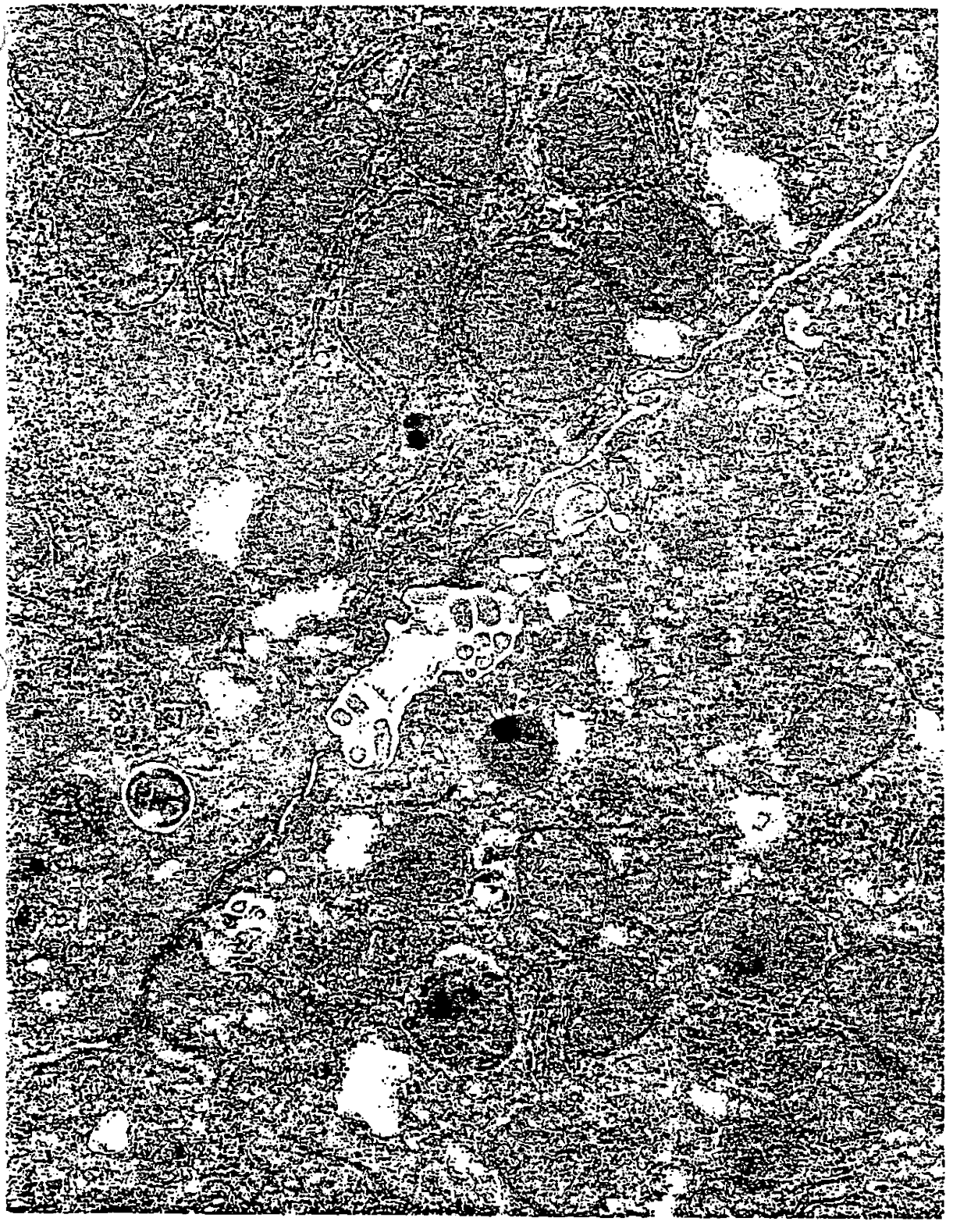


FIGURE 4. Parenchymal cell from a rat 16 days after treatment. Large amounts of RER are still prominent in the cytoplasm but the SER is greatly diminished. 20,520×.

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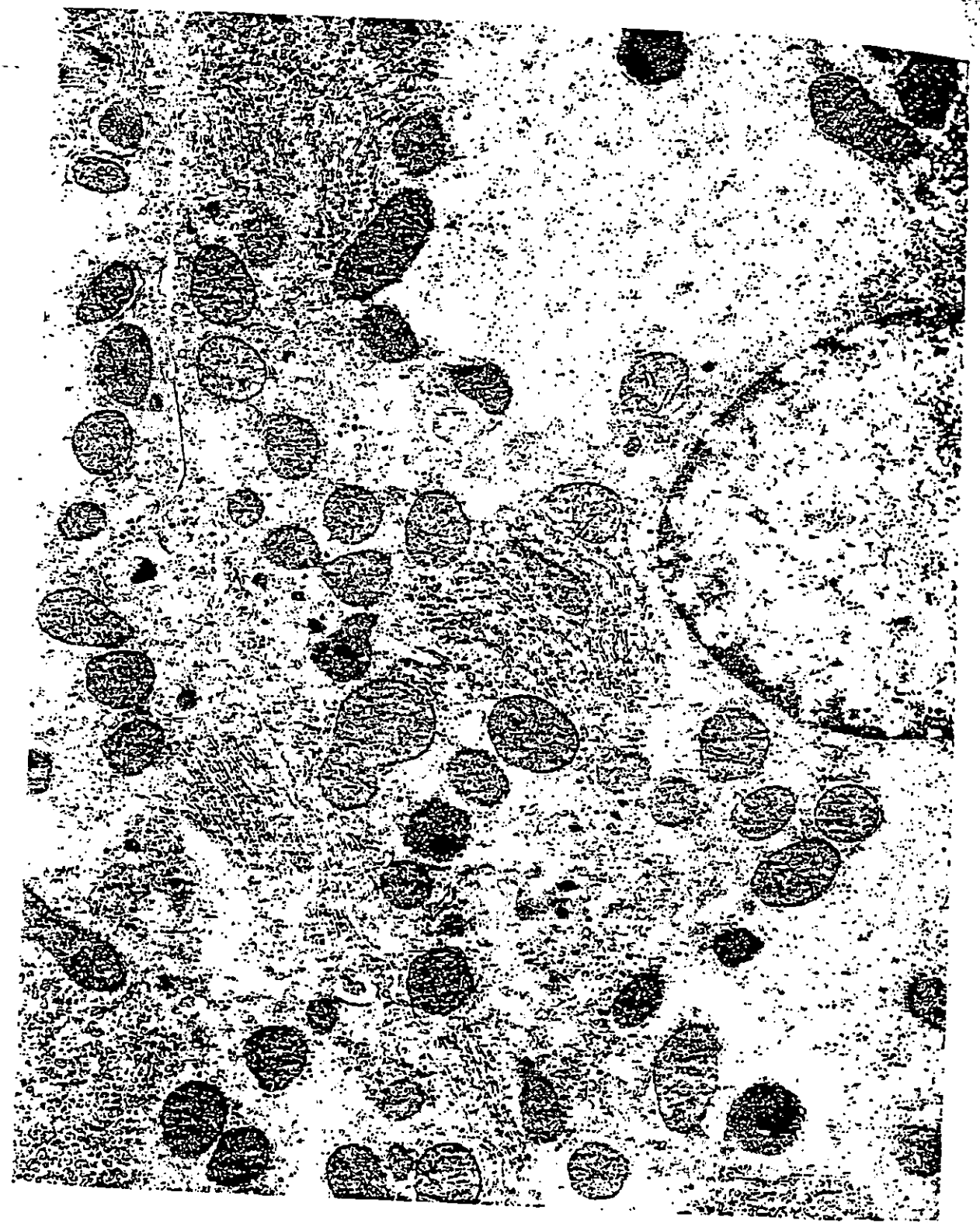


FIGURE 5. Liver cells of a rat killed 28 days after treatment containing levels of endoplasmic reticulum comparable to controls. 1510×.

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er dose of TCDD. Parenchymal cells of rats killed on day 16 contained little SER but large amounts of RER were still evident in many cells (Fig. 4). On the day 28, most liver cells of treated animals were indistinguishable from controls (Figure 5). Membranous concentric whorls of endoplasmic reticulum described in other studies (3) were never observed in liver cells of any treated animals during the course of the experiment.

Discussion

Proliferation of SER in liver cells of rats given TCDD confirms previous reports by other investigators (4). The increase of SER in parenchymal cells of treated animals used in this study was closely correlated with induction of microsomal enzyme activity in livers of these same animals (12). Similar observations have been made for a wide variety of other aromatic chlorinated compounds (7, 9, 10) and are indicative of a cellular attempt at detoxification.

Early proliferation of SER around bile canaliculi of liver cells from treated animals could suggest involvement of bile in the metabolism or excretion of TCDD. This idea is supported by the fact that increased bile secretion was noted (13) in other animals used in this study and rats given labeled octachloro dioxin excreted most of the radioactivity in the feces (14).

Alterations in RER associated with the formation of membranous concentric whorls of endoplasmic reticulum (3, 4) have been noted in livers of animals exposed to chlorinated diphenyl-*p*-dioxins in the diet. In the present study, proliferation of RER reached a maximum at day 6 following administration of TCDD and was correlated with increased RER as determined by microsomal subfractionation (12). These observations would suggest a concomitant stimulation of both RNA and protein synthesis within liver cells of treated animals. The significance and mechanism of this are unclear, but they might be related to increased synthesis of microsomal proteins (3).

The lack of membranous concentric whorl formation in liver cells of TCDD-treated rats used in this study may suggest that chronic exposure and accumulation of TCDD or its metabolites within liver cells is necessary for the phenomenon to occur. Another possibility is that other dioxins or aromatic chlorinated compounds rather than TCDD were responsible for the membranous whorls observed in earlier studies. Investigation utilizing different doses, other purified dioxin compounds, and dioxin tissue analyses may be necessary to resolve this question.

In conclusion, a single low-level dose of TCDD has been found to exert a profound effect on both the SER and RER of rat liver parenchymal cells. At present, the nature of these changes appears to be related to an induction phenomenon and changes in cellular RNA and protein metabolism. Many other studies are needed to examine the mechanisms responsible for these alterations in normal cellular function.

Acknowledgement

We wish to thank Mrs. Patricia Parker for her excellent technical assistance in preparing sections for electron microscopy.

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Effect of 2,3,7,8-Tetrachlorodibenzo-p-dioxin on the Immune System of Laboratory Animals

by J.G. Vos,* J.A. Moore,* and J.G. Zinkl*

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the most toxic compounds known. TCDD and other chlorinated dibenzodioxins have been associated with occupational chloracne in workers engaged in the manufacture of technical chlorophenols and their derivatives such as the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (*1*), in lethal liver necrosis in rabbits (*2*), and in the chick edema disease (*3*). More recently TCDD has been shown to be highly teratogenic (*4*).

Laboratory studies have found TCDD to cause severe atrophy of the thymus at sublethal dose levels (*5, 6*). Because the thymus is the central lymphoid organ for cell-mediated immunity and other environmental chemicals have produced immunosuppression (*7*), studies to test the immune response in TCDD-treated laboratory animals were conducted. Methods selected to assess cell-mediated immunity were delayed type hypersensitivity to tuberculin in guinea pigs and rats, and a local graft versus host reaction in mice. The effect of TCDD on the humoral immunity was also studied in guinea pigs.

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Materials and Methods

Studies on the Effects of TCDD on Humoral and Cell-Mediated Immunity in Guinea Pigs

In each of two experiments, groups of 10 female Hartley strain guinea pigs received 8 weekly doses of 0, 0.008, 0.04, 0.2, or 1.0 μg TCDD/kg body weight. The TCDD in an acetone–corn oil mixture was administered orally in a volume of 1 ml/kg body weight. The animals (mean weight 256 g, ranging between 208 and 301) were housed in groups of five and allowed free access to food and water. Body weights were determined weekly. The guinea pigs were killed with carbon dioxide gas. Heart blood was used for determination of total leukocyte (using a Coulter Counter, Model B) and differential leukocyte counts. The pathologic effects of TCDD, except for the effects on lymphoid organs and skin (second experiment) are reported elsewhere (*6, 8, 9*).

In one experiment, the effect of TCDD on humoral immunity was determined by measuring guinea pig response to a subcutaneous injection of tetanus toxoid. The purogenated toxoid (Lederle Laboratories, Pearl River, New York) was administered in a volume of 0.1 ml into the right hind foot pad at day 28 (1 Lf. tetanus toxoid, aluminum phosphate-adsorbed) and again at day 42 (1 Lf. tetanus toxoid, unadsorbed). Blood was collected (*10*) on days 35 and 49,

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and from the heart at the end of the experiment. The serum tetanus-antitoxin concentrations were determined by using a modified single radial immunodiffusion technique (*11*); the agar contained appropriate dilutions of tetanus toxoid and the serum (10 μl) was allowed to diffuse from the well.

The effect of TCDD on cell-mediated immunity was studied in a second experiment by measuring the delayed-type hypersensitivity to tuberculin. Guinea pigs were sensitized by a subcutaneous injection (0.05 ml) of an oil suspension containing killed *Mycobacterium tuberculosis* $H_{37}Ra$ (complete H_{37} Ra adjuvant) (Difco Laboratories, Detroit, Michigan) in the hind foot pad at day 35. The delayed hypersensitivity was tested by intradermal tuberculin injections [1.25 μg tuberculin PPD (Parke-Davis, Detroit, Michigan) in 0.1 ml diluent] on days 47 and 54. The diameter (mm) of the skin reactions was determined 24 and 48 hr after the tuberculinations. In addition, the thickness of the reaction was determined by measuring the thickness of the tuberculin reaction and of the normal skin. Subtraction of the latter from the former value gave the thickness of the tuberculin reaction. To evaluate a possible indirect immunosuppressive effect by adrenocortical hyperfunction, pooled serum samples were analyzed for cortisol and corticosteron levels by using the double isotope derivative method (*12*) (New England Nuclear, Boston, Massachusetts).

TCDD Effect on Cell-Mediated Immunity in Rats

Groups of 10 random bred female albino rats (CD stock, mean weight 185 g, ranging from 165 to 201) were given oral doses, weekly for 6 weeks, of 0, 0.2, 1.0, or 5.0 μg TCDD/kg body weight. A skin test, similar to that in guinea pigs, was performed by injecting 0.05 ml oil suspension of killed *Mycobacterium tuberculosis* in the right hind foot pad at day 28 and by injecting 5 μg tuberculin PPD in 0.1 ml diluent into the skin of the shaved flank at day 42. The diameter and thickness of the reactions were measured after 24 and 48 hr. Half of the animals of each group were euthanatized and necropsied at day 45. The weight of lymphoid organs and adrenals was determined as were total and differential leukocyte counts. Tissues, including the skin at the site of the tuberculin injection, were processed for histology. Liver pathology is published elsewhere (*8*).

Effect of TCDD on Cell-Mediated Immunity in Mice.

In this experiment, the graft versus host activity of donor (C57B1/6) spleen cells was measured by injecting them into the right hind foot pad of hybrid recipient mice, $B_6D_2F_1$, obtained by mating the parental strains C57B1/6 and DBA-2 which differ from each other at the major histocompatibility [H-2] locus. In this situation, donor cells are tolerated by the hybrid because the graft does not possess any antigens which are foreign to the host, but the donor cells react against DBA-2 antigen of host cells. The weight of the enlarged injected lymph node, which is due to the immunological reaction, compared with the weight of the uninjected left popliteal nodes (right/left ratio) was the parameter used for the graft versus host activity of the donor spleen cells (J. A. M. Kerckhaert, personal communication, 1972).

Groups of five to seven male, 2-month-old donor mice (housed individually, mean weight 24.4 g, ranging between 22.0 and 27.2 g) were orally dosed weekly with 0, 0.2, 1.0, 5.0, or 25 μg TCDD/kg body weight (in a volume of 0.1 ml per 20 g body weight) for 4 weeks. After 4 weeks, the animals were killed with carbon dioxide gas. Thymuses were weighed and processed for histology. Cell suspensions were made from the pooled spleens of each donor group. Spleens were minced with scissors and the fragments were gently pressed through a nylon gauze (200 μ pore diameter). The cells were washed with a MEM (Eagle) suspension culture medium (Flow Laboratories, Rockville, Maryland) containing 100 units of penicillin and 100 μg streptomycin per milliliter. Cell suspensions were counted with a Coulter Counter. Viability was determined with eny-

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hrosin B (0.4% in PBS). Final cell suspensions contained 2×10^8 viable nucleated cells ml medium. These donor cells (0.05 ml, 1×10^7 viable cells) were injected subcutaneously by using a 0.25 ml syringe with a 25-gauge needle into the right hind foot pad of 10–12-week-old male recipient animals. The recipient mice were killed after 7 days. Right (injected) and left (uninjected) popliteal lymph nodes were removed, trimmed free from adipose tissue, and weighed to an accuracy of 0.05 mg. The right/left ratio was determined. In a preliminary study, 10 control lymph nodes weighed 0.495 ± 0.185 mg (SD) and the lymph node weights of 10 recipients injected with 5×10^6 viable

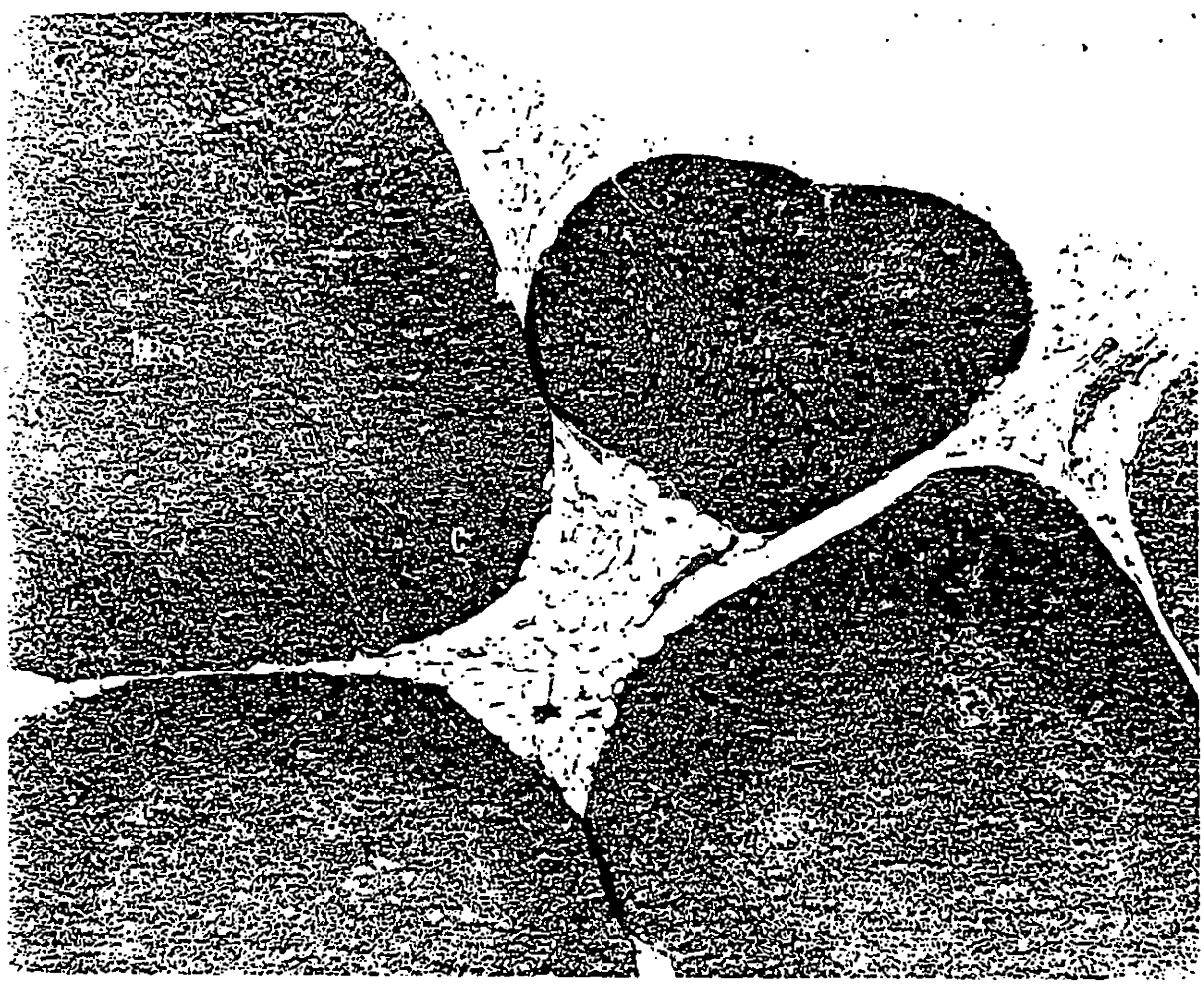


FIGURE 1. Thymus of a control guinea pig from the skin test experiment. Note the densely packed lymphocytes in the cortex (C) and the Hassall bodies (arrow) in the medulla (M). Hematoxylin and eosin; 61 ×.

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syngeneic F_1 hybrid cells were 0.495 mg ± 0.136, thus demonstrating that lymph node enlargement after 7 days is not due to a drainage of cells but to a graft versus host reaction. Recipients injected with C57-B1/6 cells showed a massive proliferation of lymphoid cells on microscopic examination of the lymph nodes.

The data obtained in the four experiments are presented as mean values and standard deviations. Dunnett's multiple comparisons test (*13*) was used to make treatment con-

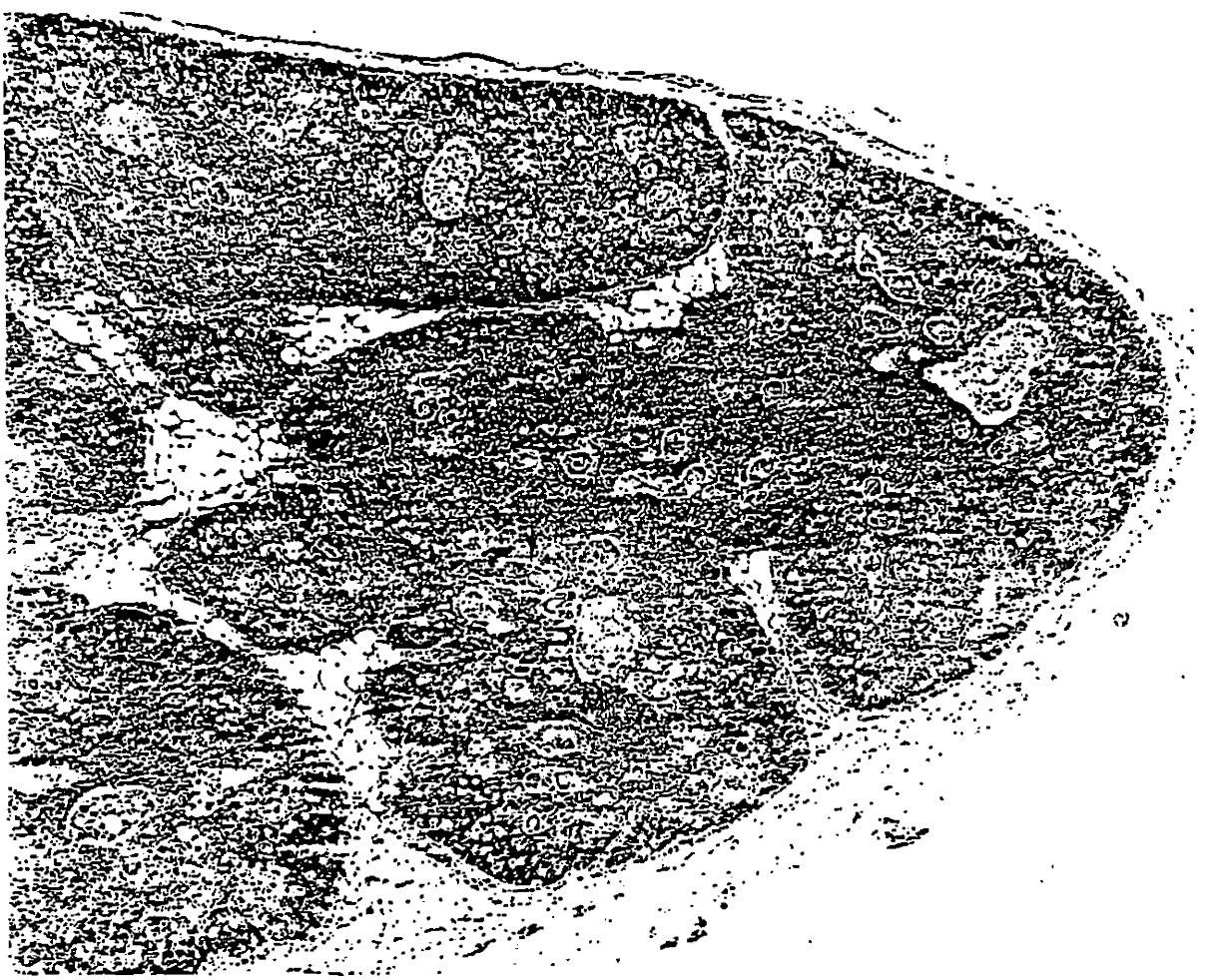


FIGURE 2. Thymus of guinea pig that was killed when moribund on day 27 after receiving four weekly oral doses of 1 μg TCDD/kg. A severe cortex atrophy (C) can be seen, with destruction of lymphocytes that are phagocytized by macrophages ("starry-sky" appearance). Large cystic Hassall bodies (arrow) filled with polymorphonuclear leukocytes are present in the medulla (M). Hematoxylin and eosin; ca. ×.

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trol comparisons, usually two-sided, except for skin reaction variables (one-sided). In addition, a nonparametric test (Jonckheere's test) (*14*) was used to test for monotonic dose–response relationships. In the case of organ weights, only the organ/body weight ratios were tested.

Results

Experiments with Guinea Pigs

All guinea pigs treated with TCDD at the 1 μg/kg level died or were killed when moribund between 24 and 32 days (mean 28 days). They showed severe weight loss (*6*), lymphopenia (9), and depletion of the lymphoid organs, especially the thymus (*8*). Microscopically (Figs. 1 and 2), there was a severe atrophy of the thymic cortex with considerable destruction of lymphocytes, the nuclear debris being engulfed by macrophages ("starry sky"). Large cystic Hassall bodies, filled with polymorphonuclear leukocytes, were seen in the medulla.

All animals at the 0, 0.008, 0.04, and 0.2 μg/kg levels survived in both experiments. Body weights, organ weights and leukocyte counts are given in Tables 1 and 2. Weight gain was significantly lower in both 0.2 μg/kg groups. Absolute thymus weight was significantly reduced at the 0.04 (experiment 1) and 0.2 μg/kg (experiments 1 and 2) dose levels, but the relative (organ to body weight ratio) weights only at the 0.2 μg/kg levels. The absolute weights of the superficial cervical lymph nodes (experiment 1) were significantly decreased in the 0.2 μg/kg group. At this level, the relative adrenal weights were significantly increased in both studies. Total leukocyte values were significantly decreased in the 0.04 μg/kg group of experiment 1 and in the 0.2 μg/kg group of ex-

Table 1. Body weights, organ weights, and leukocyte counts of guinea pigs treated with TCDD for 8 weeks (tetanus toxid stimulation).[a]

	Weekly TCDD dose				Dose-response test
	0	0.008 μg/kg	0.04 μg/kg	0.2 μg/kg	
Final body weight, g	580.2 ± 34.3	597.2 ± 37.5	551.9 ± 48.9	497.1 ± 37.8 [b]	$P < 0.01$
Organ weights, mg					
Thymus	901 ± 246	741 ± 163	672 ± 161 [c]	476 ± 70 [b]	
Spleen	1036 ± 280	858 ± 144	870 ± 174	793 ± 133	
Cervical lymph nodes	224 ± 35	202 ± 33	199 ± 38	179 ± 32 [c]	
Right popliteal lymph node	33.4 ± 13.7	36.8 ± 11.8	38.9 ± 12.4	30.5 ± 12.4	
Adrenals	294 ± 33	294 ± 42	302 ± 38	285 ± 36	
Organ/body weight ratio × 10^3					
Thymus	1.54 ± 0.39	1.24 ± 0.27	1.21 ± 0.24	0.96 ± 0.14 [b]	$P < 0.01$
Spleen	1.79 ± 0.50	1.44 ± 0.22	1.58 ± 0.30	1.59 ± 0.23	NS
Cervical lymph nodes	0.386 ± 0.055	0.338 ± 0.053	0.361 ± 0.064	0.362 ± 0.073	NS
Right popliteal lymph node	0.0584 ± 0.0257	0.0612 ± 0.0182	0.0704 ± 0.0201	0.0613 ± 0.0268	NS
Adrenals	0.507 ± 0.047	0.490 ± 0.049	0.548 ± 0.060	0.595 ± 0.064 [c]	$P < 0.01$
Total leukocytes × 10^3 per mm^3	6.41 ± 1.88	5.05 ± 1.16	4.85 ± 1.24 [c]	4.91 ± 0.99	$P < 0.05$
Lymphocytes × 10^3 per mm^3	4.16 ± 1.47	2.39 ± 0.73 [b]	2.87 ± 1.03 [c]	2.59 ± 0.46 [c]	$P < 0.05$

[a] Mean values ± SD, 10 animals per group except at the 0.2 μg/kg level (7 animals).
[b] $P < 0.01$.
[c] $P < 0.05$.

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periment 2. Significantly decreased lymphocyte counts were found in experiment 1 at all 3 dose levels. Significant monotonic dose–response relationships were determined for body weights (decrease), relative thymus weights (decrease), relative adrenal weights (increase), and total leukocyte and lymphocyte counts (decrease). Serum cortisol and corticosteron values that were measured only in experiment 2 (Table 2) were the same in all four groups. At microscopic examination of the lymphoid organs and adrenals, no effects were seen except for slight cortical atrophy of the thymus at the 0.2 μg/kg level.

Tetanus Toxoid Stimulation: Guinea Pigs

Serum tetanus antitoxin concentrations are given in Table 3. Seven days after the first tetanus toxoid injection (day 35) there was a small but significant increase at the 0.008 and 0.04 μg/kg levels. Serum antitoxin values were significantly decreased at the 0.2 μg/kg level in the secondary response (days 49 and 56), but there was no significant effect at the 0.008 and 0.04 μg/kg dose levels. Weights and histology of the injected right popliteal lymph nodes were the same in all four groups.

Skin Test: Guinea Pigs

The diameter and thickness of the skin reactions, measured 24 and 48 hr after tuberculination are given in Table 4. The diameter of the skin reactions in the 0.04 μg/kg group were significantly reduced when measured 48 hr after the first, and 24 and 48 hr after the second intradermal tuberculin injection. At the 0.2 μg/kg level, all values (diameter and thickness skin reaction) were significantly decreased. The dose response test showed also a highly significant decrease of all data. Microscopically (Fig. 3a, 3b),

Table 2. Body weights, organ weights, leukocyte counts, and serum corticosteroid levels of guinea pigs treated with TCDD for 8 weeks (skin test).[a]

	Weekly TCDD dose				Dose–response test
	0	0.008 μg/kg	0.04 μg/kg	0.2 μg/kg	
Final body weight, g	572.9 ± 64.5	573.3 ± 64.2	529.6 ± 61.4	473.1 ± 41.6[b]	$P < 0.01$
Organ weights, mg					
Thymus	760 ± 208	819 ± 216	649 ± 133	409 ± 139[b]	
Right popliteal lymph node	163 ± 99	130 ± 33	90 ± 61	82 ± 25	
Adrenals	290 ± 42	295 ± 36	267 ± 41	286 ± 28	
Organ/body weight ratios × 10^4					
Thymus	1.32 ± 0.30	1.43 ± 0.31	1.23 ± 0.22	0.86 ± 0.28	$P < 0.01$
Right popliteal lymph node	0.278 ± 0.141	0.229 ± 0.059	0.171 ± 0.114	0.176 ± 0.059	$P < 0.05$
Adrenals	0.506 ± 0.044	0.519 ± 0.070	0.505 ± 0.064	0.612 ± 0.102[c]	$P < 0.05$
Total leukocytes × 10^3 per mm^3	8.84 ± 4.21	8.23 ± 2.48	7.26 ± 1.66	6.14 ± 2.91[c]	$P < 0.01$
Lymphocytes × 10^3 per mm^3	4.27 ± 2.29	4.23 ± 1.31	3.72 ± 1.17	3.63 ± 2.37	$P < 0.05$
Corticosteroids (pooled samples), μg/100 ml					
Cortisol	93.3	92.5	85.2	88.2	
Corticosteron	1.4	1.2	1.5	1.3	

[a] Mean values ± SD, 10 animals per group.
[b] $P < 0.01$.
[c] $P < 0.05$.

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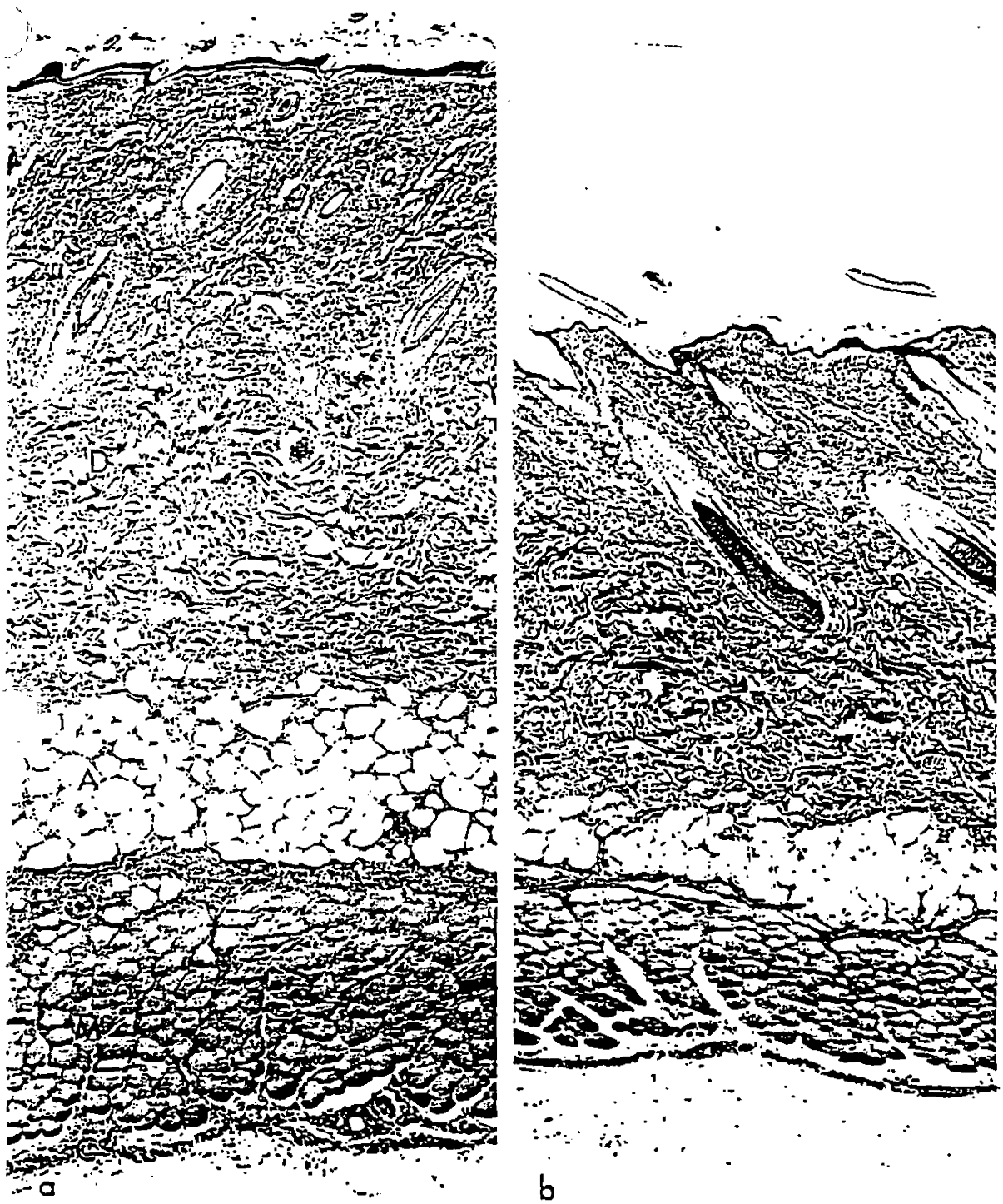


FIGURE 3. Forty-eight hour skin reaction to 1.25 μg tuberculin PPD in guinea pigs sensitized 17 days earlier with 0.05 ml of an oil suspension containing killed *Mycobacterium tuberculosis H₃₇ Ra:* (*a*) control animal, note the edema and diffuse cellularity of the dermis (D) and the infiltration both diffuse and around small vessels in the subcutaneous adipose tissue (A), muscle (M) and connective tissue (C); (*b*) guinea pig treated with 8 weekly doses of 0.2 μg TCDD/kg. There is less edema and less cellularity in the dermis and the focal cellular infiltration is much smaller. Hematoxylin and eosin; 52 ×.

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there was less cellularity and less edema in the skin sections of the 0.2 μg/kg groups which explains the reduced thickness of the skin at the site of the tuberculination. The cellularity as found in the controls consisted mainly of mononuclear cells, both diffuse and around vessels, was clearly decreased in the dermis, subcutaneous adipose tissue, muscle, and connective tissue of the 0.2 μg/kg treated animals. In contrast to the former experiment, there was a significant decrease (dose-response test) in the relative weight of the popliteal lymph node of the injected right hind leg (Table 2). Granulomas, probably caused by the adjuvant injection, were seen in all these lymph nodes.

Experiment with Rats

All animals survived the experimental period. Body weights, organ weights, and leukocyte counts of the animals killed after 45 days are given in Table 5. Body weight and absolute and relative thymus weight were significantly reduced at the 5.0 μg/kg level. A significant increase in relative spleen weight is also seen at that dose level. The effect of TCDD on adrenal weight in the rat differed from the response in the guinea pig

Table 3. Serum antitoxin values as measured by single radial immunodiffusion of guinea pigs treated with TCDD for 8 weeks (tetanus toxoid stimulation).[a]

Weekly dose of TCDD, μg/kg	Tetanus antitoxin concentrations, International Units/ml		
	Primary response (day 35)	Secondary response	
		Day 49	Day 56
0	5.55 ± 0.69	259.7 ± 99.7	246.0 ± 92.8
0.008	6.42 ± 0.64[b]	225.4 ± 98.5	265.4 ± 79.9
0.04	6.71 ± 0.61[c]	203.2 ± 57.7	230.0 ± 51.6
0.2	5.29 ± 0.82	149.2 ± 32.8[c]	175.8 ± 47.8[b]
Dose-response test	NS	$P < 0.01$	$P = 0.05$

[a] Mean values ± SD, 10 animals per group, except at the 0.2 μg/kg level (7 animals). The animals were injected with tetanus toxoid at days 28 and 42. Antibody concentrations were measured at days 35, 49, and 56 (7, 21, and 28 days after the first tetanus toxoid injection).
[b] $P < 0.05$.
[c] $P < 0.01$.

Table 4. Skin reaction (delayed hypersensitivity to tuberculin) of guinea pigs treated with TCDD for 8 weeks.[a]

Weekly dose of TCDD, μg/kg	Diameter skin reaction, mm				Thickness skin reaction, mm	
	Tuberculineation on day 47		Tuberculination on day 54		(tuberculination on day 54)	
	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
0	18.20 ± 1.87	15.15 ± 1.72	19.45 ± 1.23	15.20 ± 1.90	2.23 ± 0.49	1.73 ± 0.43
0.008	17.50 ± 1.35	13.85 ± 1.67	18.45 ± 1.23	13.85 ± 1.73	2.10 ± 0.60	2.00 ± 0.54
0.04	16.80 ± 1.83	12.90 ± 1.31[b]	17.65 ± 0.91[c]	13.40 ± 1.13[b]	1.93 ± 0.46	1.38 ± 0.34
0.2	12.90 ± 2.56[c]	8.80 ± 2.98[c]	15.20 ± 2.91[c]	8.95 ± 3.25[c]	1.13 ± 0.43[c]	0.63 ± 0.41[c]
Dose-response test	$P < 0.01$	$P < 0.01$.	$P < 0.01$.	$P < 0.01$.	$P < 0.01$.	$P < 0.01$

[a] Mean values ± SD, 10 animals per group. The animals were sensitized on day 35 with 0.05 ml of an oil suspension containing killed *Mycobacterium tuberculosis*. Intradermal tuberculination (1.25 μg tuberculin PPD) was performed on days 47 and 54. Skin reactions were measured 24 and 48 hr after tuberculination.
[b] $P < 0.05$.
[c] $P < 0.01$.

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Absolute adrenal weights decreased significantly in the rat at the 1.0 and 5.0 μg/kg dose levels. The dose-response test for the decrease in relative adrenal weights was highly significant. Also, the total leukocyte and lymphocyte counts in the rat differed from the response in the guinea pig. There was no lymphopenia, but some increase of both cell counts, showing a slight but not significant dose-response pattern. Microscopically, the only effect seen in the lymphoid organs and adrenals, was a slight to moderate cortical atrophy in the thymuses of the 5 μg/kg group. Also, the cellularity of the cortex was somewhat less dense. The results of the measurements of the skin reactions are given in Table 6. Also in contrast with the guinea pig, there was no effect on the

Table 5. Body weights, organ weights, and leukocyte counts of rats treated with TCDD for 6 weeks (skin test).[a]

	Weekly TCDD dose				Dose-response test
	0	0.2 μg/kg	1.0 μg/kg	5.0 μg/kg	
Final body weight, g	261.6 ± 28.2	264.0 ± 17.4	263.6 ± 25.6	225.0 ± 17.1[b]	$P < 0.05$
Organ weights, mg					
Thymus	318 ± 112	285 ± 50	289 ± 16	132 ± 39[b]	
Spleen	507 ± 85	498 ± 95	536 ± 53	564 ± 79	
Cervical lymph nodes	43.0 ± 16.1	40.0 ± 10.3	37.6 ± 7.6	37.4 ± 7.3	
Adrenals	80.6 ± 10.6	71.2 ± 7.2	61.2 ± 11.2[b]	55.0 ± 13.8[c]	
Organ/body weight ratios × 10^3					
Thymus	1.20 ± 0.32	1.09 ± 0.27	1.10 ± 0.13	0.59 ± 0.16[c]	$P < 0.05$
Spleen	1.94 ± 0.24	1.89 ± 0.35	2.04 ± 0.15	2.53 ± 0.48[c]	$P < 0.05$
Cervical lymph nodes	0.163 ± 0.049	0.153 ± 0.043	0.142 ± 0.016	0.167 ± 0.032	
Adrenals	0.312 ± 0.059	0.269 ± 0.014	0.231 ± 0.028	0.242 ± 0.044	$P < 0.01$
Total leukocytes × 10^3, per mm^3	5.88 ± 2.35	7.98 ± 4.41	7.68 ± 2.32	10.65 ± 5.00	NS
Lymphocytes × 10^4, per mm^3	4.13 ± 1.63	5.94 ± 2.80	5.53 ± 1.89	7.65 ± 3.11	NS

[a] Mean values ± SD, five animals per group.
[b] $P < 0.05$.
[c] $P < 0.01$.

Table 6. Skin reaction (delayed hypersensitivity to tuberculin) of rats treated with TCDD for 6 weeks.[a]

Weekly dose of TCDD, μg/kg	Diameter skin reaction, mm		Thickness skin reaction, mm	
	24 hr	48 hr	24 hr	48 hr
0	14.65 ± 2.94	10.95 ± 2.53	2.10 ± 0.45	1.46 ± 0.30
0.2	15.15 ± 2.49	9.80 ± 2.74	1.90 ± 0.59	1.42 ± 0.44
1.0	15.40 ± 1.93	10.30 ± 2.52	2.23 ± 0.68	1.58 ± 0.64
5.0	14.75 ± 1.83	10.45 ± 2.99	1.88 ± 0.70	1.65 ± 0.54

[a] Mean values ± SD, 10 animals per group. The animals were sensitized on day 28 with 0.05 ml of an oil suspension containing killed *Mycobacterium tuberculosis*. Intradermal tuberculination (5 μg tuberculin PPD) was performed on day 42. Skin reactions were measured 24 and 48 hr after tuberculination.

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diameter the thickness, or histological appearance of the tuberculin reactions in the rats.

Experiment with Mice

One animal of the 25 μg/kg group died after 24 days. Body and thymus weights of the donor mice are given in Table 7. There was no difference in the final body weights of controls and TCDD-treated animals due to lower initial weights of the controls, but there was a significant effect on weight gain at the 25 μg/kg level. Absolute and relative thymus weights were signficantly reduced at the 5.0 and 25 μg/kg dose levels. Mean thymus weight in the high dose group was only 13% of the mean weight of the controls. Microscopically (Figs. 4 and 5), there

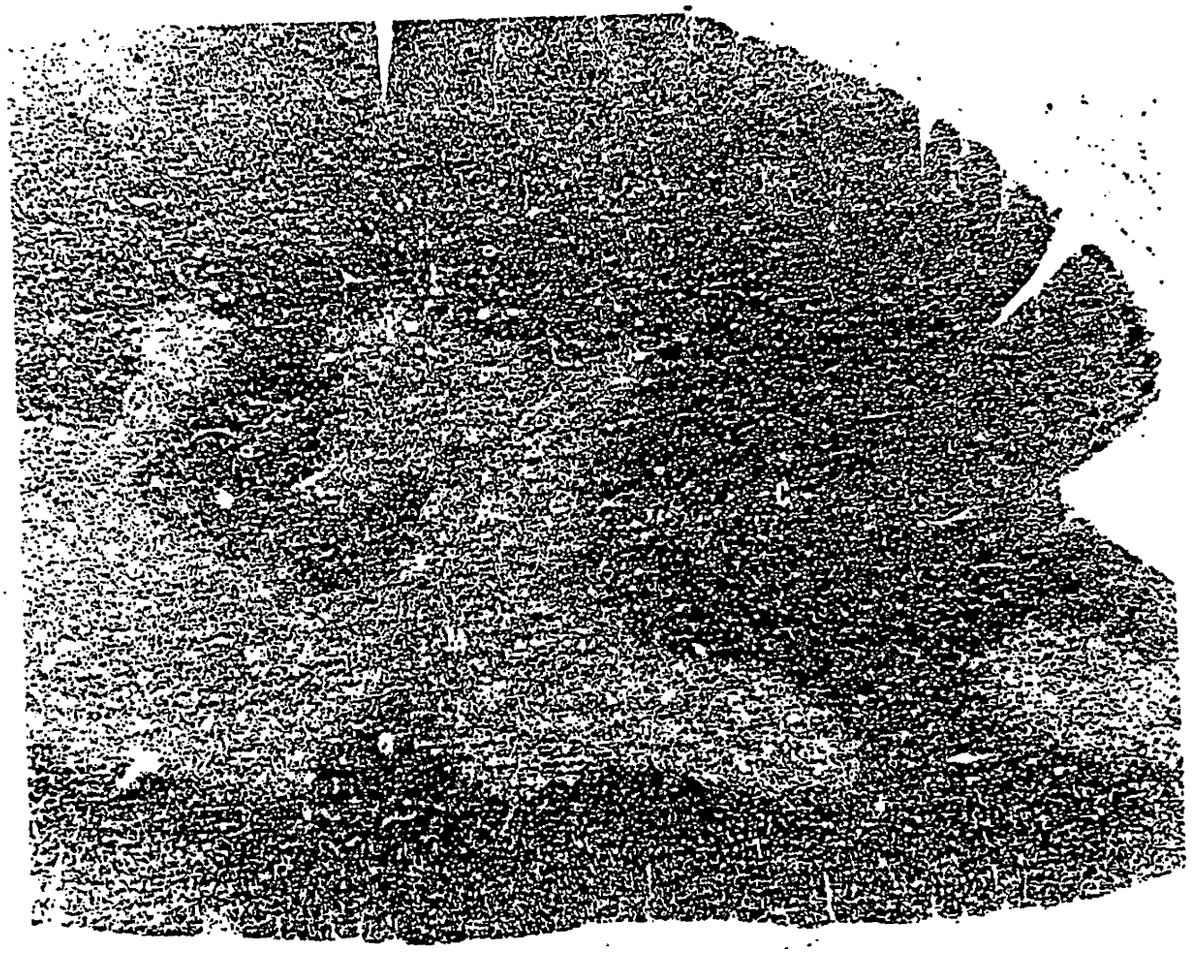


FIGURE 4. Thymus of a control mouse with the cortex at C and the medulla at M. Hematoxylin and eosin; × 61.

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was a nearly complete loss of the thymic [illegible]. The cellularity of the remaining cor[illegible] is less dense and there was destruction of lymphocytes. Spleens of the animals receiving 25 μg/kg TCDD were very small. The yield of spleen cells was too small to inject a sufficient number of recipients. As shown in Table 8, injection of 1×10^7 viable nucleated spleen cells from the 5 μg/kg group resulted in a highly significant decreased graft versus host activity when injected into the hind foot pad of the recipients ($B_6D_2F_1$). The mean popliteal lymph node ratio in the recipients injected with cells from the 1

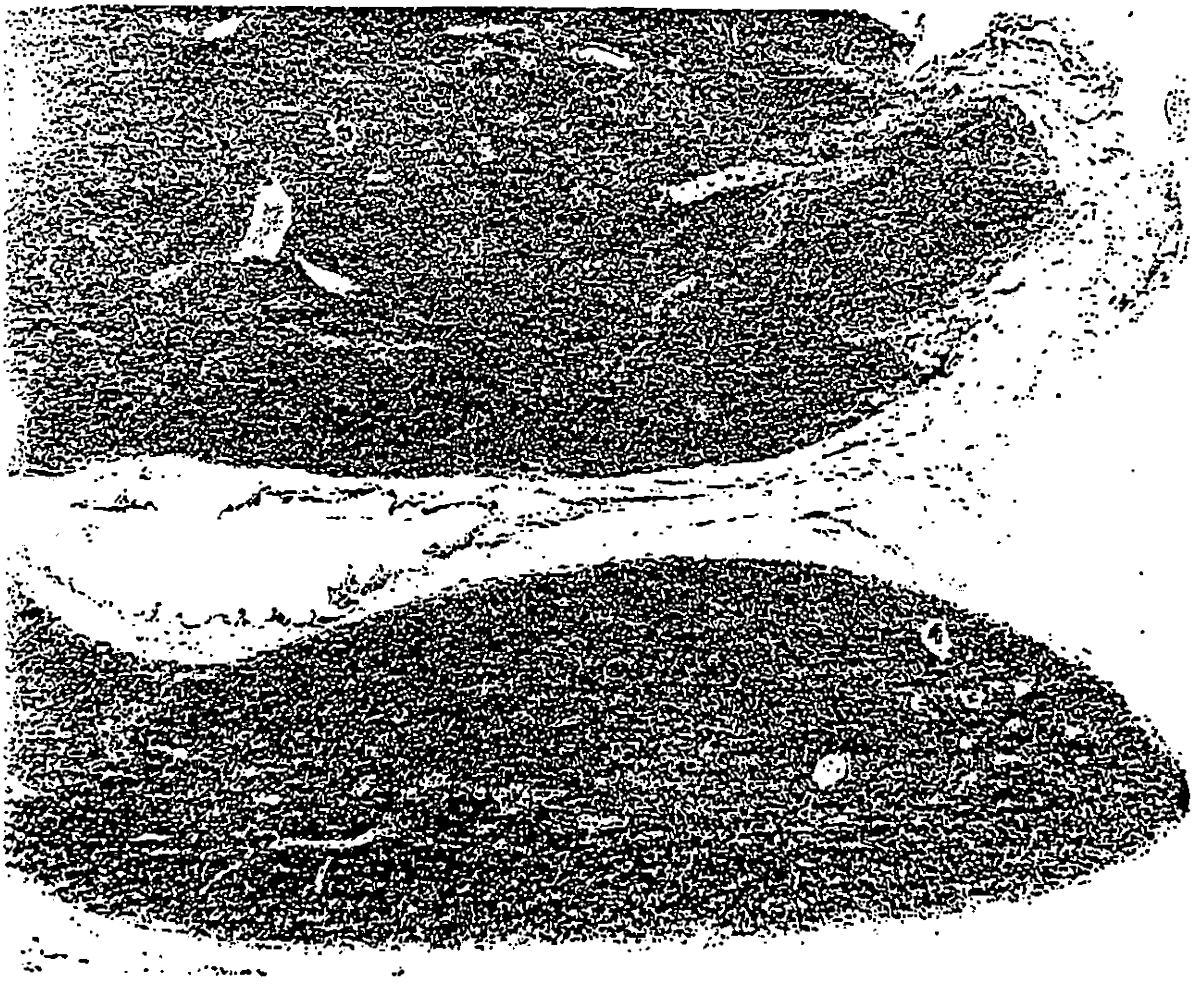


FIGURE 5. Thymus of a mouse that was killed after receiving four weekly oral doses of 25 μg TCDD/kg. Severe cortex atrophy is present; the cellularity in the remaining cortex is less dense making a distinction between cortex and medulla difficult at this magnification. Hematoxylin and eosin; 61 ×.

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Table 7. Body and thymus weights of donor mice treated with TCDD for 4 weeks.[a]

Weekly dose of TCDD, μg/kg	Final body weight, g	Weight change, g	Thymus, mg	Thymus/body weight ratio × 10³
0	26.50 ± 0.29	+2.24 ± 0.42	51.8 ± 4.1	1.95 ± 0.14
0.2	25.84 ± 2.80	+1.26 ± 2.09	41.2 ± 14.6	1.57 ± 0.46
1.0	26.34 ± 2.38	+1.78 ± 1.27	40.8 ± 10.0	1.56 ± 0.33
5.0	25.76 ± 2.10	+1.04 ± 2.13	29.0 ± 5.8[b]	1.12 ± 0.15[b]
25	22.02 ± 2.44	−1.92 ± 2.69[b]	6.7 ± 9.1[b]	0.31 ± 0.09[b]
Dose-response test	NS			$P < 0.01$

[a] Mean values ± SD, five or six animals per group.
[b] $P < 0.01$.

Table 8. Graft versus host activity of spleen cells from TCDD-treated donor mice injected into recipients.[a]

Donor TCDD dose, μg/kg	Recipients No.	Recipients Right/left ratio Popliteal lymph nodes
0	10	5.83 ± 4.32
0.2	9	5.82 ± 2.47
1.0	12	3.62 ± 1.47
5.0	14	1.98 ± 0.72[b]
Dose-response test		$P < 0.01$

[a] Mean values ± SD. Recipients (C57B1/6 × DBA-2, F-1) were injected in the right hind foot pad with 1 × 10⁷ viable nucleated donor (C57B1/6) spleen cells.
[b] $P < 0.01$.

μg/kg donor group was 38% lower than the control value. There was a highly significant monotonic dose-response relationship.

Discussion

As shown in Table 4 and Figure 3, it is clear that TCDD suppressed the cell-mediated immunity in guinea pigs at the 0.2 and 0.04 μg/kg levels (measuring the delayed hypersensitivity to tuberculin). The vulnerability of the lymphopoietic system, having a high mitotic activity, is clearly demonstrated by the thymus atrophy and lymphopenia (Tables 1 and 2, Figs. 1 and 2). In this context, it is worth mentioning that inhibition of mitosis has been observed in dividing endosperm cells of the African blood lily when exposed to TCDD (*15*). Also, there was a significant reduction in the relative weights of the stimulated popliteal lymph nodes in the skin test experiment (Table 2) Humoral immunity, measuring the antibody production against tetanus toxoid, was slightly depressed in the guinea pig at the 0.2 μg/kg level (Table 3). There was no effect on the weights of the stimulated popliteal lymph nodes in this experiment (Table 1).

In the second guinea pig experiment, there was no difference in serum cortisol and corticosteron concentrations between the different groups (Table 2). Microscopically, there was no effect on the adrenals in the 0.2 μg/kg groups. Therefore, it is likely that the increased relative adrenal weights (Tables 1 and 2) are only due to a decrease in body weight gain and not due to adrenocortical hyperfunction. Thus, indirect immunosuppression by stimulation of adrenocortical activity can be excluded. Besides the effect on the lymphoid system, there was only minor pathology in guinea pigs receiving a lethal dose of TCDD, i.e., mild liver injury, hyperplasia of the bladder epithelium, hemorrhages, atrophy of the zona glomerulosa of the adrenal cortex (*8*) and thrombocytopenia (*9*).

Analogous to the situation in the guinea pig, there was suppression of the cell-mediated immunity in the mouse. The graft versus host activity of donor spleen cells was significantly suppressed at the 5.0 μg/kg level. Donor cells of the 1 μg/kg group gave a graft versus host response that was 62% of the control value (Table 8). Thymus atrophy (Table 7, Figs. 4 and 5) and lymphopenia

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were sensitive indices for TCDD exposure. As in the case of the guinea pig, liver pathology was mild (*8*).

In contrast to the guinea pig and mouse studies, there is quite a different situation in the rat. Cell-mediated immunity (delayed hypersensitivity to tuberculin) was not suppressed (Table 6). Possibly, this test is not sensitive enough to detect an immunosuppressive effect in rats. Also, except for thymic atrophy at sublethal dose levels, there was no lymphopenia (Table 5). In addition, there was a dose-related decrease in both absolute and relative adrenal weight (Table 5), indicating an adrenal hypofunction. However, in view of the marked induction by TCDD of glucuronyl transferase in rat liver (*16*), an important enzyme involved in the metabolism of corticosteroids, adrenocortical hyperfunction regulated through the hypothalamus-pituitary-adrenocortical axis would be expected. An inhibitory effect on adrenal hypertrophy, induced by surgical trauma, was seen in rats treated with PCB (unpublished data) and with *p,p'*-DDT; in this latter study interference with the feedback mechanism of glucocorticoid hormones homeostasis was proposed (*17*).

In rats, exposed to lethal concentrations of TCDD, there was severe liver injury (degenerative and necrotic changes), thyroid pathology, hemorrhages (*8*), platelet depression, increased serum bilirubin values, and increased SGOT and SGPT activities (*9*). Liver damage can be considered to be the major cause of death in rats exposed to TCDD. In both guinea pigs and mice, there was only mild liver injury (*8*). The most significant findings are seen in the lymphoid system. Suppression of the cell-mediated immunity might be the major cause of death in these species. To test this hypothesis, a study is indicated to determine whether lymphoid (thymus) cell grafts are capable to protect mice from dying when exposed to concentrations of TCDD that are lethal for the control mice. Also, it would be worthwhile determining whether there is a difference in lethal TCDD levels in mice kept under sterile and under conventional or SPF conditions. Experiments will be conducted to determine the effect of *in utero* exposure of TCDD upon the cell-mediated immune reponse of the offspring.

Summary

In two experiments, groups of 10 female guinea pigs were dosed weekly for 8 weeks with 0, 0.008, 0.04, 0.2, or 1.0 μg/kg TCDD/kg body weight to test the cell-mediated and humoral immunity. All animals at the 1 μg/kg level died or were killed when moribund, they showed severe weight loss, lymphopenia,, and atrophy of the lymphoid organs. Weight gain was depressed at the 0.2 μg/kg level. Cell-mediated immunity was assessed by vaccination of the remaining animals with an oil suspension of killed *Mycobacterium tuberculosis*. Thymus atrophy and lymphopenia was observed. The diameters of the skin reactions, measured 24 and 48 hr after tuberculination, were significantly reduced at the 0.2 and 0.04 μg/kg levels. Indirect immunosuppression by stimulation of adrenocortical activity was excluded. The humoral immune system was stimulated in a second experiment by tetanus toxoid injections at days 28 and 42. Serum tetanus antitoxin concentrations were slightly depressed in the 0.2 μg/kg level at days 49 and 56.

A skin test, similar to that in guinea pigs, was done in rats treated weekly for 6 weeks with 0, 0.2, 1.0, and 5.0 μg TCDD/kg body weight. Animals at the 5 μg/kg level had significant lower body, thymus and adrenal weights. No effect was found on the skin reactions.

Cell-mediated immunity was tested in mice in a graft versus host assay. Groups of donor mice (C57B1/6) were treated weekly for 4 weeks with 0, 0.2, 1.0, 5.0, and 25 μg TCDD/kg body weight. Weight gain was depressed at the 25 μg/kg level. There was a remarkable thymic atrophy. Parental strain spleen cells (up to the 5.0 μg/kg group) were injected into the feet of hybrid recipients (C57B1/6 × DBA-2 F-1). The weights of the draining popliteal lymph node, as a parameter for the graft versus host activity, were significantly lower in the animals injected

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with spleen cells from the 5 μg/kg donor group.

It is concluded that TCDD at sublethal dose levels suppresses the cell-mediated immunity in both guinea pigs and mice. Humoral immunity was slightly suppressed in the guinea pig. The possible role of immune suppression in the death of TCDD-treated guinea pigs and mice is discussed in view of the absence of major pathologic effects except in the lymphoid system.

Acknowledgements

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Carcinogenesis Bioassay of Chlorinated Dibenzodioxins and Related Chemicals

by Maurice E. King,* Alan M. Shefner,* and Richard R. Bates †

Introduction

Chlorinated dibenzo-*p*-dioxins have been identified as trace contaminants of herbicides and pesticides. They may be formed as by-products in the manufacture of chlorinated phenols and, for this reason, were implicated in outbreaks of chick edema disease (1). Fat trimmings obtained from animal hides preserved with dioxin-containing chlorophenol products and subsequently used in chicken feed contained the chick edema factor. Chloracne had been earlier observed in workers who had handled technical quality 2,4,5-trichlorophenol (2). Occasionally the condition was complicated by liver involvement and psychopathological changes. Pure 2,4,5-trichlorophenol had no effect when tested in an animal bioassay system. Tetrachlorodibenzodioxin (TCDD), which was isolated from the technical mixture, produced an effect at a concentration of 0.005%, however (13). In addition, TDCC is the contaminant of the herbicide 2,4,5-T that produced teratogenic effects in rats (4). Subsequent work with pure TCDD has indeed shown teratogenic and embryotoxic effects in rats and mice (5–7).

The fact that the chlorinated dioxins are highly toxic, teratogenic, and acnegenic does not of necessity indicate they are carcinogens as well. However in view of the widespread use of products that might contain dioxins as contaminants and their extreme stability under environmental conditions, examination of their possible long-term effects is imperative. This program was initiated to determine the chronic toxicity and potential carcinogenicity of a series of chlorinated dibenzodioxins by oral administration and skin application. The unsubstituted, 2, 7-dichloro- and octachlorodibenzodioxins are relatively innocuous with regard to toxicity. The 2,3,7-trichloro-, 2,3,7,8-tetrachloro- and hexachlorodibenzodioxins, however, are quite toxic and require the use of specialized facilities to minimize the possibility of human exposure. This report describes our results to date with the nontoxic dioxins. Work is being initiated with the toxic dioxins as they become available.

Experimental

The octachlorodibenzodioxin used in these studies was prepared by Spectratec Inc., Washington, D. C. The other dioxins were synthesized by the Chemistry Division of IIT Research Institute. Swiss-Webster and B6C3F1 mice and Osborne-Mendel rats were obtained from contract supported colonies at Charles River Breeding Laboratories through arrangement with the Mammalian Genetics and Animal Production Section, National Cancer Institute.

Skin carcinogenesis studies will be continued for a total of 78 weeks. Each treatment

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group consists of 30 male and 30 female Swiss-Webster mice that are shaved weekly. In testing for complete carcinogenicity 0.2 ml of a solution of the test compound dissolved in acetone is applied three times weekly to the backs of mice. The octachloro-, dichloro-, and unsubstituted dibenzodioxin solutions in acetone contain 0.2, 3.0, and 80 mg/ml, respectively. For the study of promotion activity, each mouse was initially treated with 50 μg dimethylbenzanthracene (DMBA) 1 week prior to initiation of test compound application.

Osborne-Mendel rats and B6C3F1 mice are being used in the oral administration studies. Groups of 50 male and 50 female mice and 35 male and 35 female rats are receiving the dioxins at levels of 1% and 0.5% of the diet, and similar groups are receiving 1% and 0.5% dioxane in their drinking water. In the case of the supposedly nontoxic octachlorodibenzodioxin, in which numerous animal deaths occured after 20 weeks of feeding, additional animals were placed on test at lower dietary levels.

Results

The results for the skin carcinogenesis study to date are shown in Table 1. None of the dioxin-treated mice in the complete carcinogenesis study exhibited skin tumors, although subcutaneous tumors are present in two mice treated with octachlorodibenzodioxin. Histopathological results are not yet available for all mice that have died. Of those that have been examined, however, a malignant lymphoma of the lymphocyte type was found in a mouse treated with unsubstituted dioxin. No other significant lesions were found in any of the mice from the complete study.

The promotion study has yielded apparent skin tumors in male mice treated with unsubstituted dibenzodioxin or dichlorodibenzodioxin. As the animals showing these skin lesions are still alive, histologic confirmations of this observation is not yet available. A plasmacytoma has been found in one mouse from the dichlorodibenzodioxin promotion group.

Acetone was originally included in the complete study as a solvent control, since the dioxins are dissolved in it for skin application. No skin tumors have been found in either surviving mice or in tissue from those that have died. The acetone promotion groups, however, were started 3 months later, and skin tumors have been confirmed by microscopic examination of skin of a male mouse that died. A reticulum cell malignant lymphoma was found in one of the females that was examined, but there were no other significant lesions.

The dioxins can be considered as derivatives of 1,4-dioxane, and this compound was included in the studies because of the structural similarity. One carcinoma not microscopically confirmed and a subcutaneous tumor are apparent in the surviving mice of the complete study. Tissues from those that died revealed a reticulum cell malignant lymphoma but no evidence of skin lesions.

The dioxane promotion groups are of particular interest since their response unexpectedly rivaled that of the croton oil positive controls in both mortality and mice exhibiting papillomas. The activity of croton oil and dioxane as promoting agents following DMBA initiation is shown in Figures 1 and 2. Weekly counts of papillomas and suspect carcinomas were made by gross examination. The fraction of mice bearing skin tumors is nearly identical for both materials, as is the time course for tumor development (Fig. 1). However, croton oil treatment led to a much higher multiplicity of skin tumors per mouse than did treatment with dioxane (Fig. 2). Carcinomas were produced in both treatment groups in direct proportion to the number of papillomas present.

Histopathological results from the two treatments differed in that effects observed with croton oil were primarily neoplastic. A majority of the dioxane-treated mice had liver lesions of a mild nature (megalocytosis, occasional distended bile canaliculi, occasional necrotic centrolobular necrosis, cuffed triad vessels, general mononuclear periportal infiltration, and mild peripherolobular fibrosis). The distribution of preneoplastic and

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Table 1. Summary of dioxin skin carcinogenesis data.

Compound	Treatment	Week of test	Sex	Survivors: Number	Tumor response: Papillomas	Tumor response: Suspected carcinomas	Tumor response: Subcutaneous tumors	Pathological results
Octachloro-dibenzodioxin	Complete	60	M	20	0	0	1	No papilloma or significant pathology
			F	28	0	0	1	
	Promotion	59	M	24	0	0	3	No papilloma or significant pathology
			F	29	0	0	1	
Unsubstituted dibenzodioxin	Complete	59	M	24	0	0	0	No papilloma, 1 malignant lymphoma
			F	24	0	0	0	
	Promotion	58	M	25	1	3	2	No papilloma or significant pathology
			F	29	0	0	0	
Croton oil	Promotion	65	M	0	—	—	—	Neoplastic lesions of skin and lungs
			F	1	0	1	1	
Dichlorodibenzodioxin	Complete	64	M	17	0	0	0	No papilloma or significant pathology
			F	27	0	0	0	
	Promotion	63	M	23	5	2	2	No papilloma, 1 plasmacytoma
			F	25	0	0	0	
Acetone	Complete	62	M	24	0	0	0	No papilloma or significant pathology
			F	28	0	0	0	
	Promotion	50	M	29	8	1	1	1 papilloma, 1 malignant lymphoma
			F	26	0	0	0	
Dioxane	Complete	60	M	22	0	0	1	No papilloma, 1 malignant lymphoma
			F	25	0	1	0	
	Promotion	59	M	4	2	3	2	Neoplastic lesions of skin, lungs, and kidney
			F	5	2	3	0	

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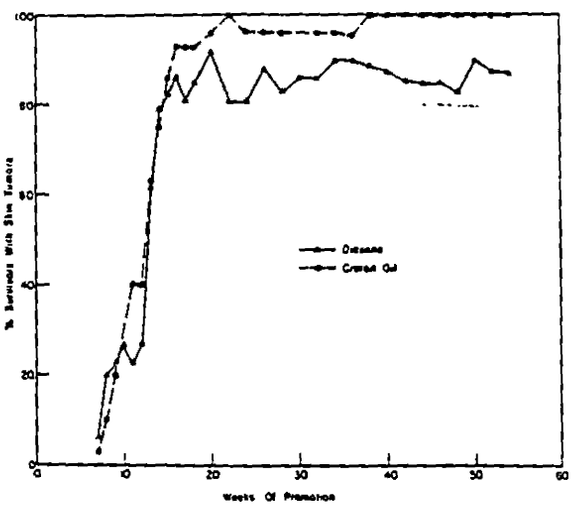


FIGURE 1. Comparative rate of promotion activity for croton oil and dioxane on male mice.

neoplastic lesions in the dioxane-treated group is shown in Table 2. In treated skin, conditions ranged from hyperplasia to dermal fibrosarcoma. Squamous cell carcinoma of the nasal septum was observed in one animal which had skin papilloma. Of nine mice with lung tumors, seven had malignant lung lesions, of which three were considered metastatic from other sites.

The average weights of controls and test animals receiving the dioxins in their diet and dioxane in water are shown in Figures

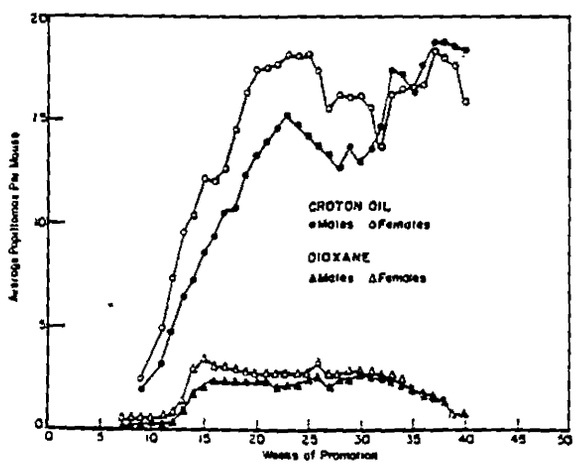


FIGURE 2. Promotion activity of 1% croton oil and dioxane on Swiss-Webster mice.

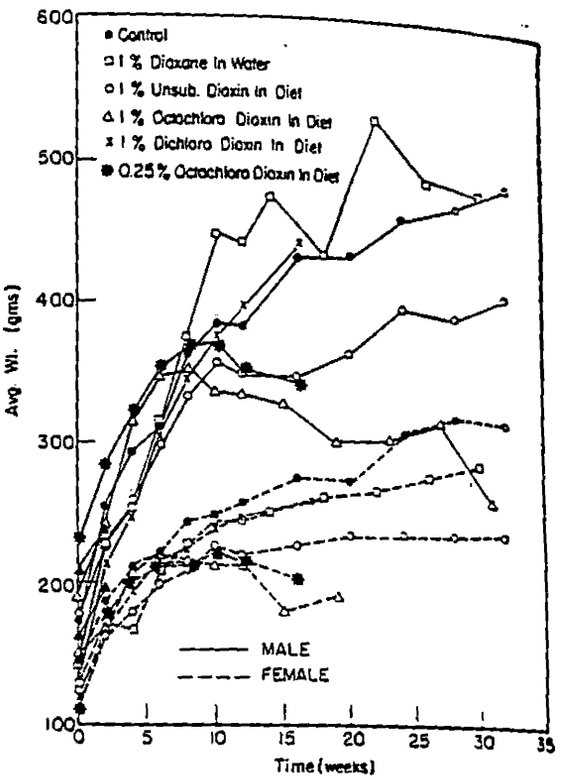


FIGURE 3. Average weight of treated female and male rats.

3 and 4 for rats and Figures 5 and 6 for mice. The stimulatory effect of dioxane on weight gain in male mice and rats is most interesting, especially in view of the skin carcinogenesis results. The effect is not as marked in females; however, after 10 weeks the weight gain of female mice at 0.5% exceeds that of the controls. Generally, the dioxins cause a decrease in growth rate although dichlorodibenzodioxin, which has not been on test as long as the other compounds, does not appear to affect growth rate at the levels tested.

A summary of the results to date in the feeding studies is shown in Table 3. Despite the stimulation of growth in surviving animals, dioxane has caused an appreciable mortality in rats but has had little effect on mice. Chronic bronchopneumonia and chronic murine pneumonia above the background level were the major changes observ-

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Table 2. Distribution of preneoplastic and neoplastic lesions in 15 mice treated with dioxane following DMBA initiation.

Type of lesion	Number of mice with specific lesions							
	Skin	Nasal septum	Trachea	Lung	Spleen	Kidney	Liver	Pericardium
Hyperplasia and/or hyperplasia	6			1				
Papilloma	2							
Carcinoma *in situ*				1				
Squamous cell carcinoma	2	1		2				
Bronchial adenomatiod lesion				3				
Alveolar adenoma				4				
Bronchiolar or alveolar carcinoma				3				
Fibrosarcoma	3			1	1		1	
Undifferentiated sarcoma	1			1		1		
Malignant lymphoma								
Lymphocyte type			1	1	1	1		
Reticulum cell sarcoma				1	1	1		1

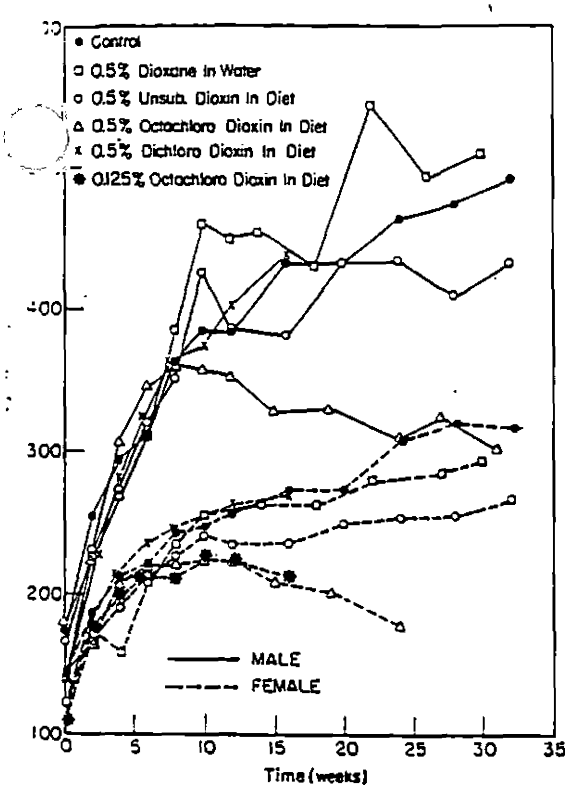


FIGURE 4. Average weight of treated female and male rats.

ed in the rats that have been examined. Appreciable mortality has also been observed in female mice that are receiving 1% unsubstituted dioxin. Hepatotoxicity was the major effect noted, but one mouse exhibited broncheolar mucosal hyperplasia. The rats that received this compound exhibited hepatotoxicity and chronic murine pneumonia.

Toxicity in animals fed octachlorodioxin was evidenced by both growth depression and mortality. A distinct difference in the susceptibility of the sexes to the compound was observed for mice and rats. Thus all male mice died by 10 and 8 weeks, respectively, in the 1% and 0.5% levels while, at 37 weeks, there were 5 female 1% survivors and 45 female survivors at 0.5%. On the other hand, all female rats died by 22 and 25 weeks, respectively, in the 1% and 0.5% groups while the last males died at 32 and 37 weeks. New test groups were set up at 0.25% for all animals and at 0.125% for female rats and male mice since these groups appeared to be more drug-sensitive. Even at these lower dose levels, all of the mice have died.

Rats and mice fed octachlorodibenzodioxin had changes ranging from early hepatotoxic

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Table 3. Summary of dioxin oral administration data.

Compound	Sex	Rats (35 per group) Week of test	Survivors	Significant pathology: Lung	Significant pathology: Liver	Mice (50 per group) Week of test	Survivors	Significant pathology: Lung	Significant pathology: Liver
Controls	M	34	35			31	50		
	F	34	35			31	50		
1% Dioxane	M	42	24	8/11	1/11	40	50		
	F	42	20	3/5	2/5	43	49		
0.5% Dioxane	M	42	26	6/6	1/6	40	49		
	F	42	32	3/3	1/3	43	49		
1% Unsubstituted	M	42	33	2/2	2/2	39	48		
dibenzodioxin	F	42	32	1/2	2/2	39	29	3/14	7/14
0.5% Unsubstituted	M	42	31	3/4	1/4	34	50		
dibenzodioxin	F	42	35			39	49		
1% Dichloro-	M	17	35			17	49	0/1	0/1
dibenzodioxin	F	17	35			17	48	1/2	1/2
0.5% Dichloro-	M	17	35			17	50		
dibenzodioxin	F	17	35			17	49		
1% Octachloro-	M	32	0	1/5	5/5	10	0	0/5	5/5
dibenzodioxin	F	22	0	0/5	5/5	37	5	0/5	6/6
0.5% Octachloro-	M	37	0	1/5	5/5	8	0	0/5	5/5
dibenzodioxin	F	25	0			37	45		
0.25% Octachloro-	M	17	28	1/2	2/2	17	1		
dibenzodioxin	F	17	15	0/3	3/3	15	0	2/2	1/2
0.125% Octachloro-	M	—	—	—	—	9	0		
diobenzodioxin	F	17	30			—	—	—	—

lesions (diffuse vacuolar degeneration, fatty metamorphosis, megalocytosis, necrosis, and cholangiolar epithelial hyperplasia) to circhosis with disrupted lobule architecture, necrosis, fibrosis, parenchymal regeneration and cholangiolar proliferation. There were areas of bronchiolar mucosal hyperplasia in the lungs of two rats, and one of these rats also had bronchiolar adenomatoid lesions. In one mouse, there were bronchiolar mucosal hyperplasia and metaplasia and one bronchiolar carcinoma in which signs of early squamous change could be seen.

Discussion

The preliminary results reported here are by no means conclusive with regard to the carcinogenicity of the chlorinated dioxins. The oral studies indicate primarily hepatotoxicity, especially in the case of octachlorodibenzodioxin. A supplementary analysis report from Midwest Research Institute provides a possible explanation for some of the toxicity observed. The batch of octachlorodibenzodioxin used in preparation of the diets was found to contain hexachlorodibenzodioxin at a level of less than 0.1%. A dietary level of 1% octachlorodibenzodioxin which contained this contaminant would provide a hexachlorodibenzodioxin intake of approximately 150 μg/day for rats and 50 μg/day for mice. Chronic toxicity values for this compound are not available. However, in preliminary experiments at Dow Chemical Co. (*5*), hexachlorodibenzodioxin at 100 μg/kg/day for 10 days caused growth depression, liver pathology, and embryotoxicity in rats. Tests with animals receiving the first batch of octachlorodibenzodioxin will continue as long as there are survivors. Additional groups of animals at dietary levels of 0.5% and 0.25% octachlorodibenzodioxin shown on analysis not to contain the hexachlorodibenzodioxin contaminant have been started. After 6 weeks, many of the male mice at 0.5% have died, thereby indicating

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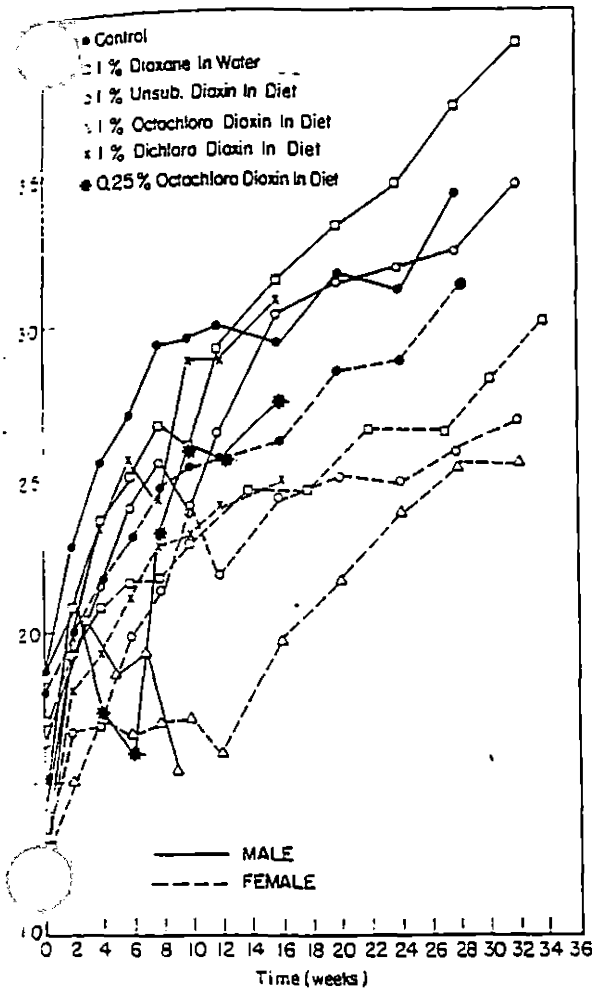


FIGURE 5. Average weight of treated female and male mice.

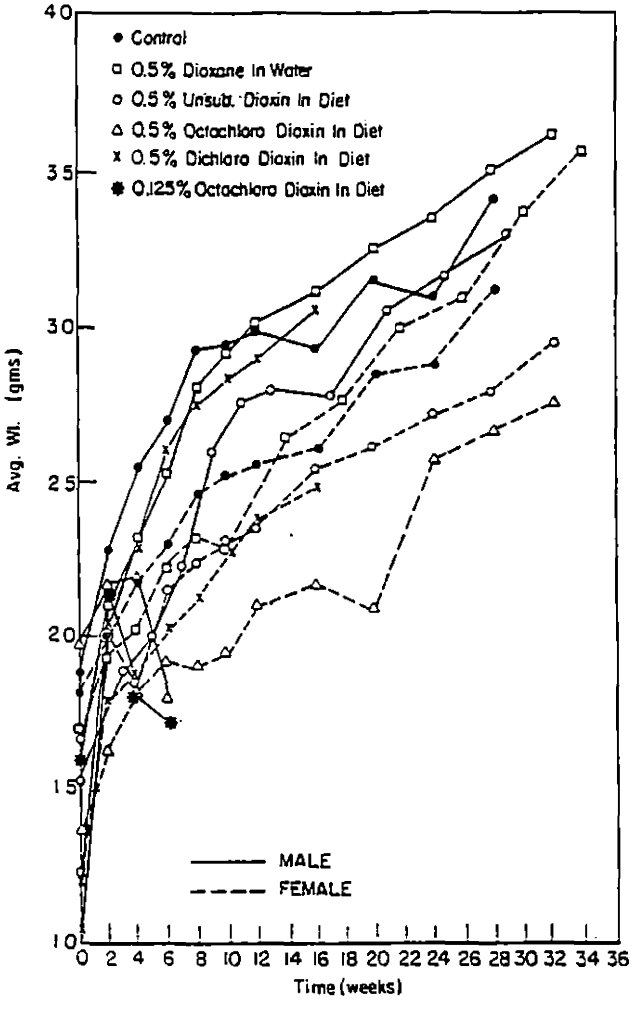


FIGURE 6. Average weight of treated female and male mice.

possible toxicity of the octachlorodibenzodioxin itself. A comparative feeding and intubation study is currently underway to determine if the octachloro causes a decrease in dietary intake.

The skin carcinogenesis assay for the nontoxic compounds is nearing completion, and no skin tumors have been observed in the complete carcinogenesis studies. The nature of the internal growths must be determined at necropsy. The fact that only male mice have exhibited skin tumors in the promotion studies can probably be attributed to the additional factor of wounding. As the mice are housed in groups of 10, there is considerable fighting among the males, resulting in scars on the backs of the less aggressive animals. Boutwell (*8*) has shown that wound healing plays an integral role in the promotion of skin carcinogenesis, and fighting among males treated with unsubstituted and dichlorodibenzodioxin as well as acetone may have contributed to the promotion of skin tumors. No explanation can be given at this time for the lack of tumors in the octachlorodioxin group, since this compound was also dissolved in acetone.

The positive control group treated with

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DMBA and croton oil responded as expected in terms of skin tumor promotion. The extent and variety of the lesions produced by DMBA and dioxane were unexpected, however, previous studies have shown dioxane to be a hepatocarcinogen (*9*) and to induce carcinomas in the nasal cavity of rats receiving dioxane in drinking water (*10*). No previous reports of dioxane as a promoting agent in skin carcinogenesis could be found. Because of the use of dioxane as a solvent in industry and in histology laboratories for tissue processing, the results obtained are of potential significance in relation to possible carcinogenic hazards to humans.

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Chlorinated Dibenzodioxins and Pentachlorophenol

by R.L. Johnson,* P.J. Gehring,† R.J. Kociba,† and B.A. Schwetz†

Introduction

Pentachlorophenol is a registered antimicrobial agent whose principal use is for the preservation of wood. Typical commercial pentachlorophenol contains a variety of substances which are considered to be "inactive" from the aspect of antimicrobial efficacy. Consequently, pentachlorophenol is sold as an antimicrobial agent with 95% active ingredients and 5% "inert" ingredients. Analysis of acceptable commercial pentachlorophenol is shown in Table 1.

The "caustic insolubles," sometimes referred to as the "nonphenolic or neutral impurities," include chlorinated dibenzo-*p*-dioxins and chlorinated dibenzofurans (*1*). Recently developed analytical technology has allowed quantitation of hexachlorodibenzo-*p*-dioxins and octachlorodibenzo-*p*-dioxin in pentachlorophenol. Portrayed in Table 2 are concentration ranges for these two chlorodibenzo-*p*-dioxins in samples of currently available commercial grade pentachlorophenol. Techniques capable of detecting 0.05 ppm showed no 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in any sample of pentachlorophenol examined by us. The absence of this compound in pentachlorophenol is not surprising, because the appropriate precursors for its formation are not present.

Table 1. Commercial pentachlorophenol composition.

	Content %
Pentachlorophenol	85–90
Tetrachlorophenol	4–8
Trichlorophenol	<0.1
Higher chlorophenols	2–6
Caustic insolubles (maximum)	1

Table 2. Concentration ranges of some chlorinated dioxins in commercial pentachlorophenol.

Chlorinated dibenzo-*p*-dioxin	Concentration range, ppm
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	None
Hexachlorodibenzo-*p*-dioxins	9–27
Octachlorodibenzo-*p*-dioxin	575–2510

Heptachlorodibenzo-*p*-dioxin and hexa-, hepta- and octachlorodibenzofurans have been qualitatively detected in commercial pentachlorophenol. However, the lack of appropriate standards for these materials does not allow their quantitation.

Severe toxicological responses have been attributed to certain chlorodibenzo-*p*-dioxins (*2*). For example, the LD_{50} of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin ranges from 0.6 μg/kg in male guinea pigs to 115 μg/kg in rabbits of mixed sexes. A benzene solution of this agent containing as little as 0.04 μg/ml produces acne in the rabbit ear bioassay. Very high embryotoxicity and the production of edema in chicks are other properties of this material. The no-effect

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dose levels for these latter activities are 0.03 and 0.1 μg/kg-day, respectively. Although pertinent for perspective, it is re-emphasized that this agent is not a contaminant of pentachlorophenol.

Limited lethality data are available on hexachlorodibenzo-*p*-dioxins and octachlorodibenzo-*p*-dioxin. The former material killed 1 of 2 and 0 of 2 male rats given overdoses of 100 and 10 mg/kg, respectively. Doses of 1 g/kg and 4 g/kg of octachlorodibenzo-*p*-dioxin failed to kill female rats and male mice, respectively.

With regard to acnegenic activity, chloroform solutions containing 10 to 50 μg/ml hexachlorodibenzo-*p*-dioxin are active while preparations of octachlorodibenzo-*p*-dioxin are inactive.

Hexachlorodibenzo-*p*-dioxin administered to pregnant rats at a dose of 100 μg/kg-day has been found teratogenic, while doses of 1 or 10 μg/kg-day produced only subcutaneous edema. A dose of 0.1 μg/kg/day was not associated with untoward effects in embryos or fetuses. Studies to date have revealed that octachlorodibenzo-*p*-dioxin is essentially devoid of untoward activity in the embryo and fetus.

Daily doses of 10 and 100 μg/kg hexachlorodibenzo-*p*-dioxin produce a positive response in the chick edema bioassay, while doses of 0.1 and 1.0 μg/kg are negative. As with the other untoward effects previously referred to, octachlorodibenzo-*p*-dioxin appears to be devoid of this activity.

Although the principal chlorodibenzo-*p*-dioxin contaminant of pentachloropenol, octachlorodibenzo-*p*-dioxin, in the amounts normally present does not appear to present a significant hazard, the presence of hexachlorodibenzo-*p*-dioxin as well as the other contaminants in pentachlorophenol previously mentioned give rise to concern. In at least one instance, the product literature warns that frequent skin contact may result in an acneform dermatitis (*3*). However, documentation of such occurrences is unavailable. In addition, it has been stated that contamination of fat used in the diet of chickens with pentachlorophenol may be responsible for the "toxic fat" syndrome.

In response to the concern about the toxicological significance of the nonphenolics in pentachlorophenol, we conducted studies to determine whether their presence in the product may contribute to its toxicological properties. After finding that the toxicological properties of the nonphenolics could, indeed, be detected by toxicological evaluations, acnegenic response, chick edema assay, and 90-day dietary feeding studies in rats, we set out to develop a product which was a "toxicological mimic" of pure pentachlorophenol. Before presenting the results of our toxicity studies, it should be emphasized that pentachlorophenol is an economic poison, and although we believe we have developed the capability to produce a product in which the contaminants do not contribute to its potential hazard, it remains an economic poison.

Experimental

The rabbit ear bioassay test was conducted according to published procedures (*2*, *4*). The chick edema bioassay test was conducted according to the procedure described in the official methods of analysis of the Association of Official Agricultural Chemists (*5*). The feeding studies utilized the Spartan strain of Sprague Dawley rats which were maintained on diets formulated to supply the various dose levels for 90 days. Parameters monitored in these feeding studies included the following: body weights, food consumption, appearance and demeanor of the rats, routine hematologic and urinary parameters, routine serum enzymes, terminal organ weights and gross and histopathologic examination of tissues.

Results

The concentration, of hexa- and octachlorodibenzo-*p*-dioxin in the commercial pentachlorophenol sample utilized in the toxicological studies reported herein are given in Table 3. Table 4 is a compilation of the toxicological data on this sample of

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nercial pentachlorophenol. A positive response was noted in both chick edema and rabbit ear bioassays. In the 90-day rat feeding study, untoward effects were noted in a majority of the parameters monitored. Hematological examination revealed a depression of erythrocytes, hemoglobin, and packed cell volumes at a dose level of 30 mg/kg-day pentachlorophenol. Clinical chemistry alterations included an elevation of serum alkaline phosphatase at 30, 10, or 3 mg/kg-day and a depression of serum albumin at 30 or 10 mg/kg-day. The weights of liver and kidneys were increased at 30, 10, or 3 mg/kg-day. Pathologic examination revealed minimal focal hepatocellular degeneration and necrosis at 30 mg/kg-day. Thus, it is evident that commercial pentachlorophenol induced untoward effects in each of the three toxicological tests.

Table 3. Concentrations of chlorinated dioxin "indicators" in commercial pentachlorophenol utilized in toxicological evaluations.

Chlorinated dioxin	Concentration, ppm
Octachlorodibenzo-*p*-dioxin	1980
Hexachlorodibenzo-*p*-dioxins	19

A chemically pure pentachlorophenol having no detectable concentrations of any chlorinated dioxins was subjected to the same toxicological tests. The toxicological data on this chemically pure pentachlorophenol, summarized in Table 5, include negative responses in both the chick edema and rabbit ear bioassays. In the 90-day rat feeding study, the only changes noted were increased liver weights at 30 or 10 mg/kg-day and increased kidney weights at 30 mg/kg-day. However, in contrast to commercial pentachlorophenol, gross and histopathological alterations did not accompany these increases in organ weights. Thus, by utilizing the results of these three tests, it may be concluded that the presence of the contaminants in commercial pentachlorophenol may be detected by toxicological evaluation.

An analysis of a sample of pentachlorophenol representative of that which we are capable of producing is shown in Table 6.

Table 4. Toxicological data on sample of commercial pentachlorophenol.

Study	Result [a]
Chick edema bioassay	+
Rabbit ear bioassay	+
Rat feeding study	
Food consumption	−
Body weight	−
Hematology	
30 mg/kg-day	+
10 mg/kg-day	−
3 mg/kg-day	−
Urinalysis	−
Clinical chemistry	
30 mg/kg-day	+
10 mg/kg-day	+
3 mg/kg-day	+
Liver weight	
30 mg/kg-day	+
10 mg/kg-day	+
3 mg/kg-day	+
Kidney weight	
30 mg/kg-day	+
10 mg/kg-day	+
3 mg/kg-day	+
Pathology	
30 mg/kg-day	+
10 mg/kg-day	−
3 mg/kg-day	−

[a] + denotes effect; − denotes no effect.

The toxicological data on this sample of pentachlorophenol are summarized in Table 7. Both the chick edema and rabbit ear bioassays gave negative responses. In the 90-day rat feeding study, the only unequivocal changes were increased liver weights at 30 or 10 mg/kg-day and increased kidney weights at 30 mg/kg-day. There were no gross or histopathological alterations noted.

To reiterate the toxicological findings on these three samples of pentachlorophenol, a comparison is provided in Table 8.

Commercial pentachlorophenol gave positive responses in both the chick edema and rabbit ear bioassays; in contrast, the chemically pure pentachlorophenol and the improved pentachlorophenol both gave negative responses in these bioassays.

In the rat feeding studies, commercial pentachlorophenol was associated with hema-

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Table 5. Toxicological data on sample of chemically pure pentachlorophenol.

Study	Results [a]
Chick edema bioassay	−
Rabbit ear bioassay	−
Rat feeding study	
Food consumption	−
Body weights	−
Hematology	−
Urinalysis	−
Clinical chemistry	−
Liver weight	
30 mg/kg-day	+
10 mg/kg-day	+
3 mg/kg-day	−
Kidney weight	
30 mg/kg-day	+
10 mg/kg-day	−
3 mg/kg-day	−
Pathology	−

[a] + denotes effect; − denotes no effect.

Table 6. Concentrations of chlorinated dioxin "indicators" in improved pentachlorophenol utilized in toxicological evaluations.

Chlorinated dioxin	Concentration, ppm
Octachlorodibenzo-*p*-dioxin	26
Hexachlorodibenzo-*p*-dioxins	1±0.1

Table 7. Toxicological data on sample of improved pentachlorophenol.

Study	Results [a]
Chick edema bioassay	−
Rabbit ear bioassay	−
Rat feeding study	
Food consumption	−
Body weights	−
Hematology	−
Urinalysis	−
Clinical chemistry	−
Liver weights	
30 mg/kg-day	+
10 mg/kg-day	+
3 mg/kg-day	−
1 mg/kg-day	−
Kidney weights	
30 mg/kg-day	+
10 mg/kg-day	−
3 mg/kg-day	−
1 mg/kg-day	−
Pathology	−

[a] + denotes effect; − denotes no effect.

tologic changes, clinical chemistry alterations, liver damage, plus liver and kidney weight increases at dose levels of 30, 10, or 3 mg/kg-day.

The results of the feeding studies with improved pentachlorophenol gave results which closely paralleled the results obtained with the chemically pure pentachlorophenol, wherein changes were limited to increased liver and kidney weights at the higher dose levels.

Table 8. Comparative evaluation of toxicological data obtained on pentachlorophenol (PCP) samples.[a]

Study	Commercial PCP	Chemically pure PCP	Improved PCP
Chick edema bioassay	+	−	−
Rabbit ear bioassay	+	−	−
Rat feeding study			
Hematologic depression	+	−	−
Clinical chemistry alterations	+	−	−
Liver damage (histopathology)	+	−	−
Liver weight increase			
30 mg/kg-day	+	+	+
10 mg/kg-day	+	+	+
3 mg/kg-day	+	−	−
Kidney weight increase			
30 mg/kg-day	+	+	+
10 mg/kg-day	+	−	−
3 mg/kg-day	+	−	−

[a] + denotes effect; − denotes no effect.

Discussion and Summary

The toxicological data have enabled us to conclude that a commercial pentachlorophenol conforming to the "improved" pentachlorophenol would not elicit chick edema and chloracne. In addition, the histopathological effects resulting from the impurities in commercial pentachlorophenol would be eliminated.

The toxicological findings discussed in this paper have been utilized as part of an application for registration of new pentachlorophenol. This application has been approved by the Environmental Protection Agency in accordance with the requirements of the Federal Insecticide, Fungicide and

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Rodenticide Act. The composition specifications for this new commercial pentachlorophenol are cited in Table 9.

In conclusion, it is feasible to produce a pentachlorophenol in commercial quantities which by comparative evaluations mimics pure pentachlorophenol in toxicological responses.

Table 9. **Composition and specifications of improved pentachlorophenol.**

	Content
Pentachlorophenol	88–93%
Tetrachlorophenol	12– 7%
Trichlorophenol	<0.1%
Higher chlorophenols	0.1%
Chlorinated dioxins	
Octachlorodibenzo-*p*-dioxin	30 ppm (max.)
Hexachlorodibenzo-*p*-dioxins	1.0 ppm (max.)

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Toxicity of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) in Aquatic Organisms*

by Richard A. Miller,† Logan A. Norris,‡ and Clifford L. Hawkes‡

Herbicides are particularly important in modern forest management as foresters attempt to make fullest use of a constantly shrinking production base. In forestry, (2,4,5,-T) 2,4,5-trichloro-phenoxyacetic acid is used to control undesirable woody species that compete with more desirable timber-producing conifers for light, space, moisture, and nutrients. Herbicide applications can result in markedly increased conifer growth, but such applications must not result in degradation of environmental quality (*1*). In evaluating hazards, scientists have focused on the herbicide, but biologically significant contaminants like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) must also be considered (*2*).

Herbicides may enter streams by several processes. Direct application or drift of spray materials to surface waters will occur only briefly during the application, but they may cause high concentrations of pollutant in streams. Herbicides could also move to streams in mass overland flow during periods of intense precipitation, but this seldom occurs on forest lands because the infiltration capacity of the forest floor is much greater than most rates of precipitation. Leaching through the soil profile is a slow process capable of only moving small amounts of herbicides short distances and offers little potential for serious stream pollution (*3*).

Studies in the Northwest indicate most contamination of forest streams by herbicides results from drift or direct application of chemical to the water surface. Detectable quantities of 2,4-D and 2,4,5-T have not been found in western streams during fall and winter months after spray applications to nearby forest lands the previous spring (*4*). In spray operations involving the use of 2,4,5-T, we expect small amounts of TCDD will enter the water with the herbicide during application. While some information is available on the toxicity of 2,4,5-T to aquatic organisms, little is known about the toxicity of TCDD.

We conducted chronic toxicity tests to assess the hazard to aquatic organisms which may be exposed to TCDD in water or food after the use of 2,4,5-T in forestry (*5*). Some of the toxic characteristics of TCDD in food and water to several major classes of aquatic organisms are reported here.

Materials and Methods

The organisms tested were three species of fish: guppies (*Poecilia reticulutas*), coho or silver salmon (*Oncorhynchus kisutch*),

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Table 1. Summary of test procedures to determine the toxicity of TCDD in aquatic organisms.

Expt. no.[a]	Organism	Container[b]	Water volume, l.	Water temperature, °C	Exposure regime: Level, Ppt in water	Exposure regime: Level, ng/g Bw[c]	Exposure regime: Duration, hr	Total observation period, days	Feeding regime	Experimental design[d]	Number of replications[e]
1[f]	Guppies (10–40 mm)	1-gal WMJ	3	20	0 100 1,000 10,000		120	37	Tubifix worms, *ad lib.* post exposure period	CRD	3 $n=20$
2	Snails (adult and juvenile)	1-gal WMJ	3	23–27	0 200		1152	48	Elodea, OMP[g] *ad lib.* during exposure period	CRD	4 $n=7$
3	Worms (40 mm)	8-in. culture dish	1	23–27	0 200		1176	55	OMP[g] 1/week, during exposure period	CRD	4 $n=20$
4	Mosquito larvae	8-in. culture dish	1	23–27	0 200		408	39	Yeast 2/week, during exposure period	CRD	4 $n=20$
5	Salmon (7.25 g wet weight)	5-gal WMJ	17	12–18	56 100 560 1,000	13.1 23.4 131.3 234.0	24 48 96	76	OMP[g] 3/week, post exposure period	SPF	4 $n=20$
6	Salmon (1.33 g wet weight)	5-gal WMJ	17	12–18	5.6 11.5 28.0 56.0	7.1 14.1 35.7 71.0	24 48 96	33	OMP[g] 3/week, post exposure period	SPF	4 $n=10$
7	Salmon (3.51 g wet weight)	5-gal WMJ	17	12–18	0.056 0.56 5.6 56.0	0.054 0.54 5.4 54.0	24 48 96	59	OMP[g] 3/week, post exposure period	SPF	4 $n=5$
8	Salmon, (TCDD recovery from water, 2.9 g wet weight)	5-gal WMJ	17	12–16	0 50		24 48 96	—	None	CRD	4
9	Rainbow trout	5-gal	18	11–13	2.3[h]	0.0063[i]	672	28	CDH[j] 2/day	CRD	5

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(1.6 g oven dry weight)[k]	aquaria	2,3 2,3	6.3 [i] 6,300 [i]	during exposure period

[a] Expts. 1–8 are static water tests. Salmon experiments (5–8) were all combinations of levels and durations of exposure listed.
[b] WMJ = wide-mouth jar.
[c] Nanograms TCDD per gram wet body weight.
[d] CRD = completely randomized; SPF = split plot factorial.
[e] n = beginning number of organisms per treatment per replication.
[f] Data of Norris and Miller (*10*).
[g] OMP = Oregon moist pellet.
[h] Concentration in food (dry weight).
[i] Weight TCDD tank per week, in nanograms.
[j] Casein, dextrose, herring oil, fish ration (*6*).
[k] Dry weight derived from size–weight relationship for young coho salmon (*7*).

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rainbow trout (*Salmo gairdineri*); and three aquatic invertebrates: a snail (*Physa* sp.); a worm (*Paranais* sp.); and mosquito larvae (*Aedes aegypti*). Guppies and mosquitoes were obtained from Oregon State University laboratory cultures, salmon and trout from State of Oregon fish hatcheries, and worms and snails from local streams.

The TCDD (98.7% 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) was obtained from the Dow Chemical Company.

Treatment regimes are summarized in Table 1 for each experiment. In static water test 1 with fish, we expressed exposure levels as nanograms (10^{-9} g) TCDD per gram total body weight of organism as well as in parts TCDD per 10^9 parts water. We do not imply the former are specific body burdens of TCDD but rather the amount of chemical in the container relative to fish biomass at the beginning of the experiment. In some cases, the initial TCDD concentration in the water is also given for reference, but these are of limited value in interpreting the static water toxicity test results because the TCDD concentration did not remain constant and cannot be related to organisms exposed in large bodies of water. We found dose–response relationships in fish were more easily expressed in terms of weights of toxicant and organism biomass.

Static Water Toxicity Tests

For static water toxicity tests, animals were acclimatized for at least 48 hr before they were exposed to TCDD in well water in glass containers (Table 2). TCDD in acetone (maximum 0.3 ml acetone/l.) was added slowly and mixed by stirring and vigorous aeration of water. Control organisms were exposed to an equivalent amount of acetone. At the end of the exposure period, the animals were placed in fresh well water containing no dioxin for the duration of the observation period. During the observation period, after TCDD exposure, the fresh water flow rate through the container with salmon was 3 l./hr. Water for guppies was exchanged once each 14 days. Salmon were weighed at the beginning of each experiment and at death. Guppy body length was measured at death. Oven-dry weights of worms were made at the end of the observation period.

Table 2. Characteristics of test water.

Constituent	Concn level, ppm	
	Static tests	Feeding tests
Calcium	12.0	14.0
Silica	6.9	27.0
Magnesium	7.8	5.1
Sodium	7.7	5.6
Potassium	1.45	0.6
Bicarbonate	79.0	77.0
Carbonate	0.0	0.0
Sulfate	5.1	
Chloride	4.3	4.0
Nitrate	6.1	0.3
Iron	0.03	0.32
Dissolved solids	132.0	95
Hardness	64.0	56.0
Specific conductance, μmko	164.0	132
pH	6.9	7.7

TCDD Recovery in Static Water Toxicity Tests

To determine TCDD recovery from water containing salmon, TCDD, 0 or 900 ng in 3.6 ml acetone, was added to 17 l. of well water containing 10 coho salmon averaging 2.9 g wet weight each. All conditions were as in experiments 5–8 (Table 1). A total of 12 containers were spiked with TCDD. Each container was sampled only once.

Water samples (1.8 l.) were collected 24, 48, and 96 hr after addition of chemical, and TCDD was determined by gas chromatography*. Each sampling time was replicated four times. Samples of water from containers with salmon but no dioxin were also analyzed to verify adequacy of the cleanup procedure.

*Slightly modified from "A tentative method for analysis of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in pond water." Personal communication, 3/12/71, Dow Chemical Company, Midland, Michigan. Details of analytical procedure available on request.

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Chronic Oral Toxicity Tests

For determination of chronic oral toxicity, we exposed young rainbow trout daily to various levels of TCDD in their food. TCDD, to 99% saturation in acetone, was added to the oil base of a casein-dextrose-herring oil fish food, slightly modified from Lee et al. (6). Acetone was removed from the oil by vacuum evaporation, leaving an average of 0.3% acetone in the dry fish food. The fish food contained 2.3 ppm, 2.3 ppb, or 2.3 ppt TCDD; exposure levels are in Table 1.

Two hundred young rainbow trout selected for uniformity of size were randomly assigned among 20 aquaria which received fresh water at the rate of 9 l./hr. The 20 aquaria were assigned at random among one control and three treatments in five replications. Fish were acclimatized to the aquaria–flowing water systems and TCDD-free food for 3 weeks before beginning the experiment. Preweighed food given daily at 0900 hr contained the daily dose of TCDD. At 1500 hr daily, food without TCDD was given *ad libitum*, and total daily consumption was recorded. To determine growth, fish were photographed once each week, and fish size index (the product of fish length and depth) obtained from a sideview photograph. In other experiments with coho salmon, sideview area was highly correlated with dry weight (7). We will establish a similar relationship for rainbow trout and express fish size in dry weight in later reports.

Results and Discussion

TCDD Recovery in Static Water Toxicity Tests

The TCDD level in water with young salmon declined significantly with time (Fig. 1). Regression analysis indicated recovery between 24 and 96 hr was linear with time (8):

$$Y = 63.1 - 13.5X$$

where Y is percentage recovery of TCDD, X is time in hours after addition of TCDD to water containing coho salmon; $r^2 = 0.86$.

TCDD concentration decreased more rapidly between 0 and 24 hr than between 24 and 96 hr, which may suggest more than one mechanism of loss was operative. The rapid loss of TCDD during the first 24 hr may largely be the result of adsorption phenomena which rapidly attain equilibrium. This hypothesis is supported by results from a similar test in which fish were not included and TCDD recovery was 60.0% 4 hr after addition of the chemical.

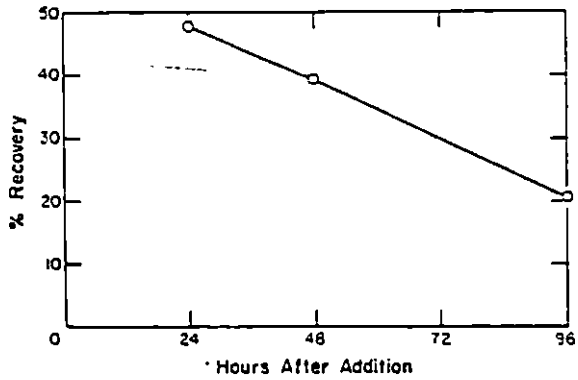


FIGURE 1. Average recovery of TCDD from water spiked with 50 ppt TCDD and containing 10 young coho salmon (four replications).

The fate of TCDD in the system is not known, but we suspect a combination of uptake by fish, adsorption on glass and suspended organic matter, and possibly loss due to aeration. Organisms in our static water toxicity tests were exposed to rapidly declining levels of TCDD because exposure solutions were not replenished. The exposure levels in Table 1 are the initial exposure levels, no adjustment being made for possible changes in TCDD concentration with time.

Fish Symptoms after TCDD Exposure

A difficulty in studying the toxicity of TCDD to fish is that the response to the chemical is not immediate. In most static water test procedures, observations would have been terminated after 96 hr (9). In our tests, initial response to the chemical did not occur for 5 to 10 days after the beginning of the exposure period, and mortality often extended over the next 2 months.

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Fish exposed to toxic levels of TCDD in water or food showed a declining interest in feeding. Salmon reduced feeding 8 days after TCDD exposure, while guppies responded in 5 days. Affected animals often spit food out shortly after taking it in. Growth of salmon exposed to TCDD in water was markedly inhibited (Fig. 2).

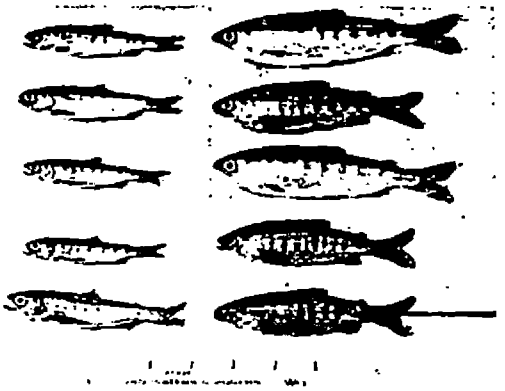


FIGURE 2. TCDD—exposed (13.1 ng/g, 96 hr) and control young coho salmon 80 days after beginning of exposure period.

Skin discoloration and fin necrosis began to appear 15 and 30 days after initial exposure of guppies and salmon, respectively (Fig. 2). Complete loss of the caudal fin occurred in both guppies and salmon. Areas showing skin discoloration often became the site of attack for disease organisms. In salmon, large fungal growths completely encircled some animals and inhibited swimming. Erosion of the upper jaw was seen in guppies surviving 1 to 2 months after exposure but not in salmon. Prior to death, fish often remained close to the bottom of the test containers and showed very little movement. There was no definite pattern prior to death; some fish that appeared perfectly healthy one day were dead the next day while other apparently diseased individuals remained alive for weeks. We detected no differences in behavior between treated and control invertebrate organisms.

Toxicity of TCDD in Water to Young Coho Salmon

Effect of level and duration of exposure—The deaths among exposed salmon frequently did not occur for 10 days after the beginning of the exposure period, regardless of exposure level (Fig. 3). In experiment 5, the effects of exposure to more than 23 ng TCDD/fish wet weight (23 ng/g) for 24 hr was irreversible, and most fish died within 60 days. The effects of level of exposure were quite marked while the effects of duration of exposure were less prominent.

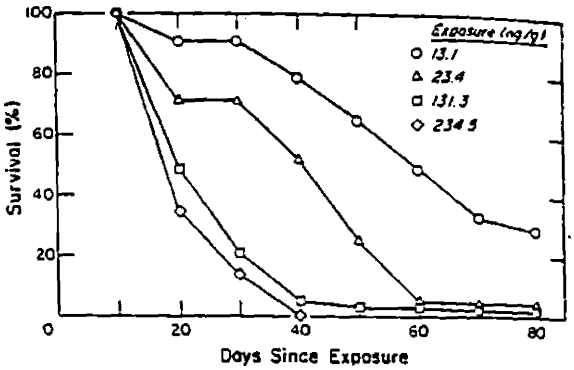


FIGURE 3. Survival of young coho salmon (experiment 5) after exposure to TCDD in water. Values are means for 24, 48, and 96 hr exposure (four replications).

In experiment 7, smaller salmon were used as we attempted to identify the minimum threshold response level for TCDD in water (Table 1). The pattern of delayed mortality observed in experiment 5 was also prominent in experiment 7 (Fig. 4). Exposure to TCDD levels of 54 ng/g for 24 hr or longer was irreversible and killed all fish within 40 days. Exposure to 5.4 ng/g resulted in 55% mortality during the 60-day observation period. Levels of TCDD as low as 0.054 ng/g caused 12% mortality in the 60-day exposure period compared to 2% mortality on controls. It appears these lower levels may be approaching the minimum threshold-response level. The duration of exposure appears less important than levels of exposure in determining mean survival time (Fig. 5).

For statistical analysis, data were expressed as days to death and subjected to multivariate analysis of variance. Mean survival time was significantly reduced with increasing TCDD exposure levels in experi-

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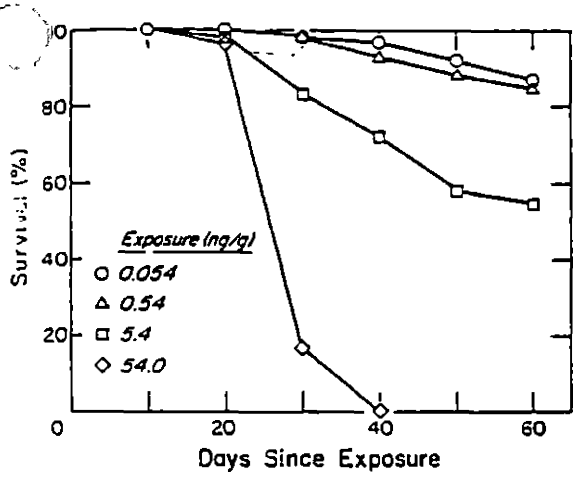


FIGURE 4. Survival of young coho salmon (experiment 7) after exposure to TCDD in water. Values are means for 24, 48, and 96-hr exposure (four replications).

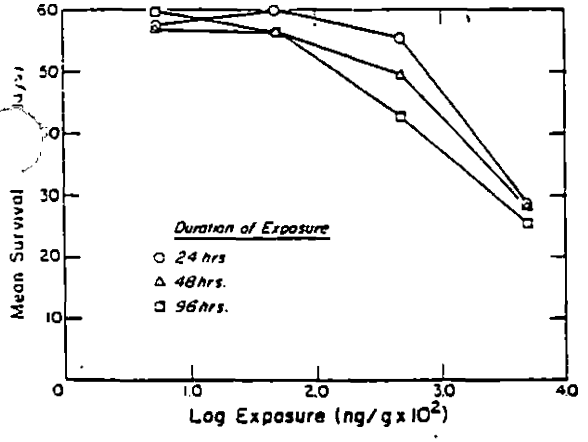


FIGURE 5. Influence of duration of exposure to TCDD on mean survival time of young coho salmon (experiment 7) (four replications).

ments 5, 6, and 7 ($P < 0.01$) Figs. 3 and 4). The duration of exposure effect was less marked, but was significant in experiments 5, 6, and 7 ($P < 0.05$). The duration of exposure-concentration interaction was not significant in any experiment. We feel the duration of exposure effect in salmon may be more pronounced as the minimum threshold–response level is approached and as the duration of exposure is reduced. A duration of exposure effect was not observed in guppies, but the levels of exposure were 2–20 times as great as with coho salmon in experiment 5.

Effect of size of fish on survival time—In both salmon and guppies, larger fish survived for longer periods than smaller fish after TCDD exposure. In some earlier work (*10*), mean survival time was plotted as a function of body length for TCDD exposed guppies ranging from 10 to 40 mm in length (Fig. 6). The regression equation was linear and highly significant ($P < 0.01$). Body length accounted for 93% of the variation of the dependent variable. A similar effect was observed in salmon when data from experiments 5, 6, and 7 were combined (Fig. 7). Time to 50% mortality for salmon exposed to 10 ng for 96 hr was determined graphically for each experiment. Regression analysis showed that the effect of body weight on survival time was linear and significant ($P < 0.01$):

$$Y = 13.8 + 7.7\,X$$

where Y is time, in days, to 50% mortality, X is body wet weight, in grams; $r^2 = 0.87$. Similar responses have been reported for other toxicants (*11*). The ability to tolerate environmental stresses increases with increasing body mass and age, up to a point, in many organisms. Toxicant uptake, storage, and detoxification probably change with fish age, lipid levels, and gill surface area–body mass ratios (*12*).

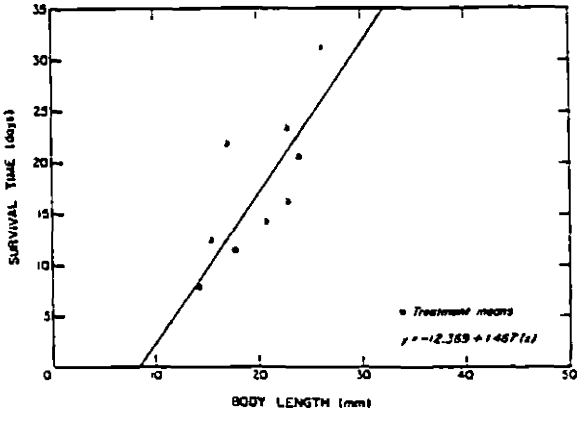


FIGURE 6. Effect of body length on mean survival time of guppies exposed to 100, 1,000, and 10,000 ppt TCDD for 120 hr (*10*) (three replications).

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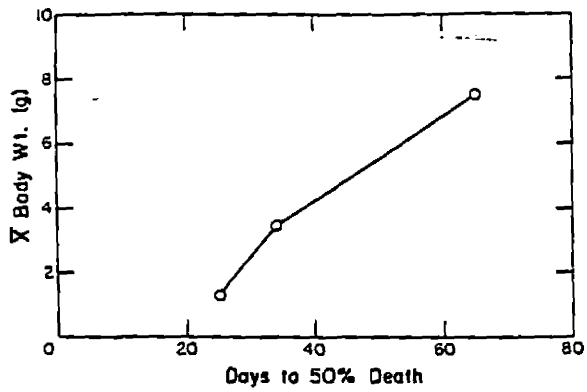


FIGURE 7. Effect of body weight on time to 50% mortality in young coho salmon exposed to 10 ng TCDD per gram wet body weight. Values are means for 24, 48, and 96 hr exposure (four replications).

Toxicity of TCDD in Water to Invertebrate Aquatic Organisms

In these tests, we exposed representatives from the class Insecta, a mosquito larvae; the class Oligochaeta, a worm; and the class Gastropoda, a pulmonate snail to TCDD in static water toxicity tests.

Toxicity to mosquito larvae—In tests with mosquitoes, we observed the maturation of larvae from the second instar through pupation during and after 17-day exposure in water which originally contained 0 or 0.2 ppb TCDD. There were no significant differences in total pupation or the rate of pupation among treated and control mosquitoes during the 30-day test period (Fig. 8).

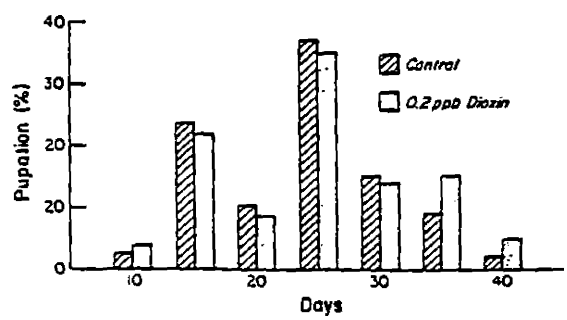


FIGURE 8. Pupation of mosquitoes exposed to 0 or 200 ppt TCDD for 17 days in water (four replications).

Toxicity to snails—Adult pulmonate snails deposited numerous egg cases in containers of well water which originally contained 0 or 0.2 ppb TCDD during a 36-day exposure period. Snail eggs completed development in the original exposure solution, and live juvenile snails and empty juvenile snail shells were counted 48 days after the beginning of the experiment. There was no significant difference between the survival of treated and control adult snails (Fig. 9).

Differences in the total snail hatch between treated and control organisms were observed in each replication, but variation among replications reduced the statistical sensitivity of these tests ($P=0.056$). Differences in the percentage survival of young snails were not significant. TCDD appeared to have its major impact on the reproductive success of snails rather than on survival of either adult or juvenile forms, in that the major effect was on total number of juvenile snail shells (Fig. 9).

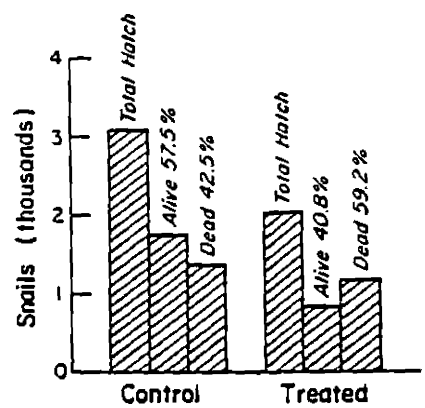


FIGURE 9. Total hatch and survival of juvenile snails from egg masses deposited in 0 or 200 ppt TCDD in water during a 36-day adult snail exposure period. Counts were made 48 days after the beginning of the exposure period.

Toxicity to aquatic worms—Adult Oligochaete worms were exposed to 0 or 0.2 ppb TCDD in water for 55 days. Animals were counted at 30, 48, and 55 days after the beginning of the exposure period. At 55 days, total and mean dry weights were determined.

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Table 3. Toxicity of TCDD in water to Oligochaete worms.

TCDD, ppt	Number of worms				Biomass, mg dry wt	
	Initial	30 days	48 days	55 days	Total at 55 days	Mean individual at 55 days
0	80	233	409	414	374	0.90
200	80	195	310	266	193	0.73

Exposure of worms to TCDD resulted in a decrease in the total number of worms present at the end of the 55-day exposure period ($P < 0.05$) (Table 3). Reductions in total worm biomass between treated and control organisms occurred in each replication, but variation among replications reduced the statistical sensitivity of this test ($P = 0.057$). TCDD exerted its principal effect on reproduction rather than growth of individual worms.

Toxicity of TCDD in Food to Young Rainbow Trout

Young rainbow trout (10/aquaria) were exposed to 0, 6.3 pg, 6.3 ng, or 6.3 μg TCDD per tank per week in food (Table 1). The TCDD-containing ration was offered each morning, and TCDD-free food was offered each afternoon. Survival was tallied daily, and growth was measured weekly.

There were no deaths among fish exposed to TCDD in the first 28 days of the experiment, but deaths began to occur in fish exposed to 6.3 μg TCDD per tank per week after 33 days of exposure. The appetite of fish receiving this dose began to decline after 10 days, and by 14 days fin necrosis was apparent. No loss of appetite or fin erosion occurred in fish exposed to lower levels of TCDD.

We observed no differences in the growth of fish receiving 0, 6.3 pg, or 6.3 ng TCDD per tank per week during the first 28 days of the experiment (Fig. 10). The growth of fish receiving 6.3 μg TCDD per week departed markedly from the others after 7 days, and they lost weight for the remaining 21 days of the exposure period. The data, expressed as the fish size index (product of fish length and body depth) were subjected to analysis of variance to test for differences among fish size after 28 days of exposure. There were no differences in the size of fish receiving 0, 6.3 pg, or 6.3 ng TCDD per tank per week. The difference between this group of fish and fish receiving 6.3 μg TCDD per tank per week was highly significant ($P < 0.01$).

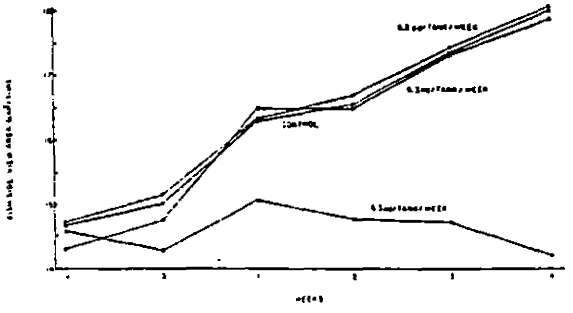


FIGURE 10. Average fish size index (length × body depth) of rainbow trout receiving TCDD in food daily (five replications).

Our data indicate TCDD in food can cause growth reduction and mortality in fish. The oral threshold response level for exposure periods up to 28 days is greater than 6.3 ng TCDD per tank per week. Additional experimentation is necessary to define more precisely the oral threshold response levels and to determine the impact of long-term chronic exposure in food to fish.

TCDD Residues in the Forest

TCDD residues have not been reported in either terrestrial or aquatic components of the forest, but we are not aware of any serious sampling efforts. 2,4,5-T and other herbicides have been reported in Northwest forest streams (4). Existing stocks of 2,4,5-T may contain up to 0.5 ppm TCDD, but new formulations must contain less than 0.1 ppm TCDD. We calculated the levels of TC-

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DD which might be in forest streams after the aerial application of 2,4,5-T assuming the principal route of entry was drift or direct application of spray materials to the stream surface. Levels of 2,4,5-T are not expected to exceed 0.1 ppm if applications are carefully controlled (Table 4). A more complete determination of threshold response levels will be required, however, before an adequate assessment of TCDD hazard to stream organisms can be made.

Conclusions

TCDD in water or food is toxic to fish. The effects of exposure for 24–96 hr of young salmon to TCDD in water at levels greater than 23 ng/g is irreversible, and death results in 10–80 days. Duration of exposure is less important than level of exposure except as threshold response levels are approached. The critical exposure period may be somewhat less than 24 hrs in static water toxicity tests in which TCDD concentration may change markedly with time. Small fish are more sensitive than large fish on an equivalent exposure level basis. TCDD in food at 2.3 ppm markedly reduced growth of young rainbow trout (10/aquaria) exposed to 6.3 μg TCDD per tank per week for 4 weeks. TCDD at 0.2 ppb had no effect on pupation of mosquito larvae, but reduced the reproductive success of a pulmonate snail and an Oligochaete worm.

Table 4. TCDD in streamwater after aerial application of 2,4,5-T to forest land.

2,4,5-T in streamwater, ppm	Anticipated TCDD in streamwater, ppt	
	Level 1[a]	Level 2[b]
1.0	0.5	0.1
0.1	0.05	0.01
0.05	0.025	0.005
0.01	0.005	0.001
0.005	0.0025	0.0005

[a] Level 1: 2,4,5-T contains 0.5 ppm TCDD.
[b] Level 2: 2,4,5-T contains 0.1 ppm TCDD.

Our research has established some important toxicity characteristics of TCDD in fish, but considerable work remains to be done. Establishment of minimum threshold response levels during long- and short-term exposure are important. The impact of previous and current TCDD exposure on long-term growth and reproduction of fish needs attention. Information on its movement, persistence, and fate of TCDD in aquatic systems will be required to adequately assess the impact of TCDD in streams. Serious attempts to determine TCDD residues in various parts of the natural aquatic ecosystem are badly needed. The most sensitive analytical techniques and positive means of residue identification will be necessary.

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Acute and Chronic Oral Toxicity of Chlorinated Dibenzofurans to Salmonid Fishes

by V. Zitko,* D.J. Wildish,* C. Hutzinger,† and P.M.K. Choi‡

A median mortality of 120 ± 30 days occurred among juvenile Atlantic salmon (*Salmo salar*), fed dry fish food containing ..7, 5.7, 2.8, and 9.1 μg/g wet weight of 2,8-, tri-, tetra-, and octachlorodibenzofuran, ctively (*1*). Only octachlorodibenzofuran was detected in the dead fish, and the level was 0.03 μg/g in the muscle and 0.21 μg/g in the gut (both values on wet weight basis). The fate of the lower chlorinated dibenzofurans was not known and additional experiments, described in this paper, were carried out with immature brook trout (*Salvelinus fontinalis*), fed relatively high levels of 2,8-dichlorodibenzofuran.

Experimental

The preparation of 2,8-dichlorodibenzofuran was carried out as described (*2*). Three crystallizations were necessary to remove tri- and tetrachlorodibenzofuran from the product.

Ten immature brook trout, tagged with colored tags were kept in running water (2 l./min) in a 200-l. Fiberglas tank and fed twice a day commercial fish food (Trout Chow, Ralston Purina Company) in the form of floatable pellets and in clear No. 3 gelatin capsules (E. Lilly & Company). Both pellets and capsules contained approximately 100 mg of food. Three fish, each fed on the average 700–800 mg of food per day, also received gelatin capsules containing crystalline 2,8-dichlorodibenzofuran. Once crystallized product, still containing a trichlorodibenzofuran and a small amount of a tetrachlorodibenzofuran was fed to one fish. By accident, one fish from the former group received one capsule containing the mixture of chlorinated dibenzofurans. The feeding schedule of 2,8-dichloridibenzofuran and the initial and final weights of the fish are presented in Figure 1. The fish were sacrificed as indicated and kept frozen (−14°C) until analysis. Fish R was isolated after the final capsule (10.3 mg) in a small tank containing 4 l. of water. Water was changed after 24, 48, 72, and 144 hr and processed as described (*3*) to detect possible hydroxylated metabolities of 2,8-dichlorodibenzofuran.

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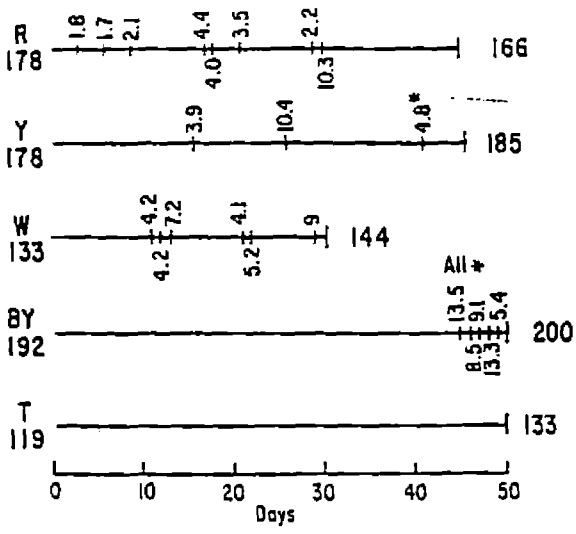


FIGURE 1. Feeding schedule. Initial and final weights (g) of fish are on the left and right margin, respectively. Administered amounts of 2,8-dichlorodibenzofuran (mg) are indicated by vertical lines. The asterisks (*) indicate administration of once-crystallized 2,8-dichlorodibenzofuran containing tri- and tetrachlorodibenzofuran.

Samples of the white lateral muscle, taken between the dorsal fin and the lateral line, and whole livers were extracted with hexane and the extracts were cleaned up by column chromatography on alumina and silica (*4*). Polychlorinated biphenyls (PCBs) were separated from chlorinated dibenzofurans by column chromatography on alumina (*5*) Gas chromatographic analysis was performed as described (*4*). A DuPont CEC 21-110B mass spectrometer was used to record mass spectra. Lipid concentrations refer to hexane-extractable lipid.

Results and Discussion

The levels of PCBs and lipid in the tissues, liver weight, and sex of the fish are summarized in Table 1. PCBs were of the Aroclor 1254 variety and had to be separated before the quantitation of chlorinated dibenzofurans. As an example, the GLC patterns of the Y liver extract are presented in Figure 2. The tracing *A* was obtained on the hexane eluate from the silicia column. After the removal of PCBs in the first fraction from the alumina column (2% methylene chloride in hexane, tracing *B*),

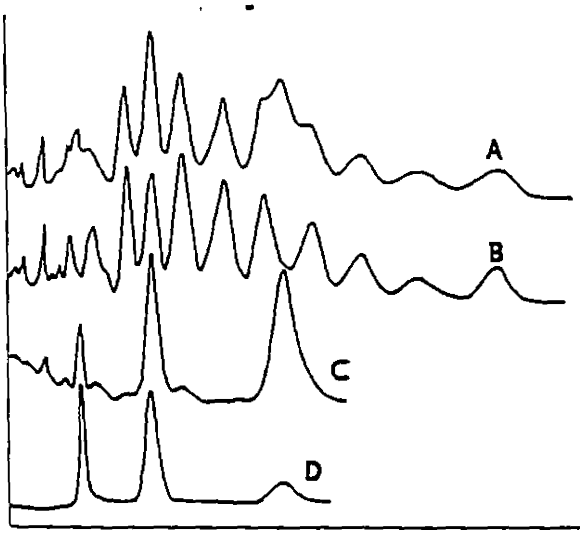


FIGURE 2. GLC patterns of Y liver extract: (*A*) hexane eluate from the silica column, containing a mixture of PCBs and chlorinated dibenzofurans and separated by chromatography on alumina to yield the PCB fraction (*B*) and the dibenzofuran fraction (*C*); (*D*) GLC pattern of once-crystallized 2,8-dichlorodibenzofuran (first peak from the left), containing tri- and tetrachlorodibenzofuran (second and third peaks, respectively).

Table 1. Chemical and biological characteristics of fish.

Fish	PCBs, μg/g wet weight*		Liver weight, % of body weight	Lipid, %		Sex (immature)
	Muscle	Liver		Muscle	Liver	
R	0.05	0.25	0.75	0.32	3.05	Male
Y	0.05	0.34	3.12	0.40	14.45	Male
W	0.15	0.35	1.86	2.98	8.01	Male
BY	0.08	0.12	1.77	0.42	2.71	Female
T	0.06	0.33	1.96	0.67	8.60	Female

* Aroclor 1254.

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Table 2. Relative accumulation of 2,8-di-, tri-, and tetrachlorodibenzofurans.

Sample	Peak height ratios		
	2,8-Dichlorodibenzofuran	Trichlorodibenzofuran	Tetrachlorodibenzofuran
Fed preparation	1	0.92	0.18
Y muscle	1	0.40	0
Y liver	1	2.06	1.87
BY muscle	1	0.87	0.28
BY liver	1	1.04	0.36
T liver	1	1.11	1.16

chlorinated dibenzofurans appeared in the second fraction (20% methylene chloride in hexane, tracing *C*). The tracing *D* of the once-crystallized 2,8-dichlorodibenzofuran is presented for comparison.

Table 3. Administered and tissue levels of 2,8-dichlorodibenzofuran.

Fish	2,8-Dichlorodibenzofuran, μg/g wet weight		
	Administered	Found	
		Muscle	Liver
R	361	0.052	0.307
Y	107	0.048	0.150
W	254	0.340	0.400
BY	260 *	0.230	1.04
	—	—	0.146

* ce-crystallized preparation

It can be seen that the relative concentration of the tri- and, particularly, of the tetrachlorodibenzofuran is higher in the isolated mixture than in the administered preparation. The relatively higher accumulation of these compounds was observed in both fish Y and BY, and also in the liver of fish T, which was not actually fed chlorinated dibenzofurans, but was present in the tank during the feeding of all fish (Table 2).

The administered doses of 2,8-dichlorodibenzofuran and the detected tissue residues are presented in Table 3. In all cases the tissue residues are very low, 0.01–0.13% of the administered dose in the muscle and 0.08–0.4% in the liver. The feeding history affects the residual levels. Thus fish W, sacrificed 1 day after dosing contains higher levels of 2,8-dichlorodibenzofuran than fish R, which received a similar last dose and a higher total dose, but was sacrificed only 15 days after the last dose.

Mass spectra of the crude mixtures of organic compounds isolated from the water, containing excreta of fish R, indicate the presence of a dichlorodibenzofuran (M^+ 236) and of a hydroxydichlorodibenzofuran (M^+ 252).

According to the feeding data, 2,8-dichlorodibenzofuran has a low acute toxicity to immature brook trout, since no mortality resulted even after administering a single dose at a level as high as 122 mg/kg. The low acute toxicity and residual levels of 2,8-dichlorodibenzofuran may be due to the poor absorption of the compound in the gut and to its excretion in the form of a conjugated hydroxy derivative.

Acknowledgement

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The Search for Chlorinated Dibenzofurans and Chlorinated Dibenzodioxins in Wildlife Populations Showing Elevated Levels of Embryonic Death

by Gerald W. Bowes,* Bernd R. Simoneit,† A.L. Burlingame,† Brock W. de Lappe,‡ and Robert W. Risebrough*

Introduction

Various kinds of reproductive abnormalities have been documented among wildlife species in North America. All are associated with areas of high agricultural or industrial activity and have been shown, or are assumed to be, pollutant-induced. Selected wildlife populations may therefore serve as the best indicators of the presence in the environment of compounds which have deleterious effects on organisms at very low concentrations, such as several of the chlorinated dibenzo-*p*-dioxins and chlorinated dibenzofurans.

The best documented of the abnormalities observed in wildlife is the shell thinning of eggs of raptorial and fish-eating species of birds. Contemporary samples show a significant increase in the variance of such parameters as shell weight, shell thickness and an index of shell thickness when compared with samples obtained prior to 1940 (*1*). Most if not all of the increased variance can be explained, however, by a function of the concentration of the DDT compound 1,1-dichloro-2,2-bis(*p*-chlorophenyl)-ethylene (*p,p'*-DDE) (*2*).

Embryonic mortality is reducing the reproductive success of several bird species, including the merlin, *Falco columbarius*, and prairie falcon, *Falco mexicanus*, of western Canada (R. Fyfe, personal communication), the osprey, *Pandion haliaetus*, of the northeastern United States (P. Spitzer, personal communication), the herring gull, *Larus argentatus*, of the Great Lakes region (*3*), and the common terns, *Sterna hirundo*, of Lake Ontario (M. Gilbertson, personal communication). In 1972 virtually no young hatched in the colonies of the latter two species on Lake Ontario (M. Gilbertson, personal communication). The embryotoxicity of commercial PCB has been attributed to chlorinated dibenzofuran contaminants in the preparations (*4–6*). These compounds, associated with or derived from PCB, are therefore a possible cause of the embryonic death observed in wild bird populations. Other

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pollutants which could contribute to the mortality, including the organochlorine insecticides and their derivatives, are invariably present in these samples; some of the mortality could also be an indirect result of the DDE-induced shell thinning.

A low incidence of birth defects has been documented in a colony of common terms in Long Island Sound (*7*). A proportionately larger number were found in 1972 in a common tern colony in Lake Ontario (M. Gilbertson, personal communication). The same kinds of birth defects—primarily those of the beak, eyes and feet—have been produced experimentally by the chlorinated dibenzodioxins (*8*).

The sea lions, *Zalophus californianus*, inhabitating the coastal waters of southern California, have shown an incidence of premature births in recent years which is judged to be substantially higher than the expected normal. A group of females giving birth to premature pups in 1970 had higher levels of both the DDT and PCB compounds than did a group of full term parturient females (*9*). There is no evidence of fetal death in this population; rather, the abnormality consists of a premature onset of partus. It is therefore unlike the fetal mortality and resorption observed among domestic mink, *Mustela vison*, that are fed either coho salmon, *Oncorhynchus kisutch*, obtained from Lake Michigan, or a diet supplemented with the commercial PCB preparation Aroclor 1254 (*10*). The pattern of fetal mortality and resorption observed among the mink is very similar, however, to that found in female rats on a diet containing 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (*11*). PCB levels in the sea lion population of southern California are among the highest found in North American mammals (*9*): they therefore provide favorable material in which to look for the associated chlorinated dibenzofurans.

Chlorinated dibenzodioxins and dibenzofurans were looked for in tissues of marine fish and birds from the Bay of Fundy and were not detected (*12*). The detection limits, however, were substantially above those known to be toxic to either rats (*11*) or chicks (*8*).

In the current study we are examining the hypothesis that several kinds of abnormalities, as yet unexplained, may be caused by chlorinated dioxins or benzofurans that have accumulated in the aquatic food webs of polluted areas; the initial results are reported in the present paper.

Materials and Methods

The initial material selected for examination consisted of a pooled collection of 45 herring gull eggs and pooled samples of sea lion blubber and liver. The herring gull eggs were obtained in the spring of 1972 from Scotch Bonnet island in Lake Ontario. Very few of the eggs laid in this colony have hatched in recent years (M. Gilbertson, personal communication). The sea lion samples had been obtained from nine females that had just given birth to premature pups on San Miguel Island, California, in the spring of 1972, during a study undertaken by the U.S. National Marine Fisheries Service, Seattle, and the Naval Underseas Center, San Diego.

Preparation of Standards

Clophen A-60, a German PCB preparation containing 60% chlorine, was selected as a source of chlorinated dibenzofurans on the basis of the earlier work by Vos et al. (*5*). A subsample of the Clophen A-60 in which the chlorinated dibenzofurans had been detected (Lot No. 912434) was provided to us by J. G. Vos. The first phase of the isolation procedure followed is similar to that outlined by Vos et al. (*5*). A 550-mg portion of the Clophen was dissolved in 400 ml of hexane and added to a Florisil column in 50-ml portions. The Florisil was activated and freed of contaminants by methods previously described (*13*). Each portion was allowed to sink to the surface at an elution rate of 5–10 ml per minute. The Florisil (180 g) was packed in a glass column with a Teflon stopcock and an outside diameter of 44 mm. As the last portion of the PCB solution eluted to the Florisil surface, a 400-

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portion of hexane was added to the column. The first 50-ml portion was used to rinse the walls and was allowed to elute to the surface as before. The hexane was followed successively by 400-ml portions each of 5% diethyl ether–hexane, 25% diethyl ether–hexane, and acetone. A cleaner preparation of the chlorinated dibenzofurans isolated from Clophen has been found in an acetone fraction after elution with 25% diethyl ether–hexane (J.G. Vos, personal communication).

The diethyl ether and acetone fractions were evaporated just to dryness under a stream of dry nitrogen, and the residue taken up in hexane. This step was repeated twice. Each of the three fractions was then placed on a micro activated alumina column (Fisher alumina, Cat. No. A-540), to further separate the suspected contaminants from PCB (*14*). In order to remove all of the PCB, the volume of the first eluting solvent system, 1% methylene chloride–hexane, was increased to 15 ml, and that of the second, 20% methylene chloride–hexane, 10 ml. Chlorinated dibenzo-dioxins appear in the latter fraction, and it is probable that the chlorinated dibenzofurans do as well (M.L. Porter and J.A. Burke, personal communication).

This fraction was then evaporated just to dryness, and the residue taken up in hexane. This step was repeated twice. Gas chromatographic analysis and fraction collection for subsequent mass spectrometric identification of components is described below.

Preparation of Environmental Samples

The herring gull eggs were individually weighed, the length and breadth measured for subsequent shell thickness index determination, and the contents pooled and freeze-dried for 5 days. Total wet weight was 3121 g, and total freeze-dried weight 567 g. The dried eggs were ground with sodium sulfate (2:1) in a heavy glass mortar, and an appropriate amount was placed in a glass thimble for extraction in a Soxhlet apparatus (extra-large size, Corning Glass Works No. 415070) fitted to a 2000-ml flask containing 1400 ml hexane–acetone azeotrope. Each portion was extracted for at least 8 hr. This procedure was repeated until the entire sample was extracted. The original solvent was left in the flask and was used for the extraction of all portions. The total extraction period was 119 hr.

The solvent was then concentrated with the use of a rotary evaporator, transferred to a 1000-ml graduated cylinder, and the volume adjusted to 1000 ml with hexane. Lipid determinations were then made. Three 3-ml aliquots were transferred from the cylinder to each of three preweighed aluminum pans (3.0 ml volume in a 5.0-ml disposable pipet), allowed to air-dry, then placed in a 100°C oven for 30 min. After cooling, the dishes containing the lipid were weighed. The total lipid recovered was 250 g.

The clean-up procedure used on the remaining sample extract was adapted from that detailed in Stanley and LeFavoure (*15*). The extract was taken from the graduated-cylinder in four 200-ml portions with a 50-ml volumetric pipet. Each of the four 200-ml portions was passed over 350 g of Celite–sulfuric acid in a Buchner funnel, followed by three 500-ml elutions with hexane. The cleaned extract was concentrated for subsequent fractioning into various chlorinated hydrocarbon components (see below).

The maximum amount of lipid placed on each Davidow column of unit 15 gCelite–9 ml sulfuric acid-9 ml fuming sulfuric acid was kept to 4 g.

Sea lion liver samples from nine females were weighed, pooled, and freeze-dried for 5 days. The total wet weight was 425.1 g, and the total freeze-dried weight was 121.4 g. The dried samples were ground with sodium sulfate (1:8), and a portion placed in a Soxhlet apparatus for extraction. Conditions were similar to those outlined above for herring gull eggs. The total extraction time for all portions was 72 hours. Solvent was concentrated as above, and aliquots taken for lipid determination. The lipid recovery was 16.6 g. The remainder was divided into two portions. Each was passed over 60 g of Celite-sulfuric acid, followed

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by three 150 ml elutions with hexane. The cleaned-up extract was concentrated for further fractionation and analysis.

Sea lion blubber samples were from eight of the nine females sampled for liver. The total wet weight was 493 g. Small slices were ground with sodium sulfate (1:6), and the entire mixture was transferred to a Soxhlet apparatus (giant size, Ace Glass, Inc., Vineland, N.J., Cat No. 6810, size H; thimble, Cat. No. 6812, size H). The sample was extracted for 4 days with 6300 ml hexane–acetone azeotrope placed in the 12000-ml flask. The extract was concentrated, aliquots taken for lipid determination, and one quarter of the total was cleaned up and prepared for subsequent fractionation and analysis. The total lipid recovery was 420 g.

Fractionation of Biological Extracts

Each of the three extracts (herring gull eggs, sea lion liver, and sea lion blubber) was divided in two, and each portion was placed on a large Florisil column (180 g) and fractionated by elution with the four solvent systems described for the Clophen, except that the volume of hexane was increased to 800 ml. Subsequent fractionation on the alumina column did not remove enough PCB interference in the 20% methylene chloride eluate. Chlorinated residues from this eluate (dissolved in hexane) were then placed on a smaller activated Florisil column (8 g, 10 mm ID), and eluted with smaller volumes of the same solvent sequence: 150 ml hexane, 200 ml 5% diethyl ether-hexane, 200 ml 25% diethyl ether–hexane, 200 ml acetone. These fractions, evaporated to dryness and taken up in hexane, were then placed on alumina columns and chlorinated hydrocarbon components separated as before.

Gas Chromatography

Extracts were analyzed with the use of an electron-capture (EC) ^{63}Ni Tracor MT-220 gas chromatograph. The stationary phase was 3% OV-1 on 100-120 mesh Supelcoport, placed in a 6 ft × 4 mm. I D Pyrex column. Other operating parameters included: oven temperature, 200° C; inlet temperature, 225° C; detector temperature, 285° C; column flow rate 60 cc/min; purge flow rate, 50 cc/min; carrier and purge gas, nitrogen.

Several of the extracts were further fractionated by collection of individual peak components with the use of a 10:1 splitter (Western Scientific, Danville, California). Glass capillary tubes, bent to a V-shape, were immersed in a beaker of liquid nitrogen during collection of the peak components through a septum.

Mass Spectrometry

The samples were dissolved in 5 μl of methylene chloride and transferred onto the direct inlet probe of the mass spectrometer, where the solvent was allowed to evaporate. The probe was rapidly introduced into the ion source and multiple scans were recorded. Both low and high resolution mass spectrometric analyses were carried out on a GEC-AEI MS-902 mass spectrometer. For high resolution the instrument was used on line to an XDS Sigma 7 computer as described by Burlingame (*16*, *17*) and Burlingame et al. (*18*). The ion source operating conditions were: resolution, 10,000; ionizing current, 500 μA; ionizing voltage, 50 eV; temperature, 200–220°C. The scan rate was 16 sec per decade with a clock rate of 24 kHz. Multiple scans were taken during each analysis and then sum-averaged together during data reduction. For low resolution the instrument was scanned either on line to an XDS Sigma 2 computer (*19*) or manually with an ultraviolet-sensitive strip-chart recorder.

Results and Discussion

The pooled sample of herring gull eggs was found by GC-electron capture analysis to contain 35 ppm DDE and 300 ppm PCB on a wet weight basis or 440 ppm and 3700 ppm, respectively, on a lipid basis. The PCB content was therefore approximately 0.9 g. The sea lion liver sample contained 12 ppm DDE and 3 ppm PCB on a wet weight basis or 300 ppm and 80 ppm, respectively, on a lipid basis. The PCB content of the sample was therefore approximately 1 mg. DDE

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PCB-concentrations in the blubber were 42 and 62 ppm on a wet weight basis, 630 and 76 ppm on a lipid basis. The PCB content of the entire sample was therefore 30 mg, of which 7.5 mg was used for the extraction process.

The electron capture chromatogram of the 20% methylene chloride–hexane extract of Clophen A-60 is shown in Figure 1. The components of peaks A–G were collected for individual analysis by low resolution mass spectrometry. Retention times of the peaks relative to dieldrin, and the identity of the compounds identified are listed in Table 1. All of the peaks were identified as chlorinated dibenzofurans; no other significant components were detected. Compounds having the same retention time relative to dieldrin as peaks A and C have previously been identified by Vos et al. (*5*) as tetrachloro- and pentachlorodibenzofuran, respectively. In the present study, peak B, consisting of two components on the EC gas chromatogram, was identified as tetrachlorodibenzofuran,

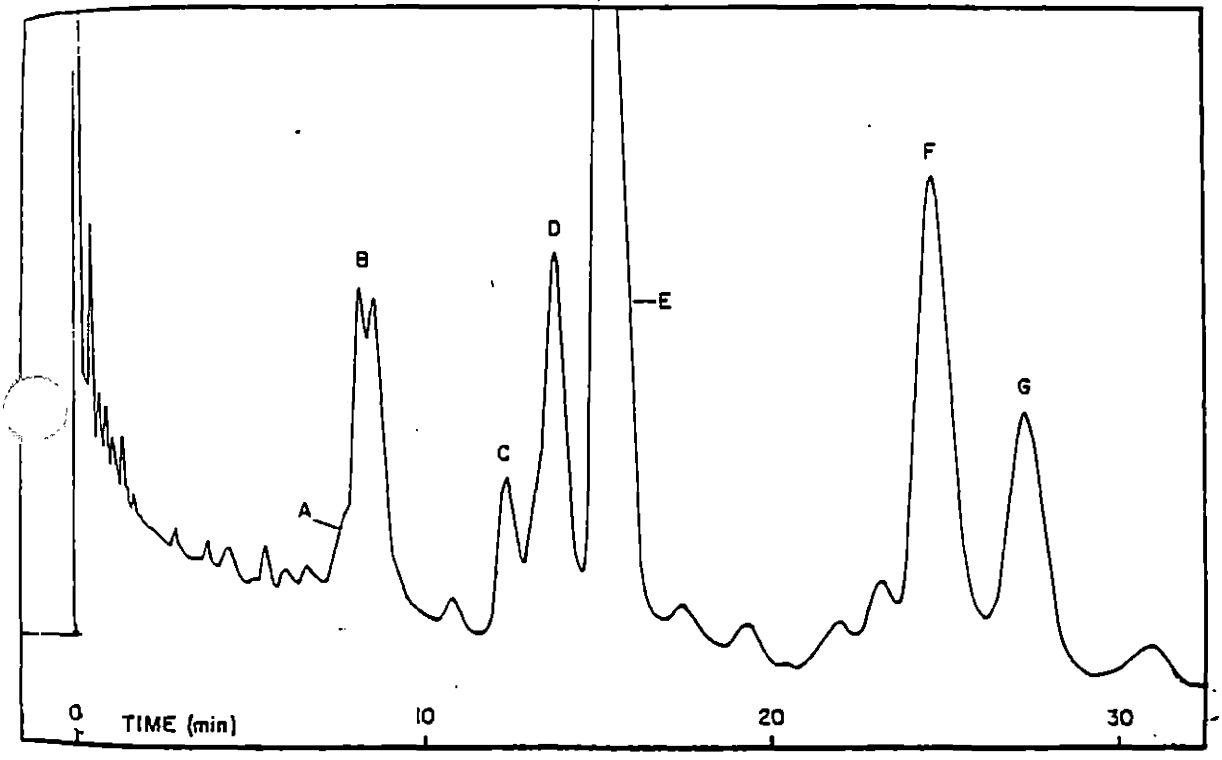


FIGURE 1. Gas chromatogram 3% OV–1 of Clophen A–60, 20% methylene chloride-hexane fraction.

Table 1. Relative retention times and identification of chromatograph peaks shown in Figure 1.

Peak	Retention time, relative to dieldrin	Composition	Mass		
A	1.49	$C_{12}H_4O^{35}Cl_4$	304	Tetrachlorodibenzofuran	
B	1.60	"	"	"	"
C	2.38	$C_{12}H_3O^{35}Cl_5$	338	Pentachlorodibenzofuran	
D	2.64	"	"	"	"
E	2.95	"	"	"	"
F	4.73	$C_{12}H_2O^{35}Cl_6$	372	Hexachlorodibenzofuran	
G	5.24	"	"	"	"

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probably isomers of peak A. In addition, peaks D and E were identified as pentachlorodibenzofurans and F and G as hexachlorodibenzofurans. Representative mass spectra of a tetra- (B), penta- (E), and hexachlorodibenzofuran (F) are shown in Figure 2. Tetrachlorodibenzofuran fragments were as follows (cf. Fig. 2*a*): the molecular ion M·

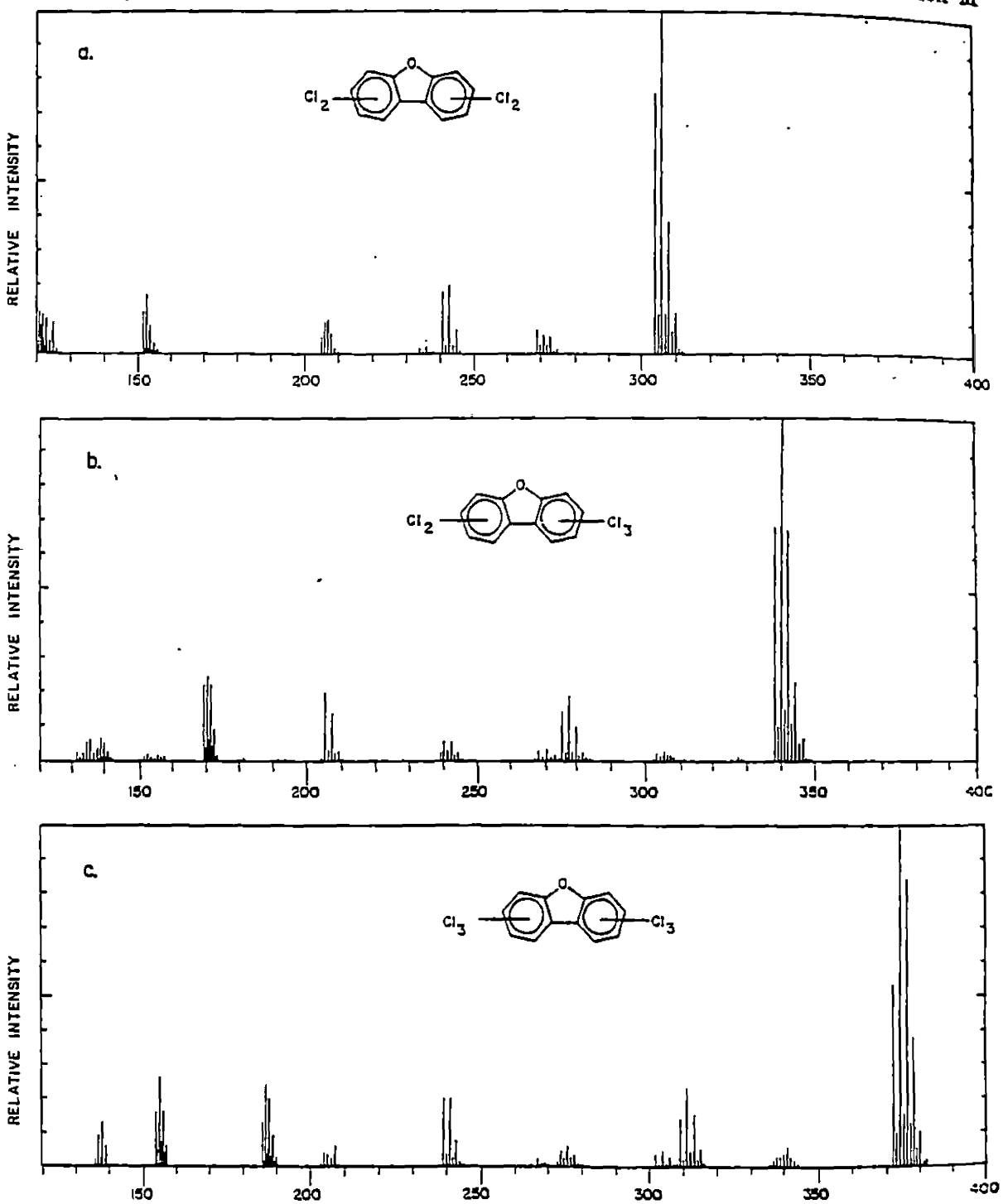


FIGURE 2. Low-resolution mass spectra of compounds isolated from Clophen A–60: (*a*) tetrachlorodibenzofuran; (*b*) pentachlorodibenzofuran; (*c*) hexachlorodibenzofuran.

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found at *m/e* 304–312, loss of Cl yields the peaks at *m/e* 269–275, subsequent elimination of CO results in *m/e* 241–245 and double Cl loss yields the minor peaks at *m/e* 23[illegible]–238, which on loss of CO and CHO yield the group at *m/e* 205–210. The doubly charged species of the molecular ion are found at *m/e* 152–156, and the strong loss of Cl followed by CO is confirmed by the doubly charged peaks at *m/e* 120–122. The pentachlorodibenzofuran and the hexachlorodibenzofuran fragment were identified in an analogous fashion as described above (cf. Figs. 2*b* and *c*, respectively).

The elemental composition of these compounds was confirmed by high-resolution mass spectrometric analysis (*20, 21*) of the total mixture. Peaks of the homologous series $C_{12}H_{8-n}OCl_n$ were only detected for $n=4$–6, and other halogenated compounds (e.g., chloronaphthalenes) were present as trace constituents.

Sixteen major peaks were present on the EC chromatogram of the second 5% diethyl ether–hexane fraction of the gull eggs, derived from the first 5% diethyl ether–hexane fraction eluted from the large Florisil column. Heights of these peaks were comparable to those of the Clophen A-60 peaks shown in Figure 1. Extract volumes and volumes injected into the gas chromatograph were also comparable. This extract was therefore chosen for analysis by low resolution mass spectrometry. The components of all 16 peaks were isolated. Retention times relative to dieldrin on the OV-1 column were: A, 1.38; B, 1.56 and 1.73; C, 2.14; D, 2.32; E, 2.52; F, 2.72; G, 3.14; H, 3.66; I, 4.21; J, 4.65; K, 4.98; L, 5.20; M, 5.95; N, 6.75; and O, 8.20. No chlorinated dibenzofurans were detected. The majority of the peaks represented chlorine-containing compounds with mass numbers from 362 to 508 that have not yet been identified.

Peak G was subjected to high-resolution mass spectrometric analysis (HRMS) and was found to be mainly a hexachloronaphthalene ($C_{10}H_2{}^{35}Cl_6$, *m/e* 331.8252; $C_{10}H_2{}^{35}Cl_5{}^{37}Cl$, *m/e* 333.8275; $C_{10}H_2{}^{35}Cl_4{}^{37}Cl_2$, *m/e* [illegible].8202) Fragment ions due to loss of two Cl where also present in these data.

Peak N was also subjected to HRMS analysis. A group of intense peaks was observed at *m/e* 436.8744, 438.8743, 440.8758, 442.8733, and 444.8622. The compound structure has not yet been identified.

The entire 5% diethyl ether–hexane fraction of the gull eggs that had been derived from the initial acetone fraction was examined by HRMS. No chlorinated dibenzofuran or dibenzodioxin compounds were detected.

None of the appropriate sea lion liver extracts produced EC peaks of sufficient height to merit analysis by mass spectrometry. Components of individual peaks of the 5% diethyl ether–hexane fraction of the initial acetone fraction, and of the 25% diethyl ether–hexane fraction derived from the initial 25% diethyl ether–hexane fraction of the sea lion blubber, were isolated for low resolution mass spectrometry; a portion of the latter extract was also analyzed by HRMS. No chlorinated compounds of interest were detected, in part because of the high hydrocarbon content of the samples.

A more exhaustive examination of these and other wildlife samples is evidently required to permit a conclusion about the presence of either chlorinated dibenzodioxins or chlorinated dibenzofurans in the North American environment.

Acknowledgement

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TCDD-Induced Changes in Rat Liver Microsomal Enzymes

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Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a contaminant of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), is extremely toxic (*1*), although the mechanism of toxicity is not known. Other papers presented at this conference cover the spectrum of environmental and health hazards of chlorinated dibenzodioxins and dibenzofurans. It should suffice to say here that these compounds are teratogens (*2–4*) in rodents, and the extensive use of 2,4,5-T, especially in Vietnam, has focused concern on their potential health hazards. Recently TCDD was shown to be an inducer of δ-aminolevulinic acid synthetase in the chick embryo (*5*) and also to decrease hexobarbital sleeping times in rats (*6*). These reports prompted us to investigate the effects of sublethal doses of TCDD on activities of hepatic microsomal and mitochondrial enzymes. The microsomal enzymes include components that are involved in the detoxication of foreign compounds and the regulation of many endogenous compounds such as the steroid hormones (*7*). Microsomal constituents and activities investigated in this study were: cytochrome P-450, cytochrome b_5, benzpyrene hydroxylation, aniline hydroxylation, aminopyrine demethylation, benzphetamine demethylation, ethylmorphine demethylation, NADPH cytochrome c reductase, β-glucuronidase, and UDP glucuronyltransferase. We also monitored possible changes in oxidative phosphorylation rates in rat liver mitochondria to determine if the toxic action of TCDD could be related to disruptions in bioenergetic pathways.

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Materials and Methods

Animals

Male and female rats (Charles River, CD strain) were used in these experiments. On the day of treatment rats weighed approximately 200 g (males 6 weeks old, females 8 weeks old). TCDD was administered as a single oral dose in 0.5 ml acetone–corn oil, and controls received 0.5 ml acetone–corn oil (*8*).

Preparation of Subcellular Fractions

Rats were killed by cervical dislocation, and approximately 4.0 ml blood was immediately drawn from the dorsal aorta. Livers were removed, minced, and homogenized in 1.15% KCl buffered with 0.02*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.5, at 5°C to make a 20% (w/v) mixture. Homogenization was accomplished by using 6 strokes in a motor-driven Potter-Elvehjem homogenizer. Nuclei and cell debris were removed by centrifugation at 670*g* for 10 min and mitochondria re-

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moved by centrifugation of the 670*g* supernatant at 10000*g* for 15 min. Microsomes were pelleted by centrifugation of the post-mitochondrial supernatant at 105000 *g* for 70 min, washed once with HEPES–KCl buffer, and finally resuspended in HEPES so that 1.0 ml of microsomal suspension contained material from 0.5g liver (wet weight). Smooth- and rough-surfaced endoplasmic reticulum (SER and RER) fractions were prepared by homogenizing chopped liver sections in 0.25*M* sucrose (pH 7.0) and preparing the microsomal subfractions on discontinuous sucrose gradients by the procedure of Gram et al. (*9*), 10 ml of post-mitochondrial supernatant and 12 ml of 1.3-*M* sucrose being used. Liver mitochondria were prepared by the procedure of Nelson et al. (*10*) and resuspended in 0.25*M* sucrose so that 2.0 ml of suspension contained mitochondria from 3.0g liver (wet weight).

Assay Methods

Cytochrome P-450 was measured by its carbon monoxide difference spectra in an ACTA III spectrophotometer following reduction with dithionite, and cytochrome b_5 was measured by its difference spectra following reduction with NADH (*11*). For the determination of *in vitro* microsomal hydroxylation of aniline and demethylation of aminopyrine and ethylmorphine, the previously described incubation medium (*12*) was employed, with the exception that HEPES buffer was used instead of Tris buffer. Concentrations of substrates were: 3.5*mM* aniline, 2.5*mM* ethylmorphine, or 2.5*mM* aminopyrine in 3.0 ml incubation medium. Enzyme reactions were started by the addition of 1.5–2.0 mg microsomal protein. Benzpyrene (BP) hydroxylation and benzphetamine demethylation rates were determined by using the incubation medium of Hook et al. (*13*). There was essentially no difference when an NADPH regenerating system (*12*) or saturating concentrations of NADPH (3.1 *mM*) were used in the incubation medium. Aniline hydroxylation was quantified by the method of Kato and Gillette (*14*). Formaldehyde released by the demethylation of benzphetamine, aminopyrine, and ethylmorphine was measured by the Nash reaction (*15*). BP hydroxylation was measured by the fluorescence method of Wattenberg et al. (*16*). NADPH cytochrome c reductase was measured by the reduced cytochrome c peak at 550 nm (*17*). β-Glucuronidase was determined by the modified method (*18*) of Talalay et al. (*19*), phenolphthalein β-D-glucuronide being used as the substrate. *p*-Nitrophenol glucuronyltransferase was determined spectrophotometrically (*20*) by using 0.9*mM* p-nitrophenol, 0.8*mM* UDPGA, 10*mM* $MgCl_2$, and Triton X-100-treated microsomes (*21*). After 3 min incubation, the reaction was stopped by the addition of 5.0 ml glycine buffer, pH 10.4 (*21*). Experimental data for glucuronyltransferase were similar in all cases whether activity was measured by *p*-nitrophenol disappearance (*20*) or *p*-nitrophenyl β-D-glucuronide appearance at 312 nm (*22*). Oxidative phosphorylation rates in isolated liver mitochondria were measured polarographically with a Clark oxygen electrode. The reaction mixture contained 120*mM* KCl, 12*mM* substrate (succinic acid), 8*mM* $MgCl_2$, 5*mM* K_2HPO_4, 10.0*mM* ADP and 20*mM* glyclglycine buffer (pH 7.4). The total volume was 1.6 ml and the temperature was maintained at 30°C. Oxygen content in the vessel was calibrated by using NADH. Microsomal and mitochondrial protein contents were determined by the method of Lowry et al. (*23*).

Results and Discussion

Time–Course Studies

Male rates were administered TCDD as a single oral dose at 5 or 25 μg/kg, and hepatic microsomal enzyme activities and cytochrome contents were measured 1, 3, 9, 16, and 28 days after treatment. The purposes of this study were to determine whether TCDD affected microsomal enzyme activities and, if so, to determine the time–course alterations in enzyme activities. The LD_{50} value for TCDD is approximately 100 μg/kg (John Moore, personal communication), and no lethality of TCDD to male rats was observed at 5 or 25 μg/kg in test animals.

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niline hydroxylation—Time–course effects of TCDD on aniline hydroxylation are presented in Figure 1. Aniline hydroxylation is expressed as nanamoles *p*-aminophe-ol formed per minute per milligram pro-ein. Enzyme activity was slightly but not significantly enhanced at day 1. By day 3, hydroxylation rates were increased over 100%, and the same level of induction was observed through day 16. After day 16, enzyme activities began to return to control values, although aniline hydroxylation was still significantly elevated 38 days after treatment with 25 μg/kg TCDD.

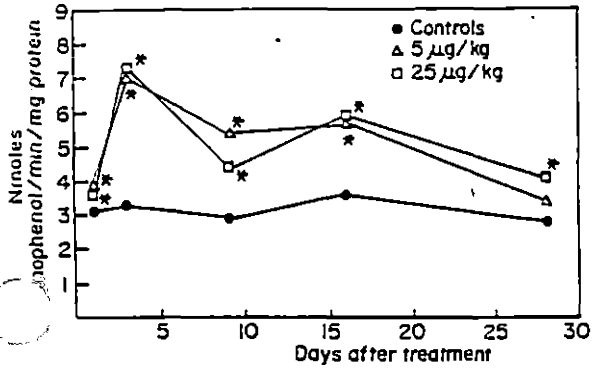


FIGURE 1. Time–course effects of a single oral dose of TCDD on liver microsomal aniline hydroxylation. An asterisk indicates that values are significantly different from controls at $P < 0.05$. $N = 3$ male rats.

Aminopyrine demethylation—The effects on aminopyrine demethylation were opposite those observed for aniline hydroxylation (Fig. 2). Specific enzyme activity was decreased approximately 30% at days 3, 9, and 16 by the 25 μg/kg dose. Values were essentially unchanged at the lower dose.

Cytochrome P-450 and b_5—Cytochrome P-450 was increased by 40% at day 1 and cytochrome b_5 was unchanged at day 1, while at day 3 contents of both microsomal cytochromes were elevated although P-450 was increased more than b_5 (Figs. 3 and 4). Nine days after treatment P-450 and b_5 were both increased by 60%. The lag period b_5 effects compared to P-450 might be

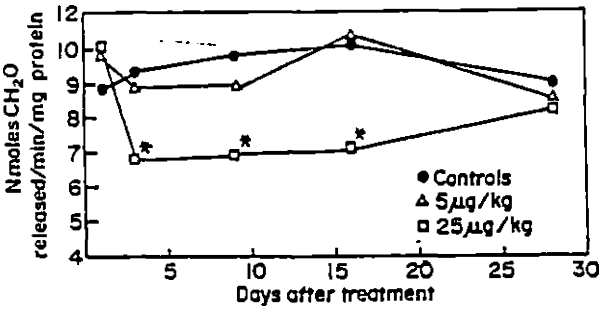


FIGURE 2. Time–course effects of a single oral dose of TCDD on liver microsomal aminopyrine demethylation. An asterisk indicates that values are significantly different from controls at $P < 0.05$. $N = 3$ male rats.

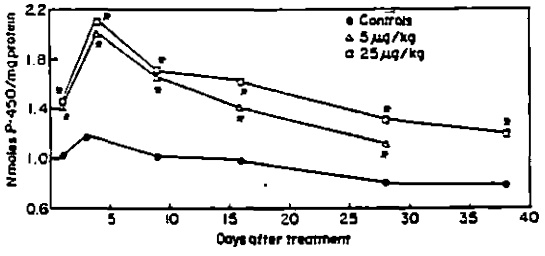


FIGURE 3. Time–course effects of a single oral dose of TCDD on liver microsomal cytochrome P–450. An asterisk indicates that values are significantly different from controls at $P < 0.05$. $N = 3$ male rats.

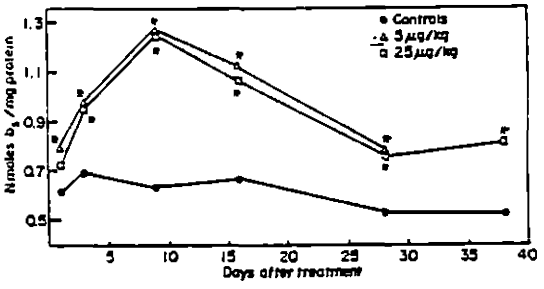


FIGURE 4. Time–course effects of a shingle oral dose of TCDD on liver microsomal cytochrome b_5. An asterisk indicates that values are significantly different from controls at $P < 0.05$. $N = 3$ male rats.

related to the slower turnover–rate of cytochrome b_5 (*24*). Thirty-eight days after TCDD treatment (25 μg/kg), b_5 content was increased by 60% and P-450 was increased by 40%. Increased P-450 content was asso-

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ciated with increased oxidative hydroxylation and decreased oxidative demethylation. This induction pattern is similar to that observed for 3-methylcholanthrene (*25*, *26*). However, TCDD appears not to shift the peak in the carbon monoxide difference spectra from 450 nm to 448 nm such as occurs with 3-methylcholanthrene induction (*26*). TCDD obviously cannot be considered a phenobarbital-type inducer, which is characterized by increased P-450 content, increased hydroxylation activity, and increased oxidative *N*-demethylation (*27*).

UDP glucuronyltransferase—Effects on glucuronyltransferase were the most striking observed in the time–course study (Fig. 5). Following TCDD treatment at 25 μg/kg enzyme activity was enhanced by 51% on day 1, 162% on day 3, 565% on day 9, 636% on day 16, 154% on day 28, and 162% on day 38. Levels of increases were slightly less at the 5 μg/kg dose compared to the 25 μg/kg dose, and time–course effects were similar. Increased glucuronlytransferase activity was not associated with changes in k_m values for substrate (*p*-nitrophenol, 0.26-*mM*) or co-factor (UDPGA, 0.58*mM*) (*28*). V_{max} in control animals was 126 nmole *p*-nitrophenol conjugated/min-mg protein compared to 539 in microsomal preparations from TCDD-treated rats (*28*). UDP glucuronyltransferase activity is phospholipid-dependent (*29*, *30*), and microsomal cholesterol has been theorized to function in the maintenance of endoplasmic reticulum structure (*31*). However, TCDD elevation of glucuronyltransferase activity does not appear to be related to alterations in total microsomal phospholipid and cholesterol levels (*28*), although individual microsomal phospholipids have not been quantified following TCDD treatment. Divalent cations and detergents are *in vitro* activators of microsomal glucuronyltransferase (*21*) and the effects of TCDD might be related to detergentlike actions on the endoplasmic reticulum or to mobilization of endogenous magnesium or other stimulatory divalent cations. However, the magnitude of the effect on glucuronyltransferase was the same whether glucuronyltransferase was measured in the presence or absence of Mg^{-2} or Triton X-100 (*28*). These data suggest that TCDD effects on glucuronyltransferase are not related to morphological alterations in endoplasmic reticulum structure, although this possibility has not been excluded. However, at this stage it appears that the possibilities that best fit the experimental data are related to increased enzyme synthesis or decreased degradation rates. TCDD induction of ALA synthetase in the chick embyro was blocked by cycloheximide (*5*) but data from protein-synthesis inhibition experiments would be difficult to obtain in rats due to the lag period in TCDD induction and the rapid toxicity of most antimetabolities. Elevation of glucuronyltransferase occurred in kidney microsomes as well as liver microsomes (*28*), although distribution studies have demonstrated that liver accumulates OCDD-Cl^{36} equivalents at much higher levels than kidneys (*32*). TCDD did not affect glucuronyltransferase when added directly to the incubation medium at 10^{-6} *M*.

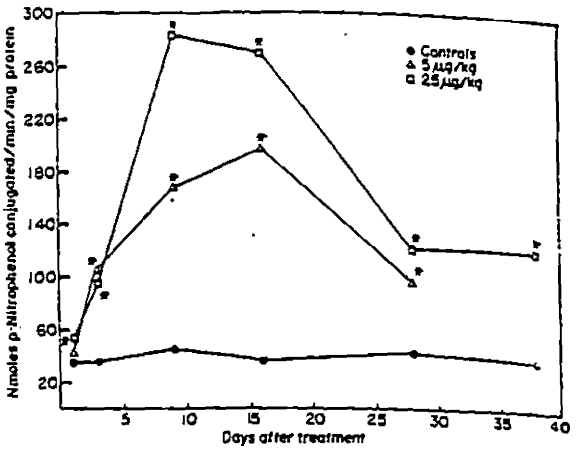


FIGURE 5. Time–course effects of a single oral dose of TCDD on liver microsomal UDP glucuronyltransferase. An asterisk indicates that values are significantly different from controls at $P < 0.05$. $N = 3$ male rats.

Microsomal protein—Since enzyme activities and cytochrome contents were measured on a per-milligram protein basis, it was of importance to measure time–course effects on microsomal protein contents. Previous

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reports show that chronic exposures to chlorinated triphenyl compounds markedly increased microsomal protein contents (*33*). Hepatic ultrastructural studies of TCDD-treated rats revealed general proliferation of RER and increases in SER in specific hepatic areas (*34*). In our studies, microsomal protein contents were not significantly changed till 28 days after a single TCDD treatment when levels were enhanced by 60% (Table 1). Therefore, enzyme activities per gram liver had essentially the same relative values between control and TCDD-treated rats as when enzyme activities were calculated per milligram protein.

Other enzymes—NADPH cytochrome c reductase and β-glucuronidase were not affected at either dose or at any period during the time–course experiment.

Liver function—Possible hepatotoxicity was monitored by serum orinthine transcarbamylase activities (*35*). Results show that after rats received 5 or 25 μg TCDD/kg there were no indications of liver damage at any time period.

–Response Studies

Dose–response relationships were examined in both male and female rats to obtain information concerning sex differences of hepatic microsomal responses to TCDD and to determine what is the lowest dose that results in induction of microsomal enzymes. Enzymes investigated in these studies included those used in the time–course experiment plus BP hydroxylation, benzphetamine demethylation, and ethylmorphine demethylation. These enzymes were added so that a more extensive comparison could be made on the different effect of TCDD on oxidative hydroxylations and demethylations. Single doses of TCDD at 0.2, 1.0, 5.0, and 25 μg/kg were used, and microsomal enzymes assayed 3 days after treatment.

Table 2. Changes in activities of male rat liver microsomal enzymes following a single oral dose of TCDD.[a]

Enzyme	Relative change from control values (100)[b] TCDD, 0.2 μg/kg	TCDD, 1.0 μg/kg	TCDD, 5.0 μg/kg	TCDD, 25.0 μg/kg
Cytochrome P–450	119	162[c]	184[c]	193[c]
Cytochrome b_5	101	139[c]	167[c]	195[c]
Aminopyrine demethylation	103	90	86[c]	72[c]
Benzphetamine demethylation	101	68[c]	70[c]	59[c]
Ethylmorphine demethylation	101	79	77	—
Aniline hydroxylation	124[c]	160[c]	202[c]	198[c]
Benzpyrene hydroxylation	102	163[c]	163[c]	467[c]
Glucuronyl-transferase	138[c]	167[c]	385[c]	471[c]
Protein	112[c]	113	105	126[c]

[a] Rats were killed 3 days after TCDD treatment at various TCDD dose levels. Each value is derived from four animals.

[b] Control values ± S.D. were: cytochrome P–450, 0.66 ± 0.08 nmole/mg protein; cytochrome b_5, 0.40 ± 0.01 nmole/mg protein; aminopyrine demethylation, 9.1 ± 0.4 nmole formaldehyde released/min–mg protein; benzphetamine demethylation, 8.1 ± 1.5 nmole formaldehyde released/min–mg protein, ethylmorphine demethylation, 15.9 ± 3.0 nmole formaldehyde released/min–mg protein; aniline hydroxylation, 3.1 ± 0.5 nmoles *p*-aminophenol formed/min–mg protein; glucuronyltransferase, 49.1 ± 0.8 nmoles *p*-nitrophenol conjugated/min–mg protein; microsomal protein, 25.3 ± 2.7 mg/g liver.

[c] Significantly different from controls at $P<0.05$.

Table 1. Time–course effects of a single oral dose of TCDD on microsmal protein.[a]

Dose, TCDD, μg/kg	Microsomal protein after TCDD treatment, mg/g liver 1 day	3 days	9 days	16 days	28 days
0	16.8 ± 1.7	16.8 ± 0.5	18.1 ± 3.0	19.3 ± 1.8	17.6 ± 2.6
5	18.4 ± 3.3	19.0 ± 5.4	21.8 ± 2.2	21.2 ± 0.9	28.2 ± 3.1[b]
25	17.8 ± 3.6	23.1 ± 3.2[b]	19.1 ± 1.2	20.6 ± 3.5	29.4 ± 3.5[b]

[a] Values at various times after TCDD treatment; $N = 3$ male rats.

[b] Significantly different from controls at $P < 0.05$.

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Males—Data on the effects of TCDD on male rat liver microsomal enzymes are presented in Tables 2 and 3. Increases in cytochrome P-450 and b_5 contents were not evident until animals received 1.0 μg/kg and the level of induction was dose-dependent up to 25 μg/kg. Activities of oxidative demethylation enzymes for all three substrates were decreased in a dose-dependent manner. Aniline hydroxylation was enhanced by 0.2 μgTCDD/kg (24%) and maximum increases occurred after 5.0 μg TCDD/kg (102%). Induction of BP hydroxylation was similar in magnitude to aniline hydroxylation at the three lower doses but after male rats received 25 μg TCDD/kg, BP hydroxylation was increased by 300% and aniline hydroxylation by 100%. Glucuronyltransferase was increased by 38, 67, 285, and 371% at the four doses from the lowest to highest, respectively. When enzyme activities per nmole cytochrome P-450 were calculated, oxidative demethylation values of the three substrates tested were significantly decreased (50–70%) at TCDD doses of 1.0 μg/kg or greater (Table 3). Hydroxylation values per P-450 unit were essentially unchanged, with the exception that BP hydroxylation per unit of P-450 increased by approximately 100% following a dose of 25 μg TCDD/kg.

Table 4. Changes in activities of female rat liver microsomal enzymes following a single oral dose of TCDD.[a]

Enzyme	Relative change from control values (100)[b]		
	TCDD, 0.2 μg/kg	TCDD, 1.0 μg/kg	TCDD, 5.0 μg/kg
Cytochrome P-450	126[c]	153[c]	196[c]
Cytochrome b_5	122[c]	131[c]	158[c]
Aminopyrine demethylation	120[c]	131[c]	120[c]
Benzphetamine demethylation	115	112	118
Benzpyrene hydroxylation	783[c]	1225[c]	1403[c]
Glucuronyltransferase	257[c]	506[c]	487[c]
Protein	94[c]	108[c]	115[c]

[a] Rats were killed 3 days after TCDD treatment at various TCDD dose levels. Each value derived from four animals.

[b] Enzyme activities were expressed as indicated in Table 2. Control values ± S.D. were; cytochrome P-450, 0.45 ± 0.06 nmole/mg protein; cytochrome b_5, 0.36 ± 0.03 nmole/mg protein; aminopyrine demethylation, 4.4 ± 0.3 nmole formaldehyde/min-mg protein; benzphetamine demethylation, 2.34 ± 0.45 nmole formaldehyde/min-mg protein; benzpyrene hydroxylation, 0.06 ± 0.01 nmole/mg-min protein; glucuronyltransferase 23.9 ± 5.4 nmole/min-mg protein; and microsomal protein (21.0 ± 5.0 mg/g liver).

[c] Significantly different from controls at $P<0.05$.

Table 3. Effect of TCDD on mixed function oxidase activity per cytochrome P-450 unit in male rat liver microsomes

Enzyme	Activity, nmole substrate metabolized/nmole P-450				
	0	TCDD, 0.2 μg/kg	TCDD, 1.0 μg/kg	TCDD, 5.0 μg/kg	TCDD, 25.0 μg/kg
Aminopyrine demethylation	13.7 ± 1.0	12.4 ± 1.5	7.6 ± 0.8[b]	6.4 ± 0.7[b]	4.6 ± 0.3[b]
Benzphetamine demethylation	12.2 ± 1.8	10.9 ± 2.3	5.1 ± 0.9[b]	4.6 ± 0.4[b]	—
Ethylmorphine demethylation	23.8 ± 1.5	21.3 ± 3.8	11.7 ± 2.2[b]	10.0 ± 1.6[b]	—
Aniline demethylation	3.4 ± 0.2	3.2 ± 0.7	3.8 ± 0.1	3.7 ± 0.3	3.5 ± 0.6
Benzpyrene hydroxylation	0.80 ± 0.14	0.72 ± 0.17	0.80 ± 0.03	0.71 ± 0.07	1.95 ± 0.20[b]

[a] Rats sacrificed 3 days after TCDD treatment at various dose levels. $N = 4$ male rats.

[b] Significantly different from controls at $P<0.05$.

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Table 5. Effect of TCDD on mixed function oxidase activity per cytochrome P-450 unit in female rat liver microsomes.[a]

Enzyme	Activity, nmole substrate metabolized/nmole P-450			
	0	TCDD, 0.2 μg/kg	TCDD, 1.0 μg/kg	TCDD, 5.0 μg/kg
Aminopyrine demethylation	9.9 ± 1.5	9.3 ± 0.2	8.4 ± 0.6	6.1 ± 1.0[b]
Benzphetamine demethylation	5.1 ± 1.0	4.7 ± 0.6	3.7 ± 0.7	3.1 ± 0.4[b]
Ethylmorphine demethylation	7.3 ± 1.4	6.4 ± 0.5	4.4 ± 0.2[b]	3.4 ± 0.7[b]
Benzpyrene hydroxylation	0.13 ± 0.05	0.8 ± 0.1[b]	1.0 ± 0.21[b]	0.96 ± 0.16[b]

[a] Rats sacrificed 3 days after TCDD treatment. $N = 4$ male rats.
[b] Significantly different from controls at $P < 0.05$.

Females—Data on the effects of TCDD on female microsomal enzymes are presented in Tables 4 and 5. Female rats were more susceptible to TCDD induction of BP hydroxylation and glucuronyltransferase than male rats (Table 4). This sex difference was quite evident following a dose of 0.2 μg/kg; male liver microsomal glucuronyltransferase increased 38% and female liver microsomal glucuronyltransferase increased 157%, male BP hydroxylation increased 2% and female BP hydroxylation increased 683%. In control animals, BP hydroxylation rates were eight times greater in liver microsomes from males compared to females, but in TCDD-treated rats activities of liver microsomal BP hydroxylase were approximately the same in both sexes. Glucuronyltransferase activity of liver microsomes from males was twice that of females in controls, but in TCDD-treated rats (0.2 μg/kg) activities were higher in microsomes from females than males. Oxidative demethylation activites were two to four times as high in microsomes from control males compared to females. Female hepatic microsomal *N*-demethylations were slightly increased by TCDD, whereas corresponding enzyme activities in male hepatic microsomes were decreased although *N*-demethylation rates were still higher in TCDD-treated males compared to TCDD-treated females at all dose levels. Since maximum elevation of P-450 in females, as in males, was approximately 30%, *N*-demethylations per unit P-450 were decreased, but in general the observed decrease in *N*-demethylations per P-450 unit in females was not as much as that in males.

These data demonstrate that TCDD markedly increases activity of some microsomal enzymes, particularly glucuronyltransferase and BP hydroxylase, and that female rats are more susceptible to action of TCDD than males. Increases in activity of microsomal enzymes after a dose of 0.2 μg TCDD/kg is quite significant in comparison to doses required for effects by other inducing agents. TCDD is approximately 100,000 times more potent an inducing agent than phenobarbital or 3-methylcholanthrene on a μg/kg basis in rats. In addition to our studies, Hook et al. (*36*) have shown that a single TCDD dose of 0.2 μg/kg to female rats increased biphenyl 2-hydroxylation by approximately 900% and biphenyl 4-hydroxylation by approximately 100%. Therefore, an oral dose of 40 ng TCDD to 200 g rats markedly increases activity of microsomal enzymes. Norback et al. (*32*) report that 95% of labeled TCDD is excreted in the feces following oral administration. Therefore, it appears that only a small portion of the administered TCDD reaches the liver, although it is possible that much of the fecal radioactivity has been added via biliary excretion and that intestinal absorption rates might vary with dose. ALA synthetase activity was increased in chick embryos by extremely low concentrations of TCDD (*5*), but in the same group of rats used in our studies, hepa-

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tic ALA synthetase was not changed (*37*) by doses 50 times that needed to increase UDP glucuronyltransferase, benzpyrene hydroxylation, and biphenyl 2-hydroxylation. The extreme sensitivity of microsomal enzymes to TCDD body burdens suggest that alterations in activities of these enzymes could be related to the toxic action of TCDD and its teratogenic effects by disrupting normal steriod regulation. Adrenal size is not enlarged in TCDD-treated rats (*38*), indicating that if steriod excretion is enhanced the compensatory feedback mechanism controlling steroid synthesis may not be operative.

Effects of TCDD on SER and RER

Effects of TCDD on the distribution of microsomal components in SER and RER of male rats are summarized in Table 6. SER to RER ratios were decreased in all parameters tested following TCDD treatment (25 μg/kg). *N*-Demethylation ratios (SER:RER) were approximately 2.4 in controls compared to 0.7 in treated rats. Specific demethylation activities were decreased by 75% in SER and were essentially unchanged in RER. BP hydroxylation was elevated in both SER and RER, but induction was greater in RER resulting in decreased SER:RER from 1.77 to 1.19. Glucuronyltransferase was also markedly increased in both subfractions (SER, 200%; RER, 300%) and SER:RER decreased from 0.56 to 0.37. Microsomal protein also exhibited decreased SER:RER following TCDD treatment although the change was not significant. These changes in SER:RER ratios are similar to those seen after 3-methylcholanthrene treatment (*26*). Ultrastructural studies revealed overall RER proliferation and SER proliferation in isolated regions of rat liver hepatocytes (*34*).

Effects on Oxidative Phosphorylation

The gradual wasting of animals that precedes death in TCDD-exposed animals suggested that toxicity might be an expression of bioenergetic disturbances. However, oxidative phosphorylation rates in isolated rat liver mitochondria from TCDD-treated rats (5 or 25 μg TCDD/kg) did not significantly differ from those in controls (Table 7). Parameters investigated were state 3 respiration, state 4 respiration, respiratory control (R.C.) (state 3/state 4) and ADP:O. The only difference observed between control and treated rats was greater uncoupling rates of the treated group on storage at 4° C.

Table 6. Submicrosomal distribution of male rat liver microsomal enzymes following a single oral dose of TCDD (25 μg/kg).[a]

Enzyme[b]	Control			TCDD		
	SER	RER	SER:RER	SER	RER	SER:RER
Aminopyrine demethylation, nmole HCHO/min–mg	9.3 ± 1.0	4.0 ± 0.6	2.34	2.6 ± 0.1	3.6 ± 0.7	0.73[c]
Benzphetamine demethylation, nmole HCHO/min–mg	9.1 ± 1.7	3.8 ± 0.4	2.38	2.1 ± 0.1[c]	3.5 ± 0.4	0.61[c]
Benzpyrene hydroxylation, nmole/min–mg	0.46 ± 0.09	0.26 ± 0.08	1.77	2.4 ± 0.3[c]	2.0 ± 0.2[c]	1.19[c]
Glucuronyl-transferase, nmole/min–mg	69.8 ± 17.2	124.5 ± 31.9	0.56	205.8 ± 22.8[c]	556.7 ± 87.9[c]	0.37[c]
Protein, mg/g liver	5.9 ± 0.5	12.8 ± 0.6	0.46	6.2 ± 0.4	16.8 ± 1.8[c]	0.37[c]

[a] Rats were killed six days after TCDD treatment. $N = 4$ male rats.
[b] Enzyme activities expressed as outlined in footnotes b to Table 2.
[c] Significantly different from controls at $P<0.05$.

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Table 7. Effects of a single oral dose (25 μg/kg) of TCDD on oxidative phosphorylation rates in rat liver mitochondria.[a]

Time after treatment, days	State 3 respiration, ng-atom O/min-mg protein	State 4 respiration, ng-atom O/ min-mg protein	R.C.	ADP:O
Controls	138 ± 14	34 ± 5	4.06	1.85 ± 0.06
1	133 ± 7	31 ± 3	4.33	1.88 ± 0.04
3	159 ± 22	37 ± 7	4.27	1.71 ± 0.09
6	145 ± 4	37 ± 2	3.92	1.73 ± 0.08
9	127 ± 6	31 ± 2	4.10	1.73 ± 0.14
16	140 ± 13	36 ± 1	3.89	1.78 ± 0.21
28	146 ± 5	37 ± 3	3.95	1.75 ± 0.07

[a] Succinate used as the substrate; each value (mean ± S.D.) derived from an average of four male rats.

Summary

Male or female rats were administered a single oral dose of TCDD at 0.2, 1.0, or 5.0 μg/kg, and activities of hepatic microsomal enzymes were monitored three days after treatment. Our data demonstrate that TCDD has an extremely potent effect on some microsomal enzymes, particularly glucuronyltransferase and benzpyrene hydroxylase, hat female rats may be more susceptible DD actions than males. Marked increases in hepatic enzyme activity in female rats was observed following a single oral dose of 0.2 μg TCDD/kg (LD_{50}=100 μg/kg) (glucuronyltransferase + 157%, benzpyrene hydroxylation + 683%), and the levels of induction increased with dose so that after 5.0 μg TCDD/kg glucuronyltransferase was induced 500% and benzpyrene hydroxylation 1400%. TCDD also increased cytochrome P-450, cytochrome b_5, and aniline hydroxylation, whereas oxidative demethylations of aminopyrine, ethylmorphine, and benzphetamine were decreased. NADPH cytochrome c reductase and β-glucuronidase were unaffected by any TCDD dose. Time–course studies revealed that increases reached a plateau 3 days after TCDD treatment following an initial lag period. Increased levels were maintained at the day 3 values through day 16 after which time activities began to return to normal although effects were still evident 38 days after treatment. Subfraction of microsomes into SER and RER revealed that TCDD markedly decreased SER to RER ratios in all parameters tested. There was no biochemical or histologic evidence of hepatotoxicity nor any effects on oxidative phosphorylation rates in liver mitochondria. These studies indicate that hepatic microsomal enzymes are extremely sensitive to TCDD body burdens.

Needs for Further Research

Our studies to date have served only to characterize the effects of TCDD on hepatic microsomal enzymes following animal exposures. The need for future research related to TCDD-microsomal interactions are many, and the following represents a list of some of the most urgent research needs: (1) study microsomal effects of *in vitro* addition of TCDD and related compounds directly to the incubation medium; (2) compare inductive properties of TCDD with 3-methylcholanthrene and other inducers; (3) determine structural requirements for induction by using structural analogs of TCDD as effectors of microsomal enzymes; (4) determine if TCDD induces extrahepatic microsomal enzymes and determine levels of induction in species other than the rat; (5) determine if induction of microsomal enzymes is related to increased synthesis, decreased degradation, or membrane effects; (6) determine rates of *in vivo* metabolism and excretion of test compounds, including steriods, following TCDD treatment; (7)

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determine if maternal exposure of TCDD induces fetal enzymes; and (8) study possible relationships between TCDD induction of microsomal enzymes and teratogenic effects.

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Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin on Drug Metabolism and Hepatic Microsomes of Rats and Mice

by J.B. Greig* and F. De Matteis*

Introduction

It has been reported (*1*) that one effect of a single, intra-peritoneal dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) is to cause a reduction in the duration of action of the drug 2-amino-5-chlorobenzoxazole (zoxazolamine) in the rat. This effect is maximal with doses of dioxin above 100 μg/kg and significant even at 5 μg/kg. Our own work showed that oral dosage had a similar effect but also that the duration of action of hexobarbitone was considerably prolonged (*2*). The results described here extend these observations.

Materials and Methods

Animals

Rats of the albino Porton strain bred in these Laboratories and weighing 180–200 g (male 6–8 weeks and female 7–10 weeks old) had free access to water and diet 41B unless otherwise indicated.

Mice of the C57BL/6 and DBA/2 strains were bred in these laboratories and kept on Sterolit bedding (Mineral & Chemical Corporation of America, Menlo Park, New Jersey, U.S.A.) for at least 3 weeks before use. They had free access to diet and water.

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Dosages

Dioxin, prepared as described elsewhere (*3*), was administered as a solution (100 μg/ml) in Arachis oil. Control animals received an equivalent volume of oil. Zoxazolamine (McNeil Laboratories, Inc.) was dissolved in 1*N* HCl (1.2 ml/100 mg), diluted with 0.9% NaCl to 10 mg/ml and administered IP at 100 mg/kg. 5-Cyclo-hex-1′-enyl-1,5-dimethylbarbituric acid (hexobarbitone) sodium (May & Baker Ltd.) was dissolved in water at 50 or 25 mg/ml and administered IP at 150 mg/kg (male rats), 75 mg/kg (female rats), 100 mg/kg (male and female mice). *dl*-Ethionine (Koch-Light) was dissolved in water (20 mg/ml) and administered as indicated in the text.

Microsomal Preparations

Liver microsomes were isolated either as described by Bond and De Matteis (*4*), except that the microsomes were sedimented at 105,000*g*, or by the calcium/sucrose method (*5*) adapted to the extent of sedimenting the calcium-treated microsomes at 1900*g* and washing them once in a 0.0125*M* sucrose solution containing 8*mM* $CaCl_2$. The final preparations were suspended in 0.1*M* phosphate buffer (pH 7.4) containing 1*mM* Na_2-EDTA (1.4 −7.0 mg protein/ml). Protein was estimated by the biuret method (*6*) and cytochrome P-450 (or P-448) by using an

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extinction coefficient of 91/*mM*–cm (*7*) for the absorbancy change between 450 (448) and 490 nm of the CO difference spectrum of a $Na_2S_2O_4$-reduced suspension.

Assays

Plasma barbiturate levels following hexobarbitone administration were measured by the method of Chromý and Babjuk (*8*).

Hexobarbitone oxidase was measured *in vitro* by the modified (*9*) method of Brodie et al. (*10*).

Zoxazolamine hydroxylase was measured by the methods of Burns and his colleagues (*11*, *12*) with minor modifications.

Difference Spectra

Difference spectra were recorded on a Unicam SP 1800 spectrophotometer with oxidized microsomal suspensions (2.7 ml, 1.4–2.2 mg protein /ml) in each 1-cm cuvet and the addition of either aniline (5–200 µl, 0.22-*M* in 0.1*M* phosphate buffer, pH 7.4) or hexobarbitone sodium (5-100 µl, 0.054*M* in water) to the sample cuvet and of an equal volume of the appropriate solvent to the reference cuvet. Pyridine difference spectra were recorded with $Na_2S_2O_4$-reduced microsomal suspensions (2.7 ml) and 0.3 ml of either 0.5*M* aqueous pyridine or water added to the cuvets.

The wavelength of cytochrome P-450 (or P-448) maximum absorption was measured on a Cary 14 spectrophotometer calibrated with a holmium filter.

Statistics

Results are quoted as the mean ± S. E. M. and were analyzed by Student's *t*-test except where the nature of the results necessitated the use of a ranking test.

Results and Discussion

Effect of Dioxin Administration on the *In Vivo* Action, and *In Vitro* Metabolism of Drugs in the Rat

One or 3 days after being given a single oral dose of dioxin rats show a shortening of the zoxazolamine paralysis time but an increase in the duration of action of hexobarbitone (*2*). The effect on the hexobarbitone sleeping time becomes progressively more marked with time until, 2 weeks after dosing, rats sleep over 4 hr and some of them die without waking (Table 1).

Table 1. Effect of dioxin on the hexobarbitone sleeping time of male rats.[a]

Time after dosing	Sleeping time, min [b]		Increase over controls, *P* %	
	Dioxin (200 µg/kg, PO)	Solvent		
12 hr	32.9 ± 3.5 (6)	37.7 ± 3.3 (5)	—	NS
24 hr	40.0 ± 3.1 (6)	27.4 ± 1.8 (6)	46	<0.01
72 hr	75.5 ± 5.9 (6)	33.6 ± 3.3 (6)	125	<0.001
1 week	125.6 ± 14.1 (6)	21.5 ± 2.9 (5)	480	<0.001
2 weeks	> 244 (6)	25.2 ± 2.7 (6)	870	0.0022 [c]

[a] Hexobarbitone sodium administered as described in Materials and Methods section.
[b] Numbers of animals in parentheses.
[c] By ranking test.

In view of the prolonged reduction of food intake of rats dosed with dioxin (*3*) it is possible that a starvation effect (*13*) might contribute significantly to the increased hexobarbitone sleeping times at later stages in the intoxication (1 or 2 weeks after dosing). However earlier experiments (*2*) had suggested that, 1 day after dosing with dioxin, the reduced food intake was not the sole cause of the prolongation of the hexobarbitone sleeping time. This interpretation is open to criticism, since in these experiments the animals had not been prefasted before dioxin administration and the dioxin might have slowed down the absorption of the residual food in the stomach and intes

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Accordingly groups of eight female rats were starved for 39 hr and then dosed with dioxin (200 μg/kg, PO) or oil. Following 24 hr further starvation the hexobarbitone sleeping time was measured; the value for the dosed group, 89.0 ±3.8 min, was significantly higher than that of the control group, 63.2 ±5.3 min, ($P < 0.005$). Thus the early effect of dioxin on the sleeping time cannnot be entirely due to differences in food consumption or absorption between control and treated animals. Therefore in all following experiments we have used rats which had been given a single dose of dioxin (200 μg/kg) 1 or 3 days previously.

It was considered possible that dioxin might prolong the hexobarbitone sleeping time by altering either the sensitivity of the nervous system or the distribution of the barbiturate within the body. These possibilities were ruled out by an experiment in which the sleeping times of groups of dioxin-treated and control rats were measured and, immediately after their waking, blood was collected for the analysis of plasma barbiturate levels (*8*). Table 2 indicates that, although there was a significant increase in the sleeping time of the dosed animals, the waking plasma barbiturate levels of the two groups were not significantly different.

Table 2. Effect of dioxin on the sleeping time and waking plasma barbiturate level of female rats.[a]

Treatment	N	Sleeping time, min	Plasma barbiturate, μg/ml
Dioxin	6	99.3 ± 19.8	67.9 ± 2.2
Controls	6	45.9 ± 4.2	64.5 ± 2.9
P		<0.025	NS

[a] Rats were dosed with dioxin (200 μg/kg, PO) or oil and 3 days later the sleeping time induced by hexobarbitone sodium (75 mg/kg, IP) was measured. Immediately after waking the animals were anesthetized (ether) and blood collected from the heart. Plasma barbiturate was measured by the method of Chromy and Babjuk (*8*).

Three days after a single oral dose of either dioxin or oil to male or female rats the liver microsomes were isolated and incubated with an NADPH-generating system and either hexobarbitone or zoxazolamine (Table 3). With either sex, following dioxin treatment, there was a significant increase in the amount of zoxazolamine metabolized, and with males there was a significant decrease in the quantity of hexobarbitone oxidized. With the microsomes from female rats the control level of hexobarbitone metabolism was lower than the males and, although in the treated livers it was lower still, the difference was not significant. Apart from this, the results agree with the *in vivo* experiments and indicate that dioxin modifies the duration of the pharmacological action of both zoxazolamine and hexobarbitone by changing the rate of metabolism of these drugs by the liver microsomes.

Table 3. Effect of dioxin treatment on the metabolism of hexobarbitone and zoxazolamine by rat liver microsomes.[a]

Treatment	Sex	Zoxazolamine metabolized, nmole/mg microsomal protein/hr [b]	Hexobarbitone metabolized, nmoles/mg microsomal protein/hr [b]
Dioxin	M	32.2 ± 4.2 (10)	31.0 ± 23.9 (5)
Controls	M	15.7 ± 2.0 (10)	192.0 ± 18.7 (5)
		P <0.005	<0.001
Dioxin	F	46.9 ± 7.9 (5)	29.4 ± 47.8 (5)
Controls	F	6.3 ± 5.4 (5)	109.2 ± 49.7 (5)
		P <0.005	NS

[a] Rats (180–200 g) received dioxin (200 μg/kg) or oil PO and were killed 3 days later. Microsomes were isolated by the calcium/sucrose method (see Materials and Methods) and the assays performed essentially as described elsewhere (*9–12*).

[b] Numbers of animals in parentheses.

Effect of Dioxin Administration on the Liver Content and Spectral Properties of Microsomal Cytochrome P–450

Besides oxygen and NADPH, it is known that three other components are required for the reconstitution of a system capable of metabolizing drugs *in vitro*. These are a lipid, phosphatidyl choline; a flavoprotein, NADPH/cytochrome P-450 reductase, and a hemoprotein, cytochrome P-450 (or P-448) (*14–16*). We have investigated the effect of dioxin treatment on cytochrome P-450 of rat liver microsomes. Groups of male

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Table 4. Changes in liver weight and microsomal protein and cytochrome P-450 induced by dioxin treatment of male rats.*

Treatment	Weight wet liver, g/100 g BW	Microsomal protein, mg/g liver	Microsomal cytochrome P-450		
			nmoles/g liver	nmoles/mg protein	λ_{max} (nm)
Dioxin	4.34 ± 0.12	26.5 ± 0.9	55.1 ± 1.5	2.08 ± 0.07	447.6 ± 0.04
Controls	3.21 ± 0.06	27.3 ± 0.6	28.1 ± 0.7	1.03 ± 0.02	449.6 ± 0.08
	P <0.001	NS	<0.001	<0.001	<0.001

* Rats received dioxin (200 μg/kg, PO) or oil and 3 days later were killed and liver microsomes isolated at 105,000g (see Materials and Methods). Each value is the mean of five observations.

rats received an oral dose of either dioxin or oil and three days later were killed and their liver microsomes isolated. Table 4 indicates that there was a significant increase in the weight of wet liver from the treated animals. There was no increase in the microsomal protein content, expressed per gram of tissue, but the cytochrome content doubled. Further, the wavelength of the peak maximum of the cytochrome spectrum was shifted from 450 nm to 448 nm. Such a shift is a feature of the microsomal enzyme induction brought about by compounds such as 20-methylcholanthrene (*17*). Since this inducer stimulates the metabolism of aromatic compounds, including zoxazolamine (*18*) this change correlates well with the observed stimulation of zoxazolamine metabolism both *in vivo* and *in vitro*. However it should be remembered that, if the CO/cytochrome P-448 complex has an extinction coefficient more than four times that of P-450, as has been reported (19), then there has in fact been a decrease in hemo-protein concentration.

The interaction of the reduced cytochrome P-450 of liver microsomes with the ligands ethyl isocyanide or pyridine is known to result in the formation of a difference spectrum with two peaks in the region 400–460 nm (*20*). The relative intensity of these two peaks, related to the absorption at 500 nm, is dependent on the pH of the suspending medium (20). In the case of liver microsomes prepared from animals pretreated with 20-methylcholanthrene and therefore containing P-448 rather than P-450, the curve of pH dependence of the peak height ratio is so shifted that the peaks are of equal intensity at a lower pH (*21*). The pH dependence of the pyridine difference spectra of microsomes from methylcholanthrene-, dioxin- or oil-treated rats was measured and is shown in Figure 1. It can be seen that the curves due to dioxin or methylcholanthrene treatment are similar to each other but distinct from that of the control.

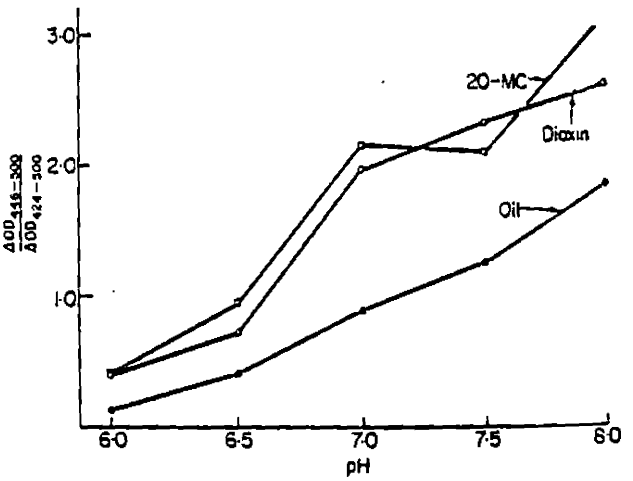


FIGURE 1. Rats (male, 180–200 g) received dioxin (200 μg/kg, PO) or Arachis oil (2 ml/kg, PO) 3 days before killing. 20-Methylcholanthrene was injected (20 mg/kg, 10 mg/ml in oil, IP) 3 and 2 days before killing. All were starved for 24 hr before decapitation. Portions of liver microsomes equivalent to 0.4 g of wet liver were isolated by the calcium/sucrose method (*5*) and suspended in 0.1M PO_4 buffer (10 ml, containing 1mM Na_2EDTA) of the appropriate pH. Pyridine difference spectra were measured as stated in Materials and Methods.

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It is known that, in the oxidized state, the cytochrome P-450 of liver microsomes will interact with various substrates of the drug metabolizing system to produce characteristic difference spectra (*22*). We have studied the effect of dioxin pretreatment of rats on the interaction of aniline and hexobarbitone with rat liver microsomes. With aniline the control microsomes showed a normal Type II spectral change; this was intensified in the case of the microsomes from treated rats (Fig. 2). Such a change is consistent with the increased cytochrome P-448 in these preparations. However, a double reciprocal plot of the spectral change/nmole of P-450 (P-448) against aniline concentration indicated that there was an increase in K_s (binding affinity constant) and in the maximal Type II spectral change (Fig. 3). A similar effect has been reported following methylcholanthrene induction (*23*).

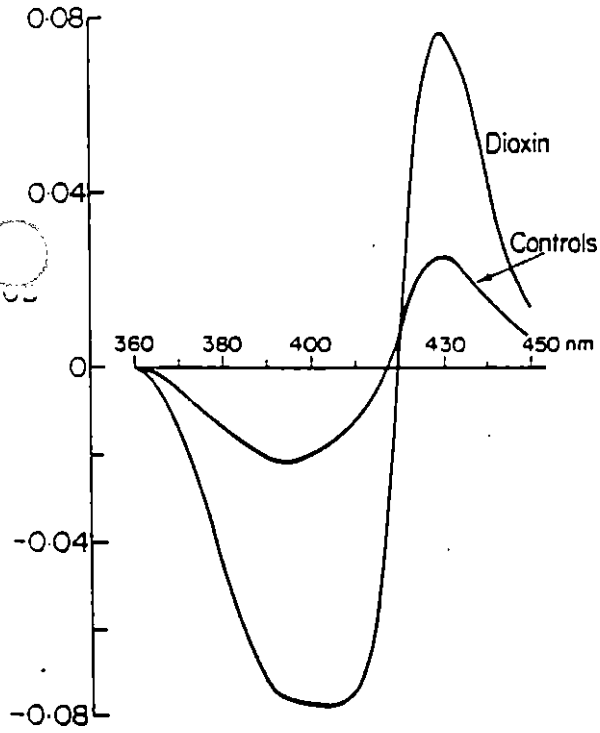


FIGURE 2. Rats (male, 180–200 g) received dioxin (200 μg/kg) or oil PO 3 days before killing. They were starved for 24 hr before isolation of liver microsomes at 105,000g. Preparations from two animals were combined. Difference spectra (aniline 0.41*mM* final concentration) measured as described in Materials and Methods on suspensions containing microsomes equivalent to 0.073g wet liver/ml.

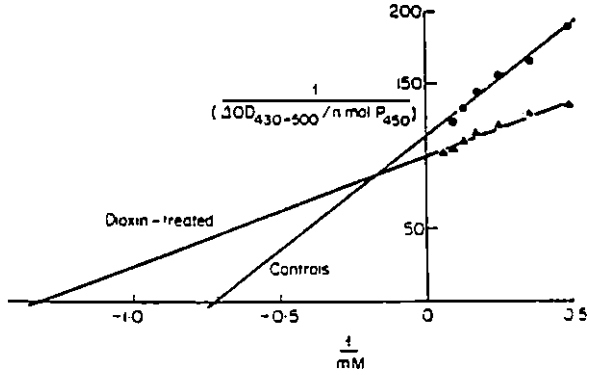


FIGURE 3. Rats (male, 170–190 g) received dioxin (200 μg/kg) or oil PO 3 days before killing. Liver microsomes, equivalent to 2.8 g wet liver, were isolated by the calcium/sucrose method (*5*) and were suspended in 0.1*M* PO_4 buffer (25 ml, pH 7.4) containing 1*mM* EDTA. Aniline concentrations in the cuvet (see Materials and Methods) were varied from 2.0 to 15.2*mM*. Each point is the mean of observations on five animals. On each axis the mean intercepts for groups of dosed and control animals were significantly different ($P < 0.05$). An extinction coefficient of 91/*mM*–cm (*7*) was used in the estimation of cytochrome P–450 (–448). The spectral changes observed are related to the total cytochrome content of the cuvet.

The interaction of hexobarbitone with microsomes from control animals produced the expected Type I difference spectra (*22*) as illustrated in Figure 4. However the microsomal preparations from rats which had received dioxin 3 days previously consistently gave a difference spectrum with a peak at 412 nm and a trough at 380 nm. This type of spectrum has been termed a modified Type II spectral change (*22*) and has been observed following methylcholanthrene pretreatment (*24*), but only with microsomal preparations from female rats. A decrease in the intensity of the Type I difference spectrum due to hexobarbitone has been reported for methylcholanthrene-treated male and female rats (*23*).

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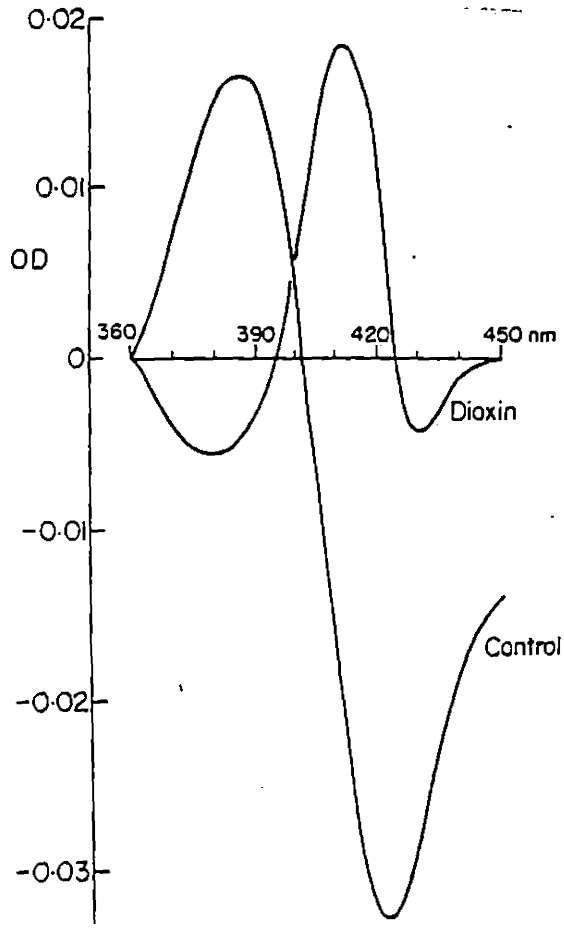


FIGURE 4. All details as in the legend to Figure 2 except that hexobarbitone was added to the sample cuvetts to a concentration of 0.79*mM*.

Comparison of the Effects of 20-Methylcholanthrene and Dioxin on Zoxazolamine Action in Mice of the DBA/2 Strain

All the changes of the properties of the liver microsomes from dioxin-treated rats are consistent with dioxin being a powerful inducer of the methylcholanthrene type. It has been reported that in mice of the DBA/2 strain the levels of hepatic aryl hydrocarbon hydroxylase are unaffected by methylcholanthrene (*25*) or benz[*a*]anthracene (*26*) treatment. Indeed when DBA/2 mice (male, 20–30 g) were injected with methylcholanthrene or oil as described by Nebert et al. (*25*) we found, 24 hr after injection, no significant difference between the zoxazolamine paralysis times of the treated and control groups (58.0 ±5.1 min, $N = 6$ and 59.3 ± 3.9 min, $N = 6$, respectively). This contrasts with C57BL/6 mice (male, 18–26 g) in which methylcholanthrene pretreatment significantly reduced the paralysis time (dosed: 15.0 ± 1.0 min, $N = 9$, controls: 79.5 ± 5.3 min, $N = 9$; $P < 0.0001$). However in both strains a single oral dose of dioxin (200 μg/kg) given 3 days before zoxazolamine significantly lowered the paralysis time (DBA/2, male, dosed: 10.5 ± 0.8 min, $N = 6$, controls: 65.7 ± 4.2 min, $N = 5$, $P < 0.001$; for C57BL/6 see Table 5).

Table 5. Effect of dioxin on sleeping and paralysis times of C57 BL/6 mice.[a]

Treatment	Sex	Sleeping time, min[b] After 3 days	Sleeping time, min[b] After 10 days	Sleeping time, min[b] After 20 days	Paralysis time after 3 days, min
Dioxin	M	52.6 ± 6.2 (7)	60.6 ± 4.7 (7)	>159.7 (7)	7.0 ± 0.7 (4)
Oil	M	71.3 ± 3.8 (6)	60.2 ± 7.7 (4)	53.4 ± 7.3 (6)	46.8 ± 3.5 (5)
		P <0.05	NS	<0.01[c]	<0.001
Dioxin	F	52.6 ± 6.5 (7)	—	—	5.9 ± 0.6 (5)
Oil	F	65.1 ± 8.4 (7)	—	—	37.7 ± 4.0 (7)
		P NS			<0.001

[a] Mice (11–27 g) received dioxin (200 μg/kg) or oil PO and hexobarbitone sodium or zoxazolamine after the stated intervals.
[b] Numbers of animals in parentheses.
[c] By ranking test.

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Assuming that, under these conditions, the duration of zoxazolamine action is entirely governed by the rate of metabolism in the liver it would appear that the apparent genetic noninducibility of hepatic aryl hydrocarbon hydroxylase in certain mouse strains is not an absolute trait but dependent on the inducer used. This is in agreement with the observations (*26*) that this enzyme, although present in smaller amounts in extrahepatic tissues, is inducible in such organs of strains in which the liver enzyme is unaffected.

Investigation of the Relationship between Stimulation of Zoxazolamine Metabolism and Inhibition of Hexobarbitone Metabolism

Although in the rat dioxin produces simultaneous and divergent effects on the metabolism of zoxazolamine and hexobarbitone by liver microsomes, it is not known whether these two effects are related to each other. An alternative would be that dioxin has two separate and distinct effects: the induction of zoxazolamine hydroxylase and the depression of hexobarbitone oxidase. The following two experiments indicate that, after dioxin treatment, a stimulation of zoxazolamine metabolism can be observed in the absence of any inhibition of hexobarbitone metabolism.

In mice of the C57BL/6 strain, as in the rat, a marked reduction of the zoxazolamine paralysis time was observed at 3 days after dioxin treatment. However, at this time the hexobarbitone sleeping time was either unchanged (in female mice) or shortened (in male mice); a prolongation was seen in males only at 20 days (Table 5).

Table 6. Effect of ethionine on dioxin-induced alterations in sleeping time and paralysis time in rats.[a]

Sex	Route of administration			Sleeping time, min[b]	Paralysis time, min[b]
	Dioxin	Oil	Ethionine		
M	IP	—	—	75.4 ± 13.9 (5)[c]	—
	—	IP	—	36.0 ± 2.2 (5)	—
M	IP	—	PO	41.3 ± 3.6 (5)	84.0 ± 6.5 (5)[d,e]
	—	IP	PO	45.0 ± 3.1 (5)	>383 (5)
M	PO	—	IP	44.7 ± 3.1 (6)	—
	—	PO	IP	42.0 ± 3.4 (6)	—
F	IP	—	PO	83.0 ± 9.4 (6)[f]	119.2 ± 9.4 (6)[e,f]
	—	IP	PO	136.4 ± 7.2 (6)	>480 (6)

[a] Animals received *dl*-ethione (200, 100, and 100 mg/kg) at $t = 0$, 4, and 8 hr, respectively; dioxin (200 μg/kg) or oil at $t = 0.5$ hr and sleeping or paralysis times were measured at $t = 24.5$ hr. All were starved during the experiment.
[b] Numbers of animals in parentheses.
[c] Significantly different from controls at $P < 0.05$.
[d] Significantly different from controls at $P < 0.01$.
[e] By ranking test.
[f] Significantly different from controls at $P < 0.005$.

The effect of dioxin on hexobarbitone metabolism can also be suppressed in rats by administering *dl*-ethionine together with the dioxin. Along with a single oral dose of dioxin male rats were given a series of injections of *dl*-ethionine, an inhibitor of protein synthesis which has been reported as preventing the induction of drug metabolising enzymes by either 20-methylcholanthrene (*27*) or phenobarbitone (*28*). It was found (Table 6) that, whereas the ethionine was ineffective in preventing the reduction of paralysis time in dioxin-treated animals, it did abolish the prolongation of hexobarbitone sleeping time due to dioxin treatment. When the routes of administration

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of dioxin and ethionine were interchanged there was still no difference in hexobarbitone metabolism.

When the experiment was carried out with female rats, in which ethionine is a more effective inhibitor of protein synthesis (*29*), the duration of both paralysis and sleep of the dioxin-treated animals was shorter than that of the controls (Table 6). Thus although these doses of ethionine are not effective in blocking the induced metabolism of zoxazolamine (*27*) in either sex, they are capable of preventing or even reversing the effect of dioxin on hexobarbitone metabolism.

Conclusions

1. The divergent effects of dioxin on the duration of action of hexobarbitone and zoxazolamine in the rat *in vivo* (*2*) are a consequence of changes in hepatic metabolism of these drugs.

2. Dioxin causes alterations in the properties of cytochrome P-450 of rat liver microsomes which are similar to those produced by methylcholanthrene.

3. It is the most effective stimulator of aromatic hydroxylation known, see (*1*), and can apparently overcome a genetic resistance to hepatic microsomal enzyme induction in mice of the DBA/2 strain.

4. The effects of dioxin on hexobarbitone and zoxazolamine metabolism can be separated by the use of C57BL/6 mice or ethionine-treated rats and might be due to two separate modes of action.

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Studies of the Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin on Mammalian Hepatic δ-Aminolevulinic Acid Synthetase

by James S. Woods*

Introduction

The toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a contaminant formed during the manufacture of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) is well known (*1-4*). The widespread utilization of this compound has caused increased concern about the potential health hazards created by the presence of TCDD in the environment. The articles which accompany this account, indeed, attest to the highly toxic nature of TCDD in both laboratory animals and man.

Interest in TCDD as a potential porphyrogenic agent arose when porphyria cutanea tarda, a form of hepatic porphyria, occurred in industrial workers associated with the manufacture of 2,4,5-T (*5*). Hepatic porphyria is a syndrome characterized by a variety of symptoms including the overproduction and excretion of porphyrins, pigmentation of the skin, photosensitivity, and intestinal and neurological disorders. The disease is characterized biochemically by an increase in the activity of the mitochondrial enzyme δ-aminolevulinic acid (ALA) synthetase, which is the first and rate-limiting enzyme in the heme biosynthetic pathway (*6*) (Fig. 1). The possibility that TCDD has porphyrogenic properties has been indicated by the recent observation that TCDD is a potent inducer of hepatic ALA synthetase in chick embryos (*7*). There is, however, no evidence to indicate that TCDD produces similar effects in mammalian species. The chick embryo system is exquisitely sensitive to the effects of agents which induce ALA synthetase (*8*). Previous studies from our laboratory (*9, 10*), on the other hand, have shown that mammalian species demonstrate a striking variability in their response to porphyric agents, especially at different stages of development.

These studies were undertaken, therefore, to determine the possible porphyrogenicity of TCDD in mammalian species and to assess further the utility of the chick embryo liver system as an indicator of the potential porphyrogenic effects of environmental agents in mammals.

Materials and Methods

ALA synthetase activity was determined by two procedures. In liver homogenates

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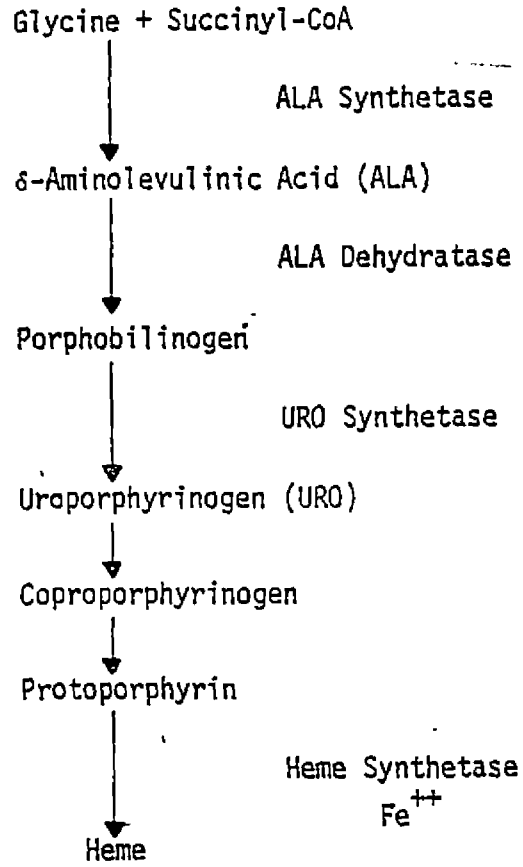


FIGURE 1. Heme biosynthetic pathway.

ALA synthetase activity was assayed using the ion exchange chromatography technique described by Mauzerall and Granick (*11*), with liver homogenates prepared for incubation as described by Marver et al. (*12*). ALA synthetase activity in subcellular fractions and at various stages of enzyme purification was measured using the procedure described by Scholnick et al. (*13*). In the latter case the incubation medium was modified to include $10^{-4}M$ GTP in addition to the prescribed substrates.

The method of Scholnick was also used for the isolation and 50-fold purification of ALA synthetase from porphyric rat liver. Hepatic subcellular fractions were prepared as described by Hayaski et al. (*14*). Protein concentrations were assayed by the method of Lowry et al. (*15*).

All test animals were treated orally with a standard solution of TCDD (10 μg/ml) dissolved in a corn oil/acetone mixture (6:1) 24 hr prior to sacrifice unless otherwise indicated. Two groups of control animals were used in all *in vivo* experiments. The first group received only the corn oil/acetone mixture in an amount equivalent to that in which TCDD was administered to test animals. The second group was treated subcutaneously with allylisopropylacetamide (AIA) (400 mg/kg), which is a well known and potent inducer of ALA synthetase in mammals (*12, 16*).

TCDD was obtained from Dow Chemical Company, Midland, Michigan. AIA was a gift from Hoffmann-LaRoche, Nutley, New Jersey. All animals and other chemicals were obtained from standard sources.

Results and Discussion

Initial studies were designed to determine the potential porphyrogenic effects in rats of TCDD when administered in doses up to 25 μg/kg, the reported LD_{50} for this species (*17*). Male rats were treated with a single 5 or 25 μg/kg dose of TCDD, and hepatic ALA synthetase activity was assayed at periods up to 28 days thereafter. In animals receiving a single dose of AIA, ALA synthetase activity increased to approximately seven times the control level after 24 hr and returned to control levels by the third day after treatment. On the other hand, TCDD did not significantly alter ALA synthetase activity, as measured in whole liver homogenates, during any part of the test period in any of the animals. All measurements of enzyme activity were within the range observed in controls.

It has been recently determined (*14*) that ALA synthetase is a mitochondrial enzyme but is synthesized extramitochondrially on the cytoplasmic ribosomes. The enzyme is subsequently incorporated into the mitochondria, where it becomes active. ALA synthetase activity may be altered by agents which interfer with any aspect of this process. It was, therefore, of interest to determine if TCDD might influence the subcellular localization of ALA synthetase and thereby alter the regulation of hepatic hem-

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Table 1. Subcellular distribution of hepatic ALA synthetase in normal and AIA- and TCDD-treated rats.[a]

Group	Treatment	ALA, nmole/mg protein-hr ± S.E.			
		Mitochondrial fraction	9,000*g* supernatant	105,000*g* supernatant	Microsomal fraction
1	Corn Oil	0.52 ± 0.09	0.27 ± 0.08	0.24 ± 0.10	0.03 ± 0.01
2	AIA	2.26 ± 0.80	1.44 ± 0.40	1.47 ± 0.30	0.28 ± 0.10
3	TCDD	0.58 ± 0.10	0.34 ± 0.06	0.15 ± 0.43	0.01 ± 0.01
4	AIA + TCDD	2.91 ± 0.87	1.37 ± 0.51	1.17 ± 0.51	0.27 ± 0.08

[a] Rats were treated with TCDD (25 μg/kg) and/or AIA (400 mg/kg) 24 hr prior to sacrifice.

atopoiesis in a manner which could not be detected when ALA synthetase activity was measured in whole liver homogenates. The results of these experiments are seen in Table 1. Analysis of the subcellular distribution of ALA synthetase in adult rat liver reveals a 2:1 distribution of activity between mitochondrial and postmitochondrial fractions. Most of the postmitochondrial activity is retained in the 105,000*g* supernatant fraction with very little found in the microsomes. Control rats in the group treated with AIA showed substantial increases in ALA synthetase activity in all fractions but relatively little alteration in the distribution of activity between fractions after 24 hours. TCDD administered alone at 25 μg/kg doses or together with AIA caused no significant alteration of ALA synthetase activity from that observed in control groups. In addition, TCDD did not affect the induction of ALA synthetase by AIA nor alter the subcellular distribution of the enzyme during induction. Similar observations were made when TCDD was administered in 100 mg/kg doses or when these experiments were conducted with female rats or mice.

In vitro tests of the possible effects of TCDD or ALA synthetase activity were conducted in liver homogenates, isolated mitochondria and on ALA synthetase purified 50-fold from porphyric rat liver. In enzyme incubation mixtures containing TCDD in concentrations ranging from 10^{-9} to $10^{-4}M$ no discernable effects on the enzyme activity could be observed.

Finally, the potential porphyrogenic effects of TCDD during the perinatal period were investigated. These studies were conducted in fetal rats which were 3 days from delivery, (−3 day), and on two groups of newborn rats, 4 and 12 days after delivery on the day of sacrifice. Newborn rats were treated orally with 25 μg/kg doses of TCDD, whereas fetal rats were treated by way of the mother. Livers from mother rats served as adult samples. The results are shown in Table 2. In no case did ALA synthetase activity in TCDD-treated rats differ from that observed in untreated animals. Fetal ALA synthetase is typically five to eight times that of the adult (*9*) and declines to adult levels shortly after birth. Refractoriness to induction of ALA synthetase is observed until the activity approaches that of the adult (*10*; *18*). At no stage of development, however, did the ALA synthetase activity in TCDD-treated rats significantly differ from that observed in controls.

These results are of particular interest in view of the potent induction of ALA synthetase activity produced by TCDD in the chick embryos. The lack of a significant effect of TCDD on ALA synthetase in mammals suggests that major differences exist among these species in the biological mechanisms which determine the ultimate pharmacological disposition of chemicals such of TCDD. Species variations in drug distribution, biotransformation, and excretion are well known (*19*). On the other hand, major differences in the mode of action of TCDD may also reflect alterations in the regulation

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Table 2. Study of potential effects of TCDD on ALA synthetase during development in rats.[a]

Group	Treatment	ALA, nmole/hr-g liver ± S.E.			
		−3 day	+4 day	+12 day	Adult
1	Corn oil	241 ± 31	94 ± 7	50 ± 16	45 ± 5
2	TCDD	253 ± 47	76 ± 21	60 ± 8	44 ± 13
3	AIA	248 ± 28	118 ± 12	113 ± 13	347 ± 17

[a] Pregnant and newborn rats were treated 24 hr prior to sacrifice with TCDD (25 μg/kg) or AIA (400 mg/kg).

of hepatic heme synthesis in these species. Striking variations in the developmental aspects of ALA synthetase regulation have already been described. The chick embryo liver is one of the most sensitive systems available in which to study the induction of ALA synthetase (*8*). In contrast, our previous studies (*10*) have shown that fetal mammals are totally insensitive to the effects of drugs which induce or repress hepatic ALA synthetase in chick embryo or in adult animals. Moreover, fetal mammalian ALA synthetase activity is significantly elevated in comparison with that of the adult (*9*), whereas the control level of ALA synthetase in 17-day old chick embryo liver is only one-third that measured in the adult chicken (*20*).

Differences in porphyrin metabolism in these two species is also suggested by the failure of a wide variety of drugs which induce hepatic porphyrin accumulation in chick embryos to do so in mammalian liver (*21*). Among the drugs capable of causing significant increases in porphyrin levels in chick embryo liver are 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC), glutethimide, methsuximide, secobarbital, methylprylon, and mephenytoin. Of this group, only DDC is capable of producing porphyrin accumulation in mice. In addition, it has been recently indicated that uroporphyrinogen synthetase, another enzyme in the heme biosynthetic pathway, may play a rate-limiting role in hepatic heme synthesis in certain strains of mice (*22*).

It therefore appears that significant differences exist among these species with regard to various steps in the regulation of hepatic heme synthesis and porphyrin metabolism. These differences, along with variations in the capacity to metabolize and distribute drugs such as TCDD, may account for the differences in susceptibility of these species to the porphyrogenic effects of TCDD and perhaps to other environmental contaminants.

The foregoing considerations attest to the increasingly prevalent observation that many environmental agents are hazardous not only by virtue of their inherent toxicity but also because of their specificity of action in different species. While utilization of non-mammalian test systems may provide, in some cases, a sensitive indication of the potentially toxic effects of certain drugs and chemicals in mammals, this study demonstrates the necessity for developing test procedures which will more clearly predict the deleterious effects of environmental contaminants in mammalian species, especially in man.

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Effect of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin on the Biliary Excretion of Indocyanine Green in Rat

by Shang W. Hwang*

Chlorinated dibenzodioxins have been found as contaminants of various technical chlorinated compounds such as 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), which are widely used in agriculture. The dioxin contaminants may be involved in various pathologic states resulting from exposure to technical chlorinated compounds (*1, 2*). Liver necrosis has been observed in the animals which were treated with derivatives of chlorophenol (*3*) or other chlorinated compounds (*4*), and it was suggested that liver necrosis-causing factors were present in these compounds. In view of the above observation, the present study was undertaken to investigate whether 2,3,7,8-tetrachloro-*p*-dibenzodioxin (TCDD) has any effect on the hepatobiliary function of rat. Biliary excretion of indocyanine green (ICG) was used as the index of function. The dye was chosen because it is completely and rapidly excreted by normal liver into the bile by an active process (*5*).

Materials and Methods

Male random bred CD rats weighing 350–400 g were treated PO with a single dose of 25 μg/kg or 5 μg/kg TCDD in acetone and corn oil. The controls received an equivalent volume of the vehicle. At day 1, 7, and 16 after treatment, the effect of TCDD on the bile flow and the biliary excretion of ICG was examined. Animals were first anesthetized with penobarbital Na (50 mg/kg) IP. Through an abdominal incision, renal pedicles were ligated, and the common bile duct was cannulated with a blunt 23-gauge hypodermic needle shaft attached to an 8-in. piece of PE 50 tubing. Bile was collected for a 20-min period, and the amount collected was measured by weighing. At the end of 20 min, freshly prepared indocyanine green in aqueous solvent (Hynson, Westcott and Dunning, Inc.) was injected at a dose of 6.25 mg/kg into the femoral vein, and the bile was collected for another 20 min. The body temperature was monitored with a telethermometer and was maintained at 37° C by warming with an incandescent lamp. At the end of the experiment, blood was withdrawn by heart puncture and the liver was excised. The ICG concentration in bile, plasma, and liver was determined by measuring its absorption at 805 nm. The rate of ICG disappearance from plasma was also determined by measuring the dye concentrations in a series of plasma samples which were withdrawn by heart puncture 1, 2, 5, 8, 12, and 20 min after ICG injection from groups of control and TCDD-treated rats.

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Results and Discussion

Bile flow during the first 20 min of the experiment increased after TCDD treatment as shown in Figure 1. The initial flow rate continued to increase through the 16th day, and rats receiving 25 μg/kg TCDD had higher flow rates than those receiving 5 μg/kg TCDD.

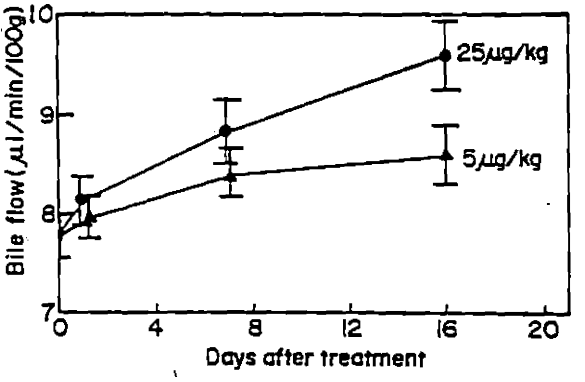


FIGURE 1. Effect of TCDD on bile flow. Each point is the mean ± SEM of four animals. Difference from control is significant ($P < 0.005$) for the 7th and 16th day after 25 μg/kg TCDD treatment and 16th day after 5 μg/kg TCDD treatment.

The increase in bile flow could be due to the increasing secretion of water by the hepatic cell or due to the decreasing reabsorption of water along the bile duct. Both mechanisms were possible, but further investigation will be needed for clarification.

TCDD also caused an increase in liver weight as shown in Figure 2. The increase in liver weight as expressed in grams liver weight per 100 g body weight was also dose-related since the higher dose caused a greater weight increase. Whether this increase in liver weight has any effect on the bile flow is not known. Similar results of bile flow increase and liver weight increase were observed in rat after phenobarbital treatment (*6*), and both TCDD and phenobarbital are potent microsomal enzyme inducers.

ICG excretion was also affected by TCDD treatment. The total amount of ICG excreted during 20 min after injection of the dye was decreased significantly as shown in Figure 3. Less hepatic excretion of the ICG was observed with the larger dose of TCDD than with the smaller dose. The rate of excretion of this dye was still markedly suppressed by the 16th day after treatment.

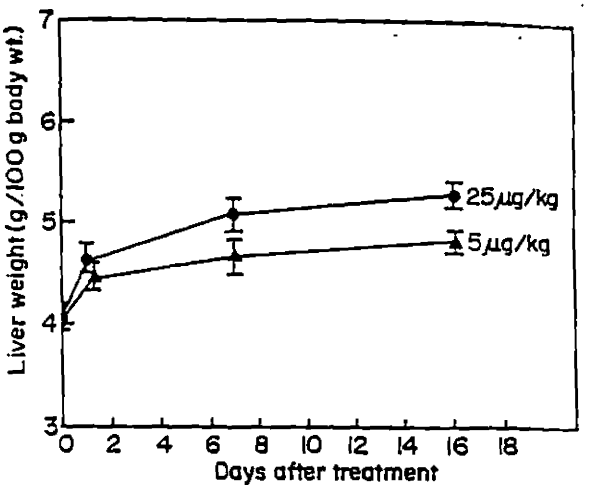


FIGURE 2. Liver weight of rat after TCDD treatment. Each point is the mean ± SEM of four animals. The difference is significant ($P < 0.05$) for the 7th and 16th day after treatment of either 5 μg/kg or 25 μg/kg TCDD.

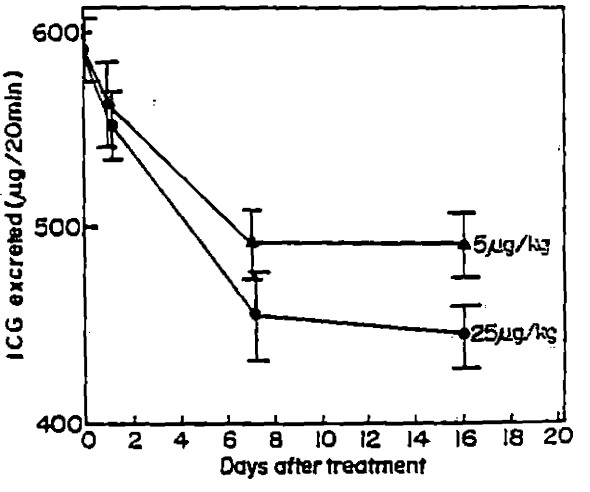


FIGURE 3. Effect of TCDD on ICG biliary excretion. The dose of ICG was 6.25 mg/kg, and the bile was collected for 20 min after ICG injection. Each point is the mean ± SEM of four animals. The difference from control is significant ($P < 0.05$) for the 7th and 16th day after treatment by either 5 μg/kg or 25 μg/kg of TCDD.

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Table 1. Effect of TCDD on liver uptake and biliary excretion of ICG.

Time after TCDD treatment, days	ICG concentration in plasma, μg/ml *		ICG concentration in liver, μg/g *		ICG concentration in bile, μg/ml *	
	TCDD 5 μg/kg	TCDD 25 μg/kg	TCDD 5 μg/kg	TCDD 25 μg/kg	TCDD 5 μg/kg	TCDD 25 μg/kg
Control	1.0 ± 0.09	1.9 ± 0.09	28.5 ± 0.9	28.5 ± 0.9	1130 ± 83	1130 ± 83
1 day	2.3 ± 0.10	2.75 ± 0.29	29.5 ± 2.3	29.0 ± 1.8	1015 ± 79	999 ± 64
7 days	3.0 ± 0.23	3.45 ± 0.15	37.5 ± 3.6	31.0 ± 2.1	869 ± 66	710 ± 45
16 days	3.1 ± 0.46	3.90 ± 0.58	41.0 ± 5.4	35.0 ± 3.6	860 ± 59	624 ± 46

* Dose of ICG was 6.25 mg/kg. Concentrations were determined 20 min after ICG injection. Each value is the mean ± SEM from four animals.

The concentrations of ICG in plasma, liver, and bile were also analyzed separately for each animal 20 min after dye injection. The results (Table 1) showed that concentration of ICG in bile of TCDD-treated rat was lower than that of the control, and the ICG levels in plasma and liver of TCDD-treated rats were higher than that of the control. A greater depression of ICG concentration in bile and a greater retention of ICG in blood were caused by 25 μg/kg dose of TCDD. In contrast, animals treated with 5 μg/kg TCDD accumulated more ICG in liver than e animals treated with 25 μg/kg TCDD. o sign of recovery was shown by the 16th day after treatment.

Bile-to-plasma, bile-to-liver and liver-to-plasma concentration ratios of ICG were also separately calculated and compared, as shown in Figure 4. Bile-to-plasma and bile-to-liver ratios decreased after TCDD treatment, and the higher dose decreased these ratios even further. Liver-to-plasma ratio decreased only after 25 μg/kg but not after 5 μg/kg TCDD pretreatment. TCDD appeared to inhibit both ICG uptake by the hepatic cell and the active secretion of ICG by the hepatic cell. Inhibition of both steps could result in the decreased total ICG excretion and lowered bile-to-plasma and bile-to-liver ICG concentration ratios as found.

The excretion of ICG from hepatic cell to bile was probably inhibited to a similar extent by both 25 μg/kg and 5 μg/kg TCDD. However, the inhibition of ICG uptake by the hepatic cell might be more dose-dependent; the inhibition was greater with 25 μg/kg than with 5 μg/kg TCDD, so the liver cumulated less ICG after 25 μg/kg TCDD

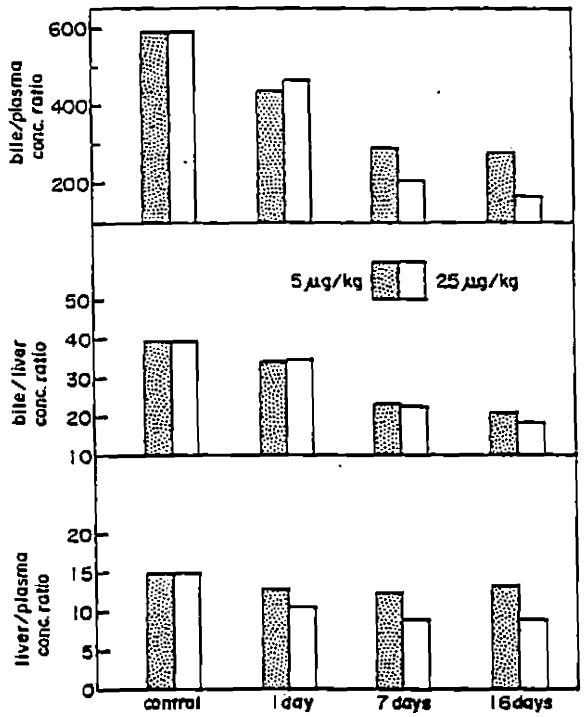


FIGURE 4. Change of ICG concentration ratios after TCDD treatment. Each bar represents the mean of four animals. The difference from control is significant ($P < 0.05$) for all ratios at 7th and 16th day after TCDD treatment except the liver/plasma (L/P) ratios after 5 μg/kg TCDD treatment.

treatment, and liver-to-plasma ICG concentration ratios decreased in animals treated with 25 μg/kg but not 5 μg/kg TCDD. Control animals should take up ICG into liver faster than TCDD-treated animals, but the secretion from liver to bile was even faster compared to the treated animals, so the control animals accumulated less ICG in both plasma and liver.

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The rate of disappearance of ICG in plasma decreased after TCDD treatment, as shown in Figure 5. The 25 μg/kg dose gave a greater reduction of disappearance rate than did the 5 μg/kg dose, and the rate was lower at the 16th day than at the 7th day after treatment. The decreasing rate of ICG disappearance in plasma gave further evidence that ICG excretion was damaged by the TCDD treatment.

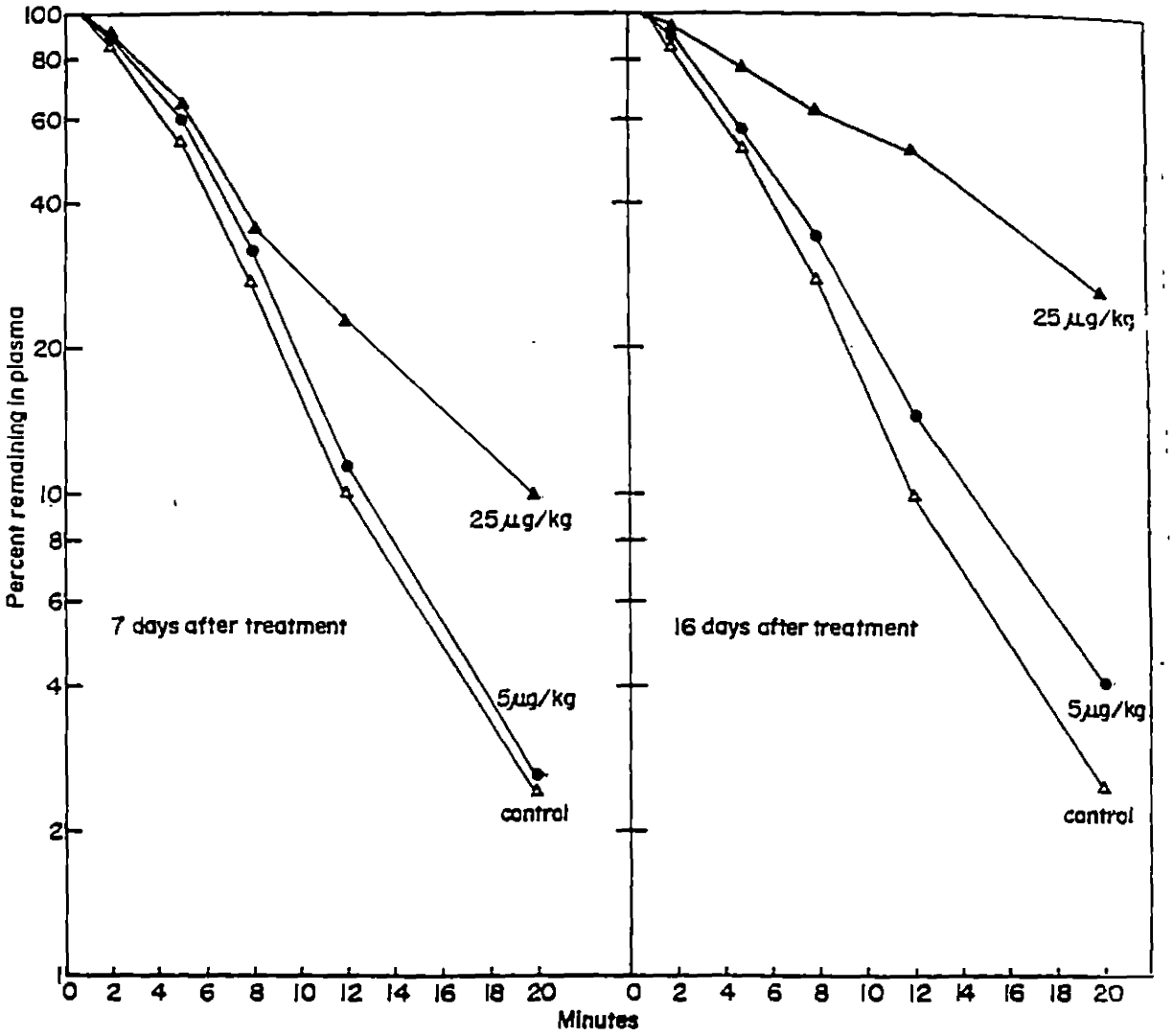


FIGURE 5. Effect of TCDD on the ICG disappearance rate in plasma. Each point is the mean of three rats.

Summary

From the above observations, it was concluded that TCDD inhibited hepatobiliary excretion of ICG, and the inhibitory effect appeared to be a long-lasting one. Many anionic compounds, including endogenous substances such as bilirubin, are actively secreted through the similar mechanism by the hepatobiliary system. The decreased ICG excretory ability might also apply to other anionic compounds, and the etiology of reported cases of jaundice and porphyria after exposure to TCDD might be partly accounted for by the decreased biliary excretory ability.

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Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin on Drug Metabolism and Hepatic Microsomes of Rats and Mice

by J.B. Greig* and F. De Matteis*

Introduction

It has been reported (*1*) that one effect of a single, intra-peritoneal dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) is to cause a reduction in the duration of action of the drug 2-amino-5-chlorobenzoxazole (zoxazolamine) in the rat. This effect is maximal with doses of dioxin above 100 μg/kg and significant even at 5 μg/kg. Our own work showed that oral dosage had a similar effect but also that the duration of action of hexobarbitone was considerably prolonged (*2*). The results described here extend these observations.

Materials and Methods

Animals

Rats of the albino Porton strain bred in these Laboratories and weighing 180–200 g (male 6–8 weeks and female 7–10 weeks old) had free access to water and diet 41B unless otherwise indicated.

Mice of the C57BL/6 and DBA/2 strains were bred in these laboratories and kept on Sterolit bedding (Mineral & Chemical Corporation of America, Menlo Park, New Jersey, U.S.A.) for at least 3 weeks before use. They had free access to diet and water.

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Dosages

Dioxin, prepared as described elsewhere (*3*), was administered as a solution (100 μg/ml) in Arachis oil. Control animals received an equivalent volume of oil. Zoxazolamine (McNeil Laboratories, Inc.) was dissolved in 1*N* HCl (1.2 ml/100 mg), diluted with 0.9% NaCl to 10 mg/ml and administered IP at 100 mg/kg. 5-Cyclo-hex-1′-enyl-1,5-dimethylbarbituric acid (hexobarbitone) sodium (May & Baker Ltd.) was dissolved in water at 50 or 25 mg/ml and administered IP at 150 mg/kg (male rats), 75 mg/kg (female rats), 100 mg/kg (male and female mice). *dl*-Ethionine (Koch-Light) was dissolved in water (20 mg/ml) and administered as indicated in the text.

Microsomal Preparations

Liver microsomes were isolated either as described by Bond and De Matteis (*4*), except that the microsomes were sedimented at 105,000*g*, or by the calcium/sucrose method (*5*) adapted to the extent of sedimenting the calcium-treated microsomes at 1900*g* and washing them once in a 0.0125*M* sucrose solution containing 8*mM* $CaCl_2$. The final preparations were suspended in 0.1*M* phosphate buffer (pH 7.4) containing 1*mM* Na_2-EDTA (1.4 –7.0 mg protein/ml). Protein was estimated by the biuret method (*6*) and cytochrome P-450 (or P-448) by using an

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extinction coefficient of 91/*mM*–cm (*7*) for the absorbancy change between 450 (448) and 490 nm of the CO difference spectrum of a $Na_2S_2O_4$-reduced suspension.

Assays

Plasma barbiturate levels following hexobarbitone administration were measured by the method of Chromý and Babjuk (*8*).

Hexobarbitone oxidase was measured *in vitro* by the modified (*9*) method of Brodie et al. (*10*).

Zoxazolamine hydroxylase was measured by the methods of Burns and his colleagues (*11, 12*) with minor modifications.

Difference Spectra

Difference spectra were recorded on a Unicam SP 1800 spectrophotometer with oxidized microsomal suspensions (2.7 ml, 1.4–2.2 mg protein /ml) in each 1-cm cuvet and the addition of either aniline (5–200 µl, 0.22-*M* in 0.1*M* phosphate buffer, pH 7.4) or hexobarbitone sodium (5-100 µl, 0.054*M* in water) to the sample cuvet and of an equal volume of the appropriate solvent to the reference cuvet. Pyridine difference spectra were recorded with $Na_2S_2O_4$-reduced microsomal suspensions (2.7 ml) and 0.3 ml of either 0.5*M* aqueous pyridine or water added to the cuvets.

The wavelength of cytochrome P-450 (or P-448) maximum absorption was measured on a Cary 14 spectrophotometer calibrated with a holmium filter.

Statistics

Results are quoted as the mean ± S. E. M. and were analyzed by Student's *t*-test except where the nature of the results necessitated the use of a ranking test.

Results and Discussion

Effect of Dioxin Administration on the *In Vivo* Action, and *In Vitro* Metabolism of Drugs in the Rat

One or 3 days after being given a single oral dose of dioxin rats show a shortening of the zoxazolamine paralysis time but an increase in the duration of action of hexobarbitone (*2*). The effect on the hexobarbitone sleeping time becomes progressively more marked with time until, 2 weeks after dosing, rats sleep over 4 hr and some of them die without waking (Table 1).

Table 1. Effect of dioxin on the hexobarbitone sleeping time of male rats.[a]

Time after dosing	Sleeping time, min [b]		Increase over controls, *P* %
	Dioxin (200 µg/kg, PO)	Solvent	
12 hr	32.9 ± 3.5 (6)	37.7 ± 3.3 (5)	— NS
24 hr	40.0 ± 3.1 (6)	27.4 ± 1.8 (6)	46 <0.01
72 hr	75.5 ± 5.9 (6)	33.6 ± 3.3 (6)	125 <0.001
1 week	125.6 ± 14.1 (6)	21.5 ± 2.9 (5)	480 <0.001
2 weeks	> 244 (6)	25.2 ± 2.7 (6)	870 0.0022 [c]

[a] Hexobarbitone sodium administered as described in Materials and Methods section.
[b] Numbers of animals in parentheses.
[c] By ranking test.

In view of the prolonged reduction of food intake of rats dosed with dioxin (*3*) it is possible that a starvation effect (*13*) might contribute significantly to the increased hexobarbitone sleeping times at later stages in the intoxication (1 or 2 weeks after dosing). However earlier experiments (*2*) had suggested that, 1 day after dosing with dioxin, the reduced food intake was not the sole cause of the prolongation of the hexobarbitone sleeping time. This interpretation is open to criticism, since in these experiments the animals had not been prefasted before dioxin administration and the dioxin might have slowed down the absorption of the residual food in the stomach and intes-

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Accordingly groups of eight female rats were starved for 39 hr and then dosed with dioxin (200 μg/kg, PO) or oil. Following 24 hr further starvation the hexobarbitone sleeping time was measured; the value for the dosed group, 89.0 ±3.8 min, was significantly higher than that of the control group, 63.2 ±5.3 min, ($P < 0.005$). Thus the early effect of dioxin on the sleeping time cannnot be entirely due to differences in food consumption or absorption between control and treated animals. Therefore in all following experiments we have used rats which had been given a single dose of dioxin (200 μg/kg) 1 or 3 days previously.

It was considered possible that dioxin might prolong the hexobarbitone sleeping time by altering either the sensitivity of the nervous system or the distribution of the barbiturate within the body. These possibilities were ruled out by an experiment in which the sleeping times of groups of dioxin-treated and control rats were measured and, immediately after their waking, blood was collected for the analysis of plasma barbiturate levels (*8*). Table 2 indicates that, although there was a significant increase in the sleeping time of the dosed animals, the waking plasma barbiturate levels of the two groups were not significantly different.

Table 2. Effect of dioxin on the sleeping time and waking plasma barbiturate level of female rats.[a]

Treatment	N	Sleeping time, min	Plasma barbiturate, μg/ml
Dioxin	6	99.3 ± 19.8	67.9 ± 2.2
Controls	6	45.9 ± 4.2	64.5 ± 2.9
P		<0.025	NS

[a] Rats were dosed with dioxin (200 μg/kg, PO) or oil and 3 days later the sleeping time induced by hexobarbitone sodium (75 mg/kg, IP) was measured. Immediately after waking the animals were anesthetized (ether) and blood collected from the heart. Plasma barbiturate was measured by the method of Chromy and Babjuk (*8*).

Three days after a single oral dose of either dioxin or oil to male or female rats the liver microsomes were isolated and incubated with an NADPH-generating system and either hexobarbitone or zoxazolamine (Table 3). With either sex, following dioxin treatment, there was a significant increase in the amount of zoxazolamine metabolized, and with males there was a significant decrease in the quantity of hexobarbitone oxidized. With the microsomes from female rats the control level of hexobarbitone metabolism was lower than the males and, although in the treated livers it was lower still, the difference was not significant. Apart from this, the results agree with the *in vivo* experiments and indicate that dioxin modifies the duration of the pharmacological action of both zoxazolamine and hexobarbitone by changing the rate of metabolism of these drugs by the liver microsomes.

Table 3. Effect of dioxin treatment on the metabolism of hexobarbitone and zoxazolamine by rat liver microsomes.[a]

Treatment	Sex	Zoxazolamine metabolized, nmole/mg microsomal protein/hr [b]	Hexobarbitone metabolized, nmoles/mg microsomal protein/hr [b]
Dioxin	M	32.2 ± 4.2 (10)	31.0 ± 23.9 (5)
Controls	M	15.7 ± 2.0 (10)	192.0 ± 18.7 (5)
		P <0.005	<0.001
Dioxin	F	46.9 ± 7.9 (5)	29.4 ± 47.8 (5)
Controls	F	6.3 ± 5.4 (5)	109.2 ± 49.7 (5)
		P <0.005	NS

[a] Rats (180–200 g) received dioxin (200 μg/kg) or oil PO and were killed 3 days later. Microsomes were isolated by the calcium/sucrose method (see Materials and Methods) and the assays performed essentially as described elsewhere (*9–12*).

[b] Numbers of animals in parentheses.

Effect of Dioxin Administration on the Liver Content and Spectral Properties of Microsomal Cytochrome P-450

Besides oxygen and NADPH, it is known that three other components are required for the reconstitution of a system capable of metabolizing drugs *in vitro*. These are a lipid, phosphatidyl choline; a flavoprotein, NADPH/cytochrome P-450 reductase, and a hemoprotein, cytochrome P-450 (or P-448) (*14–16*). We have investigated the effect of dioxin treatment on cytochrome P-450 of rat liver microsomes. Groups of male

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Table 4. Changes in liver weight and microsomal protein and cytochrome P-450 induced by dioxin treatment of male rats.*

Treatment	Weight wet liver, g/100 g BW	Microsomal protein, mg/g liver	Microsomal cytochrome P-450		
			nmoles/g liver	nmoles/mg protein	λ_{max} (nm)
Dioxin	4.34 ± 0.12	26.5 ± 0.9	55.1 ± 1.5	2.08 ± 0.07	447.6 ± 0.04
Controls	3.21 ± 0.06	27.3 ± 0.6	28.1 ± 0.7	1.03 ± 0.02	449.6 ± 0.08
	P <0.001	NS	<0.001	<0.001	<0.001

* Rats received dioxin (200 μg/kg, PO) or oil and 3 days later were killed and liver microsomes isolated at 105,000g (see Materials and Methods). Each value is the mean of five observations.

rats received an oral dose of either dioxin or oil and three days later were killed and their liver microsomes isolated. Table 4 indicates that there was a significant increase in the weight of wet liver from the treated animals. There was no increase in the microsomal protein content, expressed per gram of tissue, but the cytochrome content doubled. Further, the wavelength of the peak maximum of the cytochrome spectrum was shifted from 450 nm to 448 nm. Such a shift is a feature of the microsomal enzyme induction brought about by compounds such as 20-methylcholanthrene (*17*). Since this inducer stimulates the metabolism of aromatic compounds, including zoxazolamine (*18*) this change correlates well with the observed stimulation of zoxazolamine metabolism both *in vivo* and *in vitro*. However it should be remembered that, if the CO/cytochrome P-448 complex has an extinction coefficient more than four times that of P-450, as has been reported (19), then there has in fact been a decrease in hemo-protein concentration.

The interaction of the reduced cytochrome P-450 of liver microsomes with the ligands ethyl isocyanide or pyridine is known to result in the formation of a difference spectrum with two peaks in the region 400–460 nm (*20*). The relative intensity of these two peaks, related to the absorption at 500 nm, is dependent on the pH of the suspending medium (20). In the case of liver microsomes prepared from animals pretreated with 20-methylcholanthrene and therefore containing P-448 rather than P-450, the curve of pH dependence of the peak height ratio is so shifted that the peaks are of equal intensity at a lower pH (*21*). The pH dependence of the pyridine difference spectra of microsomes from methylcholanthrene-, dioxin- or oil-treated rats was measured and is shown in Figure 1. It can be seen that the curves due to dioxin or methylcholanthrene treatment are similar to each other but distinct from that of the control.

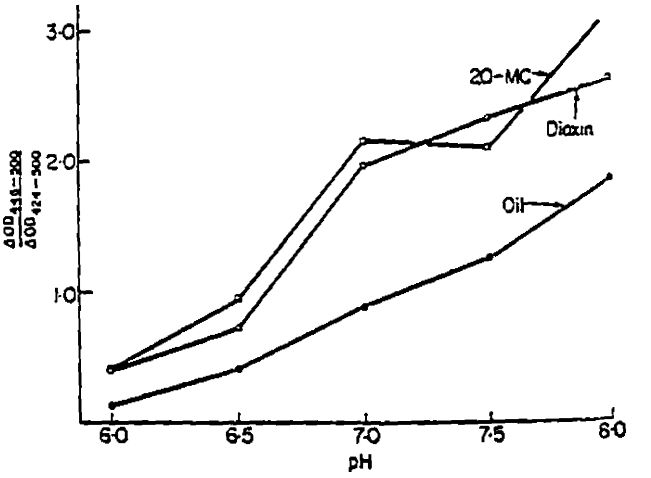


FIGURE 1. Rats (male, 180–200 g) received dioxin (200 μg/kg, PO) or Arachis oil (2 ml/kg, PO) 3 days before killing. 20-Methylcholanthrene was injected (20 mg/kg, 10 mg/ml in oil, IP) 3 and 2 days before killing. All were starved for 24 hr before decapitation. Portions of liver microsomes equivalent to 0.4 g of wet liver were isolated by the calcium/sucrose method (*5*) and suspended in 0.1M PO_4 buffer (10 ml, containing 1mM Na_2EDTA) of the appropriate pH. Pyridine difference spectra were measured as stated in Materials and Methods.

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is known that, in the oxidized state, the cytochrome P-450 of liver microsomes will interact with various substrates of the drug metabolizing system to produce characteristic difference spectra (*22*). We have studied the effect of dioxin pretreatment of rats on the interaction of aniline and hexobarbitone with rat liver microsomes. With aniline the control microsomes showed a normal Type II spectral change; this was intensified in the case of the microsomes from treated rats (Fig. 2). Such a change is consistent with the increased cytochrome P-448 in these preparations. However, a double reciprocal plot of the spectral change/nmole of P-450 (P-448) against aniline concentration indicated that there was an increase in K_s (binding affinity constant) and in the maximal Type II spectral change (Fig. 3). A similar effect has been reported following methylcholanthrene induction (*23*).

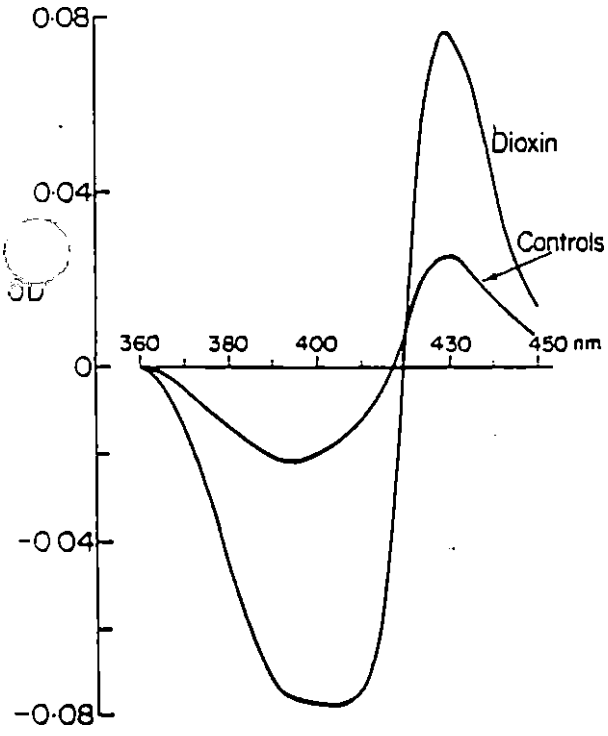


FIGURE 2. Rats (male, 180–200 g) received dioxin (200 μg/kg) or oil PO 3 days before killing. They were starved for 24 hr before isolation of liver microsomes at 105,000g. Preparations from two animals were combined. Difference spectra (aniline 0.41*mM* final concentration) measured as described in Materials and Methods on suspensions containing microsomes equivalent to 0.073g wet liver/ml.

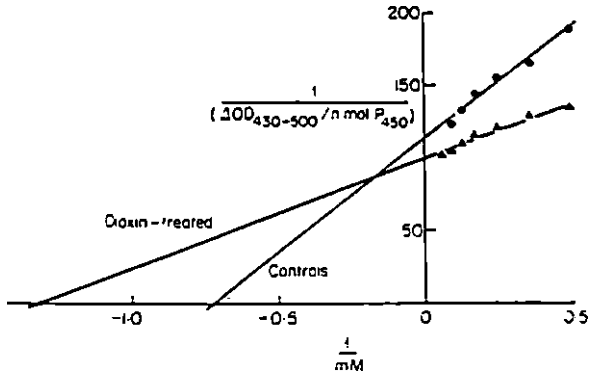


FIGURE 3. Rats (male, 170–190 g) received dioxin (200 μg/kg) or oil PO 3 days before killing. Liver microsomes, equivalent to 2.8 g wet liver, were isolated by the calcium/sucrose method (*5*) and were suspended in 0.1*M* PO_4 buffer (25 ml, pH 7.4) containing 1*mM* EDTA. Aniline concentrations in the cuvet (see Materials and Methods) were varied from 2.0 to 15.2*mM*. Each point is the mean of observations on five animals. On each axis the mean intercepts for groups of dosed and control animals were significantly different ($P < 0.05$). An extinction coefficient of 91/*mM*–cm (*7*) was used in the estimation of cytochrome P–450 (–448). The spectral changes observed are related to the total cytochrome content of the cuvet.

The interaction of hexobarbitone with microsomes from control animals produced the expected Type I difference spectra (*22*) as illustrated in Figure 4. However the microsomal preparations from rats which had received dioxin 3 days previously consistently gave a difference spectrum with a peak at 412 nm and a trough at 380 nm. This type of spectrum has been termed a modified Type II spectral change (*22*) and has been observed following methylcholanthrene pretreatment (*24*), but only with microsomal preparations from female rats. A decrease in the intensity of the Type I difference spectrum due to hexobarbitone has been reported for methylcholanthrene-treated male and female rats (*23*).

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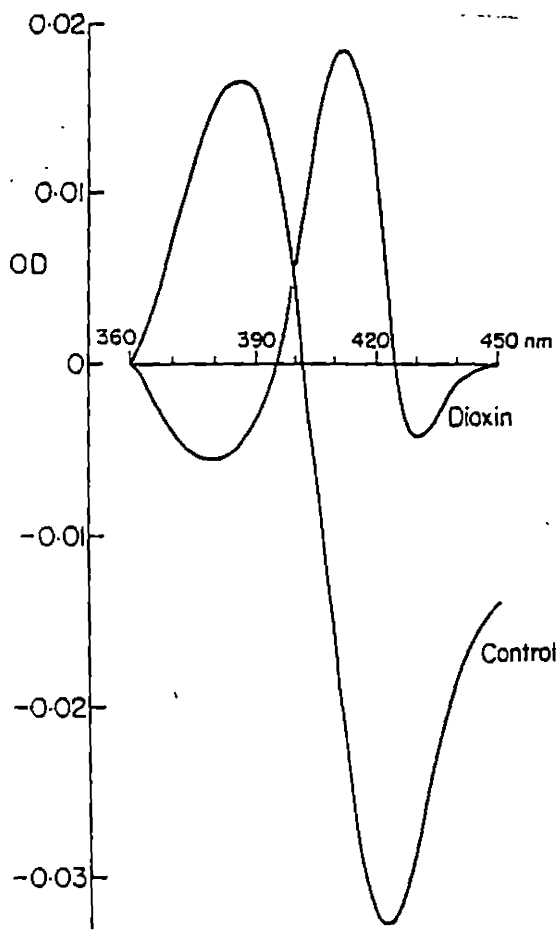


FIGURE 4. All details as in the legend to Figure 2 except that hexobarbitone was added to the sample cuvetts to a concentration of 0.79*mM*.

Comparison of the Effects of 20-Methylcholanthrene and Dioxin on Zoxazolamine Action in Mice of the DBA/2 Strain

All the changes of the properties of the liver microsomes from dioxin-treated rats are consistent with dioxin being a powerful inducer of the methylcholanthrene type. It has been reported that in mice of the DBA/2 strain the levels of hepatic aryl hydrocarbon hydroxylase are unaffected by methylcholanthrene (*25*) or benz[*a*]anthracene (*26*) treatment. Indeed when DBA/2 mice (male, 20–30 g) were injected with methylcholanthrene or oil as described by Nebert et al. (*25*) we found, 24 hr after injection, no significant difference between the zoxazolamine paralysis times of the treated and control groups (58.0 ±5.1 min, $N = 6$ and 59.3 ± 3.9 min, $N = 6$, respectively). This contrasts with C57BL/6 mice (male, 18–26 g) in which methylcholanthrene pretreatment significantly reduced the paralysis time (dosed: 15.0 ± 1.0 min, $N = 9$, controls: 79.5 ± 5.3 min, $N = 9$; $P < 0.0001$). However in both strains a single oral dose of dioxin (200 μg/kg) given 3 days before zoxazolamine significantly lowered the paralysis time (DBA/2, male, dosed: 10.5 ± 0.8 min, $N = 6$, controls: 65.7 ± 4.2 min, $N = 5$, $P < 0.001$; for C57BL/6 see Table 5).

Table 5. Effect of dioxin on sleeping and paralysis times of C57 BL/6 mice.[a]

Treatment	Sex	Sleeping time, min[b] After 3 days	Sleeping time, min[b] After 10 days	Sleeping time, min[b] After 20 days	Paralysis time after 3 days, min
Dioxin	M	52.6 ± 6.2 (7)	60.6 ± 4.7 (7)	>159.7 (7)	7.0 ± 0.7 (4)
Oil	M	71.3 ± 3.8 (6)	60.2 ± 7.7 (4)	53.4 ± 7.3 (6)	46.8 ± 3.5 (5)
		P <0.05	NS	<0.01[c]	<0.001
Dioxin	F	52.6 ± 6.5 (7)	—	—	5.9 ± 0.6 (5)
Oil	F	65.1 ± 8.4 (7)	—	—	37.7 ± 4.0 (7)
		P NS			<0.001

[a] Mice (11–27 g) received dioxin (200 μg/kg) or oil PO and hexobarbitone sodium or zoxazolamine after the stated intervals.
[b] Numbers of animals in parentheses.
[c] By ranking test.

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assuming that, under these conditions, the duration of zoxazolamine action is entirely governed by the rate of metabolism in the liver it would appear that the apparent genetic noninducibility of hepatic aryl hydrocarbon hydroxylase in certain mouse strains is not an absolute trait but dependent on the inducer used. This is in agreement with the observations (*26*) that this enzyme, although present in smaller amounts in extrahepatic tissues, is inducible in such organs of strains in which the liver enzyme is unaffected.

Investigation of the Relationship between Stimulation of Zoxazolamine Metabolism and Inhibition of Hexobarbitone Metabolism

Although in the rat dioxin produces simultaneous and divergent effects on the metabolism of zoxazolamine and hexobarbitone by liver microsomes, it is not known whether these two effects are related to each other. An alternative would be that dioxin has two separate and distinct effects: the induction of zoxazolamine hydroxylase and the depression of hexobarbitone oxidase. The following two experiments indicate that, after dioxin treatment, a stimulation of zoxazolamine metabolism can be observed in the absence of any inhibition of hexobarbitone metabolism.

In mice of the C57BL/6 strain, as in the rat, a marked reduction of the zoxazolamine paralysis time was observed at 3 days after dioxin treatment. However, at this time the hexobarbitone sleeping time was either unchanged (in female mice) or shortened (in male mice); a prolongation was seen in males only at 20 days (Table 5).

Table 6. Effect of ethionine on dioxin-induced alterations in sleeping time and paralysis time in rats.[a]

Sex	Route of administration			Sleeping time, min[b]	Paralysis time, min[b]
	Dioxin	Oil	Ethionine		
M	IP	—	—	75.4 ± 13.9 (5)[c]	—
	—	IP	—	36.0 ± 2.2 (5)	—
M	IP	—	PO	41.3 ± 3.6 (5)	84.0 ± 6.5 (5)[d,e]
	—	IP	PO	45.0 ± 3.1 (5)	>383 (5)
M	PO	—	IP	44.7 ± 3.1 (6)	—
	—	PO	IP	42.0 ± 3.4 (6)	—
F	IP	—	PO	83.0 ± 9.4 (6)[f]	119.2 ± 9.4 (6)[e,f]
	—	IP	PO	136.4 ± 7.2 (6)	>480 (6)

[a] Animals received *dl*-ethione (200, 100, and 100 mg/kg) at $t = 0$, 4, and 8 hr, respectively; dioxin (200 μg/kg) or oil at $t = 0.5$ hr and sleeping or paralysis times were measured at $t = 24.5$ hr. All were starved during the experiment.
[b] Numbers of animals in parentheses.
[c] Significantly different from controls at $P < 0.05$.
[d] Significantly different from controls at $P < 0.01$.
[e] By ranking test.
[f] Significantly different from controls at $P < 0.005$.

The effect of dioxin on hexobarbitone metabolism can also be suppressed in rats by administering *dl*-ethionine together with the dioxin. Along with a single oral dose of dioxin male rats were given a series of injections of *dl*-ethionine, an inhibitor of protein synthesis which has been reported as preventing the induction of drug metabolising enzymes by either 20-methylcholanthrene (*27*) or phenobarbitone (*28*). It was found (Table 6) that, whereas the ethionine was ineffective in preventing the reduction of paralysis time in dioxin-treated animals, it did abolish the prolongation of hexobarbitone sleeping time due to dioxin treatment. When the routes of administration

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of dioxin and ethionine were interchanged there was still no difference in hexobarbitone metabolism.

When the experiment was carried out with female rats, in which ethionine is a more effective inhibitor of protein synthesis (*29*), the duration of both paralysis and sleep of the dioxin-treated animals was shorter than that of the controls (Table 6). Thus although these doses of ethionine are not effective in blocking the induced metabolism of zoxazolamine (*27*) in either sex, they are capable of preventing or even reversing the effect of dioxin on hexobarbitone metabolism.

Conclusions

1. The divergent effects of dioxin on the duration of action of hexobarbitone and zoxazolamine in the rat *in vivo* (*2*) are a consequence of changes in hepatic metabolism of these drugs.

2. Dioxin causes alterations in the properties of cytochrome P-450 of rat liver microsomes which are similar to those produced by methylcholanthrene.

3. It is the most effective stimulator of aromatic hydroxylation known, see (*1*), and can apparently overcome a genetic resistance to hepatic microsomal enzyme induction in mice of the DBA/2 strain.

4. The effects of dioxin on hexobarbitone and zoxazolamine metabolism can be separated by the use of C57BL/6 mice or ethionine-treated rats and might be due to two separate modes of action.

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Studies of the Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin on Mammalian Hepatic δ-Aminolevulinic Acid Synthetase

by James S. Woods*

Introduction

The toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a contaminant formed ing the manufacture of the herbicide 5-trichlorophenoxyacetic acid (2,4,5-T) is well known (*1-4*). The widespread utilization of this compound has caused increased concern about the potential health hazards created by the presence of TCDD in the environment. The articles which accompany this account, indeed, attest to the highly toxic nature of TCDD in both laboratory animals and man.

Interest in TCDD as a potential porphyrogenic agent arose when porphyria cutanea tarda, a form of hepatic porphyria, occurred in industrial workers associated with the manufacture of 2,4,5-T (*5*). Hepatic porphyria is a syndrome characterized by a variety of symptoms including the overproduction and excretion of porphyrins, pigmentation of the skin, photosensitivity, and intestinal and neurological disorders. The disease is characterized biochemically by an increase in the activity of the mitochondrial enzyme δ-aminolevulinic acid (ALA) synthetase, which is the first and rate-limiting enzyme in the heme biosynthetic pathway (*6*) (Fig. 1). The possibility that TCDD has porphyrogenic properties has been indicated by the recent observation that TCDD is a potent inducer of hepatic ALA synthetase in chick embryos (*7*). There is, however, no evidence to indicate that TCDD produces similar effects in mammalian species. The chick embryo system is exquisitely sensitive to the effects of agents which induce ALA synthetase (*8*). Previous studies from our laboratory (*9, 10*), on the other hand, have shown that mammalian species demonstrate a striking variability in their response to porphyric agents, especially at different stages of development.

These studies were undertaken, therefore, to determine the possible porphyrogenicity of TCDD in mammalian species and to assess further the utility of the chick embryo liver system as an indicator of the potential porphyrogenic effects of environmental agents in mammals.

Materials and Methods

ALA synthetase activity was determined by two procedures. In liver homogenates

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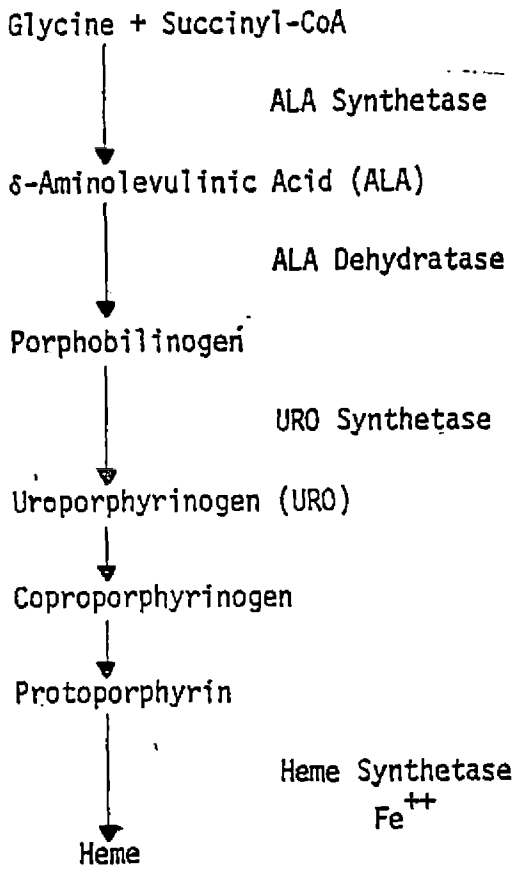


FIGURE 1. Heme biosynthetic pathway.

ALA synthetase activity was assayed using the ion exchange chromatography technique described by Mauzerall and Granick (*11*), with liver homogenates prepared for incubation as described by Marver et al. (*12*). ALA synthetase activity in subcellular fractions and at various stages of enzyme purification was measured using the procedure described by Scholnick et al. (*13*). In the latter case the incubation medium was modified to include $10^{-4}M$ GTP in addition to the prescribed substrates.

The method of Scholnick was also used for the isolation and 50-fold purification of ALA synthetase from porphyric rat liver. Hepatic subcellular fractions were prepared as described by Hayaski et al. (*14*). Protein concentrations were assayed by the method of Lowry et al. (*15*).

All test animals were treated orally with a standard solution of TCDD (10 μg/ml) dissolved in a corn oil/acetone mixture (6:1) 24 hr prior to sacrifice unless otherwise indicated. Two groups of control animals were used in all *in vivo* experiments. The first group received only the corn oil/acetone mixture in an amount equivalent to that in which TCDD was administered to test animals. The second group was treated subcutaneously with allylisopropylacetamide (AIA) (400 mg/kg), which is a well known and potent inducer of ALA synthetase in mammals (*12, 16*).

TCDD was obtained from Dow Chemical Company, Midland, Michigan. AIA was a gift from Hoffmann-LaRoche, Nutley, New Jersey. All animals and other chemicals were obtained from standard sources.

Results and Discussion

Initial studies were designed to determine the potential porphyrogenic effects in rats of TCDD when administered in doses up to 25 μg/kg, the reported LD_{50} for this species (*17*). Male rats were treated with a single 5 or 25 μg/kg dose of TCDD, and hepatic ALA synthetase activity was assayed at periods up to 28 days thereafter. In animals receiving a single dose of AIA, ALA synthetase activity increased to approximately seven times the control level after 24 hr and returned to control levels by the third day after treatment. On the other hand, TCDD did not significantly alter ALA synthetase activity, as measured in whole liver homogenates, during any part of the test period in any of the animals. All measurements of enzyme activity were within the range observed in controls.

It has been recently determined (*14*) that ALA synthetase is a mitochondrial enzyme but is synthesized extramitochondrially on the cytoplasmic ribosomes. The enzyme is subsequently incorporated into the mitochondria, where it becomes active. ALA synthetase activity may be altered by agents which interfer with any aspect of this process. It was, therefore, of interest to determine if TCDD might influence the subcellular localization of ALA synthetase and thereby alter the regulation of hepatic hem-

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Table 1. Subcellular distribution of hepatic ALA synthetase in normal and AIA- and TCDD-treated rats.*

Group	Treatment	ALA, nmole/mg protein-hr ± S.E.			
		Mitochondrial fraction	9,000g supernatant	105,000g supernatant	Microsomal fraction
1	Corn Oil	0.52 ± 0.09	0.27 ± 0.08	0.24 ± 0.10	0.03 ± 0.01
2	AIA	2.26 ± 0.80	1.44 ± 0.40	1.47 ± 0.30	0.28 ± 0.10
3	TCDD	0.58 ± 0.10	0.34 ± 0.06	0.15 ± 0.43	0.01 ± 0.01
4	AIA + TCDD	2.91 ± 0.87	1.37 ± 0.51	1.17 ± 0.51	0.27 ± 0.08

* Rats were treated with TCDD (25 μg/kg) and/or AIA (400 mg/kg) 24 hr prior to sacrifice.

atopoiesis in a manner which could not be detected when ALA synthetase activity was measured in whole liver homogenates. The results of these experiments are seen in Table 1. Analysis of the subcellular distribution of ALA synthetase in adult rat liver reveals a 2:1 distribution of activity between mitochondrial and postmitochondrial fractions. Most of the postmitochondrial activity is retained in the 105,000g supernatant fraction with very little found in the microsomes. Control rats in the group treated with AIA showed substantial increases in ALA synthetase activity in all fractions but relatively little alteration in the distribution of activity between fractions after 24 hours. TCDD administered alone at 25 μg/kg doses or together with AIA caused no significant alteration of ALA synthetase activity from that observed in control groups. In addition, TCDD did not affect the induction of ALA synthetase by AIA nor alter the subcellular distribution of the enzyme during induction. Similar observations were made when TCDD was administered in 100 mg/kg doses or when these experiments were conducted with female rats or mice.

In vitro tests of the possible effects of TCDD or ALA synthetase activity were conducted in liver homogenates, isolated mitochondria and on ALA synthetase purified 50-fold from porphyric rat liver. In enzyme incubation mixtures containing TCDD in concentrations ranging from 10^{-3} to $10^{-6}M$ no discernable effects on the enzyme activity could be observed.

Finally, the potential porphyrogenic effects of TCDD during the perinatal period were investigated. These studies were conducted in fetal rats which were 3 days from delivery, (−3 day), and on two groups of newborn rats, 4 and 12 days after delivery on the day of sacrifice. Newborn rats were treated orally with 25 μg/kg doses of TCDD, whereas fetal rats were treated by way of the mother. Livers from mother rats served as adult samples. The results are shown in Table 2. In no case did ALA synthetase activity in TCDD-treated rats differ from that observed in untreated animals. Fetal ALA synthetase is typically five to eight times that of the adult (*9*) and declines to adult levels shortly after birth. Refractoriness to induction of ALA synthetase is observed until the activity approaches that of the adult (*10*, *18*). At no stage of development, however, did the ALA synthetase activity in TCDD-treated rats significantly differ from that observed in controls.

These results are of particular interest in view of the potent induction of ALA synthetase activity produced by TCDD in the chick embryos. The lack of a significant effect of TCDD on ALA synthetase in mammals suggests that major differences exist among these species in the biological mechanisms which determine the ultimate pharmacological disposition of chemicals such of TCDD. Species variations in drug distribution, biotransformation, and excretion are well known (*19*). On the other hand, major differences in the mode of action of TCDD may also reflect alterations in the regulation

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Table 2. Study of potential effects of TCDD on ALA synthetase during development in rats.*

Group	Treatment	ALA, nmole/hr-g liver ± S.E.			
		−3 day	+4 day	+12 day	Adult
1	Corn oil	241 ± 31	94 ± 7	50 ± 16	45 ± 5
2	TCDD	253 ± 47	76 ± 21	60 ± 8	44 ± 13
3	AIA	248 ± 28	118 ± 12	113 ± 13	347 ± 17

* Pregnant and newborn rats were treated 24 hr prior to sacrifice with TCDD (25 μg/kg) or AIA (400 mg/kg).

of hepatic heme synthesis in these species. Striking variations in the developmental aspects of ALA synthetase regulation have already been described. The chick embryo liver is one of the most sensitive systems available in which to study the induction of ALA synthetase (*8*). In contrast, our previous studies (*10*) have shown that fetal mammals are totally insensitive to the effects of drugs which induce or repress hepatic ALA synthetase in chick embryo or in adult animals. Moreover, fetal mammalian ALA synthetase activity is significantly elevated in comparison with that of the adult (*9*), whereas the control level of ALA synthetase in 17-day old chick embryo liver is only one-third that measured in the adult chicken (*20*).

Differences in porphyrin metabolism in these two species is also suggested by the failure of a wide variety of drugs which induce hepatic porphyrin accumulation in chick embryos to do so in mammalian liver (*21*). Among the drugs capable of causing significant increases in porphyrin levels in chick embryo liver are 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC), glutethimide, methsuximide, secobarbital, methylprylon, and mephenytoin. Of this group, only DDC is capable of producing porphyrin accumulation in mice. In addition, it has been recently indicated that uroporphyrinogen synthetase, another enzyme in the heme biosynthetic pathway, may play a rate-limiting role in hepatic heme synthesis in certain strains of mice (*22*).

It therefore appears that significant differences exist among these species with regard to various steps in the regulation of hepatic heme synthesis and porphyrin metabolism. These differences, along with variations in the capacity to metabolize and distribute drugs such as TCDD, may account for the differences in susceptibility of these species to the porphyrogenic effects of TCDD and perhaps to other environmental contaminants.

The foregoing considerations attest to the increasingly prevalent observation that many environmental agents are hazardous not only by virtue of their inherent toxicity but also because of their specificity of action in different species. While utilization of non-mammalian test systems may provide, in some cases, a sensitive indication of the potentially toxic effects of certain drugs and chemicals in mammals, this study demonstrates the necessity for developing test procedures which will more clearly predict the deleterious effects of environmental contaminants in mammalian species, especially in man.

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Effect of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin on the Biliary Excretion of Indocyanine Green in Rat

by Shang W. Hwang*

Chlorinated dibenzodioxins have been found as contaminants of various technical chlorinated compounds such as 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), which are widely used in agriculture. The dioxin contaminants may be involved in various pathologic states resulting from exposure to technical chlorinated compounds (*1*, *2*). Liver necrosis has been observed in the animals which were treated with derivatives of chlorophenol (*3*) or other chlorinated compounds (*4*), and it was suggested that liver necrosis-causing factors were present in these compounds. In view of the above observation, the present study was undertaken to investigate whether 2,3,7,8-tetrachloro-*p*-dibenzodioxin (TCDD) has any effect on the hepatobiliary function of rat. Biliary excretion of indocyanine green (ICG) was used as the index of function. The dye was chosen because it is completely and rapidly excreted by normal liver into the bile by an active process (*5*).

Materials and Methods

Male random bred CD rats weighing 350–400 g were treated PO with a single dose of 25 μg/kg or 5 μg/kg TCDD in acetone and corn oil. The controls received an equivalent volume of the vehicle. At day 1, 7, and 16 after treatment, the effect of TCDD on the bile flow and the biliary excretion of ICG was examined. Animals were first anesthetized with penobarbital Na (50 mg/kg) IP. Through an abdominal incision, renal pedicles were ligated, and the common bile duct was cannulated with a blunt 23-gauge hypodermic needle shaft attached to an 8-in. piece of PE 50 tubing. Bile was collected for a 20-min period, and the amount collected was measured by weighing. At the end of 20 min, freshly prepared indocyanine green in aqueous solvent (Hynson, Westcott and Dunning, Inc.) was injected at a dose of 6.25 mg/kg into the femoral vein, and the bile was collected for another 20 min. The body temperature was monitored with a telethermometer and was maintained at 37° C by warming with an incandescent lamp. At the end of the experiment, blood was withdrawn by heart puncture and the liver was excised. The ICG concentration in bile, plasma, and liver was determined by measuring its absorption at 805 nm. The rate of ICG disappearance from plasma was also determined by measuring the dye concentrations in a series of plasma samples which were withdrawn by heart puncture 1, 2, 5, 8, 12, and 20 min after ICG injection from groups of control and TCDD-treated rats.

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Results and Discussion

Bile flow during the first 20 min of the experiment increased after TCDD treatment as shown in Figure 1. The initial flow rate continued to increase through the 16th day, and rats receiving 25 μg/kg TCDD had higher flow rates than those receiving 5 μg/kg TCDD.

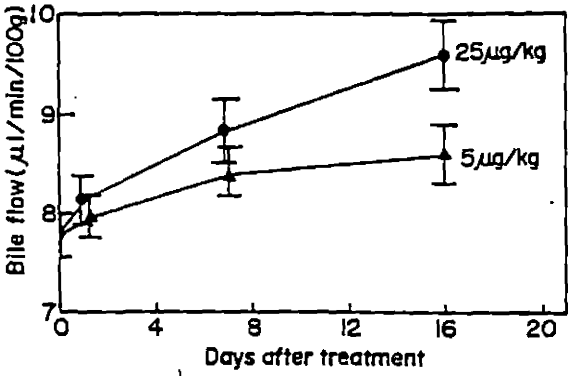


FIGURE 1. Effect of TCDD on bile flow. Each point is the mean ± SEM of four animals. Difference from control is significant ($P < 0.005$) for the 7th and 16th day after 25 μg/kg TCDD treatment and 16th day after 5 μg/kg TCDD treatment.

The increase in bile flow could be due to the increasing secretion of water by the hepatic cell or due to the decreasing reabsorption of water along the bile duct. Both mechanisms were possible, but further investigation will be needed for clarification.

TCDD also caused an increase in liver weight as shown in Figure 2. The increase in liver weight as expressed in grams liver weight per 100 g body weight was also dose-related since the higher dose caused a greater weight increase. Whether this increase in liver weight has any effect on the bile flow is not known. Similar results of bile flow increase and liver weight increase were observed in rat after phenobarbital treatment (*6*), and both TCDD and phenobarbital are potent microsomal enzyme inducers.

ICG excretion was also affected by TCDD treatment. The total amount of ICG excreted during 20 min after injection of the dye was decreased significantly as shown in Figure 3. Less hepatic excretion of the ICG was observed with the larger dose of TCDD than with the smaller dose. The rate of excretion of this dye was still markedly suppressed by the 16th day after treatment.

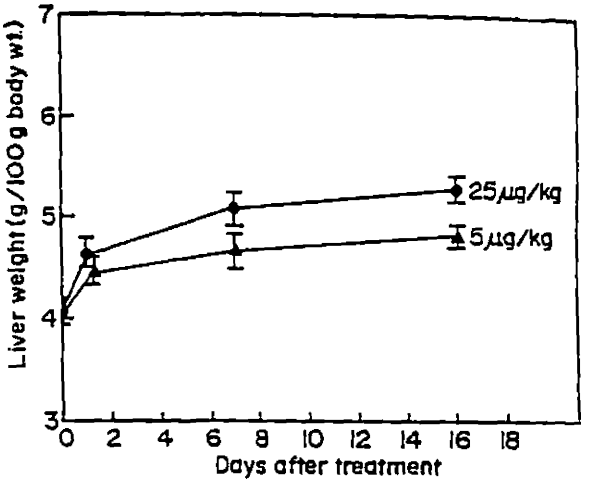


FIGURE 2. Liver weight of rat after TCDD treatment. Each point is the mean ± SEM of four animals. The difference is significant ($P < 0.05$) for the 7th and 16th day after treatment of either 5 μg/kg or 25 μg/kg TCDD.

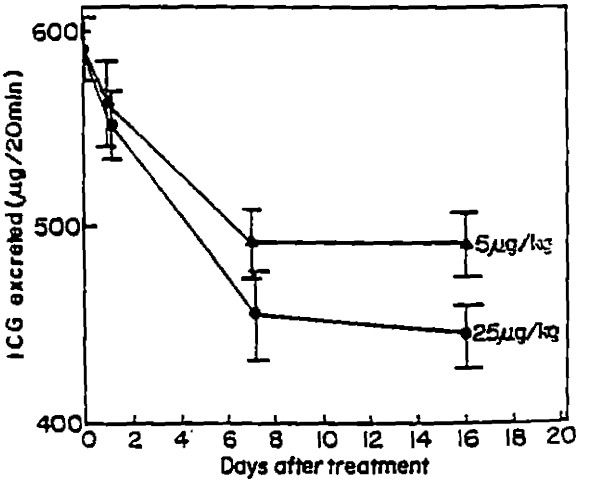


FIGURE 3. Effect of TCDD on ICG biliary excretion. The dose of ICG was 6.25 mg/kg, and the bile was collected for 20 min after ICG injection. Each point is the mean ± SEM of four animals. The difference from control is significant ($P < 0.05$) for the 7th and 16th day after treatment by either 5 μg/kg or 25 μg/kg of TCDD.

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Table 1. Effect of TCDD on liver uptake and biliary excretion of ICG.

Time after TCDD treatment, days	ICG concentration in plasma, μg/ml [a]		ICG concentration in liver, μg/g [a]		ICG concentration in bile, μg/ml [a]	
	TCDD 5 μg/kg	TCDD 25 μg/kg	TCDD 5 μg/kg	TCDD 25 μg/kg	TCDD 5 μg/kg	TCDD 25 μg/kg
Control	1.0 ± 0.09	1.9 ± 0.09	28.5 ± 0.9	28.5 ± 0.9	1130 ± 83	1130 ± 83
1 day	2.3 ± 0.10	2.75 ± 0.29	29.5 ± 2.3	29.0 ± 1.8	1015 ± 79	999 ± 64
7 days	3.0 ± 0.23	3.45 ± 0.15	37.5 ± 3.6	31.0 ± 2.1	869 ± 66	710 ± 45
16 days	3.1 ± 0.46	3.90 ± 0.58	41.0 ± 5.4	35.0 ± 3.6	860 ± 59	624 ± 46

[a] Dose of ICG was 6.25 mg/kg. Concentrations were determined 20 min after ICG injection. Each value is the mean ± SEM from four animals.

The concentrations of ICG in plasma, liver, and bile were also analyzed separately for each animal 20 min after dye injection. The results (Table 1) showed that concentration of ICG in bile of TCDD-treated rat was lower than that of the control, and the ICG levels in plasma and liver of TCDD-treated rats were higher than that of the control. A greater depression of ICG concentration in bile and a greater retention of ICG in blood were caused by 25 μg/kg dose of TCDD. In contrast, animals treated with 5 μg/kg TCDD accumulated more ICG in liver than the animals treated with 25 μg/kg TCDD. No sign of recovery was shown by the 16th day after treatment.

Bile-to-plasma, bile-to-liver and liver-to-plasma concentration ratios of ICG were also separately calculated and compared, as shown in Figure 4. Bile-to-plasma and bile-to-liver ratios decreased after TCDD treatment, and the higher dose decreased these ratios even further. Liver-to-plasma ratio decreased only after 25 μg/kg but not after 5 μg/kg TCDD pretreatment. TCDD appeared to inhibit both ICG uptake by the hepatic cell and the active secretion of ICG by the hepatic cell. Inhibition of both steps could result in the decreased total ICG excretion and lowered bile-to-plasma and bile-to-liver ICG concentration ratios as found.

The excretion of ICG from hepatic cell to bile was probably inhibited to a similar extent by both 25 μg/kg and 5 μg/kg TCDD. However, the inhibition of ICG uptake by the hepatic cell might be more dose-dependent; the inhibition was greater with 25 μg/kg than with 5 μg/kg TCDD, so the liver accumulated less ICG after 25 μg/kg TCDD

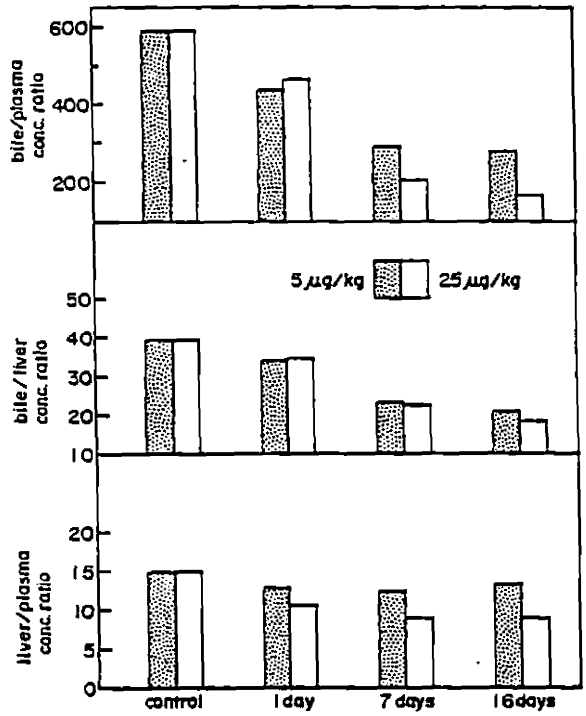


FIGURE 4. Change of ICG concentration ratios after TCDD treatment. Each bar represents the mean of four animals. The difference from control is significant ($P < 0.05$) for all ratios at 7th and 16th day after TCDD treatment except the liver/plasma (L/P) ratios after 5 μg/kg TCDD treatment.

treatment, and liver-to-plasma ICG concentration ratios decreased in animals treated with 25 μg/kg but not 5 μg/kg TCDD. Control animals should take up ICG into liver faster than TCDD-treated animals, but the secretion from liver to bile was even faster compared to the treated animals, so the control animals accumulated less ICG in both plasma and liver.

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The rate of disappearance of ICG in plasma decreased after TCDD treatment, as shown in Figure 5. The 25 μg/kg dose gave a greater reduction of disappearance rate than did the 5 μg/kg dose, and the rate was lower at the 16th day than at the 7th day after treatment. The decreasing rate of ICG disappearance in plasma gave further evidence that ICG excretion was damaged by the TCDD treatment.

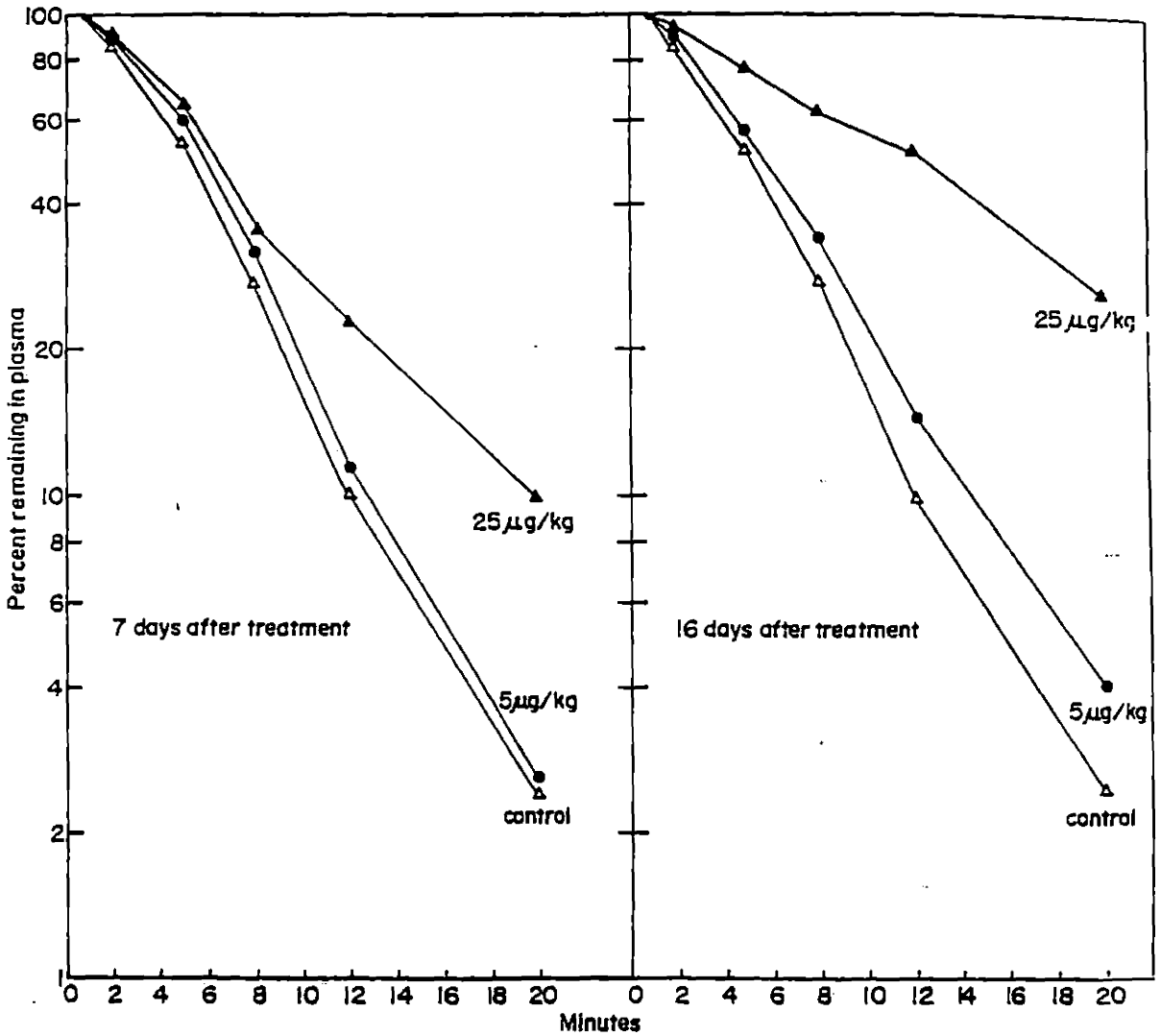


FIGURE 5. Effect of TCDD on the ICG disappearance rate in plasma. Each point is the mean of three rats.

Summary

From the above observations, it was concluded that TCDD inhibited hepatobiliary excretion of ICG, and the inhibitory effect appeared to be a long-lasting one. Many anionic compounds, including endogenous substances such as bilirubin, are actively secreted through the similar mechanism by the hepatobiliary system. The decreased ICG excretory ability might also apply to other anionic compounds, and the etiology of reported cases of jaundice and porphyria after exposure to TCDD might be partly accounted for by the decreased biliary excretory ability.

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Biological Responses of the Nonhuman Primate, Chicken, and Rat to Chlorinated Dibenzo-*p*-dioxin Ingestion*

by D.H. Norback † and J.R. Allen †

In 1958 Schmittle et al. (*1*) reported the development of hydropericardium and ascites in poultry following ingestion of feeds containing industrially contaminated fats. The toxic component was demonstrated to have the chlorinated dibenzo-*p*-dioxin (CDD) structure by Cantrell et al. in 1969). Experimental animal studies in our oratory have shown that CDD administration causes varying responses in different animal species. Gastric hyperplasia and ulceration, hydropericardium, ascites, reduced spermatogenesis, focal liver necrosis, decreased hematopoiesis, skin lesions, and eventual mortality have been demonstrated in nonhuman primates (*3*). Chickens succumbed very rapidly to the same dietary concentration with hydropericardium, hydrothorax, and ascites. They also developed liver necrosis, hypoplastic testes, and altered capillary permeability and decreased hematopoiesis (*4–6*). The rat was more resistant to the morbid effects of CDD but developed a hypertrophied liver composed of enlarged hepatocytes with a proliferated intracellular membrane system (*7*). The results of these investigations are reviewed and the variable responses and pathogenesis of lesions are discussed.

The material used in the investigations was crude industrial fat capable of producing hydropericardium, ascites, and death in the chicken. Gas–liquid chromatographic and nuclear magnetic resonance analysis of the materials demonstrated bi-, tri-, tetra-, penta-, hexa-, and heptachlorodibenzo-*p*-dioxin present in the material, with the tetrachlorinated compound comprising 64% (mass) of the total dioxins present. In addition, rats were given radioactive octachloro- or radioactive tetrachlorodibenzo-*p*-dioxin in separate experiments (*8, 9*).

Macaca mulatta monkeys were given a diet that contained varying quantities of the crude industrial fat. The percentage of fat that allowed survival of the nonhuman primate for 100 days produced 50% mortality in chickens within 15 days. The survival time of the monkeys was inversely related to the percentage of the CDD-containing fat given to the animals. Clinical and pathologic changes occurring at their demise were similar regardless of concentration of material in their diet. At death all had developed ascites, hydropericardium, and anasarca. Prior to their demise the monkeys developed a decrease in total serum protein

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from 7.5 to 5.4 g/100 ml and a decrease in the percentage albumin from 61% to 35%. There was a decrease in hematocrit from 41% found in the control animals to 16% in the experimental group, a decrease in the white blood count from 6.8×10^3 per mm^3 to 3.0×10^3, and a decrease in the red cell count from 6.5×10^6 per mm^3 to 2.5×10^6. The hemoglobin values were correspondingly reduced. Analysis of the sternal bone marrow showed a hypoplastic bone marrow with diminished myeloid and erythroid cells being replaced by fatty tissue. The lymphoid tissue of the spleen and lymph nodes was hypoplastic.

The skin changes of the monkeys included alopecia and subcutaneous edema which progressed from the eyelids to the remainder of the face, eventually involving the subcutaneous tissue of the trunk, extremities, and scrotum. Microscopically, there was edema of the dermal layer with disarray of the collagen fibers. Hair follicles, particularly of the face, contained numerous keratin cysts with hyperplasia of the epithelium (Fig. 1).

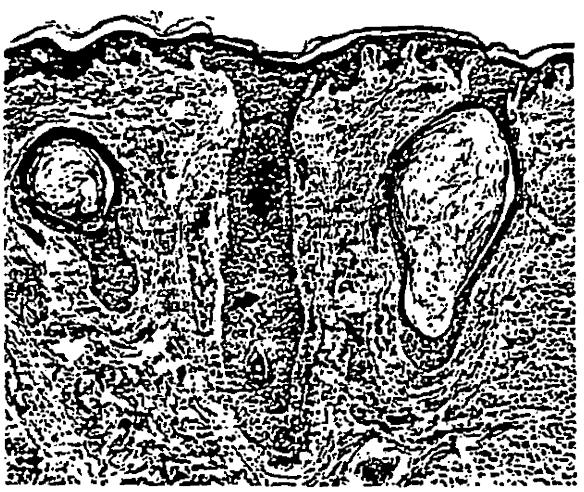


FIGURE 1. Hair follicles, particularly of the face and eyelids, of monkeys fed CDD contained numerous keratin cysts. Light micrograph of skin fixed with formalin and stained with hematoxylin and eosin. ×15.

The seminiferous tubules of the testes contained abundant spermatogonia and Sertoli cells. However, there was a decreased number of primary and secondary spermatocytes, and spermatids were inapparent in most instances (Fig. 2). The interstitial cells of Leydig appeared normal.



FIGURE 2. In a monkey fed CDD, seminiferous tubules of testes contained decreased numbers of primary and secondary spermatocytes without spermatids. Spermatogonia, Sertoli cells, and interstitial cells were normal in appearance. Light micrograph of testicular tissue fixed with formalin and stained with hematoxylin and eosin. ×115.

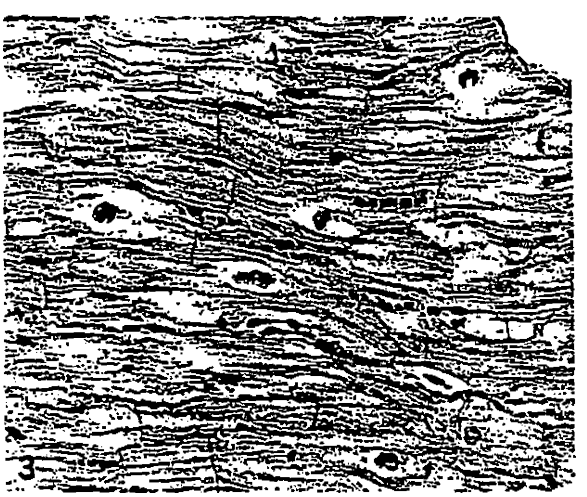


FIGURE 3. Cardiac fibers in the hearts of monkeys fed CDD were hypertrophied, and the myofilaments were widely separated by the increased intracellular fluid. Light micrograph of heart fixed with formalin and stained with hematoxylin and eosin. ×115.

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The heart was dilated, particularly in the right chamber, and an increase in the circumference of both the tricuspid and mitral valves was noted. Microscopically, hypertrophic muscle fibers were separated by fluid (Fig. 3). Electron microscopic examination demonstrated separation of the myofibrils and swelling of the mitochondria with widely separated cristae (Fig. 4). In over 60% of the experimental monkeys, marked hypertrophy of the gastric mucosa occurred in the fundic and pyloric regions. The hypertrophied mucosal layer penetrated the muscularis mucosae to form crypts and mucin-containing cysts in the submucosa (Fig. 5). In the same areas, gastric ulcerations of the mucosal layer were present (Fig. 6).

The livers were moderately yellow. On microscopic examination, enlarged multinucleated hepatocytes and fat vacuoles were apparent. Terminally the animals developed centrilobular necrosis and bile duct hyperplasia. Changes in the biliary tree were observed as proliferation and stratification of the bile duct epithelial cells of the small ducts within the portal area and in the larger ducts, including the common bile duct running through the head of the pancreas (Fig. 7). Electron microscopic examination of the hepatocytes demonstrated hypertrophied cells with numerous autophagosomes and fat droplets, an increase in the smooth endoplasmic reticulum with a decrease in the rough endoplasmic reticulum, and swollen mitochondria (Fig. 8). In the morbid animals the parenchymal cells showed numerous degenerative changes. Many of the cells



FIGURE 4. Myofibrils of dilated cardiac fibers within the heart of a monkey fed CDD were separated, and the mitochondria were moderately swollen. Electron micrograph of heart fixed with Veronal acetate-buffered osmium tetroxide solution and stained with uranyl acetate. ×9,700.

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FIGURE 5. (*a*) Normal glands of the gastric mucosa are separated by the muscularis mucosae from the submucosa in this section of a stomach taken from a control monkey. (*b*) Following CDD ingestion, marked hypertrophy of the gastric mucosa occurred in the fundic and pyloric regions. Crypts and mucin-containing cysts within the submucosa are extensions of the hypertrophied mucosa which penetrated the muscularis mucosae. Light micrograph of stomach fixed with formalin and stained with hematoxylin and eosin. (*a*) ×30; (*b*) ×25.

were shrunken and electron-dense, while others were swollen and very lucent.

Of the experimental animals evaluated, the chicken was the most sensitive to the toxic effects of CDD. Low levels of the compounds resulted in a decrease in growth rate and the development of hydropericardium, hydrothorax, and ascites, resulting in death of the animals. Following doses sufficient to cause 50% mortality within 15 days, hemoglobin was reduced from 10 g/100 ml to 6 g/100 ml, hematocrit was reduced from 31% to 18%, and the total serum protein decreased from 3.4 g/100 ml to 2.3 g/100 ml. The percentage albumin also decreased in these animals. The animals developed large hearts, and on microscopic examination the cardiac fibers were separated by edematous fluid. A lymphocytic infiltrate was observed between the myocardial fibers and in the perivascular areas. The capsule of the liver was thickened with adherent fibrinous material. Lymphoid hyperplasia was apparent in the portal areas, and the hepatic cells were infiltrated with fat (Fig. 9). Electron microscopic examination demonstrated viable cells dispersed among degenerative cells. Degenerative changes of hepatocytes and Kupffer cells included electron dense cytoplasm, irregular mitochondria, and indistinct nuclear envelopes. The Sertoli cells and spermatogonia of the semi-

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FIGURE 6. Gastric ulcerations of the hypertrophied mucosal layer were present in the stomachs of monkeys receiving CDD. Ulcers were particularly prevalent over areas of epithelial cell-lined submucosal cysts. Edema of the submucosa, portion of a submucosal cyst, fibrotic granulation tissue, a fibrinous exudate and overhanging edges of the mucosa are present. Light micrograph of stomach fixed with formalin and stained with hematoxylin and eosin. ×35.

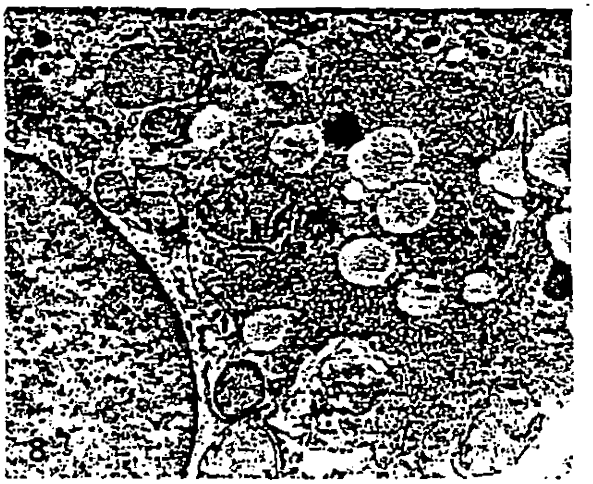


FIGURE 8. Livers of monkeys fed CDD contained hypertrophied cells with swollen mitochondria, increased numbers of lipid droplets, and a proliferation of smooth endoplasmic reticulum. Electron micrograph of liver tissue fixed in Veronal acetate-buffered osmium tetroxide solution and stained with uranyl acetate. ×8500.

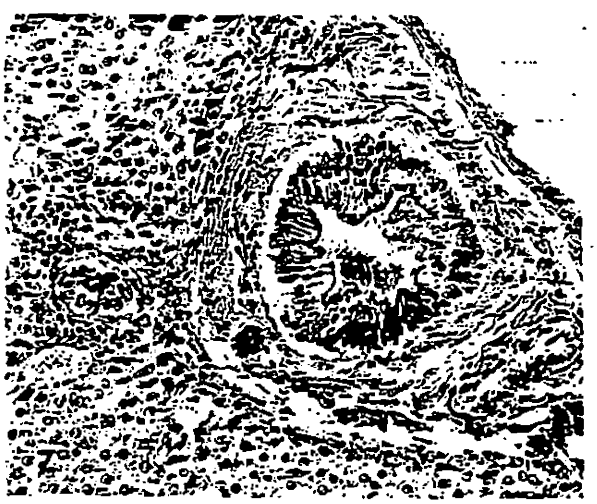


FIGURE 7. Following CDD ingestion by the monkeys, bile duct hyperplasia occurred within the liver. Tall columnar epithelial cells, many of which appeared stratified, replaced cuboidal cells found in the normal bile duct and epithelial folds extended into the lumen. Light micrographs of liver tissue fixed in formalin and stained with hematoxylin and eosin. ×115.

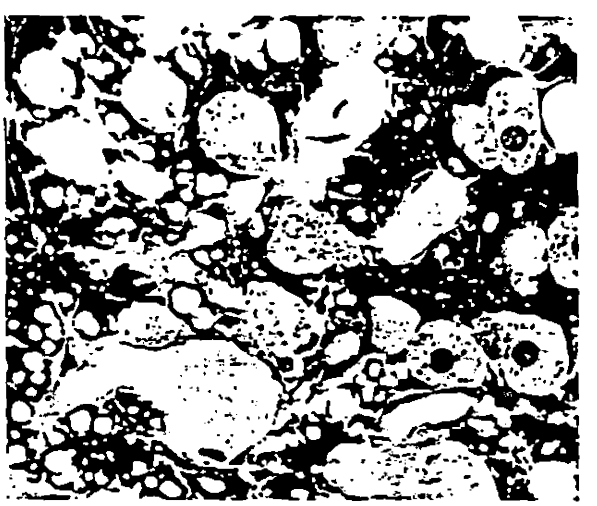


FIGURE 9. Light and dark staining cells were present in the livers of chickens fed CDD. Numerous fat droplets infiltrated the hepatocytes. Light micrograph of liver tissue fixed in Veronal acetate-buffered osmium tetroxide solution and stained with toluidine blue. ×610.

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niferous tubules within the testes were normal; however, there was a reduction in the number of primary and secondary spermatocytes, and no spermatozoa were present. Gastrointestinal changes were not observed. The perfusion of the mesenteric vessels with ferritin, thorium dioxide, iron oxide, and carbon black demonstrated a decided alteration in capillary permeability of these experimental animals.

Following administration of approximately five times the concentration of the CDD-containing fat in the diet sufficient to cause hydropericardium, ascites, and focal necrosis of the liver in chickens and in nonhuman primates, the rat developed liver alterations with a 50% mortality at 80 days. At 6 weeks, enlarged livers contained hypertrophied hepatocytes with an increase in droplets and a higher quantity of extractable lipid. The histologic pattern of the livers consisting of sinusoids separated by single sheets of hepatocytes radiating from the portal areas to the central veins was maintained. Within the large hepatocytes a proliferation of the smooth endoplasmic reticulum and reorganization of the parallel cisternae of the rough endoplasmic reticulum to form large agranular concentric membrane arrays was demonstrated electron microscopically (Fig. 10).

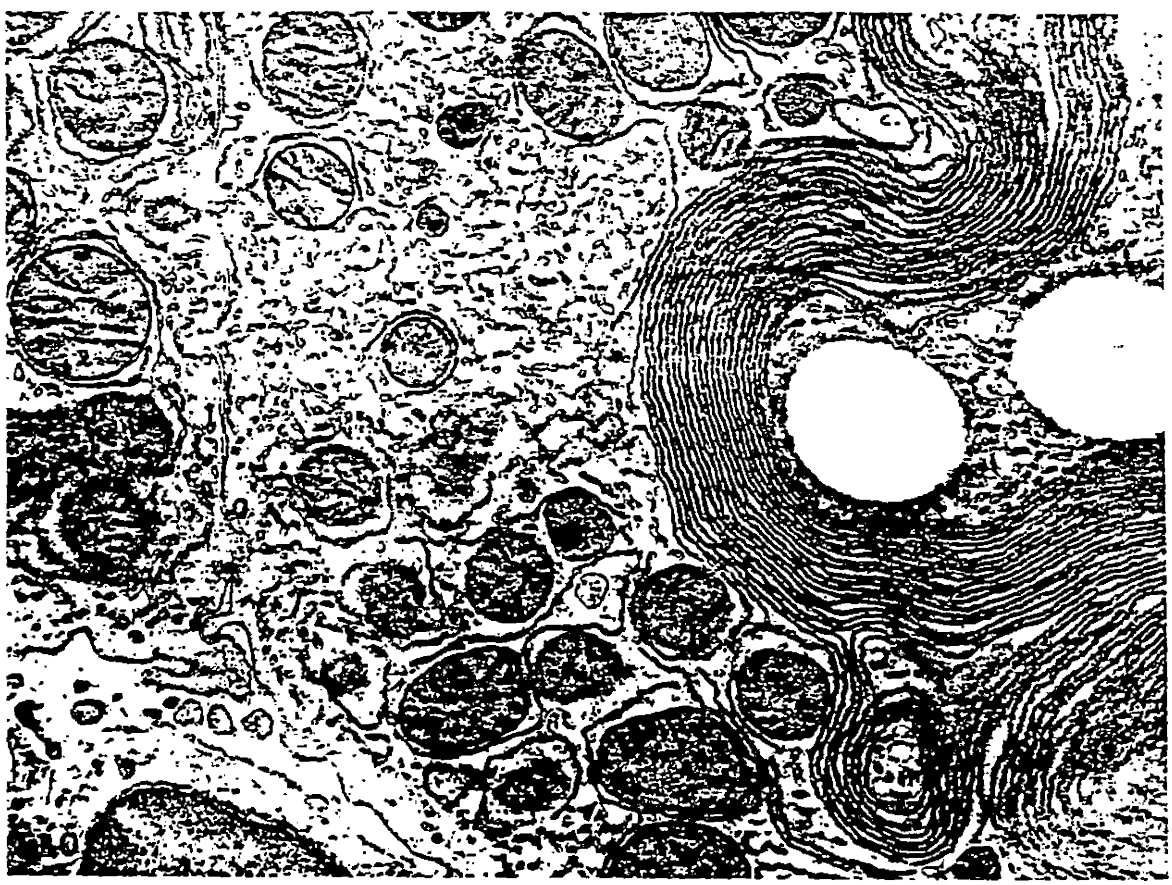


FIGURE 10. Hepatocytes of rats which ingested CDD developed a proliferated smooth endoplasmic reticulum consisting of numerous vesicles and concentric arrays of agranular membranes. The number of lipid droplets was increased. Electron micrograph of liver tissue fixed in Veronal acetate-buffered osmium tetroxide solution and stained with uranyl acetate. ×20,400.

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Administration of tetrachlorodibenzo-*p*-dioxin (1 μg/day) resulted in a 50% mortality of the rats at 21 days. The morphologic appearance of the livers was similar to that produced by ingestion of the crude CDD-containing lipid material by rats. The smooth endoplasmic reticulum was proliferated, and large concentric membrane arrays were present.

Over a 21-day period of administration of labeled octachlorodibenzo-*p*-dioxin to rats (100 μg/rat/day, approximately 12.4 mg/kg administered over 21 days), 93% of the compound passed unabsorbed through the gastrointestinal tract. An additional 5% was excreted in a lipid-soluble form in the urine. The administration of the octachloro compound produced few morbid alterations in the rats. The animals continued to gain weight and maintained normal activities and gross appearance. Approximately 50% of the material present within the body tissues was located in the liver. Other reservoirs containing radioactivity at lesser levels were the adipose tissue, skeletal musculature, and skin. Over 95% of the CDD present within the liver was located in the microsomal fraction with equal distribution within the rough and smooth fractions.

Discussion

The data from these experimental animal studies have shown that CDD administration causes varying responses in the chicken, monkey, and rat. The chicken develops extreme morbidity and mortality at dietary concentrations that are only mildly toxic to rats while the monkey is intermediate in its response to the CDD.

The chicken and monkey developed ascites, hydrothorax, hydropericardium, and anasarca; however, the rat failed to develop increased extracellular fluid. These modifications in the fluid content of the tissues and body cavities were attributed in part to hepatic degenerative changes and altered capillary permeability of the chicken and monkey. The low serum albumin, a direct result of hepatic dysfunction, was associated with decreased osmolarity of the blood and subsequent extravasation of the fluid. In addition, the capillaries were demonstrated to be more permeable to colloidal particles before the decline in serum protein was sufficiently severe to produce an accumulation of fluid in the tissues.

Gastric hyperplasia and ulceration were limited to the nonhuman primate. Hyperplastic changes are thought to be related to the chronic irritation following ingestion of the compounds and other closely related chlorinated aromatic hydrocarbons (*10*). The dysplastic histologic and cytologic pattern as demonstrated by the invasion of the mucosal cells through the muscularis mucosae and the stratification of the epithelial cells within the cysts are changes suggestive of an eventual neoplastic transformation.

Monkeys developed widespread alopecia, moderate hyperkeratosis, follicular keratin cysts, and hyperplasia of the epithelium of the hair follicles, particularly of the face. However, the skin of rats or chickens was not altered appreciably.

Hypoplasia of the lymph tissue and bone marrow was present in all three animal species. However, the blood-forming tissues of the chicken and the monkey were affected earlier and more severely than were those of the rat. As a result of these changes in the lymph tissue and bone marrow, the animals became anemic and displayed a progressive leukopenia. Due to the reduced resistance of these animals they became prime hosts for opportune pathogens which in many instances were responsible for their death.

Hypoactivity of the seminiferous tubules of the testes was associated with chronic intoxication of the monkeys and chickens. Young chickens exposed to low levels of the dioxins experienced retardation in the development of the testes and at maturity were of normal size with hypoplastic gonads. There were no other alterations in growth, blood elements, or histologic appearance of the tissues.

Enlargement of the liver occurred in all animals used in these investigations. Increased size was related to the cell hyper-

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trophy resulting from proliferation of the smooth endoplasmic reticulum and accumulation of lipid within the cytoplasm of the hepatocytes. The chicken rapidly developed widespread liver necrosis; similar degenerative changes occurred at a less rapid rate in the liver of the monkey, and the rat was very resistant to hepatic necrosis.

Radioactive studies which determined tissue and cell fraction levels of the CDD in the rat and possibly in other animal species demonstrated the proliferated hepatic endoplasmic reticulum present in animals following exposure to the CDD may serve not only as a source of enzymes to enhance the metabolism of foreign substances but may also function as an area of localization for these toxic compounds. The presence of a large portion of the ingested CDD within the microsomal fraction of the hepatic tissue may be one explanation as to why the rat is able to tolerate larger doses of the dioxins. The localization of the dioxins in these membranes may prevent their movement to other tissues of the body that are more susceptible to the toxic effects of these compounds. Although the specific reason for the difference in response of various animal species to the dioxins has not been established, further studies on absorption, metabolism, body distribution, excretion, or sensitivity of the tissues to the toxic effects of the dioxins are avenues of research that will likely clarify these questions.

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Excretion and Tissue Distribution of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in the Rat

by Walter N. Piper,* James Q. Rose,† and Perry J. Gehring †

The compound, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), is highly toxic. The LD_{50} for male and female rats given a single oral dose is 23 and 45 μg/kg, respectively (*1*). Adverse effects have been observed in a teratology study in which pregnant rats were given oral doses of 0.125–2.0 μg/kg-day TCDD from day 6 through day 15 of gestation (*2*). The adverse effects were increased fetal mortality, early and late resorptions and intestinal hemorrhage in the fetuses. No adverse effects were noted at the 0.03 μg/kg-day level.

In humans and rabbits, contamination of the skin with TCDD produces chloracne-like lesions (*3, 4*). This disease is characterized by the appearance of hyperkeratosis, papules, comedones and cysts.

There is no available information on the absorption, excretion or tissue distribution of TCDD in animals. Therefore, this study was done to determine the excretion and tissue distribution of radioactivity derived from TCDD-^{14}C following a single oral dose of the labeled compound.

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Methods

Animals

Male Spartan strain Sprague-Dawley rats weighing 165–210 g were used. The rats were acclimated to the environment of the metabolism cages 5 days prior to dosage. Food and water were provided *ad libitum* throughout the experiment.

^{14}C-Tetrachlorodibenzo-*p*-dioxin

Uniformly labeled TCDD-^{14}C was synthesized at the Radiochemistry Research Laboratory of The Dow Chemical Company. The specific activity was 2.8 μCi/mg. Mass spectrometric and gas–liquid chromatographic analyses of the TCDD^{14}C sample indicated a purity of 93.3 and 95.0%, respectively.

Dosage

TCDD was dissolved in acetone. Subsequently, one part of the acetone solution was added to and mixed with nine parts of USP corn oil. The acetone–corn oil solution of TCDD was given to rats in 5 ml/kg amounts by intubation. This volume of the solution provided a dose of 50 μg/kg and 0.14 μCi/kg TCDD-^{14}C.

Sample Collection

After administering the solution containing TCDD-^{14}C, the rats were placed in all-

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glass Roth metabolism chambers which were equipped for separate collection of urine, feces, and expired air. The CO_2 in the exiting air stream was trapped by bubbling it through a 3:7 ethanolamine–2-methoxyethanol mixture.

Radioactivity Analysis

Samples of urine, feces, and tissues were oxidized to $^{14}CO_2$ by combustion and analyzed for radioactivity (*5*). By using this method, the recovery of radioactivity from samples spiked with ^{14}C was 95 ± 5%. To determine the radioactivity expired as CO_2, 5-ml aliquots of the solution used to trap the CO_2 were added to 15 ml of a scintillation counting solution containing 4 g 2,5-diphenyloxazole (PPO) and 0.1 g 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP) per liter of 1:1 toluene–2-methoxyethanol. Samples were counted for radioactivity in a Nuclear Chicago Mark II liquid scintillation counter, and quench corrections were made by use of the internal standard technique.

Results

The percentage of the total dose of radioactivity excreted daily in the feces, urine, or expired air over a 21-day period following a single oral dose of TCDD-^{14}C is shown in Figure 1. Approximately 30% of the ^{14}C activity was excreted in the feces during the first 48 hr. Most of this probably represents unabsorbed TCDD-^{14}C. Over the remaining 19 days, 1–2% per day of the ^{14}C activity was excreted in the feces. A total of 53.2 ± 3.8% of the administered dose was excreted via the feces over the 21-day period. The total cumulative amount excreted in the urine and expired air was 13.2 ± 1.3% and 3.2 ± 0.1%, respectively.

To determine the overall rate of clearance of ^{14}C administered as TCDD from the body, the total cumulative amount of ^{14}C excreted in feces, urine, and expired air at the end of each day was subtracted from the total dose administered to the animal. These values, representing the percentage of the total dose remaining in the animal at the end of each day, were then plotted

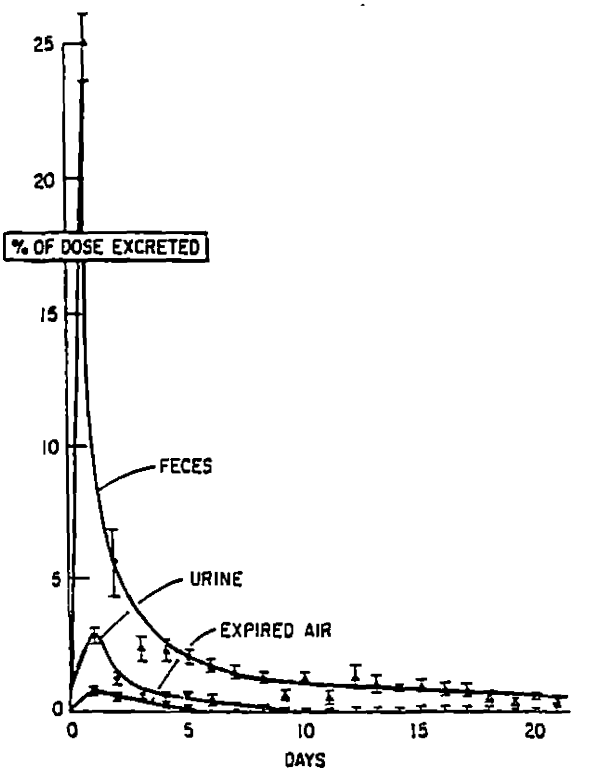


FIGURE 1. Excretion of ^{14}C activity by rats following a single oral dose of 50 μg/kg (0.14 μCi/kg) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Each point represents the mean ± SE for three rats.

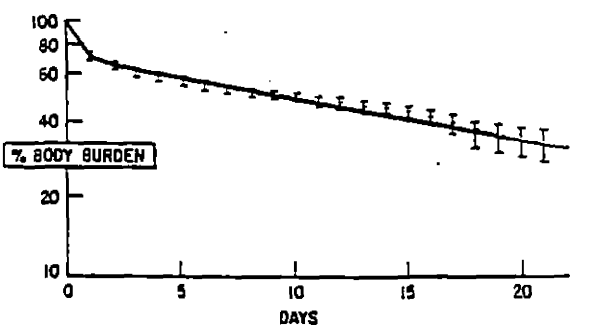


FIGURE 2. Clearance of ^{14}C activity from the body of rats given a single oral dose of 50 μg/kg (0.14 μCi/kg) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Each value represents the mean ± SE for three rats.

semilogarithmically as a function of time (Fig. 2). Except for the first 2 days following administration, the clearance of ^{14}C activity from the body followed apparent first-order rate kinetics. The half-life for

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clearance, $t_{1/2}$, was 17.4 ± 5.6 days. As previously indicated, it was assumed that the relatively large amount excreted during the first 2 days had not been absorbed. Therefore, these values were not used in calculating the clearance rate.

Analyses of tissues indicated that the ^{14}C activity derived from TCDD and/or its breakdown products was located chiefly in the liver and fat (Table 1). The percentage of the dose per gram of liver 3, 7, and 21 days following administration was 3.18, 4.49 and 1.33%/g, respectively. Comparable values for fat were 2.60, 3.22, and 0.43%/g.

Smaller concentrations of ^{14}C activity were found in other tissues: muscle, testes, lungs, heart, skin, spleen, stomach, pancreas, brain, bone, kidneys, and adrenals (Table 2). Standard errors as large as the mean suggest that some of the values presented in Table 2 may be a result of experimental error: adrenals, 3 days; bone, 7 days; spleen, 21 days; pancreas, 21 days. Radioactivity exceeding background in these tissues at the indicated time was detected in only one of three rats.

The value given in Table 2 for adrenals 21 days following administration suggests that this tissue may concentrate TCDD-^{14}C and/or a metabolite. This observation is very likely due to experimental error. The disintegrations per minute (dpm) above background for this tissue were only 17, 29, and 90. Since the total amount of tissue available for analysis was less than 20 mg, the multiplication factor may have magnified the error manyfold.

Total recovery of the administered ^{14}C activity was determined for those rats used in the 21-day experiment. The ^{14}C activity remaining in the unused carcass was determined by analyzing an aliquot of a homogenate of the remaining carcass. The recovery was 96.8 ± 3.0%.

Table 1. ^{14}C activity expressed as percentage of dose per gram in the liver and fat of rats 3, 7, and 21 days following a single oral dose of TCDD-^{14}C.[a]

	Activity, %/g[b]		
Tissue	3 days	7 days	21 days
Liver	3.18±0.21 (47%)[c]	4.49±0.62 (45%)[c]	1.33±0.70 (11%)[c]
Fat	2.60±0.48	3.22±0.63	0.43[d]

[a] Dose: 50 μg/kg (0.14 μCi/kg); 3 rats/observation.
[b] Mean ± standard error.
[c] Percentage of the total dose found in the entire liver.
[d] Mean for 2 rats.

Table 2. ^{14}C activity expressed as percentage of dose per gram in various tissues of rats 3, 7, and 21 days following a single oral dose of TCDD-^{14}C.[a]

	Activity, %/g[b]		
Tissue	3 days	7 days	21 days
Muscle	0.38±0.01	0.21±0.05	0.20±0.12
Testes	0.38±0.03	0.36±0.10	0.11±0.09
Lungs	0.27±0.02	0.39±0.14	0.06±0.05
Heart	0.20±0.03	0.40±0.16	0.09±0.05
Skin	0.19±0.10	0.19±0.10	0.09±0.04
Spleen	0.15±0.02	0.95±0.53	0.22±0.22
Stomach	0.16±0.05	0.10±0.00	0.02±0.02
Pancreas	0.11±0.06	0.16[c]	0.16±0.16
Brain	0.06±0.00	0.13±0.09	0.01±0.01
Bone	0.09±0.03	0.42±0.42	0.08±0.08
Kidneys	0.00[d]	0.34±0.17	0.00[d]
Adrenals	0.79±0.79	0.02±0.02	3.69±1.77[e]

[a] Dose: 50 μg/kg (0.14 μCi/kg); 3 rats/observation.
[b] Mean ± standard error.
[c] Mean of 2 rats.
[d] No activity above background in all 3 rats.
[e] This large value may be an experimental error. The activity above background for the adrenals of the three rats was 17, 29, and 90 dpm. Since the total amount of tissue was less than 20 mg, the multiplication factor may have magnified the error manyfold.

Discussion

In the study reported herein, the tissue distribution and excretion of ^{14}C activity have been evaluated in rats following a single oral dose of TCDD-^{14}C. Almost 30% of the dose administered was eliminated via the feces during the first 48 hr following treatment. The excretion of ^{14}C activity via the feces after the first 48 hr ranged from 1 to 2%/day. It appears that TCDD is incompletely absorbed from the gastrointestinal tract. The ^{14}C activity derived from the ab-

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sorbed TCDD-^{14}C also is excreted mainly via the feces.

Once absorbed in the body, most of the ^{14}C activity derived from TCDD-^{14}C is localized in the liver and fat. The data suggest that the level in these tissues is approximately 10-fold that in other tissues. The ^{14}C level in liver and fat seemed to increase between 3 and 7 days following administration; however, the ^{14}C activity in liver and fat decreased more between 7 and 21 days than would have been predicted on assuming that the rate of clearance from these tissues would be equal to the rate of clearance from the body. Between days 7 and 21, the ^{14}C level in muscle remained essentially unchanged. Therefore, redistribution of TCDD or metabolites of TCDD may have been occurring. The apparently high level in the adrenals 21 days after administration results probably from experimental error.

The dose of TCDD given to the male rats used in this study, 50 μg/kg, was approximately twice the LD_{50} (23 μg/kg). This large dose was necessitated because of the specific activity of the TCDD-^{14}C used. Rats lost weight, and their physical condition was poor; this typifies the insidious response to TCDD (*1*). Survival of the rats for 21 days was not totally unexpected, because in previous studies on the lethality of TCDD deaths frequently occurred 20 days or more following a single oral dose of similar magnitude (*1*). With doses that do not induce untoward effects, the compound may be excreted at a different rate.

The results do not differentiate between ^{14}C activity derived from TCDD and that of possible metabolites. However, small amounts of ^{14}C activity were detected in the expired air and urine within the first 10 days following administration. This is evidence that some metabolic alteration or breakdown of TCDD occurs.

Acknowledgement

The authors thank Mr. W. W. Muelder for preparation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-^{14}C.

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Studies on the Mechanism of Toxicity of the Chlorinated Dibenzo-p-dioxins*

by Alan Poland† and Edward Glover†

Introduction

Concern about the potential health hazards resulting from environmental pollution by the chlorinated dibenzo-*p*-dioxins and dibenzofurans arises from our recognition of the extraordinary potency of these compounds as toxins and teratogens and their inadvertant dispersion in the environment as contaminants of chlorinated phenolic products. Questions concerning the extent of environment contamination and those concerning the mechanism of toxicity produced by these compounds are at present unanswerable. Several papers in this symposium have presented the historical background which led to our current understanding and concern about this problem: (1) the "chick-edema" outbreaks caused by "toxic fats" in poultry feed and the eventual isolation and identification of a hexachlorinated dibenzo-*p*-dioxin; (2) the occurrence of acne among workers in several 2,4,5-T factories and recognition of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as the etiologic agent; (3) the NCI-commissioned study on the potential teratogenicity, carcinogenicity and mutagenicity of 2,4,5-T;(4) and the widespread use of Agent Orange as a defoliant in Viet Nam and Cambodia.

The starting point of our studies was a report by Bleiberg and colleagues (*1*) that of 29 workers in a 2,4,5-T factory, all of whom had industrially acquired acne, 11 had porphyrinuria and several had overt clinical porphyria cutanea tarda. TCDD has been shown to be the causative agent of the acne; however, the cause of the porphyria was uncertain. Porphyria cutanea tarda is an acquired defect of hepatic porphyrin metabolism characterized by an overproduction of porphyrins by the liver, increased urinary excretion of porphyrins, mechanical fragility and photosensitivity of the skin (blistering in areas exposed to sunlight), hyperpigmentation, and hirusitism. We restudied the factory 5 years later and found no evidence of porphyria in the employees (*2*). The fact that this syndrome abated following measures to reduce the formation TCDD and minimize employee exposure to this contaminant, suggested that TCDD might have been the causative agent of the industrial outbreak originally reported by Bleiberg et al. (*1*).

Methods and Materials

The halogenated dibenzo-*p*-dioxins and dibenzofurans and analyses of their purity were generously provided by Dr. A. Pohland, Food and Drug Administration, Washington, D. C. and Mr. George Lynn, Dow Chemical Company, Midland, Michigan. In addition, Drs. J. Wade and A. Kende synthesized

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and analyzed a number of dibenzo-*p*-dioxins which were tested and reported elsewhere in this symposium.

Animals

Our experiments were performed in chick embryos which were routinely 15–20 days of age. The various dibenzo-*p*-dioxins were dissolved in *p*-dioxane, and 25 μl of the solution was injected into the egg through a small hole punched in the air sac. Male Sprague-Dawley rats, 70–100 *g*, were used in some experiments.

Analysis

δ-Aminolevulinic acid synthetase activity was measured as previously reported (*3*). Aryl hydrocarbon hydroxylase was assayed essentially by the method of Gielen, Goujon, and Nebert (*4*). One unit of hydroxylase activity is defined as that amount of enzyme catalyzing the formation per minute at 37°C of hydroxylated product causing fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo[a]pyrene. The assay was performed on the 10,000*g* supernatant, and results are expressed as units per milligram wet weight of liver.

Results

A variety of xenobiotics produce experimental hepatic porphyria and all have in common the ability to induce the initial and rate limiting enzyme in the heme biosynthesis pathway, δ-aminolevulinic acid synthetase (ALA synthetase). To test whether TCDD was in fact porphyrigenic, we administered the compound dissolved in 25μl *p*-dioxane to chick embryos. The embryos were sacrificed 48 hr later, and the ALA synthetase activity assayed in their livers. As seen in Figure 1, TCDD produced a dose-related increase in enzyme activity. As little as 4.66×10^{-12} mole egg (1.5 ng) produced a doubling enzyme activity and the highest level tested 1.55×10^{-9} mole/egg (0.5 μg) produced a 35-fold induction. TCDD is more potent than any other inducer of ALA synthetase yet reported by at least three orders of magnitude, and, unlike most other porphyrigenic chemicals, induction is very prolonged, most likely a

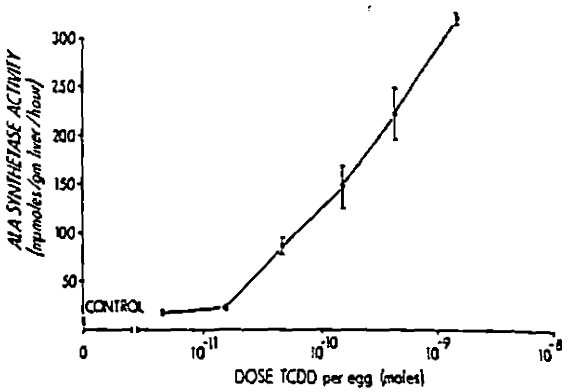


FIGURE 1. Logarithmic dose–response curve for the induction of ALA synthetase by TCDD. Chicken eggs of 17 days' gestation were injected with 25 μl of solvent (control) or solvent containing various doses of TCDD, and hepatic enzyme activity was assayed 48 hr later. The points represent the mean ± standard error of three or four groups of pooled livers.

reflection of the long biological half-life of TCDD. We next screened a series of 15 halogenated dibenzo-*p*-dioxins for their ability to induce ALA synthetase. As seen in Figure 2, all the isomers which were inducers

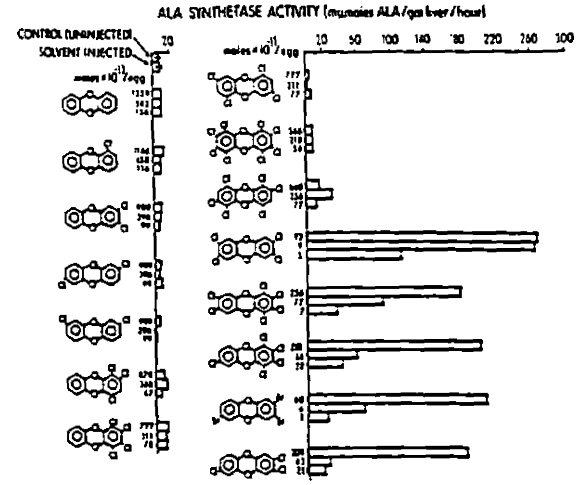


FIGURE 2. Structure–activity relationships of the halogenated dibenzo-*p*-dioxins: induction of ALA synthetase. Seventeen-day embryos were injected with the solvent (*p*-dioxane) or solvent containing the dioxin tested and enzyme activity was assayed 48 hr later. Uninjected control values ($n = 9$), do not differ appreciably from solvent injected controls ($n = 12$). Each bar for the test groups represents the value for a single group of three to five pooled livers.

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had two common properties: (1) halogen atoms occupy at least three of the four lateral ring positions (2, 3, 7, and 8) and (2) there is at least one free, nonhalogenated carbon atom. Note that octachlorodibenzo-*p*-dioxin is inactive. The compounds that were not inducers were tested at 200–400 times the molar concentration of TCDD that produced a significant response.

To the extent that toxicologic data are available (*5*), all those dioxins which are at low doses lethal, teratogenic, or produce acne, also induce ALA synthetase, and those dioxins which are not potent toxins do not induce ALA synthetase. We have also tested a limited series of dibenzofurans; the unsubstituted compound and 2,8-dichloro and octachloro derivatives all fail to induce, and a mixture of di- tri- and tetrachlorodibenzofurans is potent as an inducer of ALA synthetase. While the data are very limited, it appears the structure–activity relationship is similar in the dibenzofuran series.

As reported elsewhere in this symposium (*6*), TCDD does not induce ALA synthetase in several laboratory mammals. We have found it to be a poor inducer in the rat. This should not be interpreted to mean that the results obtained in the avian embryo have no relevance to man. For instance, many sex steroids appear to play a role in precipitating acute intermittent prophyria and porphyria cutanea tarda in man; however, while induction of ALA synthetase by these compounds can be shown in the chick embryo, it does not occur in the rat.

There is an empiric relationship observed by numerous investigators that many compounds which induce ALA synthetase also induce microsomal mixed-function oxygenase activity in the liver (also called the "drug metabolizing enzymes"). Two points are of note about this correlation: not all drugs induce both enzyme activities; also, the relationship many have a theoretical basis, in that heme is the prosthetic group of the terminal component of microsomal oxygenase, cytochrome P–450. The high concentration of cytochrome P–450 and rapid turnover relative to all other hepatic hemoproteins, accounts for a large fraction of the total heme synthesized in the control liver. Some investigators have suggested that coordinate induction of ALA synthetase, the rate- limiting step in heme synthesis, and cytochrome P–450 and microsomal oxygenase activity may have a basis in providing the extra heme necessary for forming the new cytochrome P–450. Despite this not very satisfying teleologic explanation, there is a sizable literature reporting the concommitant induction of ALA synthetase and microsomal oxygenase activity.

We next studied the effect of TCDD on microsomal oxygenase. As a measure of this enzyme complex we choose to investigate aryl hydrocarbon hydroxylase activity, because aromatic hydroxylation is induced primarily by aromatic compounds, which in our view chemically resemble TCDD.

As seen in Figure 3, TCDD produces a dose-related induction of aryl hydrocarbon hydroxylase in chick embryo liver. At the lowest dose tested, 1.55×10^{-12} mole/egg (0.5 ng) there is a nearly twofold increase in enzyme activity, and maximal induction is produced

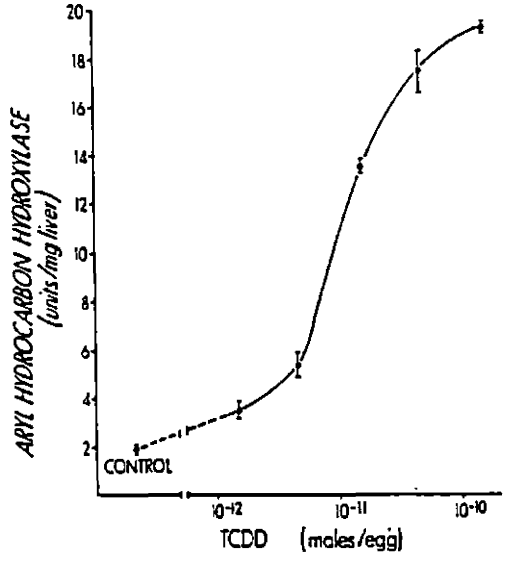


FIGURE 3. Logarithmic dose–response curve for the induction of aryl hydrocarbon hydroxylase. Eighteen-day embryos were injected with TCDD dissolved in *p*-dioxane or *p*-dioxane alone (control), and hepatic enzyme activity was assayed 24 hr later. Each point represents the mean ± standard error of four groups of pooled livers.

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by 1.55×10^{-10} mole/egg (50 ng). Following the administration of TCDD to an egg, hepatic aryl hydrocarbon hydroxylase activity rises to reach a maximum at about 18 hr, and then the elevated hydroxylase activity presists for at least 5 days.

We screened 15 halogenated dioxins for their ability to induce aryl hydrocarbon hydroxylase at three dose levels: 4.7, 47, and 470×10^{11} mole/egg (Fig. 4). The structure–activity relationship is identical to that seen with the induction of ALA synthetase: (1) the compounds which are potent inducers (induction at 4.7 or 47×10^{-11} mole/egg) have halogen atoms at least three of the four lateral ring positions and (2) they have at least one nonhalogenated ring position. There is one exception, the 1,2,4,6,7,9-hexachlorodioxin, which at high doses (470×10^{-11} mole/egg) produced a modest induction of aryl hydrocarbon hydroxylase. This compound was only 90% pure by gas–liquid chromatography, and the induction observed at the highest dose could be produced by contamination with as little as 0.1% (w/w) TCDD or an equipotent dioxin. Clarification must await the availability of a purer preparation of this hexachloro isomer.

There are certain advantages to investigating the induction of hydroxylase activity produced by TCDD in the rat, namely: one can more fully examine the duration of induction, and also the spectral changes in cytochrome P–450 accompanying aryl hydrocarbon hydroxylase induction are more fully documented in the rat. The administration

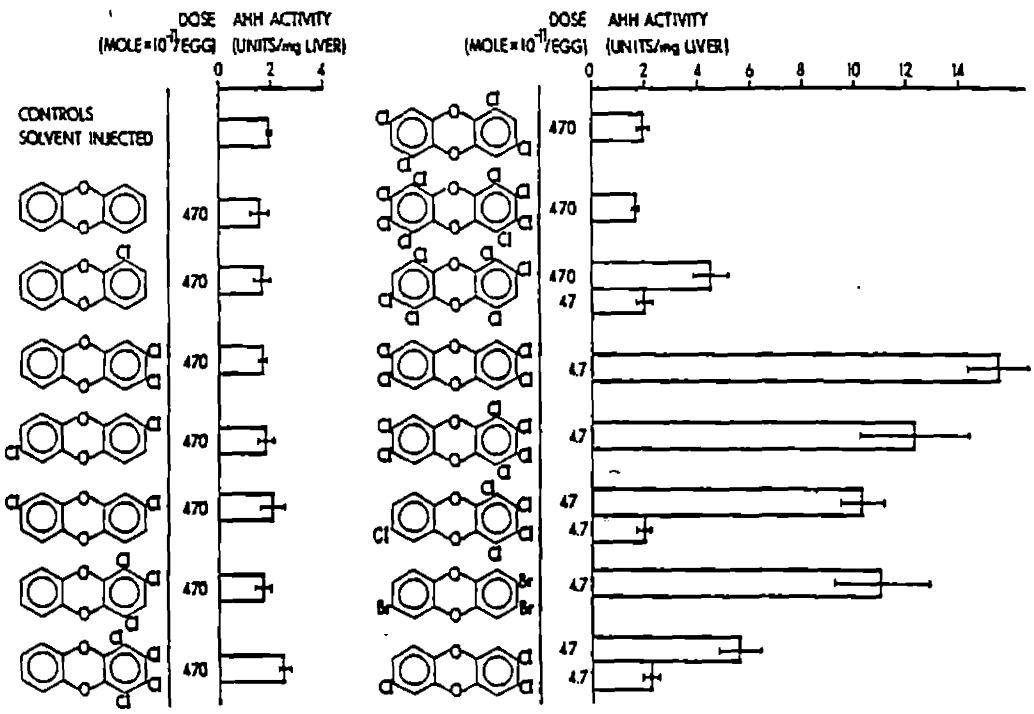


FIGURE 4. Structure–activity relationship of halogenated dibenzo-*p*-dioxins: induction of aryl hydrocarbon hydroxylase. Eighteen-day embryos were injected with 25 μl of *p*-dioxane or *p*-dioxane containing the test dioxin, and enzyme activity was assayed 24 hr later. Each bar represents the mean ± standard error of four groups of pooled livers, except the control and TCDD groups where $n = 12$.

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TCDD at a dose of 3.11×10^{-10} mole/kg produces nearly a fivefold induction in rat liver enzyme activity (Fig. 5). Maximal induction of aryl hydrocarbon hydroxylase was produced by a dose of 3.11×10^{-8} mole/kg (10 μg/kg). It is estimated that the half maximal response is elicited by a dose of 8.5×10^{-10} mole/kg (0.265 μg/kg), roughly one hundredth the dose that kills 50% of rats (*5*). 3-Methylcholanthrene, a polycylic hydrocarbon carcinogen is perhaps the most widely used compound as an inducer of aryl hydrocarbon hydroxylase. TCDD was found to be nearly 3×10^4 times as potent as 3-methylcholanthrene at inducing hydroxylase activity. Both drugs produce the same maximal degree of enzyme induction in rat liver, and the administration of both drugs together, each at a dose which produces maximal induction, elicits a response that is no greater than that produced by either drug alone.

In Figure 6 we have plotted the results of the time course of induction produced by 3-methylcholanthrene (20 mg/kg in corn oil), TCDD (10 μg/kg in *p*-dioxine) and control rats (receiving either corn oil or *p*-dioxane). Aryl hydrocarbon hydroxylase is induced to the same extent at 1 and 4 days by maximally inducing doses of each drug. However, by 8 days, hydroxylase activity in the 3-methylcholanthrene-treated rats returned to control levels, while induction persisted in the TCDD-treated rats for over 1 month. Accompanying the induction of aryl hydrocarbon hydroxylase by both drugs the following changes were noted: an increase in the total CO-binding microsomal cytochrome (cytochrome P–450 and P–448), a shift in the CO-maximum peak by difference spectroscopy from 450 nm to 448 nm, and a shift in the ratios of the peaks observed with ethyl isocyanide as a ligand by difference spectroscopy. These changes are interpreted to mean both 3-methylcholantrene and TCDD

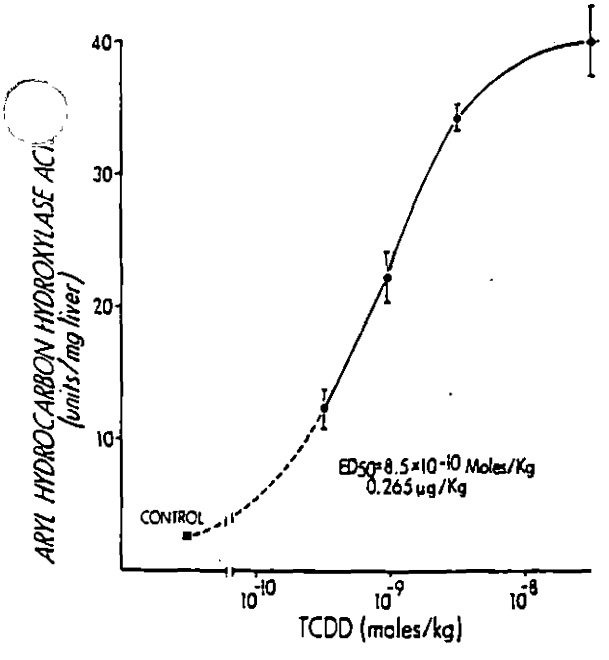


FIGURE 5. Logarithmic dose–response curve for the induction of aryl hydrocarbon hydroxylase in rat liver. Male Sprague–Dawley rats weighing about 80 g were injected intraperitoneally with TCDD dissolved in *p*-dioxane or *p*-dioxane alone (0.3 ml/kg), and hepatic aryl hydrocarbon hydroxylase activity was assayed 24 hr later. Each point is the mean ± standard error of five animals.

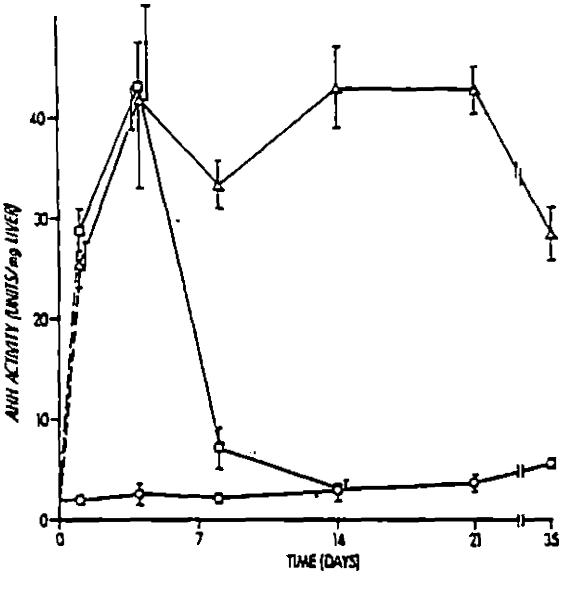


FIGURE 6. Time course of aryl hydrocarbon hydroxylase induction in rat liver following TCDD and 3-methylcholanthrene administration. Male Sprague–Dawley rats, weighing about 80 g were given a single injection of TCDD in *p*-dioxane (10 μg/kg), 3-methylcholanthrene in corn oil (20 mg/kg), or the solvents alone. Each point represents the mean ± standard error of four rats.

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induce the formation of a new type of cytochrome P–450 which contains its heme iron in a high-spin state. We have also found that both TCDD and methylcholanthrene produce induction of aryl hydrocarbon hydroxylase in a number of extrahepatic tissues.

In summary, TCDD appears to be similar to 3-methylcholanthrene as an inducer of microsomal oxygenase differing primarily in potency (by four orders of magnitude) and duration of action. The persistent induction following TCDD administration is probably a result of its long biological half-life.

Discussion

The results of our investigation demonstrate TCDD is a potent inducer of ALA synthetase and aryl hydrocarbon hydroxylase in the chick embryo liver. There is a perfect correlation between those dioxins which induce both enzymes and the toxicity data, to the extent the data are available on the various dioxins. The structure–activity relationship reveals that all dioxins which are potent inducers have halogens at three of the four lateral ring positions and at least one nonhalogenated carbon atom. The sensitivity of induction of aryl hydrocarbon hydroxylase by TCDD and other toxic dioxins suggests this response might be a very valuable screening bioassay to detect the presence of the toxic dioxins in commercial products or environmental samples. It should be emphasized that the nonspecificity of the response makes it imperative that one extract the samples tested to remove polycylic hydrocarbons, and the test is only collaborative, not definitive for TCDD and related dibenzo-*p*-dioxins.

Now, I should like to turn to the broader question of the mechanism of toxic action produced by TCDD. Any proposed mechanism of toxicity must account for several observations about TCDD (1). TCDD is a nearly planar, highly lipophilic, and a rather chemically unreactive molecule, which possesses remarkable biologic potency, and hence specificity (2). There are very large differences in susceptibility of different species to TCDD, as presented by Schwetz (*5*). The oral LD_{50} in the guinea pig is one thousandth that of the dog (3). There is a very sharp structure–activity relationship among the dioxins. The oral LD_{50} values of the 2,7-dichloro and octachloro derivatives are greater than 10^5 times that of TCDD in the rat (4). TCDD seems to produce hepatic cell necrosis, and liver insufficency is the presumed cause of death in the rat. However, multiorgan involvement in the rat has been reported at this symposium and elsewhere. Furthermore, as reported by Vos and Moore and colleagues (*7, 8*), hepatic necrosis is minimal in the mouse and guinea pig and perhaps insufficient to account for death. Thus we must account for the different pattern of histologic damage in different species (5). TCDD is remarkably slow in its toxic action leading to death. Regardless of dosage, animals die weeks after a single administration of TCDD (6). TCDD is an extraordinarily potent teratogen in a number of species (7). Finally, our investigations suggest that all dioxins which are potent toxins, as acneogens, teratogens, producing mortality or chick edema, also are potent inducers of aryl hydrocarbon hydroxylase activity. This enzyme complex is present and inducible in a number of tissues and is responsible for the aromatic hydroxylation of many xenobiotics.

It is useful to examine the proposed mechanism of toxicity for other aromatic or halogenated aromatic compounds that, like TCDD, are chemically relatively unreactive and highly lipophilic. The two most extensively investigated models are the liver necrosis produced by halogenated benzenes and the carcinogenesis produced by polycyclic hydrocarbons (*9*). Briefly the literature can be summarized as follows. The parent compound is metabolized to a very reactive arene oxide intermediate. This intermediate may then chemically rearrange to a phenol, be further metabolized to a dihydrodiol or glutathione conjugate, or, react chemically to covalently bind to various cellular macromolecules which act as nucleophiles. In the case of bromobenzene centrolobular liver necrosis, the epoxide is believed to attach to proteins, and in the case of the polycyclic

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hydrocarbon carcenogenesis the critical event is believed to be the binding of the "K-region" epoxide to DNA.

We propose a similar model for the toxicity of TCDD. The parent compound enters the cell and binds to some induction-receptor site which initiates the events which ultimately lead to the formation of more aryl hydrocarbon hydroxylase activity. TCDD is recognized by a second site in the cell, the enzyme-active center of microsomal oxygenase, and converted to a reactive metabolite, possibly an epoxide. The conversion of TCDD to its reactive metabolite is the rate-limiting step in dioxin metabolism, and this step is increased by the induction of aryl hydrocarbon hydroxylase. Some of these reactive metabolite molecules bind to cellular macromolecules producing some impairment of function which gradually produces cell death.

It is useful to examine this hypothesis; in light of the known facts concerning TCDD toxicity. The large difference in species susceptibility to TCDD might be explained by the differences in the rate of metabolism of TCDD. The multiple organ damage produced by TCDD and variable pattern of histologic damage in different species might be explained by the relative rate of formation and further inactivation of the reactive metabolite in different organs. (3) As pointed out by Gehring (*10*), there is some evidence, far from unequivocal, that the administration of C^{14}-TCDD results in unextractable radioactivity in rat liver. (4) The teratogenic effect of TCDD may be a result of mutagenesis by intercalation of the parent compound into DNA (*11*) or by intercalation and covalent binding of the metabolite, analogous to the acridine and aminofluorene compounds (*12, 13*).

This hypotesis is highly speculative and presented only to encourage further investigation. The major assumptions remain unsupported: (1) TCDD is metabolized, and (2) the metabolite covelently binds to some cellular constituent. The demonstration that both these events do or do not occur must await the synthesis of radioactive TCDD of high specific activity.

The chlorinated dibenzo-*p*-dioxins are worthy of much greater investigation, not only because the potential public health hazard they pose, but also the remarkable potency and sharply defined structure–activity relationship they demonstrate suggests an uncommon specificity of action. Ultimately, TCDD, like other potent toxins, (i.e., botulinus toxin, tetrodotoxin, organic phosphates) may become a useful biologic tool.

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Studies on the Bioaccumulation and Microbial Degradation of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

by Fumio Matsumura* and Herman J. Benezet*

While the problem of pesticidal contamination of the environment is far from being solved, considerable useful information has emerged from the research efforts made by many scientists in recent years.

First, we now know by experience that the chemicals that cause environmental problems are the ones which are extremely persistent in nature, biologically active, and easily concentrated in biological systems. Compounds which lack any of the above qualifications usually do not play any significant role in pesticidal pollution no matter how acutely toxic they are. The above analysis becomes more important, when one considers other aspects of pesticidal pollution. For instance, we are concerned about only biological effects in considering pollution, with particular emphasis on the effects on nontarget organisms.

In the case of polychlorinated dibenzo-*p*-dioxins, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the question of bioactivity is indisputable, as it is one of the most toxic compounds known to occur as a pesticidal impurity (1–3). Its chemical stability is also questionable. Thus the central question of its hazard to the environment must be studied from the viewpoint of bioconcentration in various ecosystems.

Published data on environmental fate of chlorodibenzo-*p*-dioxins are scarce at present. Zitko (*4*), for instance, could not find residues of dioxins in several aquatic animals at detection limits of 0.01–0.04 μg/g. Isensee and Jones (*5*), studied absorption and translocation of TCDD and its 2,7-dichloro analog in root and foliage, and concluded that dioxins are neither readily picked up from the soil residues nor translocated into foliage, at least in the case of soybeans and oats.

Studies by Kearney et al. (*6*) indicate that the average TCDD remaining after weathering in soil for 1 yr is of the order of 50–60% at all concentrations tested (1 to 100 ppm), while the actual field survey (*7*) on the test areas with Agent Orange (1962–1970) revealed that much less TCDD residues remaining in soil than is expected from the above figure.

In the study reported herein we have made efforts to measure the degree of bioaccumulation of TCDD in relation to well established pesticides by using several model ecosystems. The data are still preliminary, in that several model ecosystems are still being compared for their relative merits in assessing the actual impact of pesticides in

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nature. The data obtained have been, however, useful in assessing the relative tendency of a pesticide in comparison with other pesticides.

Materials and Methods

Microbial Degradation of TCDD

Microbes—Approximately 100 microbial strains which have previously shown the ability to degrade persistent pesticides were screened for their ability to degrade TCDD.

Procedure—Screening was carried out and the metabolic products were examined by thin-layer chromatography (TLC) by the method of Matsumura and Boush.(*8*).

Translocation of Pesticides

Procedure—The pesticides (0.1 μmole each) were deposited on 1 g of clean sea sand, which was placed on a column of sandy loam type soil. Water was then slowly dropped onto the surface of the sand at a rate of approximately 2 ml/hr. The water and sections of soil were extracted with chloroform.

Bioaccumulation of Pesticides

Animals—Three groups of invertebrates were used for the pesticide accumulation study: *Ostracoda* species, *Artemia salina*, and *Aedes aegypti* larvae, and one fish species, northern brook silverside, *Laludesthes sicculus sicculus*.

Pesticides—Four pesticides were selected from representative groups of important compounds: dioxin (TCDD), DDT, γ-BHC, and zectran. All compounds were ^{14}C-labeled in the benzene rings.

Procedure—Three model ecosystems were used to study bioaccumulation.

In model I, the pesticides (5 and 10 pmole) dissolved in a solvent were added directly to water along with the primary food organism, such as algae and yeast, and this mixture was then added to the aquarium containing the invertebrate test organisms.

In model II, the pesticides (20 pmole) were deposited on the inner surface of the glass container by evaporating the solvent to form a thin film. The primary food organism were grown in the container for 24 hr and then transferred along with the culture media to the aquarium containing the test invertebrate organism.

In model III, the pesticides (5 and 10 pmole) were deposited on 1 g of sand and the solvent evaporated to form a thin film on the surface of the sand particles. The sand was added to the test aquarium containing invertebrates and/or fish.

In all cases the test organisms were maintained in the aquarium at room temperature (24° C), except for the fish cultures which were maintained at 12° C. Test organisms were either homogenized in counting solution or carbonized (Model 300 Packard Tri-Carb Oxidizer), and the amount of $^{14}CO_2$ measured. Measurements of the amount of labeled material in the water, primary food organism, and on sand and glass surfaces were made by extracting with chloroform. All studies were short-term (4–7 days), in small volume containers (200 ml).

Results and Discussion

As shown in Table 1, the extent of translocation of TCDD from the sand to the organic soil layer is extremely small. Virtually no TCDD was found to leach out from the column. The mobility of TCDD in soil, therefore, must be considered much less than that of DDT. Thus, the mode of translocation of TCDD in the environment would be limited to movement of soil particles or dust-carried dispersion and biological transfer (but not plant-mediated transfer), particularly in aquatic environments.

As for the microbially mediated degradation of TCDD, our current survey indicates that such capabilities are rather rare in nature. Approximately 100 microbial strains in which the ability to degrade persistent pesticides has been previously demonstrated were screened for this purpose. Among them, only five strains showed some ability to degrade this compound (Fig. 1). We have not been able to manipulate cultural conditions

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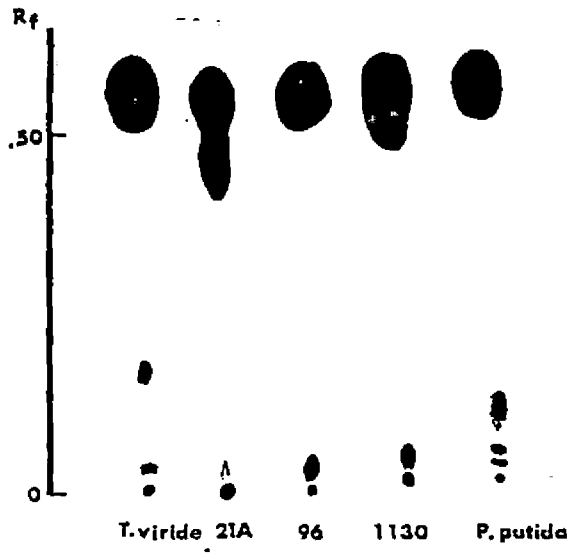


FIGURE 1. Autoradiograph of microbial degradation of TCDD. The results are shown in terms of thin-layer chromatograms of extracts from microorganisms that were incubated with TCDD. The top spots at R_f 6 are TCDD. The TLC system was: Silica gel C with carbon tetrachloride and methylene chloride (1:1) as a mobile phase. The names or the identification numbers are indicated below each chromatogram.

Table 1. Vertical translocation of pesticides from sand to organic soil.[a]

	Pesticide content, %		
	Dioxin[b]	DDT[b]	Zectran[b]
Top sand	90.41	65.01	0.07
0–0.5 cm	7.32	30.75	0.06
0.5–1.0 cm	1.04	3.51	0.08
1.0–1.5 cm	0.50	0.55	0.05
1.5–2.0 cm	0.26	0.26	0.06
2.0–2.5 cm	0.18	0.19	0.06
Water eluate[c]			
1st 50 ml	0.12	0.06	49.4
2nd 50 ml	0.08	0.04	17.6
3rd 50 ml	0.09	0.02	29.1

[a] 10 × 1.5 cm glass column.
[b] Pesticide introduced: 0.1 μmole each (33.8 μg for dioxin, 35.5 μg for DDT, and 22.2 μg for Zectran).
[c] Water eluted per day, 50 ml.

to increase the rate of degradation of TCDD in any of the microorganisms so far.

In studying the extent of biological transfer of TCDD, three different model systems were devised. In model system I, pesticides in acetone were introduced directly into water along with the primary food organisms. In model system II, pesticides were applied to the inner surface of a glass container, and the primary food organisms were grown in the container for 24 hr and were transferred to the aquarium. In model III, pesticide-coated sands were placed directly in the aquarium containing the test organisms.

In the model I experiment (Table 2), DDT behaved quite differently from other pesticides, showing high degrees of affinity to each test organism, in close agreement with the phenomenon actually observed in nature. Although this model system is simple and appears to offer a quick straightforward answer to the general tendency of pesticidal accumulation by biological systems, it has one weakness, i.e., that one is forced to work above the limit of water solubility of some of the compounds. TCDD for instance was measured at a level 100 times its water solubility. Also the extent of direct pick-up due to partitioning and food intake is uncertain. In the model II experiment, where only the portion of pesticide picked up by the primary food organisms and the media were introduced into the test aquarium, the levels of total pick-up were further reduced in the case of TCDD (but not DDT) (Table 3).

To circumvent the problem of solubility, the model III system was devised. In this way, only that portion of pesticide that is soluble should be present in water at any time. The results shown in Table 4 indicate that the rate of TCDD pick-up is extremely low in brine shrimp and fish under the experimental conditions. Mosquito larvae, which are bottom feeders, showed a surprising rate of TCDD pick up. The reaction is not at its maximal rate, since further increase in the level of the pesticide apparently increases the pick up by the larvae. Also noted is the difference between the bioconcentration pattern in fish as compared to other invertebrates. γ-BHC, in particular,

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shows high degree of concentration in fish. To study the effects of food consumption, the same test was repeated in the presence of mosquito larvae.

As expected, the level of TCDD (Table 5) in the fish increased in the presence of mosquito larvae, which are the best concentrators of TCDD among the organisms tested. On the other hand, the levels of other pesticides did not significantly change, indicating that the route through ingestion of mosquito larvae does not represent the major source of uptake in these pesticides.

It is apparent from these data that the reaction of biological concentration is greatly influenced by the external conditions and the design of the experiment, the physical and biological nature of the organisms, and by chemical characteristics of the pesticides. To facilitate understanding of the role of chemical nature of pesticides in determining the rate of bioconcentration, a comprehensive list has been prepared to illustrate their important properties (Table 6).

It can be seen here that general tendencies of bioaccumulation in invertebrate species follow closely the trend of the partition coefficients. In model II experiments, however, the values for TCDD come much lower than expected from this rule. Thus it is likely that water solubility (and solvent solubility) must play an important role where the initial pick-up is the rate-limiting factor.

It is apparent that species-specific factors play a much more important role than once

Table 2. Bioaccumulation of pesticides by aquatic invertebrates for model I (pesticides introduced directly into ambient water with the primary food organisms).

Test organisms (primary food)	Pesticide	Original concentration in water, ppb	Final concentration found in test organisms, ppb	Concentration factor
Daphnia	Dioxin	32.4	1,592	49
(algae)	DDT	35.8	44,164	1234
	Zectran	22.2	1,969	89
Ostracod	Dioxin	32.4	7,069	218
(algae)	DDT	35.8	50,771	1418
	Zectran	22.2	7,265	327
Brine shrimp	Dioxin	16.2	1,956	121
(yeast)	DDT	17.9	12,336	689
	γ-BHC	14.7	2,688	183
	Zectran	11.1	155	14

Table 3. Bioconcentration of pesticides by aquatic invertebrates for model II (primary food organisms allowed to pick up pesticide from glass surface and then given to the test organisms).

Test organism (primary food)	Pesticide	Original amount, μg (theoretical concentration, ppb)	Final concentration found, ppb		Concentration factor*
			Water aquarium	Test organisms	
Daphnia	Dioxin	6.48 (162)	0.4	879	2,198
(algae)	DDT	3.58 (179)	22.9	43,123	1,883
	Zectran	2.22 (111)	15.1	37,499	2,483
Ostracod	Dioxin	6.48 (162)	2.6	279	107
(algae)	DDT	3.58 (179)	50.8	36,391	716
	Zectran	2.22 (111)	43.5	6,177	142

*Measured against the final pesticide concentrations actually found at the end of the test.

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Table 4. Bioconcentration of pesticides by aquatic organisms for model III (pesticides introduced into system in the form of residues on sand).

Test organism	Pesticide	Amount of pesticide, μg	Concentration found, ppb		Concentration factor
			Water (including food)	Test organisms	
Brine shrimp	Dioxin	1.62	0.1	157	1,570
	DDT	1.79	0.5	3,092	6,184
	γ-BHC	1.47	5.2	495	95
	Zectran	1.11	5.0	89	18
Mosquito larvae	Dioxin	1.62	0.45	4,150	9,222
		3.24	2.40	12,000	5,000
	DDT	1.79	0.85	14,250	16,765
		3.58	1.40	30,200	21,571
	γ-BHC	1.47	6.6	1,450	220
		2.94	13.1	2,900	221
	Zectran	1.11	5.45	0	0
		2.22	10.8	89	8
Fish (silverside)	Dioxin	1.62	0	2	—
	DDT	1.79	2.1	458	218
	γ-BHC	1.47	1.8	2,904	1,613
	Zectran	1.11	4.7	213	45

Table 5. Two-step bioconcentration of pesticide by mosquito larvae, and northern brook silverside (model III).

Pesticide	Amount of pesticide, μg	Concentration found, ppb			Concentration factor	
		Water (including food)	Mosquito larvae	Fish	Mosquito larvae	Fish
Dioxin	1.62	1.3	3,700	708	2,846	54
DDT	1.79	1.1	17,900	337	16,273	306
γ-BHC	1.47	1.8	690	1080	383	600
Zectran	1.11	5	0	76	0	15

Table 6. Physicochemical characteristics of dioxin in comparison with other insecticides.

	Water solubility	Solvent solubility / Water solubility	Partition coefficient (vs. hexane)	Benzene solubility, g/100 g
Dioxin	0.2 ppb	10^7	1,000 *	0.047
DDT	1.2 ppb	10^{16}	100,000	80
Zectran	>100 ppm	10^4	100 *	—
γ-BHC	10 ppm	10^6	1,700	80

* Estimates.

suspected. For instance, the pattern of bioaccumulation and concentration in fish is quite different from those in other organisms studied, in that both γ-BHC and zectran show higher degrees of affinity than DDT and TCDD, respectively. Although the data are not sufficient to permit a definite conclusion, they suggest the possibility that water-soluble pesticides tend to accumulate in fish.

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The data indicate that TCDD is not likely to accumulate in as many biological systems as DDT. This is likely because of TCDD's low solubility in water and lipids as well as its low partition coefficient in lipids. Since microbial degradation is not expected to be a major factor, the predominant mode of elimination of this compound in the environment is photodecomposition by sunlight, (*9*).

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Environmental Generation and Degradation of Dibenzodioxins and Dibenzofurans

by D.G. Crosby,* K.W. Moilanen,* and A.S. Wong*

Introduction

Chlorinated dibenzo-*p*-dioxins and dibenzofurans have been known to chemists for many years. 2,8-Dichlorodibenzo-*p*-dioxin was first reported in 1941 (*1*); the 2,3,7,8-tetrachloro- (TCDD) and octachloro- (OCDD) analogs followed in 1957 (*2*). Extensive research by Gilman (*3*) and by Pohland and Yang (*4*) provided data on many others. Likewise, simple chlorinated dibenzofurans have been reported since the early 1930's (*5*), while experience with the more highly chlorinated ones is comparatively recent (*6, 7*).

Although the unusual toxicity of certain of these compounds was recognized at an early date through occupational illness and toxic effects on domestic animals (*8*), major concern arose only when the toxic and teratogenic properties of TCDD became apparent in widely-distributed pesticides such as 2,4,5–T (*9, 10*). Subsequent analysis (*7, 11, 12*) showed that a variety of chlorinated dibenzo-*p*-dioxins and dibenzofurans can occur as impurities from the manufacture of many industrial and agricultural chemicals based on chlorophenols and certain chlorinated aromatic hydrocarbons. To cite but a single example, production statistics (*13*) suggest that at least 50 million pounds of 2,4,5–trichlorophenol and its derivatives are manufactured in the United States each year, almost all of which contains low but detectable levels of TCDD.

The dioxins are formed most directly by heating an *o*-chlorophenol or its salts above about 200°C in either the presence or absence of a solvent. For example, heating pentachlorophenol (PCP) at 300°C for 24 hr provided a low yield of OCDD [eq. (1)], while heating the sodium salt under the same conditions provided a much higher yield (*2, 14, 15*). Reaction of the dipotassium salt of catechol with 1, 2, 4-trichlorobenzene in dimethyl sulfoxide at 175°C produced a 70% yield of 2-chlorodibenzo-*p*-dioxin (*4*); the nitro group can serve instead of chlorine as the displaced group (*4, 15*). Although dibenzofurans may be prepared by dehydration of 2,2′-dihydroxybiphenyls (*16, 17, 18*), 2-chloro-2-hydroxy- and 2,2′dichlorobiphenyls also provide them when treated with aqueous alkali (*19, 20*), analogous to dioxin formation.

Although related reactions have been suggested to operate by a free-radical mechanism (*21*), the preponderance of evidence indicates that the above processes actually represent nucleophilic substitution (*22, 23*). However, the C-Cl bond of the aromatic ring is much more stable than that in corresponding aliphatic compounds, and high temperatures are required to achieve reasonable reaction rates unless activating ring substituents such as nitro groups are present.

Other means can be used to activate the ring, for example the intermediacy of a

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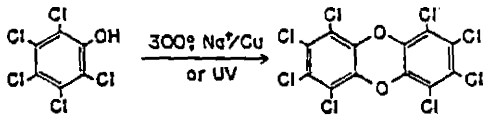
copper complex (the Ullmann reaction) (*23*). In the presence of powered copper or copper salts, a few hours at 160°C converts normally unreactive *o*-chlorophenols to dibenzo-*p*-dioxins (*3, 4, 13*). Ultraviolet light also can provide ring activation for both nucleophilic displacements and radical reaction (*24*); thus, either heat or light theoretically could transform almost ubiquitous chlorinated aromatic compounds into dioxins and dibenzofurans under environmental conditions.

Thermal Generation

Despite the suggestion (*25*) that phenol derivatives such as 2,4,5-T might be decomposed to dibenzodioxins by heat, further investigation has failed to demonstrate the conversion (*26, 27*). Actually, few circumstances are apparent in which such compounds would be subjected to both the concentration and pyrolytic conditions necessary for thermal condensation to occur; the accidental or intentional burning of herbicide-treated rangeland, for example, provides one such theoretical possibility.

However, one very real possibility does exist. A major proportion of currently produced exterior plywood and other millwork is treated with PCP, as are a number of other wood products. The burning of scrap plywood and mill wastes should provide sufficient heat and concentration to convert the fungicide into dioxins or phenoxyphenols (predioxins) whose high degree of chlorination would cause them to volatize or move with smoke rather than burn. Predioxins would be expected to form dioxins and polyphenyl ethers upon pyrolysis (*28*).

To test this concept, small chips of commercial plywood containing 53 μg/g of PCP by analysis were completely charred. The smoke and volatiles were trapped and their dioxin content compared with that of similar, solvent-extracted chips. The OCDD level in the wood was only 1 ng/g, while that in the pyrolyzate was estimated at about twice this amount, despite a persistent but nonchlorinated interference. Hepta- and hexa-



(1)

chlorodibenzo-*p*-dioxin but no TCDD were present in each sample. Identities were confirmed by gas chromatography with a mass-spectrometer detector (GC/MS).

Photochemical Generation

The ultraviolet component of sunlight is sufficiently energetic to generate free radicals by homolytic dissociation of both phenols and chlorinated aromatic compounds. The short-wavelength cutoff of ultraviolet radiation by the atmosphere is approximately 290 nm (Fig. 1), and spectral energy increases sharply above this. The homolytic dissociation energies of the Ar-Cl bond (about 80 kcal/einstein) and the ArO-H bond (about 90 kcal/einstein) correspond to wavelengths near 360 and 320 nm, respectively—clearly within the sunlight region—and free-radical mechanisms have been proposed for reactions of PCP (*29*) and other chlorophenols (*30*) in sunlight.

Blessed indeed (at least by the environmental photochemists) are those parts of the world which experience reliable, intense sunlight during most of the year. Because it varies so drastically with latitude, elevation, climate, and even the degree of air pollution, not to mention its short diurnal availability, the ultraviolet portion of solar radiation often is simulated for laboratory investigation; most of the work described here was conducted in a "sunlight-simulator" equipped with F40BL fluorescent lamps (General Electric Co.) exhibiting the spectral energy distribution also shown in Figure 1 (*31*).

It is a fundamental law of photochemistry

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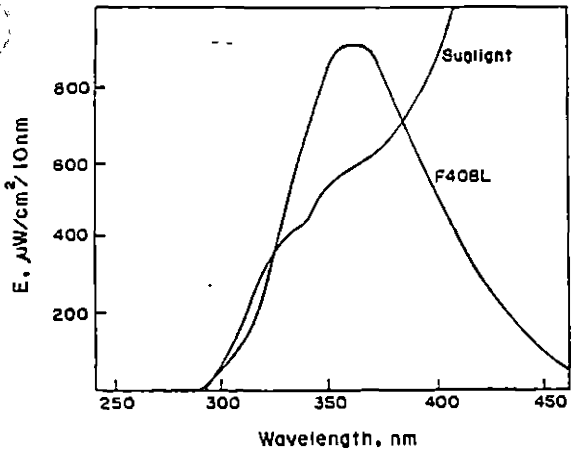
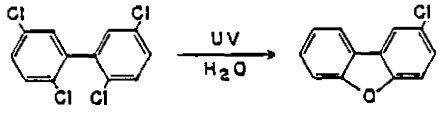


FIGURE 1. Spectral energy distribution of an F40BL fluorescent ultraviolet lamp compared with summer sunlight.

that radiation must be absorbed before reaction can occur. However, it is not necessary that the light energy absorbed by aromatic rings bring about bond scission directly. As in the previous examples, the absorption of energy can actviate the ring and facilitate nucleophilic displacements. In typical photonucleophilic reactions (*24*), chloride may be displaced from 4–CPA (4-chlorophenoxyacetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid), and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) by hydroxide ion to generate phenols, and, in accordance with earlier work of Munakata (*29*, *32*) we now find that PCP forms chlorinated catechols and resorcinols in aqueous media irradiated at sunlight wavelengths. In polychlorinated compounds, displacement of the chlorine *ortho* to the oxygen predominates.

Under these circumstances, it might be expected that a chlorophenate anion could attack the light-activated ring of another chlorophenol to form a diphenyl ether, and the reaction could be repeated intramolecularly to generate a chlorinated dibenzo-*p*-dioxin. Indeed, the irradiation of aqueous solutions of dioxin-free sodium PCP was found to generate OCDD, although only very small amounts could be detected by gas chromatography (*33*). Repeated attempts to detect TCDD after the irradiation of 2,4,5-T, 2,4,5-trichlorophenol, or sodium 2,4,5-trichlorophenate solution were unsuccessful.

The expected photonucleophilic formation of phenols from other aromatic halides such as chlorobiphenyls was demonstrated both with simple models (*34–37*) and with highly complex polychlorinated biphenyl (*PCB*) mixtures (Arochlors) (*36*). For example, irradiation of an aqueous suspension of 4,4′-dichlorobiphenyl provided 4-chloro-4′-hydroxybiphenyl and 4-chlorobiphenyl. However, as in the classical examples of synthesis cited previously (*19*, *20*), appropriately substituted chlorinated biphenyls also might be expected to form chlorodibenzofurans. Irradiation experiments with five pure 2-chlorinated biphenyls as 5 mg/l. aqueous suspensions, followed by resolution and examination of the products by GC/MS, showed that traces of 2-chlorodibenzofuran were detectable (eq. (2)], although only the 2,5-dichloro- and 2,5,2′,5′-tetrachlorobiphenyls provided identifiable amounts (a roughly steady 0.2% yield during a 7-day irradiation).



(2)

Photochemical Degradation

Our failure to detect TCDD as a product of 2,4,5-trichlorophenol photolysis can be explained on the basis of the extreme instability of the lower chlorinated dioxins to light (*38*). In either sunlight or the sunlight-simulator, 2,7-dichloro-, 2,3,7-trichloro- and 2,3,7,8-tetrachlorodibenzo-*p*-dioxins (5 mg/l. in methanol) were entirely decomposed within a few hours (Fig. 2). However, OCDD was much more stable under these conditions, which explains why traces of it could be

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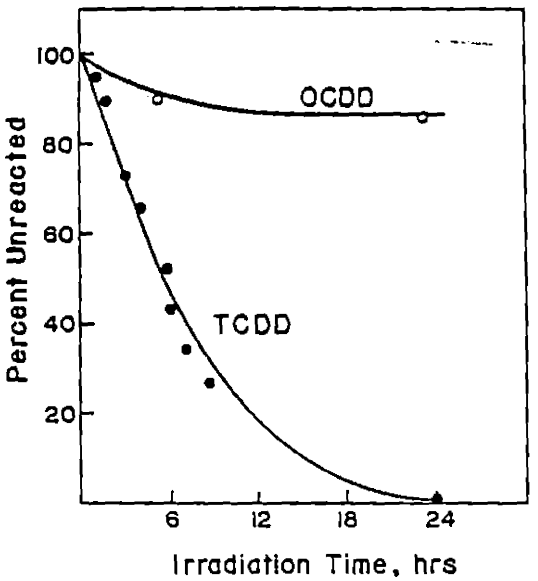
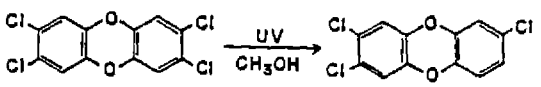


FIGURE 2. Photodecomposition rates of (○) OCDD (2.2 mg/l.) and (●) TCDD (5 mg/l.) in purified methanol under simulated sunlight (F40BL lamp).

isolated after the irradiation of PCP in water.

The rapid photodecomposition of TCDD in alcohols is accomplished by reductive dechlorination [eq. (*3*)], and an effective hydrogen donor appears absolutely necessary. Consequently, photoreduction in organic media proceeds efficiently to replace halogens one at a time with hydrogen atoms; the reduction is very sluggish in water and fails entirely in thin solid films of pure TCDD or on dry, irradiated soil surfaces (*39*, *40*). The mechanism has not been determined, although both the well-known free-radical hydrogen abstraction from solvent (*30*, *41*) and hydride transfer (*24*) are possible. OCDD photoreduction provided detectable traces of hepta- and hexachlorodioxins, and chlorinated pesticides including 4-CPA, 2,4,5-T, PCP, PCNB (pentachloronitrobenzene), and hexachlorobenzene behaved similarly, as did the chlorinated biphenyls.

On the basis of our previous experience with dioxins, biphenyls, and the other chlorinated compounds, it came as no particular surprise that model chlorinated dibenzofurans also were photoreduced in methanol



(3)

or even in water. For example, when a 5 mg/liter methanol solution of pure 2,8-dichlorodibenzofuran was irradiated in the sunlight-simulator, 2-chlorodibenzofuran was the product detected by GC/MS, and more than 95% of the starting material was photolyzed within 48 hr (Fig. 3). However, the inadvertent use of highly purified methanol in a similar experiment revealed only very slow photolysis within the same period of irradiation. The irradiation of a more concentrated solution (10 mg/l.) in the purified solvent also indicated slow decomposition; after 90 hr, the addition of 10 mg/l. of 4,4′-dichlorobenzophenone as a photosensitizer resulted in a sharp increase in the photolysis rate, although acetone did not appear to sensitize the photolysis in this instance. It appears, then, that impurities in common solvents can drastically alter photodecomposition rates.

Discussion

A remarkable level of scientific attention recently has been devoted to the chlorinated dibenzodioxins and dibenzofurans. From the smattering of articles representing the four decades preceding 1970, two recent conferences (*42*, *43*) alone have provided more than 60 research papers. While earlier work dealt largely with basic chemistry and occupational health, more recent concerns have been primarily associated with the possible environmental occurrence and environmental effects. Indeed, much of the current effort to understand and control these compounds arose because of unanswered questions about their environmental impact on humans, domestic animals, and wildlife.

Although improved manufacturing and

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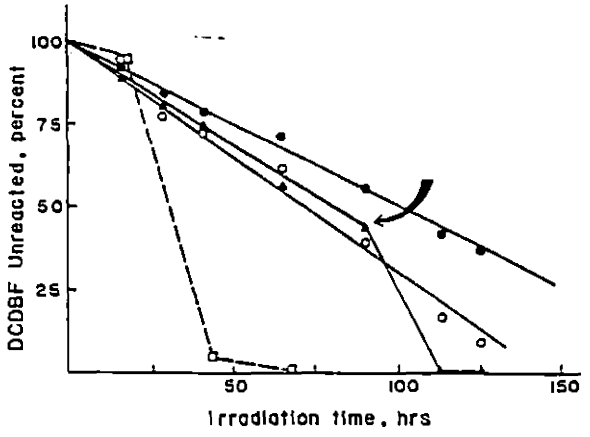


FIGURE 3. Photodecomposition rates of 2,8-dichlorodibenzofuran (DCDBF): (●,▲) in acetone-free methanol (10 mg/l.); (○) in acetone-free methanol containing added acetone (320 mg/l.); (□) in laboratory-grade methanol. The arrow indicates addition of 10 mg/l. of 4,4'-dichlorobenzophenone. Straight lines drawn for comparison only.

surveillance methods have dramatically reduced dioxin and dibenzofuran levels among industrial chemicals (*12*), there is evidence that under idealized conditions, at least, both types of compounds can be generated from commonly used chlorophenols and chlorinated biphenyls under environmental conditions. The driving force can be either the ultraviolet component of sunlight impinging upon thin films, surfaces, or solutions or heat such as might be encountered in burning wood. Conceivably but not demonstrably, phenol derivatives such as the phenoxy herbicides also might provide the raw material, but, in any case, the necessary generative reactions clearly have been demonstrated.

On the other hand, environmental persistence or accumulation theoretically could be balanced by the simultaneous destructive action of light or heat. Photolysis can be rather rapid under idealized conditions, although a hydrogen donor appears to be required, and OCDD is rather stable compared to the highly toxic TCDD. The charring temperature of wood (>350°C) is well above that frequently reported to convert PCP into OCDD, but the thermal stability of the dioxins has not been reported.

Just how probable are the environmental formation—and decomposition of dibenzodioxins and dibenzofurans? The ultraviolet component of sunlight undoubtedly is capable of energizing their degradation in the presence of organic hydrogen donors, and those donors can be expected to be present at and after the time of application as either the usual formulation solvents (such as xylene or petroleum hydrocarbons), as active constituents of the formulation (for example, the alkyl esters of 2,4-D and 2,4,5-T), or as natural organic films. Although TCDD has been reported to be stable to irradiation on soil surfaces, it was not accompanied in those experiments by a nonvolatile organic source of hydrogens as it would be in the field. It was quite stable, too, when suspended in water but rapidly degraded when benzene also was present (*39*).

Photosensitization also must be an important consideration, as demonstrated by the effect of dichlorobenzophenone on the photoreduction of chlorinated dibenzofurans. Such sensitizers have been shown to be expected under field conditions (*44–46*) and could drastically increase the photolysis rates of xenobiotics. At the least, photochemical investigations in the laboratory should recognize the possibility of sensitization, for example by impurities commonly present in even purified grades of methanol. All together, degradation appears to outrace generation in most of our laboratory studies.

However, the same solvents and active ingredients which could act as hydrogen donors and sensitizers also could protect dioxins and dibenzofurans from photolysis under practical conditions. For example, while light absorption by TCDD might be only partially obscured by the absorption of a 2,4,5-T formulation (Fig. 4) and chlorinated dibenzofurans would be little shielded by PCBs (Fig. 5), the low level of OCDD in a technical formulation of PCP or sodium PCP should remain well-protected from light (Fig. 6), although an improved, low-dioxin product (Dowicide EC-7) was somewhat less opaque. Clearly, too, OCDD is environmental-

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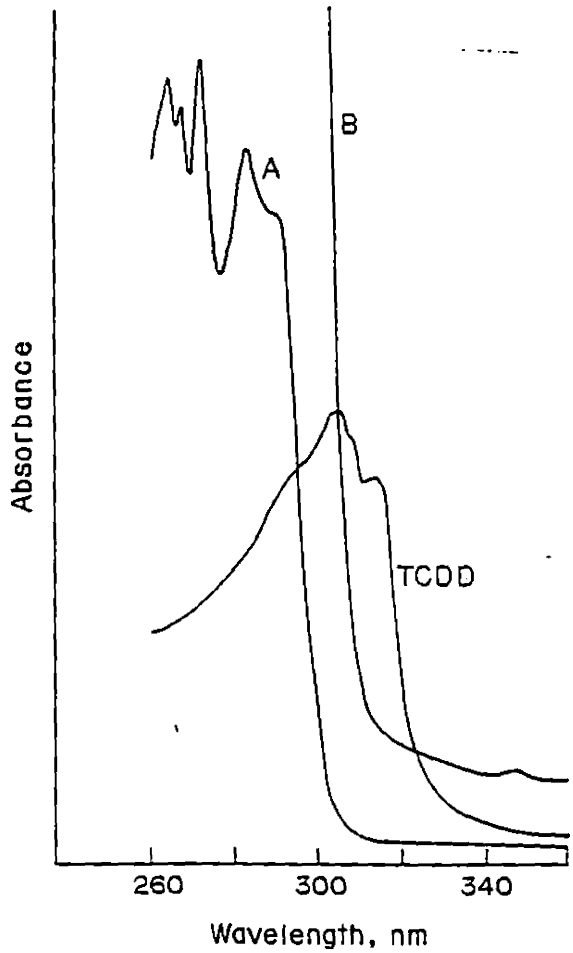


FIGURE 4. Ultraviolet absorption spectra of two concentrations (A,B) of commercial brush-killer (25.1% 2,4-D isooctyl ester and 12.0% 2,4,5-T isooctyl ester in aromatic hydrocarbon solvent) in methanol compared with that of TCDD.

ly generated—especially by heat—albeit inefficiently.

Considering the probable photolysis of TCDD to nontoxic products and the insignificant rate of dibenzofuran formation from biphenyls, it is hardly surprising that they remain undetected in environmental samples. In fact, OCDD and its immediate toxic reduction products—the hexa- and heptachlorodioxins—emerge to be of even more environmental health interest. For example, they appear to be the best candidates for both photochemical formation and stability; obviously, they are released by

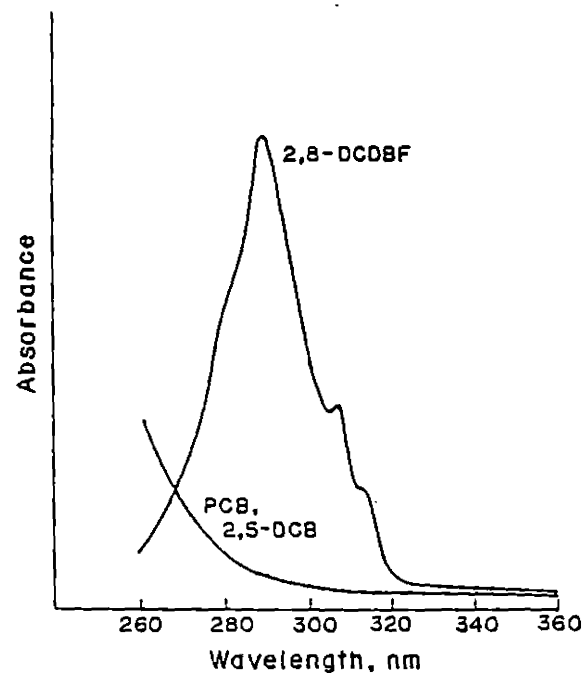


FIGURE 5. Ultraviolet absorption spectra of PCB (Arochlor 1254) and 2,5-dichlorobiphenyl in methanol compared with that of 2,8-dichlorodibenzofuran (2,8-DCDBF).

heat from common materials of construction; and the relatively innocuous OCDD readily undergoes reduction to form less chlorinated homologs which are both rather stable and toxic (*47*).

But are these compounds released, in fact? Are chlorinated dibenzo-*p*-dioxins and dibenzofurans actually generated and destroyed out there in the real world? Regrettably, no one seems to know. Experiments such as we have described generally are conducted under idealized conditions: pure compounds and solvents, artificial substrates, and even artificial sunlight. What of the sensitizers and sunscreens, the hydrogen donors and competing nucleophiles which must be present in actual environmental applications? It is apparent that rather large stakes in terms of biological research investments, manufacturing, and public health now are riding on what previously may have seemed to be only exotic details of xenobiotic chemistry.

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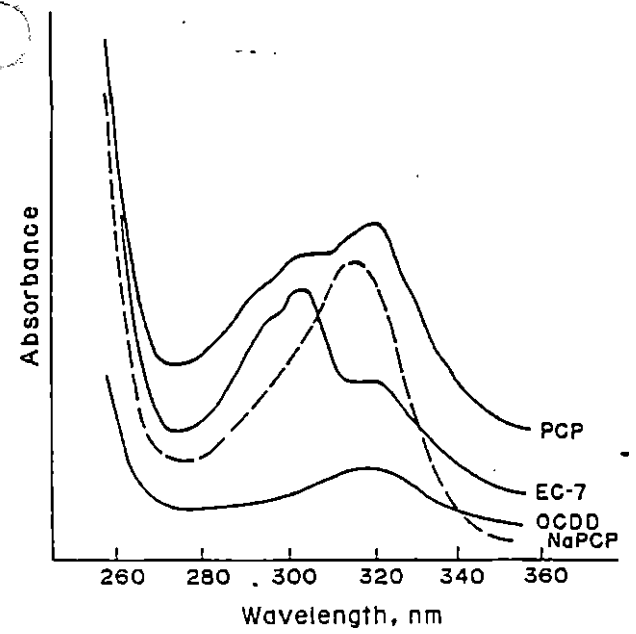


FIGURE 6. Ultraviolet absorption spectra of commercial PCP (35.3% PCP and 4.1% other chlorinated phenols in petroleum distillate) and Dowicide EC-7 (88% PCP and 12% 2,3,4,6-tetrachlorophenol) in methanol and commercial NaPCP (80-85% sodium pentachlorophenate) in water compared with that of OCDD in methanol.

Acknowledgment

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Photochemical Degradation of Di- and Octachlorodibenzofuran

by O. Hutzinger,* S. Safe,*
B.R. Wentzell,† and
V. Zitko‡

Introduction

In the last few years, it has been shown that polychlorinated biphenyls undergo decomposition on irradiation with artificial ultraviolet light sources and sunlight (*1–7*). Reductive dehalogenation of one or more chlorine atoms was a major reaction, particularly in hydrocarbon solvents. In fluorocarbon solvents or thin films, small quantities of chlorobiphenyls with increased chlorine content could also be detected. Irradiation in hydroxylic solvents gave the dechlorinated species as well as photoproducts containing oxygen. In addition, chlorinated terphenyls and quaterphenyls were also detected in some instances, and on prolonged irradiation polymeric products were formed.

It has been speculated for some time that chlorodibenzofurans may be formed from chlorobiphenyls under photochemical conditions which lead to oxygenated products. Although no chlorodibenzofurans could be detected in a number of chlorobiphenyl samples which had been exposed to sunlight for over 2 months (*4*), preliminary results indicated the formation of chlorodibenzofurans from 2,2′,4,4′,6,6′-hexachlorobiphenyl in model experiments (irradiation in methanol) (*8*).

The toxicity of pure chlorodibenzofurans has not been thoroughly investigated (*9*) but appears to be higher by several orders of magnitude than that of the chlorobiphenyls, which gives this reaction particular significance.

In this regard, the photochemical behavior of chlorodibenzofurans themselves becomes of interest from the point of view of products formed (decomposition to less toxic products) and stability. As an approximation [eq. (1)], it appears that if chlorodibenzofurans are formed from chlorobiphenyls in the environment by photochemical reaction, accumulation will be a problem if $k_1 > k_2$.

$$\text{Chlorobiphenyl} \xrightarrow{k_1} \text{chlorodibenzofuran} \xrightarrow{k_2} \text{decomposition products} \qquad (1)$$

Since a good selection of chlorodibenzofurans is not available, only preliminary investigations with two representatives, one of low (2,8-dichlorodibenzofuran) and one of high chlorine content (octachlorodibenzofuran) are reported.

Equipment and Methods

Chemicals

2,8-Dichlorodibenzofuran (*10*) and octachlorodibenzofuran (*11*) were prepared by

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literature methods. Samples for the initial experiments were provided by Dr. A. E. Pohland, (FDA, Washington, D.C.)

Irradiations

For the irradiations in solution (450 ml hexane or methanol), a Rayonet (the Southern New England Ultraviolet Co.) photochemical reactor equipped with 16 RPR-3100 lamps (310 nm) was used. Aliquots (25 ml) were taken at times specified in Figure 1. For mass spectrometric analysis of the photolysis mixture, 2,8-dichlorodibenzofuran and octachlorodibenzofuran were irradiated in methanol for 20 min.

Thin films (50 mg) of the chlorodibenzofurans coated on the inside of quartz tubes (length: 25 cm; diameter, 4 cm) were exposed to sunlight for 10 weeks in the presence of water (2 ml) during July–September 1972. Total duration of bright sunshine was ca. 580 hr (Meteorological Services, Canadian Forces Base, Shearwater, N. S., private communication).

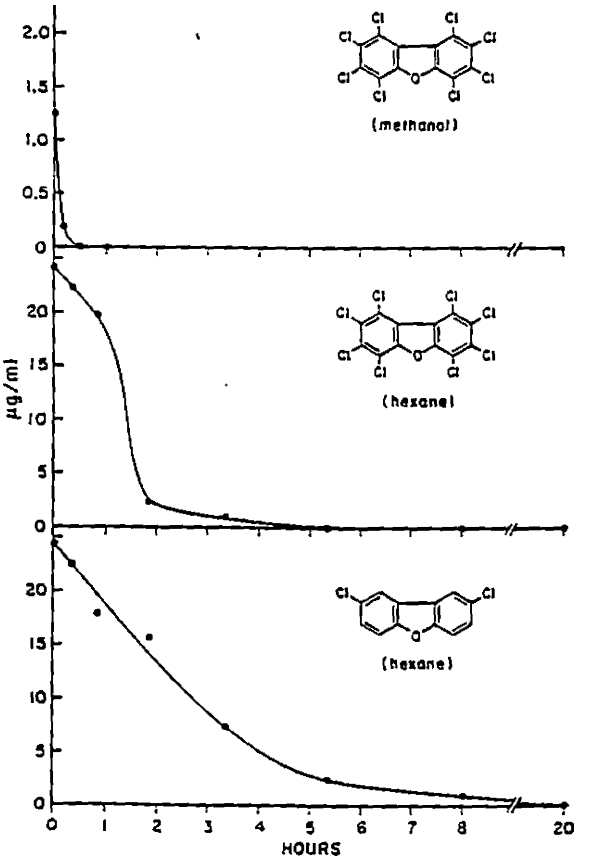


FIGURE 1. Photochemical degradation of chlorodibenzofurans in solution. Irradiation wavelength 310 nm.

Instruments

Quantitative data were obtained with a Packard model A7901 instrument equipped with 6 ft × 4 mm columns packed with either 4% SE-30 (for the 2,8-dichlorodibenzofuran) or 3% OV-210 (for the octachlorodibenzofuran) on Chromosorb W.

A DuPont/CEC 21-110B double focussing mass spectrometer was used for obtaining spectra by direct introduction. The probe was heated carefully, and spectra were recorded as the temperature was raised from 20°C to ca. 180°C.

Analysis of Samples

Quantitative analyses (photochemical stability of chlorodibenzofurans) were carried out by gas chromatography (GC) with electron capture detection on the aliquots taken from the photochemical reactor.

For the characterization of products formed, the solvent was removed from the samples and the residue chromatographed on Merck silica thin-layer plates (F-254; 0.25 mm thickness). The solvent used was hexane. For the mass spectroscopic analysis, small fractions of the total sample were transferred to a mass spectrometer sample tube. Samples from the quartz tubes were dissolved in benzene–acetone and treated as described above.

Results and Discussion

The correct numbering of the dibenzofuran ring system (Chemical Abstracts and Ring Index) is shown in Figure 2.

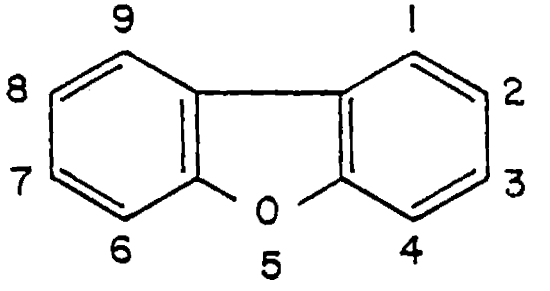


FIGURE 2. Numbering of the dibenzofuran nucleus.

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Some older systems are still in use occasionally. Calculated molecular weights (Cl = 35) for dibenzofuran and its chlorine substitution products are given in Table 1.

Table 1. Molecular weights for chlorodibenzofurans (monoisotopic formula; Cl = 35)

Formula	Molecular weight
$C_{12}H_8O$	168
$C_{12}H_7ClO$	202
$C_{12}H_6Cl_2O$	236
$C_{12}H_5Cl_3O$	270
$C_{12}H_4Cl_4O$	304
$C_{12}H_3Cl_5O$	338
$C_{12}H_2Cl_6O$	372
$C_{12}HCl_7O$	406
$C_{12}Cl_8O$	440

Irradiation of 2,8-Di- and Octachlorodibenzofuran in Solution

The relative rate of decomposition of 2,8-di- and octachlorodibenzofuran is shown in Figure 1. From these results and from the mass spectra of samples which were irradiated for 20 min it is evident that decomposition is faster in methanol than in hexane. In contrast to the chlorinated dibenzo-*p*-dioxins, where the degradation of the octachloro derivative on irradiation in methanol solution is much slower than that of the 2,7-dibenzo-*p*-dioxin (*12*), the di- and octachlorodibenzofurans show similar rates of decomposition.

For the analysis of products formed, samples which were irradiated for 20 min in methanol (short exposure) and for ca. 20 hr in hexane (long exposure) were chosen.

Thin-layer chromatography of the samples exposed for 20 min showed only two spots, a relatively weak one on the origin and a large spot with an R_f similar to those starting materials (R_f = ca. 0.55 for 2,8-dichlorodibenzofuran; R_f = ca. 0.75 for octachlorodibenzofuran).

Since organochlorine compounds which give very strong molecular ions can be analyzed by mass spectrometry in mixtures (*13–15*), samples of the irradiated products were carefully heated in the mass spectrometer probe and spectra recorded at different temperatures. Typical spectra are shown in Figures 3 and 4. No other products but those formed by dechlorination of the corresponding chlorodibenzofuran were found to be present.

No useful mass spectra could be obtained for the yellow gum which resulted from the 20-hr irradiation experiments. A large number of peaks was observed and no recognizable chlorine isotope pattern was apparent.

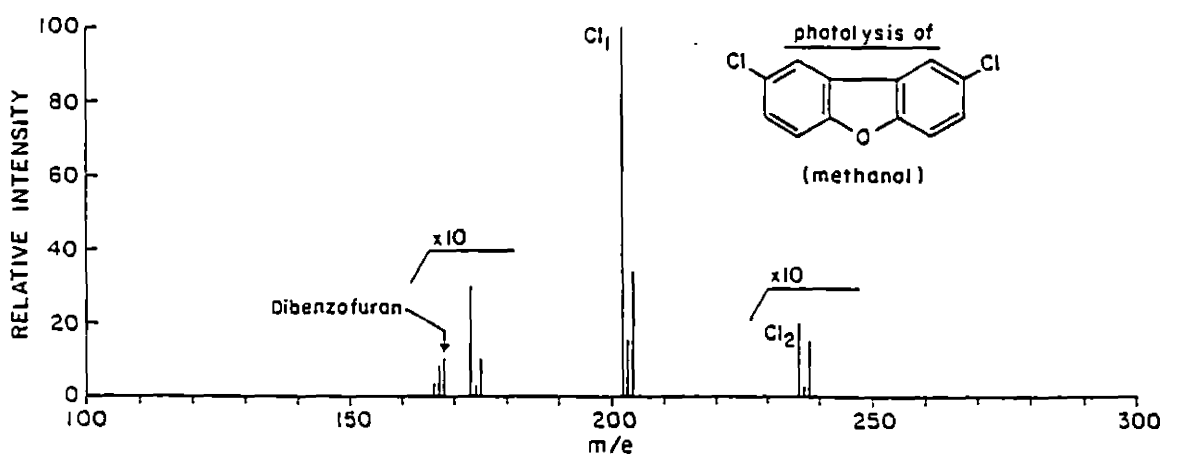


FIGURE 3. Mass spectrum (70 eV) of 2,8-dichlorodibenzofuran photolysis mixture (310 nm; 20 min; solvent methanol). Probe temperature: 40° C.

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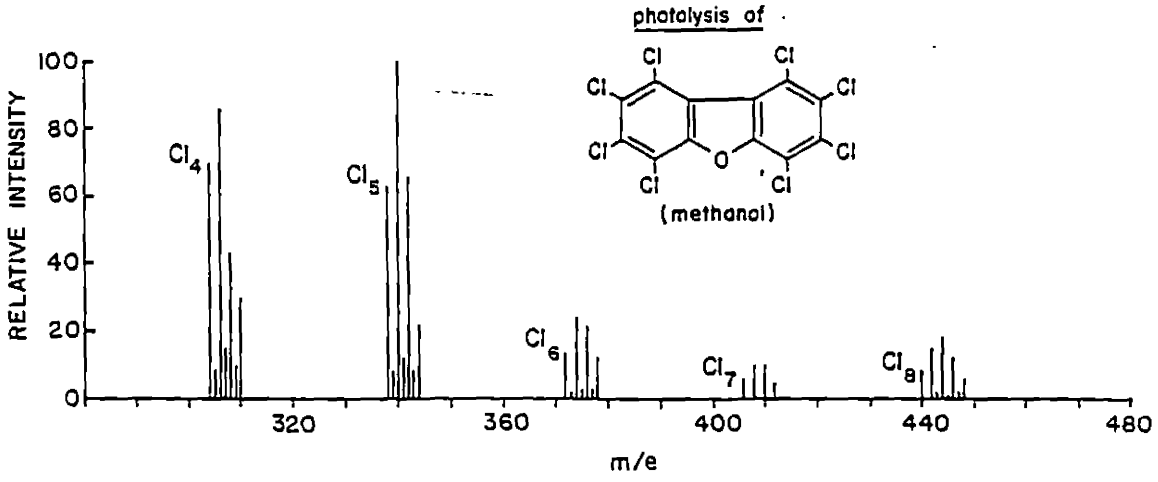


FIGURE 4. Mass spectrum (70 eV) of octachlorodibenzofuran photolysis mixture (310 nm; 20 min; solvent; methanol). Probe temperature: 80° C.

Exposure of 2,8-Di- and Octachlorodibenzofuran as Thin Film to Sunlight

The analysis by mass spectrometry was carried out as described above. Reductive dechlorination of octachlorodibenzofuran was observed to a much lesser degree than in solution. 2,8-Dichlorodibenzofuran gave, in addition to a monochloro derivative, a trichlorodibenzofuran (M^+·270).

A summary of the results of the photolysis experiments in solution and thin films is shown in Figures 5 and 6.

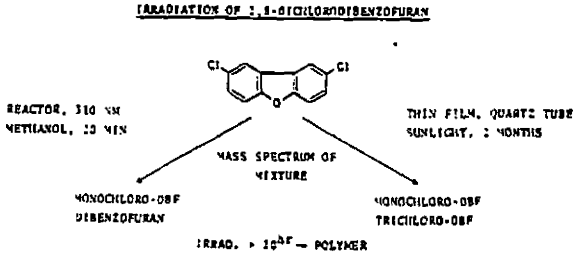


FIGURE 5. Summary of results from 2,8-dichlorodibenzofuran irradiation experiments.

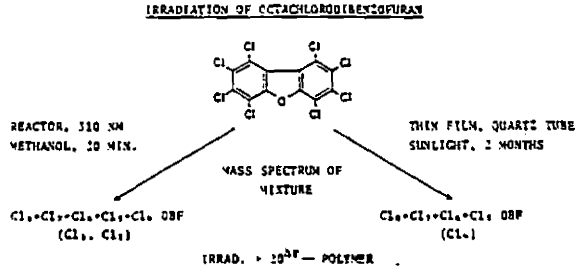


FIGURE 6. Summary of results from octachlorodibenzofuran irradiation experiments.

Summary and Conclusions

Photolysis of 2,8-di- and octachlorodibenzofuran in methanol and hexane solutions results in rapid dechlorination of the substrates with the eventual accumulation of unidentified resinous polymeric products. Dechlorination is also observed to a certain extent when thin films of these compounds were exposed to sunlight.

These preliminary data do not allow direct comparison of dibenzofuran degradation rates with rates of photochemical formation from corresponding chlorobiphenyls (*8*). In view of the photochemical lability of chlorodibenzofurans, however, it seems unlikely that accumulation of these compounds formed from chlorobiphenyls by photochemical reaction in the environment will occur.

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Tetrachlorodibenzodioxin in the Environment: Sources, Fate, and Decontamination

by P.C. Kearney,* E.A. Woolson,* A.R. Isensee,* and C.S. Helling*

Introduction

Research on the behavior and fate of pesticides in the environment has provided a number of valuable techniques for assessing the impact of any organic substance intentionally or unintentionally released in the environment. These techniques can be applied to the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to determine its behavior under comparable conditions. Important parameters affecting the environmental life history of any compound are movement, persistence, metabolism, plant uptake, translocation, photodecomposition, volatilization, and bioconcentration. Once these environmental properties are known about a compound, its anticipated behavior can be estimated, based on a comparison of information collected on registered pesticides. The modern registration process for pesticides entails a detailed accounting of the above mentioned parameters, plus information on efficacy and toxicology.

A second major consideration in assessing the impact of any organic substance in the environment is the total amount that may be present from current and past usage. For large-scale manufactured substances, production information is available if there are at least three manufacturers. Estimates on the occurrence of an impurity in any manufactured product may be extremely difficult to determine, unless this impurity has been recognized as a major contaminant and surveillance is implemented at an early stage of the production process.

Finally, once a substance has been identified as a potentially hazardous material, a safe, practical, and economically feasible decontamination process must be devised to use or destroy any known stocks.

The Agricultural Research Service became interested in the dioxin problem soon after the disclosure that it was associated with one of the older and widely used herbicides, 2,4,5-T [(2,4,5-trichlorophenoxy) acetic acid]. Our preliminary studies were directed toward identifying other sources of dioxins in pesticidal chemicals and learning something about the fate or life history of the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the environment. More recently time has been devoted toward helping in the assessment of decontamination methods that will successfully destroy TCDD. The present paper considers the sources, fate, and decontamination of TCDD in the environment.

Sources

Historically, TCDD has been associated with any process that uses 2,4,5-trichlorophenol as a starting material. A number of the currently used synthetic organic pesticides are derived from 2,4,5-trichlorophenol. In a survey of 17 pesticides examined for

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TCDD contamination, Woolson et al. (*1*) could detect TCDD only in older samples of 2,4,5-T and in one sample of silvex [2-(2,4,5-trichlorophenoxy)propionic acid]. The level of contamination in the single sample of silvex was 1.4 ppm. Of 42 samples of 2,4,5-T, 20 contained TCDD measured at a limit of sensitivity of 0.1 ppm. Of these 20 positive samples, 7 contained less than 10 ppm TCDD and 13 contained between 10 and 100 ppm TCDD. It appears, then, that TCDD contamination occurred primarily in samples of 2,4,5-T.

The United States production and domestic disappearance of 2,4,5-T for the years 1960–1970 are shown in Figure 1. The total production for this ten-year period was 106,310,000 lb of 2,4,5-T expressed as the acid equivalent. The domestic disappearance figures for 2,4,5-T include military shipments abroad, primarily as defoliants for use in Vietnam. The peak year of production was 1968, when 17.5 million lb (about 16% of the ten-year total) was manufactured. Production sharply declined in 1969, to 5 million lb. The production figures for individual manufacturers are unavailable on a yearly basis. Although current data are lacking, older available data give some indication of the extent of use of 2,4,5-T. The total use of 2,4,5-T on farms, rights-of-way, and other nonfarm uses in 1964 was about 8,912,000 lb on 7,939,000 acres in the United States. The average rate of 2,4,5-T application on all domestic acreage in 1964 was about 1 lb/acre with a range from 0.25 to 2 lb/acre.

The best estimates on dioxin content in past samples of 2,4,5-T come from an extensive survey of approximately 15 million pounds of Herbicide Orange (200 samples) conducted by the U.S. Air Force (unpublished observations, 1973). Herbicide Orange is a defoliant containing about a 50/50 mixture of the butyl esters of 2,4,5-T and 2,4-D [(2,4-dichlorophenoxy) acetic acid]. The average dioxin content of the mixture was 1.91 ppm by weight (arithmetic mean) by use of a technique sensitive to 0.05 ppm. A frequency profile for these samples is shown in Figure 2. Of the 200 samples of Herbicide Orange, 136 or 68%, contained 0.5 ppm or less of TCDD. The highest sample contained 47 ppm TCDD. In the pesticide survey conducted by Woolson et al. (*1*) a 90% decline in TCDD was noted between 1968 and 1969 from one manufacturer. However, the level was still at 2–3 ppm in the 2,4,5-T acid. It is difficult and perhaps dangerous to extrapolate the total amount of dioxin added to the environment from previous use of 2,4,5-T for the following reasons: (1) from preliminary evidence available to us, it appears that different manufacturers produced 2,4,5-T with different TCDD contents over the

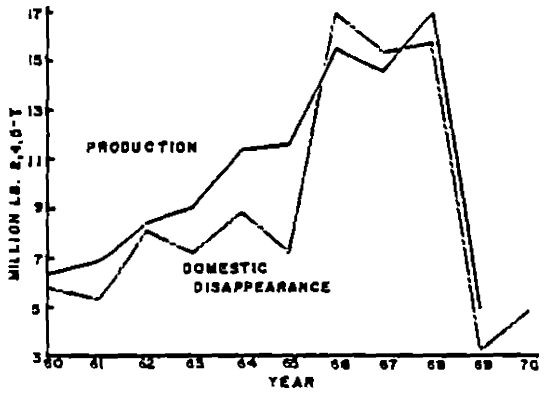


FIGURE 1. Production and domestic disappearance (includes military shipments) of 2,4,5-T acid in the U.S. (1960–1970).

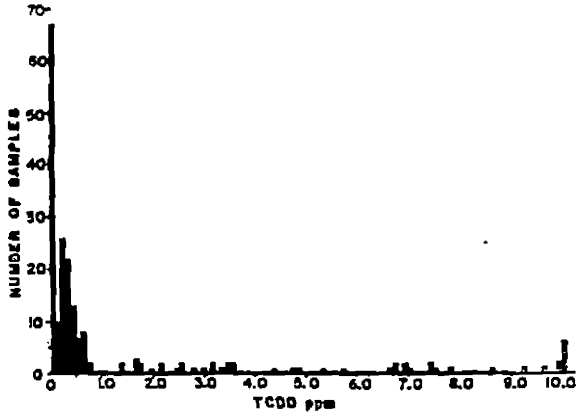


FIGURE 2. Distribution of TCDD content in 200 samples of Herbicide Orange. All samples containing more than 10 ppm appears as the last bar to the right of the figure.

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10-year period, 1960–1969; (2) total production of each manufacturer and consequently the total dioxin content on a yearly basis is unknown, (3) it is difficult, if not impossible, in certain instances to attribute specific lots of 2,4,5-T to certain manufacturers; (4) the statistical significance of the inferences drawn from 200 samples is probably too small to reach any valid conclusion on a realistic input of TCDD from herbicide applications.

In 1971 when the dioxin issue first became known, we obtained and analyzed current production samples of 2,4,5-T acid then in production by the three principal manufacturers. They contained <0.1, 2.3, and 0.1 ppm.

By 1971, industry could provide commercial samples of 2,4,5-T that met the suggested limits of less than 0.5 ppm TCDD and could probably routinely produce 2,4,5-T with a TCDD content of about 0.1 ppm. Although we can make no statement about the past input of TCDD into the environment for the reasons stated above, some predictions can be advanced as to future input if the level of TCDD is held at 0.1 ppm in 2,4,5-T. If one assumes the 1964 usage of 2,4,5-T indicates the normal peace time usage, i.e., about 8 million lb applied at 1 lb pound per acre, then the amount of TCDD reaching the soil would be equal to 0.05 mg/acre. Assuming the surface 3 in. of soil weighs 1 million lb, the concentration would be 0.1 parts per trillion.

Fate in the Environment

A number of review articles covering various aspects of dioxin in the environment (*2*, *3*) and in various environmental components including soils (*2*, *4*, *5*) sunlight (*6*), plants (*7*), and wildlife (*5*) have appeared from the USDA Pesticide Degradation Laboratory in Beltsville. A summary of these findings was presented in the review by Helling et al. (*8*) as follows.

TCDD was not photodecomposed on wet or dry soil surfaces nor was it produced photolytically from 2,4,5-trichlorophenol in water. TCDD is slowly lost in aqueous suspensions and in methanol.

Vertical movement of TCDD did not occur in a wide range of soil types. Contamination of underground water supplies seems very unlikely.

Approximately half of the TCDD applied at concentrations of 1, 10, and 100 ppm persisted in two moist soils after 1 yr under laboratory conditions.

Field applications of high rates (up to 942 lb/A) of 2,4,5-T on Lakeland sand produced no detectable (<1 ppb) TCDD residue when sampled 6 yr later to a depth of 1 m.

Soil metabolism of 2,4,5-trichlorophenol does not lead to the formation of TCDD as a condensation product.

Small quantities of TCDD (<40 ppb) were accumulated by young oats and soybeans grown on a sandy loam contaminated with TCDD (60 ppb). No TCDD was detected (<1 ppb) in mature plants or seeds grown on these same soils.

TCDD was not translocated from the point of application on the leaf surface to other plant parts. Some wash-off and possibly volatilization did occur.

Analyses of 19 eagle carcasses revealed no detectable (<50 ppb) dioxins. Admittedly more sensitive analytical techniques are needed before a clear picture of low TCDD wildlife levels can be assessed.

Because of its low solubility (3–5 ppb), relatively long persistence, lack of vertical mobility in soils, and inability to translocate in higher plants, TCDD more nearly resembles the shorter-lived chlorinated hydrocarbon insecticides in behavior than it does the more biodegradable phenoxyalkanoic acid herbicides, e.g., 2,4-D and 2,4,5-T.

Decontamination

Suspension of the use of Herbicide Orange in Vietnam has created large military surpluses that now must be used or destroyed. The U.S. Air Force has about 24 million lb of Herbicide Orange. Approximately 15 million lb contains an average of 1.91 ppm dioxin. Of the remaining material, which cannot be identified by manufacturer, approxi-

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mately 80% contains less than 0.4 ppm dioxin. The political implications associated with Herbicide Orange may preclude its use as an herbicide, even though a substantial portion of the excess stock meets currently established EPA policy. Several conventional disposal methods, in addition to some new techniques, have been investigated to determine the safest, most economical, and most practical method for disposal of this material. Three options under consideration are incineration, soil biodegradation, and chlorinolysis. Since an environmental impact statement must be filed on the ultimate method or methods used for dealing with these surplus herbicides and their dioxin contaminate, several scientific investigations have addressed their efforts toward determining the parameters needed for successful destruction of TCDD.

Incineration appears to offer one of the safest methods for complete destruction of TCDD. In the present context incineration is defined as the high temperature reaction of TCDD with oxygen conducted in a unit equipped with proper emission control devices. By the use of differential thermal analysis, Kennedy and Stojanovic (Mississippi Agricultural and Forestry Experiment Station, personal communication, 1973) have estimated that temperatures of 800–1000°C will cause complete destruction of TCDD. These estimates are in good agreement with the value of 800°C published by Langer et al. (*9*).

Additional information is needed, however, on the composition of the stack gases and particulates, as well as water emanating from any industrial incineration process involving dioxins.

A second feasible method of dioxin disposal is soil biodegradation. Our work (*4*) indicates that approximately 50% of the added TCDD had been destroyed after 1 yr in soils at reasonably high concentrations. Woolson et al. (*5*) were unable to detect any TCDD in a Lakeland sand receiving approximately 1,000 lb of 2,4,5-T per acre over a 7-yr period. No TCDD could be detected at a minimum detection limit of <1.0 ppb in core samples to a depth of 6 ft. Although TCDD is fairly recalcitrant in soils, it is immobile and thus would offer no ground water contamination problems. Soil incorporation to a depth of about 6 in. seems to offer an additional advantage, i.e., it would prevent aerial movement on soil borne particles away from the site of application.

A third method of disposal of TCDD involves a new technology called chlorinolysis. This process involves the high temperature conversion of carbon compounds in pressurized atmosphere of chlorine to form carbon tetrachloride. In the United States, chlorinolysis is still in an experimental phase. We have cooperated with the Diamond Shamrock Company in pilot studies to investigate the stability of TCDD in samples of Herbicide Orange. A detailed examination of carbon tetrachloride produced from Herbicide Orange revealed no TCDD at a level of sensitivity of 10 parts per trillion, as measured by electron-capture gas chromatography. Chlorinolysis represents a major departure from conventional disposal systems in that it converts one product into another useful resource. In addition to carbon tetrachloride, phosgene and hydrochloric acids are products of chlorinolysis. Carbon tetrachloride has a major market in the production of freon, which has wide application as a coolant and propellant.

Summary

The major source of TCDD input in the past has been from use of the herbicide 2,4,5-T. Future inputs will be minimal if the dioxin content is held at low levels (0.1 ppm) during the manufacturing process.

In the environment, TCDD behavior is similar to some of the shorter-lived chlorinated hydrocarbon insecticides. It is fairly persistent and immobile in soils, not taken up into the economic portion of plants, and slowly decomposed in water in sunlight.

Several disposal options are available for the safe decontamination of surplus stocks of 2,4,5-T containing TCDD. These include incineration, soil disposal, and chlorinolysis.

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ummary: Conference on Dibenzodioxins and Dibenzofurans, National Institute of Environmental Health Services, April 2-3, 1973

by Edward J. Burger, Jr.*

To attempt to summarize the proceedings of a meeting such as this one in any rigorous sense is clearly an unreasonably ambitious task. It is made more challenging because of the tentative character of much of the work commented upon here.

This meeting, one of a series (and I hope a growing series) for the National Institute nvironmental Health Sciences, repre- an extraordinarily useful concept. It is designed to bring together a variety of scientific bedfellows who have contemplated (and indeed investigated) a subject for its academic interest and for its very timely topical interest. These same scientists would have learned about each other eventually but, in the best tradition of science, it would have taken a long time.

By design, we are witnessing the cutting edge of scientific research for this area. This meeting has, as its avowed and very virtuous purpose, the calling out of the walls a good deal of unmatured and not totally interpreted or confirmed research. It is important to keep this tentative and unconfirmed character in mind.

One is struck, too, by the character of the research which has been reported at this meeting. There are still many gaps in our knowledge about dibenzofurans and dioxins. Yet, the thought that has gone into the design of the research reported here represents a striking degree of sophistication in many cases for which the participants should be very proud. As late as 1970 or 1971, there existed only the crudest hint of a ranking of biological activity of members of the family of chlorinated dioxins. (*1*, *2*) The reports at this conference contained descriptions of dose–response information, some beginning insight into mechanisms, and the first probings toward structure–activity relationships. I am struck by the fact that the early observations (from the occupational environment) and the early approximations of rankings have been sustained and essentially confirmed by the data reviewed at this meeting.

Chemistry, Analysis, and Chemical and Physical Properties

Dr. Langer's paper on the formation of dioxins from precursors through condensation reactions represents a good example of what we should do more of. I speak here of the attempt to predict probable and improbable behavior in the environment from knowledge of physical and chemical properties. Dr. Langer's nuptial analogy is apt in more than one way. He reminded us that chemical courtship leading to marriage (in this case, condensation) was family-specific

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and adhered to some orthodox rules. Family character, and how this is perceived by the other party to the arrangement, seems to be important. It seems to me that Dr. Langer has thrown down the challenge of confirmation for dioxin formation, reported on by some as feasible in the environment. I am sure that this challenge will be taken up.

One other point was raised by Dr. Langer and was echoed independently by others was the spurious formation of condensation products within the chambers of the very instruments used to detect them (e.g., the gas chromatograph).

What is the evidence of "weathering"—the formation in nature of dioxins or furans from chlorinated materials through the addition of energy from somewhere? Crosby et al. and Hutzinger et al. suggested that condensation reactions to form dioxins or dibenzofurans might be promoted by exposure to sunlight and presented the results of a few preliminary experiments to examine this subject. What they properly reminded us of, however, was the fact that the story does not end there. The presence of detectable condensation products depends on the dynamics of both production and ensuing decomposition. Decomposition, they reminded us, occurs typically through reduction and here depends on an available hydrogen source. (They demonstrated the point in their laboratory experiments by using a hydrocarbon medium.) Tetrachlorodibenzo-*p*-dioxin was found to be more labile than the octochlorinated member of the family. It was speculated that there was sufficient organic material in most environmental situations to assure hydrogen donors. In brief, environmental persistence seems unlikely. The evidence seems to suggest that those impurities which are found are of the less toxic varieties. However, we need more samples because of the large variety of commercial products.

A. E. Pohland et al. revealed some of the potential and the limitations of two analytic methods, electron spin resonance and visible light spectroscopy. As I heard this paper, these sounded particularly useful as confirmatory techniques. The authors cited the need for pure standards to realize the potential of their methods.

Dr. Crummett's paper is perhaps the latest in a growing series of examples of how the power of analytic methods tends to "drive" manufacturing procedures to be more rigorous and produce greater degrees of purity. His point is well made. We are all better off as a result.

It is heartening to note the similarity in degrees of resolution reported both by Crummett, et al. and by Drs. Baughman and Meselson for measurement of dioxin through mass spectroscopy. Understandably, these results rested, apparently, on an extensive clean-up procedure. (I think some may still be bothered by what I understand is a wide intrasample variability among some of the measurements.) Since they are "pushing" their art to a point near its limits, perhaps these results deserve as much confirmation as possible. Nevertheless, the power of sensitivity and resolution are indeed impressive.

Biological Effects

I think that it is extremely important to acknowledge the fact that the original biological insight into dioxins came from a series of observations made by Dr. Suskind of accidental occupational exposures in the late 1940's. The exposure was the result of accidental release of chemical intermediates in a 2,4,5-T plant in 1949 resulting in exposure of a number of workers to manifest chloracne. In 1957, Kimmig and Schulz reported chloracne among workers in 2,4,5-T plant in Germany. A third occupational incident occurred in a 2,4,5-T plant in the United States in 1964. In a way, it is somewhat disappointing that there is not more human experience reported at this meeting. I think that there is still a clouded issue, an unclear distinction between the effects of PCB, 2,4,5-T and of dioxins and furans. For example, we should somehow ascertain whether Yusho disease in Japan was a reflection of exposure to polychlorinated biphenyls or to furan impurities. Dr. Fire-

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ne reviewed the history of the contribution of the chick edema factor to our understanding of dioxins. The sleuthing done by the FDA pieced together the story of the large-scale loss of poultry (which happened first in 1957), related it to the use of tallow in poultry feed and, eventually, to the presence of dioxin impurities. Higginbotham et al. first offered a rough approximation of ranking of biological activity of dioxins which has turned out to be strikingly accurate. What has emerged from this meeting is the very wide range of toxicity for the several members of the dioxin family (perhaps as much as 10^5).

This meeting revealed some interesting (and perhaps, ultimately successful) attempts to relate chemical structure to biological activity. Several participants reminded us that to be a successful toxic dioxin, a candidate needs two halogens at the 2 and 3 positions and one at the 7 position; it also needs halogens on both benezene rings. Bromine confers more biological activity than chlorine, and chlorine more than fluorine.

I'll say very little about teratogenesis. It seems to me that a strong case can be made for clearing the air about the mechanism of teratogenesis. Is this an example of acute (embryo) toxicity with a steep dose–response curve and a demonstrable threshold? It's not clear that everyone who reports birth defects is talking about the same phenomenon. Dr. Moore, at this meeting, described some fascinating, postnatal effects of maternal exposure to TCDD through a series of cross-fostering and reciprocal cross-fostering studies of mice. These deserve further attempts at interpretation.

The effects on experimental animals are difficult to summarize completely. However, there are some underlying currents showing through:

(1) there is a variation in susceptibility among species—guinea pigs versus rats and mice; (2) there are striking sex differences; (3) delayed effects are very prominent (liver changes 2 weeks after exposure); (4) the major sites of toxic action appear to be the liver (seen as a variety of changes in liver function), the hematopoietic system (platelet depression, altered platelet function, leucocytosis and hemoconcentration) and the lymphatic system (spleen, thymus and lymph nodes). The atrophy of the thymus and the general lymphoid depletion reported at this meeting were very striking.

As for morphological alterations, the changes in the ultrastructure under the electron microscope are of course most interesting. Perhaps the most significant point is that the morphological alterations tend to follow and confirm the functional changes which were described independently. There seems to be a delay (perhaps on the order of 3 days) between exposure and manifest structural changes. The magnitude of the change is dose-related, and the changes are reversible with time. A particularly fascinating finding was that of multinucleated hepatic cells. Do these represent a stage of attempted regeneration and repair? Alternatively, are they possible precursors of neoplastic change? This conference presented little evidence that dioxins would induce or promote neoplastic changes in tissues.

Patterns of absorption into the organism and of distribution among organs once absorbed are beginning to emerge. Not unexpectedly, water and lipid solubility seems to emerge as a major influence, although clearly not the only one. For tetrachlorodibenzo-*p*-dioxin (high doses in male rats), the amount absorbed via the intestine from an ingested dose appears to be about 70%. The majority of the absorbed dose appears in the feces at a rate of 1–2% day and in the urine at a rate of 0.5% day. A small amount can be detected in the expired air (<0.1% day). The material resident in the organism is characteristically found in the adipose tissue and the liver. It is notably absent from certain other fatty tissues such as those of the central nervous system.

By contrast, for octachlorodibenzo-*p*-dioxin, only 5% achieves absorption. Again, a large share is found in the liver (50%) and in the adipose tissues (12%). Once in

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the liver, this material apparently tends to remain resident for long periods in the liver microsomes.

This meeting served to bring together a remarkable amount of work on the effect of dioxins on cellular enzymes. This was all the more remarkable, as none of this work had even been conceived of two years ago. A number of hepatic enzymes were found to be induced and a few depressed as a result of dioxin exposure. (A general caveat was voiced over what appeared to be unusually high doses of dioxin used in some of the experiments.) The degree of induction was at times striking. The experiments revealed a dose–response relationship. Again, there was an unequivocal sex difference and a characteristic latent period between exposure and induction. Effects were often long-lasting (for example, a persistent threefold increase 38 days after exposure in one experiment). Again, lipid solubility may play a large role.

The meaning of enzyme changes is as yet unclear. There are some striking differences among species. ALA synthetase, whose activity is related to the disease, porphyria, can be induced by dioxin administration in the chick embryo but apparently not in mammals. One has the impression of being very close to some insight into mechanisms yet not close enough. The combination of enzyme induction studies and changes in cellular ultrastructure could prove very helpful.

Where do we stand on our knowledge of biological activity? For furans, we know very little. It seems to me that we still must determine whether Yusho disease was a reflection of PCB exposure or a result of exposure to dibenzofuran or other impurity. For dioxins, we now are better equipped. However, we must now reconcile a number of somewhat paradoxical observations: (1) extraordinarily high degree of biological activity, especially for certain chemical forms (tetrachlorodibenzo-*p*-dioxin was pointed out to be the most potent small molecule toxin known); (2) striking species differences in activity; (3) sex differences; (4) biological activity falls off rapidly with changes in chemical structure; (5) latent period before toxic manifestations; (6) dose-related effects; (7) long-lasting but ultimately reversible effects.

Effects on Wildlife

Understandably there is less work here than one would like. The preliminary work reported by Bowes concerning survey of wildlife is a good model and should be continued. Preliminary results seemed to suggest a very wide variety of chemical species found in the animals examined with an uncertain role for dioxins and furans.

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Chlorinated Dibenzodioxins and Dibenzofurans*

by James Edward Huff† and John S. Wassom‡

The problems and universal concern about chlorinated dibenzodioxin and dibenzofuran compounds were brought to the forefront by the scientific community during the National Institute of Environmental Health Sciences' (NIEHS) Conference on this subject which was held at Research Triangle Park, North Carolina, April 2 and 3, 1973. This idea for this literature collection emanated from that scientific gathering and resulted in this annotated bibliography of 242 references. These references are categorized by year and arranged alphabetically by author. The number of references per year are: 56/1973; 67/1972; 66/1971; 24/1970; 29/1969–1934. Sources searched are summarized in Table 1. Due to the time available to complete this collection, some errors and omissions were inevitable; we apologize for these and hope those using this literature survey will supply us with past, present, and future topical reprints or citation information. We plan to maintain and up-date this file continually.

Most of the nomenclature or terms searched are listed in Table 2. Each particular author, journal, secondary abstracting service, and news copy unfortunately utilizes separate and distinct terminology when reporting on the chlorinated dibenzodioxins and dibenzofurans. As can be seen from Table 2, it is vitally important, therefore, to become thoroughly familiar with the sources being utilized before mounting a massive effort to collate all that is written or reported about a particular compound, series, or class of compounds, or subject. The magnitude of the search effort for this report is self-evident when noting all the necessary terms used.

The original papers were annotated whenever possible; some were gleaned from abstract journals. We did not alter author's remarks or conclusions; the facts are presented in these annotations as they appeared in the literature. As many salient points as possible, due to space limitations, were taken from each paper or report. Many papers and reports were referred to, after the fact, by more recent authors as having dealt intimately with the chlorinated dibenzodioxins and dibenzofurans; these were not included in this bibliography unless the dioxins or furans were mentioned specifically: most of the

* Work supported by Toxicology Information Program, National Library of Medicine; National Institute of Environmental Health Sciences; and the National Cancer Institute under contract with the Union Carbide Corporation.

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‡ Environmental Mutagen Information Center, P.O. Box Y, Bldg. 0224, Environmental Information System Office, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830.

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articles were consulted and read however to determine if these compounds were present. For example, numerous articles reporting on the adverse effects of 2,4,5-T were screened but not included because direct mention of TCDD and other dioxin derivatives was missing.

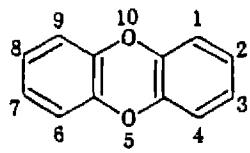
Table 1. Sources and time periods searched.

Source	Period
Multidisciplinary Information Resources	
Bibliography of Agriculture	Vol. 21 (1957) to Vol. 37(1) (1973)
Biological Abstracts	Vol. 31 (1957) to Vol. 55(8) (1973)
Biological and Agricultural Index	Vol. 19 (1964) to Vol. 24 (1970)
Bioresearch Index	Vol. 1 (1965) to Vol. 9(4) (1973)
Chemical Abstracts	Vol. 1 (1907) to Vol. 78(20) (1973)
Chemical-Biological Activities	1965 to 1971
Food Chemical News	Vol. 13 (42) 1972 to Vol. 15(9) (1973)
Health Aspects of Pesticides Abstract Bulletin	Vol. 1 (1966) to Vol. 6(4) (1973)
Health Effects of Environmental Pollutants	Vol. 1 (1972) to Vol. 2(3) (1973)
Index Medicus	Vol. 60 (1956) to Vol. 14(5) (1973)
Pesticide Chemical News	Vol. 1 (1–24) (1973)
Science Citation Index	Vol. 1 (1961) to Vol. 6 (1965); 1966 to 1972
Teratology Lookout	Vol. 3 (1972)
Toxicology Bibliography	Vol. 1 (1968) to Vol. 6(1) (1973)
Specialized Information Centers and Libraries	
Environmental Mutagen Information Center (EMIC)	
Environmental Information System Office (EISO)	
Oak Ridge National Laboratory (ORNL)	
Toxicology Information Response Center (TIRC)	
On-Line Computer Data Bases	
MEDLine	
TOXLine	
Journals	
Ambio	Vol. 1 (1972) to Vol. 2 (1973)
Archives of Environmental Health	Vol. 7 (1963) to Vol. 26(2) (1973)
Bulletin of Environmental Contamination and Toxicology	Vol. 1 (1966) to Vol. 7 (1973)
Clinical Toxicology	Vol. 1(1) (1971) to Vol. 6(1) (1973)
Environment	Vol. 12 (1970) to Vol. 15(3) (1973)
Environmental Health Perspectives	No. 1 (1972) to No. 5 (1973)
Federation Proceedings	Vol. 16(1) (1957) to Vol. 32(4) (1973)
Food and Cosmetic Toxicology	Vol. 1 (1969) to Vol. 10(6) (1972)
Journal of Agriculture and Food Chemistry	Vol. 1 (1953) to Vol. 21(2) (1973)
Journal of the Association of Official Analytical Chemists	Vol. 40 (1957) to Vol. 56 (1973)
Journal of Chromatography	Vol. 1 (1958) to Vol. 73 (1973)
Mutation Research	Vol. 1 (1964) to Vol. 10(6) (1972)
Residue Reviews	Vol. 1 (1962) to Vol. 41 (1972)
Science	Vol. 157 (1967) to Vol. 179 (1972)
Teratology	Vol. 1 (1968) to Vol. 6(2) (1972)
Toxicology and Applied Pharmacology	Vol. 1 (1959) to Vol. 24(3) (1973)

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Table 2. Terms searched.

Nomenclature	*Chemical Abstracts* registry number
	
Dibenzo-*p*-dioxin (diphenylene dioxide) (phendioxin)	262-12-4
2,3-Dichloro-	29446-15-9
2,7-Dichloro-	33857-26-0
2,3,7-Trichloro-	33857-28-2
1,2,3,4-Tetrachloro-	30746-58-8
1,3,6,8-Tetrachloro-	33423-92-6
2,3,6,7-Tetrachloro-	
2,3,7,8-Tetrachloro	1746-01-6
Pentachloro-	36088-22-9
Hexachloro-	34465-46-8
1,2,3,7,8,9-Hexachloro	19408-74-3
Heptachloro-	
1,2,3,4,6,7,8,9-Octachloro-	3268-87-9
Benzo-	
Biphenyl-	
Chlorinated	
Chlorinated dibenzodioxin(s)	
Chlorinated dibenzo-*p*-dioxin(s)	
Dibenzo-	
Dibenzo	
Chlorodibenzo dioxin(s)	
Chlorodibenzo-*p*-dioxin(s)	
Dibenzodioxin(s)	
Dibenzo-*p*-dioxins	
Dibenzo-*p*-dioxins, tetrachloro-	
Dibenzo-*p*-dioxin, other chloro-	
Dioxin	
Halogenated dibenzo dioxin(s)	
Halogenated dibenzo-*p*-dioxin(s)	
OCDD	
Polychlorinated dibenzodioxin	
Polychlorodibenzodioxin	
TCDD	
Dibenzofuran (biphenylene oxide)	132-64-9
3-Chloro-	25074-67-3
2,4-Dichloro-	24478-74-8
1,2,4-Trichloro-	24478-73-7
Tetrachloro-	23076-57-6
1,2,3,4-Tetrachloro-	24478-72-6
Pentachloro-	32076-58-7
Benzo-	
Bipheyl-	
Chlorinated	
Chlorinated dibenzofuran(s)	
Chlorodibenzo-	
Di benzo	
Dibenzo-	
Dibenzofuran	
Furan	
Halogenated dibenzofuran(s)	
Polychlorinated dibenzofurans	

1973

ANONYMOUS. Herbicides better for birds than bacteria. *Food Cosmet. Toxicol.* 11(1): 149–150 (1973).

Teratogenic effects of the 2,4,5-T contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin), are well known and the effect of this compound on liver enzymes resembles that of certain carcinogens. Dioxin has also been associated with mutagenic properties through its possible intercalation with DNA; the observed effects resemble those of acridine.

ANONYMOUS. TCDD residue disappears. *Down To Earth* 28(4): 18 (Spring 1973).

TCDD was not detected (<1 ppm) in 3-ft soil core samples in a sandy area where a total of 947 lb of 2,4,5-T per acre was applied over a 3-yr period. No TCDD residues were found (<0.05 ppm) in bald eagle tissue gathered from 15 states.

ANONYMOUS. 2,4,5-T comes to public attention again. *Pest. Chem. News* 1(19): 6–8 April 11, 1973).

EPA was asked to suspend the remaining uses of 2,4,5-T until the extent, if any, of TCDD-contaminated food chains is determined. TCDD was labeled a cumulative poison and one of the most potent agents of birth defects in animals ever discovered.

ANONYMOUS. Health hazard of dioxins still uncertain. *Chem. Eng. News* 51(16): 12 (April 16, 1973).

A selected summary report of the National Institute of Environmental Health Sciences meeting on chlorinated dibenzodioxins and dibenzofurans held at Research Triangle Park in North Carolina on April 2–3, 1973. The conference as a whole, however, seemed to raise as many questions as it answered. From the data discussed there is no doubt that these contaminants are highly toxic and teratogenic, but there is still some doubt as to how much of an actual hazard they represent to human health.

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ANONYMOUS. New look may be taken at trichlorophenol compounds. *Pest. Chem. News* 1(21): 9–10 April 25, 1973).

Work showing "surprisingly high" levels of dioxin (ppt) in fish caught in Vietnam has placed doubt on earlier government consideration about dioxin residues. It had been previously thought that the dioxin content of 2,4,5-T was so low that there was little opportunity of residues appearing. In addition to 2,4,5-T and silver, any compound using trichlorophenol intermediates "may be suspect".

ANONYMOUS. Correction. *Pest. Chem. News* 1(22): 2 (May 2, 1973).

The National Academy of Science's Advisory Committee on 2,4,5-T recommended that the registration of 2,4,5-T be restored with the following exceptions: (a) a permissible residue of not more than 0.1 ppm 2,4,5-T on edible parts of food products and in water for human consumption and (b) a limit of 0.5 ppm of contamination with TCDD, except that in all formulations to be used around the home and recreational areas, TCDD contamination should be limited to 0.1 ppm.

ANONYMOUS. Recall program, another look at trichlorophenols urged on EPA. *Pest Chem. News* 1(23): 3–5 (May 9, 1973).

The General Accounting Office urged the Environmental Protection Agency (EPA) to implement full-scale recall procedures for suspended pesticides and raised questions on the dioxin content of trichlorophenol herbicides. GAO stated that "because silvex, ronnel, erbon, and hexachlorophene can contain the same level of dioxin as 2,4,5-T and because a safe level of dioxin has not been determined, we believe EPA should establish a standard for dioxin content and prohibit the use of all pesticides containing dioxin in excess of the established standard."

BAUGHMAN, R. W., and MESELSON, M. S. An analytical method for detecting TCDD (dioxin): levels of TCDD in samples from Vietnam. *Environ. Health Perspect.* (No. 5): 27 (1973).

An analytic procedure involving extensive cleanup and mass spectroscopy detects approximately one picogram of TCDD. The method separates TCDD from DDE, PCB's, and other chlorinated hydrocarbon residues.

BOWES, G. W., SIMONEIT, B. R., BURLINGAME, A. L., DE LAPPE, B. W., PEAKALL, D. B., and RISEBROUGH, R. W. The search for chlorinated dibenzofurans and chlorinated dibenzodioxins in wildlife populations showing elevated levels of embryonic death. *Environ. Health Perspect.* (No. 5): 191 (1973).

Embryonic deaths have been recorded in the laboratory among birds treated with PCB. These deaths have been attributed to chlorinated dibenzofuran contaminants. Also, birth defects in wild populations of birds and sea lions are believed caused by chlorinated dibenzodioxins. High-resolution mass spectrometry was used to examine prepared from aborted sea lions and dead embryos of the herring gull and osprey.

CROSBY, D. G., MOILANEN, K. W., and WONG, A. S. Environmental generation and degradation of dibenzodioxins and dibenzofurans. *Environ. Health Perspect.* (No. 5): 259 (1973).

Both the chlorinated dibenzodioxins and dibenzofurans are unstable to light in the presence of organic substrates. Even if generated under environmental conditions, light provides a mechanism for rapid destruction.

CRUMMETT, W. B., and STEHL, R. H. Determination of chlorinated dibenzodioxins and dibenzofurans in various materials. *Environ. Health Perspect.* (No. 5): 15 (1973).

Chlorinated dibenzo-*p*-dioxins and chlorinated dibenzofurans can be determined in chlorinated phenols, chlorinated phenoxy herbicides, ronnel, fat, and combustion products by such analytical techniques as gas chromatography, liquid chromatography, thin-layer chromatography, and gas chromatography–mass spectrometry.

DOUGHERTY, W. H.; COULSTON, F.; GOLBERG, L. Non-teratogenicity of 2,4,5-trichlorophenoxyacetic acid in monkeys (*Macaca mulatta*). Twelfth Annual Meeting, Society of Toxicology, New York, NY (March 18–22, 1973), Abstract 9, p. 7.

Technical grade 2,4,5-T which contained less than 0.05 ppm 2,3,7,8-tetrachlorodibenzo-*p*-dioxin was administered orally to forty pregnant Rhesus monkeys daily from day 22 through day 38 of gestation. Dose levels used in the experiment were 0.05, 1.0, and 10.0 mg/kg. Hematology, clinical chemistry, and urinalysis data were recorded for all females before and at various times following treatment until parturition; no toxicity was observed. Examination of live born infants revealed no terata.

ENVIRONMENTAL PROTECTION AGENCY, PUBLICATIONS AND INFORMATION SECTION. Toxicology and pharmacology of 2,4,5-T

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(includes dioxins). Bibliography Number 73–04 (February 1973).

A bibliographic listing of 62 references pertaining to 2,4,5-T and dioxins.

ENVIRONMENTAL PROTECTION AGENCY, PUBLICATIONS AND INFORMATION SECTION. Chemistry and residues of 2,4,5-T (includes dioxins). Bibliography Number 73–05 (February 1973).

A bibliographic collection of 58 references on 2,4,5-T containing a limited number of citations on dioxins.

ENVIRONMENTAL PROTECTION AGENCY AND INSTITUTE OF RURAL ENVIRONMENTAL HEALTH. Environmental chemicals: human and animal health. Fort Collins, CO (July 23–27, 1973).

The Institute of Rural Environmental Health, Colorado State University, and the Office of Pesticide Programs, U. S. Environmental Protection Agency, conducted a one-week course on environmental problems, contaminants, toxicants, and chemicals (including dioxins); and human and animal health problems; and poisoning.

FIRESTONE, D. Etiology of chick edema disease. *Environ. Health Perspect.* (No. 5): 59 (1973).

Early work indicated that chick edema factors (CEF) were chlorinated aromatic compounds; later, the compounds were shown to belong to a family of chlorodibenzo-*p*-dioxins. Further investigation showed that (a) chlorophenols were precursors of the chlorodioxins and (b) chlorodioxins and related compounds are commonly present as minor components in commercial chlorophenols. Characteristic chick edema disease symptoms include excessive fluid in the heart sac and abdominal cavity followed by high mortality starting in the third week.

FOWLER, B., LUCIER, G., BROWN, H., and MCDANIEL, O. Ultrastructural changes in rat liver cells following a single injection of TCDD. *Environ. Health Perspect.* (No. 5): 141 (1973).

Ultrastructure changes in rat liver microsomes and mitochondria were examined at various intervals from 1 to 30 days following a single TCDD injection of 0, 5, or 25 ug/kg. No histologic difference was noted between groups. Observed changes included: proliferation of smooth endoplasmic reticulum (SER), mild increase in rough endoplasmic reticulum (RER), moderate swelling of mitochondria: at 7 days, large aggregates of SER, massive amounts of RER, and small numbers of moderately swollen mitochondria were seen from the 25 mg/kg dosed rats.

GREIG, J. B. Biochemical toxicity of TCDD in rat liver. *Environ. Health Perspect.* (No. 5): 211 (1973).

The persistent toxic effect of 2,3,7,8-tetrachlorodibenzodioxin in rats is evidenced by death as long as 15 weeks after a single oral dose. Alterations, however, in liver constitution (microsomes and cytochrome P-450) and drug metabolism (zoxazolamine and hexobarbital) occurred within 24 hr after dosing.

GUPTA, B., VOS, J., MOORE, J., ZINKL, J., and BULLOCK, B. C. Pathologic effects of TCDD in laboratory animals. *Environ. Health Perspect.* (No. 5): 125 (1973).

Gross and microscopic examinations were performed on rats, guinea pigs, and mice treated with TCDD. A spectrum of dose ranges and schedules were used. Lymphoid organs (thymus, spleen, and lymph nodes) were affected consistently. Thymus atrophy (dose related decrease in weight) was found to be a sensitive index of TCDD exposure. The most severe hepatic effects were seen in rats that received a lethal dose of TCDD. The magnitude of the degenerative and necrotic liver changes were diminished in guinea pigs and mice.

HARRIS, M., MOORE, J., and VOS, J. General biological effects of TCDD in laboratory animals. *Environ. Health Perspect.* (No. 5): 101 (1973).

Albino rats were grouped and treated with single oral doses of 0, 5, 25, 50, or 100 μg/kg TCDD in an acetone–corn oil mixture. Animals that eventually died continued to lose weight until death while survivors exhibited a depressed weight gain. Ruffled hair coat, hunched posture, inactivity, and jaundice were the overt signs seen in the high dose group. Daily oral administration of 10 μg/kg caused death in 15/16 rats with a mean time of 21.8 days. Death resulted in 9/10 female guinea pigs after receiving an oral dose of 3 μg/kg; the mean survival time was 18.1 days. A single oral dose of 1, 10, or 50 μg/kg to adult mice had no effect on appearance or body weight.

HUTZINGER, O., SAFE, S., WENTZELL, B. R., and ZITKO, V. Photochemical degradation of di- and octachlorodibenzofurans. *Environ. Health Perspect.* (No. 5): 267 (1973).

Irradiation of 2,8-dichlorodibenzofuran (low chlorine content) and octachlorodibenzofuran (high chlorine content) in hexane and methanol caused

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decomposition to compounds which are formed by reductive dechlorination as well as polar substances.

HWANG, S. W. Effect of TCDD on biliary excretion of indocyanine green. *Environ. Health Perspect* (No. 5) : 227 (1973).

Bile flow and biliary excretion of indocyanine green (ICG) in male rats 1, 7, and 16 days after receiving a single oral dose of 5 or 25 μg/kg TCDD. Bile flow rate increased at day 1 through day 16. During a 20-min. collection period, both the concentration and total ICG excreted in bile decreased. ICG disappearance rate decreased with time. These effects were dose related. In contrast, the 5 μg/kg dosed rats accumulated more ICG in liver.

JENSEN, S., and RENBERG, L. Various chlorinated dimers present in several technical chlorophenols used as fungicides. *Environ. Health Perspect.* (No. 5): 37 (1973).

The presence of 2,3,7,8-tetrachlorodibenzo-p-dioxin in 2,4,5-trichlorophenoxy acid esters originates from 2,4,5-trichlorophenol during the manufacturing process. Dimerization occurs when the phenol is produced by the action of alkali on tetrachlorobenzene. All products originating from alkali-treated chlorinated benzenes logically may contain chlorinated dibenzo-p-dioxins. Dimers in pentachlorophenol and in 2,4,6-tri- and 2,3,4,6-tetrachlorophenols from direct chlorination of phenol were presented.

JOHNSON, R. L., GEHRING, P. J., and KOCIBA, R.J. Chlorinated dibenzodioxins and pentachlorophenol. *Environ. Health Perspect.* (No. 5) : 171 (1973).

Pentachlorophenol enjoys widespread use as a wood preservative. Commercial grades have been found to contain up to 2500 ppm chlorinated dibenzo-p-dioxins. The predominant dioxin is octachlorodibenzo-p-dioxin, one of the least toxic members. Evaluating pentachlorophenol toxicity in animals revealed that some untoward effects (chloracne, chick edema disease, and histopathologic alterations) were caused by chlorinated dibenzo-p-dioxin content. Purified pentachlorophenol did not produce these effects. A new procedure was capable of producing pentachlorophenol containing lowered concentrations of chlorinated dibenzo-p-dioxin and devoid of dioxin-like toxic effects.

KEARNEY, P. C., WOOLSON, E. A. ISENSEE, A. R., and HELLING, C. S. Tetrachlorodibenzodioxin in the environment: sources, fate, and decontamination. *Environ. Health Perspect.* (No. 5): 273 (1973).

TCDD does not leach in soils, does not reside in the economic portion of plants growing in contaminated soil, degrades to about 50% after 1 yr in soils, and does not result from microbial or chemical condensation of 2,4,5-trichlorophenol in soil.

KENDE, A. S., and WADE, J. J., Synthesis of new steric and electronic analogs of 2, 3,7,8-tetrachlorodibenzo-p-dioxin. *Environ. Health Perspect.* (No. 5): 49 (1973).

Structural-activity relationships for a series of TCDD analogs were accomplished emphasizing chemical studies in an attempt to distinguish steric from electronic requirements for toxicity. Catechol condensation was used to explore the scope and limitations of polyhalobenzene electrophiles.

KING, M. E., and SHEFNER, A. M., Carcinogenesis bioassay of chlorinated dibenzodioxins and related chemicals. *Environ. Health Perspect.* (No. 5): 163 (1973).

Chlorinated dibenzodioxins were dissolved in acetone and applied to the backs of mice three times a week to assess the activity of dioxins as complete carcinogens and/or promoting agents. Octachlorodioxin caused skin tumor formation in only one female mouse. No other dioxin produced papillomas.

LANGER, H. G. Formation of dibenzodioxins and other condensation products from chlorinated phenols and derivatives. *Environ. Health Perspect.* (No. 5): 3 (1973).

Chlorodioxins are formed in a two-step condensation reaction from ortho-substituted halophenoxy radicals or anions. Reaction of chlorine with pentachlorophenol at elevated temperature proceeds by radicals; anionic condensation products result from strongly exothermic reactions of alkali metal salts of chlorinated phenols above 300° C. Reaction product distribution depends on the total number of halogen substituents, the crystal lattice arrangement of the molecule, steric effects, and an electronic effect. Dioxin formation was the major condensation product only for sodium pentachlorophenate.

LUCIER, G. W., MCDANIEL, O. S., FOWLER, B. A., FAEDER, E., HOOK, G., and SONAWANE, B. R. Studies on TCDD-induced changes in rat liver microsomal and mitochondrial enzymes. *Environ. Health Perspect.* (No. 5) : 199 (1973).

A single oral dose of 5 or 25 μg/kg was administered to male rats and time-course measurements were made on some hepatic microsomal and mitochondrial enzymes. Cytochrome P-450 and b-5 contents were increased, hydroxylation of aniline

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as induced, microsomal protein contents were increased; aminopyrine-demethylation rates were decreased, and most strikingly UDP glucuronyltransferase was increased about 8-fold.

MARTIN, R. L., PORTER, M. L., POMERANTZ, I. H. Studies on the formation potential and presence of chlorinated dibenzofurans in chlorinated biphenyls. NIEHS conference, April 2–3, 1973.

Chlorinated dibenzofurans may arise by photochemical alteration of chlorinated biphenyls under appropriate conditions; this did not occur with pentachlorobiphenyl or 2,2'-dichlorobiphenyl.

MATSUMURA, F., and BENEZET, H. J. Studies on the bioaccumulation and microbial degradation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Environ. Health Perspect.* (No. 5) : 253 (1973).

Most organisms capable of degrading other chlorinated hydrocarbons showed no ability to metabolize TCDD; a few exhibited a limited degree of TCDD-metabolizing activity. TCDD leached from sand to organic soil much less than did DDT. With pesticide-coated sand in aquaria containing various organisms, TCDD had the lowest biologic accumulation and affinity.

MESELSON, S. Vietnam dioxin contamination. Center for Short-Lived Phenomena, Event 51–73, No. 1611, Smithsonian Institute, Cambridge, Mass. (April 19, 1973).

Various fish and shellfish collected in 1970 from the Dong Nai and Saigon Rivers and along the Can Guo Coast contained dioxin; catfish had the highest concentration.

MESELSON, S. Vietnam dioxin contamination. Center for Short-Lived Phenomena, Event 51–73, No. 1627, Smithsonian Institute, Cambridge, Mass. (8 May 1973).

Samples of fish and crustaceans caught in Vietnam in September 1970 were analyzed for dioxin content by using mass spectrometry. Dioxin concentrations ranged from 18 to 814 ppt; Dong Nai river carp averaged 540 ppt dioxin.

MILLER, R. A., NORRIS, L. A., and HAWKES, C. L. Acute and chronic toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) in aquatic organisms. *Environ. Health Perspect.* (No. 5) : 177 (1973).

Guppies and coho salmon fingerlings were exposed to dioxin concentrations ranging from 0.056 ppt to more than 0.2 ppb for 24, 48, and 96 hrs. The initial concentration was found to be more important in causing death than duration of exposure. The coho salmon fingerlings threshold response level for all exposure periods was between 0.056 and 0.56 ppt dioxin. Mosquito larvae, oligochaete worms, and pulminator snails were maintained in water initially dosed with 0.2 ppb dioxin. These aquatic organisms were less sensitive than fish.

MOORE, J.; GUPTA, B.; VOS, J.; ZINKL, J. Postnatal effects of maternal exposure to TCDD. *Environ. Health Perspect.* (No. 5) : 81 (1973).

Maternal exposure of C5731/6 mice to TCDD caused dose-related variations in fetal kidney maturation and development. Thymuses were reduced in size; cystic kidneys developed. Mean body weights and thymus and spleen weights (absolute and relative) were reduced in litters whose mothers received 10 μg/kg TCDD; the 3 μg/kg dose group exhibited no weight deviations. Kidney effects were seen at both dose levels.

NEUBERT, D., ZENS, P., and ROTHENWALLNER, A. Survey of the teratogenic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in mammalian species. *Environ. Health Perspect.* (No. 5) : 67 (1973).

The frequency of cleft palate induction was used as a criterion of the teratogenic effects of TCDD. Dose-response relationships and potentiating effects of TCDD with other agents were presented.

NIEHS Conference on Chlorinated Dibenzodioxins and Dibenzofurans. National Institute of Environmental Health Sciences, Research Triangle Park, N.C. (April 2–3, 1973).

The two-way conference reviewed critically and summarized the world literature and research activities on the chlorinated derivatives of dibenzodioxin and dibenzofuran. Approximately 35 papers were presented on all aspects ranging from chemical nature to ultimate biologic effects. More than 110 scientists were in attendance. The proceedings are given in full in *Environmental Health Perspectives*, Experimental No. 5, (1973). (this issue).

NORBACK, D. H., and ENGBLOM, J. F. Chlorinated dibenzo-*p*-dioxin distribution within rat tissues and subfractions of the liver. *Fed. Proc.* 32(3) : 236 (1973) Abstract 138.

Radioactivity from orally intubated ^{36}Cl-labeled octachlorodibenzo-*p*-dioxin in rats was confined to the liver, adipose tissue, and skin after 7 weeks on a control diet; concentrations were about 20% that in tissues of rats after 21 days.

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NORBACK, D. H., ENGBLOM, J. F., and ALLEN, J. R. Chlorinated dibenzo-*p*-dioxin distribution within rat tissues and subfractions of the liver. *Environ. Health Perspect.* (No. 5): 233 (1973).

Male rats received daily for 21 days 100 μg ^{36}Cl-labeled octachlorodibenzo-*p*-dioxin by gastric intubation. Feces contained 95% of the total dose and urine 4%. Significant levels were found in the kidneys, heart, and serum. The liver contained the highest concentration per unit weight; adipose tissue had 1/3 that of the liver. Microsomes (rough and smooth fractions) had 95% of the liver radioactivity. Urine radioactivity resided in the lipid fraction.

NOVICK, S. Dioxin. *Environment* 15(4): 23–24 (May 1973).

A news item reports the detection of dioxin in fish and shellfish used for food in Vietnam.

PLIMMER, J. R., RUTH, J. M., and WOOLSON, E. A. Mass spectrometric identification of the hepta- and octa-chlorinated dibenzo-*p*-dioxins and dibenzofurans in technical pentachlorophenol. *J. Agr. Food Chem.* 21(1): 90–93 (1973).

The presence of contaminant dioxins and dibenzofurans in some samples of technical pentachlorophenol were confirmed by using mass spectrometry. Three samples collected in 1970 contained hexachlorodibenzo-*p*-dioxin (0.5 to 37 ppmw) and heptachlorodibenzo-*p*-dioxin (90 to 135 ppmw). It was reemphasized that high-resolution spectra do not provide chlorine orientation information.

PLIMMER, J. R. Technical pentachlorophenols—origin and analysis of base-insoluble contaminants. *Environ. Health Perspect.* (No. 5): 41 (1973).

Fats used as feed additives from hides preserved with technical pentachlorophenol have been implicated as a source of chick edema factor. Polychlorinated dibenzodioxins and dibenzofurans have been identified in the neutral fractions of pentachlorophenol by gas chromatography, mass spectrometry, and a combination of these two.

POHLAND, A. E., YANG, G. C., and BROWN, N. Analytical and confirmative techniques for dibenzo-*p*-dioxins based upon their cation radicals. *Environ. Health Perspect.* (No. 5): 9 (1973).

Chlorinated dibenzo-*p*-dioxins form cation radicals when dissolved in strong acids such as trifluoromethane sulfonic acid, in the presence of ultraviolet light, or an oxidizing agent like potassium nitrate. These cation radicals are quantified by using electron spin resonance and visible spectroscopic techniques. A general bathochromic shift was observed with increasing chlorine content. These shifts were dependent upon the position of the chlorine atoms.

POLAND, A. P., and GLOVER, E. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: A potent inducer of δ-aminolevulinic acid synthetase. *Science* 179(4072): 476–677 (Feb. 2, 1973).

As little as 4.66×10^{-12} mole (1.5 ng) of TCDD per egg induced hepatic-aminolvulinic acid synthetase (ALAS) activity in the chick embryo. Enzyme induction was dose-related and prolonged in time: 70% of the maximum induced activity was present 5 days after a single 150 ng dose. TCDD was linked to an outbreak of porphyria cutanea tarda where 2,4,5-T was synthesized and manufactured. At least three of the 2,3,7, and 8 positions on the ring must be occupied to induce ALAS.

POLAND, A. P., and GLOVER, E. Studies on the mechanism of action of the halogenated dibenzo-*p*-dioxins. *Environ. Health Perspect.* (No. 5): 245 (1973).

Aminolevulinic acid synthetase (ALAS) was stimulated by TCDD in the chick embryo liver: 4.66×10^{-12} mole/egg (1.5 ng) caused doubling of ALAS activity and 1.55×10^{-9} mole/egg (0.5 μg) caused a 35-fold stimulation of enzyme activity. A single dose of TCDD stimulates hepatic aryl hydrocarbon hydroxylase (AHH) and cytochrome P-450 for 35 days and more in the rat. AHH activity was induced in chick embryo liver. It is suggested that the chemically inert parent compound is not the toxic moiety, but that a highly reactive intermediate causes cell damage.

SCHWETZ, B. A., NORRIS, J. M., SPARSCHU, G. L., ROWE, V. K., GEHRING, P. J., EMERSON, J. L.; GERBIG, C. G. Chlorodibenzo-*p*-dioxin toxicology. *Environ. Health Perspect.* (No. 5): 87 (1973).

2,7-Dichlorodibenzo-(DCDD), 2,3,7,8-tetrachlorodibenzo-(TCDD), hexachlorodibenzo-(HCDD), and octachlorodibenzo-*p*-dioxin-(OCDD) were evaluated toxicology. TCDD and HCDD were acnegenic, embryo toxic (TCDD markedly so), teratogenic, and positive for chick edema factor (CEF). DCDD and OCDD were negative for acnegenicity, teratogenicity, and CEF; OCDD was embryotoxic, while DCDD was not. The lethal dose range for DCDD, TCDD, HCDD, and OCDD was g/kg, μg/kg, mg/kg, and g/kg, respectively.

STAPLEY, D. Herbicides: AAAS study finds dioxin in Vietnamese fish. *Science* 180 (4083): 285–286 (April 20, 1973).

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ish and shellfish from areas of South Vietnam that were heavily sprayed during the U. S. defoliation campaign contained significant quantities of dioxin. This is a news report on the data R. Baughman and M. Meselson presented to the NIEHS Conference on chlorinated dibenzodioxins and dibenzodifurans.

SHAPLEY, D. Herbicides: Agent Orange stockpile may go to the South Americans. *Science* 180(4081): 43–45 (April 6, 1973).

The U. S. Air Force has a surplus stockpile of 2,338,900 gal of Agent Orange (50% 2,4,5-T and 50% 2,4-D); some of these mixtures contain as much as 28 times the maximum acceptable safety limit of dioxin. Presently, dioxin concentrations permissible for new herbicides are 0.1 ppm and 0.5 ppm for stocks already manufactured.

VOS, J. G., MOORE, J. A., and ZINKL, J. Effect of TCDD on the immune system of laboratory animals. *Environ. Health Perspect.* (No. 5): 149 (1973).

TCDD at sublethal dose levels caused atrophy of the thymus, suppressed the cell-mediated immunity in both guinea pigs and mice, but did not affect the humoral immunity in guinea pigs.

WALDBOTT, G. L. *Effects of Environmental Pollutants*. C. V. Mosby Co., St. Louis, Mo., 1973.

Dioxin compounds are listed with other environmental pollutants as examples of airborne pollutants. These substances are contaminants of the popular weed killer, 2,4,5-T. Dioxins caused death and gastrointestinal hemorrhage in rat fetuses when mothers were treated with doses of 0.125 to 8 μg. The mutagenic effects are due to its intercalation with DNA. Dibenzofurans were implicated as being responsible for some of the toxic effects attributed to PCBs.

WEBBER, T. J. N., and BOX, D. G. The examination of tetrachlorvinphos and its formulations for the presence of tetrachlorodibenzo-*p*-dioxins by a gas-liquid chromatographic method. *Analyst* 98: 181–189 (1973).

A gas–liquid chromatographic analytical method was developed for use in detecting tetrachlorodibenzo-*p*-dioxin impurities in samples of the insecticide tetrachlorvinphos and its formulations. Tetrachlorvinphos is the Z- or *trans*-styrene isomer of 2-chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate. Sequential use of silica gel and aluminum oxide column chromatography followed by concentrated sulfuric acid treatment of the resultant eluate made it possible to detect TCDD levels down to a limit of 0.025 ppm with electron-capture detection. Tetrachlorodibenzo-*p*-dioxins were not detected in any of the 21 tetrachlorvinphos samples or any of its formulations selected for analysis either before or after accelerated storage at 55°C for 2 weeks.

WEISSBERG, J., and ZINKL, J. Effects of TCDD upon hemostasis and hematologic function in the rat. *Environ. Health Perspect.* (No. 5): 119 (1973).

Daily oral doses of 10 μg/kg TCDD to female rats for 10 and 14 days caused nonspecific alterations of hematopoietic function, thrombocytopenia, thrombocytoasthenia, and derangements in blood coagulation. Platelet aggregation, bleeding time, and platelet factor III activity were normal, but clot retraction was abnormal.

WILSON, J. G. Teratological potential of 2,4,5–T. *Down to Earth* 28(4): 14–17 (Spring 1973).

A chronology of the hazards of 2,4,5-T is presented. The report issued by the President's Science Advisory Committee appointed to study the 2,4,5-T question served as the basis for this review. The dioxin contaminant of 2,4,5-T, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, is discussed briefly as a toxicogen.

WOODS, J. S. Studies of the effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on mammalian hepatic δ-aminolevulinic acid synthetase. *Environ. Health Perspect.* (No. 5): 221 (1973).

No differences in hepatic δ-aminolevulinic acid synthetase activity were seen in control rats and those receiving orally 5, 25, or 100 μg/kg TCDD for up to 30 days. Mice and guinea pigs were also nonreactive. Thus, TCDD is not porphyrogenic in mammals, even at several times the LD_{50} dose levels.

ZINKL, J., MOORE, J. A., VOS, J. G., and GUPTA, B. N. Hematologic and clinical chemical effects of 2,3,7,8-tetrachlorodibenzodioxin in laboratory animals. *Environ. Health Perspect.* (No. 5): 111 (1973).

TCDD-induced changes observed in female rats after 10 days treatment with 10 μg/kg and 17 days at 1 μg/kg were increases in serum glutamic oxaloacetic transaminase. After 13 days treatment with 10 μg/kg serum glutamic pyruvate transaminase was increased. Platelet depression was observed after 10 days at all dose levels.

ZITKO, V., WILDISH, D. J.; HUTZINGER, O., and CHOI, P. M. K. Acute and chronic oral

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toxicity of chlorinated dibenzofurans to salmonid fishes. *Environ. Health Perspect.* (No. 5) : 187 (1973).

Dry fish food was fed to juvenile Atlantic salmon contaminated with a mixture of 2.7 μg/g di-, 5,7 tri-, 2.8 tetra-, and 9.1 octachlorodibenzofuran. Median mortality was 12 ± 30 days. Only octachlorodibenzofuran was found in tissues of dead fish (0.03 μg/g in muscle and 0.2 μg/g in the gut). Fish surviving 140 days feeding contained corresponding values of 0.01 and 0.02 μg/g.

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ABELSON, P. H. Pollution by organic chemicals. *Our Chemical Environment*, J. C. Giddings and M. D. Monroe, Eds., Canfield Press, San Francisco, 1972, Chap. 28, pp. 183–184.

The most toxic chlorine-containing compound known is 2,3,7,8-tetrachlorodibenzodioxine ($C_{12}H_4O_2Cl_4$), often called dioxin. The acute oral LD_{50} dose in male guinea pigs is about 10^{-4} g/kg. In spite of its toxicity, the behavior of dioxin in the food chain has not been worked out.

ANONYMOUS. TCDD residues disappear. *Agr. Res.* 21(4) : 6 (1972).

The 2,4,5-T contaminant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), can be formed during synthesis of some chlorinated phenols if high temperatures are used.

ANONYMOUS. Research heightens concern over PCB's. *Chem. Eng. News* 50(10) : 27–28 (1972).

Certain polychlorinated biphenyls may contain traces of tetrachlorodibenzofuran. A contaminant found in PCB's, not conclusively identified, has the same mass spectrum as tetrachloro-p-dibenzofuran.

ANONYMOUS. Dioxin with a bang! *Food Cosmet. Toxicol.* 10(1) : 110–111 (1972).

During a manufacturing plant explosion, dioxin was formed by the interaction of sodium 2,4,5-trichlorophenate molecules under the influence of the exothermic decomposition of sodium 2-hydroxyethoxide.

ANONYMOUS. . . . But 2,4,5–T is in the dock again. *Food Cosmet. Toxicol.* 10(5) : 722 (1972).

Negative teratogenic results have been reported for rats and rabbits treated with 2,4,5-T samples with very minute levels of dioxin impurity. Teratogenic effects have been detected, however, in three mouse strains treated with 2,4,5-T containing as little as 0.05 or 0.5 ppm dioxin.

ANONYMOUS. 2,4,5–T and dioxins accused of teratogenicity. *Food Chem. News* 13 (43) : 24–27 (Jan. 17, 1972).

Responding to challenges on 2,4,5-T cancellation, EPA concentrated its replies on lack of proof that 2,4,5-T and contaminants are not teratogens. The dose-response curves for 2,4,5-T and dioxin (TCDD) have not been determined, and the possibility of no effect levels is only a matter of conjecture.

ANONYMOUS. FDA annual report shows increase in enforcement. *Food Chem. News* 13(47) : 20 (Feb. 14, 1972).

In the herbicide section of its 1971 annual report, FDA said the toxicity of 2,4,5-T is chiefly due to dioxins.

ANONYMOUS. Senate settlement expected on pesticides bill. *Food Chem. News* 14 (27) : 53–54 (Sept. 25, 1972).

A wide range of teratogenic dioxins can be produced both in the manufacture of 2,4,5-T and during pyrolysis (incomplete combustion).

BOER, F. P., NEUMAN, M. A., and ANILINE, O. 2,8-Dichlorodibenzo-*p*-dioxin. *Acta Crystallogr.* B28(9) : 2878–2880 (1972).

Crystals of 2,8-dichlorodibenzo-p-dioxin are orthorhombic; molecules are slightly nonplanar with an unusual packing arrangement.

BOER, F. P., and NORTH, P. P. Crystal and molecular structure of 2,7-dichlorodibenzo-*p*-dioxin. *Acta Crystallogr.* B28(5) : 1613–1616 (1972).

Three-dimensional single-crystal x-ray diffraction data revealed the crystal and molecular structure of 2,7-dichlorodibenzo-p-dioxin. The C–Cl bond distance is 1.742 A, the C–O distances are 1.380 and 1.382, and the 6 C–C distances range between 1.370 and 1.397. The C–O–C angle in the heterocyclic ring is 116.3°.

BOER, F. P., VAN REMOORTERE, F. P., and MUELDER, W. W. Preparation and structure of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 2,7-dichlorodibenzo-*p*-dioxin. *J. Amer. Chem. Soc.* 94(3) : 1006–1007 (1972).

The preparation, isolation, and isometric structures of 2,3,7,8-tetrachloro and 2,7-dichlorodibenzo-p-dioxin are described.

BOER, F. P., VAN REMOORTERE, F. P., NORTH, P. P., and NEUMAN, M. A. Crystal and molecular structure of 2,3,7,8-tetrach-

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lorodibenzo-*p*-dioxin. *Acta Crystallogr.* B28(4): 1023–1029 (1972).

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was studied by using three-dimensional single-crystal x-ray diffraction. The four unique C–Cl distances range from 1.726 to 1.730 A, the 4 C–O distances from 1.377 to 1.379, and the 12 C–C bonds are all between 1.374 and 1.388. The C–O–C angles are 115.6° and 115.8°.

BRENNER, K. S., MULLER, K., and SATTEL, P. Detection and determination of 2,3,7,8-tetrachloro dibenzo-*p*-dioxin in chloro-substituted phenoxyalkane acids. *J. Chromatogr.* 64: 39–48 (1972).

Dioxin separation from the herbicide phenoxyalkane acids was accomplished by extractive distillation of the potassium salts with *n*-hexane in the Bleidner apparatus. Quantitative determination of the hexane extracts for dioxin was done by gas chromatography. Sensitivity was about 0.1 ppm.

BROOKS, G. T. Pesticides in Britain. In: Environmental Toxicology of Pesticides, F. Matsumura, G. M. Boush, and T. Misato, Eds., Academic Press, New York, 1972, pp. 61–114.

The reported teratogenic effects of 2,4,5-T in animals and the isolation of the highly toxic contaminant tetrachlorodibenzo-p-dioxin are cautionary. 2,4,5-T has been used in Europe for nearly 15 years without evidence of ill effects. More studies are needed in comparative detoxication between mammals, birds, fishes, and insects.

BROWNRIGG, J. T., EASTWOOD, D., and HORNIG, A. W. Identification of polychlorinated biphenyls in the presence of DDT-type compounds. Office of Research and Monitoring, U.S. Environmental Protection Agency, Washington, DC (Oct. 1972), EPA–R2–72–004.

Low temperature (77°K) luminescence techniques could in principle be applied to a wide variety of compounds including the highly toxic chlorinated dibenzofurans and dibenzo-*p*-dioxins.

BUU-HOI, N. P., CHANH, P-H., SESQUE, G., AZUM-GELADE, M. C., and SAINT-RUF, G. Enzymatic functions as targets of the toxicity of "dioxin" (2,3,7,8-tetrachlorodibenzo-*p*-dioxin). *Naturwiss.* 59(4): 173–174 (1972).

Following a single I.P dose of 10 mg/kg dioxin to rats, deep pertubations in several enzymatic systems were observed, i.e., a decrease in cholinesterase an increase in serum glutamic oxaloacetic acid transaminase. Effects in homeostasis indicate the liver is one of the main targets for dioxin intoxication.

BUU-HOI, N. P., CHANH, P-H., SESQUE, G., AZUM-GELADE, M.C.; Saint-Ruf, G. Organs as targets of "dioxin" (2,3,7,8-tetrachlorodibenzo-*p*-dioxin). *Naturwiss.* 59(4): 174–175 (1972).

Organ damage was found along with weight loss and hematologic effects following IP injection of 1 and 10 mg/kg dioxin to rats. Ten days after treatment, damage was observed to the liver, thymus, heart. Less damage was observed in the lungs and blood cells.

BUU-HOI, N. P., SAINT-RUF, G., and MANGANE, M. Fragmentation of dibenzo-*p*-dioxin and its derivatives under electron impact. *J. Heterocycl. Chem.* 9(3): 691–693 (1972).

Mass spectra were reported and discussed on five chloro derivatives of dibenzo-*p*-dioxin: 2,7-dichloro-, 1,6-dichloro-, 1,3,6-trichloro-, 2,3,7,8-tetrachloro-, and 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin.

CHEN, J. T. Infrared studies of chlorinated dibenzo-*p*-dioxins and structurally related compounds. Division of Pesticide Chemistry, 164th National Meeting of the American Chemical Society, New York, Aug. 27–Sept. 1, 1972, Abstract No. 12.

Data are reported on the reference infrared spectra of 24 chlorinated dibenzo-*p*-dioxins and the observed characteristic frequencies are tabulated.

COURTNEY, K. D. The teratogenic evaluation of the herbicide 2,4,5-T and dioxin. Item 4.0785, Page 1–632. Part 4. Pesticides, Environmental Protection Research Catalog, Research Information Division, U.S. EPA, Washington, D.C., Jan. 1972.

Phenoxyacetic acid herbicides and dioxins are being evaluated for teratogenic and perinatal toxic effects.

CROSBY, D. G., MOILANEN, K. W., NAKAGAWA, M., and WONG, A. S. Photonucleophilic reactions of pesticides. *Environmental Toxicology of Pesticides*, F. Matsumura, G. M. Bousch, and T. Misato, Eds., Academic Press, New York, 1972, pp. 423–433.

Photonucleophilic displacement of chloride by chlorophenoxide ion in *o*-chlorophenols introduced

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the possibility of the photochemical generation of chlorinated dibenzo-*p*-dioxins. Sunlight wavelengths exposure to sodium pentachlorophenate yielded 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin; no 2,3,7,8-TCDD resulted from irradiated 2,4,5,-T or 2,4,5-trichlorophenol.

CUNNINGHAM, H. M., and WILLIAMS, D. T. Effect of tetrachlorodibenzo-*p*-dioxin on growth rate and the synthesis of lipids and proteins in rats. *Bull. Environ. Contam. Toxicol.* 7(1) : 45–51 (1972).

Protein concentration in the rat liver was slightly reduced 3 days after dioxin treatment. This decrease was accompanied by a significant increase in ^{14}C-leucine incorporation into liver proteins. The lowest single dose of dioxin that caused an increase in rat liver weight was 0.1 μg/kg

CURLEY, A., BURSE, V. W., and JENNINGS, R. Metabolite or contaminant of Aroclor 1254® found in rat urine. Division of Pesticide Chemistry, 163rd National Meeting of the American Chemical Society, Boston, April 9–14, 1972, Abstract No. 5.

Mass spectra of urine collected from rats on dietary levels of 100 or 500 ppm Aroclor 1254 for intervals up to eight months indicated the presence of a molecular ion at m/e 304 and the characteristic isotopic cluster of 4 chlorine atoms. Others have shown the presence of tetra and pentadibenzofurans (mass numbers 304 and 338) as contaminants in the foreign products.

ENVIRONMENTAL PROTECTION AGENCY, PUBLICATIONS AND INFORMATION SECTION. Chemistry and residues of 2,4,5-T (includes dioxins). Bibliography Number 72–59 (1972).

A reference list of 43 references is given.

ENVIRONMENTAL PROTECTION AGENCY, PUBLICATIONS AND INFORMATION SECTION. Toxicology and pharmacology of 2,4,5-T (includes dioxins). Bibliography Number 72–58 (1972).

A list of 75 references is given.

EPSTEIN, S. S. Environmental pathology. A review. *Amer. J. Pathol.* 66(2) : 352–373 (1972).

Toxicity testing must not be confined to the test agent *per se*, but should be extended to its chemical and metabolic derivatives, its pyrolytic and degradation products and its contaminants and reaction products, especially when various derivatives or degradation products are of toxicologic or environmental consequence. Dioxin pyrolytic products in phenoxy herbicides are illustrative.

FIRESTONE, D., RESS, J., BROWN, N. L., BARRON, R. P., and DAMICO, J. N. Determination of polychlorodibenzo-*p*-dioxins and related compounds in commercial chlorophenols. *J. Assoc. Offic. Anal. Chem.* 55(1) : 85–92 (1972).

Twenty-one commercial chlorophenols were dissolved separately in aqueous alkali, extracted with petroleum ether, fractionated on an alumina column, and examined by electron capture gas chromatography and combined gas chromatography–mass spectrometry. The 2,3,7,8-tetrachlorodioxin was found in 3 of 6 samples of 2,4,5-trichlorophenol but not in any of the 11 samples of tetra- and pentachlorophenol. Hexachlorodioxin, present in all 8 pentachlorophenols tested, ranged from 0.17 to 39 ppm. Hexa-, hepta-, and octachlorodioxins and chlorofurans were present in most of the tetra- and pentachlorophenols.

FISHBEIN, L. Human directed aspects of PCBs. In: *Polychlorinated Biphenyls and the Environment*, Report No. ITF–PCB–72–1, (COM–72–10419), Interdepartmental Task Force on PCBs, Washington, D.C., May, 1972, pp. 122–151.

Work reported previously in the literature was summarized. Emphasis was placed on the tetra- and pentachlorodibenzofuran impurities in commercial PCB products; the tri- and tetrachlorodibenzofuran single oral liver necrotic dose (0.5 to 1.0 mg/kg) in rabbits; and the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin contaminant in 2,4,5–T and 2,4,5-trichlorophenol caused lethal liver necrosis and chloracne in rabbits at a dose range of 0.05 to 0.1 mg/kg.

FISHBEIN, L. Chromatographic and biological aspects of polychlorinated biphenyls. *J. Chromatog.* 68(1) : 345–426 (1972).

Chromatographic methods of analysis for chlorinated dibenzofurans were reviewed as part of a more extensive review of polychlorinated biphenyls.

FISHBEIN, L., and FLAMM, W. G. Potential environmental chemical hazards. Part II. Feed additives and pesticides. *Sci. Total Environ.* 1: 31–64 (1972).

The action of alkali on 1,2,4,5-tetrachlorobenzone, a by-product from lindane synthesis, produces 2,4,5-trichlorophenol which, when interacted with sodium monochloracetate, yields 2,4,5-T. The chloracnegen, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, is an impurity produced in the manufacture of 2,4,5-T: 1,2,4,5-tetra-

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orobenzene under the influence of high pressure and temperature, methanol, and sodium hydroxide, is converted to sodium trichlorophenate which reacts with another molecule of sodium trichlorophenate which reacts with another molecule of sodium trichlorophenate with high temperatures to form TCDD; or two molecules of trichlorophenol combine to form TCDD.

GOLDMANN, P. J. Severe acute chlorine acne caused by trichlorophenol decomposition products: A contribution to the perna problem. *Arbeitsmed. Sozialmed. Arbeitshyg.* 7(1): 12–18 (1972) (Ger.).

Occupational and case histories are reported describing 2,3,6,7-tetrachlorodibenzodioxin as the causative agent in 42 cases of serious skin changes, 14 cases of internal organ damage, and 7 cases of nervous system disturbances.

GREIG, J. B. Effect of 2,3,7,8-tetrachlorodibenzo-1,4-dioxin on drug metabolism in the rat. *Biochem. Pharmacol.* 21(23): 3196–3198 (1972).

Rats given a single oral 200 μg/kg dose of dioxin exhibited a decreased duration of zoxazolamine (100 mg/kg IP) induced paralysis by 54%. After 200 μg/kg oral dioxin, sleeping time induced by hexabarbital, 150 mg/kg (male rats) or 75 mg/kg (female rats), was prolonged—more than double at 3 days. These results indicate that dioxin has simultaneous stimulatory and inhibitory effects on different pathways of oxidative drug metabolism in the rat liver.

HAMMOND, A. L. Chemical pollution: polychlorinated biphenyls. *Science* 175 (4018): 155–156 (Jan. 14, 1972).

Some commercial PCB mixtures, especially those manufactured in Europe or Japan, may contain trace amounts of dibenzofurans or other toxic impurities. These may be the cause of some toxic effects of the PCBs.

HUSSAIN, S., EHRENBERG, L., LOFROTH, G., and GEJVALL, T. Mutagenic effects of TCDD on bacterial systems. *Ambio* 1(1): 32–33 (1972).

Results from three distinct bacterial assay systems showed TCDD to be mutagenic: (1) reversion to streptomycin independency in *E. coli* SD–4, (2) reversion to histidine prototrophy in *Salmonella typhimurium* strains, and (3) prophage induction in *E. coli* K–39. The results indicated that an acridine-like behavior of DNA intercalation may have caused these genetic effects.

HUSTON, B. L. Identification of three neutral contaminants in production grade 2,4-D. *J. Agr. Food Chem.* 20(3): 724–727 (1972).

Three chemical impurities of 2,4-D are profiled. These contaminants interfere with the gas–liquid chromatographic analysis of 2,4–D for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

JACKSON, W. T. Regulation of mitosis. III. Cytological effects of 2,4,5-trichlorophenoxyacetic acid and of dioxin contaminants in 2,4,5–T formulations. *J. Cell Sci.* 10: 15–25 (1972).

Inhibition of mitosis and development of cytological abnormalities observed in dividing endosperm cells of the African blood lily were believed caused by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a contaminant of 2,4,5-T, rather than the herbicide itself. In contrast to 2,4,5-T, which has no effect, dramatic inhibition of mitosis was observed in cells subjected to 0.2 to 1.0 μg/l dioxin, 0.2 μg/l dioxin plus $10^{-4}M$ 2,4,5-T, or $10^{-4}M$ 2,4,5-T containing dioxin as a contaminant. These preparations also induced formation of dicentric bridges and chromatin fusion with formation of multinuclei or a single large nucleus.

JENSEN, S. The PCB story. *Ambio* 1(4): 123–131 (1972).

The work of Vos et. al. is reported. Tetra- and pentachlorodibenzofuran impurities in commercial PCBs may have been formed from a phenolic contaminant in PCB in similar manner as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is formed from 2,4,5-T.

JENSEN, S., and RENBERG, L. Contaminants in pentachlorophenol: chlorinated dioxins and predioxins (chlorinated hydroxydiphenylethers). *Ambio* 1(2): 62–65 (1972).

Octachlorodioxin and 2-hydroxynonachlorodiphenyl ether (labeled predoxin) were identified in technical pentachlorophenate using ion exchange, diazomethane treatment, gas chromatography–mass spectrometry, and thinlayer chromatography. Predioxins have not been discovered or detected previously because: (1) clean-up procedures remove the substance, e.g., alumina column chromatography or concentrated sulfuric acid treatment of extract, (2) predoxin spontaneously forms dioxin in a gas chromatograph.

KEARNEY, P. C., WOOLSON, E. A., and ELLINGTON, C. P. Persistence and metabolism of chlorodioxins in soils. *Environ. Sci. Technol.* 6(12): 1017–1019 (1972).

Persistence of 1, 10, or 100 ppm 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was determined by electron-capture gas chromatography in soils after 20,

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40, 80, 160, and 350 days. After 1 yr, 56–63% TCDD was recovered. Neither 2,7-dichlorodibenzo-*p*-dioxin (DCDD) nor TCDD was detected in soils receiving 10, 100, or 1000 ppm 2,4-D or 2,4,5-T after 70 days. TCDD is degraded slowly in soils. TCDD and DCDD are not biosynthesized by microbial condensation reactions.

KHERA, K. S., and MCKINLEY, W. P. Pre- and postnatal studies on 2,4,5-trichlorophenoxyacetic acid, 2,4-dichlorophenoxyacetic acid and their derivatives in rats. *Toxicol. Appl. Pharmacol.* 22: 14–28 (1972).

2,4,5-T (containing less than 0.5 mg/kg 2,3,7,8-tetrachlorodibenzo-p-dioxin) induced fetopathy and skeletal anomalies in progeny from females treated with a single daily oral dose of 100 to 150 mg/kg on gestation days 6 to 15. Number of conceptions and numbers of viable and dead fetuses per litter gave no indication that *in utero* treatment of offspring with up to 100 mg/kg 2,4,5-T had impaired fertility.

KIMBROUGH, R. D. Toxicity of of chlorinated hydrocarbons and related compounds. A review including chlorinated dibenzodioxins and chlorinated dibenzofurans. *Arch. Environ. Health* 25(2): 125–131 (1972).

Trace amounts of chlorinated dibenzofurans and dibenzodioxins were identified as contaminants in many chlorinated technical compounds, e.g., 2,4,5-trichlorophenol, 2,4,5-trichorophenoxyacetic acid (2,4,5-T), and European chlorinated biphenyls (Phenoclor DP6 and Clophen A60). Toxic fat containing chick edema factor also contained chlorinated dibenzodioxins.

KINS, N., and BARANDY, J. Short method for the detection of chick edema factor in fats, oils, and fatty acids by electron capture gas chromatography. *J. Amer. Oil Chem. Soc.* 49(2): 115–117 (1972).

A modification of the official electron capture–gas chromatographic AOAC assay for chick edema factor, e.g., hexa-, hepta-, and octachlorodibenzo-p-dioxins, was presented to shorten the assay time and eliminate problems in the alumina column fractionation. In place of the alumina fractionation following 2,2,4-trimethylpentane extraction, a second sulfuric acid treatment and a caustic wash were done before the final sulfuric acid wash.

KRAYBILL, H. F. Chemical and physical properties of PCBs. In: Polychlorinated Biphenyls and the Environment, Report No. ITF-PCB-72-1, (COM-72-10419), Interdepartmental Task Force on PCBs, Washington, D.C. (May 1972). pp. 22–40.

Tetrachlorodibenzofuran (mass number 304) and pentachlorodibenzofuran (mass number 338) were identified from a fractionated PCB sample.

MAIER-BODE, H. Contribution to 2,4,5-T question. *Anz. Schaedlingskd. Pflanzenschutz* 45(1): 2–6 (1972) (Ger.).

The teratogenic effect of 2,4,5-T in an earlier U.S. experiment is attributable to the content (30 ppm) of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the test sample.

MARTIN, H. (Ed.). Pesticide Manual. Basic Information on the Chemicals used as Active Components of Pesticides, 3rd. ed. British Crop Protection Council, Worcester, England (1972).

A contaminant of 2,4,5-T (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) caused serious acne in man and produced fetal death in hamsters at 9.1 μg/kg. Modern methods of synthesis now limit the dioxin concentration in 2,4,5-T to less than 0.5 ppm.

MATSUMURA, F. Biological effects of toxic pesticidal contaminants and terminal residues. In: Environmental Toxicology of Pesticides, F. Matsumura, G. M. Boush, and T. Misato, Eds., Academic Press, New York, 1972, pp. 525–548.

Chlorinated dibenzo-p-dioxins have been classified as terminal residues-chemicals which accumulate in biologic material in the environment as a result of pesticide introduction. The presence of terminal residues in the environment is due to stable pesticides, conversion products, and chemical impurities (dioxins) that remain in the environment longer than the principal pesticides. The importance of such terminal residues in relation to the final magnitude of environmental hazardousness needs further definition.

NEUBERT, D., and DILLMANN, I. Embryotoxic effects in mice treated with 2,4,5-trichlorophenoxyacetic acid and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Naunyn-*Schmiedeberg's Arch. Pharmacol.* 272: 243–264 (1972).

Oral doses of 1 μg/kg 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDBD) in NMRI mice on days 6 to 15 of gestation produced cleft palate; embryo mortality was significant for doses of 4.5 μg/kg or more. Cleft palate without pronounced embryo lethality was produced in mice with high single doses (20–50 μg/kg) TCDBD given between days 7 and 13 of pregnancy. A maximum teratogenic effect was

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bserved on day 11 of gestation, but another peak cleft palate frequency occurred when TCDBD as given on day 8 of pregnancy.

NEUMAN, M. A., NORTH, P. P., and BOER, F. P. Crystal and molecular structure of octachlorodibenzo-*p*-dioxin. *Acta Crystallogr.* B28(8) : 2313–2317 (1972).

Three-dimensional single-crystal x-ray diffraction was used to elucidate the crystal and molecular structure of octachlorodibenzo-*p*-dioxin. The four unique C–Cl distances ranged from 1.714 to 1.718A, the two C–O distances were 1.373 and 1.374, and the six C–C bonds ranged between 1.382 and 1.396. The C–O–C angle was 115.8°.

NORBACK, D. H., and ALLEN, J. R. Chlorinated aromatic hydrocarbon induced modifications of the hepatic endoplasmic reticulum: concentric membrane arrays. Environ. Health Perspect. 1: 137–143 (1972).

Separate groups of rats were fed diets containing 1% highly chlorinated triphenyls (PCTs, Aroclor 5460), 0.02% polychlorinated biphenyls (PCBs, Arochlor 1254), and 0.002% chlorinated diphenyl-*p*-dioxin. The dioxin and PCT groups ate readily and attained 80% of control weight in 3 weeks. Liver hypertrophy varied from moderate enlargement (dioxin group) to an increase in relative liver weight 8/100 body weight for the PCB and PCT groups.

OHLAND, A. E., and YANG, G. C. Preparation and characterization of chlorinated dibenzo-*p*-dioxins. *J. Agr. Food Chem.* 20(6) : 1093–1099 (1972).

A series of 13 chlorinated dibenzo-*p*-dioxins (nine of these were new and previously unreported) was prepared containing from 1 to 8 chlorine atoms to provide pure standards of the various chlorinated derivatives for use in methods development and toxicology studies. Synthesis, utility, yield, purity, and physical and chemical properties were presented and discussed. Stability, color reactions, and infrared, ultraviolet, nuclear magnetic resonance, and phosphorescence spectra were also reported. Phosphorescence and triplet state lifetime wavelengths were dependent on the number of chlorine atoms and their positions on the dibenzo-*p*-dioxin nucleus.

RAPPE, C., and NILSSON, C.–A. An artifact in the gas chromatographic determination of impurities in pentachlorophenol. *J. Chromatogr.* 67: 247–253 (1972).

The main impurity of commercial samples of pentachlorophenol was 3,4,5,6-tetrachloro-2-(2,3,4,5,-pentachlorophenoxy)phenol; this compound underwent ring closure during gas chromatography to 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin.

SAINT–RUF, G. Formation of "dioxin" in the pyrolysis of sodium α-(2,4,5-trichlorophenoxy)-propionate. *Naturwiss.* 59 (12) : 648 (1972).

A residue of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin resulted from the pyrolysis (500°C for 5 hr) of sodium-α-(2,4,5-trichlorophenoxy)-propionate, a compound used in the synthesis of Sylvex. The dioxin impurity yield was much less than that observed during 2,4,5-T synthesis.

TASK FORCE. Polychlorinated biphenyls and the environment. Report No. ITF–PCB–72–1, (COM–72–10419), Interdepartmental Task Force on PCBs, Washington, DC (May, 1972).

This report emanated from a six-month review on the polychlorinated biphenyls by five Federal agencies. The main emphasis was PCBs; however, numerous statements and discussions centered on chlorinated dibenzodioxins and dibenzofurans. For example, fractionated samples of some PCBs of foreign manufacture contained as contaminants tetra- and pentachlorodibenzofurans. The task force recommended: (1) toxicological evaluation of a select number of representative, purified PCB isomers as well as purified trace contaminants such as the chlorinated dibenzofurans; (2) definitive mammalian elaboration of the kinetics, absorption, distribution, metabolism, and excretion of the technical PCBs as well as a number of key isomers and the chlorinated dibenzofurans; and (3) elaboration of the subcellular and intracellular actions of the technical PCBs as well as a number of representative isomers and chlorinated dibenzofurans.

VASILIU, G., and BACIU, I. Acetylation of some 2,7-dihalodibenzo-*p*-dioxins. *Rev. Chim.* (Bucharest) 23(9) : 523–525 (1972) (Rom.).

Reaction of 2,7-dichlorodibenzo-*p*-dioxin with chloroacetic acid in the presence of aluminum chloride and carbon disulfide gave the corresponding 3-monoacetyl derivative. Confirmation was by infrared spectra.

VOS, J. G. Toxicology of PCB's for mammals and for birds. *Environ. Health Perspect.* 1: 105–117 (1972).

Polychlorinated dibenzofurans or other toxic impurities in crude PCB preparations caused difficulty in interpreting toxicity studies.

VOS, J. G. Toxicology of polychlorinated biphenyls (PCB's) and impurities. *Tijdschr. Diergeneesk.* 97(22) : 1378–1385 (1972) (Neth).

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The most toxic impurities in polychlorinated biphenyls were found to be chlorinated benzofurans.

VOS, J. G., and NOTENBOOM-RAM, E. Comparative toxicity study of 2,4,5,2',4',5',-hexachlorobiphenyl and a polychlorinated biphenyl mixture in rabbits. *Toxicol. Appl. Pharmacol.* 23: 563–578 (1972).

The major acnegenic action of crude PCB mixtures originates from chlorinated dibenzofurans. The probable contribution of chlorinated dibenzofuran and pure PCB to the toxicity of technical PCB preparations in rabbits is discussed: chloracne (−furan ++, PCB +), edema formation (++, −), liver damage (++, +), and hepatic porphyria (−, ++).

WARMBRUNN, K. Considerations regarding the ban or limitation on the use of some pesticides imposed July 23, 1971. *Gesunde Pflanz.* 24(1): 6–8 (1972) (Ger).

The dioxin content of 2,4,5-T produced in Germany is approximately 1 ppm, a level at which malformation hazards are practically eliminated.

WILLIAMS, C. S. The current status of phenoxy herbicides. *Down to Earth* 27(4): 20–24 (Spring 1972).

A chronology of events from April 13, 1966 to December 6, 1971 pertaining to phenoxy herbicides is presented, with particular attention devoted to TCDD.

WILLIAMS, D. T., and BLANCHFIELD, B. J. Screening method for the detection of chlorodibenzo-*p*-dioxins in the presence of chlorobiphenyls, chloronaphthalenes, and chlorodibenzofurans. *J. Assoc. Offic. Anal. Chem.* 55(1): 93–95 (1972).

The chlorodibenzo-*p*-dioxins were chlorinated to octachlorodibenzo-*p*-dioxin and identified by electron capture–gas-liquid chromatography. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was determined at the 1 ppm level in corn oil. This method should be considered as a screen for dioxins, since it does not distinguish among the large number of isomers.

WILLIAMS, D. T., CUNNINGHAM, H. M., and BLANCHFIELD, B. J. Distribution and excretion studies of octachlorodibenzo-*p*-dioxin in the rat. *Bull. Environ. Contam. Toxicol.* 7(1). 57–62 (1972).

Absorption, distribution, and excretion of octachlorodibenzo-*p*-dioxin in the rat were examined. The AOAC method was used to analyze organs and tissues for dioxin content. The only gross pathology observed was congestion of the liver. Octachlorodibenzo-*p*-dioxin was absorbed by the rat mainly in the liver with small amounts in the adipose tissue and bile.

WILLIAMS, D. T., and BLANCHFIELD, B. J. Improved screening method for chlorodibenzo-*p*-dioxins. *J. Assoc. Offic. Anal. Chem.* 55(6): 1358–1359 (1972).

Conversion of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to octachlorodibenzo-*p*-dioxin allowed detection by electron capture–gas-liquid chromatography of as low as 0.05 ppm in corn oil. The author's previous method was modified to include different chlorination conditions and a later alumina column cleanup.

WILSON, J. G. Teratological potential of 2,4,5-T. Proceedings Twenty-Fifth Annual Meeting Southern Weed Science Society, Dallas, TX, Jan. 18–20, 1972.

A chronology of the hazards of 2,4,5-T was presented. The report issued by the President's Science Advisory Committee appointed to study the 2,4,5-T question served as the basis for this review. The dioxin contaminant of 2,4,5-T, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, was discussed briefly as a toxicogen.

WOOLSON, E. A., THOMAS, R. F., and ENSOR, P. D. J. Survey of polychlorodibenzo-*p*-dioxin content in selected pesticides. *J. Agr. Food. Chem.* 20(2): 351–354 (1972).

Electron capture–gas chromatography was used to examine 129 samples of 17 different pesticides derived from chlorophenols for polychlorinated dibenzo-*p*-dioxins. Clean-up entailed concentrated sulfuric acid extraction of impurities from hexane and mild nitration of the chlorophenol extracts. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was detected in the samples analyzed: 76% contained less than 0.1 μg/g, 7% between 0.1 to 1.0 μg/g, and 9% had greater than 10 μg/g. The 20 tri-, tetra-, and pentachlorophenols examined contained no TCDD. Phenoxy herbicides from current production had less than 0.5 μg/g TCDD.

YANG, G. C., and POHLAND, A. E. Electron spin resonance studies of cation radicals in trifluoromethane sulfonic acid. *J. Phys. Chem.* 76(10): 1504–1505 (1972).

Electron spin resonance spectra were obtained for chlorinated dibenzo-*p*-dioxins in trifluoromethane sulfonic acid (MSA); no oxidizing agents were used to produce the spectra in sulfuric acid. An oxidizing agent or ultraviolet light was needed for octachlorodibenzo-*p*-dioxin to yield a spectum in MSA; no spectrum was shown in sulfuric acid.

ZITKO, V. Absence of chlorinated dibenzodi-

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oxins and dibenzofurans from aquatic animals. *Bull. Environ. Contam. Toxicol.* 7(2/3): 105–110 (1972).

Chlorinated dibenzodioxin and dibenzofuran residues were not detected in any of the aquatic samples analyzed.

ZITKO, V., HUTZINGER, O., and CHOI, P. M. K. Contamination of the Bay of Fundy–Gulf of Maine area with polychlorinated biphenyls, polychlorinated terphenyls, chlorinated dibenzodioxins, and dibenzofurans. *Environ. Health Perspect.* 1: 47–50 (1972).

No residues of chlorinated dibenzodioxins and dibenzofurans were found in the samples analyzed: muscle and liver of white shark, eggs of double-crested cormorants and herring gulls, commercial herring oil, and ground fish herring meal. Detection limits ranged from 0.01 μg/g octachlorodibenzofuran wet tissue to 0.04 μg/TCDD g wet tissue.

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ANONYMOUS. A close look at TCDD. *Agr. Res.* 20(4): 8–10 (1971).

The toxicological properties of tetrachlorodibenzo-*p*-dioxin were investigated following the discovery that the mutagenic and teratogenic potential of 2,4,5-T may be due to this contaminant.

ANONYMOUS. Herbicides: More research on 2,4,5-T. *Chem. Eng. News* 49(20): 11 (1971).

The President's Science Advisory Committee's report on 2,4,5-T and its dioxin impurity is reviewed. The panel made a number of recommendations, including one asking for a mechanism that would temporarily restrict the use of certain registered pesticides on the basis of information that implicates the chemical as a possible health hazard, pending the collection of more conclusive information.

ANONYMOUS. Herbicides: Secret 2,4,5-T report. *Chem. Eng. News* 49(29): 15 (1971).

An advisory committee's report submitted to the Environmental Protection Agency concerning 2,4,5-T and its dioxin contaminant was criticized by a dissenting advisory committee member and the Committee for Environmental Information. Specifically stated was that a level at which TCDD is not teratogenic has not been established and that the report did not consider the consequences of the fate of TCDD in food chains and animal tissue.

ANONYMOUS. A taste of honey (flavoured with 2,4,5,-T). *Food Cosmet. Toxicol.* 9: 152 (1971).

This is a commentary on the teratogenic evaluation of 2,4,5-T by K. D. Courtney, et al.; a note added in proof indicate the 2,4,5-T contained 30 ppm dioxin. Thus, conclusions from the paper labeling 2,4,5-T teratogenic must be considered tentative.

ANONYMOUS. The PCB story unfolds. *Food Cosmet. Toxicol.* 9(4): 568–571 (1971).

Lesions resembling those produced by chick edema factor were traced to a PCB contaminant, chlorinated dibenzofurans which is present in some commercial PCBs.

ANONYMOUS. Working with 2,4,5-T. *Food Cosmet. Toxicol.* 9: 908–909 (1971).

Chloracne, characterized by inclusion cysts, comedones, and pustules, was found in 13 workers, and was correlated in severity with the presence of scarring, hyperpigmentation, hirsutism, and complaints of eye irritation. A. P. Poland et al. commented that chloracne was not correlated with occupations within plants manufacturing 2,4,5-T, duration of employment, or coproporphyrin excretion.

ANONYMOUS. Tetrachlorodibenzodioxin—intimations of carcinogenicity? *Food Cosmet. Toxicol.* 9: 909 (1971).

Although such effects as described by Buu-Hoi, et al. of the carcinomimetic activity of TCDD exist, the relationship is by no means exclusive. For example, stimulation of hydroxylating enzymes can be induced by many compounds, including BHT, which has been shown not to be carcinogenic in long-term feeding studies.

ANONYMOUS. PSAC hiccoughs over 2,4,5-T. *Nature* 231(5300): 210–211 (1971).

The President's Science Advisory Committee (PSAC) report on 2,4,5-T is questioned: justification by the PSAC concerning permissable levels of dioxin in 2,4,5-T was not based on scientific review; also, the need for establishing a dose–response curve for the teratogenicity of 2,4,5-T should have been cited.

ANONYMOUS. 2,4,5-T report attacked. *Nature* 232(5308): 218 (1971).

The response of the Committee for Environmental Information (CEI) to the scientific advisory committee's recommendation that 2,4,5-T be restored was one of strong opposition. With respect to dioxins, the CEI indicated that dioxins may accumulate in the soil and that present analytical techniques were not sensitive enough to detect them. Furthermore, lack of concrete proof that the use of 2,4,5-T was correlated with the occurrence of birth defects in Vietnam did not mean that no correlation existed.

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BAUGHMAN, R. W., and MESELSON, M. An improved analysis for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDBD). Division Pesticide Chemistry, 162nd National Meeting of the American Chemical Society, Washington, D.C., Sept. 12–17, 1971, Abstract No. 89.

Current analytical methods for the detection of potentially hazardous levels of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin are inadequate. A combined gas chromatography–mass spectroscopy method was utilized with modifications in extraction concentration and detection.

BEVENUE, A., and KAWANO, Y. Pesticides, pesticide residues, tolerances, and the law (U.S.A.). *Residue Rev.* 35: 103–149 (1971).

Politics and science surround the 2,4,5-T and hence dioxin controversy. Contents include: Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), as amended through 1969; the DDT episode; the 2,4,5-T episode: Federal Food, Drug, and Cosmetic Act (FDCA); and food imports and international pesticide control. Mention is made of the USDA's investigation of possible excessive contamination of 17 polychlorophenolic pesticides by tetrachlorodibenzo-*p*-dioxin.

BOER, F. P., NEUMAN, M. A., VAN REMOORTERE, F. P., NORTH, P. P., and RINN, H. W. X-ray diffraction studies of chlorinated dibenzo-*p*-dioxins. Division Pesticide Chemistry, 162nd National Meeting of the American Chemical Society, Washington, D.C., Sept. 12–17, 1971; Abstract No. 82.

The crystal structures of four chlorinated dibenzo-*p*-dioxin compounds (2,7-dichloro; 2,8-dichloro; 2,3,7,8-tetrachloro; and octachloro) were determined by x-ray diffraction. The structures were elucidated in order to provide absolute standards for isomeric structure assignment.

BUU-HOI, N. P., HIEN, D. P., SAINT-RUF, G., and SERVOIN-SIDOINE, J. Cancéromimetic properties of tetrachloro-2,3,7,8 dibenzo-*p*-dioxin ("dioxin"). *C. R. Acad. Sci.* (Paris) D272(10): 1447–1450 (1971) (Fr).

Tetrachloro-2,3,7,8-dibenzo-*p*-dioxin in rats caused induction of zoxazolamine hydroxylase, marked reduction in phenobarbital sedative effect, and a decrease in hepatic arginase. Dioxin therefore, has a pronounced inhibitory effect on the enzymatic systems as do the carcinogens benzo [a]-pyrene and *p*-dimethylaminoazobenzene.

BUU-HOI, N. P., SAINT-RUF, G., BIGOT, P., and MANGANE, M. Preparation, properties and identification of "dioxin" in pyrolysates of defoliants containing 2,4,5-trichlorophenoxyacetic acid, their esters, and contaminated plants. *C. R. Acad. Sci.* (Paris) D273(7): 708–711 (1971) (Fr).

Synthesis and physico-chemical properties of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and its less toxic isomer 1.3.6.8-tetrachlorodibenzo-*p*-dioxin are described. Caution was urged about the burning of wooded areas treated with 2,4,5-T or its butyl ester because of possible dioxin formation.

CAMPBELL, A. D., and FIRESTONE, D. Chick edema factor—toxic dioxins. In: International Symposium on Identification and Measurement of Environmental Pollutants, June 14–17, 1971, Ottawa, Ontario, Canada, B. Wesley, Ed., National Research Council, Canada, 1971, pp. 195–198.

The formation, occurrence, toxicity, tissue distribution in the chick, and analytical methods for the detection of polychlorodibenzo-*p*-dioxins are described. Dioxins were implicated in chick edema disease. Dioxins belong to one of the most toxic classes of chlorinated compounds known, being almost 1000 times as toxic as most pesticides.

COLLINS, T. F. X., WILLIAMS, C. H., and GRAY, G. C. Teratogenic studies with 2,4,5-T and 2,4-D in the hamster. *Bull. Environ. Contam. Toxicol.* 6(6): 559–567 (1971).

2,4,5-T commercial samples given on days 6 to 10 of organogenesis were feticidal and teratogenic in the golden Syrian hamster. The incidence of the observed effects increased with an increasing content of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Dioxin impurity caused edema and hemorrhages in newborn animals.

COMMITTEE FOR ENVIRONMENTAL INFORMATION. Critique of the report of the advisory committee on 2,4,5-T. *Environment* 13(7): 24, 29 (1971).

Criticism by the Committee for Environmental Information levied against the Advisory Committee's report on 2,4,5-T centered on the following areas: no effect levels; environmental accumulation, transmission and degradation; human hazards from

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lioxins; 2,4,5-T teratogenicity; and the benefits vs. risks of 2,4,5-T usage.

COURTNEY, C. D., and MOORE, J. A. Teratology studies with 2,4,5-trichlorophenoxyacetic acid and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Toxicol. Appl. Pharmacol.* 20(3): 396–403 (1971).

2,4,5-T and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were teratogenic in three strains of mice; combinations of these compounds did not potentiate observed results. Both produced cleft palates and kidney malformations. Studies with rats gave negative results with 2,4,5-T; TCDD induced kidney anomalies.

CROSBY, D. G., WONG, A. S., PLIMMER, J. R., and WOOLSON, E. A. Photodecomposition of chlorinated dibenzo-*p*-dioxins. *Science* 173(3998): 748–749 (1971).

2,4,5-T and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin homologs photodecompose rapidly in an alcohol solution exposed to artificial light and natural sunlight. The decomposition rate was correlated with the degree of chlorination. Negligible photodecomposition was observed in aqueous suspensions and on wet or dry soil.

DAVRING, L., and SUNNER, M. Cytogenetic effects of 2,4,5-trichlorophenoxyacetic acid on oogenesis and early embryogenesis in *Drosophila melanogaster*. *Hereditas* 68(1): 115–122 (1971).

2,4,5-T with dioxin contaminants less than 0.1 ppm was evaluated in a wild-type *Drosophila* population, Canton-S 109. Adult flies were exposed to 250 ppm 2,4,5-T in their food within or later than 24 hr of eclosion. Results indicated that this 2,4,5-T formulation effected early oogenesis and caused chromosome disturbances which could result in sterility.

ELVIDGE, D. A. The gas-chromatographic determination of 2,3,7,8-tetrachloro-*p*-dioxin in 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2,4,5-T ethyl-hexyl ester, formulations of 2,4,5-T esters and 2,4,5-trichlorophenol. *Analyst* 96(1147): 721–727 (1971).

A gas chromatographic method is described for the detection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin impurities in herbicides. Dioxin recoveries ranged from 89 to 98%; limit of detection was 0.05 ppm.

EMERSON, J. L., THOMPSON, D. J., STREBING, R. J., GERBIG, C. G., and ROBINSON, V. G. Teratogenic studies of 2,4,5-trichlorophenoxyacetic acid in the rat and rabbit. *Food Cosmet. Toxicol.* 9(3): 395–404 (1971).

Treatment of rabbits and rats with 2,4,5-T containing 0.5 ppm 2,3,7,8-tetrachlorodibenzo-*p*-dioxin did not cause any teratogenic or embryotoxic effects. Rats received 1, 3, 6, 12, or 24 mg 2,4,5-T/kg on days 6 to 15 of pregnancy. Rabbits received 0, 10, 20, or 40 mg 2,4,5-T/kg on days 6 to 18 of pregnancy.

FIRESTONE, D. Determination of polychlorodibenzo-*p*-dioxins (dioxins) in chlorophenols and lipids. Division Pesticide Chemistry, 162nd National Meeting of the American Chemical Society, Washington, D.C., Sept. 12–17, 1971; Abstract No. 80.

High resolution mass spectrometry was reported to offer a sensitive method for detecting specific dioxins after adequate sample cleanup, e.g., chromatography and gas chromatography.

FIRESTONE, D. FLICK, D. F., RESS, J., and HIGGINBOTHAM, G. R. Distribution of chick edema factors in chick tissues. *J. Assoc. Offic. Anal. Chem.* 54(6): 1293–1298 (1971).

Chickens fed toxic animal fat containing 9.9 ppm chlorinated dibenzo-*p*-dioxins excreted over 90% the ingested hexa-, hepta-, and octachlorodioxins. Decreased chlorination resulted in greater body retention. Residues were found in many tissues but was most prominent in the liver.

HANSEN, W. H., QUAIFE, M. L., HABERMANN, R. T., and FITZHUGH, O. G. Chronic toxicity of 2,4-dichlorophenoxyacetic acid in rats and dogs. *Toxicol. Appl. Pharmacol.* 20: 122–129 (1971).

2,4-D preparations, having undetectable levels of 2,7-dichlorodibenzo-*p*-dioxin or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, were tested for chronic toxicity in rats and dogs. No significant effect on growth, survival, organ weight, or hematologic values were noted.

HAYS, H., and RISEBROUGH, R. W. The early warning of the terms. *Natural History* 80(9): 39–46 (Nov. 1971).

Possible health implications to humans and wildlife from environmental pollution with chlorinated dibenzofurans are reviewed.

HELLING, C. S. Pesticide mobility in soils. II. Applications of soil thin-layer chromatography. *Soil Sci. Soc. Amer. Proc.* 35(5): 737–743 (1971).

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Relative mobility by thin-layer chromatography and diffusion in moist and air-dry soils were used to test pesticide movements. The two chlorinated dibenzo-*p*-dioxins were immobile.

HOLDEN, C. Critics weigh EPA herbicide report, find it wanting. *Science* 173(3994): 312 (1971).

The EPA's Advisory Committee on 2,4,5-T recommended that the ban on 2,4,5-T be lifted with certain qualifications, i.e., no more than 0.1 ppm 2,4,5-T in drinking water and formulations containing no more than 0.1 ppm dioxin. The Committee for Environmental Information voiced five major objections.

ISENSEE, A. R., and JONES, G. E. Absorption and translocation of root and foliage applied 2,4-dichlorophenol, 2,7-dichlorodibenzo-*p*-dioxin, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Agr. Food Chem.* 19(6): 1210–1214 (1971).

Measured uptake of ^{14}C-labeled 2,7-dichloro- and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin from nutrient solution, soil, and foliage by oats and soybeans indicated that accumulation of TCDD in plants via the soil is unlikely.

JOHNSON, J. E. The public health implications of widespread use of the phenoxy herbicides and picloram. *Bioscience* 21 (17): 899–905 (Sept. 1, 1971).

A review presenting the myriad historical aspects of the dioxin–2,4,5-T(and other herbicides) picture. The potential adverse health effects from phenoxy herbicides were documented. One conclusion reported was that impurities—particularly the chlorodibenzo-*p*-dioxins—can be an important factor, but these can be controlled by proper manufacturing techniques.

JOHNSON, J. E. Safety in the development of herbicides. *Proc. Ann. Calif. Weed Conf.* 23: 43–67 (1971).

Experiments using 2,4,5-T alone and in combination with dioxin allowed the conclusion that commercially produced 2,4,5-T containing less than 1 ppm dioxin does not present a hazard to health.

JOHNSON, J. E. Safety in the development of herbicides. *Down to Earth* 27(1): 1–7 (1971).

After the discovery of highly toxic dioxin residues in the herbicides 2,4,5-T, precautionary control measures were instituted to insure greater quality control in pesticide production. Pregnant rats were treated orally with 2,4,5-T at doses of up to 100 mg/kg/day. No symptoms of teratology were produced in the offspring. Dioxin was toxic to embryos at 0.125 μg/kg/day, whereas 0.03 μg/kg/day was below the "no-effect" level. Some aspects of production and costs are discussed.

KEARNEY, P. C., and WOOLSON, E. A. Persistence and metabolism of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in soils. Division Pesticide Chemistry, 161st National Meeting of the American Chemical Society, Los Angeles, March 28–April 2, 1971; Abstract No. 21.

TCDD applied 1, 10, and 100 ppm to two soils held at 30°C remained relatively unchanged (80–85% of original samples were recovered) after 20, 40, 80, and 160 days. Incubation of 10, 100, and 1000 ppm, 2,4-dichlorophenol, and 2,4,5-trichlorophenol in soils for 70 days produced no detectable di- or trichlorodioxins.

KEARNEY, P. C., ISENSEE, A., HELLING, C. S. WOOLSON, E. A., and PLIMMER, J. R. Environmental significance of chlorodioxins. Division Pesticide Chemistry, 162nd National Meeting of the American Chemical Society, Washington, D.C., Sept. 12–17, 1971; Abstract No. 90.

Studies on TCDD under controlled environmental conditions revealed that TCDD was persistent and immobile in soil, not readily absorbed by plants, subject to photodecomposition, and slowly degraded to polar metabolites. Other studies revealed that the environmental contamination is small and not detectable in biological samples.

KHERA, K. S., and RUDDICK, J. A. Perinatal effects of dibenzodioxins in Wistar rats. Division Pesticide Chemistry, 162nd National Meeting of the American Chemical Society, Washington, D.C. Sept. 12–17, 1971; Abstract No. 87.

Oral administration of 16 to 0.125 μg/kg 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to pregnant rats on days 6–15 of gestation caused a reduction in viable litter size and a high incidence of resorptions. No effect was observed at the 0.125 level. Histological examination of fetuses revealed brain and intestinal hemorrhages and edema of subcutaneous tissue.

KHERA, K. S., HUSTON, B. L., and MCKINNEY, W. P. Pre- and postnatal studies on 2, 4,5-T, 2,4-D, and derivatives in Wistar rats. *Toxicol. Appl. Pharmacol.* 19:369–370 (1971).

Results from teratogenic studies with rats treated with 2,4,5-T, 2,4-D, and several derivatives were

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either negative or inconclusive. The 2,3,7,8-tetrachlorodibenzho-*p*-dioxin levels in these preparations were not known in all cases, but most were suspected to be less than 0.5 ppm.

LANGER, H. G., BRADY, T. P., DALTON, L. A. SHANNON, T. W., and BRIGGS, P. R. Thermal chemistry of chlorinated phenols. Division Pesticide Chemistry, 162nd National Meeting of the American Chemical Society, Washington, D.C., Sept. 12–17, 1971; Abstract No. 83.

At the decomposition temperatures of chlorinated phenols and derivatives, the following conditions are ideal for dioxin formation: (a) high concentration of the phenol, (b) high degree of chlorination of the phenol, and (c) presence of base.

LOEFROTH, G. Environmental poisons, man and the physician. *Laekartidningen* 68 (18): 2113–2133 (1971) (Swed.).

Possible adverse health effects from environmental poisons demands caution. Tetrachlorobenzo-*p*-dioxin was mentioned for its adverse effects of fetal development.

MILNES, M. H. Formation of 2,3,7,8-tetrachlorodibenzodioxin by thermal decomposition of sodium 2,4,5-trichlorophennate. *Nature* (London) 232(5310): 395–396 (1971).

Hydrolysis of 1,2,4,5-tetrachlorobenzene in methyl alcohol with sodium hydroxide at high pressures or in ethylene glycol at 1 atm formed sodium monochlorophenate. This allowed exothermic decomposition of 2,4,5-trichlorophenate sodium to 2,3,7,8-tetrachlorodibenzodioxin.

MUELDER, W. W., and SHADOFF, L. The preparation of uniformly labeled ^{14}C-2,7-dichlorodibenzo-*p*-dioxin and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Division Pesticide Chemistry, 162nd National Meeting of the American Chemical Society, Washington, D.C., Sept. 12–17, 1971; Abstract No. 78.

2,7-Dichlorodizenzo-*p*-dioxin was prepared from isotopic potassium 2,4-dichlorophenate uniformly labeled with ^{14}C. Chlorination of the dioxin in a chloroform solution containing $FeCl_3$ and I_2 gave a mixture of tri-, tetra-, and pentachlorosubstitution products. Purification by digestion in boiling chloroform, fractional sublimation, and recrystallization from anisole yielded a product containing 92% 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

PIPER, W. N., and ROSE, J. Q. The excretion and tissue distribution of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the rat. Division Pesticide Chemistry, 162nd National Meeting of the Amercian Chemical Society, Washington, D.C., Sept. 12–17, 1971; Abstract No. 88.

TCDD was administered to male rats in a single oral dose of 50 μg/kg. The primary excretion route was via the feces and the highest tissue concentration was in the liver.

PLIMMER, J. R., CROSBY, D. G., WONG, A. S., and KLINGEBIEL, U. I. Photochemistry of dibenzo-*p*-dioxins. Division Pesticide Chemistry, 162nd National Meeting of the American Chemical Society, Washington, D.C., Sept. 12–17, 1971; Abstract No. 85.

Ring closure and photochemical reaction of *o*-chlorophenols yield dioxin. Dibenzo-*p*-dioxins were decomposed in solution by light or sunlight. Absorption of dioxin on soil reduced its rate of photodecomposition.

PLIMMER, J. R., and KLINGEBIEL, U. I. Riboflavin photosensitized oxidation of 2,4-dichlorophenol: assessment of possible chlorinated dioxin formation. *Science* 174(4007): 407–408 (1971).

Chlorophenols in aqueous solution were exposed to the action of light in wavelengths greater than 280 nm. Products were characterized by gas chromatography and mass spectrometry. Possible chlorinated dibenzo-*p*-dioxins from ring closure of a 2-phenoxyphenol derivative were not detected in the photolysis products. Under environmental conditions, dioxins are unlikely products of lower chlorinated phenols or phenoxy-alkanoic acids.

POHLAND, A. E., YANG, G. C., and HANSEN, E. A. The preparation and characterization of chlorinated dibenzo-*p*-dioxins. Division Pesticide Chemistry, 162nd National Meeting of the American Chemical Society, Washington, D.C., Sept. 12–17, 1971; Abstract No. 77.

Twelve chlorinated dibenzo-*p*-dioxin standard compounds containing from one to eight chlorine atoms were prepared as standards to develop analytical techniques and to use in toxicity studies. The wavelength associated with the observed phosphorescence and the triplet state lifetime were dependent upon the number of chlorine atoms and their positions on the dibenzo-*p*-dioxin nucleus.

POLAND, A. P., SMITH, D., METTER, G., and POSSICK, P. A health survey of workers

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in a 2,4-D and 2,4,5-T plant. *Arch. Environ. Health* 22:316–327 (1971).

A study and review of employee health in a plant producing 2,4,5-T and 2,4-D. Chloracne was found in 13/73 male workers and was believed caused by chlorinated dioxins. The plant has begun several programs to reduce dioxin levels in 2,4,5-trichlorophenol; the contaminant concentration has dropped from 10 to 25 ppm to 1 ppm.

PORTER, M. L., and BURKE, J. A. Separation of three chlorodibenzo-*p*-dioxins from some polychlorinated biphenyls by chromatography on an aluminum oxide colum.

Chlorodioxins (2,3-di-, 2,3,7-tri-, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) were separated from polychlorinated biphenyls by column chromatography on aluminum oxide. Recoveries of both dioxins and the polychlorinated biphenyls were approximately 100%.

RISEBROUGH, R. W. Determination of polychlorinated biphenyls in environmental samples. In International Symposium on Identification and Measurement of Environmental Pollutants, Ottawa, Ontario, Canada, June 14–17, 1971, B. Westley, Ed., National Research Council, Canada, 1971, pp. 147–153.

Even though it is not known whether dibenzofurans can be formed from environmental residues of polychloronated biphenyls (PCB), they should be considered as a part of the PCB problem. Dibenzofurans have been found in commercial PCB mixtures sampled from France, Germany, and the United States in concentrations ranging from 5 to 20 ppm. The European samples had higher levels than the U.S. sample.

ROLL, R. Teratogenic effect of 2,4,5-T [2,4,5-trichlorophenoxyacetic acid] in mice. *Food Cosmet. Toxicol.* 9(5): 671–676 (1971) (Ger).

2,4,5-T having a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin content of less than 0.1 ppm caused embryotoxic effects and a significant increase in the incidence of cleft palate in mice. Concentrations used in the study ranged from 35 to 130 mg/kg 2,4,5-T/dioxin which were given orally on days 6–15 of pregnancy. The teratogenic "no-effect" level of 2,4,5-T/dioxin was found to be 20 mg/kg.

ROWE, V. K., NORRIS, J. M., SPARSCHU, G. L., SCHWETZ, B. A., and GEHRING, P. J. Toxicology of chlorinated dibenzo-*p*-dioxins. Division Pesticide Chemistry, 162nd National Meeting of the American Chemical Division, Washington, D.C., Sept. 12–17, 1971, Abstract No. 86.

The toxicology of several chlorinated dibenzo-*p*-dioxins were investigated in rats, guinea pigs, and rabbits. The compounds studied were 2,7,-dichlorodibenzo-*p*-dioxin (DCDPD); 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDPD); hexachlorodibenzo-*p*-dioxin (HCDPD), and octachlorodibenzo-*p*-dioxin (OCDPD). TCDPD was found to be the most toxic of the compounds investigated. DCDPD and OCDPD were the least toxic. For instance, little if any fetotoxic effects were observed with these two compounds; doses of 2 to 4 g/kg were not lethal to rats. On the other hand, TCDPD and HCDPD induced chloracne, fetotoxicity, and teratogenic effects.

SCHWETZ, B. A., SPARSCHU, G. L., and GEHRING, P. J. The effect of 2,4-dichlorophenoxyacetic acid (2,4-D) and esters of 2,4,D on rat embryonal, foetal and neonatal growth and development. *Food Cosmet. Toxicol.* 9:801–817 (1971).

The teratogenicity of 2,4-D was investigated in the rat. Formation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the manufacture of 2,4-D is not theoretically possible. The 2.4-D sample used was analyzed for the TCDD contaminant with a method having a 0.2 ppm sensitivity and none was found. Doses of 12.5, 25, 50, 75, and 87.5 mg/kg 2,4-D were administered orally on days 6–15 of gestation. High dose levels caused signs of embryotoxicity and fetotoxicity.

SOMERS, E., and SMITH, D. M. Source and occurrence of environmental contaminants. *Food Cosmet. Toxicol.* 9(2): 185–193 (1971).

The sources, occurrences, and some of the toxicological effects of polychlorinated biphenyls and chlorodioxins were discussed. Chlorophenols exposed to high temperatures may form chlorinated derivatives of dibenzo-*p*-dioxin. Inadequate methodolgy prevents analysis for these contaminants in foods.

SPARSCHU, G. L., DUNN, F. L., LISOWE, R. W., and ROWE. V. K. Study on the effects of high levels of 2,4,5-trichlorophenoxyacetic acid on foetal development in the rat. *Food Cosmet Toxicol.* 9(4): 527–530 (1971).

Commercial-grade 2,4,5-T containing 0.5 ppm 2,3,7,8-tetrachlorodibenzo-*p*-dioxin did not induce a teratogenic response when administered orally to rats on days 6 to 15 of gestation at a dosage level of 50 mg/kg/ day.

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ARSCHU, G. L., DUNN, F. L., and ROWE, V. K. Study of the teratogenicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the rat. *Teratology* 4: 247 (Abstract) (1971).

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was administered orally to pregnant rats during days 6 to 15 of gestation in doses of 0, 0.03, 0.125, 0.5, 2.0, and 8.0 g/kg/day [sic]. No effect was observed on the fetus or mother at the 0.03 dose level. However, at the 0.125 level and above, fetal mortality, early and late resorptions, and fetal intestinal hemorrhage occurred. The effects became more pronounced with the increase in dose. Maternal toxicity was noted beginning at the 0.5 level which also increased with the dose.

SPARSCHU, G. L., DUNN, F. L., and ROWE, V. K. Study of the teratogenicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the rat. *Food Cosmet. Toxicol.* 9(3): 405–412 (1971).

Investigations of the teratogenicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats revealed that this compound is capable of inducing a high degree of adverse effects in the fetus and mother. Doses of 0, 0.03, 0.125, 0.5, 2.0, and 8.0 μg kg/day were given orally to pregnant rats during days 6 to 15 of gestation. Effects such as fetal mortality, intestinal hemorrhage, and early and late resorptions began appearing at the 0.125 level and increased with dose. It is suggested that the teratogenic effects of 2,4,5-T observed in other studies may have been due to this contaminant (30 ppm).

STEHL, R. H., PAPENFUSS, R. R., BREDWEG, R. A., and ROBERTS, R. W. The stability of pentachlorophenol and chlorinated dioxins to sunlight, heat, and combustion. Division Pesticide Chemistry, 162nd National Meeting of the American Chemical Society, Washington, D.C., Sept. 12–17, 1971; Abstract No. 92.

Degradation of pentachlorophenol in the environment could theoretically lead to the formation of chlorinated dibenzo-*p*-dioxins. Several simulated environmental conditions were checked for possible dioxin formation. Pentachlorophenol-treated wood and paper indicated no increase in dioxins when burned: likewise photolysis of sodium pentachlorophenol yielded only a trace of the compound. 2,7-Di- and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin were rapidly decomposed under artificial sunlight, whereas octachlorodibenzo-*p*-dioxin was not.

STEHL, R. H., WILKE, E., PAPENFUSS, R. R., and MATALON, R. Determination of nonphenolic impurities in chlorinated phenols and related compounds. Division Pesticide Chemistry, 162nd National Meeting of the American Chemical Division, Washington, D.C., Sept. 12–17, Abstract No. 81.

Analytical techniques for the detection of neutral impurities (chlorinated dibenzo-*p*-dioxins) in chlorinated phenols, were ion-exchange chromatography for removal of the matrix, liquid-liquid chromatography for separation of the various neutral components, followed by gas chromatographic examination of the separated components using electron capture detection.

STORHERR, R. W., WATTS, R. R., GARDNER, A. M., and OSGOOD, T. Steam distillation technique for the analysis of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in technical 2,4,5-T. *J. Assoc. Offic. Anal. Chem.* 54(1): 218–219 (1971).

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was separated from 2,4,5-T by steam distillation and analyzed by microcoulometric gas–liquid chromatography.

THOMPSON, D. J., EMERSON, J. L., and SPARSCHU, G. L. Study of the effects of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) on rat and rabbit fetal development. *Teratology* 4:243 (Abstract) (1971).

2,4,5-T was investigated for its effects on fetal development in the rat and rabbit. Doses ranging from 1 to 50 mg/kg were administered during days 6 to 15 of gestation. No clinical or gross pathology was observed. Detailed examination of the visceral and skeleton revealed no teratogenic effects. However, rats receiving 100 mg/kg during day 6 to 10 of gestation did produce maternal toxicity and death, early fetal resorptions, fetal toxicity, but no malformations.

CURRIER, W. F., GRAHAM, C., GRATKOWSKI, H., and NORRIS, L. A., U.S. Forestry Service. Report on background information for the phenoxy herbicides 2,4-D—2,4,5-T—2,4,5-TP. U.S. Forestry Service Report, pp. 1–165 (1971).

A through review is given on the phenoxy herbicides, 2,4-D (2,4-dichlorophenoxyacetic acid), 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), and 2,4,5-TP [2-(2,4,5-trichlorophenoxy)propionic acid], and their potential contaminant, e.g., dioxin.

VINOPAL, J. H., YAMAMOTO, I., and CASIDA, J. E. Preparation of tritium-labeled 2,3,-7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structure-activity investigations of

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TCDD and other related dibenzo-*p*-dioxins. Division Pesticide Chemistry, 162nd National Meeting of the American Chemical Society, Washington, D.C., Sept. 12–17, 1971, Abstract No. 79.

Preparation of tritium-labeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin was investigated. One approach used was the tritiation of 2,4-dichlorophenol or 2,4,5-trichlorophenol, lithiummetalation and subsequent aqueous hydrolysis of a chlorinated dibenzo-*p*-dioxin, and the catalytic reduction of a chlorinated dibenzo-*p*-dioxin.

WESTING, A. H. Herbicides as agents of chemical warfare: Their impact in relation to the Geneva Protocol of 1925. *Environ. Affairs* 1(3): 578–586 (1971).

The use of herbicides as chemical warfare agents is reviewed with respect to the Geneva Protocol of 1925. The total impact of these agents on the environment and man is not usually known or considered before widespread use. For example, 47 million lb of 2,4,5-T was used in Indochina over an 8-yr period before it was discovered that about 1000 lb dioxin was also being applied as an unsuspected contaminant.

WILLIAMS, D. T., and BLANCHFIELD, B. J. Thin layer chromatographic separation of two chlorodibenzo-*p*-dioxins from some polychlorinated biphenyls and organochlorine pesticides. *J. Assoc. Offic. Anal. Chem.* 54(6): 1429–1431 (1971).

Thin-layer chromatography was used for the isolation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and octachlorodibenzo-*p*-dioxin from polychlorinated biphenyls and organochlorine pesticides. After isolation, these dioxins were analyzed by the electron capture–gas chromatography.

WILSON, J. G., BOUTWELL, R. K., DAVIS, D. E., DOST, F. N., HAYES, W. J., KALTER, H., LOOMIS, T. A., SCHULERT, A., STERLING, T. D., and BOWEN, D. L. Report of the Advisory Committee on 2,4,5-T to the Administrator of the Environmental Protection Agency. (Submitted May 7, 1971).

The 2,4,5-T Scientific Advisory Committee's report to the U.S. Environmental protection Agency was stimulated after experimental results and environmental indicators pointed to a potential health threat to humans. This nine-member committee was selected from a list of names supplied by the National Academy of Science. The Committee recommended by an 8 to 1 vote to restore registration of 2,4,5-T with certain conditions; those applicable to TCDD were: (1) a limit of 0.5 ppm TCDD on existing 2,4,5-T inventories and 0.1 ppm on future 2,4,5-T production, and (2) conduct specific research on TCDD for potential soil accumulation and food drain magnification.

WILLIAMS, C. S. The status of 2,4–D, 2,4,5-T, Silvex and MCPA herbicides. *Down to Earth* 26(4): 12–15 (1971).

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the causative agent in experiments reporting terotogenic effects from 2,4,5-T. TCDD was found in 2,4,5-T formulations but not in 2,4-D samples. The precursor for 2,4,5-T is 2,4,5-trichlorophenol which is prepared from 1,2,4,5-tetrachlorobenzene; the high temperature and alkaline conditions necessary to produce this compound also favor dioxin formation. Since 2,4-D is formed by direct chlorination of 2,4-dichlorophenol and not by alkaline hydrolysis of 1,2,4-trichlorobenzene, no dioxin impurities are theoretically possible.

WOOLSON, E. A., REICHEL, W. L., and YOUNG, A. L. Dioxin residues in lakeland sand and eagle samples. Division Pesticide Chemistry, 162nd National Meeting of the American Chemical Society, Washington, D.C., Sept. 12–17, 1971. Abstract No. 91.

Between 1962 and 1969 a lakeland region in Florida was treated with 912 lb 2,4,5-T per acre. To assess the ecological importance of chlorinated dioxins in the environment, samples of soil to a depth of 3 ft. and tissues from eagles were analyzed for dioxins by electron capture-gas chromatography. No dioxins were detected at a minimum detection limit of 50 ppb for the eagle tissue samples and 5 ppb for the soil samples. Small residues of about 20 ppb 2,4,5-T were found in soil samples.

YANG, G. C., and POHLAND, A. E. Cation radicals from chlorinated dibenzo-*p*-dioxins. Division Pesticide Chemistry, 162nd National Meeting of the American Chemical Society, Washington, D.C., Sept. 12–17, 1971. Abstract No. 84.

Characteristic blue coloration and electron spin resonance (ESR) spectra of cation radicals were obtained following dissolution of chlorinated dibenzo-*p*-dioxins in trifluoromethanesulfonic acid. The mono- and dichlorodibenzo-*p*-dioxins showed well resolved ESR by hyperfine lines; the tetrachloro analogs exhibited single broad ESR lines.

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ABELSON, P. H. Pollution by organic chemicals. *Science* 170(3957): 496 (30 Oct. 1970).

When manufacture of 2,4,5-T is controlled carefully, dioxin contamination is less than 1 ppm. Dioxin was identified in 1962 as the culprit of damage and death in 1957 of uncounted numbers of chicks. Dioxin produces neurological disturbances and is teratogenic. The acute LD_{50} for male guinea pigs is 0.000001 g/kg.

ANONYMOUS. HEW, USDA hold firm; 2,4,5-T ruling postponed. *Chem. Eng. News* 48(7): 11–12 (1970).

Because early studies indicated that most of the adverse toxicological effects of 2,4,5-T were due to high dioxin contamination (27 ppm) of test samples, the USDA did not feel obligated to cancel registration of currently manufactured 2,4,5-T containing about 1 ppm dioxin. Preliminary tests have shown that 2,4,5-T containing 1 ppm dioxin is not teratogenic. However, problems may still exist, since excessive heat exposure of the tri- or pentachlorophenols, used as intermediates in 2,4,5-T production, liberates dioxins.

ANONYMOUS. Government steps up pressure on pesticides; recent joint action restricting use of 2,4,5-T herbicide portends changes in pesticide regulations and testing. *Chem. Eng. News* 48(18): 60–61 (1970).

Evidence from Congressional hearings catalyzed the government's eventual suspension and cancellation of consumer and food crop usage of 2,4,5-T and curtailment of its use in Vietnam. HEW and the USDA issued a joint statement declaring that both 2,4,5-T and its dioxin contaminant may cause birth defects. The pesticide industry reacted adversely to these actions and statements.

ANONYMOUS. Pesticide policies scored at Ag meeting. *Chem. Eng. News* 48(42): 7 (1971).

The politics and pressures brought about by the 2,4,5-T/dioxin controversy between government and industry were reviewed. The Chairman of the Secretary's Pesticide Advisory Committee (SPAC) castigated both the federal government and the pesticides industry for failure to avoid the panicky crises which brought about an "era of chemical McCarthyism."

ANONYMOUS. The strange case of the government vs. 2,4,5-T. II. *Farm Chem.* 133(3): 26 (1970).

A review of the government involvement in the 2,4,5-T controversy which began after preliminary research reports indicated 2,4,5-T was teratogenic. These government studies were instituted in an attempt to determine whether 2,4,5-T or its dioxin contaminant was the causative agent of these effects.

ANONYMOUS. The 2,4,5-T identification parade. *Food Cosmet. Toxicol.* 8: 596–597 (1970).

Inconsistent test data on the teratogenicity of 2,4,5-T were thought resultant of the level of its chief contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Levels of 27 ppm have been found in some 2,4,5-T samples.

ANONYMOUS. Defoliants, deformities: What risk? *Med. World News* 11(9): 15–17 (1970).

Fertilized chicken eggs were injected with 2.5 pg (2.5×10^{-12} g) (50 ppt) of 2,3,6,7-tetrachlorodibenzo-*p*-dioxin. The dioxin caused leg deformities, cleft palates, and beak defects in 11 of 15 survivors from 25 eggs. Unhatched birds showed tissue edema, necrotic livers, and deformities similar to hatched birds.

ANONYMOUS. Another herbicide on the blacklist. *Nature* 226(5243): 309–311 (1970).

A commentary was presented to clarify the controversy of whether 2,4,5-T or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin was the causative teratogenic and mutagenic agent. Hearings with government and industrial representatives were held before the Subcommittee on Energy, Natural Resources and the Environment of the Senate Committee on Commerce.

ANONYMOUS. The tangled tale of 2,4,5-T. PANS 16(3): 421–422 (1970).

Use of 2,4,5-T has been restricted where foodstuffs for human consumption might be contaminated. The possibility exists that dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) may be the contaminant in 2,4,5-T formulations or that there may be a synergistic effect between 2,4,5-T and dioxin. Hypothetically, if dioxin contaminated the 2,4,5-T reported in food in the U.S. to the extent of 1 ppm, it would take 100 years for a normal person to accumulate a dose 600 times less than that which produced toxic effects during the pregnancy of a rat.

COURTNEY, K. D., GAYLOR, D. W., HOGAN, M. D., FALK, H. L., BATES, R. R., MITCHELL, I. Teratogenic evaluation of 2,4,5-T. *Science* 168(3933): 864–866 (May 15, 1970).

2,4,5-T samples were evaluated in rats and mice for teratogenicity. Results indicated that 2,4,5-T s.c. or orally administered was teratogenic and fetotoxic

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to the mouse and orally to the rat. Cleft palate and cystic kidneys were among the effects observed. A note added in proof indicated that the 2,4,5-T samples contained 30 ppm 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

DARSOW, G., and SCHNELL, H. Chlorodibenzo-*p*-dioxins. Ger. Patent 1,930,259, Appl. 13 June 1969, Granted 17 Dec. 1970, 10 pp (1970).

Chlorodibenzo-*p*-dioxins were prepared from tetra- or hexachlorobenzene. The compounds were useful for flameproofing polymers, e.g. polyesters.

DAY, B. E. 2,4,5-T and government decisions. *Proc. West. Soc. Weed Sci.* 23: 4–6 (1970).

Unpublished material which influenced the 2,4,5-T ban was held as questionable and suspect since preparations tested contained 27 ppm dioxin. It was stated that 2,4,5-T containing 1 ppm dioxin is not teratogenic; therefore, the alarm about the adverse effects of 2,4,5-T was unjustified.

EMERSON, J. L., THOMPSON, D. J., GERBIG, C. G., ROBINSON, V. B. Teratogenic study of 2,4,5-trichlorophenoxyacetic acid in the rat. *Toxicol. Appl. Pharmacol.* 17: 317 (1970).

The teratogenicity of 2,4,5-T containing 1 ppm of 2,3,7,8-tetrachlorodibenzo-*p*-dicxin was evaluated in rats. No teratogenic effects from 1,3,6,12, or 24 mg/kg/day 2,4,5-T or other pathological signs were observed. These results do not substantiate the adverse effects observed with 2,4,5-T containing 27 ppm dioxin.

EPSTEIN, S. S. A family likeness. *Environment* 12(6): 16–25 (July/August, 1970).

The human and environmental implications of the widespread chemical exposure problem have been accentuated by the increased use of herbicides during the past ten years. For example, although recently dioxin impurities in 2,4,5-T have been reduced, dioxin is still present; even minute concentrations are potential hazards. The analysis, formation, and teratogenic and other toxicological consequencies of dioxins were reviewed.

EPSTEIN, S. S. Testimony on teratogenic effects of 2,4,5-T formulations. U.S. Senate Hearing before the Subcommittee of Energy, Natural Resources and the Environment of the Committee on Commerce (April 15, 1970).

Polychlorophenol contaminants and their dioxin pyrolytic products in phenoxy herbicides are of toxicologic and environmental consequence. Some dioxins are highly toxic and teratogenic at the microgram per kilogram level; most dioxins are, however, toxicologically uncharacterized.

GALSTON, A. W. Herbicide usage. *Science* 168 (3939): 1607 (1970).

The phenoxyacetic acid herbicides should not be used until all questions regarding the teratogenicity of 2,4,5-T have been resolved. A tetrachlorodibenzodioxin impurity found in some commercial preparations of 2,4,5-T was the teratogenic agent in 2,4,5-T samples tested for teratogenicity by the Bionetics Research Laboratories. The possibility that phenoxyacetic acids may be degraded in plants and soil or by fire or bright sunlight into dioxin-like teratogens should be studied. Extensive research should be accomplished on all aspects of the biomedical and environmental consequences of dioxins.

Hearings Before the Subcommittee on Energy, Natural Resources and the Environment of the Committee on Commerce, U. S. Senate, Serial 91–60, Governmental Printing Office, Washington, D.C. (1970).

An impurity in 2,4,5-trichlorophenol was found to be 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a compound with a high mammalian toxicity and teratogenic effect.

HORWITZ. W. (Ed.). Official Methods of Analysis, 11th Ed. Association of Official Analytical Chemists, Washington, D.C., 1970, p. 468 (28.109).

The official first action gas chromatographic method for chick edema factor is presented in Section 28 on oils and fats. Fat, oil, fatty acid, or lipid is treated with H_2SO_4, and extracted with petroleum ether. The extract is purified on an Al_2O_3 column, further treated with H_2SO_4, and examined by electron capture-GLC. Peaks with retention times relative to aldrin (R_a) between 8 and 45 indicate presence of chick edema factors (hexa-, hepta-, and octachlorodibenzo-*p*-dioxins).

KEARNEY, P. C. Paper presented before a joint (United Kingdom, Canada, and United States) meeting on Pesticides, Washington, D.C., (November 5, 1970).

The Agricultural Research Service, USDA, began a program to assess the significance of chlorinated dioxin impurities in currently registered pesticides. Theoretically, any pesticide with a chlorinated phenoxy nucleus or which is derived from a chlorinated phenol precursor could contain chlorinated dioxin contaminants.

GENP 011879

784640

RESS, J., HIGGINBOTHAM, G. R., and FIRESTONE, D. Methodology for chlorinated aromatics in fats, oils, and fatty acids. *J. Assoc. Offic. Anal. Chem.* 53(3): 628–634 (1970).

Following review of the current status of chemical and biological methods for chlorophenols and chlorinated dibenzo-*p*-dioxins in fats, oils, and fatty acids, the modified electron capture-gas liquid chromatography method for the detection of chick edema factors was recommended for adoption as official first action to replace all existing gas-liquid chromatography methods.

ROBSON, J. M. Testing drugs for teratogenicity and their effects on fertility: The present position. *Brit. Med Bull.* 26(3): 212–216 (1970).

The problems of interpreting test results involving teratogenic compounds were outlined using data gathered on the teratogenicity of 2,4,5-T and its dioxin contaminant.

SPARSCHU, G. L., DUNN, F. L., and ROWE, V. K. Teratogenic study of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the rat. *Toxicol. Appl. Pharmacol.* 17(1): 317–318 (1970).

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was studied to determine whether this 2,4,5-T impurity could account for fetal abnormalities in test animals. Rats received 0, 0.03, 0.125, 0.5, 2.0, and 8.0 μg/kg body weight/day on days 6 to 15 of gestation. At the 0.5 level, the number of fetuses was reduced, and the number of resorptions and fetal deaths was increased.

VOS, J. G., and KOEMAN, J. H. Comparative toxicologic study with polychlorinated biphenyls in chickens with special reference to porphyria, edema formation, liver necrosis, and tissue residues. *Toxicol. Appl. Pharmacol.* 17: 656–668 (1970).

Briefly stated were the concerns and evidence pertaining to dioxins, particularly to the chick edema factor and whether PCBs and/or the dioxins were the culprit(s).

VOS, J. G., KOEMAN, J. H., VAN DER MAAS, H. L., TEN NOEVER DE BRAUW, M. C., and DE VOS, R. H. Identification and toxicological evaluation of chlorinated dibenzofuran and chlorinated naphthalene in two commercial polychlorinated biphenyls. *Food Cosmet. Toxicol.* 8(6): 625–633 (1970).

Three commercial polychlorinated biphenyl compounds (Phenoclor DP6, Clophen, and Aroclor 1260) were analyzed for impurities. In two of these (Phenoclor DP6 and Clophen A60) pentachlorodibenzofuran and tetrachlorodibenzofuran were among the polar compounds found.

1969

CANTRELL, J. S., WEBB, N. C., and MABIS, A. J. Identification and crystal structure of a hydropericardium-producing factor: 1,2,3,7,8,9-hexachloro-dibenzo-*p*-dioxin *Chem. Abstr. Acta. Crystrallogr.* B25(1): 150–156 (1969); 70: 51805u (0000).

Chemical identity of hexachlorodibenzo-*p*-dioxin, one of the toxic substances capable of producing the chick edema disease, was established by x-ray crystallography of a sample of edema-inducing lipid material.

RIED, W., and ENG, J. T. S. Reactions with diazocarbonyl compounds. *Ann. Chem.* 727: 219–221 (1969) (Ger.); *Chem. Abstr.* 72: 12458e (0000).

o-Quinone diazides added benzene with nitrogen elimination to yield chloro substituted dibenzofurans: 1,2,3,4-tetrachloro-, 1,2,3,-trichloro-, and 1,3-dichloro-.

1968

HIGGINBOTHAM, G. R., HUANG, A., FIRESTONE, D., VERRETT, J., RESS, J., and CAMPBELL, A. D. Chemical and toxicological evaluations of isolated and synthetic chloro derivatives of dibenzo-*p*-dioxin. *Nature* 220(5168): 702–703 (1968); *Chem. Abstr.* 70:18509c (0000).

Commercial chlorophenols including 2,4-dichlorophenol, 2,4,5,-trichlorophenol, and 2,3,4,6-tetrachlorophenol, were pyrolyzed to chlorinated dibenzo-*p*-dioxins. These were examined by electron capture-gas liquid chromatography and tested bibliogically by the chick embryo assay. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was prepared by direct chlorination of dibenzo-*p*-dioxin. These dioxins proved to be chick edema factors (hydropericardium factors) and toxic.

1967

ANONYMOUS. Search for chick edema factor. *Chem. Eng. News* 45(5): 10 (Jan. 30, 1967).

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GENP 011830

Chicks afflicted with edema, or hydropericardium, suffer an accumulation of fluid in the heart sac and gross kidney and liver damage; as little as 5 μg can kill a chick. X-Ray crystallographers have determined the molecular structure of one of the toxic compounds known as the chick edema factor. Single-crystal structural analysis showed that the structure is 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin. Two crystals, each about 0.2×0.1×0.1 mm and weighing about 3μg were recrystalized from a benzene – hexane solution. The final data showed that the 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin molecules are nearly planar, and are packed in the 044 crystallographic planes, with an interplanar separation of about 3.3 A. No unusual bond lengths or angles were apparent.

1965

COX, J. M., WRIGHT, B. A., and WRIGHT, W. W. Thermal degradation of poly(phenylene oxides). *J. Appl. Polymer Sci.* 9(2): 513–522 (1965).

The thermal degradation in vacuum of various phenylene oxide polymers and copolymers was studied by a weight-loss method. Thermal stability decreased with increasing substitution in the aromatic nuclei. This may be due to the presence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, formed as an alternate reaction product. Chloro derivatives were more stable than the corresponding bromo derivatives, except for the tetra-substituted phenylene oxides.

KULKA, M., Perhalodibenzo-*p*-dioxins. Can. Patent 702,144, Appl. May 3, 1961; Granted Jan. 19, 1965, 13 pp. (1965)

Octahalodibenzo-*p*-dioxins, useful as chemical intermediates, biocides, and flame retardants, were prepared by using a pentahalophenol in the presence of a catalytic amount of halogen or a halogenated cyclohexadienone at 200–400°C. An 83% yield was attained for octachlorodibenzo-*p*-dioxin.

1962

JONES, E. L., and KRIZEK, H. A technique for testing acnegenic potency in rabbits, applied to the potent acnegen 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Invest. Dermatol.* 39: 511–518 (1962).

Hyperkeratinization of the inner surface of the rabbit ear was induced by topical application of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in acetone. The weight of keratin served as a criterion of acnegenic activity.

KULKA, M., Octahalodibenzo-*p*-dioxins. Belg. Patent 616,197, Appl. May 2, 1961; Granted July 31, 1962, 9 pp. (1962)

Octahalodibenzo-*p*-dioxins were prepared by heating pentahalophenols in the presence of catalytic amounts of halogen or halocyclohexadiene. For example, 40 g pentachlorophenol and 2 g 2,3,4,4,5,6-hexachloro-2,5-cyclohexadienone in 120 ml 1,2,4-trichlorobenzene was refluxed at 213°C to give 30 g of octachlorodibenzo-*p*-dioxin.

1961

BAUER, H., SCHULZ, K. H., and SPEIGELBERG, U. Occupational intoxications in manufacturing chlorophenol compounds. *Arch. Gewerbepath. Gewerbehyg.* 18: 538–555 (1961).

Thirty-one workers in an industry manufacturing 2,4,5-trichlorophenol by sodium hydroxide saponification of 1,2,4,5-tetrachlorobenzene exhibited acne and symptoms of psychopathologic disturbances. Solutions of 0.01 to 0.05% of 2,3,6,7-tetrachlorodibenzo-*p*-dioxin painted on rabbit ears produced acne; oral administration of 0.05–0.1 mg/kg caused serious liver damage.

KULKA, M. Octahalodibenzo-*p*-dioxins. *Can. J. Chem.* 39: 1973–1976 (1961).

Nearly 100% conversion from pentahalophenols to 1,2,3,4,6,7,8,9-octahalo-dibenzo-*p*-dioxins was accomplished by using reaction initiators such as halogens or halogen-generating compounds.

1960

DENIVELLE, L., FORT, R., and VAN HAI, P. Octachloro- and octabromodibenzo-*p*-dioxins and decachloro- and decabromodiphenyl ether. *Bull. Soc. Chim. France* 1960. 1538–1543 (1960).

Pentachlorophenol was pyrolyzed at 300°C extracted with carbon tetrachloride and aqueous alkali, recrystalized from phenyl nitrite, and yielded "perchlorophenylene oxide" (melting point of 326°C). Steric considerations dictated ring fusion in the 1,2-position leaving octachlorodibenzo-*p*-dioxin the only acceptable structure for perchlorophenylene oxide. Sodium 2,4-dichlorophenate gave dichlorodibenzo-*p*-dioxin.

KAUPP, J., and KLUG, H. Perchlorodiphenylene dioxide. Ger. Patent 1,092,480, Appl. June 27, 1956, Granted Nov. 10, 1960 (1960).

Octachlorodibenzo-*p*-dioxin was prepared by heating pentachlorophenol and 2,3,4,4,5,6-hexachloro-2,5-cyclohexadien-1-one; the yield was 84%.

GENP 011831

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1959

TOMITA, M., UEDA, S., and NARISADA, M. Dibenzo-*p*-dioxin derivatives. XXVII. Synthesis of polyhalodibenzo-*p*-dioxin. *Yakugaka Zasshi* 79: 186–192 (1959).

Pentachlorophenol was heated at 300°C for 12 hr. Recrystallization of the product from benzene, yielded octachlorodibenzo-*p*-dioxin needles.

1958

SANDERMANN, W., CASTEN, R., KRASTING, W., and PIEPER, J. Studies on wood-protection chemistry. IX. Investigations concerning oily blue stain protection agents. *Holzforsch. Holsverwert.* 10(4): 57–66 (1958).

Compounds formed by heat treatment of pentachlorophenol such as octachlorodiphenylene oxide were inactive for preventing blue stain. Tetrachlorodiphenylene oxide was especially toxic to man and beast.

1957

GILMAN, H., and DIETRICH, J. J. Halogen derivatives of dibenzo-*p*-dioxin. *J. Amer. Chem. Soc.* 79: 1439–1441 (1957).

Dibenzo-*p*-dioxin was synthesized and reacted with chlorine gas to form 2-chlorodibenzo-*p*-dioxin; a similar run coupled with ultraviolet light gave 2,7-dichlorodibenzo-*p*-dioxin.

KIMMIG, J., and SCHULZ, K. H. Chlorinated-aromatic cyclic ethers as a cause of the so-called chloracne. *Naturwiss.* 44: 337–338 (1957) (Ger.).

Trichlordibenzo furan, tetrachlorodibenzofuran, and 2,3,6,7-tetrachlorodibenzodioxin were especially effective acne-inducing agents on rabbit ears. 2,3,6,7-Tetrachlorodibenzodioxin proved most toxic; the characteristic changes were achieved with concentrations of 0.01 to 0.002%. Higher concentrations caused liver necrosis and death. Single oral administration of 0.05 to 0.1 mg/kg body weight caused death in 1–2 weeks. Autopsy revealed necrosis and fatty degeneration of the liver. Pure 2,4,5-trichlorophenol and pentachlorophenol did not cause chloracne.

KIMMIG, J., and SCHULZ, K. H. Occupational acne (so-called chloracne) due to chlorinated aromatic cyclic ethers. *Dermatologia* 115 (4): 540–546 (1957) (Ger.).

The agent causing occupational chloracne in 31 employees of chlorophenol producing factories was found to be 2,3,6,7-tetrachlorodibenzodioxin. The toxic action in animals was due to the alkaline hydrolysis product of 1,2,4,5-tetrachlorobenzene. Tetrachlorodibenzo-*p*-dioxin and tri- and tetrachlorodibenzofuran were active skin irritants. 2,3,6,7-Tetrachlorodibenzodioxin formation from sodium trichlorophenolacetate was established as was isolation from the by-products.

SANDERMANN, H. S., CASTEN, R., and STOCKMANN, H. Pyrolysis of pentachlorophenol. *Chem. Ber.* 90: 690–692 (1957).

Octachlorodiphenylene dioxide was formed after pentachlorophenol was heated at 300°C for 24 hr, distilled at 320°C, and crystallized from benzene or extracted with benzene and fractional crystallization. 2,3,7,8-Tetrachlorodiphenylene dioxide was active against insects and wood-destroying fungi; octachlorodiphenylene dioxide was inactive.

SCHULZ, K. H. Clinical and experimental studies on the etiology of chloracne. *Arch. Klin. Exp. Derm.* 206: 589–596 (1957).

The acnegenic properties occurring as a refractive dermatologic condition in factory workers involved in the production of chlorinated aromatic compounds were ascribed to TCDD, an unwanted side product in the sythesis of 2,4,5-T.

1955

OITA, K., JOHNSON, R. G., and GILMAN, H. The chlorination of dibenzofuran and some of its derivatives. *J. Org. Chem.* 20: 657–667 (1955).

Step-by-step reaction procedures were presented for the chemical synthesis of chlorinated dibenzofurans, i.e., 2-chloro-, 2,8-dichloro-, 3-chloro-, and a chlorodibenzofuran.

1954

CERNIANI, A., PASSERINI, R., and RIGHI, G. Ultraviolet spectra of some dibenzofurans. *Boll. Sci. Fac. Chim. Ind. 12:* 75–79 (1954).

2-Chloro- and 3-chloro-dibenzofurans were characterized by ultraviolet spectrometry. These derivatives had little effect on spectra of the parent compound.

1953

JULIA, M., and BAILLARGE, M. Growth factors in plants. III. (1-Carboxymethyl-2-naphthoxy)acetic acid and (2-carboxymethyl-4-chlorophenoxy) acetic acid. *Bull. Soc. Chim. France.* 640–643 (1953).

In the preparation of 2,4-dichlorophenol, a portion of the mixture was insoluble in benzene–water; the substance was extracted with boiling ligroine and recrystallized to yield 2,7-dichlorodibenzo-*p*-dioxin.

784643

GENP 011832

1952

SHIBATA, I. S., NATORI, S., and SUMI, Y. Antibacterial effects of lichen substances and their related compounds. *J. Pharm. Soc. Japan* 72: 1333–1336 (1952).

The chlorinated dibenzofurans synthesized and tested for antibacterial activity included: 2-chloro-, 3-chloro-, 2,8-dichloro-, and 3,8-dichloro-. The chlorine atom, when in the 8-position of the dibenzofuran ring, increased its antibacterial effect.

1951

TOMITA, M., and WATANABE, W. Antibacterial activity of some organic compounds in vitro. II. Antibacterial activity of dibenzo-*p*-dioxin, phenoxathiin, and 1,4-benzodioxan derivatives on *Mycobacterium tuberculosis, Staphylococcus aureus,* and *Escherichia coli. J. Pharm. Soc. Japan* 71: 1204–1206 (1951).

2,7-dichlorodibenzo-*p*-dioxin was tested for antibacterial activity and found to be inactive.

1941

HIGASI, K. The molecular structures of diphenylene dioxide and phenoxathliin as revealed by dipole-moment data. *Sci. Papers Inst. Phys. Chem. Research* 38: 331–340 (1941).

2,6-Dichlorodiphenylene dioxide exhibited a dipole moment of 0.62 Debyes. This indicated a folded molecule and no optical isomers.

HIGASI, K., and UYEO, S. Polarity and molecular structure of diphenylene dioxide. *J. Chem. Soc. Japan* 62: 396–399 (1941).

2,6-Dichlorodiphenylene dioxide was found to have a small but finite dipole movement. This molecule and the parent probably fold along the line joining the two oxygen atoms.

UEO, S. 2,6-Dichlorodiphenylene dioxide. *Bull. Chem. Soc. Japan* 16: 177–179 (1941).

Synthesis of 2,6-dichlorodiphenylene dioxide, a light yellow compound with a melting point of 207°C and a dipole moment of 0.62 was described. The dipole movement of 2,6-dichlorodiphenylene dioxide was somewhat less than the dipole moment of diphenylene dioxide which was 0.64.

1939

British Thompson-Houston Co. Ltd. Electric insulation. Brit. Pat. 506,560 (May 31, 1939).

Direct chlorination of diphenylene oxide in the presence of a catalyst produced solid, waxlike products of high dielectric constants; i.e., mixture of chlorinated diphenylene oxide and pentachlorobiphenyl.

CLARK, F. M. Chlorinated diphenylene oxide composition for treating paper in capacitors, U.S. Patent 2,198,473 (April 23, 1939).

Chlorinated diphenylene oxide containing 1 to 4 atoms of chlorine per molecule imparts a constant electrical capacity when used to impregnate paper in capacitors.

1936

BELL, F. Pyrolysis of chlorophenols. *J. Chem. Soc.* 1936: 1244.

o-Chlorophenol was pyrolyzed at 180–340°C; diphenylene oxide and 3,6-dichlorodiphenyl oxide were isolated from the distillate.

1934

GILMAN, H., BROWN, G. E., BYWATER, W. G., and KIRKPATRICK, W. H. Dibenzofuran. III. Nuclear substitutions. *J. Amer. Chem. Soc.* 56: 2473–2477 (1934).

2-Chloro- and 2,8-dichlorodibenzofuran were synthesized. Dibenzofuran and chlorine in carbon tetrachloride gave 38% of the 2,8-dichloro derivative which had a melting point of 185°C. Dibenzofuran in alcohol at 60°C gave the 2-chloro derivative which had a melting point of 102.5°C.

GENP 011833

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Erratum

The following errors were made in the article by Ulrich R. Hoegg, cigarette smoke in closed spaces, experimental issue no. 2, October 1972.
(1) Figure 2, left coordinate should read co(ppm) rather than co(ptm)
(2) References 40–57 were omitted and are as follows:

40. National Center for Health Statistics. 1970. Changes in Cigarette Consumption Between June 1966 and August 1968. U. S. Public Health Service. Monthly Vital Statistics Report, Health Interview Survey Data. 12:1-4.
41. Boaz, J. N. (Ed.) 1970. Architectural Graphic Standards, Wiley & Sons, Inc. New York, p. 13.
42. Am. Soc. Heat. Rad. Air. Eng. 1964. Guide and Data Book: Applications. Ashrae. New York. p. 261.
43. Am. Soc. Heat. Rad. Air. Eng. 1963. Guide and Data Book: Fundamentals and Equipment. Ashrae. New York. p. 432.
44. Ibid. p. 260.
45. Callender, J. H., (Ed.) 1966. Time-Saver Standards: A Handbook of Architectural Design. McGraw Hill Book Company, New York, pp. 734 and 749.
46. Turk, A. 1963. Measurements of Odorous Vapors in Test Chambers: Theoretical. Am. Soc. Heat. Rad. Air. Eng. Journal. 5:55-58.
47. Am. Conf. Gov. Ind. Hyg. 1970. Industrial Ventilation. A Manual of Recommended Practice. 11th Edition, p. 2-1.
48. Atkinson, W. O. 1970. Production of Sample Cigarettes for Tobacco and Health Research. In: Griffith. R. B. (Director) Proceedings of the Tobacco and Health Conference, Conference Report—2. University of Kentucky, Tobacco and Health Research Institute, 2:28-30.
49. Wartman, W. B., Cogbill, E. C. and Harlow, E. S. 1959. Determination of Particulate Matter in Concentrated Aerosols. Analytical Chemistry. 31:1705-1709.
50. Morse, K. M., Bumsted, H. E. and Janes, W. C. 1971. The Validity of Gravimetric Measurements of Respirable Coal Mine Dust. Amer. Indust. Hygiene Assoc. Journal. 32:104-114.
51. Benner, J. F. 1970. Tentative Summary of Leaf and Smoke Analysis of the University of Kentucky Reference and Alkaloid Series Cigarettes. In: Griffith, R. B. (Director) Proceedings of the Tobacco and Health Conference, Conference Report—2. University of Kentucky, Tobacco and Health Research Institute. 2:30-34.
52. Brief, R. S. 1960. Simple Way to Determine Air Contaminants. Air Engineering. 2:39-41.
53. Owens, D. F. and Rossano, A. T. 1969. Design Procedures to Control Cigarette Smoke and Other Air Pollutants. Am. Soc. Heat. Rad. Air. Eng. Transactions, p. 93-102.
54. Johansson. C. R. and Ronge, H. 1965. Acute Irritation Effects of Tobacco Smoke in Room Air. Nordisk Hygienisk Tidskrift. 46:49-50.
55. Vernot, E. H. 1968. Contaminant Trapping and Effect of Surface Adsorption on Atmospheric Concentration of Contaminants. In: Proceedings of the 4th Annual Conference on Atmospheric Contamination in Confined Spaces, 10-12 September. (AMRL—TR—68—175).
56. Morrow, P. E. 1964. Evaluation of Inhalation Hazards Based Upon the Respirable Dust Content and the Philosophy and Application of Selective Sampling. Am. Indust. Hygiene Assoc. Journal. 25:213-236.
57. J. Air Pollution Control Assoc. 1971. Environmental Protection Agency Sets National Air Quality Standards. 21:352-353.

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GENP 011834