Table 5. Mortality of 2,4-D and 2,4,5-T exposed workers during the follow-up period of 1972-1976. Expected numbers of deaths are based on the death rates of the total Finnish male population.

Cause of death (age, years)	Exposure started in the years						Total		
	1955-1965			1966-1971					
	Observed number of deaths (O)	Expected number of deaths (E)	Person-years	(O)	(E)	Person-years	(O)	(E)	Person-years
Malignant neoplasms									
15-24	-	0.0	52	-	0.1	1635	-	0.1	1687
25-34	-	0.1	728	-	0.3	1567	-	0.4	2295
35-44	-	0.4	918	-	0.5	1256	-	0.9	2174
45-54	1	1.5	937	-	2.0	1233	1	3.6	2170
55-64	4	4.2	690	2	4.1	671	6	8.3	1361
65-74	1	3.8	294	-	0.8	64	1	4.6	358
75 and over	-	0.2	9	-	-	-	-	0.2	9
Total	6	10.2	3628	2	7.8	5826	8	18.1	9454
All natural causes of death									
15-24	-	0.0		-	0.3		-	0.3	
25-34	-	0.5		-	1.0		-	1.5	
35-44	2	2.2		1	3.1		3	5.3	
45-54	3	8.1		6	10.6		9	18.7	
55-64	7	15.7		6	15.3		13	31.0	
65-74	8	15.4		1	3.3		9	18.7	
75 and over	-	1.2		-	-		-	1.2	
Total	20	43.1		14	33.6		34	76.7	

Table 6. Mortality of 2,4-D and 2,4,5-T exposed workers by employer during the follow-up period of 1972-1976. Expected numbers of deaths are based on the death rates of the total Finnish male population.

Cause of death	State Railways		Highway Authority		Forestry Authority		Power company	
	Observed deaths (O)	Expected deaths (E)	(O)	(E)	(O)	(E)	(O)	(E)
All malignant neoplasms	7	7.2	1	3.6	-	6.4	-	0.9
Respiratory cancer	4	3.1	-	1.5	-	2.6	-	0.3
All natural causes of death	15	29.8	9	15.6	7	27.7	3	3.6

Table 7. Localisation of malignant neoplasms among 2,4-D and 2,4,5-T exposed workers.

Observation period	Localisation					Total
	Lung	Larynx	Stomach	Caecum	Prostate	
1955-1971	7	2	4			13
1972-1976	4	1	1	1	1	8

(9)

Strictly Confidential

Chlorinated Dibenzo-p-dioxins and Dibenzofurans: Review of Toxicology and Enzyme Induction and Consideration of Mechanism of Toxicity.

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Alan Poland

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The chemistry, toxicological and biochemical effects of the chlorinated dibenzo-p-dioxins and dibenzofurans have been extensively investigated in the past few years, and this information has been thoroughly reviewed in a number of symposia and reports. Despite these efforts, at present little is known about how these compounds exert their toxicity. In this report I would like to briefly outline the major toxic actions of these compounds, consider selected biochemical effects and propose a model for their mechanism of toxicity.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the prototype of the group; it is the most potent toxin and the most thoroughly studied. The major toxic effects produced by TCDD compound are summarized below.

1) Chloracne - The most frequent sign of toxicity observed among chemical workers exposed to TCDD has been chloracne, comedone formation, with or without cysts and pustules, often widespread in body distribution, and very persistent and refractory to treatment. Chloracne has been produced experimentally by TCDD in humans, monkeys, rabbits, and hairless mice. The lesion is characterized by hyperkeratosis, acanthosis, and keratinous material producing cystic dilitation of sebaceous glands.

2) Hepatotoxicity - The liver toxicity produced by TCDD varies greatly with the species. In the rabbit and the rat, there is severe liver damage, with parenchymal cell necrosis, fatty infiltration, hyaline bodies, formation of multinucleate giant cells and signs of hepatic dysfunction such as hyperbilirubinemia and hypoalbuminemia. Hepatic damage is less extensive in mice, and minimal and focal in guinea pigs, the species most sensitive to the toxic effects of TCDD. Hepatic porphyrin accumulation and/or porphyria was observed reported in mice, rats, and humans exposed to TCDD.

3) Thymus and Lymphoid Organs - TCDD produces a dose related decrease in the mass of the thymus and a less dramatic change on the lymph nodes and lymphoid tissue of the spleen. There is a rapid atrophy of the cortical cells (T-cells) of the thymus, and in the rat, mouse and guinea pig a suppression of the cell-mediated immune response.

4) Teratogenesis - TCDD is embryotoxic and teratogenic to fetal mice, rats, and guinea pigs. Fetal resorption, cleft palate, renal lesions, and edema of the soft tissue have been reported.

5) Chick edema - In newborn chicken, TCDD produces an edematous syndrome characterized by anasarca, and hydropericardium.

6) Other effects - TCDD and other dioxins have been reported to cause a number of other types of toxicity: neuromuscular effects, epithelial atrophy, metaplasia, and hyperplasia, hemorrhage in several tissues, gonadal atrophy, hyperlipidemia, depression of the blood forming elements in bone marrow; and lesions of the endothelium, myocardium, and kidney.

Qualitatively and quantitatively the histopathology produced by TCDD differs with different species, and with acute versus chronic administration. Following the administration of a lethal dose of TCDD, the animal loses weight, exhibits decreased motor activity, and eventually dies after a period of several weeks. Sensitivity to the lethal effects of TCDD varies greatly with species: the acute oral LD_{50} is 1 ug/kg in guinea pigs; 22 ug/kg in male rats; 45 ug/kg in female rats; 115 ug/kg in the rabbit; 100-250 ug/kg in the mouse; and greater than 300 ug/kg in the dog.

Pharmacokinetics

Following acute or chronic administration of TCDD to a rat, the drug is accumulated primarily in the liver and to a lesser extent the fat, eliminated largely in the feces with a whole body half-life of about 21 days. Numerous attempts to demonstrate the metabolism of TCDD in vitro or isolate a

metabolite *in vivo* have failed, however chronic feeding studies using radiolabeled TCDD suggest a more polar radiolabeled compound appears in urine in amounts too small to identify chemically. Direct demonstration of the metabolism of TCDD is lacking, but if metabolism occurs, it proceeds very slowly. The acute toxicity of TCDD appears to be due to the parent compound.

Mutagenicity and Carcinogenicity

There are two reports that TCDD is mutagenic in bacteria, but no consensus has been reached on this. The data is amply reviewed in the IARC report.

Two recent reports indicate that chronic life-time administration of low levels of TCDD to rats is associated with an increased incidence of neoplasia. The study by the Dow Chemical Co. (as yet to be published) states: "In those rats receiving 0.1 ug/kg/day (but not 0.001 or 0.01 ug/kg/day) there were discernable increases in the incidences of hepatocellular carcinomas and squamous cell carcinomas of the lung, hard palate/nasal turbinates or tongue". Van Miller, Lalich and Allen reported tumors in rats fed TCDD 5, 50, 500, 1000 and 5000 ppt in the diet. The low dose 5 ppt is equal to 0.14 ng/kg/day or a life time dose of 95 ng/kg. In contrast to the Dow study, these authors found neoplasms at a 700 fold lower concentration of TCDD in the diet, the neoplasms were of many types, and the incidence of neoplasia was not dose-related.

While these two reports suggest that the chronic administration of TCDD is associated with an increase incidence of neoplasms, they do not indicate whether TCDD is an initiator or a promoter. This consideration is particularly important because we lack unequivocal evidence that TCDD is a mutagen and that TCDD is metabolized, and there is no evidence that TCDD and/or its metabolite(s) covalently bind to macromolecules.

Induction of Aryl Hydrocarbon Hydroxylase Activity

The administration of TCDD and other halogenated dibenzo-p-dioxins and dibenzofurans stimulates a number of enzyme activities, most notably in the liver. I will confine my remarks primarily to the induction of hepatic aryl hydrocarbon hydroxylase (AHH) activity also called benzo(a)pyrene hydroxylase. AHH activity is one measure of microsomal monooxygenase function. The microsomal enzyme complex which consists of NADPH-cytochrome P-450 reductase, and several cytochrome P-450 subspecies (or isoenzymes), is responsible for the metabolism of many lipophilic xenobiotics to more polar metabolites.

Since many chlorinated lipophilic compounds which have long biological half-lives, such as DDT, dieldrin, lindane, and the polychlorinated biphenyls stimulate various hepatic microsomal monooxygenase activities, it comes as no great surprise that the chlorinated dibenzo-p-dioxins and dibenzofurans also have this capacity. However, rather than regarding this biochemical effect as common place, I wish to suggest that it is telling us something about the mechanism of toxicity of TCDD and its congeners.

In the chicken embryo, TCDD produced a dose-related increase in hepatic AHH activity, with an ED_{50} (dose that produced one half the maximal response) of 0.3 nmol/kg. In testing an initial series of 15 halogenated dibenzo-p-dioxin congeners, a well-defined structure-activity relationship was observed: The congeners which induced AHH activity had 1) halogen atoms in at least three of the four lateral ring positions (2,3,7 and 8); a) at least one carbon atom was unsubstituted (octachlorodibenzo-p-dioxin was inactive); and 3) the order of potency of substitution was Br> Cl > F, NO_2. TCDD also produced a dose-related increase in hepatic δ -aminolevulinic acid synthetase

(ALAS) activity in the chicken embryo, and the structure-activity relationship for the induction ALAS activity by the 15 halogenated congeners was the same as that observed for induction of AHH activity. Many more halogenated dibenzo-p-dioxins and dibenzofurans have been studied for their capacity to induce AHH activity in the chicken embryo liver or in a hepatoma cell culture (Bradlaw and Firestone) and the structure-activity relationship outlined above has prevailed.

The most significant finding was that there was an excellent correlation between the potency of chlorinated dibenzo-p-dioxin congeners to induce AHH activity (and ALAS activity) and their toxic potency. The work of Schwetz and his coworkers suggests that for a given chlorinated dibenzo-p-dioxin which has a certain lethal potency (low LD_{50}), it has a corresponding potency as a teratogen, acnegen, and potency in producing chick edema. The recent study by McConnell et al. on the comparative toxicity of 13 chlorinated dibenzo-p-dioxins in mice and guinea pigs support this idea, that is, the relative potency (or rank order) of a congener to produce one toxic response is a good indicator of its relative potency (or rank order) to produce other toxic manifestations.

Hepatic Cytosol Binding Protein

Recently, a macromolecular binding species has been characterized in the hepatic cytosol fraction of rat and mouse liver which has the in vitro binding properties predicted for the receptor for the induction of AHH activity based on the in vivo biology. Namely: 1) ^{3}H-TCDD binds to this cytosol protein reversibly with a high affinity (Kd = 0.27nM) comparable to the ED_{50} for hepatic AHH induction (ED_{50} in mice $\sim$1 nmole/kg); 2) the binding affinity of halogenated dibenzo-p-dioxins and dibenzofurans for this protein in vitro corresponds to their potency to induce hepatic AHH activity in the chicken embryo; 3) other compounds, such as the polycyclic aromatic hydrocarbons, which induce AHH activity and cytochrome P_1-450 also compete for this cytosolic binding protein, but compounds which

induce other types of microsomal monooxygenase activities (e.g., phenobarbital) and steroids fail to bind. Thus it would appear this cytosolic binding protein is the receptor for the induction of AHH activity.

Structure-Activity Relationship

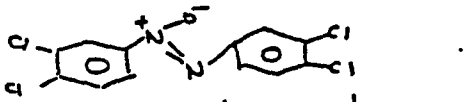
The structure-activity relationship for the induction of hepatic AHH activity and for binding to the hepatic cytosol binding species has been extended to other classes of chlorinated aromatic compounds, and again there appears to be an excellent correlation between their capacity to induce AHH activity and their potency to produce specific toxic effects similar to those produced by TCDD (e.g., chloracne, thymus atrophy, and chick edema).

3,4,3'4'-Tetrachloro azoxybenzene (TCAOB) and azobenzene (TCAB) are potent acnegens formed as trace contaminants in the synthesis of 3,4 dichloroaniline or herbicides based on this compound. At high doses in animals, TCAB is reported to produce thymic involution and liver damage similar to TCDD. Both TCAOB and TCAB are potent inducers of hepatic AHH activity and bind to the hepatic cytosol binding protein with a high affinity. Congeners such as 3,5,3'5'-tetrachloro azoxybenzene and azobenzene fail to induce AHH activity, fail to bind to the hepatic cytosol species, and fail to produce chloracne.

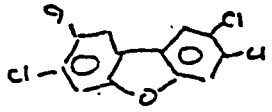
Of 16 halogenated biphenyl tested, only 3,4,3'4'-tetrachloro-, 3,4,5,3'4'5'-hexachloro- and 3,4,5,3'4'5'-hexabromo-biphenyls induced hepatic AHH activity and bound to the hepatic cytosol binding species. The 3,4,3'4'-tetrachlorobiphenyl has been reported to produce chloracne. McKinney et al. found that of five hexachlorobiphenyls tested in chickens, 3,4,5,3'4'5'-hexachlorobiphenyl was by far the most toxic, and the only one to produce significant chick edema and involution of the thymus.

From these studies we can generalize about the receptor site for binding halogenated aromatic compounds and initiating the induction for AHH. 2,3,7,8-Tetrachlorodibenzo-p-dioxin, 2,3,7,8 tetrachlorodibenzofuran, 3,4,3'4'-tetrachloroazoxybenzene and 3,4,3'4'-tetrachlorobiphenyl are approximate isostereomers.

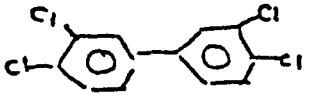
2,3,7,8-Tetrachlorodibenzo-p-dioxin



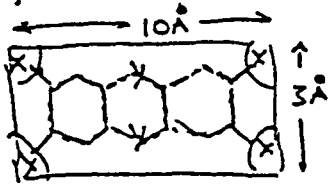
3,4,3'4'-tetrachloroazoxybenzene



2,3,7,8-Tetrachlorodibenzofuran



3,4,3'4'-tetrachlorobiphenyl



All of these compounds are planar or can assume a nearly planar configuration with halogens at 4 corners of a rectangle approximately 3Å by 10Å. We have synthesized a number of other tetrahaloheterocyclics - such as anthracene, phenanthrene, biphenylene, and phenoxazine - all of which bind to the hepatic cytosolic binding species and induce AHH activity.

It remains to be proven whether all halogenated aromatics which bind to this receptor and induce AHH activity do produce TCDD-like toxicity. At present to the extent the data is available, I am not aware of any exceptions. However we must not forget many nonchlorinated polycyclic aromatic hydrocarbons bind to this site and induce AHH activity, but it has not been noted they elicit any TCDD-like toxicity.

A Model for Mechanism of Toxicity

The case I have tried to present is that there is a striking correlation for chlorinated dibenzo-p-dioxin, dibenzofurans and other chlorinated aromatic congeners between their potency to induce AHH activity and their toxic potency to produce specific lesions (chloracne, thymic involution, chick edema). This correlation is an appropriate starting point in considering a molecular mechanism of toxicity.

A) Older Theories - AHH Induction Per Say 1) It has been suggested that the chronic stimulation of microsomal monooxygenase activity produce by TCDD might result in excessive inactivation of endogenous compounds (e.g., steroids) or produce excessive harmful metabolites of endogenous compounds. 2) It has been suggested that TCDD was metabolized to some reactive intermediate which covalently bound to some essential macromolecules analogous to the mechanism of hepatotoxicity proposed for bromobenzene, phenacetin and isoniazid. If the rate limiting step were the formation of the reactive metabolite of TCDD, then congeners which induce AHH activity, might stimulate their own metabolism and flood the system with an excess of reactive metabolites. Both of these hypotheses can probably be rejected on the weight of the available evidence. But while it doesn't appear that induction of AHH activity per se is deleterious, we still must explain its correlation with toxicity.

B) Coordinate Gene Expression

So far we have focused only on the induction of AHH activity, but it is known that the administration of TCDD or one of the classical polycyclic aromatic hydrocarbon inducers such as 3-methylcholanthrene (MC) stimulates not only AHH activity but a number of other enzyme activities.

Both TCDD and MC have been shown to induce the following hepatic enzymes: cytochrome P_1-450 mediated microsomal monooxygenase activity, δ-aminolevulinic acid synthetase, aldehyde dehydrogenase, glutathione-s-transferase B, UDP-glucuronosyl transferase, and DT-diaphorase. Very likely there are more enzymes which are coordinately expressed by one of these inducers.

From genetic studies in inbred strains of mice (not reviewed here) it appears the Ah locus codes for the cytosolic receptor protein and this protein when combined with the inducing compound (e.g. TCDD) initiates the coordinate expression of at least AHH, UDP-glucuronosyltransferase, and DT-diaphorase, and perhaps many more structural genes.

We suggest that the cytosolic binding protein serves as the cellular recognition site for TCDD and like compounds, and the binding of these compounds to the receptor serves as a master switch which initiates the expression (or repression) of numerous noncontiguous structural genes. This is analogous to the battery of genes which are coordinately expressed by the steroid hormones.

Our hypothesis is that the <u>sustained</u> expression (or repression) of one or more of the genes controlled by the Ah locus (the TCDD-receptor complex) leads to the toxic manifestations characteristically associated with the chlorinated dibenzo-p-dioxins. Thus induction of AHH activity is merely a signal that this gene battery is activated. This hypothesis: 1) appears to fit all the observable data, and while it does not indicate a specific biochemical lesion, 2) it does permit further investigation to support or reject the proposal, and 3) it does suggest we might be able to intervene at the level of the receptor (by a competitive antagonist) without knowing the ultimate biochemical lesion.

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TOXICOLOGY OF PHENOXY HERBICIDES AND HAZARD ASSESSMENT OF THEIR USE IN REFORESTATION

By Frank N. Dost, Ph. D.

(Toxicologist, Associate Professor of Veterinary Medicine,
Environmental Health Science Center,
Oregon State University)

U.S. Department of Agriculture
Forest Service, California Region (Region 5)
630 Sansome Street
San Francisco, California 94111

May 1978

TOXICOLOGY OF PHENOXY HERBICIDES AND HAZARD ASSESSMENT OF THEIR USE IN REFORESTATION

Frank N. Dost, Ph. D.

FOREWORD

This report was prepared by Dr. Frank N. Dost (Toxicologist, Assoc. Prof. Vet.Medicine, Environmental Health Science Center, Oregon State University) under a contract with the U.S. Forest Service. The report was designed to provide a comprehensive evaluation of the hazard to human health of phenoxy herbicides, specifically to include 2,4-D, 2,4,5-T, and Silvex as used in forest, range, and brushland management on the National Forests in California.

Dr. Dost reviewed our present environmental statements on forest reestablishment, rangeland enhancement, and brushland management and analyzed risks and provided suggestions on how to best minimize hazards to human health. He reviewed all available published and unpublished reports on research and studies of herbicide effects. His work was reviewed by 16 scientists in related fields, and their comments were considered in his final report

This report is part of our current effort to do the best possible job in *updating* and, where appropriate, *revising* the Forest Service environmental statements for forest reestablishment, rangeland enhancement, and brushland management.

Douglas R. Leisz

DOUGLAS R. LEISZ
Regional Forester

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Dr. Hugh Black, Associate Professor
Forest Wildlife Ecology
Dept. of Forest Management
Oregon State University
Corvallis, Oregon 97331

Dr. R. K. Boutwell
Professor of Oncology
McArdle Laboratory for Cancer Research
University of Wisconsin
Madison, Wisconsin 53706

Dr. Don Crosby
Dept. of Enviromental Toxicology
University of California
Davis, California 95616

Ephraim Kahn, M. D., Chief
Dept. of Public Health
Epidemiological Studies Lab
2151 Berkeley Way
Berkeley, California 94794

William Griffith
California Dept. of Fish & Game
987 Jedsmith Drive
Sacramento, Ca. 95819

Dr. Keith Maddy
California Department of Food & Agriculture
1220 N. Street
Sacramento, California 95814

Dr. Wilbur McNulty
Oregon Regional Primate Center
505 N. W. 185 Avenue
Beaverton, Oregon 97005

Thomas Milby, M.D.
Stanford Research Institute
2150 Shattuck Avenue
Berkeley, California 94706

Donald Morgan, M.D.
Agricultural Medical Institute
Oakdale, Iowa 52319

Dr. Logan Norris
U.S. Forest Service, PNW
3200 Jefferson Way
Corvallis, Oregon 97331

Dr. Mike Newton
Dept. of Forest Mgt.
Oregon State University
Corvallis, Oregon 97331

Dr. Steve Radosevich
Dept. of Botany
University of California
Davis, California 95616

Dr. Rojer Sandquist
U.S. Forest Service
Region 6

Fay Shon
U.S. Forest Service, PNW
3200 Jefferson Way
Corvallis, Oregon 97331

Dr. Brian Sturgess
U.S. Forest Service
Region 5

Dr. James Witt, PH.D.
3223 N.W. Glywood Circle
Corvallis, Oregon 97330

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TOXICOLOGY OF PHENOXY HERBICIDES AND HAZARD ASSESSMENT OF THEIR USE IN REFORESTATION

Introduction

The phenoxy herbicides 2,4,5-trichlorophenoxy acetic acid (2,4,5-T), 2,4-dichlorophenoxyacetic acid (2,4-D), and 2-(2,4,5-trichlorophenoxy)propionic acid (2,4,5-TP silvex) are important agents for reforestation site preparation and for release of conifer plantings. To be used, the compounds must be inserted into a segment of the environment that may be occupied by humans or may have a potential for migration to points of human contact or consumption. This document is a review of the pertinent literature describing biological effects and environmental behavior of the three herbicides, and of 2,3,7,8-tetrachlorodibenzo-p-dioxin, an extremely toxic contaminant found in ~~small~~ trace quantities in 2,4,5-T and silvex.

For various reasons, the greatest public attention has been focused on 2,4,5-T and its unique contaminant, TCDD. Since 1969 public and scientific attention to TCDD has overshadowed the specific concerns about 2,4-D, which has no TCDD, and about 2,4,5-T and silvex, which are contaminated.

The unusual nature of the TCDD contaminant has made a special case of the herbicide 2,4,5-T, and has made it the subject of an unusually vehement public, political, and scientific controversy. An enormous scientific effort has evolved in an attempt to learn about the unique character of the compound, and to provide a rational basis for regulatory decisions. Hopefully, this effort to bring the existing literature into focus will aid in the decision-making process.

As matters presently stand, there is a division in the collective public and scientific minds on the safety and benefits of use of the phenoxy herbicides. As in any controversy, both views include unreasonable people, but the bulk of the argument is among sincere citizens and competent scientists on either side. It is my hope that the reviews and assessments in this document are dispassionate and without bias; otherwise it will be of limited utility.

In preparing this review, emphasis has been placed on information about the toxic properties of the respective agents. An assessment of hazard necessarily considers along with biological effects, factors governing the probability of exposure, and the probability that the chemical once contacted will enter the organism.

With the exception of the section on TCDD, I have chosen to touch only lightly upon the environmental behavior of the compounds. This information about phenoxy herbicides is well established and is much less complex than the behavior of TCDD. A more thorough treatment of the environmental behavior of TCDD is necessary here because: 1) this contaminant is clearly the most important of the four compounds under study; 2) the high toxicity demands thorough understanding of the compound as it exists outside the target of toxic effect; 3) the environmental chemistry of TCDD is incompletely studied and subject to some controversy, whereas that of the herbicides themselves is well established.

The organization of this review and assessment includes sections reviewing the scientific literature on TCDD, 2,4,5-T, 2,4-D, and 2,4,5-TP (silvex), an assessment of the hazard potential of their distribution in the environment, recommendations on some aspects of methods of use of

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the herbicides and recommendations of needed research. A shortened version of the review and assessment is also attached. Organization within the review differs slightly among the chemical sections because of differences in research emphasis or the character of the agent itself. In certain instances, for example in considering the enzyme induction capacity of TCDD, an area may be dealt with that is not entirely germane to the issue of hazard but of considerable importance in understanding the biology of the compound.

A number of useful reviews of interaction of phenoxy herbicides or TCDD with animal systems have been or are being published. The most recent one by Leng (1977), discussing metabolic fate of phenoxy acids in domestic animals, Mercier (1976) in response to the Seveso accident which spread large amounts of TCDD through several Italian communities, and McQueen et al. (1977) refuting alleged association between herbicide use and fetal abnormalities in humans. McKenzie et al. (1975), Norris et al. (1972), Buhler (1977), and the EPA position paper arising from the Dioxin Working Group (presently in draft) are also all of considerable value.

TOXICOLOGY OF TCDD

History of Human Exposure

Probably the first clinical description of human TCDD exposure arose from the study by Kimmig and Schulz (1957) of workers in chlorophenol factories in Germany. The principal symptom was a persistent skin lesion (chloracne; so-called because it is characteristic of a number of related compounds and was at one time thought to be caused by free chlorine.)

The active agent was established as TCDD, and animal studies showed that the same lesions appeared on rabbit ears after application of as

little as 0.002% TCDD (Kimmig and Schulz, 1957). High topical doses and oral doses of 50 µg/kg or greater caused liver necrosis. A similar industrial intoxication in France was reported by Dugois and Colomb (1957). Pathology of the skin lesions is well described by Kimbrough (1974).

A study of 29 phenoxy herbicide workers by Bleiberg et al. (1964) disclosed chloracne and a high frequency of uroporphyrinuria, and tissue deposition of porphyrins related with hyperpigmentation and skin fragility. This assemblage of symptoms is commonly called porphyria cutanea tarda. The excessive production and excretion of porphyrins apparently results from a hyperactivity of the enzyme δ-amino levulinic acid (ALA) synthetase, the first and rate limiting step in porphyrin formation. Porphyrins are eventual components of heme proteins, such as hemoglobin and cytochromes. Effects on ALA synthetase and other enzyme systems will be discussed in another section. Poland et al. (1971) examined the same factories later, after somewhat more satisfactory industrial hygiene measures were established. Among the 73 male workers observed, no clinical porphyria was apparent even though some degree of chloracne still persisted in most of the subjects. There was also apparently a significant psychological effect as measured by the Minnesota Multiphasic Personality Inventory.

The absence of porphyria and persistence of chloracne in the later study has led Poland et al. (1971) to conclude that while both may be caused by TCDD, the mechanisms are probably ~~dissimilar~~ independent.

Several severe accidental exposures to TCDD have occurred. In several cases, runaway chlorophenol plant reactions caused widespread exposures within the factory or in the surrounding community. An accident in Germany in 1953 exposed a large number of workers said to have suffered liver, kidney, splenic, eye, cardiovascular, and nervous symptoms. A

worker who apparently was in extensive contact with a contaminated segment of the plant five years after the accident became ill and later died. An explosion in a Dutch plant in 1963 released 30-200 g of TCDD into the main gallery of the factory exposing 50 people. In the subsequent two years, four died, but no clear association with TCDD intoxication could be shown (Hay, 1976). An explosion in a British plant in 1968 resulted in 79 cases of chloracne (May, 1973). In Czechoslovakia a pentachlorophenate production stream overheated under pressure, producing large amounts of TCDD (Jirazek et al., 1974). (It should be noted in this and some earlier German work a different numbering convention was used. 2,3,6,7-TCDD and 2,3,7,8-TCDD are the same compound.) Of 80 exposed workers, 76 developed chloracne, porphyria cutanea tarda, disorders of plasma lipids, hepatic damage, neural lesions, and neurasthenia.

An explosive reaction in a plant at Seveso, Italy, in 1976 scattered TCDD, apparently in kilogram amounts, over a wide area that included several communities. Details are still not widely disseminated but great numbers of animals were killed and many people were made ill. A review of the incident was prepared by Mercier (1976) a few months after the incident but the slow onset and persistent effects did not permit a detailed assessment of the damage. Since that time the Seveso disaster has been given unprecented publicity and as might be expected this tragedy has been sensationalized in press coverage, in part because there may have been an effort to minimize or cover up the extent of the problem.* There were apparently defects in the administration of the plant that may have resulted in the accident itself, and management of public warning and information early after the accident was questionable. Further contributing to the extensive public attention and sensationalizing of

* since recovery from the initial phenolic burns, there has been nothing more than chloracne in ~ 30 children; no birth defects or abortions other than voluntary.

DOW 0009176

see papers by Reggiani & Tschmann-Duplessis - good reviews.

the incident is a virtual absence of objective reporting in the scientific literature. As far as I am aware, there has been no scientific evaluation of the aftermath in publication since the review by Mercier (1976), shortly after the accident.

waste oil from process

In the U.S., TCDD extracted from hexachlorophene production was stored in oil, then mistaken for waste lubricating oil and sprayed on several horse arenas and farms in Missouri for dust control. The incidents occurred in spring and summer of 1971. On one horse breeding farm, 62 of 85 horses exercized in the sprayed area became ill and 48 died, the last in January, 1974, two and a half years later. Hundreds of birds and rodents died, along with a number of dogs and cats. Several children were exposed through play in the arena soil. One developed a severe hemorrhagic cystitis and others showed clear evidence of chloracne. All recovered. The TCDD concentration in the soil was later established at more than 30 ppm, a truly massive amount. (This concentration amounts to 120 lb per acre per foot of depth, assuming 4×10^6 lb soil/acre-foot.) The details of the latter event are described in detail by Carter et al. (1975) and Kimbrough et al. (1977).

30 ppm = 30 lb TCDD/A in top 3 inches of soil or 300 million pounds of 2,4,5-T containing 0.1 ppm TCDD per acre.

All of these exposures were to a mixture of TCDD with other agents. Possibly the only published account of exposure to pure TCDD is the description by Oliver (1975) of laboratory contact by three individuals associated with synthesis experiments. The individuals all had utilized routine protective procedures but sufficient contact developed nonetheless. The exposures were not suffitient to cause porphyrinuria or liver damage but serum cholesterol was raised. In two cases personality changes and neural disorders developed two years after exposure. The amount of TCDD contacted is not known.

7328

0000085

General Toxicity in Laboratory Animals

This section is a review of data on effects of TCDD other than reproduction and teratology, carcinogenesis, and its enzyme inducing properties upon mammals. These will all be considered independently, as will studies of tissue distribution and metabolism.

Schwetz et al. (1973) have determined lethalities for several species. The guinea pig is exceedingly sensitive, with an LD_{50} of only 0.6 µg/kg; rats are less sensitive; LD_{50} for males is about 20 µg/kg and for females the LD_{50} is in excess of 40 µg/kg. Rabbits are still less sensitive at more than 100 µg/kg. Dogs were found to require even higher effective doses. In these studies, responses of mice were highly erratic and were not included. McConnell et al. (1977a) have determined a single 30 day LD_{50} for mice of 284 µg/kg, and 2 µg/kg for guinea pigs.

An important characteristic of TCDD is a very long delay before lethality, often 2-3 weeks and occasionally 7-8 weeks.

The pathology associated with TCDD toxicity may include thymic atrophy, liver necrosis, and degeneration in the kidney and thyroid. The guinea pig is unique in that it suffers limited hepatic damage, even at doses that cause substantial thymus depletion (Gupta et al., 1973). The degree of thymic atrophy is generally dose dependent; the studies of Gupta et al. (1973) did not establish a zero effect dose, but the lowest dose in the rat of 1 µg/kg/day for 31 days caused moderate thymus and liver damage, and kidney and thyroid degeneration. Guinea pigs treated with 0.2 µg/kg weekly for eight weeks had limited thymus damage and no other pathology. Studies of guinea pigs at lower doses suggest a no observed effect level at 0.008 µg/kg/weekly for eight weeks, a total of 0.064 µg/kg, although there was a slight decrease in lymphocytes at that dose (Vos et al.,

what about the immune response?

1973). Animals given tetanus toxoid at the fourth week to measure humoral immunity were found to have somewhat lower thymus weights than controls, although the difference was not significant.

According to the data of Kociba et al. (1976) the no observed effect level in rats given TCDD in the diet 5 days a week for 13 weeks is 0.001 µg/kg/ day, totaling 0.065 µg/kg, almost identical to the value found in guinea pigs. Again, thymus weight appeared to be the most sensitive parameter. The Kociba study dealt with a very broad range of clinical, chemical, and hematological measurements. At a dose of 1.0 µg/kg/day, 2 of 10 rats died and weight gain decreased sharply, erythrocyte numbers and volume decreased, reticulocytes increased in males. In females thrombocytes decreased and total leukocytes increased. Differential counts were not altered. Urinary porphyrins, creatinine and δ amino levulinic acid (ALA) all increased. Serum bilirubin, BUN and alkaline phosphatase activity also were elevated. At an intake of 0.1 µg/kg/day red cell numbers and volume were still slightly decreased in males and porphyrin, ALA, bilirubin, and alkaline phosphatase activity was elevated in females. No changes in blood parameters occurred below these levels.

0.01 mg/kg/day = 0.65 mg/kg in 13 weeks. Also 0.001 mg/kg/day for 2 years = 0.73 mg/kg over lifetime

In a similar study Zinkl et al. (1973) found no changes in SGPT activity, bilirubin, or erythrocytes after 31 days of treatment at 1.0 µg/kg/day and no change in SGOT activity, cholesterol, or serum protein at 0.1 µg/kg/day. There was a modest depression in blood glucose at the lower dose rate.

total dose 31 mg/kg 3.1 mg/kg

Mice treated with 1.0 µg/kg/week for six weeks were found to have depleted thymus weight (Vos et al., 1974) but no changes were evident at the lowest dose, 0.2 µg/week. Hematological changes occurred only at a dose of 25 µg/kg/week, for six weeks. Some changes in serum proteins did occur at an intake of 0.2 µg/kg/weekly. Unfortunately, there are no data suggesting the magnitude of the no-effect single dose in any species.

6 mg/kg 1.2 mg/kg 150 mg/kg 1.2 mg/kg

for what parameters?

Studies in primates have been limited. McNulty administered TCDD at a rate of 20 parts per billion in the diet to a single monkey and caused its death in a few days. A 10-fold lower concentration permitted survival for about two months. Total intake was estimated as about 6 µg/kg (McNulty, 1977). A study of eight female Rhesus monkeys fed 500 parts TCDD per trillion has recently been completed in the laboratory of J.R. Allen. The animals showed dermatitis and menstrual irregularities within six months after initiating treatment. One monkey died prior to the seventh month and by nine months of exposure, all survivors experienced a severe anemia and leukopenia. Food intake was normal but all of the animals lost weight. After 11 months, five of the eight monkeys died, all with severe anemia and destruction of blood forming tissues. The total dose was estimated at about three µg/kg (Allen et al., 1977a, 1977b) or about .3 µg/month.

A number of questions arise from these data. The monkey studies are limited, but they do suggest the possibility that the lethal dose is similar regardless of the time over which it is administered. The implications are quite interesting. If it is true that TCDD dosage is time independent in the monkey, then the effect is truly cumulative. No chemical is known to have such properties, and if TCDD behaves in this fashion, any estimate of acceptable dose must assume additive effects over long periods. TCDD does not remain in the body of monkeys (Van Miller et al., 1976) or other species (Van Miller et al., 1976; Fries and Marrow, 1975; Rose et al., 1976). A cumulative effect, if it exists, must then entail irreversible effects remaining after the molecule leaves active sites. TCDD is apparently not metabolized to a significant extent (Rose et al., 1976; Vinopal and Casida, 1973; Van Miller et al., 1976) so it is conceivable as well that a single molecule could interact several times before

leaving the body. A possible mechanism may be intercalation of the planar TCDD molecule into DNA (Hussain, 1973), production of misinformation and departure of the agent to interact at another DNA site.

Data in other species, especially the guinea pig do not support the idea of extensive cumulative effect. A dose rate of 0.2 µg/kg/weekly for eight weeks provided a total dose almost three times the LD_{50}, but no deaths occurred and organ changes, while measurable, were not severe (Vos et al., 1973). Rats also accepted about three times the LD_{50} of 20 µg/kg over 13 weeks, without lethality. Some mice, on the other hand, died at a total dose less than the acute LD_{50}, distributed over a four week period (Vos et al., 1974).

There are a few other biological responses to TCDD that appear after high doses of from 10-25 µg/kg. One of the more interesting is a depressed capacity for biliary excretion. TCDD sharply decreases removal of PCBs from the liver via the bile (Yang et al., 1977) and decreases plasma clearance of ouabain into bile (Hamada and Peterson, 1977). In the latter work, steroidal inducers of microsomal enzymes reversed the TCDD effect. Whether this effect has potential significance in the response to secondary toxicity can only be speculated upon. In any case the doses required for effect are ~~quite high~~ and probably not attainable under realistic environmental conditions.

Because TCDD causes increased microsomal foreign compound metabolizing activity in the kidney cortex (Fowler et al., 1975, 1977), Pegg et al. (1976) evaluated proximal tubular function in intoxicated rats. They found little change at any but large doses (25 and 50 µg/kg).

There have been few observations of metabolic changes caused by TCDD. Incorporation of ^{3}H-sodium acetate into liver lipid fractions seems to be

impaired at TCDD doses above 0.1 µg/kg (Cunningham and Williams, 1972), but the significance of this effect has apparently not been explored further.

Evidence of thymic atrophy after TCDD treatment has stimulated more direct study of effects upon the immune responses. Vos and Moore (1974) and Vos et al. (1973) found lymphocytic depletion in the cortex of the thymus and depressed cellular immunity in rats and mice exposed during gestation and the post-natal period. Guinea pigs were similarly affected (Vos et al., 1973). Thigpen et al. (1975) attempted to translate the effect into terms of infectivity and found that doses of 1 µg/kg or more increased mortality and time to death of mice infected with Salmonella bern. Course and severity of disease due to pseudorabies virus was not altered by TCDD. With all the study of TCDD effects, no pattern of pathology, tissue distribution, and biochemical or physiological change has emerged that is consistent enough to suggest a mechanism of lethal effect. McConnell et al. (1977a) quote papers in preparation by Van Logten et al. which show no association of death in TCDD intoxication with adrenal or pituitary hormones or malnutrition.

Pathologic Morphology Resulting From TCDD Intoxication

Much of the more pronounced tissue change resulting from TCDD has been observed following high acute doses of the compound. There is some question whether the acute damage seen represents that observed in a low level longer term experiment or in a field exposure.

Before TCDD as such became an issue Norback and Allen (1969) studied the membrane changes in liver following ingestion of hexachlorohexahydrophenanthrene, a constituent of so-called "toxic fat." The lesions found under electron and light microscopy anticipated the damage later to be

found after TCDD. Most striking was a great increase in the smooth endoplasmic reticulum (SER), with reorganization into a concentric arrangement, and the rough ER became folded and rolled over a period of days to form dense arrays of concentric separate, not spiraling, paired membranes. The authors speculate that SER proliferation in response to intoxication is related to acceptance of enzymes synthesized by the RER. The increased membrane mass may also be a means of sequestering chlorinated hydrocarbon in the lipid phase of the membrane in close proximity to foreign compound metabolizing enzymes. (This paper contains very useful schematic representations of the membrane development sequence.) The same authors later reported similar lesions in rats following administration of 1 µg TCDD/kg/day for 21 days (Norback and Allen, 1973). They found hypoplasia of lymph tissue and bone marrow with progressive anemia and leukopenia. The resultant reduced resistance caused substantial losses due to infection. Monkeys and chickens in the same study were much more sensitive in the latter parameters than rats. They also developed extensive fluid accumulation in all body cavities, probably due to decreased osmotic activity of blood due in turn to serum protein decreases. (Relative sensitivity of chickens has also been noted by Greig et al. (1973).) Monkeys were subject to extensive skin lesions, apparently corresponding to the chloracne of humans, but rats and chickens did not suffer skin damage. Monkeys and chickens also exhibited degeneration of the testes and decreased activity of seminiferous tubules (see also Allen et al., 1975).

Large doses of TCDD (single doses of 50 or 100 µg/kg; 16 to 31 daily doses of 10 µg/kg) to the rat caused severe liver and thymus damage, with icterus and disseminated hemorrhages, especially in the myocardium. The

Be consistent in time - either parlor present, not both.

liver cells increase in size and cellular regenerative attempts occur. Renal tubule cells became vacuolated. No pathological change occurred at doses of 0.1 µg/kg/day over 31 days, at 1.0 µg/kg weekly for 6 weeks or 5 µg/kg in a single dose (Gupta et al., 1973). Fowler et al. (1973) studied the progressive changes in rats over 28 days following a single dose of 5 or 25 µg TCDD/kg. Increased SER, roughly dose responsive, was evident by day three, especially in cells adjacent to biliary drainage. Both RER and SER were greatly increased by day 9, SER was almost at normal levels by day 16, and both were indistinguishable from those of control animals by day 28. Jones and Butler (1974) carried out a similar experiment with rats given a single 200 µg/kg treatment. (A massive dose, considering the minimum effective dose.) Most change was found in centrilobular cells, with degeneration and necrosis of parenchymal cells evident within a week after treatment. They suggest that the multinucleate cells seen in increasing numbers through two months after treatment are formed through membrane disturbance and coalescence of hepatocytes. By 10 weeks some recovery was evident but fibrosis of central veins and sinus dilation still prevailed. No mention of lethality was made, although the dose was on the order of five (female) to ten (male) times the LD_{50}. Longer term treatment of rats (0.001-1.0 µg/kg/day, 5 days weekly x 13) caused almost complete thymus involution at a dose rate of 1.0 µg/kg/day, somewhat decreased cortical thymocyte and lymphoid cell numbers after 0.1 µg/kg/day. Splenic morphology was essentially unchanged at 1.0 µg/kg/day. The latter dose also caused some gross edema, and one of five males had decreased spermatogenic activity. Females at the highest dose tended toward formation of cuboidal epithelium in the uterus and decreased size and numbers of corpora lutea. Ovarian tissue was also abnormal. At all

total 3.1 ug/kg
2 6 ug/kg
2 5 ug/kg

10 X LD50

Did animals survive? If so, the material administered was not likely TCDD.

total of 65 ug/kg

6.5 ug/kg

lower doses no effects were observed (Kociba et al., 1976). Two year continuous feeding studies at TCDD intakes of 0.001 to 0.1 µg/kg/day have just been terminated and histopathologic evaluation is not complete. Gross examination showed that in some rats given 0.1 or 0.01 µg TCDD/kg/day there were increased numbers of nodules in the liver (R.J. Kociba, Dow Chemical Company, preliminary status report; personal communication).

Lesions generally similar to those seen in rats were found in mice given single oral doses up to 200 µg/kg and weekly doses up to 25 µg (Vos et al., 1974). At a dose rate 0.1 µg/kg/week for 6 weeks decreased thymus weight occurred. At 25 µg/kg/week, serum globulins were decreased, and porphyria and bile duct epithelial proliferation occurred. Similar changes were also reported following treatment of mice with an LD_{50} (30 day) TCDD (McConnell et al., 1977a).

McConnell et al. (1977b) treated rhesus monkeys with heavy single doses of TCDD (in excess of 70 µg/kg) which were fatal in every case. Integumental changes were pronounced, anemia and lymphopenia and an increased neutrophil count followed treatment. Liver, adrenal and kidney appeared to increase in weight relative to body weight, but this effect may have been due to general wasting. With the skin hyperplasia, similar changes occurred in the epithelium of some hollow organs. The major difference from effects in similarly treated rats was the absence of significant liver lesions.

Chronic low level intoxication (500 ppt in diets, about 0.01 µg/kg/day) of monkeys until death at 7-12 months caused anemia and pancytopenia, with extensive hemorrhage. Bone marrow and lymphatic tissues were hypoplastic, and epithelial structures were hyperplastic (Allen et al., 1977).

The pathological changes resulting from absorption of TCDD by horses and cats during the Missouri horse arena episode have been recently described in detail by Kimbrough et al. (1977). The most pronounced and consistent findings were the generalized hepatic fibrosis and necrosis and gastric ulceration. There was an appreciable incidence of tubular degeneration and abscesses in the kidney and capsular thickening of the spleen. Lesions in cats were generally similar.

Effects of TCDD Upon Reproductive Functions

Of the many toxic responses to TCDD, the potential for teratogenicity has received perhaps the greatest notoriety. The early findings of 2,4,5-T teratogenicity raised a considerable public consciousness and the finding that the herbicide was contaminated by high levels of TCDD, also found to cause fetal malformation, focused additional attention on the contaminant. In evaluating this aspect of TCDD hazard it should be noted that the teratogenic potential of a toxicant can be considered in two ways. The absolute teratogenic dose of TCDD to the pregnant animal on a body weight basis is very low, as is true of all TCDD effects. A more realistic way of relating the teratogenic potential, however, is by comparison with the dose required to cause general toxic effects in the mother. For example, in the guinea pig, TCDD teratogenesis has not been studied because the teratogenic dose is apparently higher than the lethal dose.

This section is limited to studies of relatively pure TCDD upon reproductive function in experimental animals. There is reference elsewhere to reproductive studies of 2,4,5-T and silvex, which contain at least some TCDD.

Teratogenic defect resulting from TCDD are rather specific in experimental animals; the principal effects are increased frequency of cleft

palate (reported only in mice) and an abnormality in which the central collection region of the kidney becomes enlarged, with an associated fluid accumulation (Neubert et al., 1973). Limited limb deformation may be seen in a few animals (Sparschu et al., 1971). Intestinal hemorrhage is also seen; this effect is not teratogenic but rather is direct toxic consequence of fetal exposure to TCDD. Fatty degeneration of fetal livers is in the same category (Becker, 1974) as is subcutaneous edema and delayed ossification.

In teratogenicity studies of rodents the usual procedure is to administer intoxicant at some point during, or throughout days 6-15 of pregnancy, which covers the organ forming period. Sparschu et al. (1971) found that the lowest effective dose of 1.25 µg/kg/day caused some fetal mortality, resorptions, and intestinal bleeding. Skeletal abnormalities were limited to delayed ossification. Renal defects tended to occur at about the same frequency regardless of dose. These changes are all a result of direct toxicity to the fetus and are not developmental. Intestinal hemorrhage has been reported in rat fetuses at maternal dose rates of 0.25 µg/kg/day (Khera and Ruddick, 1973). At higher doses up to 8 µg/kg/day the same kind of effects occurred with greater frequency; 0.5 µg/kg/day caused decreased maternal weight gain and higher doses resulted in severe toxicity.

Courtney and Moore (1971) examined rats and three mouse strains and found that a dose of more than 1 µg/kg/day was required to produce kidney defects in rats, and the cleft palate and kidney changes in mice appeared at about 3 µg/kg/day, over days 6-15 of gestation. The C57Bl mouse strain was much more sensitive than the others tested. A somewhat larger group of NMRI mice showed a cleft palate frequency of about 3% (Neubert and

10 days

total dose

2.5 µg/kg

80 µg/kg

5 µg/kg

10 µg/kg

30 µg/kg

the real change is a delay in normal maturation. See paper!

Gaines from NCTR on 2,4,5-T.

16

7338

Dillman, 1972), similar to the low frequency groups of Courtney and Moore. The period of maximum sensitivity for TCDD is at the eleventh day of gestation, as distinguished from dexamethasone, for example, which is most effective at day 13 (Neubert et al., 1973).

Total 0.09 µg/kg

Continuous administration of 0.001 µg/kg/day to male and female rats for 90 days prior to mating and through weaning of offspring produced no effect on fertility (Murray et al., 1977). The study continued through three generations, with each preceeding generation sacrificed at weaning of its offspring; the third generation was maintained until two years from the beginning of treatment of the f_0 generation. Toxicity and poor litter survival dictated termination of feeding at 0.1 µg/kg/day; 0.01 µg/kg/day caused decreased fertility in generation f_1 and f_2 but not f_0. f_2 and f_3 litters were smaller and growth and survival were decreased after treatment at 0.01 µg/kg/day, but no pathological change was observed in liver, kidney, or thymus.

Total 0.03 to 30 µg/kg

~~More recently,~~ Smith et al. (1976) have treated CF-1 mice at doses ranging from 0.001 µg to 3.0 µg/kg/day through days 6-15 of gestation. There was no maternal toxicity at any dose. Cleft palate appeared at 1 µg, renal dilatation appeared at 3 µg/kg/day. No significant effects occurred at 0.1 µg.

total 250 to 4000 µg/kg in mice

A ~~recent~~ report by Courtney (1976) described results of treating CD-1 mice with high doses of TCDD (25-400 µg/kg/day) on days 7-16 of gestation. These very high doses caused no maternal deaths during the gestation period, but resulted in high fetal mortality. The nature of observed terata were typical of TCDD intoxication, but the enormous doses render the study useless for hazard evaluation. However, the

substantially greater incidence of terata after subcutaneous administration, compared with that following oral TCDD suggests that hepatic metabolism may affect TCDD more than has been believed. A dose rate of 25 µg/kg/day orally caused 3% incidence cleft palate; the same dose subcutaneously caused 82% incidence of cleft palate.

also excretion of part of oral dose without absorption

total = 250 ug/kg

There has been little research on effects of TCDD on primate reproduction. Experimental animals are in limited supply and the numbers of offspring are so low that a satisfactory study is almost impossible.

Allen et al. (1977) fed TCDD to eight female rhesus monkeys at a concentration of 500 ppt in the diet, which provided an intake of about 0.3 µg/kg/month. Skin lesions, endocrine and menstrual changes occurred in all of the subjects by 3 months. Six females of the eight were bred after 7 months of treatment; one monkey died before mating and another was excluded from the reproductive study. Three of the animals conceived; two aborted, and one completed a normal pregnancy. Two of the three barren animals were bred four times, the other was bred twice. This dose rate is on the order of 0.01 µg/kg/day and comparison with the rat study of Murray et al. (1977) suggests that reproductive toxicity in monkeys may be somewhat greater than in rats, but is by no means proportional to the lethal dose.

0.01 ug/kg/day

total 0.9 ug/kg

2.1 ug/kg

7340

Carcinogenic and Mutagenic Potential of TCDD

Van Miller and Allen (1977) have evaluated pathological changes in rats fed TCDD in dietary concentrations ranging from 1 ppt to 1000 ppb, beginning at animal weights of about 60 g. Estimated weekly intakes of TCDD ranged from 0.0003 µg/kg at a dietary level of 1 ppt to 2 µg/kg at 5 ppb. Actual intake of TCDD at dietary concentrations of 50-1000 ppb was not clear. At 65 weeks the abdominal viscera of surviving animals

1 million ppt

per week = 0.00009 to 0.3 ug/kg/day for 65 wk

5000 ppt

50,000 to 1,000,000 ppt

total dose = 0.02

130 ug/kg

appears on a per-day basis for comparison with chronic studies

you might also mention the Innes study using 2,4,5-T containing ~30 ppm of $TCDD^{18}$ — negative findings

0000097

3 to 600 ug/kg/day

an increase in de novo synthesis of enzyme, rather than activation of preexisting protein or decreased degradation of protein (Haugen et al., 1976). Whether the effect of TCDD on inducible enzymes has implications on other aspects of protein synthesis can only be speculated upon. Poland and Glover (1975) showed about a 10-fold difference in the dose necessary to cause induction. Through the finding that heterozygous offspring of C57Bl/6J (responsive) and DBA/2J (non-responsive) strains are intermediate in sensitivity, they support the contention of Poland and Glover (1975) that the difference is a receptor site mutation. Later studies have strengthened that premise in finding that hepatic accumulation of ^{14}C-TCDD administered intraperitoneally was greater in responsive than non-responsive strains (Poland et al., 1976). In the same study, in vitro examination of binding in the soluble fraction of liver cells showed that specific binding sites exist, and that their affinity correlates with the genetic capability for induction. Furthermore, tests of an extended series of halogenated dioxins and dibenzofurans showed that cytosolic binding corresponded closely with the induction potency of the chemical.

Niwa et al. (1975) have measured AHH induction by TCDD in a variety of cell cultures. They used 10 established cell lines, human lymphocytes, and primary fetal cultures from chick, rat, rabbit, hamster, and four strains of mice. Generally, kinetics of TCDD induction in each cell type is similar to that of 3-MC, and the induction was inhibited by actinomycin-D and cyclohexamide, which are inhibitors of protein synthesis. They found no relation between inducibility and cytotoxicity of TCDD, which is further evidence that TCDD itself is not metabolized. The sensitivity of some cell lines is such that the authors suggest use

of H-4-II-E cells (derived from Reuber hepatoma H-35 in rats) as a bio-assay, with a suggested sensitivity below 1 pMole (∿ 0.0003 µg) in 3 ml of culture medium.

The same kind of genetic differences have been studied in human lymphocyte cultures, which represent the genetic background of the cell donor and are therefore useful in making relatively non-invasive studies of potential human response to intoxication. The sensitivity to TCDD induction of AHH is about 50-fold greater than to 3-MC induction, considerably less than the 30,000-fold difference in responsive mice (Kouri et al., 1974). Atlas et al. (1976) have carried these studies forward with lymphocytes from human tissues, but have as yet dealt only with 3-MC, not TCDD. Part of the stimulus for examining human cells lies in the suggestion that extent of induction at contact sites (i.e., skin, lung) may relate to the probability of cancer initiation by activated carcinogens (Kellerman et al., 1973). Although TCDD is as yet not established as a carcinogen such research may provide suggestions about the extent of expected variations in other TCDD responses in humans.

2,4,5-T

General Toxicity in Laboratory Animals

Apparently the first published account of 2,4,5-T acute toxicity was that of Drill and Hiratzka (1953). The LD_{50} for dogs was about 100 mg/kg; the principal symptom was a mild incoordination. When fed five days weekly for 90 days, doses up to 10 mg/kg/daily were without evident effect, but 20 mg/kg/day was lethal to all 4 treated animals between 11 and 75 days after the first dose. Symptoms were limited to muscle twitching and impaired swallowing. A field study by Grigsby and Farwell

(1950) [as quoted by Rowe and Hymas (1954)] exposed a variety of stock on pasture immediately after spraying 2,4,5-T at 2-4 times usual levels, with no effect.

Rowe and Hymas (1954) summarized the available data at that time and estimated the acute oral median lethal dose for male rats to be about 500 mg/kg, male mice 389 mg/kg, and guinea pigs 381 mg/kg. LD_{50}s for various esters were higher than for the acid. A sheep fed the propyl glycol butyl ester of 2,4,5-T died after 369 daily doses of 100 mg/kg and another sheep and a cow died after seven daily doses of 250 mg/kg (Palmer and Radeleff, 1964). The triethylamine salt of 2,4,5-T at 100 mg/kg caused no observable effect after 481 days of treatment at 100 mg daily. As single animal observations these can be considered rough estimates only, but they convey the generally high doses necessary to cause harm in ruminants. 2,4,5-T has been shown to cause decreased volatile fatty acid production in vitro at concentrations of 500 µg/ml or greater (Kutches et al., 1970); this concentration is probably greater than can be maintained by a survivable daily dose of 2,4,5-T.

Subchronic (90 day) feeding of male and female rats caused no effects below 30 mg/kg/day, but body weight and food intake were depressed after treatment at 100 mg/kg/day. Alkaline phosphatase and SGPT were elevated and erythrocyte count and hemoglobin were decreased. The histopathologic findings were limited and inconsistent (~~McCollister and Kociba, 1970~~). Rats were able to tolerate 186 mg/kg/day treatment with mixed mono-, di-, and tripropylene glycol butyl ether esters of 2,4,5-T over 90 days but developed some indications of toxicity. At a dose of 18.6 mg/kg no evidence of toxicity was observed (Dow Chemical Company, 1961).

7343

0000118

A two year study of rats given 3 to 30 mg 2,4,5-T/kg/day has very recently been completed by Dow Chemical Company. Much of the analysis has yet to be completed, including morphologic pathology. Animals that received 30 mg 2,4,5-T/kg/day were found to have increased urinary porphyrin excretion after 4 months of treatment, and this change continued through the entire 2 year period. No changes were found in any other hematologic, urinary, or clinical chemistry measurement at that rate of intake, and the increased porphyrin excretion did not occur at 10 or 3 mg/kg/day (R.J. Kociba, Dow Chemical Company, preliminary status report; personal communication).

A feeding study of reindeer was prompted by allegations that a high incidence of death and abortion occurred in 1970 in a herd after use of phenoxy herbicides the previous year. Fifteen of thirty pregnant reindeer were fed birch leaves which had been sprayed with a 2,4-D/2,4,5-T mixture, through a 1.5 month period late in gestation. The daily dose of phenoxy acid was about 1 mg/kg/day. No clinical chemical changes could be detected, nor did any changes appear at autopsy either in the adult animal or the full term fetus (Erne, 1976).

The toxic effects of 2,4,5-T in rodents have been studied in much more detail than the gross toxicity studies already described. Highman et al. (1976a, 1976b) established that lethal doses in pregnant or nonpregnant mice caused myocardial lesions, bone marrow aplasia, and lymphocytic depletion in thymus, spleen, and lymph nodes, and that animals which remained apparently healthy despite similar doses did not suffer the same severity of lesions. A mild hemolytic anemia did occur in such animals. Pregnancy did not augment the toxic effect. It was also clear that major differences exist in the response of specific strains. Many NCTR mice were

seriously affected at doses below 60 mg 2,4,5-T/kg/day (6-9 days of treatment while most CRBL mice remained unaffected at doses as high as 90-120 mg/kg/day at the same time. The histopathology, hematology, and blood chemistry changes in treated mice were also studied. In many monitored animals, some myocardial fibers were found to be pale and swollen, with longitudinal striations. The condition was rarely seen in treated apparently non-intoxicated animals. Necrotic changes were also often seen in the outer myocardium. High doses caused thymic atrophy, with almost no lymphocytes in the cortex and increased numbers in the medulla (this effect was enhanced in late pregnancy by the normal tendency to cortical involution in pregnant mice). Spleens were often atrophied, and thyroid follicles were enlarged with enlarged epithelial cell. In the liver, glycogen was often depleted. Blood chemistry changes appeared not to be marked.

ridiculous dosage rate

2,4,5-T has been found to cause increased liver weight in rats at doses of 167-334 mg/kg over a two-day period, apparently through stimulation of protein and RNA synthesis, but not as newly formed 2,4,5-T metabolizing enzymes. The change reverses after withdrawal (Chang et al., 1974; Rip and Cherry, 1976). Liver nuclei isolated from treated animals were more active in RNA synthesis than those from controls. 2,4,5-T at a concentration of 4 mmolar is capable of sharply inhibiting in vitro incorporation of mevalonate-^{14}C into non-saponifiable lipids by rat liver (Olson et al., 1974). If data obtained in mice by Nony et al. (1976) can be applied, this concentration is approachable at single doses of 50 to 100 mg/kg, which are survivable by most species.

4 x 0.251 g/liter = 1000 mg/kg = 1000 ppm

?

Koschier and Berndt (1976a, 1976b, 1976c) have described the effect of 2,4,5-T upon renal physiology. Large doses of 2,4,5-T appear to

impair secretion of itself by decreasing the activity of the organic acid transport system in the proximal tubule. Organic base transport was also depressed. Small doses of 2,4,5-T were excreted very rapidly, but large daily doses led to renal depression and retention of the compound. The authors consider the data to support the conclusion that transport of phenoxy herbicides is an active process.

The psychopharmacology of 2,4,5-T does not seem to be a popular field of study. In the one study found, single doses of up to 100 mg/kg were given on day 7, 8, or 9 of pregnancy. The male offspring of females given the highest dose exhibited more exploratory open field behavior, but no difference was found in females. The highest doses caused decreased litter size but no increase in malformations (Sjödén and Söderberg, 1972).

Poultry seem relatively insensitive to 2,4,5-T. Whitehead and Pettigrew (1972) found that a single oral dose of ppm 900 mg/kg to 4 week old chicks caused 40% lethality. However, feeding of 1000 ppm ~~mg~~ 2,4,5-T ~~per kg~~ in the diet ~~daily~~ for three weeks to chicks, beginning at one day of age caused only some slowing of growth; 5000 ppm ~~mg/kg~~ in diet was lethal. At levels not causing gross toxicity, plasma calcium and magnesium were not affected. Given a choice, the birds rejected the treated diet in favor of non-contaminated food. Turkeys fed 2,4,5-T at a rate equivalent to 62 mg 2,4,5-T acid daily for 11 days were unaffected (Roberts and Rogers, 1957). Bjorklund and Erne (1971) introduced various 2,4,5-T derivatives into water and feed of chickens, quail, pheasants, and ducks. In water the LC_{100} of the triethanolamine salt for chickens over a 29 week period was 1000 ppm acid equiv. (about 200 mg/kg). In the diet of other species over

a 7-day period, the LC_{50} was in excess of 2000 ppm. Kenaga (1975) has extensively reviewed avian toxicity and safety of birds in areas treated with herbicides and concludes that the no observed effect levels are substantially above amounts that might be contacted in a field application.

Effects of 2,4,5-T on Reproduction

The teratogenic potential of 2,4,5-T has received wide publicity since 1969 when allegations were made that its use as a military defoliant had caused fetal malformations in the Vietnamese population. A succession of studies over the next three years confirmed the teratogenicity of 2,4,5-T, but the implication of the herbicide in any increase in birth defects has not been supported. The initial report of 2,4,5-T teratogenic effect (Courtney et al., 1970; Bionetics Research Laboratories, 1970) described cleft palate and cystic kidneys at doses of 46 and 113 mg/kg/day on days 6 through 14 of gestation. Of the two lesions, cystic kidney appeared to be a more sensitive indicator. The 2,4,5-T used in the study was found to contain 30 ppm 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and the respective toxicities of the two agents were still unclear at that time. The data were also criticized for an unusually high and variable incidence of embryotoxicity in control animals (Neubert and Dillman, 1972).

by subcutaneous injection in DMSO

Emerson et al. (1971) evaluated a commercial 2,4,5-T with less than 0.5 ppm TCDD and found that 24 and 40 mg/kg/day through days 6-15 of gestation caused no teratogenesis in rats. Sparschu et al. (1971) found that 50 mg was also nonteratogenic, although there was a slight increase in incidence of delayed skull ossification. (Such alteration in development is not considered teratologic, because the abnormality disappears with age. One criterion of teratogenic effect is irreversibility.)

Treatment at 100 mg/day for days 6-10 caused generalized maternal toxicity, with only 4 survivors of 25 treated animals. Intestinal hemorrhage, considered a common fetotoxic but not teratologic lesion, was found in only one pup in this study. Khera and McKinley (1972) found a similar dose response.

In mice the effective dose is similar to that in rats. At 50 mg 2,4,5-T/kg/day, through days 6-15 of gestation, the frequency of cleft palates was increased from 4.7% to 20% and resorption frequency increased. The incidence was raised to 73% by a dose of 110 mg/kg/day with a decrease in fetal weight (Bäge et al., 1973). The high dose also caused a two-fold increase in rib and vertebral malformation, but only marginal increase in dilated renal pelvis. Neubert and Dillman (1972) found increased cleft palate in NMRI mice at doses over 200 mg/kg/day through days 6-15, increased embryo lethality at doses over 45 mg/kg/day reduction in fetal weight above 15 mg/kg/day. Strain differences in mice are significant. The dose causing at least one cleft palate in 50% of litters was 25 mg/kg/day in the most sensitive of 5 strains tested and 105 mg/kg/day in the least sensitive (Gaines et al., 1975). Roll (1971) found detectable teratogenesis at doses above 35 mg/kg/day through days 6-15 of pregnancy. The no-effect level with respect to teratogenesis was established as 20 mg/kg/day.

ridiculous dosage rate

Hamsters are less sensitive to cleft palate, but do have a somewhat higher incidence of delayed head ossification which is not generally defined as a teratologic manifestation (Collins and Williams, 1971). The doses ranged from 40-100 mg/kg and there was a considerable difference among samples of 2,4,5-T of different sources and levels of TCDD contamination.

Fetal rabbits are apparently unaffected by maternal doses up to 40 mg 2,4,5-T/kg/day through days 6-18.

Ruminants are apparently also quite resistant to teratogenic effects of 2,4,5-T. Binns and Balls (1971) fed 100 mg 2,4,5-T/kg to 11 ewes from the 14th to 36th day of gestation, and fed 100 mg 2,4,5-T(PGBE)kg to another group during the same period. No evidence of deformity appeared in any of the lambs. The TCDD content was 1 ppm.

The no apparent effect levels in rodents reported in the preceeding studies contrast sharply with data in abstracts of reports by Konstantinova (1974a, 1974b), who is said to have found that 4.2 mg/kg/day of the butyl ester of 2,4,5-T through the entire gestation period caused embryotoxicity, nervous and hematologic change and histopathology in the mother. A dose rate of 0.42 mg/kg affected growth and development, nervous, liver and kidney functions and lowered fertility. The succeeding generation was found to have minor changes in organ weights. (Direct translations of these papers are not available; data quoted is obtained from Chemical Abstracts.)

There has been limited study of the teratogenic effect of 2,4,5-T in primates. Dougherty et al. (1975) administered 0.5, 1.0, and 10 mg/kg/day to groups of 10 pregnant rhesus monkeys from day 22 through 38 of gestation. The high dose was established after finding that 12 mg or more 2,4,5-T/kg daily for 18 days caused vomiting and weight loss in monkeys of both sexes. No teratogenesis occurred, although there were 1 or 2 abortions, premature births, or neonatal deaths in all groups, including the control.

* Include Wilson's study in monkeys too. 7849

The importance of TCDD in the teratogenicity of 2,4,5-T was examined soon after the contamination was recognized. Courtney and Moore (1971) tested 2,4,5-T with 0.5 and 0.05 ppm TCDD, 2,4,5-T and TCDD together and

The entire section on 2,4,5-T should precede the section on TCDD & put the dosage levels into more reasonable perspective

TCDD alone in mice. It was evident that the concentrations used or addition of 1 µg TCDD/kg/day through days 6-15 did not alter the apparent teratogenicity of 2,4,5-T. Neubert and Dillman (1972) reached a similar conclusion, suggesting that to potentiate 2,4,5-T effect on pregnant mice at least 1.5 ppm TCDD is necessary. Collins and Williams (1971) observed additive effects of 2.9 and 45 ppm TCDD contamination of 2,4,5-T administered to hamsters, but many of the changes appeared characteristic of primary TCDD intoxication.

in contrast to current levels of 0.02 ppm or less in 2,4,5-T and ester products.

Nutritional status seems to have little influence on the effect of 2,4,5-T on reproductive functions. Hall (1972) fed 250 and 1000 ppm 2,4,5-T with diets containing 20% and 60% casein. Resorption and stillbirth incidence and litter size were similar in all groups, although fetal organ and placenta weights were decreased in the high 2,4,5-T, high protein group. Effects of low protein intake were augmented by the high concentration of herbicide.

Incubating eggs have been considered to be particularly vulnerable to herbicides because they remain in one place, and because the eggshell is porous. A number of studies of 2,4-D effect on eggs have appeared since 1967, with conflicting results (see section on 2,4-D), but 2,4,5-T has not received much attention. Somers et al. (1973) sprayed hen eggs with a formulation containing 2,4,5-T and 2,4-D in a treatment equivalent to 11.2 kg/hectare (10 times normal field application rates) prior to incubation without effect on hatchability or early survivability of chicks. They then treated eggs of the pheasant, as a genetically more heterogeneous species, in the same way, without adverse effect. Entry of herbicide into the egg was verified analytically. The treatment did, however, increase weight gain in male chicks during the first four weeks of life (Somers et al.,

1974). The LD_{50} of 2,4,5-T in DMSO, injected directly into the air space of chicken eggs at doses up to 125 mg/kg has been calculated to be 62 mg/kg, and in acetone carrier, 133 mg/kg (Strange et al., 1976). No teratogenic effect was evident. The solvent toxicity was shown to be substantial, but it seems clear that acquisition of an effective dose of 2,4,5-T by an egg in the field is highly unlikely.

In fact, immersion of hen eggs in 1% 2,4,5-T was ineffective, and a 5% solution was only moderately effective. The eggs were immersed for 10 seconds, then returned to incubation (Gyrd-Hansen and Dalgaard-Mikkelsen, 1974).

Insects may be more sensitive; Dävring and Sunner (1971) and Dävring (1975) have shown that while Drosophila are highly resistant to lethal effects of 2,4,5-T ester (LD_{50} = 4700 ppm in the diet), 1 ppm caused disturbed egg follicle development and chromosomal defects in developed oocytes.

A study on a species of killifish has shown that concentrations of 20 ppm have substantial teratogenic effect. The embryos developed several cardiovascular anomalies, and occasional eye and splenic defects. The 20 ppm treatment reduced hatchability to less than 50%, and 25 ppm allowed less than 5% to hatch. No anomalies were found after treatment with 14 ppm 2,4,5-T, and the hatch was reduced only 4% (Schreiweis and Murray, 1976).

Carcinogenic and Mutagenic Potential of 2,4,5-T

Probably the most important issue about any chemical introduced into the environment by human activity is the possibility that it may increase the incidence of cancer in the human population. The probability of muta-

genic activity is of almost equivalent concern, both because of the possibility of genetic alteration and because mutagenesis may be a useful predictor of carcinogenic activity. The relationship is by no means constant. However, McCann et al. (1975) have assembled data from a number of laboratories using microbial systems ("Ames test") and find that 85% of known carcinogens are positive, 10% of non-carcinogens are active. This is not to say that almost every agent identified as a mutagen will be carcinogenic, but the test should become an aid in deciding priorities for direct screening.

Only a limited number of experimental studies of 2,4,5-T carcinogenicity have been made. A screening program for 120 pesticides and industrial chemicals was reported by Innes et al. (1969). Eleven of the agents caused an elevated incidence of tumors; none of the phenoxy herbicides, including 2,4,5-T, caused increased tumor formation at a dose previously determined to be the maximum tolerable daily dose over 20 days, without lethality (21.5 mg/kg/day for 2,4,5-T). Muranyi-Kovacs et al. (1976) treated two strains of mice with 80 ppm 2,4,5-T (TCDD content 0.05 ppm) in the diet for more than 500 days. Daily dosage was about 12-15 mg/kg. In one strain survival time was significantly decreased in males, in the other significantly increased in females. An apparently significant small increase in tumor incidence occurred with the increase in life span. The authors are concerned that altered life span may confound the analysis, but consider that 2,4,5-T can not yet confidently be considered non-carcinogenic, and requires further analysis.

A two year study of rats given up to 30 mg 2,4,5-T/kg/day has just been terminated by Dow Chemical Company, but the pathological evaluation had not been completed at the time of this preparation.

7352

see Dow's RPAR response for more info

One epidemiological study has been made of tumor incidence in a group of Swedish railroad employees. It was possible to isolate groups who had handled phenoxy acids exclusively, and a cohort of 207 persons with at least 45 days of exposure, totaling 1747 person-years. No increase in tumor incidence could be detected in these workers although increased incidence appeared in groups exposed to other herbicides (Axelson and Sundell, 1974).

Mutagenic assessments have been carried out on a number of systems. Buselmaier et al. (1973) were not able to show 2,4,5-T induced mutations in Salmonella typhimurium G46 his-, and Serratia marcescens a21 Leu- and a31 his- in a host mediated assay in mice. Serum from animals treated orally with 2,4,5-T also did not induce mutants in S. typhimurium his- (Styles, 1973). Anderson et al. (1972) were unable to demonstrate any mutagenic properties of 2,4,5-T or other phenoxy herbicides when tested against eight histidine requiring mutants of S. typhimirium.

Sex linked lethal tests of adult male Drosophila showed 2,4,5-T to be negative, although fertility was decreased (Vogel and Chandler, 1974). Majumdar and Golia (1974) were able to show an increase in sex linked recessive lethal mutations after 15 day feeding on 1000 ppm 2,4,5-T. A non-significant increase was apparent after feeding 250 ppm.

In gerbils six-day feeding of up to 150 mg 2,4,5-T/kg produced no increase in chlorosomal abnormalities, but higher doses did cause some increase (Majumdar and Hall, 1973). Jenssen and Renberg (1976) tested 2,4,5-T for mutagenicity by seeking micronuclei in erythrocytes of mouse bone marrow, which is a high resolution detection system. The system was cytogenetically negative, although some mitotic depression was detected. It was noted that only about 5% of circulating 2,4,5-T found its

way into the cells, which was suggested as indicating a limited hazard due to lack of access.

A limited survey of pesticide applicators who had been in contact with a variety of agents showed some increase in chromatid gaps and breaks during the spray season (Yoder et al., 1975). Recovery in the off season reduced frequency below that of controls, suggesting an augmented repair system. 2,4,5-T was the least frequently used agent, and it is not likely that any observed effect can be attributed to it.

Dow Chemical Company has maintained a program of continuing health monitoring of 2,4,5-T production workers. Cytogenetic evaluations of 52 men in early 1970 were reported to be negative (Johnson, 1971), and a second analysis in 1974 also found no evidence of abnormality (Kilian, 1975). An interesting finding, however, was that the fraction of each exposed group found to have no abnormal cells was above 80%, while of the control group only 74% had no abnormal cells.

Absorption, Distribution, Metabolism, and Excretion of 2,4,5-T

2,4,5-T has a rather short residence time in all species. A cow given 450 mg of 2,4,5-T acid in four daily doses excreted the entire amount in urine in six days (St. John et al., 1964). Erne (1966a, 1966b) administered 100 mg of the amine salt/kg to rats and swine and found plasma half time for the rat to be three hours, and 10 hours for pigs. Kidney, liver, lungs, and spleen concentrations of 2,4,5-T could occasionally be forced above plasma levels, but apparently none of the material entered the brain or adipose tissue. Tissue half lives ranged from 5-30 hours. When the agent was administered repeatedly plasma levels tended to lessen, with increased excretion. Of the 2,4,5-T in blood, about

20% was in erythrocytes. 2,4,5-T is excreted more slowly by mice than by rats, at a rate of 1-4% of the original dose per hour (Zielinski and Fishbein, 1967).

By overloading sheep (four 250 mg/kg doses) it is possible to produce residues in tissues. Maximum accumulations were about 100 ppm in fat and muscle. In each case residues were found in the acid form regardless of the form in which it was fed (Clark et al., 1970). The latter finding is at some variance with Clark et al. (1971), in which the PGBE ester of 2,4,5-T was reported to remain as the ester in urine and tissues. Cattle fed 0.15 and 0.75 mg 2,4,5-T propylene glycol butyl ether esters/kg/day for 32 weeks, produced no increase over background residue. Clark et al. (1971) later reported feeding sheep 2000 ppm 2,4,5-T in the diet and finding 1.0 ppm 2,4,5-T in muscle. No residue could be detected 7 days later. Deer browsing on land treated for reforestation were found not to contain significant tissue residues; measurable 2,4,5-T was found in stomach contents, urine, and feces up to 43 days after herbicide application (Newton and Norris, 1968). The implication, again, is that sustained field intake will not cause detectable tissue residues.

Grunow et al. (1971) found about 50-70% of administered 2,4,5-T in urine, and found that a substantial fraction emerged as derivatives, one of which was identified as N(2,4,5-trichlorophenoxy acetyl) glycine. The taurine conjugate and 2,4,5-trichlorophenol have also been identified (Grunow and Böhme, 1974). Nony et al. (1976) have measured 2,4,5-T, its glycine amide and alkaline hydrolyzable conjugates in blood, urine, and feces. The proportion of metabolites is quite low in blood, and increases substantially in urine from 24-72 hours after administration of the herbicide. In the feces the glycine amide is found in only limited amounts, but other conjugates may account for as much as 25% of the total recovery.

Research with labeled 2,4,5-T in rats showed that the half-life (T/2) of plasma clearance was about 4.5 hours and essentially dose independent below doses of 50 mg/kg but increased as dosage increased to 19.4 hrs at 100 mg/kg and 25.2 hr at 200 mg/kg. The T/2 of a 5 mg/kg dose in dogs was much longer, 77 hours. About 90% of the 2,4,5-T in blood was reversibly bound to plasma protein, over a wide range of concentrations. It was found also that as the dose was elevated, a small amount of fecal excretion took place. Detectable, minute amounts of ^{14}C emerged in the respiratory gases (Piper et al., 1973). Fang et al. (1973) also showed a non-dose dependent T/2 at lower doses. Nony et al. (1976) found in excess of 10% of the total excreted material in feces. The extent of protein binding has been further defined by Kolberg et al. (1973); bovine serum albumin binds 13-14 moles 2,4,5-T/mol BSA.

As suggested by the slower T/2, the percentage of dose excreted/day decreased sharply at the higher doses, indicating a limit in excretory capacity by the kidney. Rat kidney tissue slices can concentrate 2,4,5-T about 15 times, slices from dogs can concentrate about 9 times. Berndt and Koschier (1973) found an energy dependent uptake of phenoxy herbicides by renal cortical tissue. Metabolic inhibitors depress the process. Acetate and lactate as substrates enhance the accumulation. Increased 2,4,5-T in the medium slows the concentrating capacity of the kidney.

The above work was later carried forward by resorting to intravenous injection of 2,4,5-T in order to take advantage of useful pharmacokinetic models (Sauerhoff et al., 1976). Doses of 5 mg/kg and 100 mg/kg were used, and samples were taken through 36 hours (5 mg) and 72 hours (100 mg). After 5 mg/kg rats excreted about 50% of the body burden every 12 hours, in spite of a 4.3 hour plasma T/2, further illustrating the concentrating

capacity of the kidney. At 100 mg/kg the plasma T/2 was 23.1 hours for the first 36 hours, then was comparable to the animals given a lower dose.

In humans (Gehring et al., 1973) found that a 5 mg/kg dose was excreted with a T/2 of 23 hours for both plasma and whole body clearance. Essentially all of the administered material emerged in urine unchanged.

The phenoxy acids are relatively strong organic acids, which is one of the reasons they are excreted in large part as the parent molecule. The possibility of hydrolysis by rumen flora or by tissue enzymatic processes was first considered by Wright et al. (1970) who found that after feeding of 2(2,4,5-trichlorophenoxy) ethyl-2,2-dichloropropionate to sheep, 2,4,5-trichlorophenol residues could be detected in tissues. Clark et al. (1975) measured phenoxy acid and phenol residues in muscle, fat, liver, and kidney of sheep fed 2000 ppm 2,4,5-T for 28 days, and found 1.00, 0.27, 2.29, and 27.2 ppm 2,4,5-T and 0.13, <0.05, 6.1, and 0.9 ppm 2,4,5-trichlorophenol, respectively. Seven days after withdrawal, 2,4,5-T residues were well below 0.1 ppm or below the detection limit of 0.05 ppm. The phenol remained at significant levels in both liver (4.4 ppm) and kidney (0.81 ppm).

According to Koschier and Berndt (1976c) the rate of excretion rises almost immediately to about 80% of input in animals treated daily, then continues to rise for 7-9 days until excretion approximately equals input, where it remains through the duration of treatment. The rate of administration was 20 mg/kg/day. As dose rate increased the initial percentage excreted was less, but 7-9 days was still required to reach a rate equal to input. An apparent over-shoot then took place for 5-6 days.

The markedly different rates of 2,4,5-T excretion by rats and dogs was studied in vitro by Hook et al. (1974). They found that kidney of

both species actively accumulated 2,4,5-T by a saturable, oxygen dependent process which was depressed by other organic anions. Rat kidney had a greater dependence on potassium, and dog kidney increased accumulation of 2,4,5-T in the presence of acetate. The capacity of kidney to transport the standard test anion, para-amino hippuric acid (PAH), can be competitively inhibited by 2,4,5-T, indicating that both are transported by the same mechanism. The authors concluded that the greater capacity for PAH transport by rat tissues accounts for the difference in retention between rats and dogs. Apparently lower renal excretion is also responsible for the extended half-time of 2,4,5-T in new-born rats observed by Fang et al. (1973); Hook et al. (1974) have found that kidneys of 10 day old rats are much less efficient than adults. Subsequent work by the same group (Hook et al., 1976) indicated that plasma binding in dogs was more tenacious than in rats.

The high specificity of 2,4,5-T in causing cleft palate has been investigated by Dencker (1976) as a distribution phenomenon. The sensitive period has been shown to be late in fetal development, during days 12-13 (Neubert and Dillman, 1972). Palatal closure occurs very late in organogenesis and is complete at about day 15.

Dencker (1976) points out that no unusual uptake of 2,4,5-T in these structures occurs, but overall uptake in the fetus is greatly increased between days 11 and 18. It may well be that this increase, at a time when formation of most other structures have progressed far enough that they are not sensitive, is responsible for cleft palate.

It is possible for 2,4,5-T to reach milk if a sufficient level is included in the diet. At 30 ppm in the diet for two weeks, ~~followed by one week on untreated feed~~, no 2,4,5-T or trichlorophenol was found at

confusing

a detection level of 0.05 ppm; at 100 ppm trichlorpphenol was barely detectable. At 1000 ppm 0.31-0.54 ppm 2,4,5-T and 0.16-0.27 ppm trichlorophenol was found in milk, and somewhat less was found in cream (Bjerke et al., 1972). These figures can be put in the perspective of forage consumption. Residues on grass are on the order of 100-150 ppm/lb/acre per pound applied per acre (Morton et al. 1967). immediately after application. These amounts drop sharply to about 20% of initial levels during the initial two weeks post-application. This factor, with the rapid excretion characteristic of 2,4,5-T make even limited human exposure through dairy products or meat highly unlikely unless products are obtained immediately after heavy spraying. (Lang, 1972).

Behavior of 2,4,5-T in the Environment and Effects on Submammalian Species

One factor in the hazard potential of an herbicide is determined by its persistence after application and its movement and reactions in soil, water, or in plants.

Appearance of 2,4,5-T in forest streams has been shown to result only if the herbicide was introduced directly to the stream, and diminished from about 0.1 ppm to less than 0.01 ppm within one day after treatment (Norris, 1967). Norris (1968) also found that heavy rains six months after treatment did not move detectable 2,4,5-T into streams.

The herbicide is usually degraded on the forest floor quite rapidly. Norris (1969) found that nearly 90% disappeared in two months, although cold weather, sterile soil, and lack of moisture may extend the period of degradation over as much as 9 months. The trichlorophenol hydrolysis product of 2,4,5-T degrades faster than the parent compound (Alexander and Aleem, 1961). Evaluation of several soils from South Vietnam indicates that concentrations on the order of those expected after a typical forest application should be virtually gone in seven weeks (Byast and Hance, 1975).

More than 20% of the radioactive carbon of labeled 2,4,5-T had become incorporated in the soil, but actual residues of herbicide were usually about 1%. 2,4,5-T has also been found short lived in various Texas soil by Bovey and Baur (1972). Altom and Stritzke (1973) studied 2,4,5-T in three Oklahoma soils and found half-times of 24, 14, and 21 days. The shorter time was on grass lands, the others were forests.

Leaching tests show that 2,4,5-T bound in soil remained in the upper 6 inches of test columns even after application of 4.5 inches of water (Wiese and Davis, 1964).

The various esters of 2,4,5-T are often intrinsically more toxic than the parent acid, probably because they are more fat soluble. In an aqueous system, however, they hydrolyze rapidly to the acid, and the rate is enhanced by soil and microorganisms (Teasley and Williams, 1970, as quoted by Kenaga, 1974). It seems reasonable to expect a fairly rapid hydrolysis in any system with some water present. Also on plant leaves, and grasses (Norton, 1967).

In essence, 2,4,5-T disappears relatively rapidly after application, probably within two months in a forest environment. The evidence also seems firm that 2,4,5-T deposited on the ground will not move into a water course, and will not leach into deeper layers.

It is difficult to separate physical behavior after application from effects on lower organisms, because there is a very close interface between an agent bound to soil or in water, and insects or fish. Lower organisms are of direct importance in assessing environmental impact of any introduced chemical. Food chain effects may predict repercussions at higher levels, and specifically sensitive organisms may be useful indicators of pollution. Ecological displacement of any kind must be accepted only after careful examination, whether secondary to the intended

impact of the chemical on plant species or as a result of direct toxic effect of the applied chemical.

The various derivatives of 2,4,5-T differ in toxicity to fish. The parent herbicide, the amine salts and isooctyl esters are relatively limited in toxicity, but the propylene glycol butyl ether and butoxy ethanol esters are quite toxic to some species. There is a large number of gross effect studies on various aquatic species; these generally use an end point of death or immobilization. Shellfish studies measure shell growth as an index of effect.

Standardization of methods has not received as much attention as seems justified. Study periods vary from 24-96 hours, water temperatures sometimes appear outside the normal acceptable range for the species, and most evaluations seem to be carried on under static conditions. Nonetheless, the relative gross effects can be useful in reference to a given application in the field. As a very rough approximation, a 2 kg/hectare application over a water course would produce a concentration of about 2 mg/L or 2 ppm, if an assumption of dilution in the upper 10 cm of water is accepted. Flowing water will diminish the concentration below detectable limits in a few hundred yards (Evans and Duseja, 1973), and application over deeper water will dilute accordingly.

Concentrations of 2,4,5-T or its derivatives found to have no effect are listed below as acid equivalents:

	Species	Exposure Period	No Effect Acid Equivalent Concentration ppm	Reference
2,4,5-T	killifish	48 h	50	Butler, 1963
	mullet	48 h	50	Butler, 1963
	sea lamprey	72 h	2	Applegate et al., 1957
2,4,5-T Na salt	bluegill	12 d	46	Hiltibran, 1967

2,4,5-T butyl ester	trout	24 h	4.1	Applegate et al., 1957
	bluegill	24 hr	4.1	Applegate et al., 1957
	lamprey	24 h	4.1	Applegate et al., 1957
2,4,5-T isooctyl ester	bluegill	8 d	7	Hiltibran, 1967
	green sunfish	8 d	7	Hiltibran, 1967
	bluegill	12 d	0.7	Hiltibran, 1967

Median lethal concentrations of 2,4,5-T derivatives are similarly tabulated:

	Species	Exposure Period (hours)	LC_{50} Acid Equivalent Concentration ppm	Reference
2,4,5-T DMA salt	bluegill	48	144	Hughes and Davis (1963)
2,4,5-T TEA salt	bluegill	24	54	Davis and Hughes (1963)
2,4,5-T Oleic-1,3-propylene diamine salt	bluegill	48	2.9	Davis and Hughes (1963)
2,4,5-T isopropyl ester	bluegill	48	1.7	Davis and Hughes (1963)
2,4,5-T isooctyl ester	bluegill	48	31	Hughes and Davis (1963)
2,4,5-T butoxyethanol ester	bluegill	48	1.4	Hughes and Davis 1963
2,4,5-T PGBE esters	bluegill	48	17	Hughes and Davis, 1963
	spot	24	0.21	Cope, 1965
	harlequin	48	1.0	Alabaster, 1969

While the more complex esters of 2,4,5-T tend to greater toxicity in water at pH 6.5, they hydrolyze to the parent acid in about a day (Teasley and Williams, 1970, as quoted by Kenaga (1974).

Effects of 2,4,5-T on other aquatic organisms have also been measured. In flowing salt water 1 ppm 2,4,5-T caused no mortality in brown shrimp and 2 ppm was without effect on oyster shell growth (Butler, 1963). The PGBE ester of 2,4,5-T at 0.14 ppm (0.09 ppm acid equivalent) caused a 50% decrease in oyster shell growth after 96 hour exposure (Butler, 1963).

Studies of terrestrial non-mammals seem to have been limited to honey bees. Honey bees are an important component of the crop cycle and in many areas are systematically managed for pollination. Phenoxy herbicides, including 2,4,5-T, fed in 60% sucrose-water solution at 10 ppm did not affect hatching, but reduced brood development when fed at 100 ppm (Morton and Moffett, 1972). When sprayed in a water carrier at field application rates, 2,4,5-T was non-toxic. (Petroleum solvents alone caused high mortality during the first day after spraying.)

Aerial spraying did not result in herbicide accumulation in honey sacs or colonies (Moffett and Morton, 1972). The point was made that the primary damage would be failure of flowers and loss of nectar, rather than direct toxicity. When fed directly to newly emerged bees, the phenoxy herbicides were essentially non-toxic at concentrations up to 1000 ppm in the diet (Morton et al., 1972).

2,4-D

Toxicity to Humans

For some reason there have been reported several incidents of 2,4-D poisoning in humans, but virtually no reports of human intoxication by 2,4,5-T. Probably the best known was a suicide by a single dose of 2,4-D DMA salt of which more than 90 mg/kg was estimated to have been retained by the victim, after extensive vomiting (Nielsen et al., 1965). An

accidental poisoning with a formulation containing 49% S-ethyldipropyl-thiocarbamate, 9% kerosene, and 36.5% 2,4-D isooctyl ester caused symptoms and laboratory findings attributable to 2,4-D intoxication. Among these were twitching and paralysis of intercostal muscles, hemoglobinuria, and myoglobinuria. No evidence of peripheral neurological damage was evident during 36 months of follow-up observations (Berwick, 1970). Goldstein et al. (1959) reported on three cases in which 2,4-D esters were absorbed through the skin, resulting in peripheral neurological changes. One patient spilled 60 ml of a 10% solution of 2,4-D ester on the forearms and did not wash. Unusual fatigue developed in a few hours, and over 10 days after exposure there was extended nausea and vomiting, with considerable weight loss. A second exposure caused similar symptoms, then pain and numbness in all digits, loss of skin from the palms, and within six weeks there was substantial general neural damage. Another patient eventually developed metacarpal pain and swelling in both hands, and later became partially paralyzed. The third experienced gastrointestinal disturbance and vertigo, then paresthesia in the arms and legs and persistent general muscle fasciculations. In two of the cases, there were second exposures which appeared to cause greater impact than the initial event. Similar cases have been described by Todd (1962), Berkeley and Magee (1963), and Wilson (1956).

Seabury (1963) attempted use of 2,4-D in treatment of a terminal case of disseminated coccidoidomycosis, on the rationale that the 2,4-D as a synthetic plant hormone might alter the course of the fungus infection. At the time of the treatment (1949) there was no therapeutic agent for the disease. In 24 treatments over a period of more than a month the dosage was raised to a final treatment of 3600 mg. No prior doses, up to 2000 mg caused any response, but the final administration caused

extreme quiescence, and fibrillation of muscles in the face and hands, followed by deep stupor and reflex failure. The patient recovered from the 2,4-D within 48 hours and died of the fungus disease about two weeks later.

General Toxicity to Laboratory Animals

One of the earliest published studies of 2,4-D toxicity was by Bucher in 1948. She found an acute LD_{50} of 280 mg/kg in mice, and found that single doses of 150-200 mg/kg would produce a myotonia persisting several hours. The animals remained awake and alert, but when moved exhibited gross incoordination. They were capable of working out of the syndrome with continued exercise, but if allowed to remain quiet the myotonia would recur. The chemical also caused diarrhea in mice at the higher doses, and gastrointestinal and upper respiratory irritation in dogs. Mice were able to tolerate daily doses of 1/2 LD_{50} for three months. Hill and Carlisle (1947) established LD_{50} values for several species: mice, 375 mg/kg; rats, 666 mg/kg; rabbits 800 mg/kg; and guinea pigs, 1000 mg/kg. They also treated monkeys and were able to give up to 214 mg/kg without severe residual effect. Twice that dose caused vomiting, incoordination, and loss of muscle tone. Rowe and Hymas (1954) summarized lethality data on 2,4-D and five salts or esters; in general the lethal doses were very high, with the exception of a few dogs given 2,4-D itself, for which the LD_{50} was estimated at 100 mg/kg. The latter data was apparently derived from the observations of Drill and Hiratzka (1953). Hansen et al. (1971) conducted a two-year feeding study on rats, at dosages of 5 to 1250 ppm 2,4-D in the diet, without appreciable change in growth, hematologic values, or organ weight. In the same study dogs were maintained for two years with little effect at doses up to 500 ppm 2,4-D.

2,4,5-T

Drill and Hiratzka (1953) found that 20 mg/kg daily was fatal in 3 of 4 dogs within 49 days of a 90-day study.

Grazing species of animals might be expected to suffer exposures in the field from eating treated vegetation. Palmer and Radeleff (1964) evaluated a series of herbicides at high doses in limited numbers of ruminants. One sheep tolerated 481 daily doses of 100 mg 2,4-D alkanolamine salt, another was unaffected by the same treatment with 2,4-D propylene glycol butyl ethyl ester. The first compound was lethal after 7-500 mg/kg doses; the second was lethal after 9-250 mg doses. A bovine tolerated 112 doses of 50 mg of the alkanolamine salt, but another developed digestive problems after 80 doses of 100 mg/kg. Palmer (1963) administered daily doses of 50-250 mg 2,4-D/kg as the alkanolamine salt to a group of steers. The steer given 100 mg/kg developed ruminal atony after 86 days, presumably as a result of the herbicide. Animals given 200 and 250 mg/kg became intoxicated in 34 and 15 days, respectively, with dessicated mucous membranes and tendency to nosebleed when restrained.

Ruminants on sprayed pasture or cows given 5.5 gm daily did not show clinical evidence of toxicity, nor did production decline. 2,4-D appeared in serum of a cow fed 5.5 gm/day for 106 days, but none was passed in milk (Mitchell et al., 1946).

Chickens and other fowl also appear relatively insensitive. Bjorn and Northen (1948) found no impairment in weight gain at doses up to 28 mg 2,4-D alkanolamine/kg three times weekly for four weeks; at 280 mg/kg there was a marked decrease in gain. Single doses of 765 mg/kg were lethal, 380 mg/kg was not. 2,4-D did not cause decrease in growth rate at dose rates up to 1000 ppm in the diet, but at 7500 ppm growth essentially stopped (Whitehead and Pettigew, 1972). The acute LD_{50} was

estimated at 900 mg/kg. Whitehead (1973) tested dietary 2,4-D at concentrations up to 100 mg/kg diet, and found that at dietary levels of 10 mg/kg diet or greater, growth rate was depressed. Because food conversion efficiency was not affected, it was suggested that palatability was affected resulting in decreased consumption. Chickens will discriminate against 2,4-D contaminated food (Whitehead and Pettigrew, 1972). The U.S. Fish and Wildlife service tested a number of chemicals by feeding to various game bird species in the early 1960s. 2,4-D acetamide given to young quail at 2500 ppm caused 72% mortality in 12 days, the butoxyethanol ester at 5000 ppm caused 28% mortality in 135 days, and 2500 ppm dimethylamine salt caused 12% death in 138 days. In older birds 2500 ppm was almost without effect after 50 days, and after 111 days of 1000 ppm feeding. Tests on Coturnix indicated a similar tolerance (Stickel, 1964). More recently, Hill et al. (1975) have shown the LC_{50} for 2,4-D acetamide in the diet of bob white, coturnix, pheasant, and mallard to be in excess of 5000 ppm. The butoxy ethanol ester and DMA salt have a similar low toxicity.

A number of other biological effects of 2,4-D have been found experimentally, usually at very high doses. Dybing and Kolberg (1967) suggested that 2,4-D was actively reabsorbed, and if so would competitively decrease the clearance of p-aminohippurate without interfering with creatinine clearance. Studies with rabbits given 100 mg priming doses, then 2 mg 2,4-D/min produced decreased PAH clearance at doses of 62-115 mg/kg. Chang et al. (1974) studied a number of effects on rat liver following treatment with 2-5 gm/kg 2,4-D over 4-7 weeks. Glycogen content was 50-100% higher, and liver nuclei synthesized RNA more actively in vitro than

2000 to 5000 mg/kg

did controls. DNA content per liver was decreased. In this work, 2,4-D was compared with 2,4,5-T; the latter compound caused increased liver weight and protein accumulation, while 2,4-D decreased liver weight and lessened RNA content, with no protein accumulation. Speculation is justified that TCDD in 2,4,5-T was responsible for the difference. A dietary supplement of 60 ppm 2,4-D to lambs had no effect on rumen liquor or sheep or serum proteins. Weight gain was slightly decreased over a 12 week feeding period (Abou-Akkada et al., 1975). The same authors (1973) had previously found that 2,4-D did not alter ruminal microbial function. At fairly high doses (80 mg/kg/day, for seven days) 2,4-D increases ^{131}I intake by the thyroid. The effect only occurs in a normally functioning thyroid gland; it was not seen after hypophysectomy or iodine depletion (Florsheim and Velcoff, 1962). The effect is apparently due to lowered thyroxine binding to serum protein (Florsheim et al., 1963) but whether this is secondary to 2,4-D binding or is a specific pharmacologic effect is not known.

There has been limited in vitro study of 2,4-D. Weiss and Beckert found stimulated mitotic activity and increased chromatin after cultured monkey kidney cells, Girardi heart cells, and trout gonad cells were exposed to 10 or 50 ppm 2,4-D for 72 hours. 2,4-D inhibits mevalonate incorporation into non-saponifiable lipids of liver, but only at concentrations in excess of 1 mM (Olson et al., 1974). Oxidative phosphorylation in rat mitochondria appears to be sensitive to concentrations as low as 10^{-4} M; at 10^{-3} M respiration was normal but P/O decreased to 20% of normal (Brody, 1952). In L929 cells in monolayer culture, 2,4-D causes triglyceride accumulation when present at a concentration of 500 µg/ml (Kolberg et al., 1972) and inhibits all growth at 50 µg/ml (Kolberg et al., 1971)

2,4-D at high doses is used also as a chemical inducer of experimental myotonia, as a model of the congenital disease (Iyer et al., 1977; Eyzaguirre et al., 1948). The action is apparently due to an increase in membrane resistance and reduced chloride transport. Electrical activity of the brain was found to be reversibly inhibited by doses of 200 mg 2,4-D/kg, in rats (Desi et al., 1962). The chemical also produces a primary myopathy that is useful in study of the family of diseases which includes white muscle disease in lambs (Heene, 1969). These effects are not of toxicologic significance except in cases of massive acute intake resulting in myoneural symptoms.

Effect of 2,4-D on Reproductive Function

As with 2,4,5-T, the possibility that 2,4-D has teratogenic potential was first studied by the Bionetics Research Laboratories study (Bionetics Research Laboratories, Inc., 1970). The data were suggestive that incidence of failed lower jaw formation was somewhat greater than that resulting from the DMSO carrier. Schwetz et al. (1971) measured teratogenic and fetotoxic effects of 2,4-D and two esters on rats. The higher daily doses (75 mg 2,4-D/kg; 75 mg propylene glycol butyl ester of 2,4-D/kg; 87.5 mg isooctyl ester of 2,4-D/kg, each just below the maternal toxic dose) caused decreased fetal weight, subcutaneous edema, delayed bone ossification and wavy ribs. Most of these changes are fetotoxic rather than teratogenic. The last two are developmental effects but have no effect on survivability. No teratogenic responses were found at any dose.

There is some difference in definition of teratogenic response among authors in the field. A variety of skeletal defects that do not interfere

with postnatal survival were found by Khera and McKinley (1972); most effects observed were wavy ribs or fused sternum. The increased incidence of these changes was evident at doses as low as 25 mg/kg/day. 2,4-D teratogenesis was studied by Bage et al. (1973) but only in presence of 2,4,5-T. The mixture caused some teratogenesis, but was less effective than 2,4,5-T alone, so the impact of 2,4-D in the system was difficult to evaluate.

Hamsters are subject to a teratogenic effect of high doses of 2,4-D. Collins and Williams (1971) found that 2,4-D from three different sources caused a low incidence of anomalies, usually fused ribs, at doses of 100 mg/kg/day through days 6-10 of gestation. There was no satisfactory dose response relationship. Dietary 2,4-D at 500 and 1000 ppm did not alter reproductive function in a three-generation, 6 litter study with rats. Percentage of pups surviving to weaning and weanling weight was decreased at 1500 ppm, however (Hansen et al., 1971).

2000 mg in 50 kg sheep = 40 mg/kg per day

Sheep are apparently not subject to 2,4-D induced teratogenesis. Binns and Johnson (1970) administered 2 grams daily for 30, 60, and 90 days following breeding and caused no malformation. There were presumably 6 sheep per group but the number in this specific experiment was not stated.

Eggs are peculiarly vulnerable to exposure to herbicides and several studies have been directed toward defining the hazard of 2,4-D to chicken or game bird eggs. An injected dose of 10 mg 2,4-D/egg caused 50% mortality, 5 mg/egg resulted in 30% loss, and at 0.5 mg/egg 90% of the eggs hatched. No deformities occurred at any dose (Dunachie and Fletcher, 1967). A later study by Dunachie and Fletcher (1970) confirmed these findings for 2,4-D and 2,4-DB (2,4-dichlorophenoxybutyric acid).

laparotomy during course of study is not acceptable protocol

were examined surgically and any observed tumors were biopsied. The diet with TCDD was then continued through the 78th week and all surviving animals sacrificed at 95 weeks.

Total dose = 0.0003 × 78 = 0.0234 mg/kg to 2 × 78 = 156 mg/kg in 78 weeks

There were 10 animals at each dose range; all those at 50-1000 ppb TCDD died by the fourth week. In the group given 1 ppt there were no tumors, but of those fed 5, 50, 500, 1000, and 5000 ppt 23 of 50 had developed tumors of several types. No control animals for this or a parallel experiment developed neoplasms. The great variety of tumors suggested to the authors the possibility that TCDD is a promoter rather than inducer of neoplastic activity, in view of the usual narrow spectrum of tumors caused by many chemical carcinogens. General pathologic changes have been described in a previous section.

need about other components in rat diet? unsaturated fats, or malonaldehyde

Two studies of carcinogenesis in rats have utilized continuous feeding of TCDD over a two-year period. IITRI One program has been completed but the analysis of histopathology and gross tumor incidence have not been completed. The other study, by Dow Chemical Company, has been completed and a preliminary report filed with EPA. Dietary levels were 21, 210, and 2200 ppt TCDD. The maximum dose caused increased tumor incidence in some organs and decreased incidence in others. There was substantial mortality resulting from general toxic effects. The intermediate dose caused moderate systemic toxicity but no tumors and the lowest dose was without effect other than slight liver changes such as induction of increased drug metabolizing capability. These latter changes were seen only in females (J. Davidson, personal communication). The reasons for differences between the Dow and Van Miller studies is not apparent; it is paradoxical that the Dow work had a ~~higher~~ normal incidence of tumors in control animals, while showing a lower incidence of effect in treated animals.

expand on this based on pre pub. copy of 2-yr study by Koiba et al.

Cytogenetic studies of TCDD by Green and Moreland (1975) suggested that the dioxin does not have potential for producing chromosomal abnormalities in bone marrow. The dose schedule provided up to 15 µg/kg/ daily for 5 days, with sacrifice on day 5. A single injection experiment with sacrifice at four weeks was also negative. Khera and Ruddick (1973) were also unable to find mutagenic activity of 2,4,5-T in a dominant lethal study.

Since it is not possible to obtain an absolutely TCDD-free preparation of 2,4,5-T, the studies of the herbicide may also be of value in considering TCDD effects. Effects of 2,4,5-T and TCDD on dividing African blood lily endosperm cells are not explicitly pertinent to animal studies, but the relation shown between herbicide and contaminant is of interest. Highly purified 2,4,5-T at 10^{-4} M was not effective, but 0.2 µg TCDD/L with or without 2,4,5-T, and commercial 2,4,5-T all caused dramatic mitotic inhibition and chromosomal aberations (Jackson, 1972) (0.2 µg/L = 200 ppt).

Hussain (1972) found that TCDD was somewhat mutagenic using the Ames procedure at doses that were intrinsically very toxic to the organisms. The authors suggested that the limited mutagenic activity was by intercalation of TCDD into DNA. Seiler (1973) has also obtained data suggesting that TCDD is mutagenic in the S. typhimurium TA 1532 strain. There was no indication of the concentrations necessary to produce effects.

Absorption, Distribution, Metabolism, and Excretion of TCDD

The biological action of a foreign compound is highly dependent on its logistics. A chemical must obviously be absorbed from the environment in order to interact, and it must be carried in blood to cells and move

to or across cell membranes to sites of reaction. The agent in its original form may interact with sensitive systems, or it may be converted to an active derivative after entering an organism. Such conversion usually takes place in the liver. A given organ or cell type may have a particular affinity or sensitivity for a toxicant and thereby be singled out for attack. The chemical nature of the agent or its product will also dictate the extent and rate of excretion in urine or bile or respiratory gases. Even with an active excretion mechanism such factors as sensitivity of bladder epithelium or an active enterohepatic circulation may result in toxic effects after elimination seems to have been accomplished.

In spite of the enormous capacity for induction of mixed function oxidases (discussed below) TCDD is metabolized to a very limited extent. Rats given a single dose of 50 µg/TCDD-U-^{14}C/kg by stomach tube excreted about 30% in feces during the two days following treatment, and then 1-2% daily over the 19 days following, to a total of 53%. Urinary-^{14}C was about 13% and respiratory excretion totalled 3.2%. After the initial 2 days, total clearance half-time (T/2) was about 17 days. Residual TCDD at 3, 7, and 21 days was greatest in liver and fat (Piper et al., 1973). The long residence time and limited respiratory and urinary excretion suggested that TCDD was essentially unchanged but this was not verified. As a general rule lipophilic compounds are either sequestered in fat, eliminated as conjugates in bile, or converted to a water soluble form and excreted by kidneys. Absence of urinary excretion therefore implies limited conversion. In a later study of rats given a lower single dose of 1 µg/kg, TCDD was identified in feces but not urine, and again, liver and fat contained the highest concentrations. T/2 was 31 days (Rose et al., 1976). In the same investigation, repeated doses 5 days a week for 7 weeks resulted in some urinary loss, but most TCDD was excreted into feces. In this program

of essentially continuous intake, T/2 was calculated as 24 days. The residual radioactivity in the liver was identified as unchanged TCDD. The report of Vinopal and Casida (1973) is suggestive that TCDD does not metabolize only because after giving a dose of 130 µg TCDD-^{3}H/kg to mice, no mention was made of tritium in the urine. Allen et al. (1975) found that over a 25 day period about 4.5% of a dose of 50 µg TCDD/kg appeared in urine. The daily fraction of total intake excreted gradually increased, which seems indicative of an unusually stable molecule, because at least a modest exchange of tritium with body water should be expected.

>LD50

The variety of indirect evidence indicating that TCDD is not metabolically altered must be considered in the perspective of other indirect evidence that metabolic change may occur. Beatty and Neal (1975) have shown that pretreatment with phenobarbital decreases and castration increases TCDD toxicity. The former treatment increases and the latter diminishes drug metabolizing capacity. The teratology study of Courtney (1976) also is suggestive that liver metabolism of TCDD occurs. An oral dose of 25 µg/kg/day through the organogenesis period caused only 3% cleft palate among the experimental fetuses, but subcutaneous administration of that dose resulted in 82% cleft palates. In view of the apparent affinity of TCDD for the increased endoplasmic reticulum following induction (Van Miller et al., 1976; Poland et al., 1976), it is possible that induction merely increases the capacity for sequestration of TCDD in a temporarily non-active system, but the remarkable difference seen by Courtney is difficult to ascribe to distribution differences only.

may just be binding in liver rather than metabolism

total 250 µg/kg

mice or rats?

not all absorbed from oral dose?

The selective distribution of TCDD into liver and fat has also been shown by Fries and Marrow (1975), Allen et al. (1975), and Van Miller et al. (1976) in rats and by Van Miller et al. (1976) in primates. The

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liver TCDD appeared to be sequestered on smooth endoplasmic reticulum (SER), principally that induced by presence of the toxicant (Allen et al., 1975). While liver storage of TCDD in monkeys was much lower than in rats, the difference appeared to be due to the characteristic lesser proliferation of SER in the monkey that has been described by Van Miller et al. (1976). Considering the difference between species in total induced ER membrane, TCDD appeared to bind to endoplasmic reticulum to about the same extent in both species (Van Miller et al., 1976).

Unlike the behavior of other chlorinated hydrocarbons in fasted or underfed animals, TCDD is not lost from adipose tissue with fat mobilization (Allen et al., 1975). In the case of PCB, for example, the fat soluble chlorinated hydrocarbon is liberated with mobilized fat and eventually may reaccumulate in the liver or other target organs. The reason for the difference is not clear, but it seems possible that TCDD may nonspecifically partition into fat, then selectively bind to adipose cell membrane protein. Liver TCDD residues do decrease during fasting, possibly because increased gluconeogenesis makes demands on available labile protein including the endoplasmic reticulum to which the TCDD may be bound. Hepatic subcellular distribution has been further defined by Poland and his associates (Poland and Glover, 1974, 1975; Poland et al., 1976; Nebert et al., 1975); and by Chhabra et al. (1974) in studies in which the genetically determined susceptibility for TCDD induced benzpyrene hydroxylase induction was found to correlate directly with the extent of TCDD binding in liver.

The T/2 for TCDD residence in the body has ranged from about 16-20 days for single dose experiments (Piper et al., 1973; Allen et al., 1975), although Rose et al. (1976) arrived at a figure of 31 days. When TCDD was

longer time - wasn't it 7 wks?

Total 6 and 18 ug/kg

administered in the diet over 12 days at dose rates of about 0.5 and 1.5 µg/kg/day, T/2 was found to be 12 days for males and 15 for females (Fries and Marrow, 1975). Rose et al. (1976) calculated a half-time of 24 days after a seven week feeding at dose rates of 1-0.01 µg/kg/day.

0.42 to 42 ug/kg

DOW0008 194

The concentration of TCDD reaches a maximum at a given dose rate. Fries and Marrow (1975) found that after 6 weeks of administration retention approached a steady state which would be 10.5 times the daily intake. Rose et al. (1976) found similarly in 7 weeks that a steady state concentration of a little more than 10 times the daily intake could be predicted, agreeing remarkably well with the Fries and Marrow estimate.

The difference in distribution between primates and rats is of interest. Seven days after equivalent doses of TCDD-^{3}H, livers of adult monkeys contained 0.09% of total dose/gm tissue, rats contained 4.54% (Van Miller et al., 1976). In adipose tissue the relationship was monkey, 0.16, rat 3.46, suggesting that storage or retention is not strictly related to binding on inducible membranes. In terms of total body distribution, the rat liver contained 40% of the dose and that of the monkey, 10%. This is a much closer ratio (4/1) than that of liver concentrations (4.54/.09). Skin as a whole stored a much higher percentage of administered TCDD, because of the much higher amount of adipose tissue, in spite of a concentration ratio (3.5/0.16) that was also quite unfavorable.

what dosage rate?

Whether these differences bear any relation to differences in sensitivity to toxic effects is not known. The difference in liver is at least associated in amount and time with the much greater capacity for induction in the rat. There are no data available to indicate whether the thymus of species like the guinea pig, which is subject to more extensive TCDD induced thymus damage, has a selective affinity for the toxicant as does the

See relative ppt levels in liver and kidney of rats fed 0.001 0.01 and 0.1 ug/kg per day for 2 years.

73'0

liver in the rat. The thymus of infant monkeys is expected to be highly active, but appeared less retentive compared with other tissues, while the adult rat thymus accumulated more TCDD/unit weight than most organs (Van Miller et al., 1976), further confusing this issue because liver damage is thought to be the primary lesion in the rat.

Each of the single administration experiments utilized heavy doses of TCDD, taking advantage of the long delay in onset of symptoms and death to obtain improvement in radiochemical detection capability. Studies in which 400 µg/kg (about 20 x LD_{50}) were administered (Van Miller et al., 1976) produced liver concentrations of about 3.6 ppm. Kociba et al. (1976) caused discernable but minor hepatic lesions at a total dose of 0.65 µg/kg given over seven weeks. The expected concentration of TCDD in liver at this intake is on the order of 20 ppb (Rose et al., 1976). A ten-fold lower dose caused no histopathologic damage. It is not surprising therefore that the beach mice studied by Young et al. (1974) which accumulated 300-500 ppt TCDD in their livers, did not show liver morphologic lesions.

Behavior of TCDD in the Physical Environment and in Submammalian Species

The extremely high toxicity of TCDD and the very small amounts of the agent present in 2,4,5-T and in the environment present a problem that is different less in philosophy than in scale. There is a tendency on the one hand to dismiss TCDD as a pollutant because the amount available at any one point or even sector is minute, and on the other to assume that any amount in excess of true zero residue is catastrophic, because of the enormous toxicity of the material. The amount of TCDD distributed on vegetation after a typical treatment is so small as to defy direct analysis and the quest for residues in biological materials has forced analytical

sensitivity closer to zero than has been the case with any other pollutant. In spite of these unique characteristics, TCDD must be considered in the same way as any other chemical: How much is present in the human environment? How persistent is it? Can it move to unintended targets? If so, will the amount assimilated by humans or other organisms reach harmful proportions?

Present manufacture of 2,4,5-T contains about 0.02 parts TCDD per million parts 2,4,5-T, although manufacturers claim only about 0.05 ppm. Some lots contain only about 0.01 ppm. A two pound per acre application of 2,4,5-T will, therefore, distribute a little less than 20 µg TCDD/acre. It is necessary to predict what may be expected to happen to that material by examining existing laboratory data.

The environmental persistence of TCDD is highly dependent on the conditions to which it is exposed. When mixed into soil, degradation was found to be very slow, with a half-life of one year (Kearney, 1972). Because the material was introduced as an acetone solution there has been some question about possible crystallization and limited access of TCDD to soil organisms. However, TCDD in 2,4,5-T deposited on leaves and exposed to direct sunlight was found to degrade with a half-life of a little more than an hour (Crosby et al., 1977). The latter study shed a good deal of light on earlier studies in which pure TCDD was carefully coated on clean glass slides, water, or dry sterile soil for exposure to sunlight, and found to be almost insensitive to photodecomposition (Plimmer et al., 1973; Kearney et al., 1972). Crosby et al. noted that reduction would proceed in methanol, and that the reaction slowed significantly in highly purified reagent. The finding that benzene accelerated the degradation of TCDD in aqueous systems (Plimmer et al., 1973) suggested that a

at rates of 1, 10 and 100 ppm equivalent to up to 5 billion pounds of 2,4,5-T containing 0.02 ppm TCDD per acre.

good hydrogen donor was essential to photodecomposition. This conclusion has been verified in more recent work (Crosby et al., 1977). In that study TCDD with 2,4,5-T was also applied on soil, and a half-life of 50-55 hours could be inferred from the data. This finding provides some suggestion about the reaction time in indirect light, because the rough surface will provide shaded areas where reaction must be slower. As yet, studies of the effect of various parts of the spectrum emerging from light transmitted through leaves, or reflected from various surfaces has not been done. The absorption maximum for TCDD is known (Crosby et al., 1973) but the relative sensitivity to degradation at that wave length apparently is not known.

Accumulation of TCDD in the biota has been studied both in the laboratory and in the field, but there are still many gaps in the needed data.

In spite of the limited solubility of TCDD (0.2 ppb) in water, it can accumulate in aquatic organisms to the extent it is available on sediments or other reservoir (Matsumura and Benezet, 1973). In their experiments, TCDD was deposited on sand, in solvent which was evaporated to leave the dioxin as a film on sand particles. The sand was placed in a small confined static aqueous system and various aquatic organisms added. Brine shrimp reached a concentration of 157 ppb, mosquito larvae concentrated TCDD to 4150 ppb and silverside fish accumulated virtually no TCDD. It may be assumed that the concentration of TCDD in water was maintained throughout the experiment by the excess dioxin residue on sand.

Sand is not a good adsorbent. Silt or clay would be better.

but solubility is only 0.2 ppb !

When sufficient TCDD to represent 162 ppb in the aquarium was ingested by algae which were then placed in an aquarium with Daphnia and with Ostracods, the latter species accumulated TCDD to concentrations of 879 and 279 ppb, respectively due to adsorption. They also showed that mosquite fish concentrate

get dynamic transfer of TCDD from sand to water to surface of fish which is better adsorbent than sand.

TCDD to a lesser extent than do the bottom feeding larvae on which they feed. It is clear that accumulation does occur, but depends on maintaining saturation of the ambient water.

Matsumura and Benezet (1975) also examined movement of TCDD which had been bound to sand, to a sandy loam soil (inorganic to organic soil). Very little TCDD was translocated by a slow leaching with water. Subsequently, a more complex food chain system of soil, water, algae, duckweed, snails, daphnids, gambusia, and catfish was assembled (Isensee and Jones, 1975). ^{14}C-TCDD bound to soil in concentrations varying from 0.0001 to 7.45 ppm was placed in aquaria. The residues found in the various species after 33 days exposure tended to peak at the Daphnia stage. Gambusia were added at the end of the exposure period with access to water and other organisms for 3 days. Catfish fingerlings were exposed for 6 days, beginning after all other organisms were removed. The catfish accumulated TCDD to roughly the concentration found in the first biological stage, the algae. Nonetheless, at a water concentration of 7 ppt TCDD a terminal accumulation ratio of more than 10,000 was found in catfish, representing TCDD concentrations of about 100 ppb (Isensee and Jones, 1975). The work also shows, however, that as soil or water concentrations decrease, so also do concentrations in the organisms of the system. Toxicity was not studied although the higher tissue accumulations exceeded mammalian toxic doses, assuming uniform distribution of dosage. The authors point out that with the long latency of TCDD toxicity, the fish may not have had time to develop lesions and die.

Ward (1976) examined the persistence of TCDD in lake water and sediments. As expected, TCDD is almost entirely bound to lake sediments and is subject to very limited but nonetheless real metabolic degradation when microorganisms were present. The slow microbial activity is enhanced

by presence of nutrients. Ward suggested that water-mediated evaporation of TCDD occurred to a limited extent.

Toxicity of TCDD to coho salmon has been assayed by Miller et al. (1973). TCDD was administered in the diet and dosage expressed as ng TCDD per gram organism mass. At 13.1 ng/g only about 30% of the experimental group survived for 80 days past exposure, and at 5.4 ng/g almost half the fish did not survive. Deaths often did not occur until 10 days after the end of exposure, and some lethally affected fish survived 60 days or more. In studies of rainbow trout there were no apparent effects at intakes of 6.3 ng TCDD/g or greater. Mosquito larvae pupated at a normal rate in water containing 200 ppt TCDD; snails reproduced normally and oligochaete worms suffered some reproductive deficit at the same concentration. Beatty et al. (1976) found Rana catesbiana tadpoles to be remarkably resistant, with no apparent lethal effect by intraperitoneal doses of 1000 µg TCDD/kg. Doses up to 500 µg/kg did not affect adult frogs, and no histopathological lesions appeared.

i.e. ppb
3,100 ppt
5400 ppt

Field studies of fish and mammals have provided evidence both supporting and contradicting claims of TCDD accumulation in higher animals. An evaluation of biota in the area used by USAF for training bomber crews in application of military defoliants showed little accumulation of TCDD in fish living in the streams of the area. The area was literally saturated with 2,4,5-T, receiving on the order of 1000 lb/acre during the peak year, containing up to 2-10 ppm TCDD. Mice in the area accumulated tissue burdens of several hundred ppt as measured at the end of the spray program, when the sandy soil had accumulated residues up to 700 ppt. These animals demonstrated no pathological changes (Young et al., 1974), which is not surprising when extrapolating the data of Rose et al.

the soil or the 2,4,5-T used ?

(1976) and Kociba et al. (1976) to relate tissue levels and effective dose rates in rats. Given the extended period of exposure, it seems possible that the mouse population could adapt through survival of non-responding strains over the estimated 30 generations of exposures. It may be also that fish strains have evolved similarly as non-accumulators of TCDD, although such adaptation seems unlikely.

Speculation. Dosages are really very low compared to all the above studies

Fish have also been analyzed in drainages from areas in Texas and Arkansas which had been extensively treated with 2,4,5-T (Shadoff et al., 1977). The Texas samples were from a pond which collected run-off from a watershed on which the herbicide was used for brush control. The Arkansas site was a pond into which adjacent rice fields were drained and from which irrigation water was obtained, in essence recycling any contaminants. Treatment was 1.25 lb 2,4,5-T/acre, 4-8 weeks prior to flooding. The cycle had been in use for 18 years. Fish from the Texas site did not contain TCDD, at detection limits of 5-7 ppt. Mud at a detection limit of 3 ppt and water at 0.1 ppt were also negative. Six human milk samples obtained from the San Angelo area near the Texas site were also negative at a detection limit of 3 ppt. No TCDD was found in the Arkansas fish samples. Meselson and O'Keefe (correspondence to Representative James Weaver, 1977) have presented preliminary findings of about 1-2 ppt TCDD in human milk from the San Angelo area, and from an area in western Oregon. using a neutral extraction procedure for the analysis (a method which has not been validated).

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Cows milk samples from 2,4,5-T-treated areas of Oklahoma, Arkansas, and Missouri were found to be negative for TCDD by Mahle et al. (1977) with detection limits on the order of 1 ppt. TCDD in beef fat was undetectable in two series of animals which had grazed on 2,4,5-T treated pastures in Oklahoma, Texas, and Missouri. In a third group of seven

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animals confined in an entirely sprayed pasture, three samples were positive at the detection limit of 3-4 ppt.(Kocher et al., 1977). TCDD has been found, however, in several beef cattle maintained in a field experiment on fields which had received 1, 2, 3, or 4 lbs 2,4,5-T/acre. Average TCDD in fat was 20.3 ppt in the 4 lb/acre group, but there were only three samples, one of which was extremely high. At 3 lb/acre the average was 12.8 ppt, again derived from some high values and many negatives. Interpretation of this data by EPA is still in process, and is subject to some argument. (Holloway said the above is incorrect).

Incorrect. See Mike Newton's letter to Jack Postle in Newport Oregon, 8/9/77

A number of wild animal samples were obtained in treated forest areas of western Oregon, several of which contained unusually high concentrations of TCDD. The unique character of the data raises some questions, since there seems to be few or no samples containing TCDD concentrations intermediate between the low background level and the 100-200 ppt values in a few animals.

analyzed in 1973 ? by inadequate method, interferences due to PCB's DDT etc ?

An issue has been raised about formation of TCDD during combustion of 2,4,5-T, and subsequent distribution in air. Stehl and Lamparski (1977) ignited grass treated with 2,4,5-T in a system where combustion was self supported and found less than 0.0002% converted to TCDD. Combustion of 2,4,5-T and trichlorophenol on filter paper led to production of even less TCDD. If the conversion of a field application were at this rate, about 2 parts of available 2,4,5-T per million would be converted, which is considerably more than the residual contaminant level. It should be remembered, however, that burning would not be done until foliage dries, and with a 1-2 week half time it is doubtful whether much herbicide would be available for conversion. Even this maximum level of conversion is probably not of great concern, since the product would be diluted with combustion gases and air in millions of cubic meters of atmosphere. The

effect of exposure to light on decomposition while in the atmosphere is unknown. Masking or wavelength shifts may have an inhibitory influence on photodegradation, but the duration of atmospheric suspension and light exposure should enhance the reaction.

Induction of Microsomal Enzymes

TCDD has been found to be a remarkably potent inducer of many enzymes which act on foreign chemicals. The effect is apparently dependent upon synthesis of additional enzyme, rather than activation of partially synthesized or otherwise inactive complete protein. TCDD also increases activity of δ-aminolevulinic acid synthetase in some species, resulting in an accumulation of porphyrins in tissues and excretory routes. The potency of TCDD for inducing some enzymes is between four and five orders of magnitude greater than that of such classical inducing agents as 3-methyl cholanthrene or phenobarbital. According to Hook et al. (1975a) TCDD induces cytochrome P-450 at doses of fewer molecules of TCDD than there are of P-450 in the liver. This property is important because the induced enzymes may increase conversion of other organic intoxicants to non-toxic products, or conversely may increase conversion of non-toxic materials to highly reactive intermediates which exert profound toxic effects. Many carcinogens are activated in this way. The changes in "drug-metabolizing" enzymes also have been used to describe profound genetic differences among animal strains, and their respective response to intoxication.

Enzyme induction may also serve as a predictor of general toxicity, since the induction potency seems well correlated with lethal, acnegenic, and teratologic potential (Schwetz et al., 1973; Poland and Glover, 1973a).

The enzymes which oxidize foreign chemicals have evolved in response to reactive groupings on molecules found in nature. While natural and synthetic chemicals may represent an almost infinite variety of structures, the building blocks are the same, and they exist in relatively limited numbers. Consequently, organisms have not had to develop an infinite number of enzymes to survive, but rather have developed enzymes specific for reactive groups.

The effects on these systems are among the more sensitive changes found to be caused by TCDD. To simplify presentation the following table identifies specific enzymes and the TCDD doses at which responses have been observed. Unfortunately, few explorations of the entire dose response curve have been made; the "no observed adverse effect" intake has usually not been determined, nor has there been an adequate study of the impact of low level chronic studies on the microsomal enzymes. Most of the data in the table refers to rats. Relatively few studies of mice have been made, and in the guinea pig the effective doses approach the lethal dose, and observed changes are minor. Hook et al. (1975b) administered 0.175 µg/kg to guinea pigs and found a slight increase in liver biphenyl-4-hydroxylase and biphenyl-2-hydroxylase, and no change in aryl hydrocarbon hydroxylase or UDP glucuronyl transferase activities.

Several generalizations appear applicable to the enzyme inductive properties of TCDD. While most microsomal enzymes are increased after TCDD, the enzymes identified as demethylating aminopyrine, benzphetamine and morphine are decreased (Lucier et al., 1973).

Females seem to be more sensitive to the inducing effects of TCDD (Lucier et al., 1975b). While effects on UDP glucuronyl transferases are substantial, steroid glucuronyl transferases are not changed. The

all very high compared to anticipated exposure.

Induct · of Foreign Compound Metabolizing Enzymes by TCDD (1)

Enzyme	TCDD Dose (µg/kg)	Extent of Initial Induction (x normal)	Duration of Increased Activity	References	Remarks (liver unless other tissue specified)
p-Nitrophenol-UDP glucuronyl transferase	3	14	>34d	Lucier et al., 1975a	pregnant rats (2)
	3	7	>34d	Lucier et al., 1975a	neonate, (3)
	25	6	73d	Lucier et al., 1975b	male rat
	5	5	>28d	Hook et al., 1975b	
	0.2	2.5	--	Lucier et al., 1973	
				Lucier et al., 1973	
Benzpyrene (aryl hydrocarbon) hydroxylase	3	14	>21d	Lucier et al., 1975a	pregnant rats (2)
	3	2		Lucier et al., 1975a	neonate, (4)
	2.5	14	--	Berry et al., 1976	pregnant rat, (5)
	2.5	100	--	Berry et al., 1976	fetal liver
	0.2	7	--	Lucier, 1973	male rats (6)
	0.2	8	--	Hook et al., 1975b	female rats (7)
	1.0	1.5	--	Hook et al., 1975b	male (7)
	25	3	--	Hook et al., 1975b	male rat liver
	25	>70	--	Hook et al., 1975b	male rat kidney (8)
	10	180	--	Poland and Glover, 1974	rat kidney
	10	10	--	Poland and Glover, 1974	rat lung
	10	100	--	Poland and Glover, 1974	rat intestine
	.08	3	--	Poland and Glover, 1974	C3H/HeN mouse
NADPH diaphorase (dehydrogenase)	90	13	>15d	Beatty and Neal, 1976	male rat liver (9)
Aniline hydroxylase	5	2	>15d	Lucier et al., 1973	
	0.2	1.2	--	Lucier et al., 1973	
Cytochrome P-450	1.0	1.6	--	Lucier et al., 1973	male, 3 day
Cytochrome B_5	1.0	1.4	--	Lucier, 1977	male, 3 day
Biphenyl-2-hydroxylase	25	13	--	Hook et al., 1975b	rat liver
	25	18		Hook et al., 1975b	rat kidney (11)

what is significant increase in induction?

Induction of Foreign Compound Metabolizing Enzymes by TCDD (cont)

Enzyme	TCDD Dose (µg/kg)	Extent of Initial Induction (x normal)	Duration of Increased Activity	References	Remarks (liver unless other tissue specified)
Biphenyl-4-hydroxylase	25	2		Hook et al., 1975b	rat liver
	25	40		Hook et al., 1975b	rat kidney
Amino levulinic acid synthetase	25	none	--	Woods, 1973	
	25	2		Goldstein, 1973	mouse (11)
	1.5 ng/egg	2		Poland and Glover, 1973c	chick embryo

[1]This table is selective and is intended to show the variety of work done, the dose ranges studied, and duration of effects. In most cases the lowest dose producing effect has been shown and data for higher doses omitted. The references noted should be consulted for greater detail.

[2]Pregnant rats, treated on day 10 of 23 day gestation, sacrificed 21 days post partum.

[3]Mother treated at day 5 of gestation; activity measured at day 21 postnatal. No increase in activity before parturition.

[4]Same as 2 except measured at day 8 postnatal.

[5]TCDD at day 17 of gestation, sacrificed at day 20.

[6]Male rats, sacrificed 3 days after TCDD.

[7]Male rats did not respond at this dose; there was a small but significant ($P < 0.05$) increase in amino pyrine demethylase and cytochrome P-450 in females at 0.2 µg TCDD/kg.

[8]Resting activity below detection limit.

[9]Maximal activity in cytosol at 7 days, still rising in microsomes at 15 days.

[10]5 µg/kg produced no effect.

[11]Resting activity very low in these tissues; factor of increase approximate.

bulk of induction occurs in liver but a similar but usually lesser increase can occur in the kidney and some other tissues (see table). In kidney, activity seems concentrated in the "outer stripe" of the medulla and in the cortex. This region contains the terminal straight segments of the proximal tubules, which appear to be the only significantly inducible cells in the kidney (Fowler et al., 1977).

The induction of aryl hydrocarbon hydroxylase (AHH) by TCDD has been very useful in explaining genetic variability in receptor and effector mechanisms. TCDD is about 3×10^4 times more potent than 3-methylcholanthrene, producing half maximal stimulation in the rat liver at 0.85 nmoles/kg and in the C3H/HeN mouse (female) at a dose of 0.42 nmoles TCDD per kg (Poland and Glover, 1974). While the susceptibility to AHH induction is about the same in the species tested (ED_5 = 0.4-1.2 nmole/kg), and the LD_{50}s differ by about two orders of magnitude, this difference does not detract from the value of the system in studying the genetics of drug response and the great problems of non-homogeneity of exposed populations.

There is known to be a profound difference in the responses of various strains of mice to AHH induction by 3-methylcholanthrene (3-MC), leading to a designation of "responsive" and "non-responsive" strains. Even at high doses, 3-MC fails to cause induction in the non-responsive animals. TCDD, however, will elicit an amplified enzyme activity in such mice (Poland and Glover, 1975; Chhabra et al., 1974).

Poland and Glover (1975) and Neubert et al. (1975) have shown that the genetically non-responsive mice have the genetic apparatus necessary for expression of the inducible activities and suggest that the difference arises from a mutant form of inducer-binding receptor with a diminished affinity for aromatic hydrocarbons. The induction process is apparently

n.c data on controls in this study.

In contrast, Lutz-Ostertag and Lutz (1970) sprayed pheasant and grouse eggs with 2,4-D at rates commonly used in the field and caused embryonic mortality and terata. Somers et al. (1973, 1974) were not able to support these findings after spraying mixtures of 2,4-D and picloram at usual field rates (2.8 kg/ha) and 2,4-D and 2,4,5-T on hen eggs (1973) and pheasant eggs (1974) at 10 times field concentrations (11.2 kg/ha). They found no "adverse effect on hatching success, incidence of malformed embryo or subsequent chick mortality". Kopischke (1972) also found no effect on pheasant eggs sprayed at field concentrations, but found that diesel fuel as a carrier blocked hatching completely. Dipping hen eggs in 1% 2,4-D for 10 seconds, then continuing incubation was similarly ineffective (Gyrd-Hansen and Dalgaard-Mikkelsen, 1974).

An interesting observation of 2,4-D distribution in mouse fetuses has been made by Lindquist and Ullberg (1971). Labeled 2,4-D given late in gestation accumulated early in the yolk sac, passed on to the fetus and was almost completely eliminated by 24 hours after administration. Distribution among tissues was non-selective, and concentrations tended to parallel those of the dam, perhaps explaining in part the lack of teratogenic effect.

Carcinogenic and Mutagenic Potential of 2,4-D

Innes et al. (1969) screened 120 compounds for tumorigenic properties in mice. 2,4-D and several of its esters were included; none caused increased tumor incidence. Hansen et al. (1971) carried out a carcinogenesis study in rats and concluded that although tumors were found, the observed incidence did not support a finding that 2,4-D is carcinogenic. There seemed to be some inconsistencies in interpretation that may have confused the issue. Apparently no other evaluations of cancer

See Hansen, Quaife and Fitzhugh.

(7389 for dogs also

potential of 2,4-D have been made. A number of mutagenic screens have included 2,4-D, however. Jenssen and Renberg (1976) found that 2,4-D would not induce increased micronuclei in mouse bone marrow erythrocyte, but the compound did slightly depress mitotic activity. Sex-linked lethality assay of 2,4-D in male Drosophila was also negative for mutagenic activity (Vogel and Chandler, 1974). Styles (1975) treated rats with 2,4-D, then used serum from the animals in a host mediated assay with histidine-requiring S. typhimurium mutants. No effect of 2,4-D was evident. In a screen of 110 compounds with the "Ames test," using eight histidine-requiring mutant strains, Anderson et al. (1972) was unable to detect mutagenic activity by 2,4-D.

other references exist – see review by BAS

Absorption, Metabolism, Tissue Distribution, and Excretion of 2,4-D

2,4-D was absorbed rapidly from the lung of rats in which the herbicide was injected intratracheally as 0.1 ml of 0.01-10 mM solution. The animals were sacrificed from one half to 120 minutes later. The half-time for 2,4-D absorption was 1.4 minutes, and concentration had little influence on the rate (Burton et al., 1974). When ingested, 2,4-D is absorbed primarily by the portal circulation; the lymphatic drainage accounts for very little 2,4-D absorption, as might be expected from the limited fat solubility of the herbicide (Sieber, 1976). Blood concentration of ^{14}C from radiolabeled 2,4-D administered orally to sheep has been shown to rise very rapidly (Clark et al., 1964). The rate of absorption of 2,4-D from the rumen is not known; the immediate elevation in blood ^{14}C suggests that at least some material was removed directly from the rumen. However, Gutenmann et al. (1963) fed 5 ppm 2,4-D to cattle and found that the concentration in the rumen contents decreased from 3.5 to 0.5 ppm over a 24 hour period, which does not suggest rapid

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absorption, but rather dilution with passage of rumen content. The chemical did not disappear in an artificial rumen.

Kohli et al. (1974) gave 5 mg orally to six human volunteers and found peak plasma concentrations by seven hours; the average plasma concentration one week later was about 10% of the peak concentration. Half time for excretion was calculated at 33 hours. inconsistent

Clinical observations of human patients who had extensive skin contact with 2,4-D showed systemic toxic responses within a few hours (Goldstein et al., 1959; Todd, 1962), indicating rapid absorption through the skin.

Application of 2,4-D as the DMA salt, isooctyl ester and butyl ester to the body surface of rabbits was relatively ineffective (Kay et al., 1965). Treatment was with 15 ml of aqueous or oil solution applied on a 4 x 3 gauze patch, covered tightly with plastic film. Contact was seven hours daily, five days a week for three weeks; concentrations of 2,4-D were 0.626 and 3.13% acid equivalent. Some animals were treated on abraded skin. A few animals died during the experiment but the pathology and symptoms were not typical of 2,4-D poisoning; most of the fatalities were among the animals with skin damage. No neurological lesions were found, nor were any other observed parameters changed. The only lesions observed were at the site of application.

There seems to be general agreement, other than the report by Kohli et al. (1974), that significant amounts of 2,4-D do not remain in an animal for much more than one or two days. Zeilinski and Fishbein (1967) compared whole body residence times of two esters of 2,4-D and the 2,4-D acid after subcutaneous injection of 100 mg/kg to mice. Of the butyl ester, 95% was gone after 6 hours, and in animals that had been pre-

treated with five similar daily doses, the rate of disappearance was further enhanced. The isooctyl ester was considerably slower to disappear, with a half time of somewhat less than four hours, and the acid itself was retained slightly longer. The authors did not specifically note whether hydrolysis of esters to 2,4-D acid was identified as part of the metabolic process, but the method appeared to account for the disappearance of all forms of the compound. As a comparison, the disappearance half-time of 2,4,5-T acid was on the order of 20 hours.

Khanna and Fang (1966) administered 2,4-D-^{14}C to male and female rats at very high (80 mg/rat) and relatively low (1 mg/rat) doses and found tissue residues to be greatest at about 6 hours, and almost undetectable by 30 hours after treatment. Extremely high concentrations were found in the stomach but there was no information about separation of stomach content from the tissue proper. No label was found in respiratory gases. A very small fraction of urinary label was found to be an unidentified metabolite, but the data indicate that almost all 2,4-D is excreted without change by rats.

Although 2,4-D binds reversibly to bovine serum albumin (Kolberg et al., 1973) and therefore probably to other plasma proteins, there seem to be no specific tissue binding sites as are found in plants. Norris (1974) has examined fish muscle and rat liver after exposure to 2,4-D, seeking specific binding sites and has concluded that none exist.

2,4-D apparently does not move into milk of lactating animals in significant amounts, whether treated by direct administration of the herbicide or by grazing on treated land (Bjerke et al., 1972; Gutenmann et al., 1963; Bache et al., 1964; St. John et al., 1964; Klingman et al., 1966). Under forcing conditions, however, (1000 ppm 2,4-D in diet), the

for 3 weeks followed by untreated feed for 1 week.

None found when animals were withdrawn from the feed for 3 days

level of 2,4-D residue in cows milk was forced up to 0.06 ppm, and were still barely detectable seven days later.

A substantial capacity for conjugation of 2,4,5-T has been described elsewhere in this report (Nony et al., 1976). Grunow and Böhme (1974) have found that the ability to conjugate 2,4-D with glycine and taurine exists but is considerably less than that for 2,4,5-T. In dogfish and flounder, however, a major fraction of urinary 2,4-D is excreted as the taurine conjugate (James and Bend, 1976), and in three of the four dogfish studied 10-20% of the label was found in bile after 48 hours.

In spite of the rapid loss of 2,4-D by fish, persistence of the herbicide or its products may be prolonged. Treatment of pond weeds with up to 9 kg/ha usually left no detectable residues in fish by 28 days post-application, even at the highest rates (Schultz and Harman, 1974). Schultz (1973) has found, however, that some unidentified products may be present in fish 2-3 months after treatment.

^{14}C fragments from microbial degradation incorporated into natural components. (see Stalling et al.)

The rapid appearance of intact 2,4-D in urine is apparently mediated by a presumably active and saturable tubular excretion mechanism (Erne and Sperber, 1974). The extensive excretion of 2,4-D in urine has been noted in steers (Lisk et al., 1963), sheep (Clark et al., 1964), rats (Sieber, 1976), and humans (Kohli et al., 1974), and apparently is rapid enough to remove all but massive exposures before significant damage can occur.

use human data - Dow

Behavior of 2,4-D in the Environment and Effects on Submammalian Species

This report is primarily concerned with effects of herbicides on non-plant species, but a cursory qualitative survey of literature on field persistence is useful in judging how long a given amount of herbicide may remain available.

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Water concentrations are a major concern, in relation both to aquatic species and to water supplies for the human population. 2,4-D applied in a watershed area appears in streams to a very limited extent (White et al., 1976; Krammes and Willetts, 1964). Movement into subsurface water flows also appears negligible (White et al., 1976). In aerated lake water 2,4-D persists up to 120 days, but in lake mud 2,4-D hydrolyzes very rapidly, because of microbial activity (Aly and Faust, 1964). Half-time of 2,4-D disappearance seems overall to be much less than two weeks in aqueous systems.

For some applications on water, relatively massive applications of up to 40 lbs/A per acre are necessary. Two reservoirs on the Tennessee River were treated at 20 and 40 lbs/acre depending on the nature of the water milfoil infestation. The treatments did not affect fish or other fauna, and had little adverse effect on most other plants. Plankton did retain significant amounts of herbicide over extended periods, and 2,4-D was detectable on occasion in finished domestic water from the reservoirs (Wojtalik et al., 1971).

In soils, 2,4-D has been found to have a half-time of 4-5 days by Altom and Stritzke (1973). White et al. (1976) found that 95% of 2,4-D in the top 0.5 cm of soil would disappear in 7 days. However, an in vitro study by Alexander and Aleem (1961) indicated that 2,4-D would be detectable for as much as 94 days in one type of soil and only 23 days in another. The preparation included 100 ml of nutrient medium and 4 mls of soil as an inoculum; whether such a system represents actual breakdown conditions in soil is possibly questionable. Sufficient material to cause detectable phytotoxicity persists for about a month, according to Mullison (1972). In areas where 2,4-D is used annually, the breakdown may be more rapid in the later years than following the first

treatment (Hurle and Rademacher, 1970). Wiersma et al. (1972) reported a 1969 national program for soil monitoring in which three samples from a total of 28 which had been treated with 2,4-D were found to have from 0.01 to 0.03 ppm 2,4-D present. It seems unlikely that 2,4-D would persist in either water or soil for more than a month.

2,4-D has been used extensively in control of aquatic weeds, particularly water hyacinth and water milfoil, and has probably been subjected to more study of aquatic toxicity than any other herbicide. A number of short term screens have been conducted with fish. Alabaster (1969) reported on 164 compounds, including 2,4-D sodium salt, which was found to have a 24 hour LC_{50} of 1160 ppm for harlequin fish, and the butoxyethylester of 2,4-D which was much more toxic with a 24 hour LD_{50} of 1 ppm, for the same species.

The 24-96 hr LC_{50} of PGBE esters of 2,4-D for rainbow trout were slightly above 1 ppm (Cope, 1964). For mullet and killifish the LC_{50} (24 hr) was 5 ppm for both the PGBE and butoxyethanol esters. The parent acid was ineffective at 50 ppm (Butler, 1963).

Oyster shell growth was 50% inhibited by 3.75 ppm butoxyethanol ester, but the acid and dimethylamine salt were not effective at 2 ppm (Butler, 1963). Exposure to the DMA salt for 24 hrs at 2 ppm caused no mortality in shrimp; 48 hour exposure caused 10% lethality. The butoxyethanol and PGBE esters caused no mortality to shrimp at 1 ppm x 48 hour. The ethyl hexyl ester of 2,4-D caused 38% decrease in oyster shell growth at 5 ppm over 96 hrs (Butler, 1965). The considerable differences in 2,4-D effect among various species were illustrated by Sanders (1970a) 2,4-D acid has a 48 hour median response concentration (TL_{50}) of 100

mg/L (100 ppm) for Daphnia and 3.2 mg/L for scud. (Crosby and Tucker (1966) found the same value for Daphnia.) The PGBE ester TL_{50} varies from 0.1 for Daphnia to 2.6 for scud to more than 100 ppm for crayfish. The DMA salt TL_{50} is more than 100 ppm in bluegill but TL_{50} for the butyl ether ester in bluegill is 1.1 ppm, and 100 ppm for crayfish.

Stonefly naiads are less sensitive to 2,4-D itself (LC_{50} x 96 hr = 15 ppm) than the butoxy ethanol ester (LC_{50} x 96 hr = 1.6 ppm) (Sanders and Cope, 1968). Sanders (1970b) has also reported a 96 hr TL_{50} for 2,4-D amine of 100 ppm for tadpoles.

The lethal effect on tadpoles is apparently limited, but Buslovich and Borushko (1976) found that 2,4-D sodium salt would inhibit metamorphosis of Rana temporaria tadpoles, and blocked thyroidin stimulation of the process. The DMA salt was effective at 2 ppm. The authors speculate that 2,4-D antagonizes thyroid hormone.

Few studies of truly chronic exposure of fish to 2,4-D have been made. Mount and Stephan (1967) compared the 96 hour LC_{50} for 2,4-D butoxyethanol ester (5.6 ppm) with various 10 month exposures, and found that 1/19th of the LC_{50}, or 0.3 ppm could be tolerated by fathead minnows without effect on growth or reproduction. Eggs were much more sensitive than adults over 48 hour exposures. Schultz (1973) placed bluegill, channel catfish, and largemouth bass in solution of 2,4-D DMA salt, labeled with ^{14}C, at concentrations of 0.5, 1.0, or 2.0 mg/L (ppm). Fish and water samples were removed at intervals up to 84 days for analysis of ^{14}C and 2,4-D content.

Considerable radioactivity remained in fish tissues but apparently none was associated with 2,4-D, suggesting extensive ~~metabolism.~~ degradation by microorganisms or sunlight. The

first tissue in which radioactivity appeared was the gall bladder of catfish and bluegills, and eventually ^{14}C appeared in every tissue analyzed. In both catfish and bluegill, the amount of radioactivity in muscle tended to increase through the collection period. No toxicity was evident in any of the exposed fish. <- due to incorporation of ^{14}C fragments into natural components (Stalling)

To relate concentrations of one ppm to field condition, an application of 2 kg/hectare (10000 M^2) to water will give a concentration of 2 mg/L or 2 ppm if the water is 10 cm deep. Deeper water will result in further dilution. If applied to flowing water, water movement will dilute the herbicide quite rapidly, and that material distributed on soil and vegetation will tend to remain in place. Obviously a spill or other accident presents different problems.

Among insects, the effects of 2,4-D on honey bees has received the most attention. Palmer-Jones (1964) has listed a number of investigators who concluded that 2,4-D is safe for bees, as well as others who have shown toxic effects, some of which may depend on the species of plant on which the herbicide is deposited. In a field investigation of a three lb/acre application Palmer-Jones found that a 22% mortality occurred in 48 hours after dusting. However, when bees at the hive were heavily dusted directly, there was no mortality, leading to the conclusion that the bees in the field were acquiring intoxicant by some mechanism other than surface contact. Moffett et al. (1972) sprayed caged bees directly with various phenoxy herbicides at a one pound/acre rate with very little effect. When included in the diet at concentrations up to 100 ppm, 2,4-D did not decrease lifetime of bees and the ester used was ineffective at 1000 ppm (Morton et al., 1972). The herbicide does cause decreased brood development when fed at 100 ppm but has no reproductive effect at 10 ppm (Morton and Moffett, 1972).

Treatment of Coccinellid beetle larvae with 2,4-D at a rate equal to 8 oz. acid equivalent/acre caused greatly increased mortality, regardless of the age of the larvae through day 12 at time of spraying (Adams, 1960).

SILVEX

There appears to be a much more limited accumulation of data on biological effects of silvex and related compounds than is available for other phenoxy herbicides. The descriptions of biological effects of silvex will be categorized in two broader sections rather than the more detailed entries prepared for the other agents.

Biological Effects of Silvex and Its Derivatives

The acute median lethal dose of silvex and its esters is quite high, and falls in a relatively narrow range among species and among the derivatives. Rowe and Hymas (1954) summarized data which had been established at that time. Silvex and its mono-, di-, and tri-propylene glycol ether esters were of almost identical LD_{50} in guinea pig of 1200 and 1350 mg/kg. The lethalities to rats were also similar at about 650 mg/kg. The rat appears to be most sensitive, and chicks and mice require doses similar to the guinea pig.

Subchronic (90 day) studies of rats fed 10-600 mg/kg/day of the PGBE ester resulted in more than 50% lethality at the highest dose. The time of death ranged from 15-85 days. Growth was depressed in rats fed 300 mg/kg. There is somewhat of a paradox in the findings, because pathology in the dead animals indicated malnutrition rather than herbicide toxicity. A paired feeding study at 300 and 600 mg/kg/day indicates that depressed growth was not entirely due to inadequate food consumption. Dose rates of

30 and 100 mg/kg/day caused an increase in liver weight but 10 mg/kg/day caused no change (Mullison, 1966). The study was followed by a 90-day feeding of up to 10000 ppm silvex (sodium salt) in the diet. 10000 ppm is 1% of the diet, and if the animals consumed 10 gm daily and weigh 300 gm, the dose rate would be on the order of 300 mg/kg/day. That dose rate was highly lethal and was abandoned, but did produce swelling and granular degeneration of hepatocytes and ultimate cellular necrosis. Renal tubule cells were swelled and vacuolated, and seminiferous tubules were degenerated. At levels above 10 ppm (about 3 mg/kg/day) some growth depression occurred. Mullison (1966) reported also fed Kurosal (potassium salt of silvex, containing 53.3% silvex acid) for two years at concentrations up to 300 ppm. The highest dose caused a slightly increased kidney/body weight ratio in males, but 100 ppm and lower dose rates caused no change in food consumption, growth, gross and microscopic morphology hematology or bone marrow. 100 ppm was stated by the author to be equivalent to 2.6 mg/kg/day of silver.

Kurosal was without effect on beagles at a feeding rate of 56 ppm (about 19 mg/kg/day) over 2 years. Females fed 190 ppm suffered some hepatic degeneration and necrosis after a year of feeding, but at the end of two years no damage could be found. At the 56 ppm intake, there were no changes over a very broad spectrum of hematologic, clinical chemistry and morphologic analyses.

The liver damage at higher levels included hepatocyte necrosis, bile duct proliferation, and bile pigment deposition throughout the liver and in the epithelium of kidney tubules.

In a subchronic study in cattle by Palmer et al. (1964), one yearling Brahma-cross fed 100 mg silvex/kg/daily died after 29 days. Two other

animals given 25 and 50 mg/kg for 73 days showed no evidence of toxicity. A 90-day experiment with 50 mg/kg/day, either by drench or by injection into a rumen fistula resulted in death of one of the latter group of three animals. Two of those treated orally developed severe inflammations in the parotid area, apparently due to local irritation.

The survey by Innes et al. (1961) screened silvex against two strains of mice for 18 months. The concentration of silvex was 121 ppm, which was the maximum tolerable dose rate, and there was no increase in tumors in either strain. The mutagenic screen by Anderson et al. (1972) did not detect point mutations resulting from silvex treatment in either T_4 phage or S. typhimurium (histidine requiring).

Silvex is teratogenic at high doses. Courtney (1975) found that 398 mg/kg/day, on days 12-15 of gestation, caused 3% cleft palate in the group given silvex in DMSO subcutaneously, and 7% where given orally in corn oil. Fetal mortality increased to 25% in the group treated subcutaneously.

The Dow Chemical Co. has also conducted a series of teratology studies with silvex. Dose rates of 75, 100 and 150 mg/kg/day from day 6 to day 15 caused several cardiovascular anomalies; 50 mg/kg/day caused retarded ossification in the sternum and skull. (Dow Chemical Co., 1972). The no adverse effect level was considered to be 25 mg/kg/day. The PGBE ester caused skeletal changes at an intake of 50 mg/kg/daily but no changes were found after 35 mg/kg/day (23 mg/kg silvex acid equivalent).

Birds seem peculiarly resistant to the herbicides. For example, DeWitt et al. (1963) fed 5000 ppm BEE ester of silvex to young bob white quail, pheasant and mallard ducks. The average lethal intakes were 9350, 9240 and 21,000 mg/kg, and time of death varied from 10 to 100 days.

Mallards were able to consume 100 ppm for 100 days with no lethality, but reproduction was impaired.

Silvex acid at 5000 ppm in the diet was tolerated for 5 days with no mortality by Coturnix. The 5 day LD_{50} for pheasants was 4500 ppm of silvex BEE ester, for bobwhite quail the LD_{50} was 30 ppm over 5 days, for Coturnix it was in excess of 5000 ppm, for pheasants LD_{50} was less than 3000, and no mallards died after 5 days on 5000 ppm. All birds were two weeks old at the initiation of the study (Heath et al., 1972).

Stickel (1964) reported US Fish and Wildlife studies in which bobwhite were fed 1000 ppm of an unspecified silvex ester; 50% were dead by day 34 with average total dose of 17000 mg/kg. Of a group fed 5000 ppm, the average daily dose was 2300 mg/kg. Half of the birds survived more than 4 days, 40% survived more than 10 days, and an additional 4% died during the remaining 14 days. The average dose to birds that survived 24 days was 54,500 mg/kg.

These findings indicate that wildfowl should not be adversely affected by any field application of silvex, or for that matter even a gross over-application.

Effect of Silvex on Aquatic and Invertebrate Species

Silvex has become an herbicide of choice for control of surface and underwater water needs. As a consequence, there has perhaps been more attention paid to its impact on aquatic species than any of the other phenoxy herbicides. There is an unfortunate diversity of experimental methods and conventions for expressing concentration and other factors which sometimes makes comparison of data difficult.

Table 1 has been constructed to simplify consideration of the various reports of toxicity to aquatic species.

Table 1. Gross Toxicity of Silvex and Its Derivatives to Fish

Species	Form of Silvex	Concentration (Acid Equiv.) ppm	Effect	Period	Reference
Bluegill	BEE ester	0.36	LC_{50}	48 h	Cope, 1964
	BEE ester	1.7	LC_{50}	48 h	Hughes and Davis, 1966
	Isooctyl ester	1.4	LC_{50}	48 h	
(young)	K salt liquid	83	LC_{50}	48 h	Hughes and Davis, 1965
	K salt granular	100	LC_{50}	48 h	Hughes and Davis, 1965
(fry)	PGBE ester	0.3	tolerated	96 h	Jones, 1962
	K salt, liquid	100	tolerated	96 h	Jones, 1962
	K salt, granular	150	tolerated	96 h	Jones, 1962
(eggs)	PGBE ester	10	no effect on hatch		Wilber and Whitney, 1973
(fry)	PGBE ester	5	100% lethal	36 h	Wilber and Whitney, 1973
	PGBE ester	1	no effect	80 days	Cope, 1964
	PGBE ester	3	liver degen.	2 weeks	Cope, 1964
	K salt	10	no effect	2 1/2 mo	Cope, 1964
	K salt	75	35% lethal	2 1/2 mo	Cope, 1964
Stoneroller (eggs)	K salt	25	95% hatch	72 h	Hiltibran, 1967
	PGBE ester	5	45% hatch	72 h	Hiltibran, 1967
Chorus frog (tadpole)	BEE	20	LC_{50}	24 h	Sanders, 1970b
	BEE	10	LC_{50}	96 h	Sanders, 1970b
Fowler's toad (tadpole)	BEE	22	LC_{50}	24 h	Sanders, 1970b

Among factors that influence toxicity to fish are the chemical form of the agent, whether the formulation is granular or liquid, and water hardness. Generally the esters are more toxic, and liquid formulations are more toxic. Data on water hardness is contradictory; but the tendency to greater toxicity in soft water seems to be associated with the salt rather than the esters (Surber and Pickering, 1962).

Distribution rates for silvex treatment of aquatic weeds may be as high as 40 lbs/acre. An assumption of dilution throughout an 8 foot depth is apparently conventional, which would give a concentration of 1.8-1.9 ppm. If this concentration of an ester formulation were to hold in a given area, it would clearly be lethal to many fish. The salt should have up to a 100 fold safety factor for fish, depending on mineral content of the water. Such application is not a consideration in forest operation, but accidental overwater dilution could occur. Terrestrial application is at rates at least 10 fold less than 40 lb/acre but many water courses are much shallower than 8 feet, thereby leaving a similar hazard. If flowing, dilution will be rapid and periods of exposure should be limited. In static water the agent will diffuse away from the application site with time except in small shallow ponds, where some damage might possibly occur

Lower aquatic organisms have also been well studied because of silvex use on water weeds. Representative though not comprehensive data is compiled in Table 2. Again, it appears that the salts of silvex are much less toxic than the more complex esters. The most vivid contrast is in the effect on Daphnia magna, which is 50% immobilized by 100 ppm silver potassium salt, and by only 0.18 ppm silvex PGBE ester. Some species, however, are particularly resistant to even the PGBE ester of

silvex. The crayfish LC_{50} is in excess of 100 ppm; for a number of other organisms this factor is less than 1 ppm.

It is not necessary to catalogue the entire literature on silvex effects on either fish or other aquatic species. The range of effective concentrations is well illustrated as well as the extent, if not details, of species differences.

Effects of silvex on terrestrial insect life have been evaluated in honey bees by USDA workers at Tucson. All of the phenoxy herbicides including silvex were found to be relatively non-toxic to bees when applied in water at field concentration (Morton et al., 1972; Moffett et al., 1972). When fed at 100 or 1000 ppm, silvex reduced brood production, but at 10 ppm, no adverse effect was found. In each case, when the toxicant was removed, the colonies regained their original reproductive efficiency (Morton and Moffett, 1972).

Metabolic Fate of Silvex in Mammals

There have been few studies of the disposition of silvex after absorption by mammals. Bjerke et al. (1972) reported a study in which dairy cows fed silvex at 1000 ppm in feed and found 0.12 ppm ~~2,4,5-trichlorophenol (TCP)~~ silver in milk and 0.16 ppm in cream. In one week off contaminated feed the concentrations decreased below 0.05 ppm. Leng (1972) in a review, reported same study plus beef animals work in which 2,4,5-TCP was not detected in milk and meat; however, residues of silvex were substantial in the liver. After feeding 300 ppm silvex 28 days, 4 ppm were found in liver, 18 in kidney, 0.6 in muscle and 0.9 in fat. After 2000 ppm in the diet, 12, 30, 2 and 4 ppm were found in the respective tissues. In a similar experiment Clark et al. (1975) found roughly similar values, with traces of 2,4,5-TCP in each tissue. In a single cow experiment, 5 ppm below validated sensitivity of method.

did not find TCP after feeding silver.

in beef animal and sheep

Above study same samples (duplicates analyzed by USDA and Dow)

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This issue is no more clouded by those viewpoints than is any other debate over chemical usage, and we are best served by dealing with the problem without these extremes.

Without question, the issue of phenoxy herbicides use has evolved to concerns about the contaminant TCDD in 2,4,5-T and silvex. Though 2,4-D has no such impurity, it has become tarred with the same brush, and it is not unrealistic to devote more space to the contaminant than any of the primary chemicals.

Because of the extremely low environmental levels and enormous intrinsic toxicity of TCDD, the most critical technical question about that chemical centers on analytical methodology. There are few laboratories with the sensitivity to deal with concentrations on the order of 10 ppt or less, and there is real disagreement about reliability of detection or measurement at such low levels. When the data are considered, some samples in which TCDD was undetectable had high detection limits, some areas with no spray history were found to have measurable TCDD. Also, many samples of similar origin differed widely. Nonetheless, the data appear to be telling us clearly that some TCDD is present in some segments of the environment, that the amounts are uncertain, and that existing residue information must be augmented if we are to understand the behavior of TCDD. With any effort presently imaginable, however, it will probably not be possible to directly monitor the amount of TCDD in the physical environment.

A proper analysis of hazard requires consideration of chemical behavior and ambient levels of the potential intoxicant, but in the case of TCDD we must rely on indirect assessment. The extent of uptake in

The most difficult problem of the several that are a part of the task of hazard assessment is, again, part of the decision pattern for any chemical, the risk-benefit analysis. Risk benefit analysis is an excellent basis for conversation but nearly useless in decision making. In the present case, there seems to be a general conviction that herbicide application is a highly useful tool in forest production. At the same time, estimates of differences in actual relative costs of tree production or retail materials, either near-term or long-term, are difficult to find. Technically, economic benefit should be an accessible factor, but it seems elusive.

Risk, on the other hand, is not quantifiable unless major and obvious effects occur; such situations make the decisions for us. If we are able to show any human illness resulting from environmental practices of chemicals, almost certainly they will be disqualified. An exception may be in effects which are certainly reversible.

As a society we have yet to learn a usable method of determining whether, or how much, human injury can be considered acceptable.

The assessments of hazard in this report are therefore opinions by one person about the potential for human harm resulting from distribution of the chemicals at minimum levels that will achieve the forestry objective. This document should be taken as a working base enabling incorporation of other opinions, and it should be subject to periodic updating, based on new findings, existing papers inadvertently missed in review, and existing papers which may become more pertinent in the light of other new findings.

experiments has not produced a "no observed effect" dose because the detailed analyses available only through interim sacrifice have not been made in primates. The group of monkeys studied in Allen's laboratories first showed clinical symptoms after three months of exposure to about 0.01 µg TCDD/kg/day, or a total dose of somewhat less than 1 µg/kg, but there is no way of knowing whether pathologic examination would have detected changes at the more than 10-fold lower dose found ineffective in the rat and guinea pig. The existing literature also does not provide access to a useful single dose no observable effect level. There is no useful data that relates specifically to human effects.

It therefore seems reasonable to accept 0.06 µg/kg as a subchronic total dose over a short term, say, one year below which no effect can be detected. From this point it follows that some attempt must be made to judge potential human exposure levels. It is clearly impossible to directly evaluate acquisition by surface contact with treated foliage. Direct analytical methods for TCDD are probably more sensitive than for any other organic compound, but measurement of foliar distribution cannot be made at field application levels.

In my opinion significant TCDD exposure by inhalation is highly improbable. An intake of 0.02 µg of TCDD would require inhalation of one g 2,4,5-T containing 0.02 ppm TCDD, along with 10 g of diluent at usual dilution rates. The volume of air in which the spray is distributed is very large. Such an intake for a 50 kg person would constitute 1/150 of the assumed no observable effect dose. The potential for inhalation of TCDD formed during combustion of 2,4,5-T is infinitely less, because of the enormous dilution in air.

and assumption of perfect retention of TCDD or its effect. In point of fact, the high TCDD concentrations are in liver and fat and such a hypothetical whole animal concentration would not be approached.

A slightly different approach by Dr. George Streisinger makes assumptions about fat concentration and the percentage of fat in, as an example, ground meat. He has concluded that either 78 or 408 (depending on different no-effect assumptions) half-pound meals would exceed the danger barrier. Dr. Streisinger included a 100/1 safety factor, however, and the specified number of meals would bring a person to within 1/100th of a possibly harmful intake.

2,4,5-T is applied on a given forest area only 1-3 times in a timber growth cycle. While it is possible for a deer to acquire significant residues in a single year, the likelihood of a long-term continuous exposure of deer would require migration among sprayed plots, or repeated treatment of a single area, and is therefore remote. Even range land is not usually treated annually.

The significance of TCDD in mothers milk is considerably greater than consumption in meat. The analyses by Dr. Meselson's group indicate the possibility that TCDD on the order of 1-2 ppt may be present in human milk. Dr. Meselson has been careful to state that while he has great confidence in the individual analyses, they are close to detection limits, and the data really asks rather than answers the question.

For the sake of discussion, a sustained output of 1 ppt in human milk could provide an infant dose approaching the no observed effect level. Assuming a liter of milk consumed daily at 100 days, total intake would be 0.1 µg. If the average weight of the infant were 6 kg, the 100 day

0.12 ppt at 60-90 days. Exposure of a nursing infant will therefore be minimal.

It appears to me therefore that the potential for harmful exposure of infants through human milk should be negligible. This route is nonetheless the most probable of the several potential means of contact, and demands a thorough analysis of human milk in exposed populations.

The data on TCDD carcinogenic potential suggest that it is not a carcinogen. It has been suggested that TCDD is a promoter of carcinogenic activity on the basis of a wide variety of tumor types developed over a long exposure period by the Wisconsin group. The recently completed Dow Chemical Co. study indicates that tumor incidence changed only at doses that were lethal to many animals over the two-year test period. The data base exists for one and probably several human epidemiological studies which could answer the question of influence on human cancer incidence. A recommendation is made later for such studies.

Of the toxicological data evaluated, perhaps the most disturbing is that of Allen et al. (1977) suggesting that the lethal dose in monkeys is similar whether the agent is administered over a short period or over several months. Time independence of dosage has never been demonstrated for a chemical. If it truly exists for TCDD, there is implication that the compound leaves permanent damage, even though it has departed from the body. In this situation, continual low level impact would cause gradually accumulating injury until clinical illness would prevail. In that sense, the idea of a no-effect level would not be applicable, and no exposure would be permissible. As with radiation, however, a small intake is possible without endangering health over a lifetime. In the case of TCDD this amount is not known, but it is finite. In view of a

The acute toxicity of 2,4,5-T is quite low and in itself is not a factor in the environmental hazard potential of the herbicide. This segment is concerned only with 2,4,5-T; the implications of the dioxin contaminant have been considered earlier. A wide variety of specific toxic changes have been found in experiments with acute or short-term repeated administration of high doses of 2,4,5-T.

2,4,5-T is teratogenic, but again, only at high doses. The dose response to 2,4,5-T is such that a large fraction of the maternal lethal dose is required to produce birth defects.

There are substantial differences in effective teratogenic doses among strains of mice. The lowest effective dose is still substantial, however, and the kind of teratogenic response remains the same. The differences among the genetically very specific mouse strains do raise the possibility of high individual sensitivity in the heterogeneous human population. This question applies to any potential effect in humans by any chemical and really constitutes a common social question that we have not learned to handle.

The excretion of 2,4,5-T by mammals is rapid, with relatively little conversion to other compounds. Initial residues after application are high enough to possibly cause some temporary slight tissue deposition of 2,4,5-T in tissues of grazing animals, if sustained for a period of more than two weeks, but environmental degradation of 2,4,5-T is rapid and the chemical does not migrate extensively, once deposited. For these reasons and because general toxic responses to the herbicide only occur at high doses, I do not consider that 2,4,5-T as used properly in forestry procedures represents a general toxic or teratogenic hazard to the human population.

The reproductive effects of 2,4-D do not appear until doses approaching the lethal level are reached, and there seems to be no reason for concern in this province.

As a potential carcinogen, 2,4-D has had very little study. The only evaluation of which I am aware was negative, in one species, which indicates that the compound is not highly carcinogenic. This finding of course can give no confidence about low level carcinogenicity. Several studies of mutagenic potential have been negative, adding some confidence.

What about Hansen et al in dogs?

Perhaps the key information lies in the rapid excretion of intact 2,4-D in the urine. This process is rapid and relatively complete. In some species a portion of the compound is converted to other forms for excretion, but the process is rapid. Rapid excretion probably is the factor that prevents 2,4-D or its products from reaching the detectable margin in milk after field exposure. It is possible experimentally to overload an animal to the extent that the material will appear in milk.

Because of its short persistence in the environment and the absence of toxic responses at any doses that might be found in the field, I believe the use of 2,4-D in practices presently considered standard is not hazardous.

There are, as with any chemical, some open questions, which are addressed in the section on recommendations. These relate primarily to epidemiological needs and clinical surveillance.

Hazard Assessment--Silvex

The general considerations applicable to 2,4,5-T may also be applied to silvex. There are some minor differences between the compounds, but the ranges of toxicity, the manner of biological disposition, and the

RECOMMENDATIONS OF STEPS WHICH SHOULD BE CONSIDERED TO ASSURE THAT EXPOSURE TO TCDD IS MINIMIZED OR PREVENTED

I have stated my opinion that 2,4,5-T can be used safely, with certain additional safeguards. I believe that opinion should be reviewed as new data emerge. A number of clinical questions now being asked have accessible answers, and these must be obtained. In addition, the toxic nature of TCDD and our inability to measure it satisfactorily in the environment dictates a very conservative attitude about its distribution. I see no inconsistency in accepting its presence in small quantities and recommending unique steps to prevent those quantities from reaching people.

To assure public protection and a more compatible relation with the public, I suggest the following considerations in designing a spray program.

1. With the consideration of health hazard, there is a political reality that needs more attention. Every treatment operation must be designed to prevent intrusion of spray onto premises not under control of the agency or firm using the chemical, as a matter of principle. I would not be surprised if Forest Service policy includes that concept, but I would also be surprised if its application is somewhat less than perfect. Personal rights are becoming more and more clearly defined, and it seems to be time to decide where such rights begin and end, and take a visible public position on the issue.

Do we have to protect agricultural workers who are trespassing on private property?

2. As a means of preventing any involuntary exposure, there should be assurances that every residence is identified, with any water sources that may be in the Forest. Non-legal residents should also be identified.

in my mind that people complaining of physical injury are in fact affected. I am skeptical that the application of herbicide causes these effects in more than a few incidents, but it is possible. Of more importance, there may be some other public health hazard operant in the area which causes the symptoms. It is even possible that other illness is brought to the attention of the victims by their immediate concern about improper herbicide intrusion on their private property.

2. The opinion of Van Miller et al. (1977) that TCDD is a promoter of carcinogenic effect by other compounds seems reasonable according to their evidence. If the forest application of 2,4,5-T containing TCDD is inserting effective levels of TCDD into the human environment, epidemiological evaluation should disclose higher than normal incidence of various forms of cancer in 2,4,5-T use areas. Lane County, Oregon, has a very effective tumor registry program, with virtually all pathology of human cancer examined by a single consortium of specialists. These data should provide evidence of any existing difference in cancer patterns from other areas. A more definitive study should be possible in rangeland or rice-growing areas where the herbicide is used annually. Epidemiological studies such as these may settle the persistent question of cancer latency, because on an area basis, the pattern of past use of herbicides should be reasonably accessible.

3. A thorough health surveillance should be made of all herbicide applicators and others with industrial or agricultural exposure to 2,4,5-T or silvex. Cytogenetic evaluation through several seasons of the year should be an integral part of the study. Design of the medical components of the survey should be by a nationally constituted panel.

ANALYTICAL REPORT

MIDLAND ANALYTICAL LABORATORIES

DOW CHEMICAL U.S.A.

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AL NUMBER: AL-50439 RESTRICTED

ACCT. NUMBER: 8004 | SUB FUNCTION:

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DATE: June 16, 1978

TO: W. B. Crummett, 574
E. L. Garfield, 607
M. B. Chenoweth, 607
L. P. McCarty, 1701
M. L. Leng, 9008

RESTRICTED REPORT, COPIES MAY BE OBTAINED FROM E.L. Garfield, Medical Dept, 607 or R.H. Stehl, 574 Building written request only.

TITLE: DETERMINATION OF PHENOXY HERBICIDES IN BLOOD SAMPLES FROM 2,4,5-T PLANT PERSONNEL

INTRODUCTION

Human blood samples were submitted by Bud Garfield, Medical Department, for a determination of phenoxy herbicides - 2,4-dichlorophenoxyacetic acid (2,4-D), 2-(2,4,5-trichlorophenoxy) propionic acid (Silvex), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). The samples were obtained from personnel working at the 2,4,5-T production facility.

EXPERIMENTAL

The samples were prepared by extraction, hydrolysis, methylation with diazomethane, and silica gel column clean-up prior to analysis by gas chromatography with electron capture detection. The experimental procedure used is detailed in report ML-AL 78-50252.

The silica gel column clean-up was changed slightly. The column used was packed with 1.5 gram dry silica gel (~11 cm x 6 mm i.d., glass). The column was eluted with 60/40 methylene chloride/hexane. The first 2 ml of eluent were collected and discarded (waste fraction). The next 13 ml of eluent were collected for analysis. Then an additional two ml were collected to check for recoveries (extra fraction). The collected fractions were evaporated to dryness. Prior to analysis, the sample residue was dissolved in 1 ml benzene. The waste fraction and extra fraction were also analyzed by dissolving the residue in 200 µl benzene.

Validation data for this procedure are also obtained in ML-AL 78-50252.

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SIGNATURE: Marcha L Langhorst

M. L. Langhorst	PHONE	BUILDING	DATE FINISHED	HOURS	RESULTS PHONED	REVIEWED BY
	6-6207	574	6/14/78	16		RH

RESULTS AND DISCUSSION

Results are detailed in Table I. Figure 1 shows a typical standard chromatogram and chromatographic conditions used. Figure 2 shows a typical chromatogram for a blood sample from an employee at the 2,4,5-T plant. Figure 3 shows the analyzed waste fraction and extra fraction. Figure 4 shows a reagent blank.

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3. Langhorst, M. L., "Determination of Phenoxy Herbicides in Goat Tissues", ML-AL 78-50080.

jdc

TABLE I

PHENOXY HERBICIDES IN BLOOD FROM 2,4,5-T PLANT PERSONNEL

Sample	CONCENTRATION FOUND (ng/gm blood) 2,4-D	Silvex	2,4,5-T
028736-75638	ND(3)	1.5	8.8
028737-75631	ND(3)	ND (0.5)	1.2
028996-J. Schaefer	ND(3)	ND (0.5)	1.8
Reagent Blank	ND(3)	ND (0.5)	ND (1)

Notes:

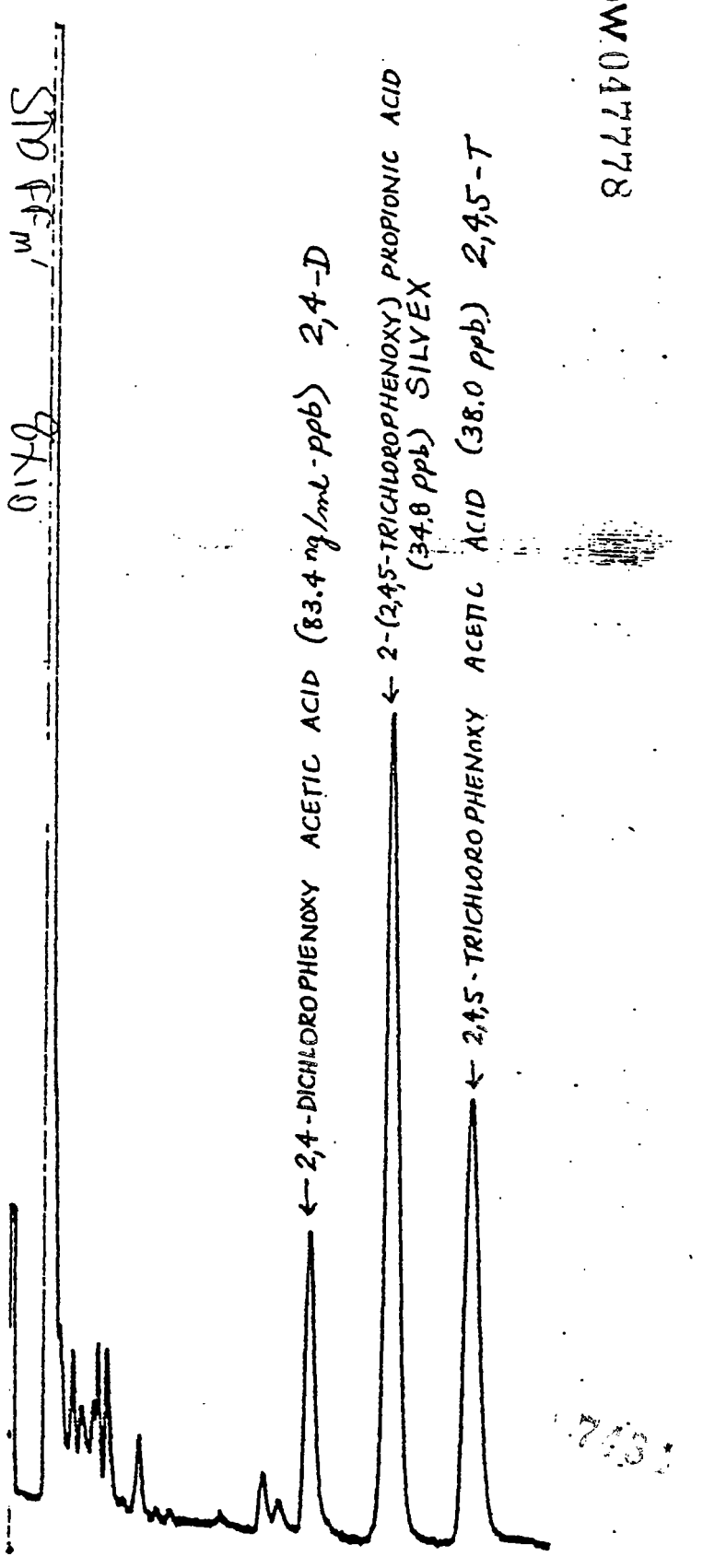
1. N.D. = not detectable with the minimum detection limit shown in parenthesis in ng/gm blood.
2. Minimum detection limits (MDL's) by GC-EC are:

Compound	M.D.L. (ng/ml)	M.D.L. (ng/gm blood)
2,4-D	17	3
Silvex	2.3	0.5
2,4,5-T	4.7	1. (assuming 5 gram blood sample)

3. Any phenoxy herbicides present in the samples as esters are hydrolyzed to acids and methylated for analysis as methyl esters. Results are reported as phenoxy herbicide acids, although the compounds may have been present in the original sample as esters or acids.

FIGURE 1 TYPICAL STANDARD CHROMATOGRAM

Operator M. L. Langhorst Date 6-14-78
COLUMN No. glass Length 9ft Dia. 2mm i.d.
Coating SP-2401 Concn. 3%
Support Supelcoport Mesh 100/120
TEMP: Col: Init. 175 °C Final ____ °C
Rate ____ °C/min. Det. 350 °C Inj. 210 °C
CARRIER GAS Nitrogen Rate 28 ml/min.
Pressures: Inlet ____ Outlet ____
Hydrogen ____ ml/min. Air ____ ml/min.
DETECTOR: Electron Capture, Ni^{63} ma. ____ volts
Scavenger ____ Rate ____ ml/min.
Sens. 8 X 10 Rec. range 1 mv.
SAMPLE STANDARD FFm' Size 2ul
Solvent hexane/benzene Concn. As labeled



DOW 0077778

0000731

DOW 0477779

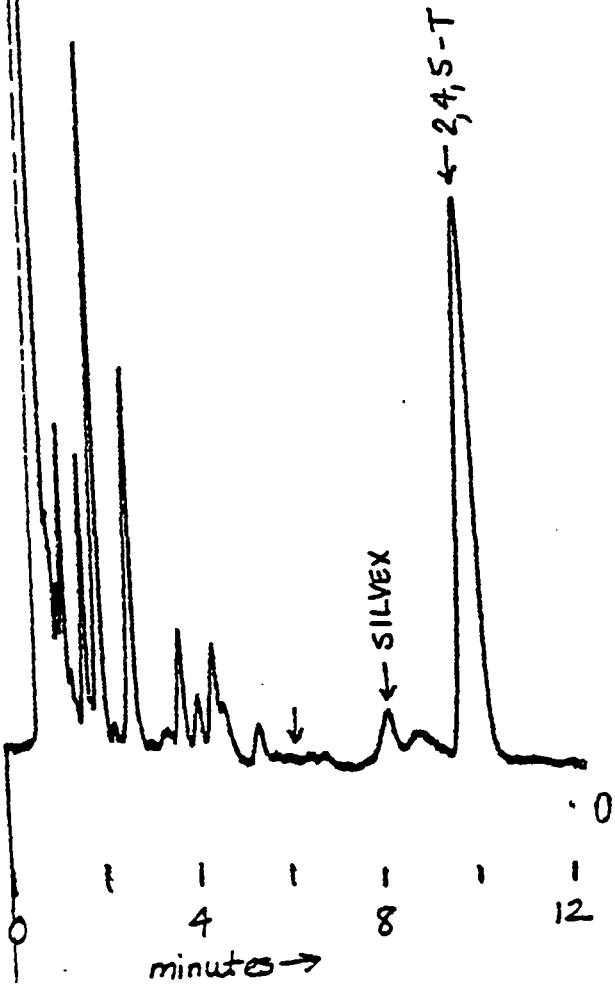
FIGURE 2.

TYPICAL CHROMATOGRAM.
BLOOD SAMPLE FROM
2,4,5-T PLANT EMPLOYEE

G.C. CONDITIONS
same as Figure 1.

Blood #2 - 8X10

SAMPLE #
028736-
75638



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0000782

FIGURE 3 WASTE FRACTION AND EXTRA FRACTION FROM SILICA GEL CLEAN-UP - GC CONDITIONS SAME AS FIGURE 1.

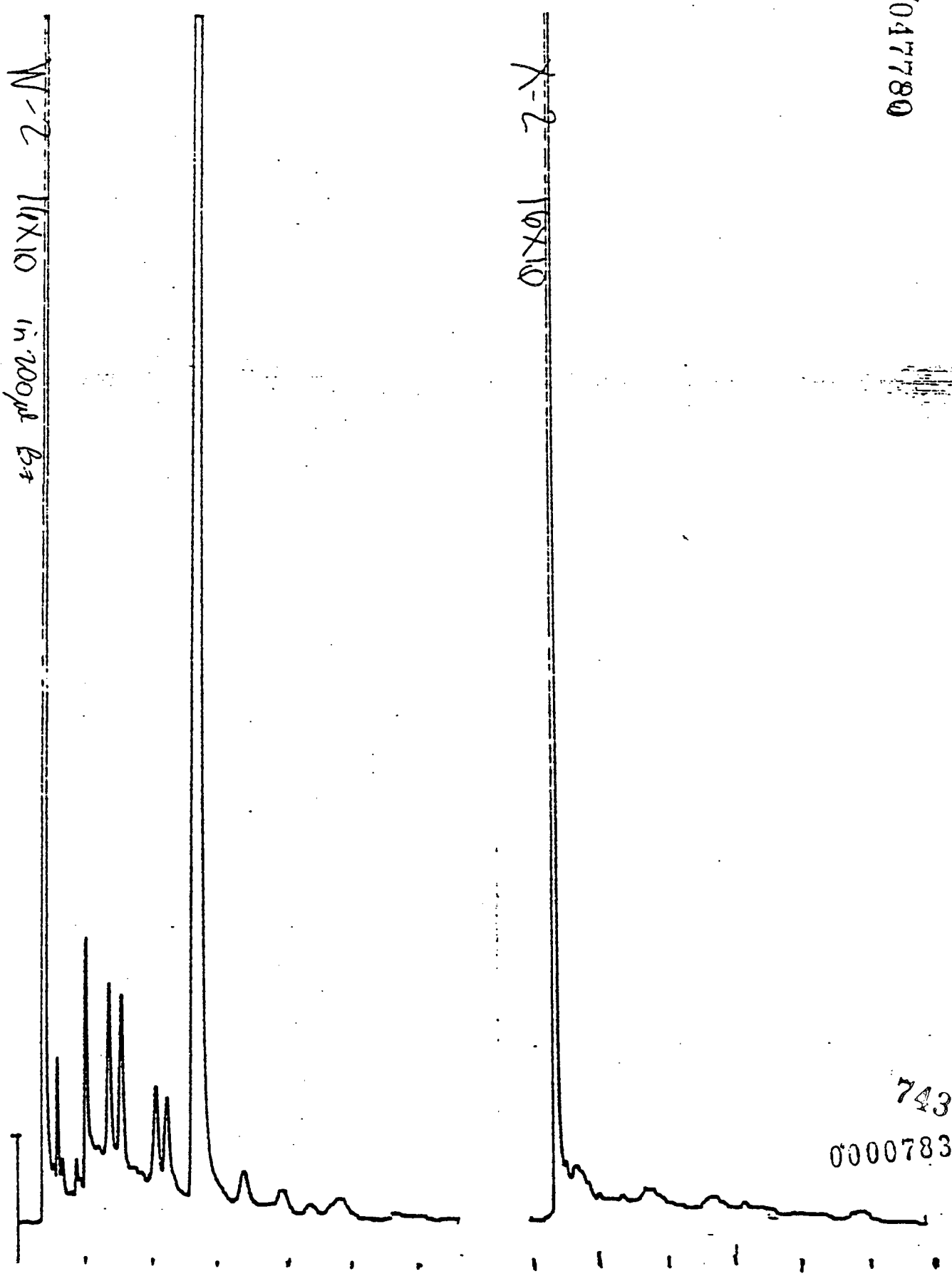
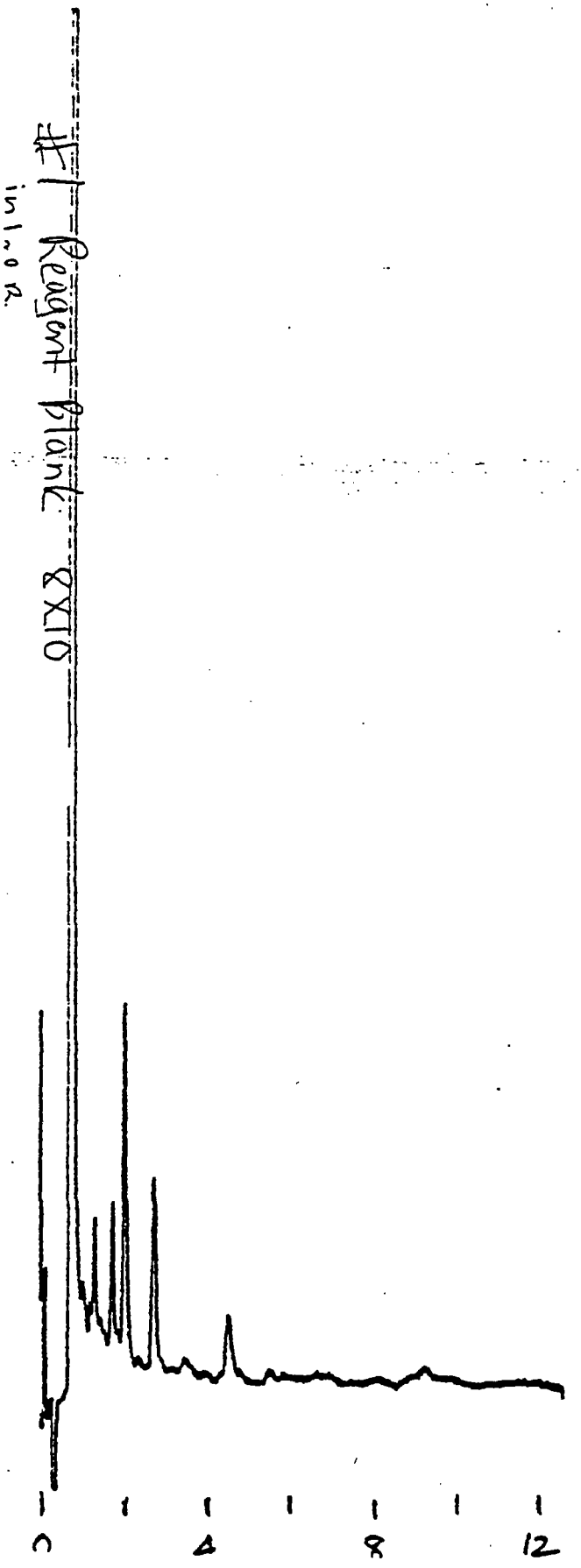


FIGURE 4

REAGENT BLANK

G.C. CONDITIONS
SAME AS FIGURE 1.



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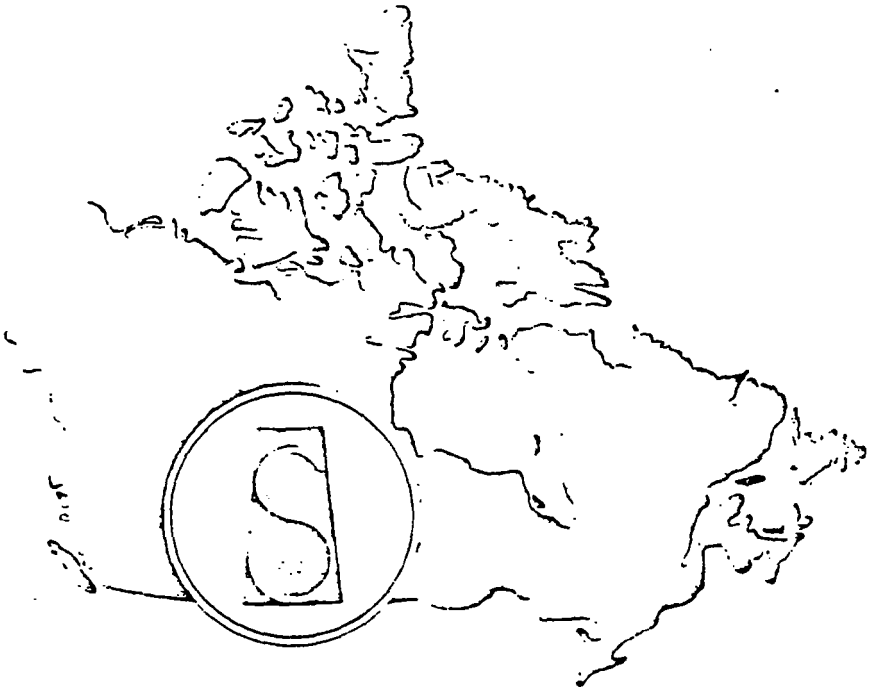
7438



OCCUPATIONAL EXPOSURE MEASUREMENT OF AN AERIAL SPRAY CREW TO A MIXED SPRAY OF 2,4-D AND 2,4,5-T

by

K. Yoshida, Senior Research Scientist
K. Wallace, Junior Research Scientist
Physics Division

SASKATCHEWAN RESEARCH COUNCIL

7439

July 1978

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PREFACE

This investigation was carried out in response to an urgent request from, Dow Chemical (Canada) Ltd. at Sarnia, Ontario, for such information particularly as it pertained to 2,4,5-T. A research contract with Dow was signed, and the completion of this report by SRC for them is the culmination of the present contractual arrangement. The report itself must be considered of a preliminary nature as the time scale did not permit a full meteorological analysis of the airflow and drift characteristics to be undertaken.

J. Maybank, Head

Physics Division

OCCUPATIONAL EXPOSURE MEASUREMENT OF AN AERIAL SPRAY CREW TO A MIXED SPRAY OF 2,4-D and 2,4,5-T

K. Yoshida and K. Wallace

INTRODUCTION

Aerial spray application of pesticide accounts for only 10% of the total spray operations in Saskatchewan, but it does include large acreages of bush and community pasture spraying. For these a mixture of 2,4-D and 2,4,5-T is commonly used.

The operation from which exposure samples were collected was by a local aerial applicator who was well acquainted with the topography of the pasture and experienced in aerial spraying. The two flagmen were beginners, locally hired. While it is sometimes difficult to pursuade such crews to cooperate in exposure sampling, in the present case they agreed. The entire operation followed routine procedures commonly practised by average aerial applicators, with the aircraft and other equipment being used in an adequately maintained condition.

The sampling team tried to avoid disturbing the spray operation so the exposure samples collected should be unbiased by sampling procedure. Standardized methods of sampling and analysis were used (see Appendix 1). The results obtained through analysis are presented, without interpretation, for the absorption of active ingredient by the human body.

DESCRIPTION OF SPRAY APPLICATION

1) Aircraft

Two Cessna Agtruck aircraft were used, one a recent model, the other about 10 years old. Nozzle settings of these two seemed adequate for 3GPA application, except that on the older plane the straight back nozzle orientation under the belly caused excessive deposition of spray to the centre rear. On the newer aircraft the belly nozzles were in the straight down position. There was no noticeable leak of spray liquid except occasionally while refilling. Details are given in Table 1.

2) Spray formulation

The application rate set by the owner of the pasture was 24 oz/acre (acid equivalent) of 2,4-D butyl ester and 8 oz/acre (a.e.) of 2,4,5-T iso-octyl ester. For improved deposition, 15 oz/500 gal. of adjuvant (Amway) and 0.5 GPA of diesel oil were mixed with the water which was pumped from a local slough.

3) Field conditions

The area sprayed was well developed pasture with shrubs scattered over a gently rolling terrain. The size of pasture sprayed for the day was approximately 1,300 acre, being roughly 2 miles in the east to west direction. The estimated number of swaths from the geometry of the pasture would be 80. Each aircraft landed for refilling around 24 times.

4) General weather Conditions

The operation lasted from 5:30 p.m. to 10:30 p.m. during which time the air temperature remained near 15°C and the relative humidity around 40-45%.

The wind was from the northwest, initially varying between 8 and 15 km/h, and decreasing toward sunset.

The operation was controlled by a field supervisor who could decide start or stop spray operation due to the weather conditions. He also supervised the action of two flagmen in the pasture, and checked the deposition pattern of spray chemical occasionally.

DESCRIPTION OF EXPOSURE SUBJECTS

1) Pilot

The pilot A (with recent model of aircraft) has been doing aerial spray for the past 20 years and seemed to be an experienced person. He agreed to wear a Gas badge and film badge on his lapel throughout the operation. He was spraying two days before the sampling day at another field, but did not spray 2,4,5-T. Pilot B has been doing aerial spraying for the past 10 years and agreed to wear gas badge. Due to mis-orientation of belly nozzles, pilot B might have been exposed to drifting chemical from the belly of aircraft (Table 2).

2) Loading and Mixing Crew

The loading attendant has several year's experience in aerial spray operation. He refused to wear the charcoal tube sampler or to provide urine specimen, but did agree to wear the badge. His duty was to open the 45 gal drums (2,4-D or 2,4,5-T concentrate) and by a hand pump to transfer the concentrate to a mixing tank, then to proceed in mixing and loading the aircraft. While the aircraft was being loaded, he stood on the upwind side of the liquid hose. On disconnecting the hose from the aircraft, there was usually a small amount of spillage.

3) Water truck operator

This person stayed with the loading attendant except when he was loading the aircraft or when she drove the water truck to the nearby slough. She also kept the records of chemical consumption and aircraft refills. She agreed to have one of the charcoal tubes in her truck and to wear gas badge and film badge. No urine sample was collected

4) Loading Assistant

This person carried a charcoal tube at his lapel position, gas badge and film badge and almost everytime followed the loading attendant and so likely had an equivalent degree of exposure. He had not been exposed to any kind of herbicide spray for the past one year. Urine samples were collected (Table 2).

5) Flagmen

Flagman A was locally-hired temporary help (a university student) inexperienced in aerial spray operation. He was exposed to other herbicidal operations 40 hours before the date of sampling (for 6 hours operation). He agreed to provide urine samples, and to wear gas badge, film badge and charcoal tube. Flagman B was also a locally hired worker with some pilot training. He was also exposed in the same way as flagman A in the previous operation, however he has no previous experience of aerial spray operation before he came to Canada from Europe (Table 2).

From the observation of the pasture (field) supervisor who drove through the field under spraying, these two flagmen were directly sprayed at the swath ends before retreating to the adjacent position, due to their inexperience in field operation.

6) Field Supervisor

This person has been supervising bush spray operations each year for the past 5 years. He stayed most of the time near the mixing station except for occasional driving to the field to observe the operation. He agreed to wear gas and film badges. No urine sample was collected (Table 2).

7) Observers

Two observers were always stationed upwind of the loading site and helped to maintain the landing strip. They never touched spray liquid and wore gas and film badges (Table 2).

SAMPLING AND ANALYTICAL METHODS

1) Dermal Exposure

Film badges were worn on clothing lapels. A 47 mm glass fiber filter (Gelman type A-E) was wetted with 1.0 ml of ethyleneglycol by pipetting and then mounted in a rectangular-based culture dish (Millipor PD1504700). The filter paper was retained in the cell by an o-ring. The purpose of wetting the surface of the filter paper is to increase the retention of chemical.

The film badges were kept with lids in an air-tight container with the lids being removed prior to the beginning of the exposure period. Afterwards the lids were replaced and the air-tight badges were transported in a cold box to the lab.

2) Respiratory Exposure

Standard size charcoal tubes (Environmental Compliance Corp. Cat. No. 226-01, NIOSH approved) were used by the two flagmen, the loading assistant, the mixer (water carrier), and by one of the pilots. Tubes were aspirated at 1.7 L.P.M. by personal sampler pumps (MSA model G), and attached at the lapel position which is within the breathing zone (60 cm radium about the nose). The flow rate of each pump was registered before and after the sampling period to obtain the mean value of flow rate.

Gas badge organic dosimeters (Abcor. Inc.) were worn by every member of the crew and by the supervisor and observers. Badges were used strictly according to the instructions supplied by the manufacturer. The expiring date of the activated carbon collection element set by the manufacturer was 10 days after the date of use (Abcor. Inc. 1977).

3) General Background

The degree of air contamination at the mixing station was monitored by using two sets of midget impingers at downwind side of the station 10 meters from the edge of chemical wagon. The inlet of impingers was maintained at 1 meter above the ground level. The impingers were filled with 15 ml of ethyleneglycol and aspirated at 2.8 L.P.M. for the entire spray operation.

4) Urine samples

Immediately after the spray operation started, the two flagmen and one loading assistant started collecting urine in a plastic bottle. Two flagmen completed 24 hour collection with nighttime passing uncollected. At exactly 24 hours after the beginning of spray operation, the last passing was collected. The loading assistant collected the entire passings of urine for the next 24 hours, and then to 48 hours, and one passing at 72 hours.

5) Chemical Analysis

The draft shield and charcoal element of the gas badge as well as the filter badge and charcoal tube were placed in 20 ml of methanol for desorption in a teflon stoppered test tube. The samples were poured through a pad of glass wool to remove any traces of charcoal, then evaporated down to 1 ml in a round bottom flask using a rotary evaporator.

Samples were then methylated using 14% BF_3-CH_3OH (5 ml) at 70°C in a water bath for twenty minutes; after cooling, 15 ml of saturated sodium chloride and 15 ml of hexane were added and the sample shaken. A suitable aliquot (usually 2-4 ul of the hexane) was injected into

For the urine samples, 100 ml of urine were passed through a column of XAD-2 resin for cleanup prior to methylation. The urine was acidified and elutriated with 50 ml of 20% acetonitrile in 0.1 M sodium bicarbonate and extracted with two 25 ml portions of di-ethyl ether. The ether was dried with Na_2SO_4 (anhydrous) and evaporated to dryness then methylated with BF_3-CH_3OH for 20 min. at 70°C. Hexane and saturated sodium chloride were added and 2 or 3 ul were ingected for quantitation of 2,4-D methyl ester and 2,4,5-T methyl ester against standards. To confirm the presence of these two esters the samples of methyl esters were butylated and the corresponding butyl esters of 2,4-D and 2,4,5-T were found and compared to standards. The procedure is given in greater detail in appendix I.

RESULTS

1) Dermal Exposure

From analysis of film badges (Table 3), it is obvious that the two flagmen were exposed to both droplet and vapor forms of the mixed spray liquid. They are the highest in density (up to 11 mg/cm^2) among the crew and observers. The values found for the 2,4-D/2,4,5-T ratio are closer to 2:1 than to the nominal 3:1 of the solution mix; this may be due to preferential evaporation in the field of the higher volitile component.

The loading attendant who was in most cases exposed to the vapor form of the chemicals received the next highest dosage, which averages close to the expected 3:1 ratio of the two chemicals. It would be primarily received in the form of vapour and not spray droplets as for the flagmen.

The exposures of other members of the crew and of the observers were considerably lower, at least as measured by the badges; they too would tend to be primarily vapour.(Table 3).

There are two major sources of dermal exposure, one being direct contact with the spray solution and the other being exposure to airborne droplets or vapor. The film badge cannot discriminate between vapour airborne and droplets as the source of contamination. Therefore these values must be considered as measures of general exposure to airborne active ingredients (assuming that there was no accidental contact with the film surface by contaminated hands or clothing. (Hays, 1977).

2) Respiratory Exposure

Both the gasbadges and the charcoal-filled tubes provided a measure of the inhalation amounts; values found for the individuals wearing each are given in Tables 4 and 5 respectively. It is possible to convert the latter into resonably equivaletn concentration values but such is not generally feasible with the former.

It can be seen that gas badge exposures were in the 2-4 mg. range for 2,4-D near the threshold limit and generally at "Trace" for 2,4,5-T (i.e. below the sensitivity limit for this chemical and technique). The charcoal tube is considered to provide somewhat more reliable data, and in Table 5 more of the samples lie above the threshold limit. Again it is seen that one flagman at least received a considerably greater dosage than did the other crew members. A surprising feature is that the ratio of 2,4,5-T to 2,4-D is close to unity, not 3:1 as in the spray mix. The reason for this is not clear, but could be due to preferential absorption, or greater ease of subsequent desorption of the former chemical.

3) Urine Excretion

The concentration of the two active ingredients in the 24 hour excretion was up to 5.11 p.p.m. (2,4-D) and 1.16 p.p.m. (2,4,5-T) with flagman A, and roughly one half these amounts with Flagman B. This is somewhat surprising inasmuch as both the dermal exposure data (Table 3) and the inhalation amounts (Table 5) indicated higher values in the vicinity of B than around A. It may be due to uncertainties in the collection of the urine samples and the inability to ensure, under

the circumstances, total collection from each individual. The amounts of 2,4-D and 2,4,5-T found in the urine for the loading assistant were generally much lower, would be expected from his exposure data. They showed a diminution with time out to 72 hours as would be expected. The metabolism of 2,4-D and 2,4,5-T has been reported elsewhere. (Kutz et al. 1977).

4) General Remarks

One of the most difficult problems encountered in the field work was how to persuade the spray crew to be volunteer exposure subjects. This is possible only once because some of them happened to be aware of the exposure problem and they tend to become suspicious on a second attempt. They are also exposed to spray chemicals intermittently, which in turn makes the continuous sampling of urine extremely difficult.

The collection and storage or extraction must be done immediately after each passing or at least immediately after every 12 hours. This was impossible with the flagmen who travel a great distance from the filling station, where sampling team was located.

The degree of exposure of flagmen is highly dependent on the nature of drifting of spray solution from an aircraft. Had the spraying been done under significantly hotter conditions higher vapour ingestions would have been likely. This problem has been studied in previous years (Maybank, et al 1978). However, further study is needed to delineate the behavior of drifting spray droplets and vapor in view of exposure determinations. (Druham and Wolf, 1962, 1963). Spray application of 2,4,5-T in the Saskatchewan pasture will continue in years to come, therefore this could be practically one of the very few of large operations in North America where exposure samples for 2,4,5-T could be obtained.

ACKNOWLEDGMENTS

This study was conducted under a short-term contract with Dow Chemical Canada Ltd. The authors would like to acknowledge the assistance provided by the following persons: Dr. A. E. Smith of Agriculture Canada Research Station at Regina for help on the analytical methods for urine, Dr. F. Kelada of Occupational Health and Safety Division of the Saskatchewan Department of Labour for general planning of exposure study and M. Peters and J. Ross of the Physics Division of SRC for general instrumentation in sampling and analysis.

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Table 1 Description of Spray Operation

1) Aircraft

Model: Cessna Agtruck X2

Cruising speed: 110 m.p.h. Height: 4-8 ft.

Tank capacity: 200 gal

Spray nozzles: D6-45 and D10-45 mixed, horizontal

Estimated swath width: 55 ft.

2) Spray Formulation

Application rate (liquid) set: 3 G.P.A.

Active ingredients: 2,4-D butyl ester 128, 2,4,5-T iso octyl 112.

Application rate (active) (oz/a): 2,4-D (24), 2,4,5-T (8)

Adjuvants used: Amway (15 oz/500 gal.) Diesel Oil (0.5 G.P.A.)

3) Field Conditions

Area sprayed: approximately 1,300 acres

Size of field: 1 mile x 2 mile

Estimated number of swaths: 80

Number of aircraft refill: 24 x 2 aircrafts

4) General Condition for spraying: Moderate to good condition

Air temperature: steady 15 deg. (C)

Relative humidity: 40-45%

Wind: NW 5-10 (m.p.h.)

Sky: partly cloudy

Operation period: 5:30 p.m. to 10:30 p.m.

TABLE 2

Crew	Sex	Age	Physical Appearance	Clothing	Smoking	Alcohol
Pilot A	M	Mid 40	Fat	Coverall hat	Moderate	Nc
Pilot B	M	Mid 30	Moderate to slim	Coverall hat	Moderate	Moderate
Loading Attendant	M	Mid 20	Slim	Coverall mitten no hat	Heavy	Heavy
Water truck operator	F	Low 20	Moderate	Coverall no hat	No	Moderate
Loading Assistant	M	Mid 40	Moderate	Fullsleeve jacket no hat	No	Light
Flagman A	M	Low 20	Slim	Coverall no hat	No	No
Flagman B	M	Low 20	Slim	Coverall no hat	No	Heavy
Field Supervisor	M	Mid 20	Slim	Fullsleeve jacket hat	Moderate	Moderate
Observer A	M	High 20	Slim	Short sleeve shirt no hat	No	Moderate
Observer B	M	High 20	Slim	Short sleeve shirt no hat	Moderate	Light

Table 3 Analysis of Dermal Exposure (film badges)

Mass Exposure Subject	Methyl ester form (µg) 2,4-D	2,4,5-T	Corrected for extraction eff (µg.) 2,4-D	2,4,5-T	Total mass in sample (µg) 2,4-D	2,4,5-T	Density in badge µg/cm^2) in ester 2,4-D	2,4,5-T
Loading Attendant	56.0	19.0	74.4	25.5	87.8	30.0	5.08	1.73
Water truck Operator	6.6	2.5	8.8	3.4	10.4	4.0	0.60	0.23
Loading Assistant	1.2	15.0	1.6	20.0	1.9	27.0	0.11	1.56
Flagman A	65.6	31.9	87.2	42.7	103.0	58.1	6.0	3.36
Flagman B	131.3	65.6	174.6	87.9	206.0	119.5	11.91	6.91
Field Supervisor	1.0	0.60	1.3	0.8	1.5	1.1	0.08	0.06
Observer A	0.6	0.75	0.8	1.0	0.9	1.4	0.05	0.08
Observer B	0.4	0.19	0.5	2.5	0.6	3.4	0.03	0.20
Correction Factor (x)	-	-	1.33	1.34	1.18	1.36	0.058	0.058

Table 4 Analysis of Gasbadges

Mass	Methyl ester form (µg)		Corrected for extraction ett. (µg)		Total mass in sample (µg)	
Exposure subject	2,4-D	2,4,5-T	2,4-D	2,4,5-T	2,4-D	2,4,5-T
Pilot A	2.0	T	2.88	T	3.40	T
Watertruck Operator	2.0	0.3	2.88	0.57	3.40	0.78
Loading Assistant	2.0	T	2.88	T	3.40	T
Flagman A	2.3	T	3.31	T	3.91	T
Flagman B	1.5	T	2.16	T	2.55	T
Field Superviser	2.0	T	2.88	T	3.40	T
Observer A	1.0	T	1.44	T	1.70	T
Correction Factor (x)	NA	NA	1.44	1.90	1.18	1.56

* Total mass in nominal formulations, 2,4-D butly ester and 2,4,5-T isocctyl ester.

Table 5 Analysis of Charcoal Tubes

Mass	Methyl ester form (µg)		Total mass in sample (µg)		Concentration ($\mu g/m^3$)	
Exposure Subject	2,4-D	2,4,5-T	2,4-D	2,4,5-T	2,4-D	2,4,5-T
Pilot A	0.21	T	0.25	T	0.57	T
Loading Assistant	0.17	0.14	0.20	0.19	0.45	0.43
Watertruck Operator	0.25	T	0.30	T	0.67	T
Flagman A	0.25	0.20	0.30	0.27	0.68	0.61
Flagman B	0.60	0.45	0.71	0.61	1.61	1.38
Correction Factor (x)	-	-	1.18	1.36	2.26	2.26

† Extraction efficiency is assumed to be 100%.

† Total mass in sample in nominal forms of active ingredients. 2,4-D butyl ester and 2,4,5-T iso-octyl ester.

† Air volume sampled during the total operation is 1.7 L.P.M. x 260 = 442ℓ.

Table 6 Analysis of Urine Excretion (Part 1)

Mass		Methyl ester form (µg)		Total mass in 100 ml (µg)		Concentration (PPM)		Total Urine	Total mass in total urine (µg)	
Exposure Subject	24 hr.	2,4-D	2,4,5-T	2,4-D	2,4,5-T	2,4-D	2,4,5-T	Vol (ml)	2,4-D	2,4,5-T
Flagman A	No. 1	480	113	652.81	133.37	6.52	1.33	-	-	-
	No. 2	360	83	489.63	97.94	4.90	0.98	-	-	-
	Mean	-	-	-	-	5.71	1.16	830	6880	1398
Flagman B	No. 1	225	50	306.02	59.01	3.06	0.59	-	-	-
	No. 2	165	35	224.42	41.31	2.24	0.41	-	-	-
	Mean	-	-	-	-	2.65	0.50	550	4818	909

. Total mass in 100 ml is in nominal forms of ester, 2,4-D butyl ester, 2,4,5-T Isoocytl ester.

. Urine collected from first 2~3 passings and final 24 hour passing.

Table 6 Analysis of Urine Excretion (Part II)

Loading Assistant		Methyl ester form (μg)		Total mass in 100 ml (μg)		Concentration (PPM)		Total Urine	Total mass in Total urine (μg)	
Time ellapsed		2,4-D	2,4,5-T	2,4-D	2,4,5-T	2,4-D	2,4,5-T	(ml)	2,4-D	2,4,5-T
24 hr.	No.1	3.0	1.13	4.08	1.33	0.04	0.01	-	-	-
	No.2	4.0	1.7	5.44	2.01	0.05	0.02	-	-	-
	Mean	-	-	-	-	0.045	0.015	1720	26.2	8.7
48 hr.	No.1	2.8	0.6	3.81	0.71	0.04	0.007	-	-	-
	No.2	3.3	0.75	4.49	0.89	0.04	0.009	-	-	-
	Mean	-	-	-	-	0.04	0.008	2300	17.4	3.5
72 hr.	No.1	1.4	0.6	1.90	0.71	0.02	0.007	-	-	-
	No.2	1.4	0.6	1.90	0.71	0.02	0.007	-	-	-
	Mean	-	-	-	-	0.02	0.007	220	0.91	0.32

. Urine collected as total of 24 hours and the next 24 hours and then one passing at 72 hours after the beginning of operation.

APPENDIX I

Determination of 2,4-D in urine

1. Scope

This method is suitable for the quantitative determination of 2,4-D in urine at the 0.05 ppm level.

2. Principle

100 ml urine sample is cleaned up using a column of XAD-2 resin. Following elution with 20% acetonitrile in 0.1 M sodium bicarbonate and acidification of the eluate, the 2,4-D is ether extracted from the acidic solution. The ether extract is evaporated to dryness and the residue is methylated using diazomethane or boron trifluride-methanol reagent. The 2,4-D methyl ester can then be quintitated using electron-capture gas chromatography.

3. Special Equipment

a) Gas chromatograph, Hewlett-Packard, Tracor, etc., equipped with an electron-capture detector and glass columns.

b) Chromatography columns, 14 mm i.d. x 400 mm long with stopcock.

4. Reagents

a) Acetonitrile, benzene, n-hexane, methanol, Glass distilled, (Caledon Laboratories Ltd., Georgetown, Ont.).

b) Hydrochloric acid, sulphuric acid, sodium bicarbonate, (reagent grade), n-butanol, (certified), (Fisher Scientific).

c) Amberlite XAD-2 resin, Mallinckrodt, (North American Scientific Co.).

d) Di-ethyl ether (Caledon Laboratories Ltd., Georgetown, Ontario).

5. G.C. Conditions

(For H-P 5713-A with nickel-63 detector)

Column ..... 1.5 m x 6 mm O.d. (4mm i.d.) glass column (on column injection) packed with Ultrabond 20M (The R F R Corp., 1 Main Street, Hope, R.I. 02831.)

Gas..... Argon-methane 95:5, at 40 ml/min

Temp..... Column and injector 150°C for methyl ester R.T. 3.5 min

165°C for butyl ester R.T. 4.0 min

Detector 300°C

6. Standard Curve

Prepare standard solutions of 2,4-D methyl and butyl esters in n-hexane. Concentration may range from 0.1 to 1.0 ng/ul. Prepare standard curve of peak heights vs concentration.

7. Analysis

Place a small plug of glass wool in the bottom of the chromatography column and add 5 gm XAD-2 resin. Wash resin down onto glass woll with distilled water until the resin in suspended in the water. Allow resin to settle to the bottom again. Wash resin with 50 ml of 20% acetonitrile in 0.1 M sodium bicarbonate. Rinse again with distilled water. Acidify 100 ml urine with 10 ml conc. HCl and add this to the column. Allow urine to pass through at about 5 ml/min. Wash resin to neutrallity with distilled water. Elute with 50 ml of 20% acetonitrile in 0.1 M sodium bicoarcnate 2 ml/min. Collect eluate in a beaker containing 5 ml. conc. HCl. Wash column with a further 25 ml distilled water after eluate has passed through and collect with eluate. Transfer eluate and wash to a separatory funnel and extract

with 2 x 50 ml di-ethyl ether. Check that aqueous phase is still acidic and discard. Evaporate ether to dryness on raotry evaporater. Traces of water are removed from the flask by azeotroping with equal portions of methanol and benzene. Quantitatively transfer residue to a suitable container and methylate with diazomethane or boron trifluride-methanol reagent. After methylation, take up ester in 25 ml of n-hexane. Inject into gas chromatograph.

8. Confirmation.

Confirmation of 2,4-D presence is obtained by either buytlation of the residue after evaporation of the ether extract or by trans-butylation of the methyl ester after GLC quantitation.

In the first case, if enough urine sample is available, the column clean-up and ether extraction of a second 100 ml urine is carried out as in Section 7. Following the evaporation of the ether extract to dryness, the residue is transferred to a test tube using a small amount of di-ethyl ether. The ether is evaporated to dryness and 2 ml n-butanol and 5 drops of concentrated sulphuric acid are added to the tube. The tube is heated in an oil bath at 100°C for one hour. Add 50 ml distilled water and 25 ml n-hexane and shake well. Dry the hexane layer over sodium chloride and inject into the gas chromatograph using 2,4-D butylester as a standard.

If urine sample is insufficient for a second extraction, the confirmation may be done by trans butylatics of the methyl ester from the initial extraction after GLC. A portion of hexane (5 ml) is transferred to tube containing 2 ml of n-butanol and the hexane evaporated off using a rotary

evaporator. 5 drops of concentrated sulphuric acid are added and the tube heated 1 hr at 100°C as before. Add 50 ml distilled water and 5 ml n-hexane and shake. Dry hexane over sodium chloride and inject into the GLC.

9. Recovery Determination

Blank urine samples are spiked at 0.1 ppm with 2,4-D acid. Urine is then cleaned up and extracted as described in Section 7. Recoveries should be in excess of 90%.

10. Comments

a) Recoveries from male urine samples ages 25-50 years have been found to be 95%.

b) Butylation and trans-butylation yields have been found to be 90-95%.

c) Urine blanks, when subjected to this clean-up and extraction, gave no interfering peaks on GLC trace.

d) In the procedure for confirmation of 2,4-D by butylation or trans-butylation it may be necessary to shake the hexane layer with a second 50 ml of water to remove all the n-butanol before drying hexane for injection into the GCL.

ANALYTICAL REPORT

MIDLAND ANALYTICAL LABORATORIES

DOW CHEMICAL U.S.A

AL NO. RESTRICTED REPORT
AL 78-50668

ACCT. NUMBER 8004 | SUB FUNCTION

LAB. NO. | PROBLEM NUMBER

DATE July 28, 1978

TO: M. B. Chenoweth, 607
E. L. Garfield, 607
M. L. Leng, 9008
W. H. Braun, 1803
L. P. McCarty 1701 & 607
J. H. Saunders, 607
W. B. Crummett, 574

TITLE DETERMINATION OF PHENOXY HERBICIDES IN URINE AND BLOOD SAMPLES FROM 2,4,5-T PLANT PERSONNEL - II.

DOW047812

INTRODUCTION

Human urine and whole blood samples were obtained from personnel working at the 2,4,5-T production facility for a determination of 2,4-dichlorophenoxy acetic acid (2,4-D), 2-(2,4,5-trichlorophenoxy) propionic acid (Silvex), and 2,4,5-trichlorophenoxy acetic acid (2,4,5-T).

EXPERIMENTAL

The samples were prepared by extraction, hydrolysis, methylation with diazomethane, and silica gel column clean-up prior to analysis by gas chromatography-mass spectrometry (GC-MS). The experimental procedure used is detailed in Report ML-AL 78-50252.

The silica gel column used was changed slightly. The column used was packed with 1.5 grams dry silica gel (~ 11 cm x 6 mm i.d. glass). The column was eluted with 60/40 methylene chloride/hexane. The first 2 ml were collected and discarded (waste fraction). The next 13 ml of eluent were collected for analysis. Then an additional two ml were collected to check for recoveries, (extra fraction). The collected fractions were evaporated to dryness. Prior to analysis, the sample residue was dissolved in 50 µl benzene.

Validation data for this procedure are detailed in ML-AL 78-50252.

RESULTS AND DISCUSSION

Results are detailed in Table I. Figure 1 shows a typical standard ion chromatogram and GC-MS conditions. Figure 2 shows typical GC-MS data for a urine sample from a 2,4,5-T plant worker.

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SIGNATURE	PHONE	BUILDING	DATE FINISHED	HOURS	RESULTS PHONED	REVIEWED
Marsha L. Langhorst Data Book AL 1207 p. 118	6-6207	574	7/13/78	37		RLB

FORM W-50340

Figure 3 shows typical GC-MS data for a blood sample from a 2,4,5-T plant worker.

The presence of Silvex was asked to be confirmed. It was questioned whether Silvex could have an interfering compound by GC-MS six-ion monitoring or whether Silvex or an interference could be created from 2,4,5-T during the sample preparation or analysis.

To check the creation of an interference during sample preparation, a urine and blood sample were spiked with high levels (1.85 µg/gm sample) of 2,4,5-T. The sample was processed through the usual procedure and analyzed. No peaks were found other than 2,4,5-T. See Figure 4.

To check if the ethyl ester of 2,4,5-T might interfere with the methyl ester of Silvex, (both have parent ions by MS at m/e = 282) by GC-MS. A sample of 2,4,5-T was ethylated with ethanolic HCl. The sample was analyzed by GC with electron capture detection and found to elute at a different retention time than Silvex. Therefore, it could not interfere by GC-MS and produce a response interpreted as Silvex.

In addition, to check if any ethyl ester of 2,4,5-T is formed during methylation, a relatively high concentration of 2,4,5-T was methylated. No peaks were detectable except the methyl ester of 2,4,5-T. This evidence is shown in Figures 5 and 6.

The identify of Silvex in urine and blood samples is confirmed by retention time, ions in the mass spectra, and ion abundance ratios (282/198).

REFERENCES

1. Marsha L. Langhorst, "Determination of Phenoxy Herbicides in Human Urine and Blood Samples - (Validation Data)", ML-AL 78-50252.

2. Marsha L. Langhorst, "Determination of Phenoxy Herbicides in Human Urine and Blood Samples (RESTRICTED REPORT)" ML-AL 78-50405.

3. Marsha L. Langhorst, "Determination of Phenoxy Herbicides in Human Urine and Blood Samples from 2,4,5-T Plant Personnel, (RESTRICTED REPORT)" ML-AL 78-50489.

td

July 28, 1978 AL 78-50668

DOW047815

TABLE I

RESULTS

PHENOXY HERBICIDES IN 2,4,5-T PLANT PERSONNEL

No.	Person	Sample Type	Concentration found (ng/gm Sample) 2,4-D	Silvex	2,4,5-T
U-1		Urine	380	21	540
U-2		Urine	520	4.1	220
U-3		Urine	510	23	310
U-4		Urine	270	11	170
B-1		Blood	2.0	N.D.(1)	8.7
B-2		Blood	8.6	N.D.(1)	5.6
B-3		Blood	9.3	N.D.(1)	7.9
B-4		Blood	3.8	N.D.(1)	2.4

SPIKED SAMPLES FOR RECOVERIES

No.	Sample Type	Spiked with 2,4,5-T	Conc found (ng/gm) 2,4-D	Silvex	2,4,5-T	% Recovery
U-1	Urine	1850 ng/gm urine	N.D.(1)	N.D.(0.5)	1540	83
B-1	Blood	1850 ng/gm blood	N.D.(2)	N.D.(1)	1400	76

NOTES: N.D. = Not detected with minimum detection limits shown in parentheses.

Minimum detection limits (MDL's) are:

Compound	in ng/ml	in ng	in ng/gm urine	in ng/gm blood
2,4-D	200	10	1	2
Silvex	100	5	0.5	1
2,4,5-T	100	5	0.5	1
		assuming 50 µl final volume	assuming 10 gm urine sample	assuming 5 gram blood sample

Methods are validated down to 3 ng/gm urine and 5 ng/gm blood.

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DOW 041816

FIGURE 1. STANDARD GC-MS DATA

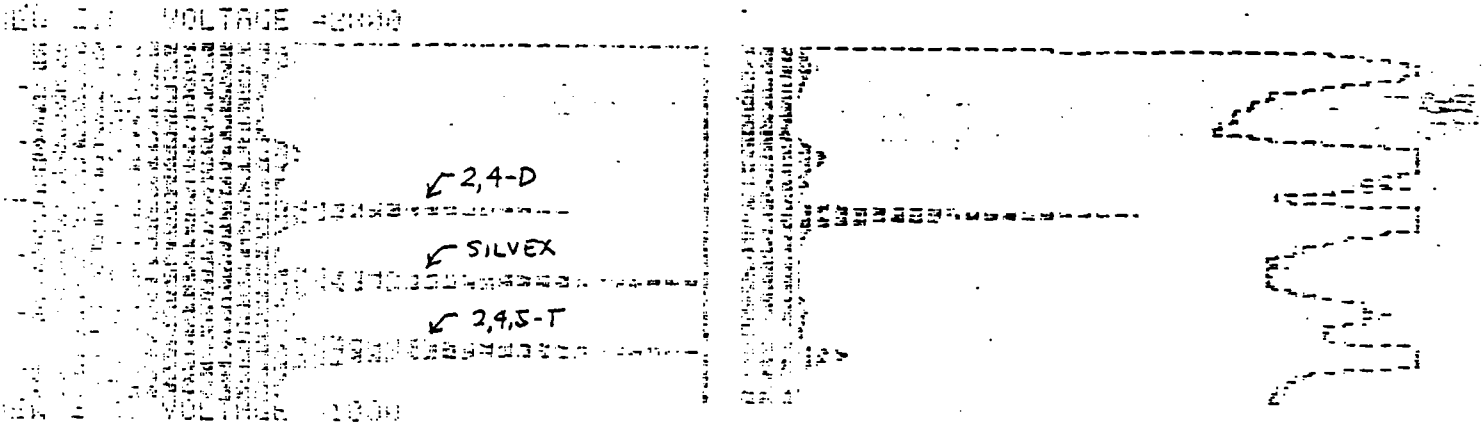
INSTRUMENT: Hewlett-Packard 5992 GC-MS
COLUMN: 9'X 2mm i.d. glass, 3% SP-2401 on 100/120 Supelcoport

STD Um'

TEMP1	TIME1	RATE	TEMP2	TIME2	PORT	SOLVT	OVEN MAX	RUN TIME	E.M. VOLTS
190.0	isothermal		190.0	50.0	210.0	2.0	210.0	20.0	1000

STANDARD Um'

2.36 µg/ml 2,4-D
1.98 µg/ml SILVEX
2.13 µg/ml 2,4,5-T
(as methyl esters)



ION	234.00	236.00	198.00	282.00	233.00	268.00	
ABUNDANCE	393.78	197.16	219.56	175.85	555.37	292.26	

ION	234.00	236.00	198.00	282.00	233.00	268.00	
ABUNDANCE	393.78	197.16	219.56	175.85	555.37	292.26	

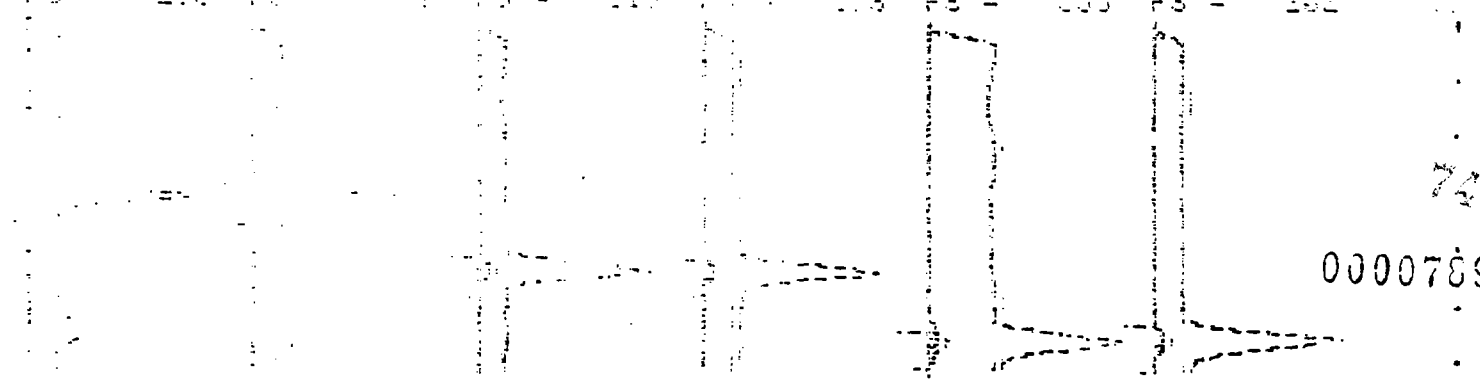


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FIGURE 2 URINE SAMPLE -

DOW 047817

4.00

SUM OF MONITORED ION ABUNDANCES
Full Scale = 100

ION 234.00
Full Scale = 50

	204.00	246.00	0.00	262.00	288.00	268.00
	283.95	192.47	93.44	32.96	404.12	144.14

4.00 Friday

	204.00	246.00	0.00	262.00	288.00	268.00
	283.95	192.47	93.44	32.96	404.12	144.14

AMOUNT INJECTED = 0.00

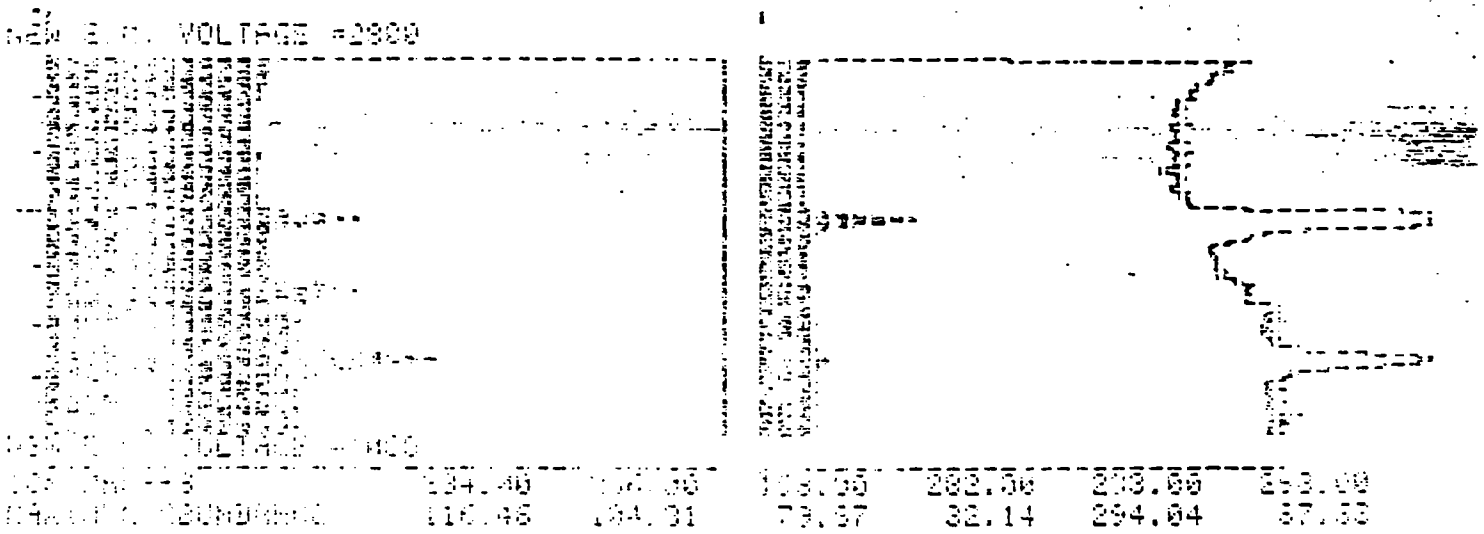
ION =283.00 ION =303.00 ION =268.00
FS = 32 FS = 404 FS = 144

0000700

FIGURE 3 BLOOD SAMPLE -

#B-4 Blood Fraction in Styrene benzene

DOW 047818



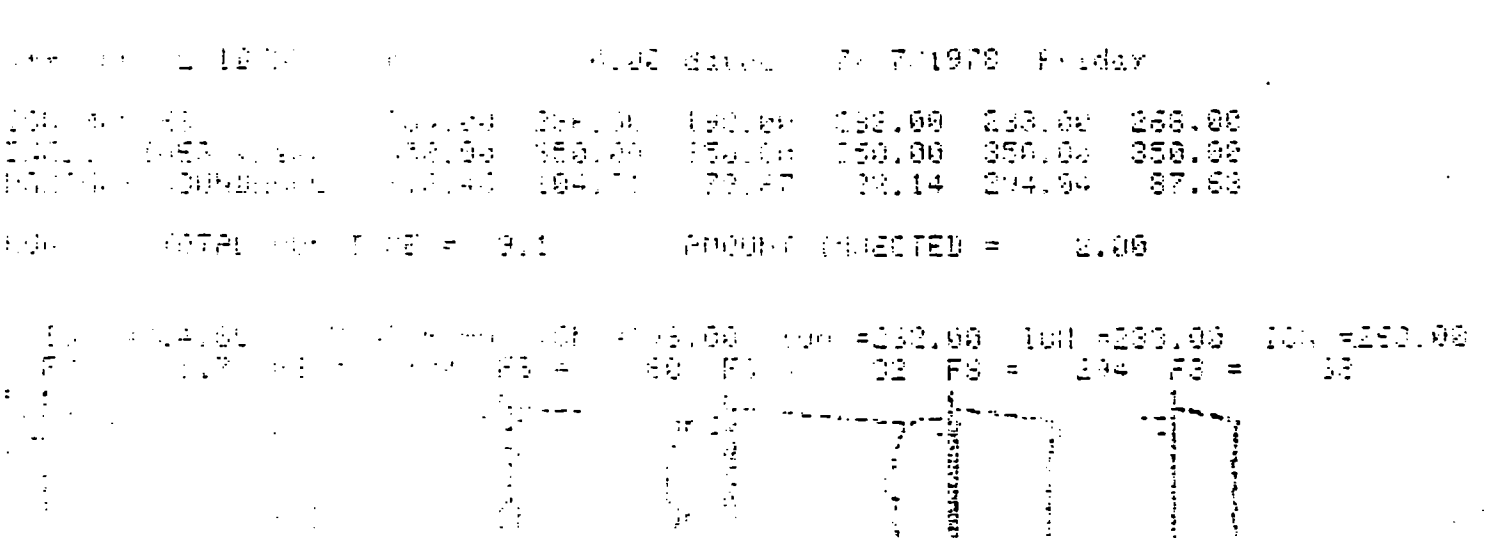


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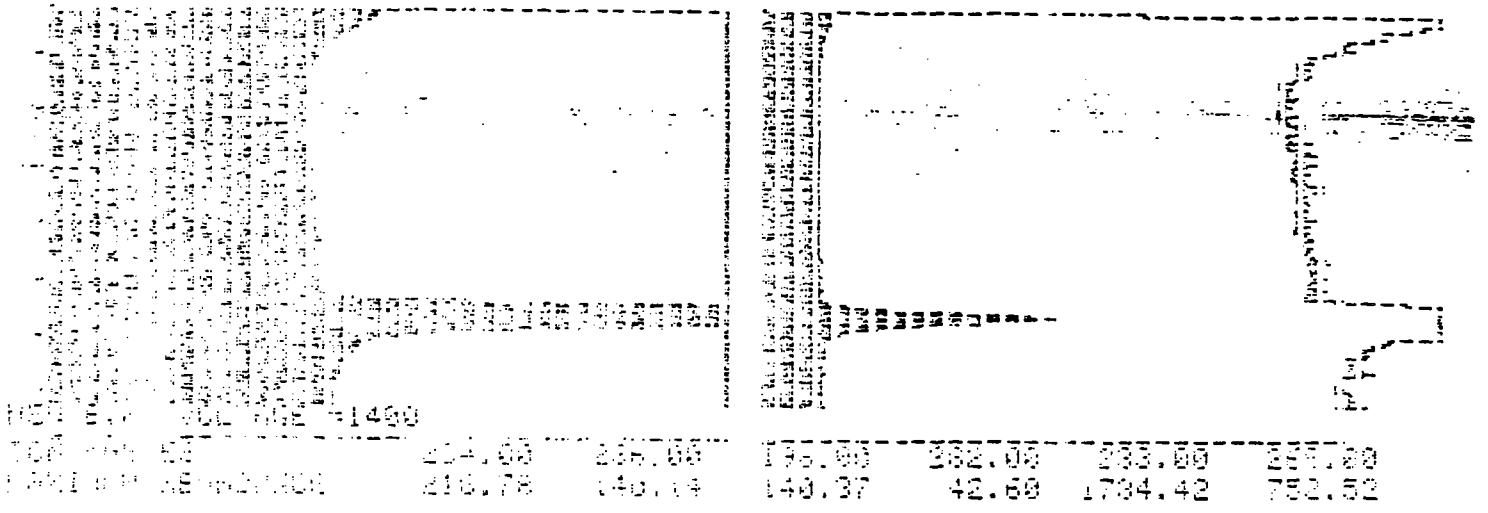
FIGURE 4 CONTROL URINE SPIKED WITH 2,4,5-T

#1 Urine + 245T in Methanol

DOW 0478199



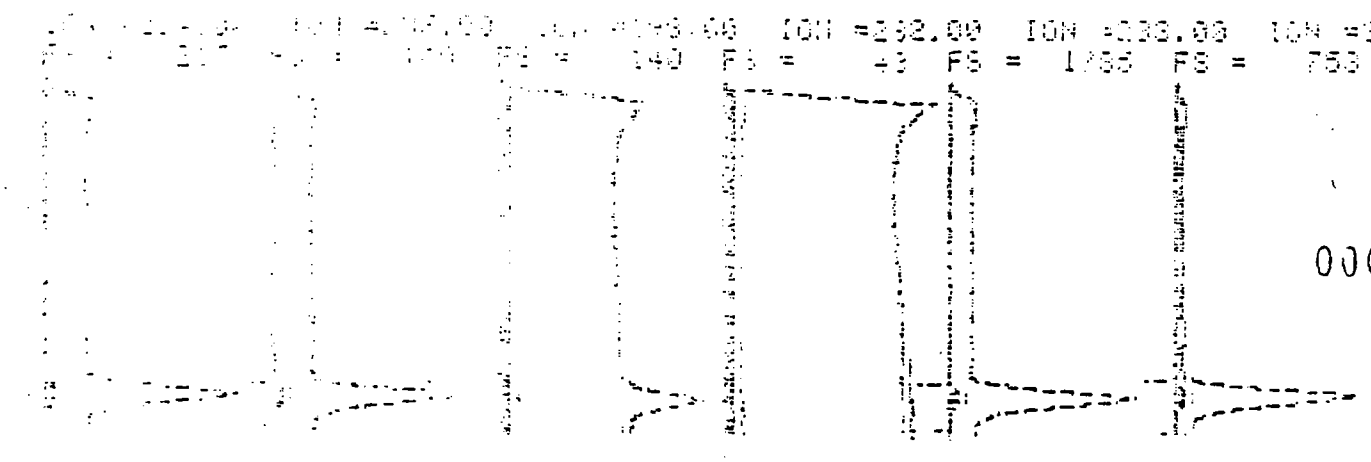


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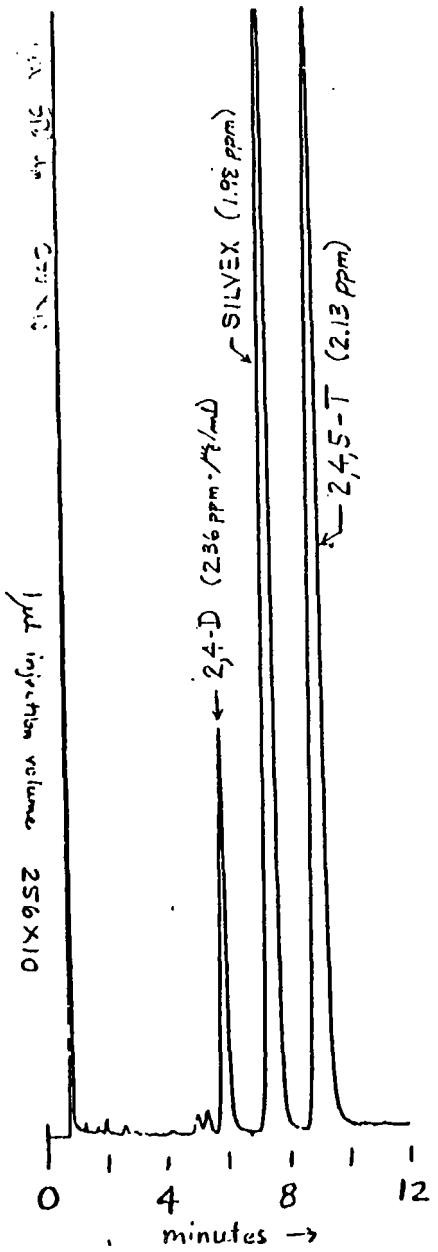
FIGURE ' RETENTION TIMES OF PHENOXY HERBICIDE STANDARD CHROMATOGRAMS AND GC CONDITIONS.

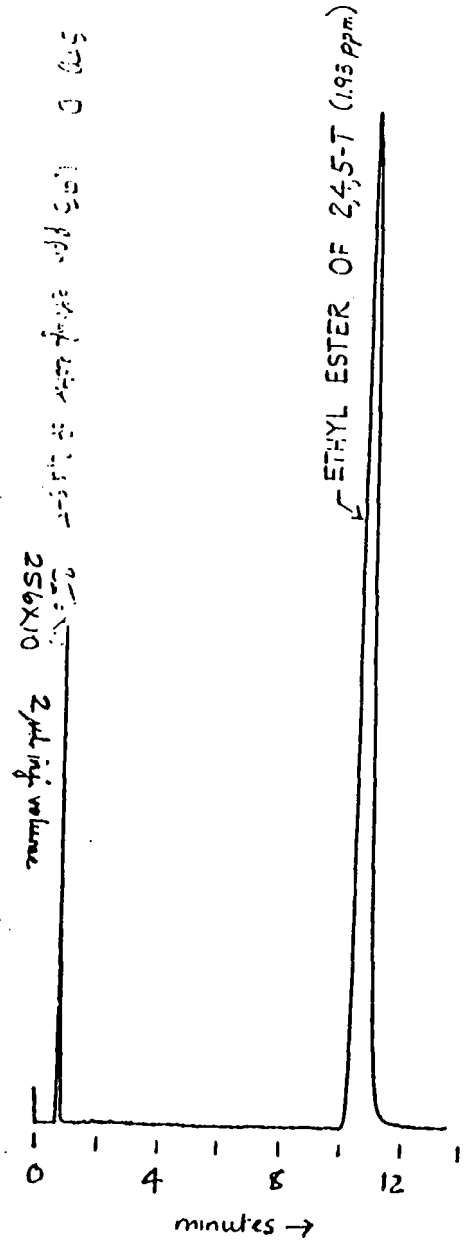
INSTRUMENT: Varian 3700 Gas Chromatograph

Operator	M.L. LANGHORST	Date	7-3-78
Column No.	glass	Length	9 feet
Dia.	2mm i.d.		
Coating	SP-2401	Concn.	3%
Support	Supelcoport	Mesh	100/120
TEMP: Col: Init.	175°	°C Final	°C
Rate	°C/min.	Det. 340 °C Inj.	210 °C
CARRIER GAS	Nitrogen	Rate	30 ml./min.
Pressures: Inlet		Outlet	
Hydrogen	ml./min.	Air	ml./min.
DETECTOR E.C. Ni 63	T.C.	F.I.D.	
Scavenger		Rate	ml./min.
Sens. As labeled	Rec.Range	1	mv.
SAMPLE: As labeled		Size As labeled	
Solvent Benzene		Concn.	

STANDARD - PHENOXY HERBICIDES AS METHYL ESTERS



STANDARD - 2,4,5-T AS ETHYL ESTER



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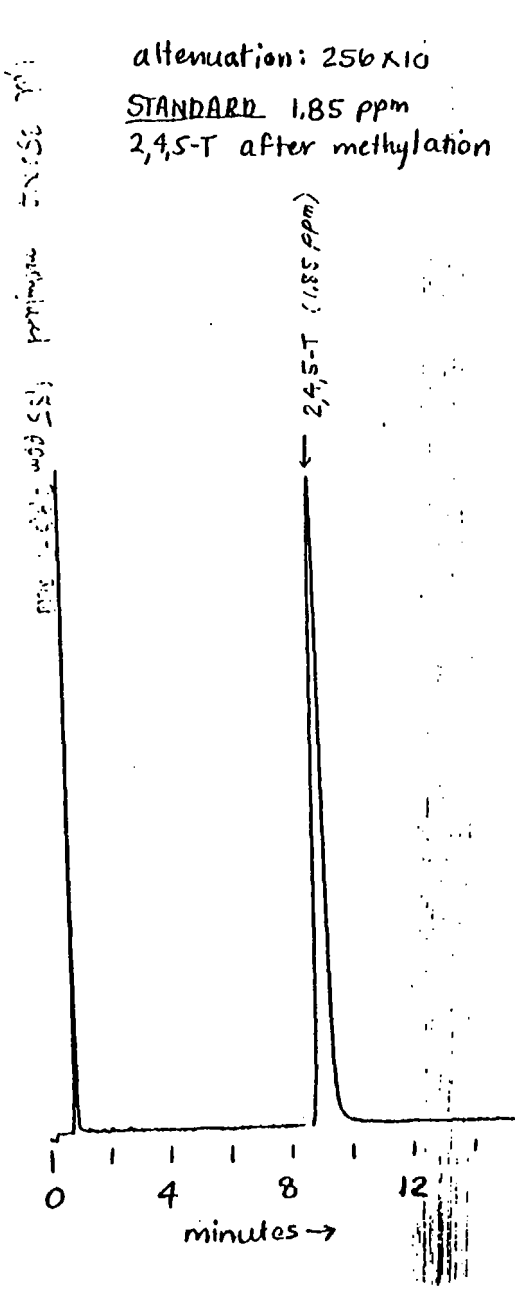
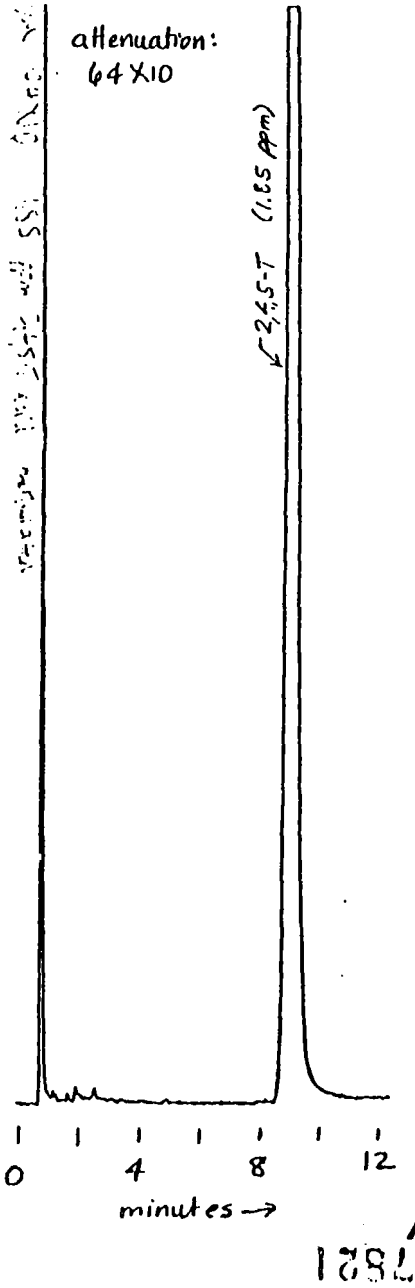
DOW 017820

FIGURE 6 CHROMATOGRAMS OF 2,4,5-T AFTER METHYLATION – SHOWING THAT NO ETHYL ESTER IS FORMED DURING METHYLATION





78-12-7

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1617-25 (1978)
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21 OCTOBER 1978

Pathological-embryological investigations in cases of abortion related to the Seveso accident

by Helga Rehder, Laura Sanchioni, F. Cefis, and A. Gropp

Correspondence: Dr. Helga Rehder or Prof. A Gropp, Dept. for Pathology of the Medical Hochschule, Ratzeburger Allee 160, D-2400 Lubeck. Dr. Laura Sanchioni or Prof F. Cefis, Laboratorio di ricerche cliniche, anatomia ed istologic patologica, Istituti elinici di perfezionamento, Via San Barnaba 8, Milano, Italy.

Summary. After the explosion accident on July 10, 1976 in Seveso (Italy), material from 30 interrupted pregnancies and from 4 spontaneous abortions was investigated by embryological and histomorphological studies. No indications of mutagenic, teratogenic or fetotoxic effects of TCDD could be found. The cases of spontaneous abortion, albeit more suspect for dioxin damage, showed different morphological alterations due obviously to a variety of causative factors independent of TCDD. On the other hand it is not possible to exclude entirely an embryotoxic effect of TCDD because in the majority of cases the fetal tissues were incomplete.

The explosion in a chemical factory of ICMESA on July 10, 1976 in Seveso, northern Italy, by which the factory and large surrounding inhabited areas were exposed to the especially toxic 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and Trichlorphenol (TCP), has aroused the attention and interest of world publicity above all on account of threatened health injuries to the inhabitants.

Factory accidents with the release of dioxin gases were already known from earlier years. In 1953, 54 workers from a chemical factory in Ludwigshafen suffered the effects of such an accident [15]. To a large degree

their illnesses were longstanding chloracne and injuries to the inner organs [9,36]. In an explosion in 1963 in a chemical factory in Amsterdam 50 workers were exposed to injurious dioxin gases [12]. In 1968 79 men, after an explosion in a chemical factory in Bolsover, England were affected [23]. Similar accidents occurred in chemical factories in the USA [2,28] as well as in Czechoslovakia [17], Austria [31], France [6,7], Russia [35] and in 1955 in Hamburg [19].

In contrast to these accidents, which are limited to the factories affected and remain essentially a work and accident medicine problem, the explosion in Seveso took on the dimension of an environmental catastrophe because the toxic dioxin gases were carried from the factory area by wind conditions and exposed bordering localities. A health danger to large groups of people was therefore possible, adults as well as children, which was already evident shortly after the accident with the first chloracne cases. In addition, exposure of animals and plants must be reckoned with and also contamination of the ground in an extensive land area for many years to come [12,13,14].

Although at the time of the accident positive, defineable embryotoxic injuries from dioxin combinations in humans were not known and in animal experiments as well [5,25, 33,34] were only little known, very quickly assumptions on possible damages to pregnant females and to unborn children with regard to the great toxicity of dioxin combinations appeared. According to one estimate, at the time of the explosion accident in the area affected, ca. 150 women were in the first trimester of pregnancy. Additional women appeared endangered in as far as pregnancy had already begun or was in the meantime begun, by delayed evacuation and insufficient separation measures. The public discussions, carried out on political, world view and religous levels, about the risks of these women bringing malformed children into the world because of the effects of dioxin, were very lively in the weeks and months after the explosion accident in Italy and outside the country as well with the general discussion about termination of the pregnancies. From this arose for the pregnant women, doctors and other affected persons and institutions, special situations of conflict.

125 women from the exposed area as well as the bordering regions, in the time up to October, 1976 decided on termination. In 30 cases Interruptio was approved

and carried out. Place of living and working of the applicant as well as other contacts with dioxin were taken into consideration. According to the measureable degree of ground contamination with dioxin, the district administration divided the southeast area bordering on Seveso into a heavily exposed Zone A and a less exposed Zone B (diagram 1). The naming of an R Zone (restrict area) came later, when small to nearly no dioxin content in the ground - and very much later than in the A and B Zones- was recognized as a biological exposure by cases of chloracne appearing or domestic animals dying.
As "contact with dioxin" was also considered transit through the heavily infected area (superstrada) and the consumption of regionally produced, possibly dioxin-containing vegetables or meat.

The fetuses removed by Interruptio as well as 4 spontaneous abortions from the Seveso region, which were all registered in the Gynecological Clinic in Milan, were also examined embryologically and pathologically-anatomically in the Department for Pathology of the Medical Hochschule in Lubeck in co-operation with the Laboratoria di ricerche cliniche, anatomia ed istologia patologica, Instituti clinici di perfezionalmento, Milan. The results of these investigations were conveyed in a

summary to the Health Officials of the Lombardy region on February 25, 1977. A detailed compilation and observation appeared also after a long interval on the explosion accident in Seveso, especially with regard to additional untested reports on congenital malformations by dioxin. The completeness of the documention is required by the far-reaching significance of the results and of public discussion.

Material Investigations (see Table 1)
Of the 30 removed fetuses by Interruptio, 3 came from the A Zone, 5 from the B Zone and 13 from the R Zone. In the remaining 9 cases the mother's place of living was outside of the danger zones, but in 7 cases could be made valid in connection with place of work, visit, travel and nutrition. In 2 cases the Interruptio occurred for psychic reasons.

The fetuses represented very different gestational ages/ The smallest, of a few millimeters in size, was judged to be 5 weeks, the largest, with a crown-heel length of 17.5cm, 20 weeks gestational age. In the majority of the cases the developmental ages were between 10 and 12 weeks.

In the 25 cases with gestational age of 5 to 12 weeks the Interruptio was by instrument curettage. There was an unavoidable dismembering of the fetus and a loss of fetal tissue during the suction during the action. As a result there were, in these cases only a few fetal organs available for pathological-anatomical examination. In one case only a foot and a small piece of liver, in another only a head segment and a vitelline sac were present. In the five larger fetuses of a total length of 13-17.5cm the Interruptio was introduced by intra-amial prostaglandin instillation and in some of these ended by extraction. Therefore here also were artificial lesions on the fetuses, for example, widespread skin defects or opening of the cranium cavity with loss of the brain. The 4 spontaneous abortions had occurred in the 11-24th week of pregnancy in the A and R Zones. In three of these cases fetuses of 11-19cm crown-heel length were present, in the fourth case there was merely an empty amniotic sac with placenta tissue.

A majority of the Interruptio and abortion material was, at the end of September, 1976, in formalin, Bouinsic solution or Carnoy solution sent to us. Thereby the useable examination techniques were limited from the beginning to the paraffin histology. Histochemical or

electron optical investigations could not be carried out since they require fresh material or a differentiated fixation technique.

In the cases in which only incomplete fetal tissue fragments were available, they were examined with the Lupen microscope* for form and for the position of the organs or organ segments within the organ assemblage. Each piece of tissue was photographed for documentation, measured and described. The material was finally imbedded in paraffin and in stages - or a series of cuts- completely worked up. Many times well over one hundred cuts were made. For the histological examination the following dyes were used; Hematoxylin-eosin, connective tissue-, Elastic- and PAS- dye, aldehyde-fuchsin dye, Esterase proof and Berlin blue reaction.

The larger, more intact fetuses were examined by X-rays for skeleton changes. This was however, unsuccessful in the 2 Carnoy-fixated cases, since the lime from the bones was dissolved by the fixative. A regular obduction

*Lupe means magnifying glass, whether this is a special type of microscope the translator does not know.

with organ section followed with help of the Lupen microscope. Each individual step was documented photographically, the organs were weighed, worked up for the paraffin histology and as described above, dyed. The brains were, in as far as they were intact, sent to Professor GULLOTTA, Neuropathological Institute of the University of Bonn (Dir. Prof. Kersting) for examination. In a to a high degree autolytic fetus of 4cm in length, cross sections were taken and examined macroscopically and histologically in series cuts.

Findings and Evaluation

A. In a <u>first group</u>, to which the majority belong, namely 23 of a total of 30 Interruptio cases (Table 1) the macroscopic and histological examinations revealed no malformations; likewise no tissue mis-differentiations or degenerative organ changes were shown. The tissue differentiation degree and the size of the fetuses were largely compatible with the length of pregnancy. Small differences of 1-2 weeks were not counted since the exact conception date is only seldom possible and also the pathological and anatomical criteria allow only an approximate estimate of the developmental age. Normally individual variations exist in the course of development.

Determination of sex was possible only in cases in which the material contained differentiated gonadal tissue or differentiated Wolffian or Muller ducts allowed an opinion (see Table 1).

B. In a second group of 6 interruption cases, changes of a different kind were morphologically detectable (see Table 1). They can be identified as artifacts, development retardation to a slight degree or as within the range of variation of normal development.

So in case 6 there was a secondary obliterated duodenum, through epithelium bridges, a condition which can appear in this age of development as a temporary appearance [10].

In case 15 over the coccygeal bone there was a subepiderman cyst, whose lumen was no longer in contact with the ventriculus terminales above it of the spinal cord. The cyst consisted of a so-called "coccygeal medullary vestige" which, after formation of the Filum terminale on whose distal end, therefore in the area of the rear neuroporus, which in the meantime has closed, persists sometimes for a while [21]. In the same case there was a defect of the ventricle septum underneath the

septum membranaceum. Because of the parietal tissue tearing and the missing endothelium lining it was understood as artifact.

A prolapse of the retina in the area of a lateral rupture of the sclera was seen in cases 8 and 17 after localization by the presence of fresh bleeding and the lack of a tissue reaction in the surrounding area as artificial, occurring at the earliest, during the curettage.

Case 27 showed, in a male decapitated fetus of 16.5cm crown-heel length, a rotation of the liver in a counterclockwise direction to the left with fixation lacking on the right diaphragm.
Displacement of the cecum in the right upper abdomen as well as partial intrahepatic gall bladder. In addition there were widespread bleedings in the lumen and wall of individual intestinal loops in the sense of a beginning hemorraging infarction. This together with the decapitation, is seen as an indication of traumatization during Interruptiom which also led to rupture of the Lig. trigonum hepatis and to dislocation of the liver and secondarily of the intestine. The fact is thus considered that a congenital misplacement of the liver is hardly isolated in appearance: either it takes

place primarily within the boundaries of a syndrome, as in Ivemark-syndrome, or it is observed secondarily in connection with a stomach wall or diaphragm defect, an omphalitis or hernia. The partly intrahepatic position of the gall bladder was also a normally frequent anomaly with no essential significance.

Case 18 showed, in comparison to the size of the fetus a still widened kidney pelvis calyx system. This can be seen as physiological up to the 10-11th week of gestation. Here it is a result of a slight development retardation. Also the differentation of the elastic fibers in the trachea seemed retarded.

C. A third group contains the 30th case as well as the 4 spontaneous abortions (cases 13,29,31 and 34: Table 1), in which with regard to a possible dioxin damage, the question of the cause of the spontaneous fetus deaths is especially urgently asked.

In Case 30 there was a great discrepancy in the anamestic data. The Interruptio took place in the 27th week of pregnancy. The female fetus, decapitated by the manipulation during the action showed a nape of the neck-heel length of only 4cm. It was to a high degree autolytic, shrunken and mummified, the placenta tissue clearly regressively changed, so that in this case a spontaneous previous death of the fetus with retention in utero a "missed abortion" must be assumed. At the time of the explosion accident the mother was in the 9th week of pregnancy. The fetus death occurred surely shortly thereafter, without morphological proof of its cause.

In case 13 was a female fetus of 17.5cm length from the 15th week of pregnancy, whose good condition of preservation points to a fetus death shortly before or during the Abortus. The numerous fresh areas of inner bleeding spoke for hypoxia as the cause of death. Widespread hematoma of the cranial soft parts obviously as a result of labor pains following amniotic sac rupture, had developed before the fetal death; the intracellular hemosiderine deposits in the region spoke for this. An indication for the cause of the early labor or the sac rupture was not to be determined from the morphological picture of fetus and placenta.

In case 29 a male fetus of 19.5cm length from the clinical 24th week of pregnancy, an infarct placenta was seen as the cause of death. The placenta was small and 4/5 of its volume was interspersed with widespread older and fresh infarctions. The fetus seemed, with regard to the given length of pregnancy, underdeveloped. This can be traced back to lack of sufficient oxygen as a result of the older placenta changes. A "missed abortion" can be excluded because of lacking maceration signs. The pronounced autolytic changes of the inner organs in this case were the result of a misplaced fixation.

Case 31 was a female fetus 11cm in length from the 12th week of pregnancy, which already had definite maceration. Here there was an anomaly in the region of the heart with an aorta valve defect as well as a longitudinal swelling of the aorta wall next to the pulmonary artery. This began directly above the aorta valve commisure and reached to the aorta curvature. Histologically there was a circumscribed texture disturbance of the elastic fibers of the aorta wall. The fetus showed, in addition, a serration anomaly of the right lung as well as a peculiar Facies with inclined lids, flat nose, a large capped on the point, tongue, as well as a receding chin. The skull was stretched, the occiput flat with a neck edema above a questionable strangulation ridge. It was to be assumed that the interpretation of the outer phanotype was injured by the advanced autolysis, so that the characteristics named can not be evaluated alone. In connection with the inner anomalies however- with all retention- it can be seen as an indication for the presence of chromosomal aberration. The fetus was strangulated by the umbilical cord and retained in utero. The placenta showed regressive changes as

well as fresh chorioamnionitis, which apparently developed after the fetal death.

Case 34 has to do with an abortion in the clinical 14th week of pregnancy. The abortus material contained, in addition to decidua tissue, however, only a small hazel nut sized amniotic sac, without content, that is without a fetus. The scanty placenta villi were hardly vascularized, hydropically swollen and showed only slight trophoblast proliferation on the surface area as well as individually inflated and vacuolized histiositic giant cells in the villi. The histological picture spoke in favor of an early abortion with resorption of the embryo as well as degeneration of the placental tissue by primary tissue unworthiness of the embryo placement. PHILLIPPE and BOUE [27] described such changes of the placenta during chromosomal aberration in early abortion so that in this case the assumption of a chromosomal anomaly of the embryo placement seems possible.

Discussion

The attempt to evaluate the described findings and the discussion of a possible embryotoxic effect of the dioxin, especially with regard to conclusions made in fear, zeal and emotion, require cool consideration. The epidemiological as well as the anamnestic and clinical data must be taken into consideration, which are partially stated in Table 1.

So, in 4 of 5 cases (cases 29,30[1], 31,34) with spontaneous fetal death, it is shown that the mothers came from the

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[1] A case of Interruption in which, however, an intrauterine fetal death had occurred earlier.

so-called R zone (restrict area) or had visited them. Although the question is asked especially urgently in these cases about dioxin damage, they are of a zone which, to be sure showed a biological infection; in which however, the chloracne cases appeared in isolated instances and very much later than in the primary exposed zones A and B. In addition, the women affected could give no information on possible contacts with dioxin, namely neither consumption of regional vegetables or meat nor actual time spent in the heavily exposed regions. Only in Case 13, belonging to the group of spontaneous abortions, had the mother resided until the evacuation on July 27, 1976 in the most heavily exposed A zone. The abortus occurred 4 weeks later and was attributed to the lack of older morphological changes in the placenta and fetus causing an early rupture of membranes. Information on symptoms as possible clinical indication of an intoxication of the mother was not present in these cases of spontaneous abortion. In case 31 a hyperemesis during the pregnancy as well as fever at the time of the abortion were present. This complaint coincides with the pathological-anatomical finding of chorioamnionitis. A slight rise of the γ-glutamyl-transferase in case 30 can be seen in connection with

the long retention in the range of a "missed abortion". As a whole the causes for abortion seem very heterogenous. If it is taken into consideration, that normally the comprehensible abortion rate of all conceptions is about 10% [8], even if the majority are early abortions, one must consider that some of these miscarriages would also have occurred without dioxin damage.

The Committee for the Organization of Health Affairs of Brianza Seveso [4] has, in a situation report on abortion frequency in the area around Seveso, taken the position, that proceeding from a general spontaneous abortion rate of 10%, in the pregnancies with terms in July to September, 1976 in some areas around Seveso, a slight but clear rise of the spontaneous abortion rate occurred, in Seveso itself nearly 20%. For the pregnancies with term in October-December, 1976 the increase of the abortion frequency became still more clear and reached almost 23%, which was also true outside of Seveso. The absolute figures are, to be sure -corresponding to the individual areas- relatively small, so that smaller deviations already cause large relation variations. Nevertheless the distribution pattern of the abortion frequency, with a rise above all in Seveso, can be seen as a possible causal connection with dioxin injury, without final conclusions being drawn at this time.

In the larger group of those cases, in which contact with dioxin was to a greater extent, there were no morphological changes in the accesible Interruptio material for examination. Also, in case 19, in which the mother had shown mild symptoms of chloracne, the fetus seemed normal by embryological-morphological criteria. Certainly the evaluation of the negative pathological-anatomical findings can be given only with the reservation that the Interruptio material, for the most part was incomplete and that the methodology of the paraffin histology imposed certain restrictions of interpretation.

A toxic substance can, depending on the time of its becoming effective during the pregnancy, exhibit different injurious patterns. It can, as a mutagen, in the embryo cells, induce a gene change or cause structural chromosome aberrations or division defects, from which chromosome anomalies of the embryo arise. An embryo cell injury of this or that type by dioxin can however, be discussed only in the cases in which conception occurred after the explosion. This excludes a case such as the one in December, 1976 where a child with Ichthyosis congenita (on which, as on many others under similar circumstances, many cases have been reported in the press)

was said to have a series of possible gene mutations caused by dioxin. In both abortion cases 31 and 34, in which a chromosomal aberration is regarded as possible to probable, conception did not occur until September. One can, however, on the other hand, not fail to take into consideration that chromosomal aberrations in spontaneous abortions are also normally frequent. Among early abortions- case 34 would fall in this category- they can make up as much as 60% [21]. In animal experiments TCDD has produced, in addition to a positive mutation test on bacteria [32] only in one plant [16] and mammal species [24] chrosome aberrations. A dominant lethal test on rats was negative [18]. TCDD is therefore seen only as a potential mutagen [30] with high species specificity, whose mutagenic effect in humans seems questionable.

An injury by a toxic substance can be called <u>teratogenic</u> which affects embryo development in the early stages and can lead to the lack of certain developments. Since in humans the formation of most organs is completed by the 6th week of gestation, a teratogenic effect of dioxin would be possible only in cases in which at the time of the accident the pregnancy was not older than 5-6 weeks. A child that was born in January, 1977 with

a rectum atresia in the region around Seveso can therefore only with difficulty be seen as as a valid victim of dioxin contamination, since the teratogenic determination period for the rectum atresia is before the 7th week of gestation.

To be sure, for TCDD in animal experiments and also with regard to its teratogenic effect, a high species specificity seems to exist. Cleft palate as well as hydronephrosis could only be produced in the mouse [5, 25] but not in rats [5,33,34], rabbits [37], and sheep [1], while only in the hamster were eye deformities brought by TCDD [3,26]. In the cases we observed there were no deformities of a comparable type, a statement which, to be sure, is again an indication of the incompleteness of most of the embryos examined. The slight broadening of the kidney pelvis in case 18, which is still seen as physiological up to the 10-11 week of gestation, was identified as a slight developmental retardation and not as a malformation.

Only the anomaly of the aorta valve with the valve defect and texture disturbance of the aorta wall in case 31 is understood as a deformity in the narrow

sense. The aorta wall lesion could occur as a result of a disturbed division of the arterial heart chamber or secondarily as brought on by the mechanical overload because of the valve anomaly. Within the range of chromosomal syndrome, aorta valve changes occur especially frequently. The additional degenerative signs present are regarded as proof for chromosomal symptom complex and the heart anomaly therefore not seen as caused by a teratogen, or at least not definitely caused by a teratogen.

A <u>fetoxic</u> injury is finally to be assumed if a substance influences a fetal organism after the 6th gestation week, therefore during tissue differentiation and the growth of the organs. To be expected are growth retardation, that is organ hyplasis or degenerative injuries to inner organs with cell necroses. These changes are especially difficult to identify in the fetus since they happen without reaction. Only in widespread necroses are clear defect formations, especially as observed in the brain, the result. Although in the material examined from Seveso, no morphologically tangible proof for such injuries existed, the difficulty lies in recognizing the real and essential limitation in the early or middle fetal stages, which works against a definitive interpre-

tation or even a complete exclusion of fetoxic injury.

In the animal experiments TCDD in mice produced a toxic liver cell fatty degeneration as well as liver cell necroses, bile duct proliferation and a raised iron content, without histologically tangible siderosis [22,39]. These changes stand in connection with a hepatic porphyry, which is seen by POLAND and GLOVER [29] as the result of a strengthened induction of the a-Aminolavalin acid synthetase by TCDD. In addition, there was an atrophy of the thymus and of the lymphatic system within the picture of a "lymphocytic depletion" [11,38]. With high TCDD doses in rat fetuses intestinal bleeding was caused [34]. In the material present, degenerative changes in the liver and thymus, also compared to fetuses of the same age not exposed to TCDD, could not be proven.

It is difficult, the present findings in hand, to make a statement on the embryo or fetotoxic effect of dioxin in humans. To be sure it can be clearly stated that an actual proof of such an effect was not accomplished; however a mutagenic, teratogenic or fetotoxic effect of TCDD can not, on the grounds of the reservations named- incompleteness of the material and limited examination techniques- be completely eliminated. This is

valid especially for the increased spontaneous abortion rate which is reported from the region around Seveso (see above) for whose cause however, on the few examined fetuses of spontaneous abortion in this report, no uniform morphological substrata could be found.

Nevertheless it can be shown, with the present investigation, which aspects are to be considered in the evaluation of malformations in the cases observed in the region of Seveso, before a connection with a dioxin injury can be substantiated. In addition it must be considered that statements without knowledge of the deformation-statistical and epidemiological situation in this region before the 10th of July, 1976 can cause a false judgment on those affected as can publicity.

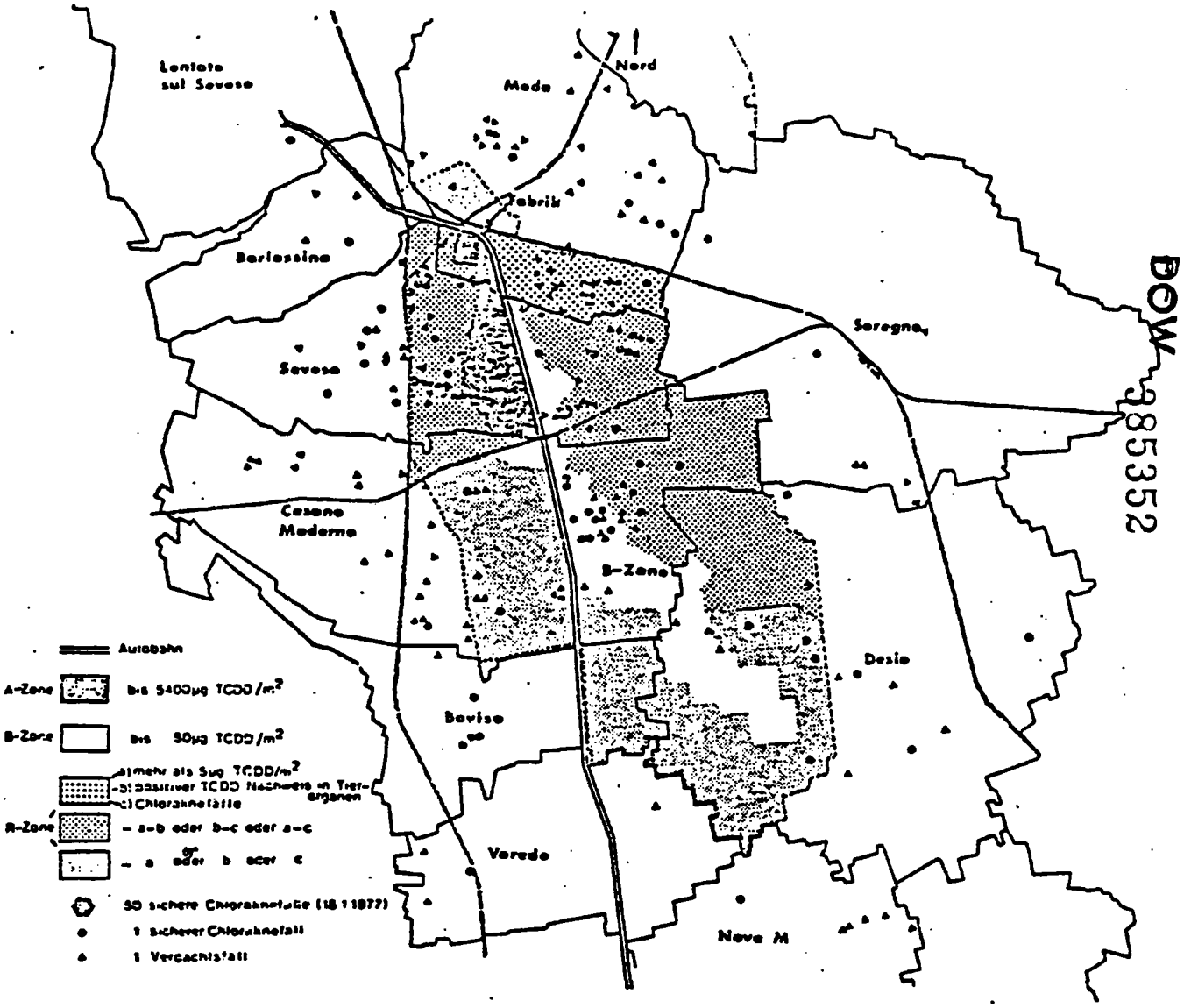

Abb. 1. Von der TCDD-Verseuchung betroffene Zonen des Gebietes Seveso [4].

diagram 1. TCDD contaminated zones in the Seveso region [4].

A zone-up to 5400 μg TCDD/m^2

B zone-up to 50 μg TCDD/m^2

R zone a)more than 5 μg TCDD/m^2
b)positive TCDD proof in animal organs
c)chloracne cases

○ 50 certain chloracne cases (1-18-77)

• 1 certain chloracne case

▲ 1 suspected case

Table 1. Previous history, circumstances and findings in individual cases (1976)

group	case no.	zone[1]	Indication of contact	age of mother	last menst. period	SSW in 10-[illegible]	Interr. or abort.	SSW at interrup.	clin. symptoms	material	dev. age wk.	finding	sex
1	1	ext.	7.19-20 work in factory then vacation	30	5-30	5^{1-4}	6-13	6^3	-	few organ fragments (foot, liver, placenta)	8	normal	-
1	2	A		22	6-12	1^3	8-13	6^4	-	some bone and organ fragments	6-7	normal	-
1	3	B		39	4-10	2^3	8-13	7^1	-	incomp. segments of embryo	7-8	normal villi edema	undiff.
1	4	B		31	4-11	2^0	8-19	7^6	-	few organ fragments	6	normal	-
1	5	ext.	travel through. food	34	6-23	2^3	8-19	6^1	-	incomp. segments of embryo	5-6	normal autolysis artifacts	-
2	6	B	food	37	6-21	0^5	8-25	7^3	-	incomp. segments of embryo	5-6	normal Duodenum oblit.	undiff.
1	7	B	urography	26	6-5	3^0	8-25	9^6	-	few organ and bone fragments	10 11	normal	-
2	8	B	psychiatr.	35	6-6	2^6	8-25	9^3	-	some organ and bone fragments	10	normal, prolapse of neuroretina as artifact	
1	9	B	travel through. food	26	6-23	0^3	8-25	8^6	headache	some organ and bone fragments	7-8	normal	
1	10	B	lived in B zone during interr. (5 children)	32	6-26	0^2	8-25	6^5	-	numerous organ and bone fragments	7	normal, slight edema of placenta	
1	11	ext.	transit in car	31	6-21	0^5	8-26	7^3	-	numerous organ and bone fragments	7	normal	
1	12	B		37	6-9	2^3	8-26	9^1	-	numerous organ and bone fragments	9-10	normal, edema of soft parts	-
3	13	A		36	?	8	?.	15(Ab.)	-	fr. fetum, 152g. 17.5cm	Hensis IV	normal, skull hematoma	
1	14	B		28	?	1^4	?	9^3	-	single bone and organ fragments	9	normal	-

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2	15	ext.	food	32	6-19	1^7	9-6	9^2	-	numerous organ and bone fragments	10	normal	♂
												(coccygeal medullary vestige.septum defect of heart	
1	16	R		31	6-15	1^4	9-6	9^6	γGT raised	insemp. segments of embryo	9-10	normal	♂
2	17	R		25	6-14	1^5	9-6	10^0	-	insemp. fragments of embryo	10	normal(scleral rupture.retina-prolapse.artific.	-
2	18	ext.	psychiatr.	36	?	5^3	?	12	-	female fetus 88g 18.5cm	Hemsin III-IV	normal (widened kidney pelvis.slight devl. retard.)	?
1	19	R	chloroma death of pat	33	6-1	5^4	9-10	12^3	chloroma	male fetus. 38g.16.5cm skin lesions.brain defect	10-11	amniotocerty	♂
1	20	ext.	psychiatr.	40	?	6^3	9-13	9^4	-	insemp. segments of embryo	10-11	normal	?
1	21	ext.	food	27	6-27	concept. 7-11	9-14	9^2	-	numerous organ and bone fragments	10-11	normal	?
1	22	A	work. food	26	?	concept. 8-1	?	7^3	-	indiv. bone and soft part fragments	?	normal	-
1	23	R-A	food	34	6-24	Concept. 7-9	9-22	10^6	-	insemp. segments of embryo	10-11	normal	?
1	24	R		32	7-16	concept. 7-23	9-29	9^4	-	numerous organ and bone fragments	9-10	normal	♂
1	25	ext.	repeated transit	32	7-16	concept. 8-1	9-29	8^3	-	indiv. segments of embryo	8-9	normal	♂
1	26	R		25	?	concept. 7-26	?	9^2	-	numerous organ and bone fragments	9-10	normal	♂
2	27	R-A	food(animals)	30	6-16	1^2	10-6	14^3	-	male decapit. fetus and head 90g. 13cm	15-16	liver rotation intrasep. gall bladder. hemorrag. infarct. of intestines.stenosis normal	♂
1	28	R-A	8 hrs after 7-10 vacation food	19	6-27	concept. 7-11	10-8	12^3	uterus too small	female.inj. decapit.fetus 36g 13cm	14-16	normal	?
3	29	R		-	5-11	6^0	11-7 abort.	23^5	uterus too small	male fetus 24g 11cm	Hemsin IV-V	normal.infarct. placenta.autolysis by wrong implantation	♂
3	30	R		26	6-28	10^3	11-14	22^1	γGT raised	insemp. autolytic fe. male fetus non maps of each-knee length	9-10	autolysis,retention regressive placenta	?
3	31	R or ext.		-	6-27	concept. 9-10/11	11-27 abort.	11^5	fever. hyperemesis	fe. fetus 34g 11cm	12-13	autolysis. clear mongoloid face, insemp.lung lapp. only 2 aorta valves texture disturb. of aorta wall	?
1	32	ext. food. transit	34	9-17	concept. 10-1	12-10	10^0	headache	insemp. segments of embryo	18	normal. indiv. hydrop.placenta villi	♂	
1	33	ext. food. transit	16	10-2	concept. 10-16	12-10	7^6	headache	indiv. organ and bone fragments	?	normal	♂	
3	34	visit in R zone		?	9-3	concept. 9-17	12-17	14^0 (Ab)	-	empty amniotic sac placenta	?	windmole° degenerative placenta tissue	-

*R zone-biological contamination, ext. =dwelling outside A or B zone

** Six-week of pregnancy(raised numbers=days)

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M. L. Ling
5/11/77

MAY 11 REC'D

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

4 MAY 1977

SUBJECT: Dioxin: Position Document

see p. 10 for Toxicological effect level = 80 - 200 ppt in beef fat

TO: Dioxin Implementation Task Force

FROM: Edwin L. Johnson
Deputy Assistant Administrator
for Pesticide Programs

When we met last fall I agreed that the Agency would put together a summary of what it had learned since withdrawal of the hearings in 1974. You have been cooperating with EPA in the conduct of a monitoring program to determine the extent and frequency of dioxin (TCDD) residues in the environment as a result of the use of 2,4,5,-T and related pesticides. The attached document is a draft of our evaluation of phase I of the Dioxin Implementation Plan and a summary of our plan for proceeding with phase II of the program. As I indicated to you last fall, this document is being provided to you in advance of a formal release for comment and suggestions. It has also been made available to the Administrators Pesticide Policy Advisory Committee for the same purpose.

We intend to make the document final and release it generally in the latter part of May and we therefore would appreciate you submitting any comments or suggestions you have for modifying this document by May 19, 1977. All correspondence should be forwarded to Mr. W. Thomas Hollaway WH-566, Office of Special Pesticide Reviews, Environmental Protection Agency 401 M Steet S.W. Washington, D.C. 20460 (Telephone No. 202/755-9336). Your cooperation in this program in the past and your suggestions for this document as well as the future directions of the program will be greatly appreciated.

Ed Johnson

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ENVIRONMENTALISTS ATTACK PROPOSAL TO REDUCE THE SCOPE OF RCRA

Environmental groups have told Environmental Protection Agency Administrator Douglas M. Costle that easing up on hazardous waste regulations to exempt generators producing less than 1,000 kg/month of hazardous wastes from the rules would "pose a severe threat to health and the environment" (See Dec. 6, Page 20).

In a Dec. 5 letter to Costle, the Environmental Defense Fund, Environmental Action, Environmental Action Foundation and Citizens for a Better Environment said that attempts to modify regulations under the Resource Conservation and Recovery Act (RCRA) in this manner "leave the public unprotected from clearly hazardous waste and run contrary to the intent of Congress as expressed in RCRA."

Moreover, they argued that by relaxing the rules to exempt generators producing as much as 1,000 kg/month of waste would mean that 4.6 billion pounds of hazardous waste would be disposed of without the benefit of environmental safeguards.

The environmentalists also objected to a proposal to exempt six industries, electroplating and metal finishing, mercury production, wool scouring, wool fabric dyeing and finishing, knit fabric dyeing and finishing and hydrofluric acid production from the RCRA regulations because, they said, some wastes from these industries are linked with cancer. For example, waste from the electroplating industry contains heavy metals which cause renal and neural damage as well as cancer, their letter said. And the fabric dyeing industry often uses carcinogenic aromatic dyes, they added.

Also emphasized by the environmentalists was the costs of cleaning up improperly disposed hazardous waste which, they said, far exceeds the cost of proper disposal. Finally, they argued that RCRA doesn't authorize the EPA to exempt industries from regulation "solely on the basis of economic concerns."

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HUMAN BIRTH DEFECTS NOT CAUSED BY 2,4,5-T OR 2,4-D EXPOSURE, REPORT SAYS

A Consultative Council appointed by the Minister of Health, Victoria, Australia, has concluded that 2,4,5-T or 2,4-D exposure did not cause a cluster of birth defects among babies in the Yarram district in 1975-76 (See Dec. 6, Page 5 and 9). Specifically, the Council concluded in its report:

"Analysis of all information available showed no evidence that these birth defects were caused by exposure to 2,4-D or 2,4,5-T.

"The normal agricultural use of 2,4-D and 2,4,5-T has not been shown to cause birth abnormalities in domestic animals nor is there evidence to connect such use with human birth abnormalities.

"Victoria lacks organized research on the epidemiology of birth defects and lacks an adequate system of surveillance of those birth defects which are not rapidly lethal."

Environmental Protection Agency officials could not be reached for comment on the conclusions and observations in the "Report of the Consultative Council on Congenital Abnormalities in the Yarram District."

The report said "Regular sampling shows that 2,4-D and 2,4,5-T do not occur in food or water supplies in Victoria." It continued:

> "Both 2,4-D and 2,4,5-T have now been used in Victoria for almost 30 years with no known detrimental effects on the health of the general public. They have a similar safety record overseas when used as aids to agricultural production. Any adverse effects which have been reported are concerned with personnel involved in the production or agricultural use of these compounds."

In the report, the Council noted that it had compared birth defect numbers in two statistical divisions with similar numbers of births and where 2,4,5-T and 2,4-D use was considerably higher in one division than in the other and found that there were no differences in the rates or types of defects. "Hence there does not appear to be a relationship between herbicide usage and perinatal death rates or type of birth defects," according to the report.

T[illegible] noted that the average level of TCDD in 2,4,5-T offered for sale in Victoria over [illegible]e past two years was 0.06 p.p.m. and that the legal maximum was 0.1 p.p.m. The report said, "It is noted that one sample (of 2,4,5-T acid) reported on in Victoria in 1977 contained 0.2 p.p.m. of TCDD in the formulation. No other sample has exceeded this figure." Observations on human effects of the pesticides discussed in the report included:

> "Although isolated reports of ill effects have been noted in connection with the agricultural use of 2,4-D and 2,4,5-T, broad experience over the thirty years in which the compounds have been used suggests that the dangers are minimal. It is noteworthy that substantiated ill effects in the general population potentially exposed to residues of the compounds have not been observed. This is not unexpected in view of the lack of 2,4,-D or 2,4,5-T residues in foodstuffs after normal agricultural usage. . .
>
> "Definitive ill-effects in connection with 2,4-D and 2,4,5-T are almost wholly related to people involved in the manufacture of the compounds and to those affected by industrial accidents, e.g. explosions in manufacturing plants. It appears that those connected with the manufacture of the compounds are at greater risk than the sprayers involved in the agricultural use of the herbicides."

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[illegible]CP SUSPENSION REQUESTED BY HEALTH RESEARCH GROUP; NO RESPONSE FROM EPA

Suspension of all uses of dibromochloropropane (DBCP) was requested last week by the Health Research Group (HRG) which anticipates having intervenor status in the cancellation hearing (See Dec. 6, Page 35). The Dec. 8 suspension request had not been answered by the Environmental Protection Agency at press time.

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Of Mice and Men—The Troubles of 2, 4, 5-T

OF ALL FORESTLAND controversies, that over the use and abuse of herbicides wins a questionable prize for being the most enduring. Now that public fears over clearcutting have abated, anxieties seem to have transferred nicely to the practice of aerially spraying herbicides in forest management. For this development, one must thank newspapers and television for their timely accounts of tumor-ridden mice and malformed fetuses, all brought on by exposure to phenoxy herbicides of one kind or another. Those who keep themselves current on the varied horrors we're capable of inflicting on ourselves will have surely heard of sleepy Oregon valleys sprayed with chemicals likened to Agent Orange, the defoliating compound once used by the Pentagon in Vietnam. There is indeed something insidious about chemicals that cause plants, animals, and even humans to drop dead. It wasn't for nothing that pone joined thalidomide as a household word in our disaster lexicon.

Aside from the irresistible appeal such stories have for reporters and broadcasters, there are good reasons we are hearing more lately about forest herbicides. Apparently the same people who give us poisonous compounds are refining their techniques for measuring the levels of poisons in them. Ten years ago the state of the art was fairly primitive. Biochemists could detect, say, only one part of poison in a million parts of herbicide. But today, Harvard University scientists claim they can detect one part in a trillion parts of herbicide, thus turning up traces where there once were thought to be none. It's called progress: science obliges us with lethal poisons, and finally with sophisticated means of measuring their toxicity, too.

Against this background of public suspicion and scientific progress comes 2,4,5-T. It is to forest pesticides what Ali is to boxing—the meanest and the greatest. For the past thirty years or so T, as the chemical is affectionately known to applicators, has proven useful for killing off brushy, broadleaf plants which often invade a forest site and then compete successfully with commercially valuable conifers for sunlight and nutrients. T works, all right, but not always where intended, and it can be harmful when it drifts onto non-target crops. One spokesman for Dow Chemical Company, which manufactures it, said, "You can stand in a vineyard and think of 2,4,5-T and the leaves will wither away." He isn't referring to the power of suggestion, either.

The use of T also happens to be relatively cheap. The reported costs of aerial application sound suspiciously low —under $50 an acre according to many users—but aerial spraying is still considerably cheaper and perhaps more effective than manual attempts to control vegetation. And even if the costs are somewhat higher than stated, the payoffs from a judicious use are undeniable. According to accepted estimates, chemical control of vegetation can on average produce a 40 percent increase in timber volume at the time of harvest. Today, when more than half the commercial forest is growing wood at less than optimum rates, many argue that herbicide use becomes essential to meeting our long-term timber needs. "A major cause of this loss [of growing capacity] is competing and undesirable vegetation," said a recent statement by the Society of American Foresters.

WONDERFUL, SAY CRITICS, but encouraging tree growth by spraying phenoxy herbicides is like curing a headache with a frontal lobotomy. In fact, not since paraquat was used to ruin marijuana fields in Mexico has chemical spraying been so roundly attacked.

Actually, it's not so much the herbicide itself that is causing the outcry, but the toxic impurity, called dioxin (TCDD), that is contained in T. Dioxin sneaks into the compound accidentally in minute amounts, during the manufacturing process. It is not a necessary ingredient, and contributes nothing to the effectiveness of the herbicide. But it's extremely poisonous to humans; some say the most poisonous synthetic compound made. From Harvard to the University of California at Berkeley, tests are confirming the draconian characteristics of dioxin, and the associated consequences of giving T to test animals in relatively massive doses. Curiously, the astonishing number of tests run on the herbicide has led to no consensus on its long-run effects on humans, animals, or the environment. The findings are sometimes contradictory—Texas Tech University scientists say dioxin isn't present in soils sprayed with T; others disagree. A U.S. Air Force study says dioxin doesn't accumulate in water or animals, Berkeley says it does, and so on. If anything, tests seem to add to the confusion; what's safe to one scientist is sinister to another.

Press and television accounts generally haven't served us very well. Two major networks recently dug up old Vietnam war footage to tighten the link between T and Agent Orange, the military defoliant. (Orange was comprised of about equal portions of T and 2,4-D, and applied in heavy doses.) Perhaps more than anything else,

cited the public outcry against herbicides. In the past two years, Senators Dale Bumpers (D-Ark.) and Gaylord Nelson (D-Wisc.) have proposed legislation to ban the use of phenoxy herbicides on public lands. The Environmental Protection Agency (EPA)—which wrested herbicide jurisdiction from the Agriculture Department and now regulates such compounds under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA)—has in the past moved to ban the use of T, changed its mind, but is now taking a second look at registration. With its testing procedures under fire, EPA initiated on April 21 its Rebuttable Presumption Against Registration (RPAR) process for the phenoxy herbicides T, 2,4-D, and silvex, a mixture of T and D. By invoking this process, EPA permitted continued use but only until all interested parties submitted evidence to rebut the agency's presumption against registering the herbicide. In plainer English, RPAR means that the continued use of T is in doubt.

Meanwhile, the Agriculture Department sought to head off a major confrontation by imposing strict guidelines on the streamside use of T. It later liberalized them under pressure, but required each proposed spraying on national forests to be cleared by Assistant Secretary M. Rupert Cutler.

WHILE POLICYMAKERS debate the merits of more or less herbicide use, and their tireless counterparts in the laboratories decide just how toxic herbicides are, it's a sure bet that the media will exploit the controversy for all it's worth, mixing creative new concoctions of fact and fantasy. Amid all this conjecture and hairpulling, important but seldom reported facts tend to belie the impression created by the doomsayers.

For example, in the casually reported link between T and Agent Orange the differences are greater than the similarities, yet never get equal time. Dioxin was of course present in Agent Orange, but only in the sense that heat is present in both blast furnaces and body temperatures. The amount of dioxin in production-grade T is legally limited to 0.1 part per million of herbicide, which runs to about 1 ounce of dioxin to 312 tons of T. And the Council for Agricultural Science and Technology (CAST) says that the dioxin amount in the T currently being manufactured is considerably less than that. Moreover, the volume of T used in Agent Orange applications was about 30 times greater per year per acre than that used today in forest management. All in all, taking the dioxin levels and volume applied per acre, the dioxin level in Agent Orange was anywhere from 3,000 to 30,000 times greater than in domestic use. Even given the tremendous amounts of dioxin sprayed in Vietnam, where the eradication of vegetative cover and the destruction of the enrivonment were deliberate military management objectives, the environmental impact is still not clear. Phillip Handler, president of the National Academy of Sciences, summed up the findings of a NAS study on herbicidal warfare in Vietnam in March 1974: "The volume of merchantable timber killed by herbicide spraying proved to be strikingly smaller than previously reported in preliminary estimates by others . . . . 2,4,5,-T and 2,4-D are rapidly excreted in unchanged form by most animals, and there is no evidence for accumulation in any tissues or in the food chain." As for reports of malformed fetuses, the NAS report found "no conclusive evidence of association between exposure to herbicides and birth defects in humans."

As more refined laboratory measurements turn up traces of dioxin in humans and in the soil, it's useful to recall the remark social commentator Irving Kristol made about class conflict in modern-day society. "If you have to measure class conflict to find it," Kristol admonished his fellow sociologists, "then there isn't any class conflict." Similarly, the reason dioxin is so difficult to measure in the soil is simply that there isn't much there. Studies suggest dioxin degrades fairly quickly anyway, by as much as 50 percent in a year. Nor does it seem to appear in plant seeds, or readily dissolve in water. It disappears rapidly in vegetation too—some tests show that 80 to 95 percent vanishes within a month.

SO ACUTE is the amount of dioxin in T that some experts have concluded that only people with suicidal tendencies and enormous appetites would ever become ill from T. A one-hundred-pound human, say these experts, would have to quickly consume all the dioxin on one acre of sprayed forest to suffer any ill effects. While this writer is not anxious to lose 65 pounds and test that claim, the example does dramatize the unlikelihood of harmful side effects from proper applications of the herbicide.

Caution should be exercised, too, when taking lab test results at face value. It is obviously unconscionable to dismiss or ignore such results, but it is also hazardous to infer from them that humans would suffer the same effects as animals. Despite the painful similarities, men really are different from mice and rats. For example, humans don't develop the propensity to grow tumors that laboratory animals often do when they're subjected to relatively high, regular doses of herbicides. In one test, 75 percent of the male mice injected with T developed tumors, but so did 78 percent of the mice who were given nothing at all. In fact, the female mice which developed tumors outlived those that did not. These results are cited by Richard Ellerby, president of the Oregon Society of Clinical Oncology, to allay the Orwellian speculation often fostered by lab tests. Besides, lab tests also show that tumors spring from steady diets of egg yolk, bracken fern, and even water. So the old Aristotelian maxim "moderation in all things" applies here, too.

At any rate, organisms in the field never receive anywhere near the dosage of either T or dioxin that lab animals get. In terms of practical application, therefore, many experts do not consider T carcinogenic at all. That conclusion isn't surprising, given the legal limitations on the amount of dioxin allowed in production-grade T, the lower rates usually achieved in practice, and the fact that most stands are sprayed intermittently, say about once every 30 years.

Whether these facts, or any others, influence EPA's judgment on registering T next spring is anyone's guess. The arguments both

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...ind con will be delivered with ...gehammer subtlety, one sus-...cts. But the conclusions of a re-...nt study, "The Phenoxy Her-...cides," by the Council for Ag-...ultural Science and Technology ...' check some of the unre-...a... invective. CAST has its detractors who point to the heavy purse of industrial money behind its research program. But CAST's independent member-scientists choose their own research projects and count among their number some well-respected authorities in the herbicide field. The abridged portion reprinted below omits ex-tations, which I deleted) largely concerns the findings on the toxicity of T, probably the aspect most crucial to the continued use of the herbicide.—*Luke Popovich*

(The Phenoxy Herbicides, Second Edition, August 1978, pages 20-22. Reprinted by permission of the Council for Agricultural Science and Technology, Ames, Iowa.)

A dioxin contaminant referred to as TCDD is ...rmed in the manufacture of the trichlorophenol used ... make 2,4,5-T and silvex. The presence and signifi-...nce of this impurity first became known about 20 ...rs ago. 2,4,5-T formerly contained TCDD at con-...ntrations of 1 to 70 ppm, the higher levels being ...fficient to cause skin eruptions called chloracne in ...dustrial workers. Although it has not been feasible to ...liminate this contaminant entirely, present produc-...on methods are able to reduce the dioxin level ...utinely to less than 0.01 ppm in commercial 2,4,5-T ...th occasional batches containing as much as 0.05 ...pm. The average level of dioxin in present U.S. ...oduction of 2,4,5-T appears to be about 0.01 ppm. ... have reduced to a thousandth of its original content ... impurity in a chemical already widely used by the ...ublic and recognized as safe would appear to be a ...fficient solution of this problem. However, it has ...ecome apparent that TCDD is one of the most toxic ...hemicals known. This poses the complexity of assess-...g the consequences of the presence of a very small ...mo... of a very toxic substance in mixtures sprayed ... to the environment.

TCDD is toxic to laboratory animals at all stages of ...fe, and it also is a weak teratogen in mice. The acute ...ral LD_{50} ranges from 0.0006 mg/kg in male guinea ...igs to 115 micrograms per kilogram in rabbits. Dogs ...e less sensitive to TCDD than rabbits.

Among the effects are skin damage, liver damage, ...emorrhage and reduced ability to cope with disease ...rganisms. Death from a lethal dose is often delayed ...everal weeks. Following ingestion by the rat, TCDD ... absorbed from the gut, localized in the liver and fat, ...nd eliminated largely via the feces but to some extent ...rough the urine. About half of the ingested material ... eliminated from the body in the first 17 days.

Birth defects develop when the chemical is adminis-...ered during the time of pregnancy when fetal organs ...e forming. The effect is directly upon the developing ...etus rather than on the genetics of the mother. TCDD ... more of a toxicant than a teratogen. It usually causes ...eath of the fetus rather than abnormalities. Because ... the narrow range of dosage between the no-effect ...evel on the fetus and the lethal effect on the mother, ...CDD is classed as a weak teratogen.

The evidence shows that TCDD is from 5,000 to ...0,000 times more toxic to mammals than 2,4,5-T, ...epending on species. Under current standards set by ...he ...ironmental Protection Agency, the content of ...CDD in commercial 2,4,5-T must be less than 1 part ... 10 million parts of 2,4,5-T. In practice the content is ...bout 1 part in 100 million. On this basis, a single toxic ...ose of TCDD would be contained in from 200 to ...0,000 toxic doses of 2,4,5-T, depending upon the test species. Thus, the current level of TCDD in 2,4,5-T does not contribute significantly to the toxicity of herbicidal preparations of 2,4,5-T.

For a person or animal to be poisoned with TCDD without first being poisoned with 2,4,5-T would require the existence of a mechanism in nature that would separate TCDD from 2,4,5-T, greatly concentrate it and make it accessible for consumption in food or feed. Any postulated mechanism must take into account what is known about the environmental fate of TCDD.

The amount of TCDD distributed in the United States in 2,4,5-T and silvex is probably no more than 1 ounce annually. This material is distributed over approximately 5 million acres at the rate of 5 micrograms per acre. The most sensitive species known is the guinea pig, which has an LD_{50} of 0.6 microgram per kilogram. If we assume that we have a grazing animal about the size of a sheep or deer (175 lb or 80 kg) and the sensitivity of a guinea pig, this animal would have to consume, without excretion, all of the treated vegetation on more than 9 acres of land to get a lethal dose. If the animal had the sensitivity of a rat, it would have to consume, without excretion, all of the treated vegetation on more than 400 acres.

TCDD falling on foliage is not absorbed appreciably by plants but is rapidly decomposed by sunlight. When TCDD on leaves is exposed to sunlight, most of the chemical decomposes the first day. Washing into the soil by rainfall plus additional slight losses by volatility serve further to dilute and disperse the remaining residue. There is evidence also that TCDD is not formed when 2,4,5-T is subject to ultraviolet radiation and that it is not formed in significant quantities from 2,4,5-T when treated vegetation is burned.

Once in the soil, TCDD residues become firmly bound to soil particles and are not appreciably taken up by plants. The residues do not leach downward but remain localized in the surface soil. Microbial degradation then comes into play and decomposes most of the remaining chemical to basic materials over a period of probably a year or two.

TCDD sprayed into waters rapidly disappears, due to vapor distillation into the atmosphere and to decomposition by sunlight provided the waters contain small amounts of organic compounds. Its low solubility in water (0.0002 ppm) causes it to migrate rapidly out of solution and to adhere to any available surface on which it may be degraded in place. When sufficient supplies of the chemical are present for a long enough time, TCDD can accumulate in algae, snails and fish at concentrations exceeding those in the ambient water. This property is not believed to be of practical concern, however, since herbicide spraying does not lead to a substantial amount of TCDD in the environment subject to accumulation. Analyses reveal that accumulation in food-chain organisms is not a prob-

has been widely investigated using methods sensitive to as little as 10 parts per trillion (ppt). Amounts of TCDD sufficient to cause direct toxicity or birth defects, however, have never been found in food or water as a consequence of proper herbicide spraying.

In an Environmental Protection Agency-directed test of 85 samples of fat from cattle grazed on pastures sprayed with 2,4,5-T, one contained TCDD at a concentration of 60 ppt, two had approximately 20 ppt, five had 5 to 10 ppt (lower than the test's reliability), and the remainder had no detectable TCDD. The significance of the positive values in this survey is questionable for various reasons including (a) inadequate information on the sources of the samples, (b) the difficulties of analyzing samples for such low concentrations of TCDD in the presence of interfering substances and (c) the failure to identify TCDD in any of the corresponding liver samples (TCDD normally accumulates in the liver concurrently with accumulation in the fat). In any event, the average levels of TCDD found in the fat and, indeed, even the highest level, represent concentrations well below the no-effect level established by EPA. In the only formal experiment designed to test the accumulation of TCDD in animals in the wild, no detectable amounts of TCDD [were found] in tissues of mountain beaver at the end of 45 to 60 days of feeding on vegetation that had been sprayed with 2 lb of 2,4,5-T per acre at the beginning of the feeding period.

Because the general public first became aware of TCDD as a very toxic contaminant in 2,4,5-T, recent poisonings by TCDD from other sources have been improperly linked to herbicides. There have been several accidents involving high-level exposure of persons to TCDD. One was an incident in Missouri involving waste oil used to keep down dust in stables. A recent and more serious incident involving TCDD occurred in Seveso, Italy. Herbicides were not involved in either of these poisonings. The incidents involved far higher levels of exposure of man and animals to TCDD than are encountered in the field use of 2,4,5-T and serve to document the safety rather than the hazard of 2,4,5-T. In the Seveso incident, for example, TCDD was released in a village at rates per acre that were millions of times greater than those that occur from 2,4,5-T treatments. People continued to live in the contaminated area for about 2 weeks after the accident. A number of animals were killed by the fall-out of phenolics and possibly by other chemicals, and there were numerous cases of human chloracne. No cases of severe human illness were attributable to exposure to TCDD, however, nor was there an increased incidence of human birth defects.

Evaluating the hazard of TCDD in the environment poses the problem of comprehending numerical values far removed from common experience. One is likely to be so overwhelmed by the very great toxicity of TCDD that he fails to comprehend the infinitesimal levels of exposure. One must recognize also the long history of safe use of 2,4,5-T containing a thousand or more times as much TCDD as at present. The evidence indicates that the TCDD contaminant in 2,4,5-T and silvex is well below levels hazardous to humans and other organisms. ■

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The Science of the Total Environment, 10 (1978) 105–115

CARCINOGENICITY TESTING OF CHEMICALS WITH PARTICULAR REFERENCE TO ORGANOCHLORINE PESTICIDES

MELVIN D. REUBER*
11014 Swansfield Road, Columbia, MD 21044 (U.S.A.)
(Received October 14th, 1977; in final form February 2nd, 1978)

ABSTRACT

The organochlorine chemicals comprise a large number of pesticides that are used widely throughout the world. The organochlorine pesticides given in the diet to mice are carcinogenic for the liver. They induce not only carcinomas of the liver, particularly at the higher doses, but also carcinomas and sarcomas in other organs in rats. They cause acute and chronic liver and kidney injury, which interferes with the development of carcinomas and sarcomas in rats.

The testing of chemicals for carcinogenicity, with particular reference to organochlorine pesticides, includes discussions on the following topics: classification of hepatic lesions in mice and rats, diagnostic criteria for malignant tumors of the liver in mice and rats, toxicity versus carcinogenicity in the testing of chemicals, a comparison of carcinomas and cirrhosis of the liver in experimental animals and humans, and the significance of laboratory carcinogenicity findings to human health.

INTRODUCTION

The organochlorines comprise a large number of chemicals that have anesthetic and narcotic action, many of which are highly toxic[1–4]. There has been wide distribution and use of the organochlorines as agricultural pesticides throughout the world. They are applied by all the conventional means using the common types of equipment on vegetables, fruit, forage, and fiber crops. Examination of large numbers of samples of specific food product classes collected in retail food stores have shown that they are contaminated by organochlorines.

The organochlorines are absorbed into the system following inhalation, ingestion, or contact with the intact skin. After ingestion into the system they are stored to some extent in the fat and liver and to lesser extent in the kidney, muscle, and other organs.

The focus in the toxicity and carcinogenicity studies has been primarily on the reaction of the liver to the organochlorine pesticides. Certainly in the mouse, where we have the greatest amount of experimental data, this is the focal point of the

* Present address: NCI Frederick Cancer Research Center, Frederick, MD 21701, U.S.A.

distinguish between hyperplasia and carcinomas, rather than to use the term "hepatoma" as employed by some pathologists. The widespread and varying use of this term over many years has left it, in my opinion, unacceptably vague.

It should be noted in reports from feeding studies that metastases from carcinomas of the liver to lung or to other organs are not reliable unless, among other things, serial sections are done. Serial sections were not done in any of the studies on the organochlorine pesticides. In addition, multiple sections of the liver are also necessary to properly evaluate the lesions, particularly local invasion of veins of the liver by carcinomas. Small carcinomas less than 5 mm, for example, might be overlooked on gross examination.

THE IMPORTANCE OF HYPERPLASTIC NODULES OF THE LIVER

Carcinomas develop in the liver of animals only after the cells pass through stages of hyperplasia. The earliest stage, referred to as "areas" of hyperplasia or as "hyperplasia" is seen during the early weeks and months of the administration of certain chemicals. The change at this stage may not progress to that of carcinoma unless the chemical or "stimulus" continues to be administered. If these hyperplastic cells continue to grow, they form hyperplastic nodules. The cells appear much the same, but have increased in number enough to form a nodule. This nodule is a mass which compresses the adjacent tissue and can be seen on careful gross examination of the liver. This hyperplastic nodule is precarcinogenic and has the capability of developing into a carcinoma even if the chemical is no longer given to the animal[25-29].

Cells in hyperplastic nodules grow in sheets. Additionally, the cells are also characterized by the following: the cells are increased in size, there are double nucleated cells, and there are mitotic figures. In nodules which progress to well-differentiated carcinomas, the cytoplasm tends to be eosinophilic; whereas in nodules which progress to poorly-differentiated carcinomas, the cytoplasm tends to be basophilic.

The rate at which the hyperplastic cells become hyperplastic nodules and carcinomas depends upon the potency of the chemical as a carcinogen, the dose of the chemical, the length of administration of the chemical, and the route of administration. Growth also varies with the species of animal, the strain of animal, the age and sex of the animal, and hormones, as well as lack of protein in the diet. This change of hyperplastic cells to nodules and of nodules into carcinomas is also dependent upon the health and nutritional status of the animal. If there are acute toxic effects, the animal will die from necrosis or instant death of cells and organs, or from acute infectious diseases. The animal can also die as a result of malignant tumors in organs other than the liver, or from chronic diseases, other than malignant tumors, in the liver or other organs.

In the analysis of data, the number of carcinomas of the liver is not the only indication that a chemical is carcinogenic. Animals may have hyperplastic nodules in the liver or other changes of importance in other organs. A full evaluation, therefore, should involve an analysis of variants taking into consideration all available data[26]. In the case of the liver, this includes the most advanced lesions of the liver, i.e.,

Hepatocellular carcinomas arise from parenchymal cells and often retain the vesicular nuclei and sometimes the eosinophilic cytoplasm. Poorly-differentiated hepatocellular carcinomas grow in sheets and generally can be fairly easily recognized. The criteria are those for anaplasia: (1) size and shape of nuclei and cells; (2) presence of abnormal mitotic figures; (3) cytoplasm usually basophilic, but can be darkly eosinophilic; and (4) nuclei vesicular with prominent nucleoli.

Well-differentiated hepatocellular carcinomas are more difficult to recognize and often are incorrectly diagnosed. Histologically these carcinomas retain many of the characteristics of normal liver cells. Criteria for well-differentiated hepatocellular carcinomas need not be those of anaplasia, but are the following: (1) formation of cords, two or several layers in thickness, with the formation of canaliculi and often prominent sinusoids with lining cells; (2) cytoplasm usually darkly eosinophilic, but can be basophilic; (3) cytoplasm either decreased or increased in amount; (4) nuclei vesicular with prominent nucleoli; and (5) absence of double nucleated cells or mitotic figures. Formation of cords with canaliculi can often be the most helpful indication of well-differentiated carcinomas. Occasional carcinomas may be made up predominantly of canaliculi and can contain bile casts. Glycogen is rarely present.

Cholangiocarcinomas retain may of the characteristics of bile duct cells, i.e., columnar cells with lightly basophilic cytoplasm and oval nuclei.

Well-differentiated cholangiocarcinomas are fairly easy to recognize. Well-differentiated cholangiocarcinomas: (1) form ducts sometimes containing mucin in the cytoplasm or lumen; (2) cytoplasm is lightly basophilic; (3) "desmoplastic" background of fibrous connective tissue; (4) nuclei are oval with inconspicuous nucleoli; and (5) lack of mitotic figures.

Poorly-differentiated cholangiocarcinomas grow in sheets and have: (1) oval cells and nuclei with some mitoses; (2) lightly basophilic cytoplasm; and (3) fibrous connective tissue stroma[57]. They appear to be attempting to form ducts.

Undifferentiated carcinomas grow in sheets. They are more anaplastic than poorly-differentiated carcinomas and have: (1) great variation in size and shape of nuclei and cells; (2) basophilic cytoplasm; (3) frequent abnormal mitoses; and (4) nucleoli prominent and often more than one.

The most frequent sarcomas of the liver are hemangioendothelial sarcomas. Well-differentiated hemangiosarcomas retain many of the characteristics of endothelial cells[58]. They are made up of (1) spindle-shaped cells with spindle-shaped nuclei; and (2) vascular spaces filled with red blood cells. Lesser differentiated sarcomas are easily diagnosed because of the anaplastic characteristics. They also form some vascular spaces. More anaplastic sarcomas without vascular spaces can be referred to as Kupffer cell sarcomas.

The liver must be examined carefully for hemangioendothelial sarcomas. They can be small and grossly resemble an area of recent hemorrhage. Sometimes the cellular areas are also obscured by the red blood cells on the histologic section.

Malignant lymphomas, particularly the reticulum cell types, are also seen in the liver[54]. Reticulum cells are large with a large nucleus with prominent nucleolus and an

from those observed in rats appeared in print. Before this statement could be corroborated, it was repeated with greater emphasis being placed upon it each time. Several years ago I carried out a transplant experiment in mice, repeating previous work in rats, to see what differences existed between these hepatic lesions in mice and rats[52].

Carcinomas of the liver developing in mice are no different, histologically, biologically, and in other respects, than carcinomas of the liver in rats and hamsters. Varying stages of hyperplasia precede the development of carcinomas of the liver in all three species. Hyperplastic lesions cannot be transplanted; carcinomas can be transplanted. The histological patterns of the carcinomas are the same. They kill the animals with or without metastases.

Carcinomas developing in the liver of animals closely resemble those seen in humans[61]. The gross and histologic patterns are the same. The well-differentiated hepatocellular carcinomas in animals and humans both metastasize to the porta hepatic lymph nodes and to the lungs. The poorly-differentiated hepatocellular carcinomas spread more widely throughout the body. Carcinomas in both humans and animals can cause death by rupturing and bleeding into the peritoneal cavity. Hyperplastic lesions are sometimes seen in humans. If humans, like animals, die from some other cause earlier in the course of the disease, hyperplastic nodules can be observed in the liver. Terminally, though, hyperplastic nodules are rare in either animals or humans.

The role of cirrhosis in the development of carcinoma of the liver in animals depends upon the chemical and the carcinogenic potency of that chemical. Chemicals that cause both carcinomas and cirrhosis, such as aminofluorenes and carbon tetrachloride, need to be given in large doses, sometimes over a long period of time. If the dose is too large, the animals will die from cirrhosis before they get the chance to develop carcinomas. Cirrhosis often accompanies the development of carcinomas in humans. That situation then is similar to that in animals, except that the toxic agent in humans is usually alcohol or viruses.

Chemicals that cause carcinomas only and not cirrhosis generally are extremely potent and can be given in much smaller doses; the length of time depends upon the chemical. Here again, the animals die early from acute diseases, such as hepatic or renal necrosis, when larger doses are administered. The organochlorine pesticides would appear to fall in the latter category with regard to animals. Chemicals such as carbon tetrachloride and mercuric chloride, when ingested in large doses by humans, cause death by either hepatic or renal necrosis. Chemicals in this group that cause carcinomas of the liver only if given in the lower doses for longer periods of time in humans have not yet been identified.

SIGNIFICANCE OF THE LABORATORY CARCINOGENICITY FINDINGS TO HUMAN HEALTH

Carcinogenesis in man can be detected or reliably predicted in either of two ways: (1) by direct observation of two groups of human beings, one exposed to a suspected carcinogen and the other protected from such agent; and (2) by one or more bioassays in experimental animals. The first type of study, labeled an epidemiological study, is

species should be deemed to have relevance for other mammalian species — including man.

Quantitatively, one must assess the relationship between the dosage required to produce tumors in test animals and that required in man. This assessment, difficult as it is, is complicated by the fact that "man" is much more variable in his physical characteristics than are most other species of mammals. One can state, however, that it is quantitatively impossible to establish any "safe level" of distribution of a carcinogen in the environment. A safe level may exist for a particular human being, but because there is such a variation among human beings, it is impossible to establish such a level for a community. Furthermore, review of the literature on carcinogenicity tests suggests that no such "null effect" threshold or "safe level" has beendetermined in test animals — much less for man — for any known carcinogen.

Given the state of carcinogenicity testing and knowledge, there can be only one safe tolerance for a carcinogen — an absolute zero tolerance — and the only way a substance shown to be carcinogenic in test animals will not be a threat to human health is simply to prevent that substance from entering the environment. To the extent that it does enter man's environment, it will constitute a very real threat to his health. Furthermore, acknowledging the difficulties involved in detecting carcinogens in the human population, this threat may not be manifested for up to 30 or 40 years, given the long latent period of many known human carcinogens.

Based upon this statement of principles of carcinogenicity testing and the significance of such tests for man, one must conclude that since the organochlorine pesticides have been shown to be carcinogenic in mammalian test animals, they must be considered as potential carcinogens for man.

CONCLUSIONS ON TOXICITY AND CARCINOGENICITY OF ORGANOCHLORINE PESTICIDES

The organochlorine pesticides given in the diet to mice are carcinogenic for the liver[19]. These hepatic tumors are malignant, invade and metastasize, and grow on transplantation to isologous hosts. Dieldrin given in the diet induces significant numbers of malignant tumors in mice at every dose tested, including the very lowest dose of 0.1 ppm[6].

The organochlorine pesticides induce not only malignant tumors of the liver in rats, particularly at the higher doses, but also carcinomas and sarcomas in other organs at doses as low as 0.5, 1 or 2 ppm in the diet[20-24]. Rats at the lowest feeding levels also develop noticeably early hyperplasia, as well as hyperplastic nodules, and occasional cirrhosis and carcinomas of the liver.

The organochlorine pesticides, when given at high doses, cause considerable acute and chronic liver and kidney injury in rats[64-66]. Necrosis of the liver and kidneys or chronic nephritis not only resulted in death of rats, but also interfered with the development of carcinomas and sarcomas. Hyperplastic nodules in the liver would most likely have progressed to carcinomas of the liver, had the rats been healthier or survived for a longer period of time.

The carcinomas of the liver and other organs and sarcomas observed in rats and

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STUDIES ON 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN-INDUCED IMMUNE SUPPRESSION AND DECREASED RESISTANCE TO INFECTION: ENDOTOXIN HYPERSENSITIVITY, SERUM ZINC CONCENTRATIONS AND EFFECT OF THYMOSIN TREATMENT

J.G. VOS, J.G. KREEFTENBERG, H.W.B. ENGEL, A. MINDERHOUD and L.M. VAN NOORLE JANSEN

National Institute of Public Health, P.O. Box 1, Bilthoven (The Netherlands)

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SUMMARY

Sublethal doses of the highly toxic chemical 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) cause thymus atrophy in several species and induce suppression of cell-mediated immunity as measured by different parameters. The selective effect on thymus is not likely caused by a cytotoxic effect on lymphocytes or by an effect on pituitary or adrenals. In the present study, mice received daily injections with thymosin in order to study whether reduced production of thymic hormones could be involved in the atrophy. Also, serum zinc concentrations were measured. As thymosin injections did not increase thymus weight and mitogenic responsiveness of thymocytes, and zinc levels were not depressed, the mode of action of TCDD-induced thymus atrophy remains unknown. It has been reported that TCDD markedly decreased resistance of mice to infection with *Salmonella bern*. In this study, data are presented that indicate that the increased susceptibility is likely due to the endotoxin content of the bacteria: pre-treatment of mice with single or repeated doses of TCDD markedly enhanced their susceptibility to endotoxin even at dose levels that did not produce thymus atrophy. Finally, possible effects of TCDD on macrophage functions were studied. As treatment with TCDD did neither impair non-specific killing and phagocytosis of *Listeria monocytogenes*, nor macrophage reduction of nitro-blue tetrazolium, it seems likely that the immunosuppression is only due to a, to date unknown, effect on T-lymphocytes, and not due to a combined effect on both T-cells and macrophages, and that the endotoxin hypersensitivity is not the result of alteration in phagocytic function of macrophages.

INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a chemical that can be

formed in small, but significant, amounts in the production of some chlorinated phenols or chemicals synthetized from chlorophenols, such as the herbicide 2,4,5,-trichlorophenoxyacetic acid. It is one of the most toxic chemicals known [1], it is a teratogen [2,35], it is associated with chick edema disease in chickens [4] and with several industrial incidents resulting in cases of chloracne in man [5,6]. TCDD exposure has been shown to induce thymus atrophy in rats, mice, guinea pigs [7—12] and monkeys [McConnell and Moore, in press]. Thymus atrophy appeared to be one of the most consistently sensitive parameters of TCDD exposure. Since lymphocytes derived from the thymus (T-cells) play a central role in cell-mediated immunity, several funciton studies of the thymus-dependent immunity have been carried out in mice, rats and guinea pigs. TCDD exposure did result in functional impairment of diffferent parameters of cellular immunity (delayed type hypersensitivity, allograft rejection, graft-versus-host activity and responsiveness of lymphocytes to the mitogens phytohemagglutinin (PHA) and concanavalin A (Con A) [11,13, Moore and Faith, in press] but the suppression of the cell-mediated immunity seems to be an age-related phenomenon, at least in the mouse and the rat where TCDD exposure during the ontogenesis of the immune system seems to be pre-requisite in order to obtain immune suppression. TCDD mimics in this respect the effect of neonatal thymectomy.

Since the TCDD-induced atrophy of the thymus can be explained in many ways and no clear-cut mode of action has been described, we were interested to examine whether a reduced production of thymic hormones by thymus-epithelial cells could be involved. Therefore, in the present study, the effect of daily administration of thymosin on TCDD-induced thymus atrophy was studied. As shown by Thigpen et al. (14), TCDD exposure markedly decreased the resistance of mice to infection with *Salmonella bern*, whereas TCDD has no significant effect on mortality in pseudorabies virus-infected mice. In the present study, data are presented that indicate that the increased susceptibility to *Salmonella* infection is very likely due to the endotoxin content of the bacteria. Since on the one hand zinc deficiency can result in severe atrophy of lymphoid organs [15,16] and on the other hand parenteral administration of zinc increases the resistance of mice to endotoxin [17], serum zinc concentrations were also measured in TCDD-exposed mice. Finally as macrophages play an important role in the immune response and in the detoxification of endotoxin, function studies of macrophages (non-specific phagocytosis and killing of *Listeria monocytogenes* and reduction of nitro-blue tetrazolium by macrophages) were also included.

MATERIALS AND METHODS

TCDD

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (purity 98.6%, Lot No. 851:144-II,

kindly provided by Dow Chemical Company, Midland, Mich., U.S.A.) was dissolved in reagent grade acetone at a concentration of 50 μg/ml. For the animal experiments, this stock solution was diluted with at least 9 parts of arachis oil to provide the different concentrations. Mice were intubated orally with 0, 1.5, 5, 15, 50 or 100 μg TCDD/kg body weight.

Animals

Specific pathogen-free outbred Swiss mice were reared at the Institute. They received food and water ad libitum. Because of the high toxicity of TCDD, all animal experiments were carried out under non-sterile conditions in plastic Trexler isolators, and all contaminated material was incinerated at temperatures over 800° C (N.V. Roteb, Rotterdam, The Netherlands).

Zinc analysis

For the determination of zinc in serum, an atomic absorption spectrometer with acetylene-air slot burner and zinc hollow-cathode lamp was used. Fifty μl of serum was diluted with 500 μl of distilled water. After mixing thoroughly 100 μl of the sample was aspirated into the flame and the resulting atomic absorption signal was recorded. Peak heights were compared with results obtained from the aspiration of aqueous standard solutions under the same conditions and zinc concentrations were calculated.

Experiment with thymosin

The effect of thymosin injections on TCDD-induced thymus atrophy was studied in mouse pups that were exposed to TCDD by postnatal maternal treatment. At birth the number of neonates was standardized to 6. Groups of 5 nursing mice received 0 or 10 μg TCDD/kg body weight (in a volume of 0.1 ml/30 g), postnatally on days 1, 4, 8, 11, 15 and 18. Calf thymosin (fraction 5, Lot No. BPM 390, kindly provided by Dr. A.L. Goldstein, Galveston, Tex., U.S.A.) was dissolved in sterile saline at a stock concentration of 15 mg/ml, passed via Millipore bacteria filter and aliquots stored at −20° C. Just prior to injection, a dilution (1 mg/ml) was made in sterile phosphate-buffered saline (PBS). In the first week, groups of 2 pups per litter received daily subcutaneous injections of a high (50 μg) or low (15 μg) dose of thymosin. In addition, 3 pups per control litter received PBS. In the second and third week, the doses were doubled and tripled respectively. The endotoxin content of the thymosin (1 mg/ml) solution was estimated with the Limulus assay [18] and contained between 12.5 and 62.5 ng endotoxin/ml. After the 22-day experimental period, the animals were killed. Zinc concentration was measured in serum.

The thymus of half of the number of mice was removed under sterile conditions. Cell suspensions were made by using a glass homogenizer, the viability was determined with a dye exclusion test and the final suspension

was adjusted to a concentration of 3×10^6 viable nucleated cells/ml in minimum essential medium (MEM, Gibco F14) buffered with Tris (0.025 M) and supplemented with 20% inactivated fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 IU/ml). A total volume of 1 ml was cultured in polystyrene tubes (Nunc, Denmark) and 12.5 μl reconstituted phytohemagglutinin (PHA, Wellcome Labs., Beckenham, Kent, U.K.), 6 μg concanavalin A (Con A, Pharmacia, Uppsala, Sweden) or 50 μl reconstituted pokeweed (PWM, Gibco) were added. After 24 h cultivation, 10 μCi of [6-^{3}H]thymidine (specific activity 600 mCi/mmole) was added. Twenty-four hours later the cells were harvested on glass fiber filters using a multiple cell culture harvester (Skatron, Lierbyen, Norway). After drying, the filters were placed into scintillation vials and 2 ml of scintillation fluid were added. Counting was done in a Packard liquid scintillation counter. Results (median value of triplicate cultures) were expressed as the difference between stimulated and non-stimulated cultures (in counts/min (cpm) per 3×10^6 cells).

Experiments with endotoxin

In 3 experiments, the effect of TCDD on the susceptibility of mice to endotoxin was studied. Since there is no major difference in the biological activity of endotoxins of *Salmonella* and *E. coli* species, *E. coli* endotoxin was used in the present study. Endotoxin (lipopolysaccharide, *E. coli*, O 127: B 8, Difco Labs, Detroit, Mich.) was dissolved in sterile saline. In two studies, 3—4 week-old male Swiss mice were intubated orally, once a week for 4 weeks, with 0, 1.5, 5, 15 or 50 μg TCDD/kg (0.1 ml/10 g body weight). Two days after administration of the final dose of TCDD, the mice were injected intravenously with 10, 20, 100, 250 or 500 μg endotoxin in 0.1 ml. The mortality due to endotoxin shock was scored after 48 h. Surviving animals were killed and examined macroscopically.

In the third experiment, 3—4 week-old female mice received a single oral dose of 0 or 100 μg TCDD/kg body weight (0.2 ml/10 g). Animals were challenged with endotoxin 5 days after intubation. In the second and third experiment, additional animals were intubated and killed to determine the effect of TCDD on body weight and on the weight of thymus and spleen. Also, serum zinc concentrations were measured in the third experiment.

Clearance of Listeria monocytogenes

The resistance of *Listeria monocytogenes* is a combination of non-specific phagocytosis and cell-mediated immunity; non-specific phagocytosis and killing can be measured shortly (within 2 days) after the intravenous inoculation of *Listeria monocytogenes* [19]. Male Swiss mice (3—4 weeks old) were intubated orally, once a week for 4 weeks, with 0 or 50 μg TCDD/kg body weight. Four days after the last intubation the animals were inoculated intravenously with 2.4×10^3 *Listeria monocytogenes* organisms (Strain L 242/73 type 4b). One or two days after the injection, the spleens were weighed,

homogenized and serial dilutions were plated to determine the viable counts of *Listeria* [20]. The number of bacteria was expressed as ^{10}log/spleen.

Reduction of nitro-blue tetrazolium

Lymphokine activated macrophages show increased glucose oxidation via the hexose-monophosphate pathway; an increase that can be quantitated by the reduction of soluble nitro-blue tetrazolium (NBT) to insoluble formazan. Krueger et al. [21] demonstrated a correlation between delayed type hypersensitivity skin tests and NBT reduction by lymphokine-activated macrophages. Similarly Engel and Sekhuis (unpublished data) showed an increase in NBT reduction in macrophages of mice that were injected with *Corynebacterium parvum*, without the addition of lymphokines to the culture chamber. In the present experiment, the effect of TCDD on NBT reduction of macrophages was determined in order to study an effect on the baseline activity of non-activated macrophages. For that purpose, 3—4-week-old male Swiss mice were intubated orally, once a week for 4 or 5 weeks with 0 or 50 μg TCDD/kg body weight. Five days after the last intubation, peritoneal macrophages were harvested in 2 washings with 2.5 ml Eagle's Basal Medium (Gibco, G 13) supplemented with 20% inactivated calf serum, 40 mM L-glutamine and 5 U heparin/ml. Of this suspension 0.5 ml was added to an 8-chambered tissue culture slide and incubated at 37° C in 10% CO_2 in air for 2 h. At this time chambers were washed 3 times to remove non-adherent cells. Of each mouse, one chamber was used for the identification of macrophages (Giemsa stain); the other was used for the NBT assay according the technique described by Krueger et al. [21]. One hundred cells were individually scored at 1000 × on the quantity of formazan precipitate as a measure of NBT reduction (no precipitate = 0; slight precipitate = 1; moderate precipitate = 2; strong precipitate = 3). Results were expressed as the sum of the scores per 100 cells.

Statistical analysis

Student's *t* test was used to make one-sided treatment—control comparisons.

RESULTS

The effect of daily thymosin injections on body and thymus weights of 22-day-old mice is given in Table I. Body and thymus weights of the 3 TCDD groups were significantly lower when compared with the control group. Treatment of TCDD-exposed pups with thymosin significantly reduced body weight, but did not alter thymus weight. Serum zinc concentrations were the same in control and TCDD-exposed pups. The responsiveness of thymus cells to the mitogens PHA, Con A and PWM is presented in Table II. In general, the response of the TCDD groups to these mitogens was approximately half

TABLE I

BODY AND THYMUS WEIGHTS[a] AND SERUM ZINC CONCENTRATIONS OF 22-DAY-OLD TCDD-TREATED MICE THAT RECEIVED DAILY INJECTIONS WITH THYMOSIN[a]

Dose of TCDD (μg/kg)	Thymosin treatment	Body weight (g)	Thymus weight (mg)	Zinc (mg/l)
0	0	13.1 ± 0.9	85 ± 8	1.1 ± 0.2
10	0	12.4 ± 0.8[b]	60 ± 16[c]	1.2 ± 0.6
10	low dose[e]	11.2 ± 1.0[c,d]	60 ± 10[c]	
10	high dose[e]	11.2 ± 1.2[c,d]	58 ± 7[c]	

[a] Mean values ± S.D. (body weight 8—10 animals, thymus weight and zinc analyses of 4—5 animals). Mice were exposed to 0 or 10 μg TCDD/kg by maternal treatment postnatally on days 1, 4, 8, 11, 15 and 18.
[b] Significantly ($P < 0.05$) different from control group (no TCDD and no thymosin).
[c] Significantly ($P < 0.001$) different from control group (no TCDD and no thymosin).
[d] Significantly ($P < 0.025$) different from thymosin control group (10 μg TCDD and no thymosin).
[e] For schedule see text.

of the response seen in the control group. Since the results are expressed on a cell-for-cell basis and thymus weight was reduced by TCDD treatment, the total response per thymus is likely even more decreased. Thymosin treatment of TCDD-exposed mice did not result in a significant increase in the mitogenic responsiveness of the thymus cells.

The results of the first two experiments with endotoxin are given in Table III. Pretreatment of mice with TCDD markedly enhanced their susceptibility

TABLE II

MITOGENIC RESPONSE OF THYMUS CELLS FROM 22-DAY-OLD TCDD-TREATED MICE THAT RECEIVED DAILY INJECTION WITH THYMOSIN[a]

Dose of TCDD (μg/kg)	Thymosin treatment	Δ PHA (cpm)	Δ Con A (cpm)	Δ PWM (cpm)
0	0	19,001 ± 10,774	20,895 ± 6,517	6,494 ± 2,647
10	0	8,097 ± 2,731[b]	10,779 ± 6,345[b]	2,108 ± 1,879[b]
10	low dose[c]	9,985 ± 8,248	14,616 ± 4,989	3,714 ± 1,485
10	high dose[c]	11,878 ± 4,677	11,244 ± 2,595[b]	2,542 ± 1,135[b]

[a] Mean values ± S.D., 4—6 animals per group. Mice were exposed to 0 or 10 μg TCDD/kg by maternal treatment postnatally on days 1, 4, 8, 11, 15 and 18. Results are expressed as the difference between stimulated and non-stimulated cultures (in cpm/3 × 10^5 cells).
[b] Significantly ($P < 0.05$) different from control group (no TCDD and no thymosin).
[c] For schedule see text.

TABLE III

EFFECT OF 4 WEEKLY DOSES OF TCDD ON THE SUSCEPTIBILITY OF MICE TO ENDOTOXIN

Dose of TCDD (μg/kg)[a]	Incidence of mortality[b]		
	10 μg endotoxin	250 μg endotoxin	
0	0/4	0/5	
1.5	0/4	1/5	
5	0/4	6/6	
15	1/4	6/6	
50	2/4	ND[c]	
	10 μg endotoxin	100 μg endotoxin	500 μg endotoxin
0	0/6	0/6	2/6
1.5	0/6	1/6	3/6
50	2/5	5/6	6/6

[a] In two experiments, male mice (3—4 weeks of age) were intubated orally, once a week for 4 weeks, with TCDD in acetone-arachis oil carrier.
[b] Intravenous injection with endotoxin (E. coli. LPS) two days after last intubation. Mortality scored after 48 h.
[c] ND = not done.

to endotoxin. After the injection of 10 μg endotoxin, 2 out of 4 animals died in the 50 μg TCDD group and 1 in the 15 μg TCDD group. Injection of 250 μg endotoxin was lethal for all mice in the 5 and 15 μg TCDD groups and caused one death in the 1.5 μg TCDD group, whereas all controls surviv-

TABLE IV

EFFECT OF 4 WEEKLY DOSES OF TCDD ON BODY AND ORGAN WEIGHTS OF MICE[a]

Dose of TCDD (μg/kg)	Body weight (g)	Thymus weight (mg)	Spleen weight (mg)
0	30.7 ± 2.2	66 ± 20	106 ± 13
1.5	28.7 ± 2.5	65 ± 12	97 ± 23
50	26.0 ± 2.3[c]	34 ± 10[c]	89 ± 8[b]

[a] Mean values ± S.D., 6 animals per group. Male mice, 3—4 weeks of age, were intubated orally, once a week for 4 weeks, with TCDD in acetone-arachis oil carrier.
[b] $P < 0.025$.
[c] $P < 0.005$.

TABLE V

EFFECT OF A SINGLE ORAL DOSE OF TCDD ON BODY AND ORGAN WEIGHTS AND ON SERUM ZINC CONCENTRATION OF MICE[a], AND ON THE SUSCEPTIBILITY TO ENDOTOXIN

Dose of TCDD (μg/kg)	Body weight[a] (g)	Thymus weight[a] (mg)	Spleen weight[a] (mg)	Zinc[a] (mg/l)
0	19.3 ± 1.8	70 ± 15	120 ± 23	1.0 ± 0.2
100	18.7 ± 2.4	51 ± 14[b]	97 ± 19[b]	1.4 ± 0.5
	Incidence of mortality[c]			
	20 μg endotoxin	100 μg endotoxin	500 μg endotoxin	
0	ND[d]	0/5	5/5	
100	5/5	5/5	ND	

[a] Mean value ± S.D., 7 animals per group. Female mice, 3–4 weeks of age, were intubated once orally with TCDD in acetone-arachis oil carrier and killed after 5 days.
[b] $P < 0.05$.
[c] Intravenous injection with endotoxin (E. coli, LPS) 5 days after intubation. Mortality scored after 48 h.
[d] ND = not done.

ed. In the second experiment, animals were given 0, 1.5 or 50 μg TCDD/kg body weight and were challenged with 10, 100 or 500 μg endotoxin. The 10 μg dose of endotoxin caused 2 deaths out of 5 animals in the 50 μg TCDD group, and a similar mortality (2 out of 6) was scored in the controls injected with 500 μg endotoxin. Thus, the susceptibility to endotoxin was approximately 50-fold enhanced in the 50 μg TCDD group when compared with the controls. Even at the 1.5 μg TCDD level there was a slightly increased mortality after injection of endotoxin. This latter dose of TCDD being more than one order of magnitude lower than the dose that caused thymus atrophy at gross inspection. As shown in Table IV, body weights and weights of thymus and spleen of mice of the 50 μg TCDD group were significantly decreased, whereas these parameters in the group intubated with 1.5 μg TCDD/kg body weight did not differ from the controls. A single oral dose of 100 μg TCDD/kg body weight significantly reduced thymus and spleen weight of female mice, killed after 5 days; but body weights and serum zinc levels were not altered (Table V). Also, this treatment enhanced the susceptibility to endotoxin: a 100% mortality occurred in the TCDD-exposed animals after injection with 20 μg LPS, whereas a similar mortality was seen in controls receiving 500 μg LPS.

The results of the study of the effect of TCDD on non-specific phagocytosis and killing of *L. monocytogenes* are given in Table VI. The number of viable *Listeria* organisms, determined one or two days after inoculation of this

TABLE VI

EFFECT OF 4 WEEKLY DOSES OF TCDD IN MICE ON BODY AND SPLEEN WEIGHTS AND ON DAY 1 AND DAY 2 SPLEEN COUNTS OF LISTERIA MONOCYTOGENES[a]

Dose of TCDD (μg/kg)	Body weight (g)	Spleen weight (mg)	Listeria count[b] (^{10}log/spleen)
Day 1 p.i.			
0	23.4 ± 1.4	140 ± 10	4.89 ± 0.40
50	21.7 ± 0.6[c]	96 ± 18[d]	4.58 ± 0.24
Day 2 p.i.			
0	21.7 ± 1.5	178 ± 23	5.13 ± 0.31
50	21.2 ± 2.4	124 ± 29[d]	5.08 ± 0.27

[a] Mean values ± S.D., 5 animals per group. Male mice, 3—4 weeks of age, were intubated orally, once a week for 4 weeks with TCDD in acetone-arachis oil carrier.

[b] Animals were inoculated intravenously with 2.4×10^3 Listeria monocytogenes organisms 4 days after the last intubation; the number of viable Listeria organisms per spleen was determined after 1 and 2 days.

[c] $P < 0.05$.

[d] $P < 0.01$.

organism, was the same in spleens from treated and control mice. Spleen weights were significantly lower in TCDD-exposed animals. In addition, body weights of mice killed one day after inoculation were significantly reduced in the TCDD group. Finally, as shown in Table VII, in two experiments TCDD did not alter the number of peritoneal macrophages, nor did it cause a significant effect on the macrophage reduction of NBT.

TABLE VII

EFFECT OF 4 OR 5 WEEKLY DOSES OF TCDD IN MICE ON NUMBER OF PERITONEAL MACROPHAGES AND ON MACROPHAGE REDUCTION OF NBT[a]

Dose of TCDD (μg/kg)	No. of peritoneal macrophages ($\times 10^6$)	Reduction of NBT[b]
Four doses		
0	1.94 ± 0.68	32.2 ± 22.2
50	2.23 ± 0.35	37.0 ± 8.9
Five doses		
0	1.81 ± 0.54	23.8 ± 17.1
50	2.08 ± 0.31	57.0 ± 14.0

[a] Mean values ± S.D., 4—5 animals per group. Male mice, 3—4 weeks of age, were intubated orally, once a week for 4 or 5 weeks with TCDD in acetone oil carrier.

[b] For details see text.

DISCUSSION

From the present results it can be concluded that thymosin treatment of mice at the employed doses does neither protect the animals from thymus atrophy nor from a reduced mitogenic responsiveness of thymocytes. The presence of a minor quantity of endotoxin in the thymosin solution could not conceal a possible increase, since treatment of mice with *E. coli* LPS has been shown to increase the PHA and Con A responsiveness of thymocytes [22]. Thus, it seems unlikely that the depletion of lymphocytes in the thymus, as induced by TCDD, is caused by a reduced production of thymic hormones, since calf thymosin has been shown in several studies to potentiate various parameters of thymus-dependent immunity [23]. Neither seems zinc deficiency to be a cause for the thymus atrophy. A direct cytotoxic action of TCDD on lymphocytes is also unlikely, since the mitogen responsiveness of mouse or rat lymphocytes was not reduced when cultured in the presence of TCDD at concentrations up to 0.02 μg/ml medium [13]. Even the presence of 0.32 μg TCDD/ml medium has been reported to be not toxic for human lymphocytes [24], whereas concentrations that are two orders of magnitude lower do induce aryl hydrocarbon hydroxylase activity in human lymphocytes [25]. In another study [26] a cytotoxic action of TCDD at a concentration of 0.48 μg/ml was reported for several mammalian cell types. These results make it unlikely that TCDD does need metabolic activation, but they do indicate that the lymphocyte depletion of the thymus and the thymus-dependent lymphoid tissue are not caused by a direct effect on lymphocytes, but rather by an indirect way. There are several conditions that can cause thymus atrophy [27]. Regarding the selective effect of TCDD on thymus, it is not likely caused by an effect on pituitary, on adrenals or by reduced food intake, since thymus atrophy did occur in TCDD-exposed rats that were hypophysectomized or adrenalectomized and in rats that were placed on a restricted diet [Van Logten, Gupta and Moore, in preparation] though it is still possible that TCDD may impair the absorption and transport of certain nutrients. In addition, serum α-fetoprotein levels are not elevated in TCDD-exposed rats [Vos and Boekestein, unpublished data], thus the observed immunosuppression is not due to a α-fetoprotein induction. In conclusion, the mode of action of the TCDD-induced thymus atrophy remains unknown.

The results of the present experiments with endotoxin show a marked increase in susceptibility to endotoxin of mice treated with a single or with repeated doses of TCDD. This sensitizing effect of TCDD for endotoxin offers a logical explanation for the decreased resistance of TCDD-exposed mice to infection with (endotoxin-containing) *Salmonella* bacteria as observed by Thigpen et al. [14]. Another explanation for the increased susceptibility to this type of infection, which is primarily dependent upon cell-mediated immunity [28] is by general suppression of the thymus-dependent immunity. This explanation is, however, less likely since increased mortality by *Salmonella* occurred at dose levels of TCDD that did not produce thymus

atrophy. The endotoxin hypersensitivity, which has to date only been studied in mice, is a phenomenon of particular interest when considered in connection with: (1) terminal hemorrhages that were seen in TCDD-exposed rats and guinea pigs [8,29] and mice [12,30], (2) thrombocytopenia, observed in rats and guinea pigs [10,31], and (3) presence of thrombi in blood vessels of heart and lung in the rat after a lethal dose of TCDD [7,8]. These lesions are also seen in endotoxin shock and since TCDD, at least in the mouse, also induces endotoxin hypersensitivity, one has to consider endotoxin shock as a possible cause of death. Regarding the mechanism of endotoxin hypersensitivity, several possibilities exist (see review of Cook et al. [32]). With regard to the endotoxin hypersensitivity produced by TCDD it would be worthwhile to investigate the role of the enzyme glutathione-S-transferase B, a cytosolic glutathione-conjugating enzyme, since sulfhydryl containing enzymes are necessary for endotoxin inactivation. TCDD has been reported to increase the activity of both hepatic and renal glutathione-S-transferase B [33].

Finally, as treatment of mice with TCDD did neither impair the non-specific killing and phagocytosis of *L. monocytogenes* nor the macrophage reduction of NBT, it seems likely that the TCDD-induced suppression of the cell-mediated immunity is only due to a, to date unknown, effect on T-lymphocytes and not due to a combined effect on both T-cells and macrophages. Similarly, the results of the experiment with *L. monocytogenes* indicate that the endotoxin hypersensitivity is not the result of alteration in phagocytic function of macrophages but, as discussed in the previous paragraph, perhaps due to impaired endotoxin detoxifying properties of macrophages.

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Review Article

Medical Problems Raised by the TCDD Contamination in Seveso, Italy

G. Reggiani

Clinical Research, F. Hoffmann-La Roche & Co.,
Grenzacher Straße 124, CH-4002 Basel, Switzerland

Abstract. The Seveso accident, where TCDD was produced and released on an urban area, raised several medical problems linked with the non-specificity of the clinical features of the effects from the exposure and the wide variety and severity of lesions and symptoms caused by this toxin in the animal experiment. The assessment of the health hazards had to rely on the detection by chemical analysis of the toxin in the environment and on the definition of an area contaminated by the toxin. Evacuation of the population was the only possible preventive measure for precluding or at least strongly reducing further exposure. Of the known clinical symptoms of the TCDD toxic effects on humans, the typical skin lesions (chloracne) appeared almost only in children and young people. The degree and intensity was mild and spontaneous healing occurred in the majority of the cases.

Transient decrease of the lymphocytes and passing signs of impairment of the liver functions have been detected by the immediately established monitoring of the health condition of the population. No overt pathology of the liver, the kidney, the blood reproductive organs, the central and peripheral nervous system, the carbohydrate, fat and porphyrin metabolism has been registered up to now.

Close control and thorough examination of the current pregnancies and their outcome did not reveal any embryotoxic or teratogenic effect imputable to the chemical.

Comparing the picture of the medical injuries caused by the Seveso accident with that of similar accidents occurred in the past in USA and Europe, both in respect of the short as well as long term toxicity, one should conclude that the TCDD exposure in Seveso has been less severe than in the previous accidents.

Key words: TCDD release – Delimitation of contamination – Human health protective and preventive measures.

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BIOASSAY OF PICLORAM FOR POSSIBLE CARCINOGENICITY

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Carcinogen Bioassay and Program Resources Branch
Carcinogenesis Program
Division of Cancer Cause and Prevention
National Cancer Institute
National Institutes of Health
Bethesda, Maryland 20014

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
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BIOASSAY OF
PICLORAM
FOR POSSIBLE CARCINOGENICITY

Carcinogenesis Program
Division of Cancer Cause and Prevention
National Cancer Institute
National Institutes of Health

CONTRIBUTORS: This report presents the results of the bioassay of picloram for possible carcinogenicity, conducted for the Carcinogen Bioassay and Program Resources Branch, Carcinogenesis Program, Division of Cancer Cause and Prevention, National Cancer Institute (NCI), Bethesda, Maryland. The bioassay was conducted by Gulf South Research Institute, New Iberia, Louisiana, initially under direct contract to NCI and currently under a subcontract to Tracor Jitco, Inc., prime contractor for the NCI carcinogenesis bioassay program.

The experimental design was determined by Drs. J. H. Weisburger[1,2] and R. R. Bates[1,3]; the doses were selected by Drs. T. E. Shellenberger[4,5], J. H. Weisburger, and R. R. Bates. Animal treatment and observation were supervised by Drs. T. E. Shellenberger and H. P. Burchfield[4], with the technical assistance of Ms. D. H. Monceaux[4] and Mr. D. Broussard[4]. Histopathology was performed by Drs. E. Bernal[4] and B. Buratto[4], and the diagnoses included in this report represent the interpretation of these pathologists.

Animal pathology tables and survival tables were compiled at EG&G Mason Research Institute[6]. Statistical analyses were performed by Dr. J. R. Joiner[7], using methods selected for the bioassay program by Dr. J. J. Gart[8]. Chemicals used in this bioassay were analyzed under the direction of Dr. H. P. Burchfield, and the analytical results were reviewed by Dr. S. S. Olin[7].

This report was prepared at Tracor Jitco under the direction of Dr. Marshall Steinberg[7], Director of the Bioassay Program; Drs. J. F. Robens[7] and R. W. Foglemen[7], toxicologists; Dr. R. L.

Schueler[7], patholigist; Ms. L. A. Waitz[7] and Ms. Y. E. Presley[7], technical writers; and Dr. E. W. Gunberg[7], technical editor, assisted by Ms. P. J. Graboske[7].

The statistical analysis was reviewed by a member or members of the Mathematical Statistics and Applied Mathematics Section of NCI (Dr. John J. Gart, Mr. Jun-mo Nam, Dr. Hugh M. Pettigrew, and Dr. Robert E. Tarone served as reviewers on an alternating basis).

The following other scientists at the National Cancer Institute were responsible for evaluating the bioassay experiment, interpreting the results, and reporting the findings:

Dr. Kenneth C. Chu
Dr. Cipriano Cueto, Jr.
Dr. J. Fielding Douglas
Dr. Dawn G. Goodman
Dr. Richard A. Griesemer
Mr. Harry A. Milman
Dr. Thomas W. Orme
Dr. Robert A. Squire[9]
Dr. Jerrold M. Ward

---

[1]Carcinogenesis Program, Divison of Cancer Cause and Prevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

[2]Now with the Naylor Dana Institute for Disease Prevention, American Health Foundation, Hammond House Road, Valhalla, New York.

[3]Now with the Office of the Commissioner, Food and Drug Administration, Rockville, Maryland.

[4]Gulf South Research Institute, Atchafalaya Basin Laboratories, P. O. Box 1177, New Iberia, Louisiana.

[5]Now with the National Center for Toxicological Research, Jefferson, Arkansas.

[6]EG&G Mason Research Institute, 1530 East Jefferson Street, Rockville, Maryland.

[7]Tracor Jitco, Inc., 1776 East Jefferson Street, Rockville, Maryland.

[8]Mathematical Statistics and Applied Mathematics Section, Biometry Branch, Field Studies and Statistics, Division of Cancer Cause and Prevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

[9]Now with the Division of Comparative Medicine, Johns Hopkins University, School of Medicine, Traylor Building, Baltimore, Maryland.

SUMMARY

A bioassay of technical-grade picloram for possible carcinogenicity was conducted by administering the test chemical in feed to Osborne-Mendel rats and B6C3F1 mice.

Groups of 50 rats and 50 mice of each sex were administered picloram in the diet at one of the following doses for 80 weeks. Time-weighted average doses for the rats were 7,437 or 14,875 ppm; those for the mice were 2,531 or 5,062 ppm. The rats were then observed for 33 weeks, the mice for 10 weeks. Matched controls consisted of groups of 10 untreated rats or 10 untreated mice of each sex; pooled controls, used for statistical evaluation, consisted of the matched-control groups combined with 30 untreated male and 30 untreated female rats or mice from similar bioassays of three other test chemicals. All surviving rats were killed at 113 weeks; all surviving mice were killed at 90 weeks. Survival was adequate for meaningful statistical analyses of the incidences of tumors in rats and mice of both sexes.

Mean body weights of the high-dose rats were lower than those of the matched controls during the first part of the study; however, beginning at approximately 80 weeks, mean weights of controls were lower than those of treated animals. Body weights of the mice were unaffected by the picloram.

In rats, a relatively high incidence of follicular hyperplasia, C-cell hyperplasia, and C-cell adenoma of the thyroid occurred in both sexes. However, the statistical tests for adenoma did not show sufficent evidence for association of the tumor with picloram administration.

An increased incidence of hepatic neoplastic nodules was observed in treated male and female rats as compared with untreated animals. This lesion is considered to be a benign tumor. In male rats the lesion appeared only in three animals of the

low-dose treatment group and was not significant when compared with the controls; however, the test for positive dose-related trend in females was significant (pooled controls 0/39, low-dose 5/50, high-dose 7/49, P = 0.016) and the incidence in the high-dose group was significant (P = 0.014) when compared with that in the pooled-control group.

There was also one hepatocellular carcinoma in a low-dose male rat and one in a high-dose female rat. In both males and females, there was a possibly treatment-related lesion of the liver diagnosed as foci of cellular alteration. The incidences of this latter lesion were, female rats: matched controls 1/10, low-dose 8/50, high-dose 18/49; male rats: matched controls 0/10, low-dose 12/49, high-dose 5/49. Thus, there is evidence that picloram affected the livers of rats of both sexes, but more particularly those of the females.

No tumors were found in male or female mice or male rats at incidences that could be significantly associated with treatment, and it is concluded that picloram was not carcinogenic for B6C3F1 mice or male Osborne-Mendel rats.

In female rats, however, the incidence of neoplastic nodules of the liver, benign tumors, was associated with treatment with picloram. It is concluded that under the conditions of the bioassay, the findings are suggestive of the ability of the compound to induce benign tumors in the livers of female Osborne-Mendel rats.

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Strictly Confidential

Coordination of epidemiological studies on the long term hazards of chlorinated dibenzodioxin....

Working Group of the NIEHS and the IARC, Lyon, 10. and 11. Januar 1...

Mortality Study of persons exposed to Dioxine after an accident on November 15, 1953 in Ludwigshafen, Germany.

Introduction:

According to the Volume 15 of the IARC-Monographs and to our know ledge, several accidents have occurred during the past decades, in which persons came into contact with trichlorophenol-dioxin, yet there have been no prospective epidemiological studies completed on persons exposed to TCDD. This report describes the findings of a long-term follow up study after an accidental chemical reaction during which TCDD had been released into a building of a factory which was then occupied by workers and also lateron was used for the ongoing production of other chemicals.

Event description:

The event happend on Nov.17th, 1953 and was described in a publication about the acute and sub-acute sequelae of the event as follows (1,2):

> During the hydrolization of 1,2,4,5-tetrachlorobenzol into 2,4,5 - trichlorophenol, which was carried out in methanol-sodium hydroxide by temperature of 180 °C and high pressure, an accidental reaction took place. Due to the explosive increase of pressure and temperatur within the autoclave an uncontrolled reaction led to "toxic chlorinated carbohydrates". This occurred in a building four stories high in which the different stories are connected by several "windows" and openings for tubes and connections. The autoclaves were located in the second floor, but also reached parts of the ceiling of the first floor, from which the feeding tubes went up to the second floor and to the autoclaves. The slit opening for these tubes was covered by a metal sheet.
>
> The reaction was followed by the development of a steam phase leavin through a broken security valve which turned into an intense vaporous haze which filled the autoclave room in short time. This haze also invaded the other rooms in all four floors and the staircase connecting all four levels of the building. After "several minutes" the clouds were dispersed or had been precipitating in the form of a visible layer, covering the appliances, walls, window glasses, doors, and other surfaces all over, so that the room was reasonably clear to be entered.

The workshop or autoclave room was situated in a fire-proof part of the building which had, throughout the four stories, a special masonry. In this special part of the building several industrial hygiene measures were taken subsequently, because of the immediate incidence of cases with chlor-acne in the workers exposed to the event.

The measures taken were:

Whitewash of the whole building (walls and staircase covered with lime)

The workers were protected by van-der-Grinten-Masks and full protective cloth as a fully gas-proof combinations.

After this crew of six painters covered all metal by a special paint , called rust-transformer, and lateron with red iron-oxide paint.

The floor was covered with Acronal, a plastic layer, and silicon-solu-tion. A second layer of Oppanol foil was followed by several washings with clearing and neutralizing chemicals. After this procedure the total surfaces were flamed and the autoclaves were boiled with benzene.

Despite this elaborate cleaning programme these measures had been ineffective as was found out lateron.

The medical history of a worker who was exposed to the contaminated area five years after the event gave evidence of a still remaining hazard at this site. In the interim time no cases of illness had been observed among the employed persons, despite the fact that a full production programme had been going on in the rooms, making use of the autoclaves, too.

The particular hydrolization process described above which precipitated the adverse chemical reaction had been discontinued since November 1953, and also had the production of trichlorophenol been abandoned.

One incident case of a health damage in 1953 is of particular interest because of its fatal outcome of the illness of the mechanic, who had been exposed to the substance. The 57-year old man used a welding kit during the repair at one of the autoclave and therefore wore all the protective cloth and mask. While heating a bearing of the stirring mechanism he several times lifted the mask to wipe off the sweat from his forehead. The heat developed by the welding flame caused a vaporization of the lubricant of the bearing. Four day after this work acute dermatological and neurological symptoms developed. Six months later the person became hospitalized for pancreatitis and liver enlargement. After nine months a tumour in the left upper abdomen and symptoms of an acute inflammatory process caused another hospitalization, during which the person died. Autopsy revealed pancreatic necrosis and perforation of stomach and bulbus duodeni, liver abscesses and chlor-acne of the trunc.

In 1968, ten years after this incidence, sufficient knowledge available then necessitated the total disrution of the whole building which was effected in early 1969 according to a detailed plan. This activity remained so far without any detectable health sequelae or acute symptoms.

The experiences with the acute and subacute clinical reactions have been presented and published at the MEDICHEM - Congresses in Ludwigshafen (1972), Haifa (1976), and recently in San Francisco as far as mortality was determined.

The presentation in Haifa considered only the medical histories of 42 resp. 55 symptom carriers for evaluation.

There was no mention of any exposure data and measurements being missing may be due to the fact that in 1953 the substance was still unidentified and could only be determined and tested in animal studies after that event.

It was, however, suggested that the typical effects seen in the cases would not seem to be caused by a substance or compound which was an intermediate or transient product formed only during the unintended chemical reaction but that the sublimate-like substance had to be suspected the agent in question.

The possibility can, therefore, not be excluded that individual multiple exposure occurred to the unsuccessfully removed sublimate in the subsequent period.

Epidemiological study:

Methodology:

The epidemiological investigation was initiated as a consequence of the discussion of these descriptive, clinical presentations and was further prompted by the recent event at Seveso.

The fatal incidence in 1958 seemed to be indication of long-term persistence of the substance in the originally contaminated area and therefore a cohort could have been composed by all those persons who possibly may have entered the rooms after 1953 until 1969. In order to have some certainty about exposure and also to establish a sufficient latency period to detect all possible fatal hazards it was decided to begin the follow-up study with the core group, i.e. all those men exposed during the accident and during the immediate cleaning and repair work. This cohort commprised 75 persons. The time elapsed since the event was almost exactly 24 years at the closing date of the study. The follow-up was successfully carried through for all 75 persons which is quite a common experience given the registration system in Germany.

In Table 1 the number of person years is given together with the person years of a comparison group (matched reference cohort).

The follow-up technique can be demonstrated.

As soon as a person has left the factory or the last residence, the follow up is extended as many times as necessary to determine the vital status. From all deceased persons the death certificates were requested from the public health offices of administrative centers.

The total of 1525 person years reflects an average observation period of almost 20 years per individual. The expected number of deaths has been calculated on the person-year principle, the reference groups for the population mortality.

Continued by „Results" of the paper of THIESS and FRENTZEL-BEYME

Problems and pitfalls:

In this study one major pitfall has been avoided which is called the healthy worker effect, because two aspects of this often fallacious element of cohort studies did not apply.
Many of the workers were already exposed for some time as being employed in the factory and the exposure to dioxin was an additional experience of a timely defined duration.
For this reason of the observed deaths with the total population mortality can be done without correction for duration of exposure.
This has been shown to be influence for results in such studies where long term exposure necessarily require long-term survivals in the cohorts which are not to be anticipated in the general population in the same time . We were not able, however, to consider the possible multiple exposure before and after the accident adequately. It is hoped, though, that the comparison with an internal reference group helps to overcome this possible influence of multiple exposure under the assumption that also in this group there existed the same possible exposures to other substances as the study group.
The selection of comparison persons at random was done manually for reason of convenience; a much more efficient and proper procedure would have been the use of a random digit generator, and the automatic selection of persons matched by age and date of entry into the factory.
One pitfall generally encountered in such kind of studies is the problem of validity of the diagnoses on the death certificates.
The attempt was made to validate the diagnoses, especially when cancer was mentioned. The result of the data collection for validation is shown in Table 3.

Conclusions:

Taking into account these problems and pitfalls and the fact, that results of epidemiological studies are usually not able to prove a causal relationship but can be considered as pointers indicating possible risk the following conclusions can be drawn:
Although based on a comparatively small absolute number a malignant neoplasms have increased consistently above expectation for the possibility of a mere chance event. The effect is, however, shown to be significant only in one age-group.
The statistical test applied to this finding indicates that the observation of an excess of stomach carcinoma in this age cannot be considered to be due to the small number of cases alone.
The mortality of all causes in the dioxin-exposed group did differ slightly from the expected deaths in several comparison groups.

This evaluation may be stipulating the need for further studies into the question whether the observed increase of cancer of the stomach in one particular age group can be duely associated with the exposure to dioxin.

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IDENTIFICATION OF SUBSTITUTION PATTERNS IN POLYCHLORINATED DIBENZO-p-DIOXINS (PCDDs) BY MASS SPECTROMETRY

Hans Rudolf Buser
Swiss Federal Research Station
CH - 8820 Wädenswil, Switzerland

and

Christoffer Rappe
Department of Organic Chemistry, University of Umea
S - 901 87 Umea, Sweden

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Introduction

Polychlorinated dibenzo-p-dioxins (PCDDs, structure see Figure 1) are a group of compounds that have been the subject of much concern during recent years. They are known as highly stable minor contaminants in a variety of products like chlorinated phenols, phenoxy acids and hexachlorobenzene. They were involved in several industrial accidents, the most recent occurring at Seveso, Italy. Moreover, they have been identified in fly ash and flue gases from municipal incinerators and industrial heating facilities.[1,2]

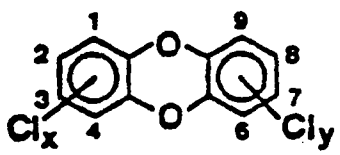

Figure 1 Structure and numbering (Chemical Abstract System) of polychlorinated dibenzo-p-dioxins (PCDDs).

In all, 75 positional isomers exist for the PCDDs containing one to eight chlorine atoms, and a number of these are known as extremely hazardous compounds. Recent work of McConnell et al. [3], Poland et al.[4] and Bradlaw[5] has shown that positional isomers vary greatly in acute toxicity and biological activity. Factors of 1000-10 000 are found for closely related isomers like the 2,3,7,8- and the 1,2,3,8-tetra-CDD. Consequently, it becomes important to identify the discrete isomers found in various products.

This identification is difficult to obtain using normal analytical techniques. With the exception of mass spectrometry, other spectroscopic methods use larger amounts than are very often available and many times there is a need for pure samples or even single crystals. Very often, identifications have to rely on GC retention times, especially in mixtures where many and different PCDDs are present. This is especially true for biological and environmental samples where the PCDDs are present only in minute quantities together with a multitude of other compounds. Even the best separation techniques such as high-resolution GC using glass capillary columns may be insufficient to separate all positional isomers of some PCDDs. In Figure 2 is a mass fragmentogram of a synthetic sample showing the elution of 10 different tetra-CDDs, all containing 2 chlorine atoms in each of the carbon rings of the dioxin molecule. Although this column gives a reasonable separation of these isomers, its efficiency for separating all 22 tetra-CDD isomers would be still far too low.

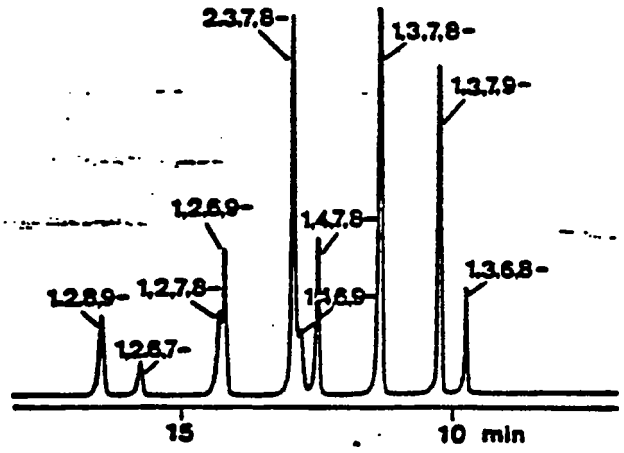

Figure 2 Mass fragmentogram (m/e 320) showing elution of 10 tetra-CDD isomers with 2:2 chlorine substitution patterns prepared by microscale pyrolysis of different trichlorophenates; 50 m OV-17 glass capillary column, 200°C, isothermal; column efficiency 140 000 theoretical plates.

Much interest has been devoted to the mass spectrometry of PCDDs. The electron-impact (EI) mass spectra allow an easy identification of PCDDs and distinguishing them from other polychlorinated compounds such as dibenzofurans, diphenyl ethers, biphenyls and naphthalenes. This identification is based on the intense molecular ions (M^+) with the expected ion clustering due to the chlorine isotopes and the characteristic fragmentation with formation of M^+-COCl and M^+-2 COCl.[6]

In the high mass region (m/e > 200) usually used for the MS identification of PCDDs, different positional isomers show no pronounced differences. However, in previous papers[7,8] we have reported some observations concerning differences in the lower mass range of different isomers for the tetra- and hexa-CDDs. The problems were related to impurities in industrial products (hexa-CDDs) and an environmental contamination at Seveso, Italy (tetra-CDDs).

In this paper, we report a more general approach that allows the identification of a substitution pattern of a PCDD by observation of its lower mass ions. As substitution pattern we define the number of chlorine atoms in each carbon ring of a dioxin molecule (see Fig. 1). From Table 1, it is evident that the number of positional isomers of a PCDD can be subdivided into smaller groups with the same substitution pattern. Consequently, the number of possible isomers can be reduced leading to a more simple identification, which is of importance also in the discussion of the possible precursors to these hazardous compounds. The partial mass spectra of 34 different PCDDs are reported. The use of this new technique is illustrated in some applications related to recent PCDD problems concerning environmental pollution and industrial products.

Table 1 Number of isomers and substitution pattern of PCDDs

Compounds	Number of chlorines in each carbon ring[a] X	Y	Number of isomers	Total number of isomers
mono-CDDs	1	0	2	2
di-CDDs	2	0	4	
"	1	1	6	10
tri-CDDs	3	0	2	
"	2	1	12	14
tetra-CDDs	4	0	1	
"	3	1	8	
"	2	2	13	22
penta-CDDs	4	1	2	
	3	2	12	14
hexa-CDDs	4	2	4	
"	3	3	6	10
hepta-CDDs	4	3	2	2
octa-CDD	4	4	1	1

[a] see Figure 1

Experimental

Reference compounds

The PCDD isomers used in the present study are listed in Table 2. Some of these isomers were obtained as reference compounds from the sources listed, others were prepared by microscale pyrolysis of different potassium polychlorophenates under conditions previously described.[7] PCDD isomers prepared by this route were characterized by MS without actual isolation. Dilute solutions of PCDDs in methylene chloride at concentrations of 5-20 ng/µl were used for GC-MS analysis.

GC-MS analysis

A Finnigan 4000 quadrupole GC-MS instrument equipped with EI source and Finnigan 6111 data system was used. The ion source conditions were 70 eV and 250°C. Samples were injected (1-5 µl, splitless) on a 50 m 0.36 mm ID OV-17 glass capillary column coupled to the MS via a platinum capillary interface. The column was temperature programmed from 100 to 160°C with 20°/min and to 250°C with 2°/min. The elution temperatures of the different PCDD isomers are given in Table 2. Mass spectra were recorded by repetitive scanning (m/e 35-460; 1.4 sec/scan) using the data syster

Results and Discussion

General remarks on mass spectrometry of PCDDs

As previously mentioned, PCDDs are readily identified by EI mass spectrometry. Through the presence of molecular ions, the ion clustering due to the chlorine isotopes and from the typical fragmentation one can distinguish them from other chlorinated pollutants. The stability of the PCDD system is indicated by intense (usually base peaks) M^+-ions and also by fairly strong doubly charged molecular ions (M^{2+}). Major fragmentation leading to ions in the higher mass range is due to the expulsion of CO and Cl· with formation of M^+-COCl and M^+- 2 COCl. Minor fragmentation is by loss of Cl·, Cl_2 and sometimes HCl from M^+ and the major fragment ions with formation of M^+-Cl, M^+-Cl_2, M^+-COCl-Cl, M^+-COCl-Cl_2, M^+-2 COCl-Cl etc.[6] Isomers of various PCDDs give very similar spectra in this mass range and differentiation between isomers on the basis of ions in this region is impossible.

In the lower mass range, the major ions present in the EI mass spectra of PCDDs are C_4-, C_5- and C_6-species, the latter formed by fission of the carbon-oxygen bonds of the dioxin system and followed by the loss of Cl· and preferably Cl_2 etc. The resulting ions do contain information on the number of chlorine atoms present in each carbon ring of the original PCDD. Isomers of a PCDD with the same chlorine substitution pattern give in this region very similar mass spectra too, but clearly different from those of isomers with a different substitution pattern. Though no exact fragmentation modes were deduced from these mass spectral data, the presence or absence of characteristic ions allows an identification of the chlorine substitution patterns of PCDDs.

Table 2 PCDD isomers investigated

Compounds	Chlorine substitution pattern	Elution temperature (50 m OV-17)	Source or route of preparation[a,b]
2-mono-CDD	1:0	169.3	Givaudan
2,3-di-CDD	2:0	177.5	Givaudan
2,7- " "	1:1	177.5	"
2,8- " "	1:1	177.5	"
1,2,4-tri-CDD	3:0	188.1	Givaudan
1,3,7- " "	2:1	186.7	"
2,3,7- " "	2:1	189.1	"
1,2,3,4-tetra-CDD	4:0	203.9	Andersson
1,2,3,8- or 1,2,3,7-	3:1	203.7	Nilsson
2,3,7,8-tetra-CDD	2:2	203.1	Linz
1,3,6,8- " "	2:2	196.2	pyrolysis of 2,4,6-tri-CP
1,3,7,9- " "	2:2	197.4	" "
1,4,6,9- " "	2:2	202.8	pyrolysis of 2,3,6-tri-CP
1,2,6,9- " "	2:2	205.6	" "
1,2,6,7- " "	2:2	207.9	pyrolysis of 2,3,4-tri-CP
1,2,8,9- " "	2:2	208.9	" "
1,2,3,4,6-penta-CDD	4:1	219.0	pyrolysis of 2,3-di-CP+PCP
1,2,3,4,7- " "	4:1	217.8	pyrolysis of 2,4-di-CP+PCP
1,2,4,7,8- " "	3:2	215.0	Firestone
1,2,3,7,8- " "	3:2	218.3	"
penta-CDDs, 3 isomers	3:2	211.7, 214.1 215.3	pyrolysis of 2,4,6-tri-CP +2,3,4,6-tetra-CP
1,2,3,4,6,8-hexa-CDD	4:2	228.5	pyrolysis of 2,3,5-tri-CP+PCP
1,2,3,4,6,9- " "	4:2	231.4	pyrolysis of 2,3,6-tri-CP+PCP
1,2,3,4,7,8- " "	4:2	232.1	pyrolysis of 2,4,5-tri-CP+PCP
1,2,3,4,6,7- " "	4:2	239.9	pyrolysis of 2,3,4-tri-CP+PCP
1,2,4,6,7,9- or 1,2,4,6,8,9-	3:3	227.0	pyrolysis of 2,3,5,6-tetra-CP
1,2,3,6,8,9- or 1,2,3,6,7,9-	3:3	229.8	pyrolysis of 2,3,4,6-tetra-CP
1,2,3,6,7,8-hexa-CDD	3:3	252.6	Firestone
1,2,3,7,8,9- " "	3:3	233.8	"
1,2,3,4,6,7,9-hepta-CDD	4:3	243.4	pyrolysis of 2,3,5,6-tetra-CP+PCP
1,2,3,4,6,7,8- " "	4:3	246.6	pyrolysis of 2,3,4,5-tetra-CP+PCP
1,2,3,4,6,7,8,9-octa-CDD	4:4	250	pyrolysis of PCP

[a] Sources : Givaudan = Givaudan Ltd., Dübendorf, Switzerland
Andersson = Dr. K. Andersson, University of Umea, Sweden
Nilsson = Dr. C.A. Nilsson, " " " "
Firestone = Dr. D. Firestone, Food and Drug Adm., Washington, D.C., USA
Linz = Stickstoffwerke Linz, Austria

[b] Abbreviations : di-CP, tri-CP, tetra-CP, PCP = di-, tri-, tetra- and pentachlorophenate

It should be pointed out that a careful observation of mass spectra in this mass range is required and may be complicated by the presence of background peaks from samples or GC columns. This may be circumvented by using such features as background subtraction and running specific mass chromatograms to determine the presence or absence of a characteristic ion in the spectrum of a PCDD.

Differences in the lower mass range of mass spectra of PCDDs

Mono-, di- and tri-CDDs

In Figure 3 we report partial mass spectra of mono-, di- and tri-CDDs with different chlorine substitution patterns.

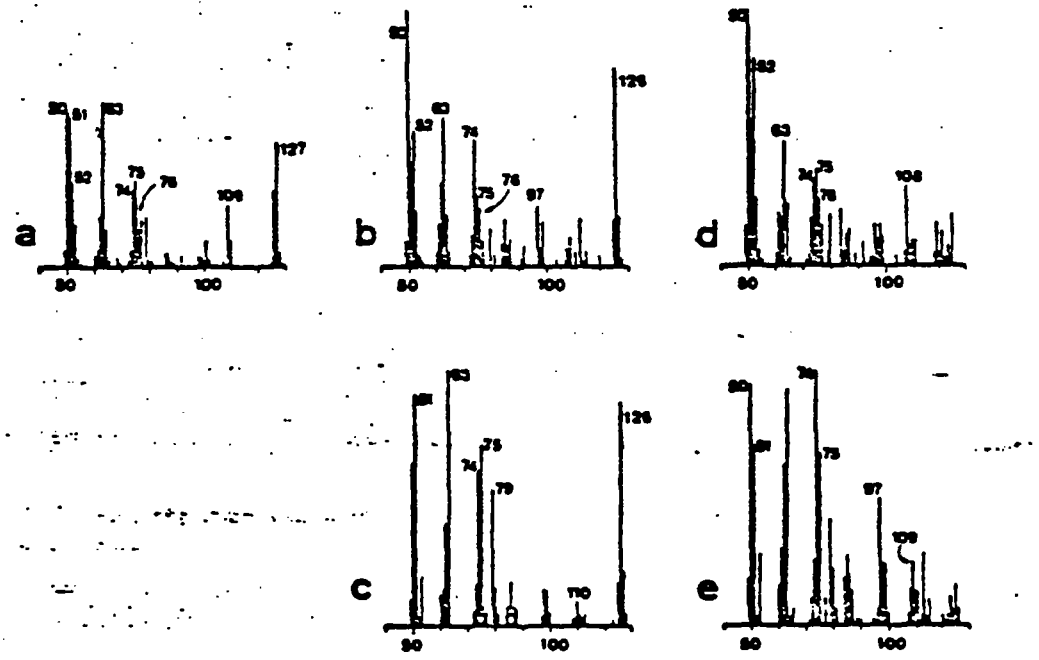

Figure 3 Partial mass spectra (m/e 40-130) of mono-, di- and tri-CDDs. a) 2-Mono-CDD (1:0); b) 2,3-(2:0) and c) 2,7-di-CDD (1:1); d) 1,2,4-(3:0) and e) 2,3,7-tri-CDD (2:1). Chlorine substitution pattern in parenthesis.

In case of the mono-CDDs, 2 isomers exist with 1:0 chlorine substitution. The partial mass spectrum of 2-mono-CDD is presented in Figure 3 a. Chlorine containing ions in the lower mass range seem to play a minor role with the exception of M^{2+} at m/e 109 and 110. The non-halogenated carbon ring with 4 hydrogen atoms (4 H) yields ions at m/e 76 ($C_6H_4^+$) and

52 ($C_4H_4^+$). Each of these ions is accompanied by peaks that result from the loss of one and two H-radicals. These ions were observed from all PCDDs containing a non-halogenated (4 H) carbon ring. The other carbon ring containing the Cl-aton can also produce the ion at m/e 75 ($C_6H_3^+$) etc.

In case of the di-CDDs, isomers of two groups exist: a first group contains both chlorine atoms in the same carbon ring (2:0), a second group one chlorine atom in each of the rings (1:1 substitution). Marked differences in the mass spectra of isomers of these two groups exist. 2,3-Di-CDD (Figure 3 b), an isomer with 2:0 chlorine substitution, yields ions at m/e 76 ($C_6H_4^+$), 52 ($C_4H_4^+$) and their accompanying ions from the non-halogenated (4 H) carbon ring. The di-halogenated (2 H) carbon ring yields ions at m/e 109 ($C_6H_2Cl^+$, minor), 97 ($C_5H_2Cl^+$) and 74 ($C_6H_2^+$).

2,7-Di-CDD (1:1 substitution, mass spectrum Figure 3 c) yields ions at m/e 110 ($C_6H_3Cl^+$, minor), 75 ($C_6H_3^+$), 51 ($C_4H_3^+$) etc. from the mono-halogenated (3 H) carbon rings. These ions were present in the spectra of all PCDDs containing carbon rings substituted by a single chlorine atom.

Tri-CDDs exist with 3:0 and 2:1 chlorine substitution. 1,2,4-Tri-CDD (Figure 3 d, 3:0 substitution) yields ions at m/e 76, 52 etc. from the non-halogenated (4 H) carbon ring. The tri-halogenated (1 H) carbon ring yields a characteristic ion at m/e 108 (C_6HCl^+). This ion is found in the mass spectra of all PCDDs containing a tri-halogenated carbon ring.

2,3,7-Tri-CDD (Figure 3 e, 2:1 substitution) yields ions at m/e 75 ($C_6H_3^+$), 51 ($C_4H_3^+$) etc. from the mono-halogenated (3 H) carbon ring. Peaks which appear at m/e 109 ($C_6H_2Cl^+$, minor), 97 ($C_5H_2Cl^+$) and 74 ($C_6H_2^+$) are characteristic of a di-halogenated (2 H) carbon ring.

Tetra-CDDs

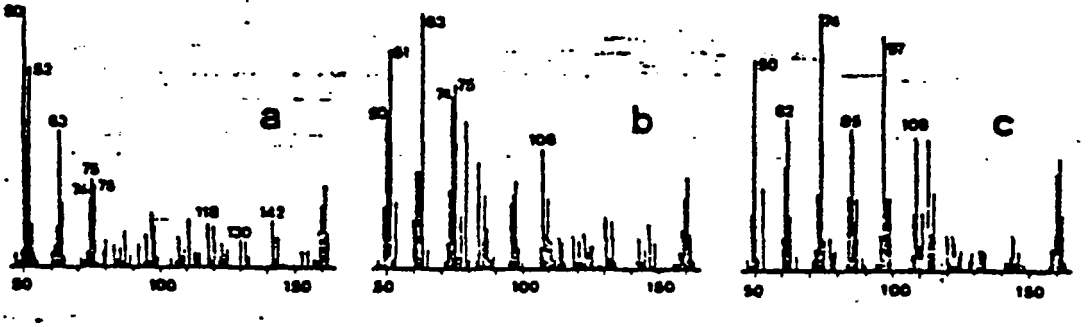

Figure 4 Partial mass spectra (m/e 45-165) of tetra-CDDs. a) 1,2,3,4-(4:0), b) 1,2,3,8-(3:1) and c) 2,3,7,8-tetra-CDD (2:2).

A total of 22 tetra-CDDs exists with 4:0, 3:1 and 2:2 chlorine substitution. The mass spectrum of 1,2,3,4-tetra-CDD (Figure 4 a) is from the only isomer of the 4:0 group. Ions at m/e 74-76 and 50-52 are indicative of a non-halogenated (4 H) carbon ring. The fully halogenated carbon ring yields chlorine containing ions at m/e 142 ($C_6Cl_2^+$), 130 ($C_5Cl_2^+$) and 118 ($C_4Cl_2^+$); these ions are found in the mass spectra of all PCDDs containing a fully halogenated carbon ring.

1,2,3,8-Tetra-CDD does belong to a group of 8 isomers with 3:1 chlorine substitution. Its mass spectrum (Figure 4 b) shows ions at m/e 75 ($C_6H_3^+$), 51 ($C_4H_3^+$) etc. from the mono-halogenated (3 H) carbon ring. The tri-halogenated (1 H) carbon ring yields the characteristic ion at m/e 108 (C_6HCl^+), common to all PCDDs containing this substitution.

The largest group of all PCDDs are the tetra-CDDs with 2:2 chlorine substitution (13 isomers). Mass spectra of 7 isomers of this group were studied and showed only minor differences. In Figure 4 c, the partial mass spectrum of 2,3,7,8-tetra-CDD is reported. The di-halogenated (2 H) carbon rings yield the characteristic ions at m/e 109 ($C_6H_2Cl^+$), 97 ($C_5H_2Cl^+$), 74 ($C_6H_2^+$) and 50 ($C_4H_2^+$); the ion at m/e 74 was the most intense ion in this mass range of all isomers of this group.

Penta- and hexa-CDDs

Penta-CDDs exist with 4:1 (2 isomers) and with 3:2 chlorine substitution (12 isomers). In Figure 5 a, we present a partial mass spectrum of 1,2,3,4,7-penta-CDD, an isomer with 4:1 chlorine substitution. The mono-halogenated (3 H) carbon ring is indicated by intense ions at m/e 75 ($C_6H_3^+$), 51 ($C_4H_3^+$) etc. The fully halogenated carbon ring is indicated by the characteristic ion at m/e 142 ($C_6Cl_2^+$).

The mass spectrum of 1,2,4,7,8-penta-CDD (Figure 5 b) shows ions at m/e 109 ($C_6H_2Cl^+$), 97 ($C_5H_2Cl^+$), 74 ($C_6H_2^+$) and 50 ($C_4H_2^+$) due to the di-halogenated (2 H) carbon ring. The tri-halogenated (1 H) carbon ring is indicated by the intense ion at m/e 108 (C_6HCl^+) and the ion at m/e 96 (C_5HCl^+).

Hexa-CDDs exist in two groups: 6 isomers with 3:3 and 4 isomers with 4:2 chlorine substitution. Four isomers of the first group and all 4 of the second group were available. Isomers of the same group yield virtually identical mass spectra. In Figure 5 c, the mass spectrum of 1,2,3,4,6,8-hexa-CDD (4:2) is shown. The di-halogenated (2 H) carbon ring is indicated by m/e 109 ($C_6H_2Cl^+$), 97 ($C_5H_2Cl^+$) and the intense ion at m/e 74 ($C_6H_2^+$) and 50 ($C_4H_2^+$); the fully halogenated carbon ring is indicated by the characteristic ion at m/e 142 ($C_6Cl_2^+$).

In Figure 5 d, the mass spectrum of 1,2,3,6,7,8-hexa-CDD, an isomer with 3:3 chlorine substitution, is shown. Tri-halogenated (1 H) carbon rings are indicated by ions at m/e 145 ($C_6HCl_2^+$), 131 ($C_5HCl_2^+$) and the very intense ion at m/e 109 (C_6HCl^+). Isomers of this group are easily distinguished from those having a 4:2 chlorine substitution pattern.

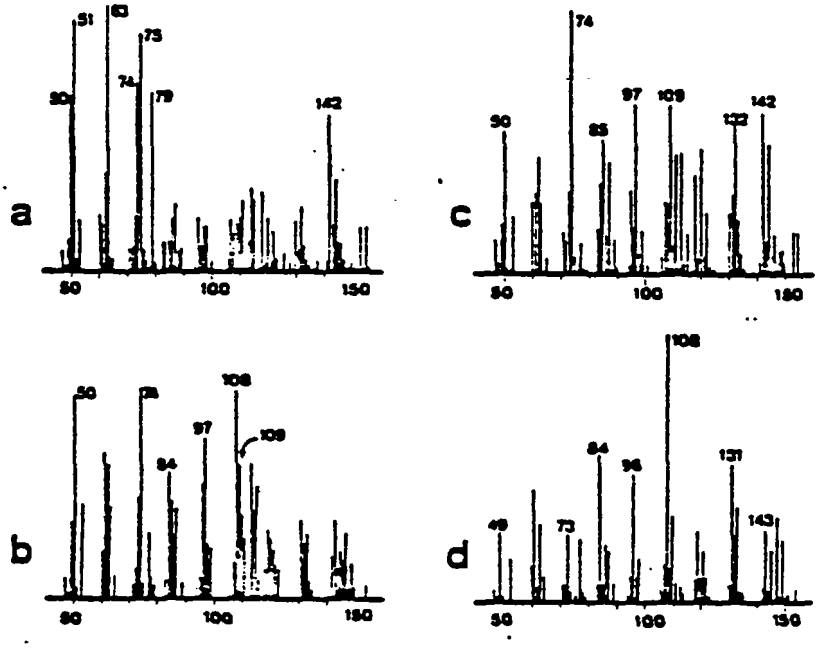

Figure 5 Partial mass spectra (m/e 40-150) of penta- and hexa-CDDs. a) 1,2,3,4,7-(4:1) and b) 1,2,4,7,8-penta-CDD (3:2); c) 1,2,3,4,6,8-(4:2) and d) 1,2,3,6,7,8-hexa-CDD (3:3).

Hepta- and octa-CDDs

Hepta-CDDs (2 isomers with 4:3 chlorine substitution) and octa-CDD (4:4) are sufficiently characterized by their higher mass ions. Nevertheless, their lower range mass spectra are included for completeness. The features outlined for the lower PCDDs are further validated by the mass spectra of the hepta- and octa-CDDs (Figure 6).

Both hepta-CDD isomers yield virtually identical mass spectra with the tri-halogenated (1 H) carbon rings indicated by the intense ion at m/e 108 (C_6HCl^+); the fully halogenated carbon ring is indicated by the ions at m/e 142 ($C_6Cl_2^+$) etc.

In case of the octa-CDD, the ion at m/e 142 ($C_6Cl_2^+$) becomes the largest peak in the lower mass range. Its presence and that of ions at m/e 130, 118 etc. is indicative of fully halogenated carbon rings.

Figure 6 Partial mass spectra (m/e 45-160) of a) 1,2,3,4,6,7,8-hepta-CDD and b) octa-CDD.

Key low mass ions of PCDDs

In Table 3, we summarise some of the characteristic ions of PCDDs in the lower mass range, that are indicative of the differently chlorinated carbon rings and allow a determination of the chlorine substitution pattern of an individual PCDD isomer.

Table 3 Characteristic ions indicative of the different chlorinated carbon rings of PCDDs

Number of chlorines on carbon ring	m/e Values and composition of some characteristic ions [a]		
0	76 ($C_6H_4^+$) 52 ($C_4H_4^+$)		
1	75 ($C_6H_3^+$) 51 ($C_4H_3^+$)	110 ($C_6H_3Cl^+$)	
2	74 ($C_6H_2^+$) 50 ($C_4H_2^+$)	109 ($C_6H_2Cl^+$) 97 ($C_5H_2Cl^+$) 85 ($C_4H_2Cl^+$)	
3		108 (C_6HCl^+) 96 (C_5HCl^+) 84 (C_4HCl^+)	143 (C_6HCl_2 131 ($C_5HCl_2^+$)
4		107 (C_6Cl^+) 95 (C_5Cl^+)	142 ($C_6Cl_2^+$) 130 ($C_5Cl_2^+$) 118 ($C_4Cl_2^+$)

[a] major ions underlined

Applications

PCDDs in fly ash

PCDDs and PCDFs ranging from the tetra- to the octachloro compounds were identified in several fly ash samples from a municipal incinerator and an industrial heating facility.[2] The major PCDDs were identified as isomers formed in the pyrolysis of the most common polychlorophenates (2,4,6-tri-, 2,3,4,6-tetra- and pentachlorophenate).[9] These identifications were based on chlorine substitution patterns from mass spectral data reported on here and by comparison of retention times of reference PCDDs on high-resolution glass capillary columns. In Figure 7 is a mass fragmentogram of a fly ash sample from an industrial heating facility showing the elution of tetra-, penta- and hexa-CDDs. The two major tetra-CDDs had 2:2 chlorine substitution (1,3,6,8- and 1,3,7,9-tetra-CDD from 2,4,6-trichlorophenate). A minor tetra-CDD had also the 2:2 substitution pattern and the same retention time as the extremely toxic 2,3,7,8-tetra-CDD. The third major tetra-CDD, eluting immediately after the 2,3,7,8-isomer, could now be identified as 1,2,3,8-(or 1,2,3,7-) tetra-CDD. This isomer with 3:1 chlorine substitution is a condensation product in the pyrolysis of 2,4-di- and 2,3,4,6-tetrachlorophenate.

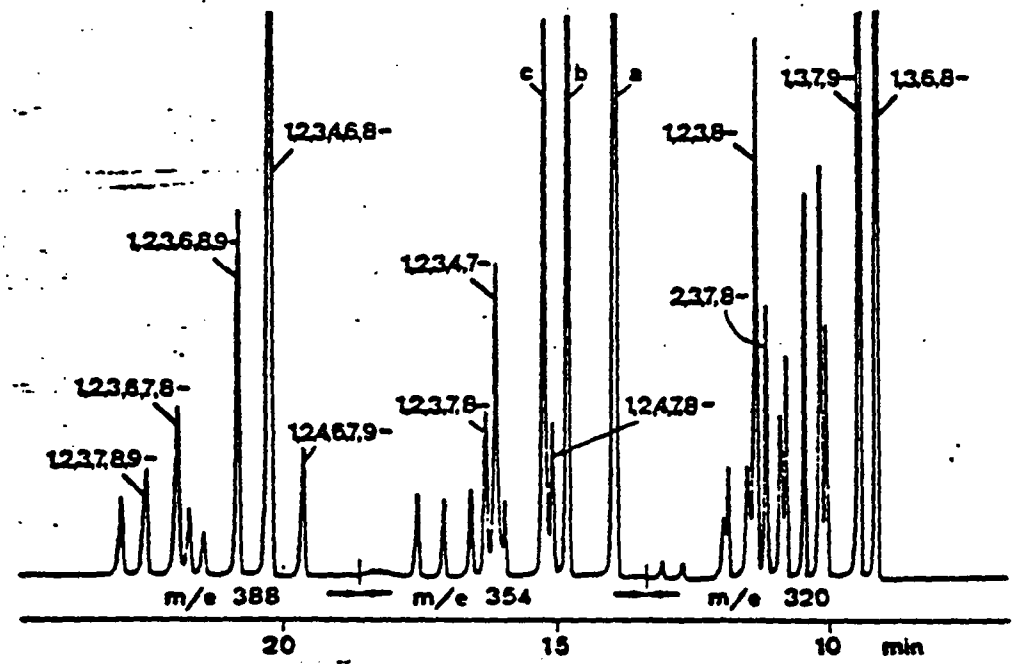

Figure 7 Mass fragmentogram (m/e 320, 354 and 368) of a fly ash sample showing elution of tetra-, penta- and hexa-CDDs; 50 m OV-17 glass capillary column, 200-240°C, 2°/min; sample injection in tetradecane.

The three major penta-CDDs (peaks a, b and c, Figure 7) found in fly ash had a 3:2 chlorine substitution pattern. They were the condensation products in the pyrolysis of 2,4,6-tri- and 2,3,4,6-tetrachlorophenate. An additional penta-CDD had a 4:1 chlorine substitution pattern and an identical retention time as 1,2,3,4,7-penta-CDD. This isomer is the condensation product in the pyrolysis of 2,4-di- and pentachlorophenate. In case of the hexa-CDDs, the main isomer had a 4:2 chlorine substitution pattern. It was identified as 1,2,3,4,6,8-hexa-CDD, a condensation product formed in the pyrolysis of 2,4,6-tri- and pentachlorophenate. Four additional hexa-CDDs in fly ash had the 3:3 chlorine substitution pattern. They were the dimerization products formed in the pyrolysis of 2,3,4,6-tetrachlorophenate (1,2,4,6,7,9-, 1,2,3,6,8,9-, 1,2,3,6,7,8- and 1,2,3,7,8,9-hexa-CDD).

PCDDs in Seveso soil

Di-, tri- and tetra-CDDs were observed in the soil at Seveso, Italy after an accident at a chemical plant producing 2,4,5-trichlorophenol.[8] As reported, the major tetra-CDD found had an identical mass spectrum (2:2 chlorine substitution) and it did co-chromatograph with 2,3,7,8-tetra-CDD on several different glass capillary columns. No identifications of other PCDDs were given in that report. It was now shown that the minor tetra-CDD present at an approximate level of 4% relative to the 2,3,7,8-isomer has a 2:2 chlorine substitution pattern, indicating its formation from trichlorophenates, presumably from 2,4,5- and an isomeric trichlorophenate impurity present or formed in the reactor at the plant. Its formation was confirmed by microscale pyrolyses with mixtures of 2,4,5- and other trichlorophenates (2,3,4-, 2,3,5-, 2,3,6- and 2,4,6-tri-CP). The main condensation product of 2,4,5- and 2,3,5- or 2,4,6-trichlorophenate (1,3,7,8-tetra-CDD) had an identical retention time (50 m OV-17 at 200°C) as the minor tetra-CDD found in Seveso soil.

The di- and tri-CDDs present in this soil had 1:1 and 2:1 chlorine substitution patterns, respectively. The di-CDD did co-chromatograph with 2,7- and 2,8-di-CDD, two isomers not separated on a 50 m OV-17 column under the conditions used. The tri-CDD did co-chromatograph with the 2,3,7-substituted isomer. Di- and tri-CDDs could have been formed in the reactor itself, possibly from tri- and tetrachlorobenzene, or by photolysis of 2,3,7,8-tetra-CDD in the environment. An analysis of the reactor content left after the accident could give the final answer.

PCDDs from photolysis of octa-CDD

Dechlorination to lower PCDDs was shown to occur by UV-photolysis of octa-CDD in organic solvents.[10,11] The major hepta-CDD formed was the 1,2,3,4,6,7,9-substituted isomer, indicating a preferential loss of lateral (2,3,7- or 8-) chlorine atoms.[11] The photolysis products were now analyzed to determine the chlorine substitution patterns of the lower PCDDs formed. The major hexa-CDD formed had a 3:3 pattern and an identical retention time as

1,2,4,6,7,9- (or 1,2,4,6,8,9-) hexa-CDD; two minor isomers had 3:3 and 4:2 chlorine substitution patterns. A major and a minor penta-CDD were formed both having 3:2 chlorine substitution patterns; the major is expected to be the 1,2,4,6,9- substituted isomer. In case of the tetra-CDDs, a major and a minor isomer were formed, both with 2:2 chlorine substitution. The major tetra-CDD did co-chromatograph with 1,4,6,9-tetra-CDD formed in the pyrolysis of 2,3,6-trichlorophenate. Sunlight photolysis of octa-CDD did yield the same isomers. The reaction scheme deduced from the data above shows that chlorine atoms are removed preferably from a lateral position on the carbon ring with the higher chlorine substitution. It further indicates that the extremely toxic 2,3,7,8-tetra-CDD is not likely being formed from the photolysis of higher PCDDs.

PCDDs in commercial pentachlorophenol

A series of commercial PCP and PCP-Na samples was analyzed for the presence of PCDDs and PCDFs.[12] Samples with high PCDD content showed, in addition to hepta- and octa-CDD, the presence of 3 hexa-CDDs in nearly constant isomeric ratios. These 3 isomers had 3:3 chlorine substitution patterns and were identified as 1,2,4,6,7,9- (or 1,2,4,6,8,9-), 1,2,3,6,8,9- (or 1,2,3,6,7,9-) and 1,2,3,6,7,8-hexa-CDD. Unexpectedly, some PCP-Na samples showed the presence of a tetra-CDD at levels of 0.1 to 0.3 ppm. This isomer had a 4:0 chlorine substitution pattern and identical retention times on several glass capillary columns as 1,2,3,4-tetra-CDD, a very unsymmetrical isomer. At present, we have no explanation for the presence of this isomer.

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79-0425. Kitchin, K. T.; Woods, J. S.* (Lab. Environ. Toxicol., NIEHS, Research Triangle Park, NC 27709) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin induction of aryl hydrocarbon hydroxylase in female rat liver. Evidence for *de novo* synthesis of cytochrome P-448. *Mol. Pharmacol.* 14(5): 890-899; 1978. (54 references)

The effects of 2,3,7,8-tetra chlorodibenzo-*p*-dioxin (TCDD) on heme synthesis and the synthesis of cytochrome P448, as reflected in aryl hydrocarbon hydroxylase activity, were determined in female rat liver. TCDD treatment did not increase the activity of δ-aminolevulinic acid synthetase, the rate limiting enzyme in heme biosynthesis. In contrast, a 23-fold increase in the level of cytochrome P448 mediated aryl hydrocarbon hydroxylase was observed 24 hr after TCDD treatment. Actinomycin D and cycloheximide, but not $CoCl_2$, completely prevented the induction of new cytochrome P448 and increased aryl hydrocarbon hydroxylase activity by TCDD. *In vitro* reconstitution of cytochrome P450 and increases in enzymatically active aryl hydrocarbon hydroxylase levels were achieved by adding hemin to whole liver homogenates from rats given TCDD (a hemoprotein inducer) and $CoCl_2$ (a heme depleter). These results suggest that TCDD induces *de novo* protein synthesis of apocytochrome P448, which combines with heme to yield new cytochrome P448 and a concommitant increase in aryl hydrocarbon hydroxylase activity. Therefore it is concluded that TCDD selectively induces the formation of cytochrome P448 leading to increased aryl hydrocarbon hydroxylase activity, and that protein synthesis, rather than heme synthesis, is the rate-limiting event in this process. (Author abstract by permission of Academic Press)

PESTICIDES ABSTRACTS

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A REVIEW OF THE GENETIC TOXICOLOGY OF CHLORINATED DIBENZO-*p*-DIOXINS *

J.S. WASSOM [1], J.E. HUFF [2,3] and N. LOPRIENO [4]

[1] *Environmental Mutagen Information Center,* [2] *Biomedical Sciences Section, Information Center Complex/Information Division, Oak Ridge National Laboratory, Oak Ridge, TN 37830 (U.S.A.);* [3] *Unit of Chemical Carcinogenesis, International Agency for Research on Cancer, 69372 Lyon Cedex 2 (France); and* [4] *Laboratory of Genetics, Institute of Anthropology, Pisa University, 56100 Pisa (Italy)*

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Summary

Information from both published and unpublished sources considered relevant to the understanding of the genetic toxicology of chlorinated dibenzo-*p*-dioxins is summarized in this review. Interest in writing this paper was stimulated by the fact that this class of compounds, particularly 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), has gained notoriety as an extreme environmental and industrial hazard. The potential for human exposure occurs in the work place when dioxins are formed during the synthesis of a number of commercially important compounds such as 2,4,5-trichlorophenoxyacetic acid, hexachlorophene, and pentachlorophenol. Environmental contamination may result from manufacturing processes and from dioxin contaminants in marketed products.

Research on dioxins as potential mutagens was initiated because of their structural similarity to acridines, a class of known intercalating agents. To date, only 4 dioxin compounds have been evaluated for mutagenicity: the di-, tetra-, and octa-chlorinated derivatives and the unsubstituted dibenzo-*p*-dioxin. Since only a few of the many possible structural forms of dioxins have been tested, no definite conclusions can be made about their potential mutagenicity. Furthermore, the positive mutagenicity and cytological effects reported thus far

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Abbreviations: BP, benzo[*a*]pyrene; DCDD, 2,7-dichlorodibenzo-*p*-dioxin; OCDD, octachlorodibenzo-*p*-dioxin; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCPE, 2,4,5-thrichlorophenoxyethanol.

with the few dioxin isomers examined seems to depend on the position of chlorine substitution. The most active form of the molecule is the 2,3,7,8-derivative (TCDD).

Data available for assessing the mutagenic potential of TCDD are conflicting and scarce. Differences in testing results reported in these studies could be attributed to solubility problems with the test chemical, treatment protocols, purity of test samples, or toxicity. Because there are conflicting data, additional experiments are needed before the mutagenic potential of TCDD and other dioxins can be determined. Studies exploring the promoting effect of dioxins on the mutagenicity of other compounds are also recommended because experiments have shown TCDD to be an extremely active liver enzyme inducing agent that enhances the mutagenicity of certain polycyclic hydrocarbons such as 3-methylcholanthrene in vitro.

The importance of discerning the hazards to human health from dioxin compounds became apparent after an accidental release of TCDD from a chemical plant contaminated the Seveso, Italy area in July 1976 *. This accident revealed that insufficient data were available to properly evaluate the long-term health risks posed by dioxin compounds. Several research projects were therefore initiated after the Seveso incident; it is hoped that many of the questions concerning the mutagenicity of TCDD and possibly of other dioxin congeners will be answered as a result of this work.

Introduction

Chlorinated dibenzo-*p*-dioxins are a group of chemical compounds which are among the most toxic and hazardous pollutants in the environment. These compounds, collectively referred to as dioxins, are impurities associated with certain end products resulting from the treatment of chlorinated benzenes at elevated temperature and pressure under alkaline conditions. The most notable contaminant of this group is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (See Fig. 1) which may be formed along with other dioxin compounds during the manufacture of several commercially important products such as the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) [25], the fungicide pentachlorophenol [39], and the germicide hexachlorophene [39].

Suspicions of the possible long-term health hazards of dioxins arose after it was found that 2,4,5-T was teratogenic in the rat and mouse [15]. Shortly thereafter it was discovered that the 2,4,5-T sample used in this study contained about 30 ppm TCDD [15]. It was primarily the report implicating TCDD as a contaminant of 2,4,5-T [15] that led to its further evaluation for teratogenicity [16] and its eventual testing for mutagenicity [21]. These and other similar reports published during the late 1960's and early 1970's also

* In January 1978, a joint International Agency for Research on Cancer (IARC)/US National Institute of Environmental Health Sciences ad hoc Working Group was convened on the Coordination of Epidemiological Studies on the Long-Term Hazards of Chlorinated Dibenzodioxins and Chlorinated Dibenzofurans. The participants recommended that IARC coordinate the follow-up of the accident-exposed individuals from five countries (IARC International Technical Report No. 78/001, 1978).

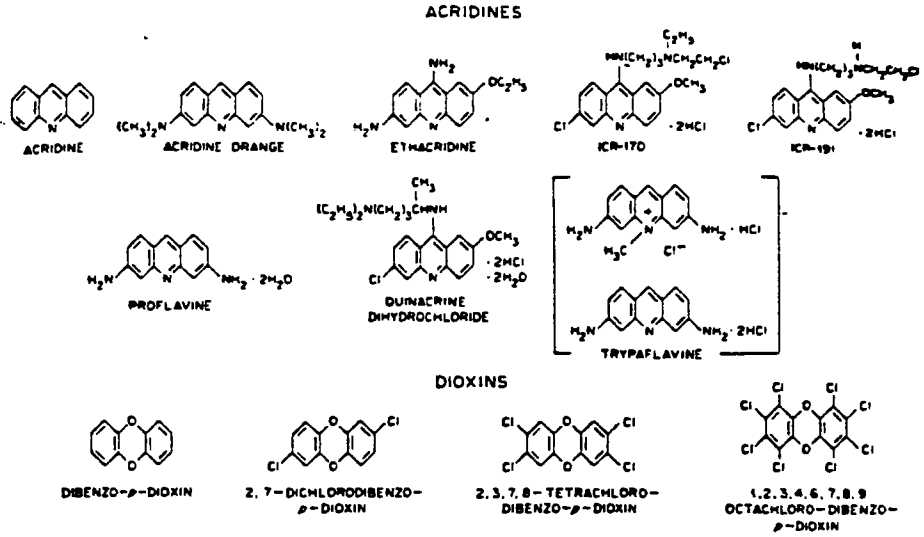

Fig. 1. Structural comparison of acridines and dioxins.

stimulated toxicological studies on other dioxin derivatives as well as studies dealing with issues such as environmental contamination and movement and analytical detection of these compounds. Detailed state-of-the art reviews summarizing work in these areas have been published [35—37,39] or are currently being prepared for publication [10,20]. Since the question of potential mutagenicity, carcinogenicity, and teratogenicity of dioxins has been raised as a result of the incident in Seveso, Italy * [51,69], we thought it important to review the information available on the genetic toxicology of these compounds.

The only theory proposed to explain how dioxins may exert their mutagenic effect is the suggestion that these compounds intercalate DNA. This mechanism was proposed due to the similarity in structure between dioxins and acridines (see Fig. 1); therefore, information comparing the mutagenicity of dioxins and acridines has been included (see "Comparative Mutagenicity of Dioxins and Acridines").

The basic dibenzo-*p*-dioxin nucleus is nearly planar and has 8 possible points of chemical substitution. From the monochloro- to the octachloro-dibenzo-*p*-dioxin, a variety of derivatives are possible and some have proven to be chemically persistent and biologically active [30]. The lethal dose and toxic manifestations are structure- and species-dependent. Of all the possible dioxin structural configurations, TCDD is the most widely known and tested. This derivative has been called one of the most potent small molecule toxins known [30]. Because of its extreme toxicity and potential mutagenicity, carcinogenicity **,

* Seveso, Italy (population approx. 17,000) is located near the city of Milan. In July 1976, the Seveso area was accidentally exposed to high levels of TCDD as a result of an explosion in a nearby chemical plant. For a comprehensive description of this accident, the reader may wish to consult the book by John G. Fuller entitled "The Poison That Fell from the Sky", Random House, New York, 1977.

** At least 24 long-term experimental carcinogenicity studies are in progress on various chlorinated dioxins (M-J. Ghess, H. Bartsch, J.E. Huff, and L. Tomatis, IARC Information Bulletin on the Survey of Chemicals Being Tested for Carcinogenicity, Number 7, Lyon, January 1978, 460 pages).

and teratogenicity [16], laboratory use should be carefully controlled and a rigid safety protocol followed [8].

There are several general articles which refer to the possible mutagenicity of dioxins [4—6,68], but the bulk of the material reviewed came from a number of published papers that contain experimental results [7,13,17,27,28,38,40,41, 43,57,64] describing the evaluation of these compounds for mutagenic and/or related cytological effects. Information from unpublished sources is also included [9,49]. Summaries of all these investigations are presented in the following section.

Genetic toxicology testing

Jackson [40] evaluated highly purified samples of 2,4,5-T * and TCDD for cytological effects in the African blood lily (*Haemanthus katherinae* Baker). Treatments involving both compounds in varying proportions were studied. In contrast to the no-effect result with a highly purified sample of 2,4,5-T, dramatic inhibition of mitosis was observed in cells exposed either to 10^{-4} molar 2,4,5-T containing 0.2—1.0 μg TCDD per liter of water ** or to a 10^{-4} molar solution of 2,4,5-T containing an unknown level of TCDD as a contaminant. Similar results were obtained when treatments were limited to TCDD alone (0.2 μg and 1.0 μg TCDD per liter of water **). These treatments also induced formation of dicentric bridges and chromatin fusion with formation of multinuclei or a single large nucleus. Because these effects were not evident in the pure 2,4,5-T sample, Jackson concluded that the cytological effects produced were due to the TCDD contaminant.

Dävring and Summer [18] reported results obtained with a commercial sample of 2,4,5-T in which dioxin contamination was less than 0.1 ppm. This formulation was evaluated for cytological effects in a wild-type *Drosophila melanogaster* population by exposing adult flies, 24 h after eclosion, to 250 ppm 2,4,5-T in food. Results indicated that this 2,4,5-T formulation affected early oogenesis and caused sterility. However, it was not unequivocally stated that the observed sterility was of genetic origin.

The formation of multinucleated cells after treatment with TCDD has also been observed in mammals [12,29,42]. For example, Greig et al. [29] treated female Porton rats with single oral doses (50—400 μg/kg) of TCDD dissolved in dimethylsulfoxide or arachis oil. Histological examination of liver cells 60 days after treatment with 100 μg/kg TCDD revealed that parenchymal cell structures were altered and many were multinucleated. No mitoses were observed in any of these multinucleated cells. The only other abnormality detected was an occasional pyknotic nucleus. These results were interpreted as indicating that TCDD had interfered with the capacity of liver cells to maintain their correct morphology, thus leading to death and/or structural disorganization. Buu-Hoi

* Further information regarding the cytological effects produced by 2,4,5-T will be available soon in a paper entitled "The Genotoxic Effects of 2,4,5-T" being prepared for publication in Mutation Res. by W.F. Grant.

** Jackson [40] reported that the maximum solubility of TCDD in water was 0.2 μg/l and that the 1.0 μg/l treatments were therefore probably subjected only to a water-saturated solution (0.2 μg/l).

et al. [12] have reported similar observations in liver and myocardial cells of Wistar rats after single intraperitoneal injections of TCDD (10 mg/kg). Khera and Ruddick [41] observed that mitotic figures in myocardial cells were rare in rats treated in utero during days 6—15 of gestation with 250—2000 μg/kg of 2,7-dichlorodibenzo-*p*-dioxin per day. Kimbrough et al. [42] have also reported multinucleated cell formation in the livers of New Zealand rabbits whose ears had been painted daily for 4 days with 0.2 ml of a 20 μg/ml TCDD solution.

Vos, Moore, and Zinkl [67] have suggested that TCDD could be a hepatocarcinogen due to its specific cytological effect on the proliferating cells of the liver. When 58-week-old male C57B1/6 mice were treated with single oral doses of TCDD (100, 150—200 μg/kg), an enlargement of liver cell nuclei was induced. The LD_{50} in these studies was calculated to be 114 μg/kg. In subacute studies, 100 4-month-old male mice were divided into two groups and either treated for two or six weeks with weekly TCDD doses of 0, 0.2, 1.0, 5.0, or 25 μg/kg. Liver cells of mice treated with six doses of 25 μg/kg showed polyploidy, vacuolization of nuclei, and an increased mitotic rate.

Green and Moreland [27] were the first to test dioxins (TCDD, 2,7-dichlorodibenzo-*p*-dioxin, and the unsubstituted dibenzo-*p*-dioxin) for their ability to induce chromosome aberrations in mammalian cells. In one of their experiments [27] the dioxin compounds were dissolved in dimethylsulfoxide and doses of 10 μg/kg were administered daily by intubation to male rats for a 5-day period. Analyses of bone marrow preparations from animals sacrificed 6 h after the last treatment were negative. In a second experiment [27], TCDD was dissolved in an anisole/corn oil solvent (1.5% v/v) and administered intraperitoneally to rats in doses of 5, 10 or 15 μg/kg. Another group of test animals received 20 μg/kg of TCDD orally from the same test solution. Animals receiving the higher doses, 15 μg/kg intraperitoneally and 20 μg/kg orally, were killed 29 days after treatment. Again, bone-marrow preparations revealed no evidence of chromosomal aberrations in any of the animals. As a result of these two studies [27], the authors concluded that the test compounds (TCDD, 2,7-dichlorodibenzo-*p*-dioxin and dibenzo-*p*-dioxin) appeared to possess no potential for producing chromosome aberrations in the bone marrow of male rats. A group of positive controls treated with triethylenemelamine (0.375 mg/kg), administered intraperitoneally and orally, and used as positive controls showed a statistically significant increase in chromosome abnormalities over untreated controls. In contrast with these earlier results, Green, Moreland and Sheu [28], in a later study, found that TCDD significantly increased the number of chromosome aberrations in rat bone marrow when a different experimental protocol was used. In this experiment [28], male and female Osborne—Mendel rats received TCDD doses of 0.25, 0.5, 1.0, 2.0 or 4.0 μg/kg by gavage twice weekly for 13 weeks. The TCDD samples used were dissolved in a solvent consisting of one part acetone to 9 parts corn oil. Bone marrow from the treated animals was assayed for mitotic inhibition and chromosome aberrations. No change in the mitotic index was observed at any dose, however, an effect was found in cells from both male and female animals when assayed for chromosome breaks. The treated female groups showed a significant increase at the 4 μg/kg level as compared with the 0.25 μg/kg level ($P < 0.01$). The treated male group showed a significant increase at the 2 μg and 4 μg/kg

levels as compared with the 0.25 μg/kg level ($P < 0.01$). The authors cautioned that even though the results were significant, the observed activity should be regarded as only weakly positive.

Czeizel and Király [17] compared the frequency of chromosome aberrations in the peripheral lymphocytes of 76 workers employed at a chemical herbicide producing factory in Budapest (Hungary), along with that of 33 control individuals. The manufacturing process at this factory favored the formation of dioxins. Of the workers surveyed, 36 had been exposed to 2,4,5-trichlorophenoxyethanol (TCPE) or Klorinol and 26 to Buvinol (a combination herbicide containing TCPE and 2-chloro-6-ethylamino-4-isopropylamino-1,3,5-triazine). The remaining 14 workers had never been engaged in the production or use of either of these herbicides. TCDD contamination found in the final herbicide products has been reported to be less than 0.1 mg/kg and generally not more than 0.05 mg/kg [66]. The frequency of chromatid-type and unstable chromosome aberrations found by Czeizel and Király [17] was higher ($P < 0.001$) in the factory workers than in the controls, regardless of whether or not they had been directly involved in production of the herbicides. However, aberrations were more frequent in workers preparing TCPE and Buvinol than in the other factory workers, but the difference was significant only for the chromatid-type effect. Chromosome aberrations have also been reported in studies of Vietnamese populations exposed to the herbicide 2,4,5-T in which TCDD was present as a contaminant [32]. In these studies, a higher incidence of chromosomal abnormalities was reported in individuals brought in contact with this herbicide as a result of forest defoliation. These studies, however, have been criticized as being statistically unsound (see Hay [31,32]).

Preliminary results of a cytogenetic investigation of TCDD-exposed individuals from the Seveso, Italy population have been reported by Tenchini et al. [64]. Examinations of the affected population were confined to women who became pregnant immediately before or after exposure to TCDD and who had chosen to abort because of the possible teratogenic effects of this compound. Chromosome analyses of maternal peripheral blood and abortive fetal tissue were scored for the presence of numerical and structural chromosome variations; no significant change was found in the chromosome number of any sample analyzed. Also, no significant increase was observed in the frequency of structural chromosome aberrations from maternal blood samples. There was, however, a higher number of structural aberrations in the fetal tissues than in maternal blood samples or fibroblasts from adult tissues, but the frequency of these aberrations did not appear to be greater than that expected to occur spontaneously in cultures of comparable cell types. Tenchini et al. [64] pointed out that these preliminary data do not answer the question of whether the higher frequencies of chromosome aberrations found in fetal tissues were due to chromosome damage caused by TCDD exposure. In another preliminary cytogenetic study related to the Seveso incident [46], no chromosome abnormalities were found in the peripheral blood cells of 90 workers selected from the chemical plant which was the source of the TCDD contamination in Seveso. Likewise, no abnormalities were found in chromosomes from peripheral blood cells of Seveso residents located in the area most severely contaminated with TCDD [46]. Hay [31] has cited results from a study done at another industrial

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concern regarding the frequency of chromosome abnormalities in workers presumably exposed to TCDD during the production of 2,4,5-trichlorophenol. In this study, 800 workers with chloracne presumably caused from TCDD exposure were investigated and the frequency of chromosome abnormalities determined. The abnormalities observed among these workers were reported to be not greater than the statistical norm.

TCDD was first evaluated for mutagenicity by Hussain et al. [38] because of its structural similarity to acridines (Fig. 1). The assay systems used were (1) *Escherichia coli* strain Sd-4 which measures reversion from streptomycin dependence to streptomycin independence, (2) *Salmonella typhimurium* strains TA1530 and TA1532 which measure reversion from histidine dependence to independence, and (3) prophage induction in *Escherichia coli* K39 cells. TCDD was mutagenic in *E. coli* Sd-4 and in Salmonella TA1532. TCDD had a weak prophage-inducing effect and did not show any mutagenic activity in Salmonella TA1530. Parallel studies with acridine orange in *E. coli* Sd-4 and acridine mustard in Salmonella TA1532 were used as positive controls. Corollary results with these control compounds, coupled with the well-documented knowledge that Salmonella strain TA1532 detects frameshift mutagens, seem to indicate that TCDD was mutagenic via intercalation with DNA.

Seiler [57] evaluated TCDD and octachlorodibenzo-*p*-dioxin (OCDD) in *Salmonella typhimurium* strains G46, TA1530, TA1531, TA1532, and TA1534 and found TCDD to be a strong mutagen in strain TA1532, a doubtful mutagen in TA1531 and TA1534, and a nonmutagen in G46 and TA1530. OCDD was reported as a questionable mutagen in strains TA1532 and TA1534 and as a nonmutagen in strains G46, TA1530 and TA1531 [57]. McCann [49] (personal communication) tested TCDD in the Salmonella system using a spot test and the standard plate test with strains TA1532, TA1535, TA1537, and TA1538, both with and without metabolic activation. In all experiments, negative results were obtained. The results [49] found with TA1532 are in conflict with those obtained by Seiler [57] in this strain. McCann's negative results with TA1532 cannot be directly compared with Hussain's et al. positive studies because these authors [38] used a liquid incubation procedure in their test protocol. The negative response which McCann [49] observed with the other Salmonella strains (TA1535, TA1537, and TA1538) adds further to the difficulty in understanding the potential mutagenicity of TCDD, especially since strains TA1537 and TA1538 are sensitive to the action of frameshift mutagens. Nebert, Thorgeirsson and Felton [53] have also reported negative findings with strains TA1535 and TA1538. The differences in results for TA1532 could be due to many factors, such as the solubility of the TCDD sample, its purity, treatment protocols, and the problems involved in handling such a toxic compound. Table 1 summarizes results from these studies.

Commoner [13] tested the unsubstituted dibenzo-*p*-dioxin in four of the Ames Salmonella strains (TA1535, TA1537, TA1538, and TA100) by the standard plate test to determine the reliability of the test system in distinguishing carcinogens from noncarcinogens. Negative results were reported for all strains with the concentrations tested (1.0, 10 and 100 μg per plate). Dimethylsulfoxide was used as the solvent in the preparation of all test samples in this study.

TABLE 1

MUTAGENICITY OF DIOXIN COMPOUNDS IN *Salmonella typhimurium* [a]

Dioxin isomer	Strains detecting base-pair substitutions [b]				Strains detecting frameshifts [b]					Reference No.
	G46	TA1530	TA1535	TA100	TA1531	TA1532	TA1534	TA1537	TA1538	
TCDD	0	0	—	0	0	—	0	—	—	49
	0	0	—	0	0	0	0	0	—	53
	0	—	0	0	0	+	0	0	0	38
	—	—	0	0	?	+	?	0	0	57
OCDD	—	—	0	0	—	?	?	0	0	57
Dibenzo-*p*-dioxin	0	0	—	—	0	0	0	—	—	13

[a] See also Table 4 for a description of the Salmonella strains.

[b] 0, not tested; —, negative results; +, positive results; ?, doubtful mutagen. Results shown were obtained using different experimental protocols.

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Further evidence for the possible intercalating reaction of TCDD with DNA has been reported by Kondorosi et al. [43]. These investigators checked TCDD and several electrophiles (methyl methanesulfonate, ethyl methanesulfonate, diepoxybutane, and ethylene dibromide) for their effect on the transfection of Qβ RNA. The loss of transfectivity in this system indicates a reaction with single-stranded nucleic acid. Intercalating agents which do not have active side-chains (e.g., alkylating groups) would be expected to give negative results in this test because they react primarily with double-stranded DNA. All the agents studied gave positive results except TCDD. Using TCDD at concentrations reported to be mutagenic in *Escherichia coli* Sd-4 and *Salmonella typhimurium* TA1532 [38], Kondorosi et al. [43] found no effect on the transfection of Qβ RNA. The authors stated that these results confirm the assumption that TCDD forms a physical complex (intercalation) with DNA rather than reacting chemically with the nucleic acid.

Toxicity and dominant lethal studies with TCDD have been reported by Khera and Ruddick [41]. Groups of 20 male Wistar rats were dosed orally with 4, 8 or 12 μg/kg TCDD per day for 7 days before mating (survival results are shown in Table 2). Surviving males were caged with two untreated virgin females for 5 days and this regimen was followed for 7 sequential mating trials. Females were killed 9 days after separation from the males and viable embryos, resorption sites, and corpora lutea were counted. No evidence was found for the induction of dominant lethal mutations during post-meiotic phases of spermatogenesis although the incidence of pregnancies from all mating trials in each treated group was reduced. Histological examination of surviving males showed normal testes, but the epididymides were inflamed with sperm granuloma formation which is analogous to changes seen in the autoimmune reaction following bacterial infection or the response of tissues to foreign bodies [41].

Other evidence that TCDD produces adverse effects in the testes has been reported by Van Miller and Allen [65]. In this study, male Sprague–Dawley rats were fed diets containing various levels of TCDD for 65 weeks. Animals receiving 0.05, 0.5 and 1.0 ppm of TCDD in their food died within 4 weeks, and autopsies showed a noticeable decrease in spermatogenesis. Seiler [58] reported that a 0.4 mg/kg dose of TCDD administered intraperitoneally to male mice produced an approximate 50% reduction in the rate of testicular DNA synthesis. TCDD and other dioxins have also been implicated in causing adverse effects in the testes of a number of other animals (mice [50], chickens [54], guinea pigs [50], and monkeys [54]). The fact that TCDD apparently reaches the testes [41,50,54,58,65] and the report that TCDD has a weak chromosome-breaking effect in rat bone-marrow cells [28] support, to some degree,

TABLE 2

SURVIVAL IN MALE WISTAR RATS AFTER TCDD TREATMENT [a]

Dose (μg/kg)	Dead animals	Survivors	Mean survival time (days)
12	20	0	17.7
8	11	9	20.1
4	2	18	36.5

[a] No mortalities were observed in the control group. Source: Khera and Ruddick [41].

TABLE 3

THREE-GENERATION REPRODUCTION STUDY OF MALE AND FEMALE SPRAGUE–DAWLEY RATS INGESTING TCDD

Daily dose ingested (μg/kg) [a]	Treatment time prior to mating (days)	Observations		
		F_0	F_1	F_2
0.1	90(F_0) 109–116 (F_1 and F_2) [b]	Decreased fertility; poor survival in F_1 offspring	c	c
0.01	90(F_0) 109–116 (F_1 and F_2) [b]	No effect on fertility; F_1 offspring and litter size appeared normal	Significant decrease in fertility; F_2 offspring had decreased survival rate and reduced growth rate; smaller litter size	Significant decrease in fertility; F_3 offspring had decreased survival rate and reduced growth rate; smaller litter size
0.001	90(F_0) 109–116 (F_1 and F_2) [b]	No effect on fertility	No effect on fertility	No effect on fertility

[a] Animals were maintained continuously on diets containing sufficient TCDD to provide the dose levels shown.

[b] Offspring comprising the F_1 and F_2 generations were potentially exposed to TCDD during gestation via placental transfer and during lactation. Animals from these groups began ingesting TCDD diets at 14–21 days of age and were mated at approximately 130 days of age.

[c] Due to adverse effects observed in F_0 parents, treatment was discountinued. Compiled from Murray et al. [52].

the possibility that TCDD may have the capacity to act as a weak dominant lethal agent. Further preliminary results reported by Murray et al. [52], in a three-generation reproduction study of Sprague–Dawley rats ingesting various levels of TCDD, adds some additional support to this possibility (Table 3). Multigeneration studies, however, are not proper indicators of dominant lethality, nor is the production of adverse testicular effects an indicator of mutagenicity. These data [52] and the data cited previously [41,50,54,58,65] suggest a need for further testing. Even with the present evidence and speculation, it is not clear whether the adverse effects produced by TCDD in the testes are the result of direct TCDD activity or the result of some other toxic response triggered by TCDD.

Modifying effects on mutagenesis

The importance of metabolism in mutagenesis studies has been considered because many compounds thought to be nonmutagenic are converted to mutagens after metabolic activation. Polycyclic hydrocarbons are a notable example. Inducible enzymes in various body organs, particularly the liver, are involved in the metabolic conversion of promutagens. Agents frequently used to increase these enzyme levels include Aroclor 1254 (a polychlorinated biphenyl), phenobarbital, and 3-methylcholanthrene.

TCDD has been reported to be a potent stimulant for hepatic aryl hydrocarbon hydroxylase activity in male rats (Sprague–Dawley), chick embryos (Leghorn), and female mice from several inbred strains (C3H/HeN, C57Bl/6J, and BALB/cJ) [55]. When compared with results obtained in the rat with

3-methylcholanthrene, TCDD is 30 000 times more potent in inducing aryl hydrocarbon hydroxylase activity [55]. Felton and Nebert [24] used liver fractions from C57B1/6N and DBA/2N mice pretreated with TCDD at doses ranging from 0.1 to 100 μg/kg to assay the in vitro mutagenicity of 3-methylcholanthrene and benzo[*a*]pyrene in the Ames Salmonella test sytem using strain TA1538. A significant increase in 3-methylcholanthrene mutagenesis was observed in test samples pretreated with liver fractions from TCDD-induced mice. The mutagenicity of 3-methylcholanthrene followed quite closely the hydroxylase activity and cytochrome P_1-450 (P 448) formation. The mutagenicity of benzo[*a*]pyrene was not affected when subjected to this same protocol.

Berry et al. [7] reported that TCDD appears to be an exceptionally potent and broad-spectrum transplacental inducing agent for carcinogen-transforming enzymes found in various tissues. Pregnant Sprague—Dawley rats were given single intraperitoneal injections of TCDD (0.2—6.0 μg/kg) dissolved in corn oil on day 17 of gestation. Animals were killed on day 20 of gestation, and homogenates were made of maternal livers, lungs, kidneys, adrenals, and placentas and of fetal livers, kidneys, and skin. These homogenates were then treated with [^{3}H]benzo[*a*]pyrene (BP) and were assayed for the effects of TCDD pretreatment on the tissue-mediated covalent binding of BP to DNA. Tissue samples from the livers, lungs, and placentas of TCDD-pretreated dams, as well as samples from livers, lungs, and skin of their fetuses, showed an increased capacity to covalently bind BP to DNA when compared with tissue samples taken from noninduced control animals. Berry et al. [7] stated that "The nature of the TCDD enhancement appeared to be tissue-specific, and moreover, the data from the binding in vitro appeared to correlate well with the metabolizing capabilities of the tissue and the metabolites formed (BP-7,8-dihydrodiol, BP-4,5-dihydrodiol, BP-9 phenol, and BP-3 phenol)".

The effect of TCDD on the covalent binding activity of benzo[*a*]pyrene to DNA and the mutagenicity of 3-methylcholanthrene raises the question of whether TCDD can influence the biological activity of not only other polycyclic hydrocarbons but other compounds as well. The significance of this possibility will not be fully understood until additional data are available.

Comparative mutagenicity of dioxins and acridines

The testing of TCDD for mutagenicity and its initial classification as a mutagen was brought about by its structural similarities to acridines (Fig. 1). The following sections summarize and compare the mutagenicity of acridine and dioxin compounds as measured in identical test systems. These results neither prove nor disprove the possibility that TCDD may act as an intercalating agent. The exact mode of action of TCDD and other dioxins will be clarified only after further experimentation.

Bacteria

Salmonella typhimurium

Tests with several genetically defined histidine mutants of *Salmonella typhimurium* are presently used in a variety of applications for the evaluation of

chemicals for mutagenicity [1–3] (Table 4). Salmonella strains used in the evaluation of TCDD, OCDD, and the unsubstituted dibenzo-*p*-dioxin are shown in Table 5, along with results for several acridine compounds.

Escherichia coli Sd-4

E. coli Sd-4 is a mutant derivative of *E. coli* B and is used to detect reversions from streptomycin-dependence to streptomycin-independence. Documentation of the molecular mechanism resulting in this mutation was not found but reportedly results from a base-pair substitution mutation [10]. See Table 6 for comparative results of TCDD with acriflavin.

Bacteriophages (prophage induction)

A search of the Environmental Mutagen Information Center data base revealed no information on the ability of acridines to activate *lambda* phage in *E. coli* K39 cells which were used to test TCDD [38]. Reports noting activation of *lambda* phage in another *E. coli* strain and negative findings for phage induction in *Salmonella thompson* were found and are shown in Table 7.

In an extensive review on prophage induction in lysogenic bacteria, Heinemann [33] listed acridine orange as having a moderate prophage-inducing ability. Hussain et al. [38] stated that TCDD had a weak prophage-inducing effect and that such results agreed with data obtained from studies with acridines. The data in Table 7 indicate that acridines, considered as a group, could be classed as weak when tested for prophage activation. Presently, insufficient data are available at this writing for a true comparison.

TABLE 4

CHARACTERISTICS OF THE AMES SALMONELLA TEST STRAINS USED TO EVALUATE THE MUTAGENICITY OF TCDD AND OTHER DIOXINS

Strain	Mutation (site in histidine operon)	Other characteristics	Type of mutation detected
G46	*his* G46	Normal lipopolysaccharide coat; DNA excision-repair present	Base substitution
TA1530	*his* G46	Partial lipopolysaccharide coat present; DNA excision-repair deficient	Base substitution
TA1531	*his* C207	Partial lipopolysaccharide coat present; DNA excision-repair deficient	Frameshift
TA1532	*his* C3076	Partial lipopolysaccharide coat present; DNA excision-repair deficient	Frameshift
TA1534	*his* D3052	Normal lipopolysacchride coat; DNA excision-repair present	Frameshift
TA1535	*his* G46	Lipopolysaccharide deficient; DNA excision-repair defective	Base substitution
TA1537	*his* C3076	Lipopolysaccharide deficient; DNA excision-repair defective	Frameshift
TA1538	*his* D3052	Lipopolysaccharide deficient; DNA excision-repair defective	Frameshift
TA100	*his* G46	Lipopolysaccharide deficient; DNA excision-repair defective; R factor plasmid present	Base substitution

Compiled from Ames et al. [1,2] and Simmon [60].

TABLE 5

MUTAGENICITY RESULTS WITH *Salmonella typhimurium* [a]

Chemical tested	Strains detecting base-pair substitutions [b]				Strains detecting frameshifts [b]				Reference No.
	his G46	TA1530	TA1535	TA100	TA1531	TA1532	TA1534	TA1537	TA1538	
TCDD	0	0	—	0	0	—	0	—	—	49
	0	—	0	0	0	+	0	0	0	38
	0	0	—	0	0	0	0	0	—	53
	—	—	0	0	?	+	?	0	0	57
OCDD	—	—	0	0	—	?	?	0	0	57
Dibenzo-*p*-dioxin	0	0	—	—	0	0	0	—	—	13
Trypaflavine	—	0	—	0	0	0	0	+	+	34,56
Quinacrine	—	—	0	0	—	+	—	0	0	11,14,44
Proflavine	—	0	0	0	0	0	0	0	+	11,48
ICR-170 [c]	—/+	0	0	0	0	+	0	+	0	11,38,45,48
ICR-191	—	—	0	0	+	+	+	+	+	2,11,14,48
Acridine orange	0	0	0	0	0	0	0	+	0	48

[a] See Table 4 for supporting information.

[b] 0, Not tested in the papers screened; —, negative results; +, positive results; ?, doubtful mutagen. Results shown were obtained using different experimental protocols.

[c] Acts as both an intercalating and an alkylating agent.

TABLE 6

MUTAGENICITY RESULTS WITH *Escherichia coli* Sd-4

Chemical tested	Results [a]	Ref.
TCDD	+	38
Acriflavine	+	19

[a] +, Positive results.

TABLE 7

PROPHAGE INDUCTION

Chemical tested	Host organism	Results [a]	Ref.
TCDD	*Escherichia coli* K39	+	38
Acridine orange	*Escherichia coli* 18	+	61
Proflavine	*Salmonella thompson* 19	—	62
Ethacridine lactate monohydrate	*Salmonella thompson* 19	—	62

[a] +, Positive results; —, negative results.

TABLE 8

MAMMALIAN DOMINANT LETHAL STUDIES

Compound	Reported results [a]	Ref.
TCDD	—	41
Acridine orange	—	47
Acriflavine	—	22
9-Aminoacridine	—	22
ICR-170 [b]	+	63
Quinacrine hydrochloride	—	22
Trypaflavine	—	22

[a] —, Negative results; +, positive results.
[b] Acts as both an intercalating and an alkylating agent.

TABLE 9

CHROMOSOME ABERRATION STUDIES IN CULTURED MAMMALIAN CELLS

Compound	Cell type	Reported results [a]	Ref.
TCDD [b]	Rat bone marrow cells	+ (weak)	28
TCDD [b]	Rat bone marrow cells	—	27
2,7-Dichlorodibenzo-*p*-dioxin	Rat bone marrow cells	—	27
Dibenzo-*p*-dioxin	Rat bone marrow cells	—	27
Proflavine	HeLa cells	+	59
Acridine	Human diploid fibroblasts	+	59
Acridine orange	Human diploid fibroblasts	+	59

[a] +, Positive results; —, negative results.
[b] Results from these experiments [27,28] were obtained using different experimental protocols, especially in the dose range tested, dosing schedule, and solvents used.

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Mammals

Dominant lethal studies

Malling and Wassom [47] in their review of the mutagenic action of chemicals reported that only 6 acridine compounds had been evaluated, at the time of their review, in the dominant lethal test. Results from these tests are shown in Table 8.

Positive results in the dominant lethal test are believed to be due to chromosome rearrangements rather than point mutations. Therefore, a mutagen that induces predominantly point mutations would go undetected in this test. Freese [26] stated that acridines produce point mutations via intercalation by adding or deleting bases in DNA. Thus, negative results would be expected if the activity of TCDD resembled the intercalating action of acridines as supported by the negative results reported by Khera and Ruddick [41]. However, the results obtained by Green, Moreland and Sheu [28] in the rat bone-marrow test, coupled with the evidence that TCDD does produce adverse effects in the testis [41,50,54,58,65], still leave the unresolved question of whether TCDD can act as a weak dominant lethal-inducing agent or cause some other type of damage to the genetic material of mammalian germ cells. Several acridine compounds are known inducers of chromosome aberrations in plants, Drosophila, and mammalian cells in culture [59], but no reports on the evaluation of acridine compounds in the rat bone-marrow test were found. Results from other mammalian studies with acridines are shown in Table 9.

Mutation research in progress

The Seveso incident revealed a lack of information concerning the health effects of TCDD. As a result, a number of research projects were initiated in an attempt to obtain a better perspective of the hazards posed by TCDD exposure. Most of these investigations are taking place in laboratories in Italy and other European countries. Information is also being collected on people or domestic animals exposed to TCDD as a result of the accident at the chemical plant [46] which caused the contamination of the Seveso area. Projects under way include fertility studies, dominant lethal studies in rabbits, translocation tests in mice, point-mutation tests using hamster cells, cytological examinations of peripheral blood from exposed individuals and exposed animals (cattle and rabbits), cytological examinations of fetal tissue from abortuses, and mitotic crossing-over and recombination studies in yeast.

Discussion and conclusion

Of the many structurally possible dioxin configurations, only 4 have been tested for mutagenicity or other related effects. These are the di- [27], tetra- [9, 27, 28, 38, 41, 49, 53, 57], and octa- [57] chlorinated derivatives as well as the unsubstituted dibenzo-*p*-dioxin [13,27]. Of these, TCDD has been the most frequently tested but the results obtained from these studies have not been conclusive. For example, positive results were reported when TCDD was tested alone for cytological disturbances in the African blood lily [40] or for

mutation induction in *Salmonella typhimurium* [38,57] and *Escherichia coli* [38]. Positive results were also reported when 2,4,5-T test samples contaminated with TCDD were evaluated for cytological effects in Drosophila [18] and the African blood lily [40]. On the other hand, negative results were reported with TCDD in *Salmonella typhimurium* conflicting with those studies reporting positive findings (see Table 1). The octachloro derivative (OCDD) was a doubtful mutagen when evaluated in the Salmonella test and the unsubstituted dibenzo-*p*-dioxin was nonmutagenic [13].

Mammalian studies with dioxins are scarce. The only data found showed that TCDD gave negative results when tested for dominant lethal effects in rats [41] and weakly positive results when assayed for the induction of chromosome aberrations in rat bone-marrow cells [28]. These data, coupled with studies showing that TCDD does reach the testes [41,50,54,58,65], indicate the possibility that TCDD could act as a weak dominant-lethal inducing agent or could cause damage to the genetic material which manifests itself in some other way. The dichloro derivative (DCDD) and the unsubstituted dibenzo-*p*-dioxin were both negative in the rat bone marrow test [27].

The cytological effects produced by TCDD in the liver of treated animals [12,29,42,67] may be attributed to the toxic effects produced by the short-term high dose levels administered in these studies and the fact that TCDD tends to concentrate in the liver.

Preliminary results obtained from cytogenetic analyses of maternal blood and abortive fetal tissue taken from persons exposed to TCDD during the Seveso, Italy accident are inconclusive [64]. Inconclusive results have also been reported [31] from studies of peripheral blood cells taken from individuals exposed to TCDD within the chemical plant which caused the accidental exposure at Seveso as well as from individuals residing in heavily contaminated areas. On the other hand, workers exposed to the herbicides Klorinol and Buvinol, which may have varying levels of TCDD contamination, had a higher incidence of chromatid breaks in peripheral lymphocytes than did nonexposed individuals [17]. Increased frequencies in chromosome aberrations were also reported in Vietnamese exposed to TCDD-contaminated herbicide 2,4,5-T, but these studies have been challenged and are of limited value [32].

The information implicating TCDD and other dioxin compounds as mutagens is scarce and conflicting; therefore, more work is required before the mutagenic potential of these compounds can be determined. Factors which have contributed to the difficulty in understanding the experimental results with dioxins, particularly the TCDD derivative, include the extremes in toxicity of these compounds and their varied sensitivities to different treatment protocols. Solubility * seems to be one of the more outstanding problems encountered when working with dioxins. To obtain comparable data, all future studies should be done under similar treatment conditions whenever practical and with

* Information on the solubility of TCDD can be found in Table 1 of the paper by W.B. Crummett and R.J. Stehl entitled "Determination of Chlorinated Dibenzo-*p*-dioxins and Dibenzofurans in Various Materials", Environ. Health Perspect., 5 (1973) 15—25. See also the paper by C. Botré, A. Memoli and F. Alhaique entitled "TCDD Solubilization and Photodecomposition in Aqueous Solutions", Environ. Sci. Technol., 12 (1978) 335—336.

samples of known purity *. Mutagenicity assay systems that are sensitive to the action of intercalating agents should be considered first when systems are being selected for additional tests. For instance, reexamination in sensitive strains of Salmonella, particularly TA1532, is needed since this strain is the only one which has shown a positive response (Tables 4 and 5). Experiments such as these will help resolve the conflicting results observed thus far in the Ames Salmonella test. Likewise, retesting for dominant lethality in mammals or testing in other in vivo mammalian systems which measure damage to the genetic material of germ cells should also be given consideration. The testing of dioxins using the multipurpose *Saccharomyces cerevisiae* strain MP-1 would also be of interest since Fahrig, Nilsson and Rappe [23] have found that the predioxins, 4,5,6-trichloro-2-(2,4-dichlorophenoxy)phenol and 4-chloro-2-(2,4-dichlorophenoxy)phenol induce forward mutations and mitotic crossing-over in this yeast strain. Pentachlorophenol (isomer purity 99%) induced some forward mutations and mitotic gene conversion in this same yeast strain, but 2,3,7,8-tetrachlorodibenzofuran, a compound which is structurally similar to TCDD, did not induce any genetic activity in this organism [23]. These observations make further testing of dioxins in this or other yeast strains which detect mitotic crossing-over, gene conversion, or forward and reverse mutations desirable (see Zimmermann [70]) **. Evidence that TCDD is a potent inducer of certain microsomal enzymes points to the need for additional studies on the promoting effect which dioxins may have on the mutagenicity of other compounds. Particular attention should be given to formulations in which dioxins are potential contaminants. A better understanding of the mutagenicity of TCDD, and perhaps other dioxins, will be obtained once studies stimulated by the Seveso tragedy have been properly evaluated.

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* See paper by O. Aniline entitled "Preparation of Chlorodibenzo-*p*-dioxins for Toxicological Evaluation", in: E.H. Blair (Ed.), Chlorodioxins — Origin and Fate. Advances in Chemistry Series No. 120, American Chemical Society, Washington, D.C., 1973, pp. 126—135.

** After this manuscript was written, Bronzetti (personal communication) [9] sent the authors results from some preliminary experiments he had done with TCDD in the yeast strain D7 of *Saccharomyces cerevisiae*. The genetic end-points of these experiments were mitotic crossing-over, gene conversion, and reverse mutation. Results from these experiments, which were done with and without metabolic activation, revealed that TCDD induced a high rate of killing, and no crossing-over or gene conversion. A weak reverse mutation inducing effect was observed as well as the presence of several "petite colonies" on the plates screened. The TCDD concentrations tested in these experiments were 1, 2, 4, 6 and 8 μg/ml. An acetone—corn oil solvent was used in the preparation of the TCDD samples.

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CHAPTER 6:

COMPARISON OF THE HEALTH EFFECTS OF AQUATIC HERBICIDES

Based on absorption and metabolism, it is possible to determine a comparative index of likelihood of injury to internal organs due to environmental exposure to aquatic herbicides. In order or increasing risk:

1. Endothall is poorly absorbed through the skin, lungs, or gastrointestinal tract unless the membrane is first damaged. It is minimally metabolized by animals or intestinal bacteria, is not lipid soluble, and has not been reported to alter metabolism.

2. Diquat is poorly absorbed through the skin, lungs, or gastrointestinal tract unless the membrane is first damaged. It is not metabolized by animals, but is metabolized by intestinal bacteria to unknown products of unknown toxicity which are partially absorbed. It is not lipid soluble and does not accumulate in the body. However, it alters metabolism in many, if not all, organs by its ability to be reduced to a free radical at the expense of NADPH and to then autooxidize, producing hydrogen peroxide or superoxide free radicals.

3. 2,4-dichlorophenoxyacetic acid is lipid soluble and rapidly absorbed through membranes of the skin, lungs, and gastrointestinal tract. It is not metabolized by animals, but its derivatives (salts, esters, etc.) are hydrolyzed to the acid. Although it is degraded by soil bacteria, it apparently is not changed by intestinal bacteria, possibly because it is absorbed too rapidly. Up to 32% of absorbed 2,4-D is found in cell nuclei. It is rapidly distributed to all tissues and is not excreted as soon as the above lipid-insoluble herbicides.

4. Dichlobenil is lipid soluble and rapidly absorbed by all routes of environmental exposure. It is metabolized by animals to a variety of known and unknown products, some of which are highly toxic uncouplers of oxidative phosphorylation.

It should be noted that this approach to risk assessment gives exactly the opposite order from an assessment based on the acute oral LD_{50}. LD_{50} is not relevant to low-dose chronic exposure, but is the basis for the hazard levels assigned to pesticide labels: caution, warning, danger, in order of decreasing lethal dose. Dichlobenil and 2,4-D formulations used for water milfoil are labeled "caution," diquat is labeled "warning," and endothall is labeled either "warning" or "danger" depending on its concentration.

None of the four herbicides has passed all of the currently accepted tests for the determination of safety of low-level exposure to humans. Endothall has been subjected to adequately designed

testing, but the final results of the mouse carcinogenicity test are not in yet, and an outside investigator has detected mutagenic potential in a very sensitive but reliable assay properly performed.

Diquat has not been adequately tested for teratogenicity in mammals, but inadequate tests suggest possible hazard, and one study in amphibians shows teratogenicity and embryotoxicity at concentrations lower than proposed for Lake Washington (Anderson and Prahlad, 1976). Chronic feeding tests have shown that diquat induces cataracts in rats at 2.5 mg/kg/day and in dogs at 5 mg/kg/day orally. Diquat was positive in one short-term carcinogen-screening test, the induction of DNA repair synthesis. It was negative for carcinogenicity in both rats and mice in less than adequate tests, which could give false negative results.

2,4-D was found to be both teratogenic and embryotoxic, with effects noted as low as 0.5 mg/kg/day. This injury was induced at even lower concentrations in the presence of a primary microbial breakdown product of 2,4-D, 2,4-dichlorophenol. 2,4-D was shown to cause point mutations in animal cells, to damage DNA in a manner similar to ironizing radiation, and to stimulate cell division. Testing of 2,4-D for carcinogenicity is inadequate according to present standards, but those inadequate tests demonstrate significant tumor increases, particularly in sarcomas of the lymphoreticular system in both rats and mice. (Inadequate studies can give false negative results, not false positives.) One study for tumor promoting activity was highly positive, using the mouse skin system. The breakdown product, 2,4-dichlorophenol, is also a good promoter of skin carcinogenesis.

Dichlobenil testing for teratogenicity and carginogenicity is so inadequate that no conclusions are justified. An adequate reproduction study indicated that dichlobenil inhibits growth of young rats at 50 ppm and decreases fertility at 100 ppm. It has not been tested for mutagenicity in any animal system, and microbial system are notoriously unreliable for chlorinated aromatic compounds.

Present testing standards are certainly minimal for the determination of safety to humans. They do not assay for tumor promoting ability or for effects on the functioning of the higher nervous system such as loss of intelligence, loss of ability to concentrate, or induction of emotional instability.

Since only the manufacturer of endothall has met these minimal standards of testing for health effects (one test not yet complete, however), but all four herbicides are registered by the Environmental Protection Agency and approved for use in public waters, an attempt was made to determine whether EPA files contained additional relevant unpublished studies which would justify registration. Catalogs of documents in the EPA files on dichlobenil and diquat were obtained from Friends of the Earth, and a request was sent to EPA under the Freedom of Information Act for all of the documents listed under

neurotoxicity, oncogenicity, chronic feeding, reproduction, and teratology. This totaled one document on dichlobenil (chronic feeding) and eight documents on diquat (neurotoxicity, oncogenicity, chronic feeding, and reproduction).

After two months, two documents on chronic feeding of diquat were received, and after six months the EPA closed the request without sending anything more. The two documents received are reproduced in their entirety below:

#123373-0007 Chronic - Oral
Rat: 500 ppm. w/w. for 16 months did not affect the rate of growth, during the growing period, and no toxic signs have been observed.

#123370-0034 Chronic toxicity
Chronic toxicity studies show that 500 ppm in the diet of young rats of both sexes produced no detectable effect.

It should be apparent even to the casual reader that these do not contain the minimum essentials of an abstract (species, strain, sex, number of animals, doses, formulations, route of administration, duration of study, effects looked for, methods of analysis of data, conclusions) and certainly provide no data appropriate for regulatory decision making. It is possible that the two reports above are from the same study, not repeated studies, reducing even further the total number of relevant studies in the EPA catalogs.

Since the primary argument of other government agencies which routinely expose the public to these herbicides is that they are registered with EPA and therefore must be safe, it should be determined whether EPA really has no data on the health effects of dichlobenil and diquat, or merely defies the law. The Federal Insecticide, Fungicide and Rodenticide Act states:

Section 10.(d)(1) All information concerning the objectives, methodology, results, or significance of any test or experiment performed on or with a registered or previously registered pesticide or its separate ingredients, impurities, or degradation products, and any information concerning the effects of such pesticide in the environment, including, but not limited to, data on safety to fish and wildlife, humans and other mammals, plants, animals, and soil, and studies on persistence, translocation and fate in the environment, and metabolism, shall be available for disclosure to the public.

Much of the information used in this report was obtained from the chemical companies and has not gone through the process of peer review required before publication by reputable scientific journals. This is also true of the unpublished manuscript and publications of government

agencies. The reader should consider this when using such information for decision making.

One final conclusion of this study must be that published reviews of this subject are extremely unreliable, quoting other reviews with no original data and thus giving the appearance that many more studies have been done than in fact have. This author welcomes correspondence concerning any additional relevant studies which may become available.

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Milwaukee Journal 11/7/80

Pesticide use is still not issue

By Richard C. Kleinitz
Journal Madison Bureau

Madison, Wis. — The state's public intervener urged the Agriculture Department Thursday to write stronger protections for ground water into proposed rules for pesticide use.

"Pesticides do not belong in our waters," Asst. Atty. Gen. Thomas Dawson stated.

At the first of a series of hearings on revision of the state's pesticide regulations (Ag 29), a spokesman for the state's vegetable processing industry argued that the proposals on overspraying, posting of treated fields and pre-spraying notice were impractical.

Alvin Randall, executive secretary of the Wisconsin Canners and Freezers Association, said it was not practical to try to eliminate all risk for all people.

Adverse effects

Likewise Russell Weisensel, executive director of the Wisconsin Agri-Business Council, suggested that as rules become more rigid they adversely affect more small farmers. He said his group wondered whether there was much benefit to be gained from the revisions.

"People have to take some of the risk themselves," Randall said.

George Klacon, of the Green Giant Corp., said people should be educated to recognize that there are pesticides being used in fields.

He said, "I don't believe people can put up enough signs for those passersby who might want to take a few ears of corn." Weisensel added that normal trespass laws should suffice.

Wants to know

Carole Ann Barth, a researcher for Citizens for a Better Environment (CBE) who said she was asthmatic, also commented on the matter of posting and prior notice.

"I don't expect the world to stop around me," she reasoned, " but I want to know what is happening around me so I can arrange my own activity."

Dawson, who intervenes for the public interest in environmental matters, asked that the rules be written to prohibit any application of pesticides that would enter the state's waters.

He noted that Wisconsin relied heavily on ground water for domes-

Turn to Hearing, Page 7

Hearing

Controlled pesticide use defended

From Page 1

tic use. He proposed that whenever the agency received credible evidence that a pesticide had been identified in ground water, an order should be issued prohibiting its further use in the recharge zone of the ground water basin.

Dawson said that aldicarb — a chemical used to treat a potato pest and which has caused problems in New York — has been detected in the Wisconsin Central Sands region. He pointed out that his proposal would ban its use until it could no longer be detected.

The proposed Department of Agriculture, Trade and Consumer Protection rules would require that residents of adjacent land be given notice of aerial spraying if requested. The same provision would apply for beekeepers when spraying is conducted within three miles of their hives.

Other revisions would restrict daytime spraying where crops or weeds visited by bees are in bloom; require a permit for use of chlordane; include municipalities as commercial applicators, and require permits and enclosed storage of pesticides.

Dawson objected that the rules did not require annual reporting of pesticide use. He said the information was needed to properly monitor human and environmental effects.

Weisensel described Dawson's proposals as unbalanced and said they caused the agency a great many problems in drafting the rules.

Dennis Dixon, Elkhorn, president of the Wisconsin Agricultural Aviation Association, said he "was not surprised that the activist minority is not satisfied."

Various speakers took issue with the statement in the proposed rules that any use resulting in overspray was negligent. Dixon said some provision should be made for equipment failure, adding that aerial application should not be singled out because there was no evidence of harm to human health.

Dixon said notice should be limited to toxic materials.

Ray Geymann, of Oconomowoc Canning Co., proposed that beekeepers be required to own or lease foraging rights in order to qualify for the prior notice protection. Furthermore, he said all beekeepers should be licensed and a fund should be developed from honey receipts to finance a system of coordinating application information and notice.

Geymann also said a 1.5 mile radius for beekeeper notice seemed more reasonable.

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CHAPTER 2:

HEALTH EFFECTS OF 2,4-D (2,4-dichlorophenoxyacetic acid) AND ITS DERIVATIVES

A. Introduction

The herbicide 2,4-D was prepared in 1941 by the interaction of 2,4-dichlorophenol, monochloroacetic acid and sodium hydroxide, and a similar process is used in its commercial production. 2,4-D is a systemic herbicide widely used for site preparation and conifer release in forestry, for control of broadleaf weeds in cereal crops and sugar cane, and on turf, pastures and non-cropland. It is also used to control the ripening of bananas and citrus fruits, to delay preharvest dropping of some fruits, and in some countries as a fungicide for the control of Alternaria rots when lemons are to be held for storage. As a component of "Agent Orange," 2,4-D was used to defoliate jungle areas in South Vietnam (IARC, 1977). The EPA has approved the use of 2,4-D, marketed as "Aqua-Kleen" by Amchem Products, Inc., for control of several aquatic plants including water milfoil. Label requirements include a "caution" against accessibility of the product to children; applying to water used for irrigation, sprays, dairy animals or domestic use; or contact with skin, eyes, and clothing.

B. Metabolism of 2,4-D

A total of nine human male "volunteers" in two different studies have been fed a single dose of 5 mg/kg of 2,4-D (Kohli et al., 1974; Sauerhoff et al, 1977) to determine its metabolism. Essentially all of the 2,4-D was absorbed from the gastrointestinal tract. It was distributed widely through the body and excreted in the urine unchanged. No metabolites were detected. No symptoms of illness or abnormal blood chemistry were detected. 95% of the dose was recovered from urine in six days.

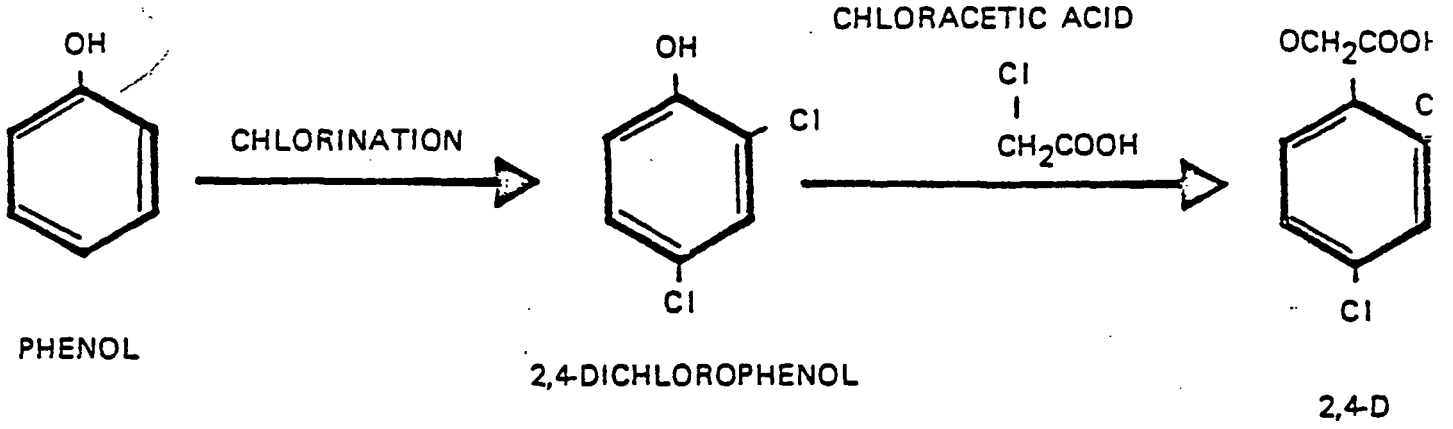
The metabolism of isotope-labeled 2,4-D in rats has been determined and is in agreement with the human studies above (Khanna and Fang, 1966). Radioactivity was found in all organs and tissues examined, with 9% to 32% in the nuclear fraction. All was unchanged 2,4-D, suggesting that the chemical in the cytosol was not peptide-bound as found in plant tissues.

2,4-D is lipid-soluble and therefore rapidly absorbed from the lung (Burton et al., 1974), when assayed in rats.

2,4-D amine salt administered orally was readily absorbed by rats, pigs, calves, and chickens, but 2,4-D butyl ester was much more slowly absorbed and the circulating chemical was all in acid form, indicating that the ester was hydrolyzed during absorption (Erne, 1966). Absorbed 2,4-D was distributed rapidly through the body with

highest levels in the liver, kidneys, lungs, and spleen. It is readily passed through the placenta in the pig and into chicken eggs.

C. Impurities and Breakdown Products



Synthesis of 2,4-D

Technical 2,4-D available in the United States is about 98% pure (IARC, 1977). The primary contaminants are bis (2,4-dichlorophenoxy) methane, bis (2,6-dichlorophenoxy) methane, and 2,2', 4,6'-tetrachlorodiphenoxymethane (Huston, 1972). No toxicological information was found on these; they are not listed in NIOSH's Registry of Toxic Effects of Chemical Substances 1977. Incomplete reaction of 2,4-dichlorophenol during synthesis of 2,4-D would leave some of this precursor contaminating the product, but no published evidence of this was found.

2,4-dichlorophenol is a major product of the breakdown of 2,4-D by microorganisms (Paris and Lewis, 1973). No. 2,4-dichlorophenol was detectable in mice which had been injected with 2,4-D acid or its butyl or isooctyl esters (Zielinski and Fishbein, 1967), but this route of administration bypasses the intestinal tract. Intestinal bacteria may be able to hydrolyze 2,4-D to 2,4-dichlorophenol also, but the metabolic studies above in section B would indicate that this does not happen. However, the abstract of a Russian study which is not available in the United States, states "in pregnant rats receiving a single 50 mg/kg oral dose of 2,4-D the metabolite 2,4-dichlorophenol was detected in the placenta, the amniotic fluid, and the embryo and in the mother's milk after delivery" (Antonenko, 1977). 2,4-D during its breakdown in the environment must contain 2,4-dichlorophenol and lead to human exposure through food and water.

20% 2,4-dichlorophenol promoted the appearance of skin tumors in mice following a single initiating dose of dimethylbenzanthracene (43% with papillomas and 11% with carcinomas after 15 weeks, vs. 7% and 0%

in initiated controls). After a longer period of application to the skin, it was able to induce tumors without prior application of the initiator (75% with papillomas and 6% with carcinomas after 24 weeks). After 39 weeks of treatment with 2,4-dichlorophenol, 62% of the mice had carcinomas. The test substance was dissolved in benzene in these experiments, and control animals were treated with benzene alone (Boutwell and Bosch, 1959). This indicates that 2,4-dichlorophenol is a skin carcinogen in mice, or at least a cocarcinogen with benzene which is not carcinogenic by itself in this system.

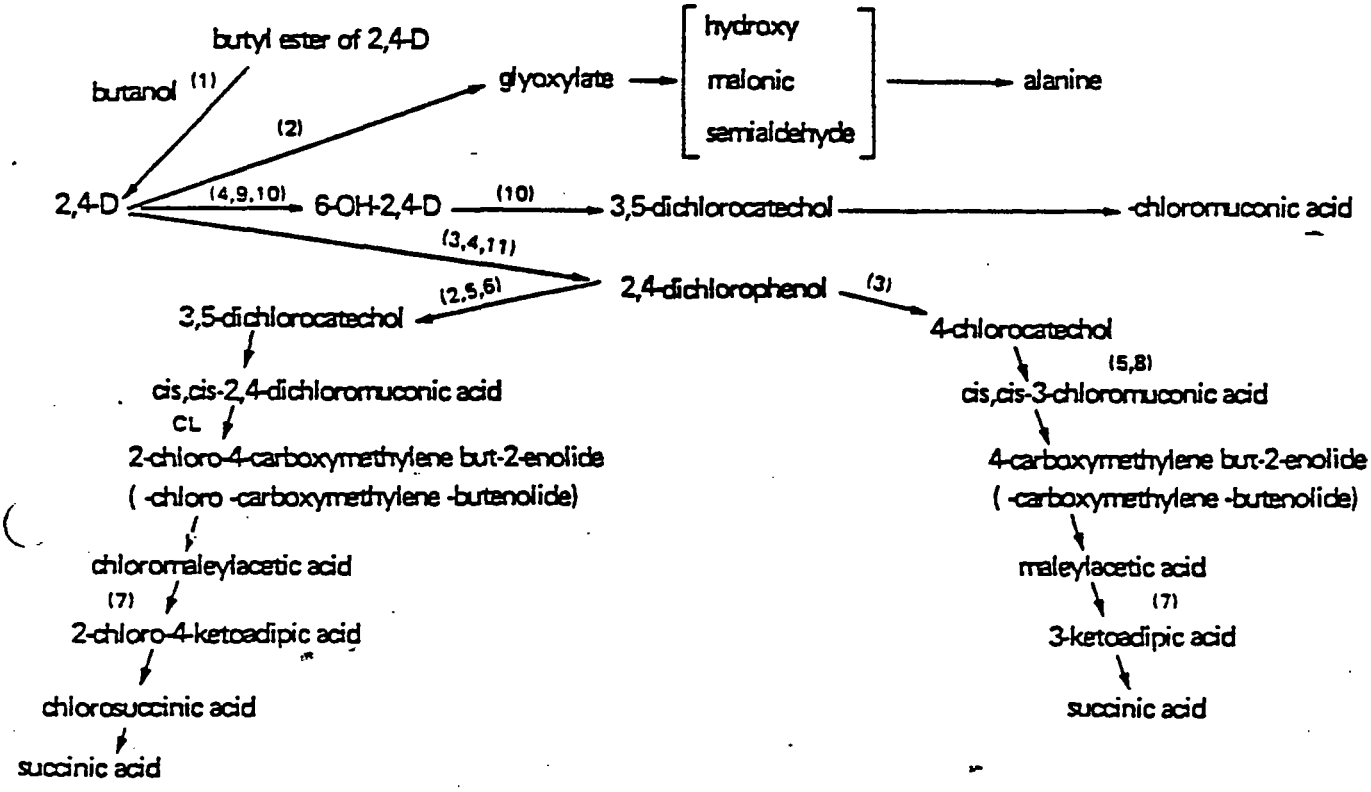


Fig. 1 Pathways of microbial degradation of butyl ester of 2,4-D. Numbers are references: *(1) Aly and Faust 1964; (2) Tiedje and Alexander 1969; (3) Steenson and Walker 1957; (4) Bell 1957; (5) Bollag et al., 1968; (6) Loos et al., 1967; (7) Duxbury et al., 1970; (8) Tiedje et al., 1969; (9) Audus 1960; (10) Fernley and Evans 1959; and (11) Evans and Smith 1954.*

SOURCE: Doris F. Paris and David L. Lewis, "Chemical and Microbial Degradation of Ten Selected Pesticides in Aquatic Systems," Residue Reviews, Volume 45, 1973, p. 107.

2,4-dichlorophenol, like other polychlorinated phenols, can form chlorodibenzo-dioxins when heated (Epstein, 1970), but not the infamous TCDD (2,3,7,8-tétrachlorodibenzo-p-dioxin) found in 2,4,5-T (Gribble, 1974). The most likely dioxin produced by heating 2,4-dichlorophenol would be 2,7-dichlorodibenzo-p-dioxin, which is reported to be minimally toxic (Schwetz et al, 1973, Dow Chemical Co.). No deaths occurred in four male mice given 2000 mg/kg or in two female rats given 1000 mg/kg. No signs of toxicity were observed in these

animals. This dioxin was negative in the rabbit ear assay for chloracne. Rats treated with 100 mg/kg/day (gavage) 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 in seven litters examined at Dow.

However, Khera and Ruddick (1973) of the Health Protection Branch, Department of National Health and Welfare, Canada, found significant teratological injury to the heart muscle in fetuses of rats given 2 or 1 mg/kg/day of 2,7-dichlorodibenzo-p-dioxin orally on days 5-14 of gestation. Microscopic examination of the heart revealed edematous separation of myofibrils that had resulted in compression thinning and fragmentation of myofibres. Mitotic figures were rare, indicating that growth of the cardiac tissue was suppressed. Such lesions were not found in rats given 0.5 or 0.25 mg/kg/day or in the controls given the solvents anisole and corn oil.

2/7-dichlorodibenzo-p-dioxin should not be formed during production of 2,4-D because the reaction is carried out at a lower temperature than is required for dioxin formation. However, it could be formed from residual unreacted 2,4-dichlorophenol if heated during storage or transit, and from the microbial breakdown product during fires in treated brush or slash.

One author has reported detecting a trace of hexachlorodibenzo-p-dioxin (HCDD) in one of 28 samples of 2,4-D tested for content of higher dioxins (Woolson et al, 1972). This was probably an artifact of laboratory contamination since there is no apparent way for this to form directly from 2,4-D production.

Other products of microbial or plant degradation of 2,4-D (Paris and Lewis, 1973; Ashton and Crafts, 1973) are not listed in the NIOSH directory (NIOSH, 1977). Microbial products include 6-hydroxy-2,4-D, various chlorocatechols, and various chloromuconic acids before finally becoming succinic acid, a normal physiological component of all cells (Paris and Lewis, 1973). Catechol has been shown to be an efficient cocarcinogen for mouse skin in the presence of minute amounts of benzo(a)pyrene, but the chlorocatechols were not tested (Van Duuren and Goldschmidt, 1976).

Esters of 2,4-D are split by microbial action to release 2,4-D acid which is degraded as described above (Aly and Faust, 1964).

D. Acute and Subacute Toxic Effects

The acute oral LD_{50} of 2,4-D for humans is reported to be 30 mg/kg, and for rats it is 375 mg/kg (NIOSH, 1977). Five incidents in which agricultural workers were poisoned by 2,4-D when they returned to the fields from one to 14 days after spraying were described by Radionov et al, 1967. Symptoms reported were consistent and included headache, weakness, dizziness, nausea and vomiting, sore

throat, irritation of the nasal mucosa, substernal pain, and loss of consciousness. Recovery took four to nine days.

Workers engaged in the spraying of 2,4-D and its derivatives from aircraft for three to five years and longer complained of rapid fatigue which usually cleared overnight. They periodically had headaches, pain in the liver and epigastric regions, and poor appetite. They had impaired taste sensitivity to salty, sour, bitter, and sweet test solutions. There was a deterioration in sensitivity to odors also. No changes in blood chemistry were found (Fetisov, 1966).

In addition to the symptoms above, peripheral neuropathy has been reported in humans hours or days after exposure to 2,4-D on the skin and probably simultaneously by inhalation (Goldstein et al., 1959; Berkley and Magee, 1963). The symptoms progressed through several weeks until pain, paresthesias, and paralysis were severe. Disability was protracted, and recovery was incomplete even after the lapse of years. Numbness and aching had extended proximally from the fingers and toes, and the patients became unable to walk because of pain and weakness. They also suffered moderately severe sensory deficits on tests of touch, pain, and temperature.

Autopsy of a human fatality due to ingestion of 2,4-D revealed widespread plaques of acute demyelination in all parts of the brain, with central petechiae (Dudley and Thapar, 1972). Multiple petechiae were seen throughout the white matter of the brain, and the kidneys were hyperemic.

The nervous system of rats, cats, and dogs was studied after 2,4-D administration parenterally (Desi et al., 1962). A reversible inhibition of cerebral electrical activity was observed in the acute experiments, and in chronic experiments the same was present to a gradually increasing degree. According to conditioned-reflex experiments, the higher nervous activity suffered severe damage. The point of attack seemed to be the reticular formation. Lesions in this region paralyze the function of the cerebral cortex.

Pretreatment of rats with 250 mg/kg 2,4-D three hours prior to administration of 9 mg/kg ^{14}C-2,2-D greatly increased the level of ^{14}C in rat brain and spinal fluid as compared to plasma level (Elo and Ylitalo, 1977). The increase was much more striking in the brain (11-fold) and spinal fluid (39-fold) than in the liver (4.5-fold). The likeliest explanation of this is that 2,4-D impaired function of the blood-brain barrier by causing capillary injuries.

Cattle and sheep which died after 5 to 34 daily oral doses of 2,4-D alkanolamine salt were necropsied (Palmer and Radeleff, 1964). Lesions included liver and kidney degeneration, the heart usually contained hemorrhages, and there was usually an excessive quantity of pericaridal fluid.

Mice acutely poisoned with 2,4-D had dark and mottled liver, kidneys, and spleen, blood-stained fluid in the upper intestine, and wide dilation of the blood vessels of the lungs, liver, and kidneys (Bucher, 1946).

In dogs that died two to nine days after a single dose of 2,4-D, symptoms included ataxia, progressive increase in spasm of the hind legs, and myotonia (Drill and Hiratzka, 1953). Sneezing, eye rubbing, and diarrhea were observed, but not vomiting, and some dogs showed evidence of meningeal irritation. Autopsy showed inflammation and necrosis of the intestinal mucosa, hepatic necrosis, and renal tubular degeneration.

Myotonia in animals exposed to acute and subacute treatment with 2,4-D has been described extensively (i.e. Bucher, 1946; Heene, 1975; Janon et al, 1978). Injection of 2,4-D into adrenalectomized and hydrocortisone-treated rats did not cause the myotonic response seen after injection into normal rats (Buslovich and Koldovskaya, 1972).

Myotonia was present in an elderly man after ingestion of a lethal dose of 2,4-D (Dudley and Thapar, 1972).

10^{-4}M 2,4-D caused uncoupling of oxidation and phosphorylation in isolated rat liver mitochondria (Abo-Khatwa and Hollingsworth, 1974), and 2,4-D inhibits synthesis of cholesterol and fatty acids in rat liver (Olson et al, 1974). It increases the quantity of RNA and protein in rat liver and stimulates RNA synthesis in isolated rat liver nuclei (Chang et al., 1974).

E. Tests for Developmental Toxicity

1. Summary of Research

Five assays for developmental toxicity of 2,4-D in rats have been published, using widely different doses and different strains of rats. Khera and McKinley of the Food and Drug Directorate of Canada used oral doses of 50 to 150 mg/kg of body weight/day during organogenesis and found skeletal malformations at 100 mg/kg/day and above. Schwetz et al of Dow Chemical Company used oral doses of 12.5 to 87.5 mg/kg/day during organogenesis and found skeletal anomalies at 75 mg/kg/day and above. They also found an increase in fetuses with subcutaneous edema at all doses, and general growth retardation.

Konstantinova et al. of the Scientific Institute of Rural Hygiene in Saratov, USSR, tested 2,4-D and its breakdown product, 2,4-dichlorophenol, separately and together, and found developmental toxicity in the form of internal hemorrhages after 50 mg/kg/day of 2,4-D and after 1 mg/kg/day of 2,4-dichlorophenol and also after a mixture of 0.1 mg/kg/day of each. Akeksashina et al of the Byelorussian Scientific Research Institute of Sanitation and Hygiene in Minsk, USSR, did two different experiments. One used intraperitoneal doses of 0.1 or 0.5 mg/kg/day throughout pregnancy and found growth retardation and

hemorrhaging into the abdominal cavity at the higher dose and vascular distention at both doses. The other experiment tested single doses of 1/2 LD_{50} given intraperitoneally on various days of gestation, and found increased resorption of fetuses with reduced litter size, growth reduction with increased size of brain ventricles, and hemorrhaging into the abdominal cavity.

A test for developmental toxicity in hamsters was done by Collins and Williams of the U.S. Food and Drug Administration. They used oral doses of 20 to 100 mg/kg/day during organogenesis and found a dose-dependent increase in fused ribs at 60 mg/kg/day and above which was significant only if repeats were pooled.

Two studies of the teratogenicity of 2,4-D in mice have been done. A large series of tests using different strains of mice, different doses, and different esters of 2,4-D was completed in 1968 by Bionetics Research Laboratories, and has now been published. Oral administration of 100 mg/kg/day of 2,4-D acid during organogenesis caused a significant increase in fetal mortality and percent of abnormal fetuses, with most of these being eye and jaw malformations.

2,4-D acid produced a significant increase in fetal abnormalities in four of six adequately-sized groups of three strains, when given subcutaneously at 100 mg/kg/day. Subcutaneous administration resulted in a significant increase in abnormal fetuses in one strain after 48, 94, 100, or 130 mg/kg/day of the isooctyl, isopropyl, butyl, and isooctyl esters respectively. This was a repeat of a previously negative study. The anomalies were mainly of the eye and jaw. All of these subcutaneous administrations were dissolved in dimethylsulfoxide, which is a teratogen for the nervous system of the hamster (Ferm, 1966). However, the DMSO controls in this mouse study showed no significant increase in developmental toxicity over untreated controls.

The other mouse study was done by Courtney of the U.S. Environmental Protection Agency. She used oral doses of 150 or 250 mg/kg/day of 2,4-D and four of its esters during organogenesis and found growth retardation at both doses and cleft palate at the higher dose. Other malformations were not assayed.

Two developmental studies on pigs have been done by Bjorklund and Erne of the Swedish Royal Veterinary College and National Veterinary Institute. A pig was fed 500 ppm of 2,4-D amine while pregnant plus six weeks after. The fifteen piglets born were underdeveloped, apathetic, and did not want to suck, and ten died the first day. No malformations were observed. In the second study, eight-week-old pigs were fed 500 ppm of 2,4-D amine for 12 months. After one month, 2 out of 5 in the experimental group developed malformations in the hooves on the forelegs, making walking difficult.

2. Conclusions

The four manifestations of developmental toxicity have all been demonstrated in animals treated with 2,4-D or its esters or amines.

Malformation: skeletal at high dose (60 mg/kg/day or above), peripheral circulatory system distended at low dose (0.1 mg/kg/day) and after single high dose (1/2 LD_{50}), eye anomalies at 100 mg/kg/day.

Malfunction: subcutaneous edema at 12.5 mg/kg/day and above, hemorrhage into soft tissues and body cavities from 50 mg/kg/day to 0.5 mg/kg/day.

Growth retardation: prenatal at 0.5 mg/kg/day and above. Postnatal after 50 mg/kg/day or when treatment continued during lactation.

Lethality: pre-implantation not reported. Post-implantation at 100 mg/kg/day and above, or after a single high dose (1/2 LD_{50}). Postnatal in progeny of rats fed 150 mg/kg/day, or in a pig fed 500 ppm.

The above summary includes both oral and intraperitoneal treatment. 2,4-D is rapidly and completely absorbed from the gastrointestinal tract and is not metabolized in animals, so the serum concentrations should be similar by the two routes. Erne, 1966, has demonstrated the ease of placental transfer of 2,4-D in the pig. 2,4-D also rapidly penetrates the placenta in rats; 24 hours after a single dose, 16.8% of that dose remains in the uterus, placentas, fetuses, and amniotic fluid (Fedorova and Belova, 1974).

The effect of 2,4-D on the circulatory system was synergistic with that of its microbial breakdown product, 2,4-dichlorophenol. When the customary 100-fold safety factor is applied to the effective dose (Wilson, 1973, p. 156), the acceptable tolerance is equivalent to a 50 kg woman drinking 40 ml of treated water (2.5 ppm) daily during early pregnancy at a time when 50% of the 2,4-D has been degraded to 2,4-dichlorophenol. The situation is not quite that simple, however, because breakdown continues to other untested compounds, and part of the 2,4-D would be degraded in plants where 2,4-dichlorophenol is not part of that pathway (Ashton and Crafts, 1973).

Many of the studies reported here were done in other countries, raising the question of the similarity of synthesis and contaminants. No studies indicated that the synthesis and purity of these products is different elsewhere. 2,4-D caused developmental toxicity in all four species tested, including studies done in four countries. It is interesting to note that the hemorrhaging found in all of the Russian rat studies using low doses was not found in any of the high dose American studies. This may be a strain difference in rats, or a difference in the protocols of observation.

F. Tests for Effects on Reproduction and Fertility

1. Summary of Research

A study by Schwetz et al., of Dow Chemical Company, claims to show that 2,4-D has no effect on fertility, but it actually contains no such experiment.

The only study of the effect of 2,4-D on reproduction and fertility was done by Hansen et al., of the U.S. Food and Drug Administration. They used rats for a three-generation study and found no effect on fertility.

2. Conclusions

On the basis of a single study in a single species, there is no indication that 2,4-D might interefere with reproduction and fertility in mammals.

G. Tests for Mutagenic Effects and Other Short-Term Tests for Cancer

1. Summary of Research

Assays of 2,4-D for both forward and reverse mutation in bacteria and bacterial virus are consistently negative (Fahrig, 1974; Shirasu et al., 1976; Simmon et al., 1977: Andersen et al., 1972). These included tests run in the presence of an activation system prepared from the livers of rats or mice. This is to be expected since 2,4-D is a chlorinated hydrocarbon and these as a class are negative in the Ames test.

2,4-D caused a significant increase in recessive lethal mutations in Drosophila melanogaster when it was fed to male flies at 1000 ppm for two weeks (Magnusson et al., 1977), but was negative when the flies were treated with a 9mM solution for 3 days (Vogel and Chandler, 1974) and after unspecified treatment (Fahrig, 1974).

0.001 mM to 1.0 mM 2,4-D with or without rat liver activation induced unscheduled DNA synthesis in SV-40 transformed human fibroblast cells in culture (Ahmed at al., 1977a).

0.01 mM 2,4-D in the medium of primary human fibroblast cultures caused DNA damage leading to increased removal and reinsertion of bases. The type of repair was that seen following ionizing radiation (Hart et al., 1977).

0.01 mM 2,4-D in the culture medium caused a significant increase in forward mutation to ouabain resistance in the Chinese hamster V79 aneuploid lung cell line (Ahmed et al., 1977b).

0.01 mM or 0.1 mM 2,4-D in the culture medium of bovine fetal muscle cells caused the mitotic cells to exhibit unipolar and tripolar

spindles and a variety of other abnormalities including malorientation of the mitotic apparatus in relation to the axis of the cell. Myoblasts in initial stages of myogenesis were noted to be in mitosis in treated cultures, suggesting that 2,4-D may have a stimulatory effect on myoblasts which normally are in a post-mitotic stage (Basrur et al., 1976).

No evidence of nondisjunction or chromosome loss was found in Drosophila after both males and females had been fed 100 ppm 2,4-D for their entire larval period (Magnusson et al., 1977). Chromosome aberrations were not detected in human lymphocyte cultures (Fahrig, 1974) but no methods are given.

No chromosome breakage activity was found 24 hours after intraperitoneal injection of 100 mg/kg 2,4-D in mice, as indicated by no detectable increase of micronuclei in erythrocytes of bone marrow (Jenssen and Renberg, 1976).

2. Conclusions

2,4-D causes point mutations in animal cells without liver activation, damages DNA in a manner similar to ionizing radiation, and stimulates mitosis. It does not cause chromosome breakage or nondisjunction.

H. Tests for Carcinogenic Effects

1. Summary of Research

Only three laboratory studies for the detection of carcinogenicity of 2,4-D or any of its derivatives appear in the literature. All other reports are discussion or reanalysis of the data of these three studies, none of which uses optimum methodology according to the guidelines in Chapter 1. All doses used were lower than the 1500 ppm used in the 3-generation reproduction study in which adults tolerated chronic feeding and even pregnancy without lethality (Hansen et al., 1971). This study indicates that the MTD for rats is at least 1500 ppm. If mice are equally tolerant to the chemical, as suggested by their similar LD_{50}, the MTD for mice should be at least 750 ppm (mice eat twice as much per day in relation to their body weight).

The study done at the U.S. Food and Drug Administration in the early 1960's comes closest to being adequately designed, with a maximum dose of 1250 ppm, 25 rats/group, and two years duration. However, only a few of the animals had adequate pathological examinations at the end of that time, so small tumors would have been overlooked. There was a high incidence of tumors in controls, which could easily disguise a carcinogenic effect in such small samples. Increased incidence of tumors was statistically significant in total malignant tumors in males, in lymphosarcoma in both males and females, and in breast neoplasms in females. This study is described and analyzed by Hansen et al., 1971, and Reuber, 1979.

A mouse carcinogenesis study was sponsored by the National Cancer Institute, also in the 1960's, and the data published years later. It consisted of both oral feeding studies and single dose subcutaneous injection studies. Two strains and both sexes were studied, but only 18 mice were included in each group and all were killed by 18 months. In the original study, strains and sexes were pooled to give a large enough sample size for statistical analysis by the chi-square method, and a significant increase in total tumors and in reticulum cell sarcoma was found after subcutaneous administration of 2,4-D isooctyl ester. No other tests were postitive using this approach, which hides strain and sex differences in sensitivity. Analysis of the data by strain and sex, using the chi-square method, indicates a carcinogenic effect of feeding the isooctyl and butyl esters of 2,4-D to females of one strain, with the reservation that the method is not totally reliable with such small samples. Analysis of the data by strain, sex, and organ system using more elegant statistical methods indicated that the induction of reticulum cell sarcoma by injection of the isooctyl ester is most significant in females of the other strain. However, the importance of this observation to human health is limited by the injection route of administration and the knowledge that 2,4-D esters taken orally are probably split into the acid form before absorption (Erne, 1966) so that circulation of the ester might not happen after contamination of humans. This study is described, analyzed, and discussed in Bionetics, 1968, Innes et al., 1969, Reuber, 1979, Mrak report, 1969, and Jurek, 1974. Its primary defect is its short duration, but the small group size and less-than-optimal oral dose given the adult animals could also contribute to false negative results. This study also had a rather high background level of tumors in the control animals.

A study at the Institute of Nutrition of the USSR Academy of Medical Sciences included three experiments. The first experiment used adequate numbers of rats and duration of feeding 2,4-D amine, but the dose was not over 1/2 MTD. The background was very low and the tumor increase insignificant. However, the publication (Arkhipov and Koslova, 1974) does not state whether the rats were autopsied and whether histopathology was done, so small tumors might not have been found. A similar experiment with mice (100/group) was also negative, with no tumors in either the control or treated animals. The third experiment involved application of the herbicide to the skin of mice (100/group), with or without prior application of a low dose of an initiator of skin carcinogenesis. Animals treated with either the herbicide alone or the initiator alone did not develop any tumors, but 17.7% of those given the sequential treatment developed skin papillomas, the premalignant lesion of skin carcinomas, indicating strong tumor-promoting activity of 2,4-D. Again, there is no report of histopathology, so it would be impossible to distinguish conclusively between papillomas and carcinomas.

2. Conclusions

2,4-D has not been tested for carcinogenicity according to present standards, but two less-than-adequate experiments give statistically significant increases in malignant tumors when analyzed by organ systems. In both mice and rats, the lymphoreticular system was most sensitive to 2,4-D carcinogenesis, but other organs also showed increases.

The high incidence of tumors in the controls of both of the American studies is of concern in discriminating between a complete carcinogen and a promoter of existing unintentionally-initiated preneoplastic cells. This situation suggests that, unless the strains used have a genetic predisposition to cancer, something in their environment was mildly carcinogenic. This could be air-borne carcinogen from other tests in the same room, chemically-polluted drinking water, or contaminated feed.

It has recently been shown that small amounts of the strongly carcinogenic nitrosamines are present in several varieties of laboratory animal feed containing contaminated fish meal, with the highest level in the diet which is recommended by NIH for use in carcinogenicity testing (Edwards, 1979). The 2,4-D testing was done too long ago to have used this particular feed formula, but the diet used also contains fish meal. In this author's laboratory, a single injection of dimethylnitrosamine causes lymphosarcoma in those Wistar rats which did not die earlier of kidney or lung carcinoma, the primary and secondary sites of action of the carcinogen when given intraperitoneally. A very minute amount of initiator is needed when the appropriate strong promoter is present.

The Russian rats and mice were apparently very resistant to spontaneous carcinogenesis or lived in a very clean environment. Although the dose used was only 1/2 MTD, the number of animals used was twice the usual number, making it likely that a carcinogenic effect would be detectable. This suggests that the carcinogenic effect demonstrated in the American studies may be primarily promotion, an effect clearly demonstrated in the third Russian experiment in the mouse skin system. Cancer initiators are tissue-specific, although the most sensitive tissue may differ between species. Promoters may be less specific, affecting more different tissues.

Both phenol and 2,4-dichlorophenol, compounds used in the synthesis of 2,4-D, are also efficient promoters of mouse skin carcinogenesis (Boutwell and Bosch, 1959). 2,4-dichlorophenol is the first-level product of the breakdown of 2,4-D by microorganisms in the environment. Second-level products are chlorinated catechols, and their parent compound catechol has been shown to be a carcinogen on mouse skin (Van Duuren and Goldschmidt, 1976), so these compounds should be highly suspect.

The conditions of the American experiments approximate the conditions of American people: wide exposure to a variety of cancer initiators and a 25% spontaneous cancer rate in lifetime studies. Over such a background, the presence of additional nonphysiologic chemicals which can stimulate the rate of development of cancers from premalignant cells must be considered a significant hazard.

I. Conclusions About Health Effects of 2,4-D

2,4-D is lipid soluble and is rapidly and completely absorbed through all normal routes of exposure. Its butyl ester, and therefore probably all of its esters, is hydrolized before absorption from the gastrointestinal tract. It is not metabolized in animals, but rapidly penetrates the placenta.

2,4-D as normally manufactured does not contain chlorodibenzodioxins because the reaction is not heated sufficiently to form them. However, 2,7-DCDD (also known as 3,8-DCDD) could be formed if the reaction mix was overheated or if partially degraded 2,4-D were heated during storage or disposal. This dioxin is far less toxic than the TCDD found in 2,4,5-T, but has been shown to be fetotoxic to the heart muscle in rats.

Many cases have been reported of human poisoning by inhalation or absorption through the skin. Damage is primarily to the nervous system, and some such symptoms are not readily repaired. Gastrointestinal symptoms and irritation of mucous membranes are also seen.

Laboratory tests have shown development toxicity of 2,4-D in four species of animals. These include all four classes of developmental toxicity (malformation, malfunction, growth retardation, and lethality) but not all four in any one experiment. They include tests of the esters and amine of 2,4-D as well as the parent compound, and both pre- and postnatal adverse effects. A synergistic toxicity of 2,4-D and its primary microbial breakdown product, 2,4-dichlorophenol, on the fetal circulatory system was demonstrated in rats. The abstract of a Russian study, which is not available in the United States, reports a survey of workers in the production of herbicides of the 2,4-D family and finds that "substantial menstrual and child-bearing functions arise manifested as higher rate of miscarriages, premature births, toxicosis of second half of pregnancy, and the menace of miscarriages during the whole pregnancy period" (Elina, 1974).

An adequately designed study indicated that 2,4-D does not interfere with fertility in rats, although other studies have shown that it causes point mutations in animal cells, damages DNA in a manner similar to ionizing radiation, and stimulates cell division.

Carcinogenicity testing of 2,4-D has been limited to three studies, none of which meets today's minimum standards. In spite of the inadequate experimental design or assay, two of these studies

demonstrated carcinogenicity to the lymphoreticular system in rats and mice. The third study was negative for complete carcinogenesis, but demonstrated the strong tumor-promoting ability of 2,4-D in the mouse skin system. In a separate study, 2,4-dichlorophenol, the breakdown product of 2,4-D, proved to be a strong promoter and probably also a weak initiator in the mouse skin system.

The positive carcinogenicity studies had high background incidences of tumors in the unexposed animals, similar to the rate in the human population of the United States, while the negative test had a negligible background. This could mean that 2,4-D is only a promoter of previously initiated, premalignant cells, not a complete carcinogen by itself. If so, it is still a significant hazard because of the similar circumstances of the human population to the high-background experiments. Several human epidemiological studies showing increased cancer rates after 2,4-D exposure have all included persons exposed to other herbicides as well, making it impossible to prove that any particular chemical was to blame.

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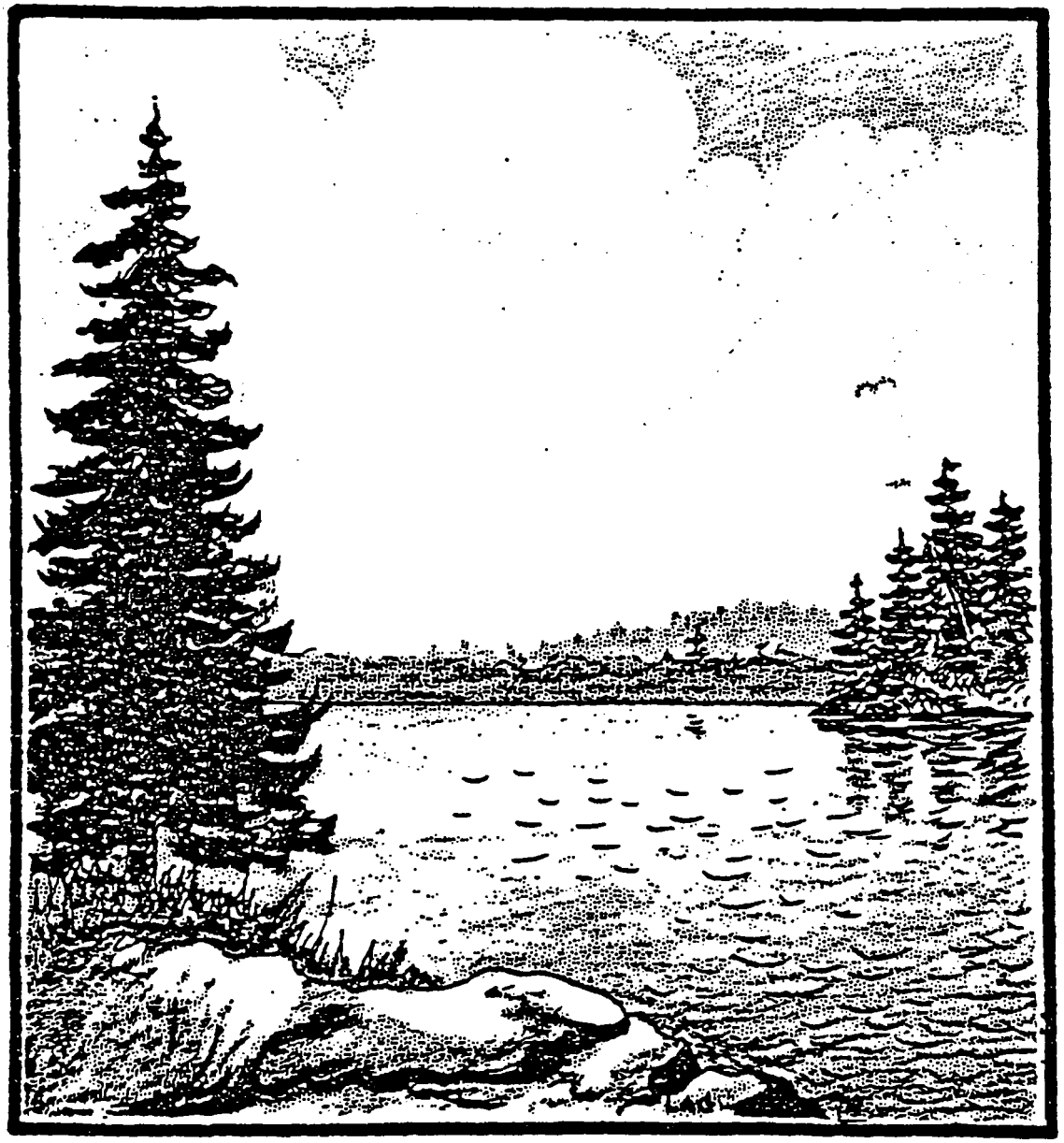
ASSESSMENT OF HUMAN HEALTH RISK ASSOCIATED WITH THE USE OF 2,4-D IN FORESTRY MANAGEMENT.



MINNESOTA DEPARTMENT OF HEALTH
717 S. E. DELAWARE ST. MINNEAPOLIS 55440

ASSESSMENT OF HUMAN HEALTH RISK ASSOCIATED WITH THE USE OF 2,4-D IN FORESTRY MANAGEMENT

Minnesota Department of Health
Division of Environmental Health
Section of Health Risk Assessment
717 S.E. Delaware Street
Minneapolis, Minnesota 55440

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INTRODUCTION

Recently, concerns have been expressed by Minnesota residents that the use of 2,4-D in forestry management may have an impact on human health and on the environment. This report is a quantitative assessment of possible human health effects resulting from the use of 2,4-D in forestry management. It does not contain an evaluation of the possible effects of 2,4-D on the wildlife population, except as a route of human exposure, or of possible effects on human health resulting from the use of 2,4-D in agriculture.

The scope of this assessment includes a literature review of the toxicology of 2,4-D and an estimation of 2,4-D exposure by a "maximally exposed individual". From this, conclusions and concerns are drawn on potential health impacts from exposure to 2,4-D and recommendations are made on the use of 2,4-D in forestry management in Minnesota.

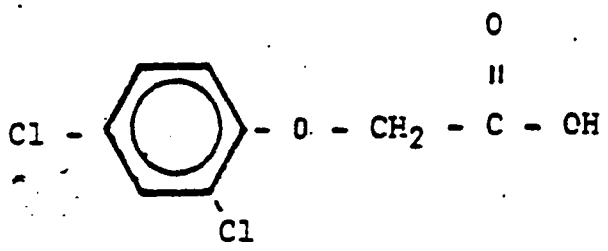
PHYSICAL AND CHEMICAL PROPERTIES OF 2,4-D

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is a member of the major chemical class Chlorinated Phenoxyalkanic Acids (see Table 1 for a partial list of other herbicides in this class).

Table 1. Chlorinated Phenoxyalkanic Acids

Common Name or Designation	Chemical Name
1. 2,4-D	(2,4-dichlorophenoxy)acetic acid
2. 2,4-DB	4-(2,4-dichlorophenoxy)butyric acid
3. 2,4,5-T	(2,4,5-trichlorophenoxy)acetic acid
4. Dichloroprop	2-(2,4-dichlorophenoxy)propionic acid
5. MCPA	[(4-chloro-o-tolyl)oxy] acetic acid
6. Silvex	2-(2,4,5-trichlorophenoxy)propionic acid

The chemical formula of 2,4-D is shown below:



Chemical and physical properties of pure 2,4-D are given below:

a. Description: Odorless white crystals

b. Boiling point: 160°C at 0.4 mm

c. Melting point: 140-141°C

d. Solubility: Soluble in 95% ethanol and in acetone, dioxane, and isopropyl alcohol; solubility in water is 540 ppm at 20°C

e. Stability: Stable up to and including its melting point

f. Reactivity: Forms salts that are soluble in water

Synthesis of 2,4-D

The syntheses of 2,4-D and 2,4,5-T are considered together because of their similarities in their production and also because of an important difference that has resulted in some confusion over the toxicities of the two herbicides.

The method of synthesis of 2,4-D is as follows (see figure 1), monochloroacetic acid is reacted with 2,4-dichlorophenol in the presence of an aqueous sodium hydroxide to form 2,4-D (U.S. EPA, 1974).

The production of 2,4,5-T is similar to that of 2,4-D except that 2,4,5-trichlorophenol is required as a starting material and this is manufactured by the basic hydrolysis of 1,2,4,5-tetrachlorobenzene. Hydrolysis is carried-out under pressure at an elevated temperature that must be carefully controlled to avoid formation of a toxic impurity, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). TCDD is one of the most toxic chemicals known. As an example, the LD_{50} value for a single dose of TCDD to guinea pigs is on the average 0.0006 mg/kg (Schwetz, et al., 1973). As a contaminant of 2,4,5-T, TCDD has generated considerable concern over the use of phenoxy herbicides. However, TCDD shcuild not be confused as a contaminant of all phenoxy herbicides.

Contaminants of 2,4-D

Of 28 samples of 2,4-D tested for content of chlorodibenzo-para-dioxins (this includes TCDD), no TCDD was found in any of the samples (Woolson, et al., 1972). However, hexachlorodibenzo-p-dioxin (HCDD) was found in one sample at a level of 10 ppm. HCDD is toxic but much less toxic than TCDD. Schwetz, et al.,(1973), observed teratogenesis in the offspring of rats treated with 100 mg/kg/day of HCDD. Maternal toxicity was also noted at this dose level. In a concurrent study, chick edema was produced

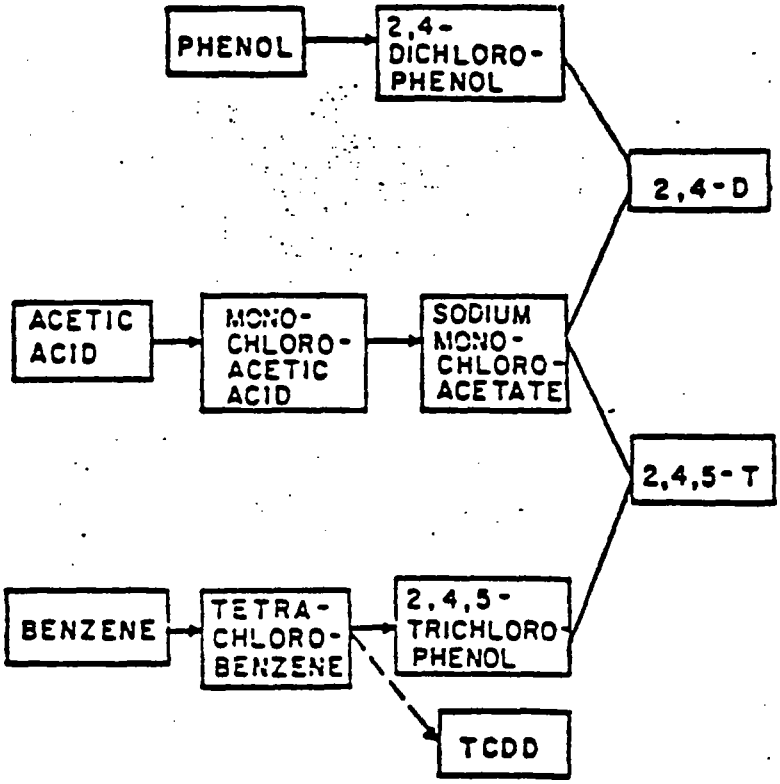


Figure 1. Diagrammatic representation of the major steps in the production of 2,4-D, 2,4,5-T. Also shown is the step where TCDD (2,3,7,8-tetrachlorodibenzo-para-dioxin) arises. No comparable material is formed in the 2,4-D process because of the different conditions for the production of the dichloro- and the trichloro-phenol.

in birds treated with 10 and 100 mg/kg-day of HCDD.

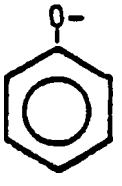
Another dioxin that may be associated with 2,4-D is 2,7-dichlorodibenzo-p-dioxin (DCDD). Schwetz, et al., (1973) found DCDD to be limited in toxicity. DCDD failed to cause death in female rats given oral doses of 1g/kg. DCDD was neither teratogenic or embryotoxic at 100 mg/kg/day.

The herbicide 2,4-D may be contaminated with isomeric 2,6-D (2,6-dichlorophenoxy acetic acid) unless careful control of the process is maintained. 2,6-D is more resistent to biological degradation than is 2,4-D (U.S.EPA, 1974).

Bis(2,4-dichlorophenoxy)methane has been identified as the major contaminant of 2,4-D (Huston, 1972). An analysis of a commercial sample of 2,4-D indicated that it contained 30 ppm of this contaminant. Smaller quantities of bis(2,6-dichlorophenoxy) methane and 2,2', 4,4'-tetrachlorodiphenoxy methane were also found. The toxicological significance of these three compounds as impurities in production grade 2,4-D is not known.

Formulations

2,4-D is a strong acid (pka 2.64) that is only slightly soluble in water and petroleum oils. Although the acid is the active form, it is normally converted to water-soluble amines or oil-soluble esters for convenience in handling and application and also to improve the effectiveness of the herbicide. To indicate the nature of the different formulations, the phenoxy group is designated as R.



Then examples of the various salts and esters are as follows:

Salts

Sodium salt, $R\text{-}CH_2COONa$

Potassium salt, $R\text{-}CH_2COCK$

Ammonium salt, $R\text{-}CH_2COONH_4$

Triethylamine salt, $R\text{-}CH_2COONH(C_2H_5)_3$

Triethylolamine salt, $R\text{-}CH_2COONH(C_2H_4OH)_3$

Alkyl Esters

Methyl ester, $R\text{-}CH_2COOCH_3$

Isopropyl ester, $R\text{-}CH_2COOC(H)(CH_3)(CH_3)$

Butyl ester, $R\text{-}CH_2COOC_4H_9$

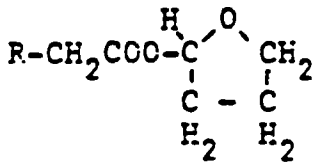
Octyl ester, $R\text{-}CH_2COOC_8H_{17}$

Heavy or Low Volatile Esters

Butoxyethanol ester, $R\text{-}CH_2COOC_2H_4\text{-}O\text{-}C_4H_9$

Propyleneglycolbutylether ester, $R\text{-}CH_2COOCH_2CH(OH)CH_2O\text{-}C_4H_9$

Tetrahydrofurfuryl ester



These formulations are then mixed with other ingredients, such as solvents, emulsifiers, thickeners, and wetting agents, to make commercial formulations for specific uses. The salt and amine forms are diluted with water and sprayed on foliage or injected undiluted into trees. Esters are sprayed as oil solutions or as emulsions with water.

The strength of commercial formulations is expressed in terms of the equivalent content of the parent acid. However, different products are not equally effective even on this basis. Esters generally have greater herbicidal activity than the amine forms.

Other studies also suggest that under normal use 2,4-D persists in soil for about a month. In moist loam soil 2,4-D applied at a rate of 1/2 to 3 lbs./acre persisted for 1 to 4 weeks, under summertime conditions in a temperate climate (Klingman, 1966). Alexander and Aleem (1961) reported that 2,4-D, at normal recommended dosages, persisted for about 1 month in two types of soil and for about 3 months in a third type.

Degradation of 2,4-D has also been studied in forest litter. In one study with red alder forest floor material, 94% of the 2,4-D was degraded in 35 days (see Figure 3)(Norris, 1971). Compared to other herbicides studied in this investigation, degradation of 2,4-D was rapid.

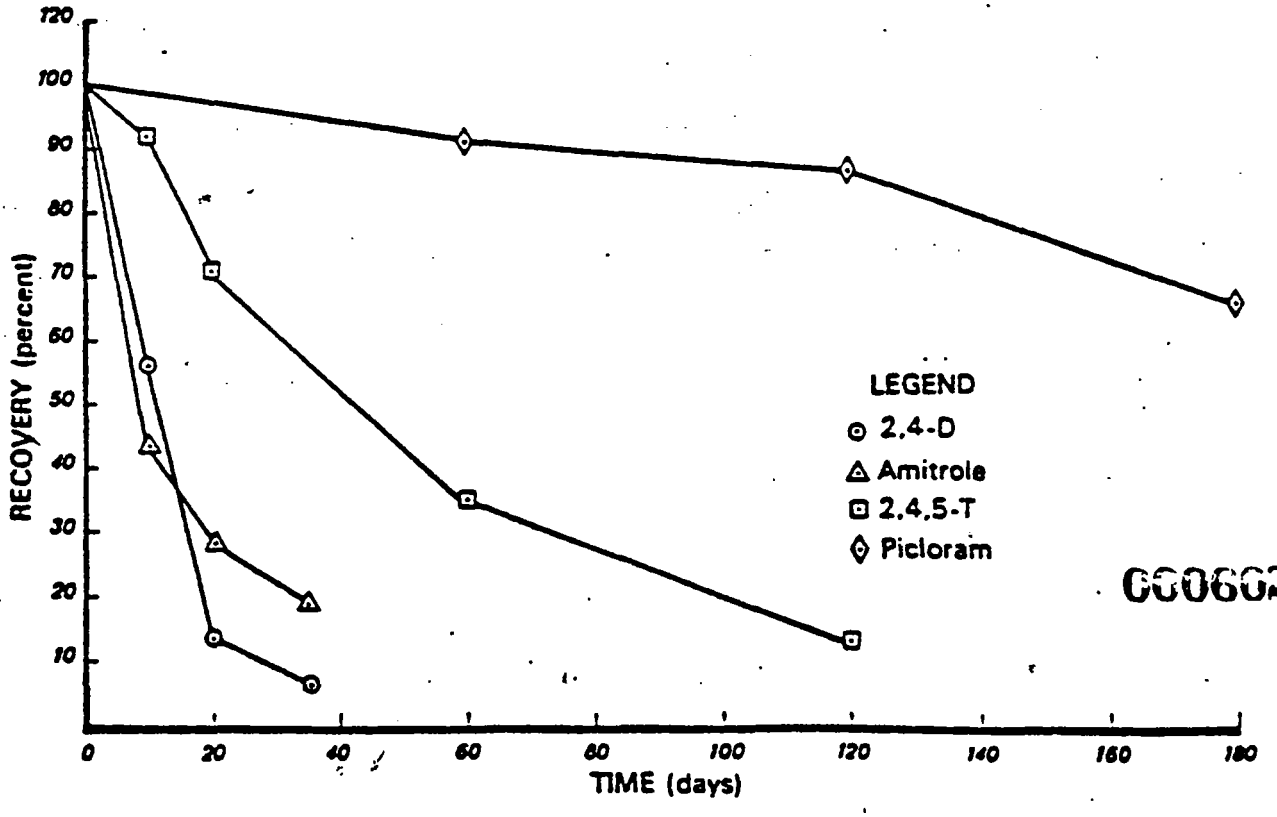


Figure 3. Recovery of 2,4-D, amitrole, 2,4,5-T, and picloram from red alder forest floor material. Amitrole, 2,4-D, and 2,4,5-T applied at 2 pounds per acre and picloram at 0.5 pounds per acre in water (Norris, 1971)

PERSISTENCE IN THE ENVIRONMENT

The persistence of 2,4-D in the soil and water is an important factor in its potential to occur as residues in foods and water or to come in contact with nontarget organisms.

Persistence in Soils

The persistence of 2,4-D in soils appears to be a function of several factors. These factors include concentration, soil type, formulation, temperature, moisture content, organic matter, and possibly microbial ecology at the site of application. Therefore, it is difficult to assign any finite limits on the time 2,4-D will remain intact. A more reasonable approach is to assign a time range in which 75 to 100% dissipation has occurred. The persistence of 2,4-D, when applied at recommended rates, is about 1 month. This is a relatively short time when compared to other herbicides (see figure 2)(Kearney, 1970).

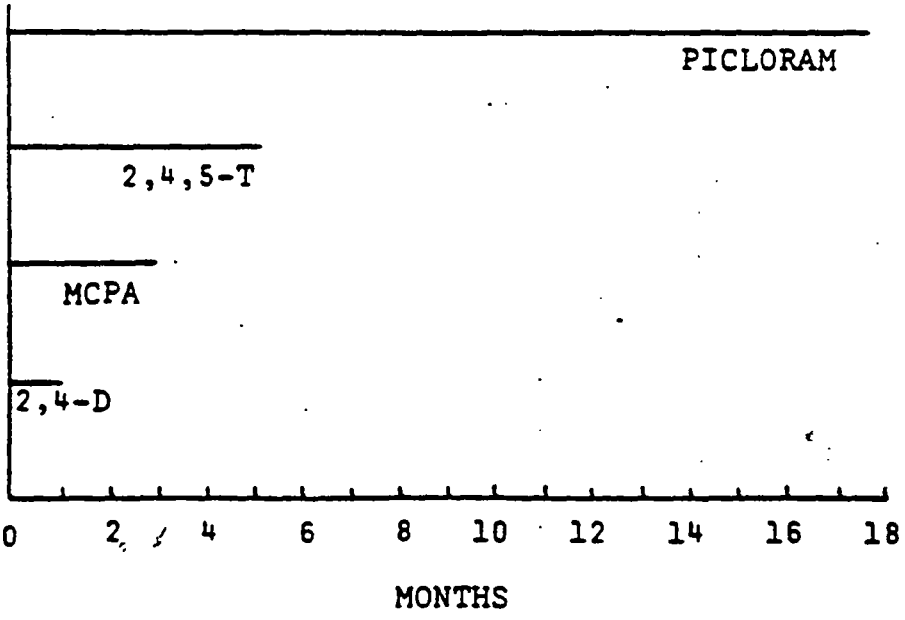


Figure 2. Persistence of herbicides in soils. Length of bar represents time required for 75 to 100% loss of the compound (Kearney, 1970).

Norris (1966) noted similar results in an earlier study where degradation of triethanol amine salts of carbon^{14}-carboxyl labeled 2,4-D applied to an alder was measured as liberated $C^{14}O_2$. More than 89% of the applied 2,4-D could be accounted for by the liberation of radioactive CO_2 in 315 hours.

In the soil in the presence of moisture, even at relatively low levels, the esters of 2,4-D are readily converted (hydrolyzed) into the acid form (Smith, 1972). The degradation of 2,4-D acid can be shown to be due primarily to the action of soil micro-organisms (Allebone, et al., 1975).

Persistence in Water

In water, 2,4-D breaks down either photochemically or under the action of aquatic micro-organisms. Light provides the energy for a variety of degradative reactions including oxidation, reduction, and hydrolysis (Allebone, et al., 1975).

In surface waters, levels of 1,000 ppb were degraded to 10 ppb within 30 days of application (House, et al., 1971, cited in Allebone, et al., 1975).

DeMorco, et al. (1967), investigating the persistence of 2,4-D in natural lake waters, found that in "warm" aerobic waters the herbicide was degraded within 6 days whereas in "cold" deoxygenated waters it persisted for up to 80 days.

Low levels (0.024 ppm) of 2,4-D were found in pond water 1 day after application of 1.33 ppm granular material (Frank and Comes, 1967). The concentration in water increased to a maximum of 0.067 ppm after 18 days and 2,4-D could no longer be recovered after 24 days. Higher concentrations (4.96 ppm) were found in the hydrosoil

after 1 day, and there tended to persist longer. Negligible residues (0.05 ppm) were detected after 55 days in the soil. The increased persistence of 2,4-D in sediment was also noted by Smith and Ison (1967). They recorded and isolated significant concentrations of 2,4-D (58.8 ppm) in sediment samples removed from a reservoir some 10 months after treatment.

Degradation in soil appears to be rapid, occurring in several weeks in most soils and forest litter. Degradation in water is also rapid; however, 2,4-D appears to persist longer in bottom sediments. The two main pathways of microbial degradation are thought to be via a hydroxyphenoxyacetic acid intermediate or via the corresponding phenol (Allebone, et al., 1975). Apparently degradation goes to completion (CO_2) although the exact mechanisms are not well understood (Norris, 1966). No information is available on the toxicity of intermediate metabolites or other possible end products of microbial or photo degradation.