Quantitative Aspects of Risk Assessment in Chemical Carcinogenesis
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Non-Linear Pharmacokinetic Parameters Need to be Considered in High Dose/Low Dose Extrapolation

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A. Introduction

Numerous recent studies, both with short-term in vitro tests and longterm animal bioassays, have led to the designation of many materials encountered in our daily lives as potential carcinogens. However, the classification of a material as a potential carcinogen does not necessarily mean that this substance, at the concentrations present in the environment, is actually a danger to human health. In order to conclude that a potential carcinogen does constitute a public health hazard, we must first perform a quantitative risk estimation for that material. In doing so, we must remember that any estimation process will always indicate some finite degree of risk, no matter how small. Thus, our aim must be to identify acceptable levels of risk, rather than to attempt to erroneously eliminate all risk.

Only with realistic risk estimates can we properly evaluate the consequences of regulatory action. For example, would it be wise to stop chlorinating drinking water to reduce cancer risk from chloroform if this action might lead to a typhus epidemic? Or, should we discontinue use of all insecticides with even the slightest suggestion of carcinogenic risk if this action would lead to crop failures and famine?

In view of the fact that literally hundreds of common industrial or natural chemicals have been indicted on some basis or another as potential carcinogens, we must insist on a careful evaluation of all the factors involved in carcinogenesis. Reasonable estimations of carcinogenic risk to man can be obtained only when we combine carefully conducted animal bioassays with a fundamental understanding of pharmacokinetic processes and biochemical mechanisms of tumorigenesis.

B. Significant Factors in Risk Estimation

I. Mechanisms of Tumorigenesis

It is becoming increasingly clear that there are at least two types of processes which can contribute to the observation of an increased incidence of tumors in an animal bioassay; genetic and epigenetic. The first of these involves reaction of the test

substance (or a metabolically activated form of the test substance) with the genetic material of the cell. This genetic mechanism is illustrated in the pioneering work of the Millers (1966, 1970) showing that many potent carcinogens are electrophiles which react with sites on the bases of DNA to disturb normal base pairing. If the altered bases are not removed by DNA repair systems before replication, they may become permanent genetic changes or mutations. Support for the somatic mutation hypothesis of chemical carcinogenesis includes the following observations: many chemical carcinogens induce mutations; the long-latent period between chemical exposure and the manifestation of cancer is consistent with effects in a macromolecule such as DNA which is capable of retaining information over a long period of time; genetic diseases with associated chromosomal abnormalities are associated often with an increased incidence of cancer; and many chemical carcinogens interact with DNA.

In addition to genotoxic effects, chemicals can also produce tumors through cytotoxic effects. In this case, tissue damage occurs to such an extent as to cause cell death and subsequent cell regeneration.

During chronic administration of cytotoxic agents, DNA replication is stimulated greatly in the affected tissues. Since there is a tiny but still finite chance for error in each replication cycle, the net effect is to increase the spontaneous mutation rate.

Another consequence of increasing cell division is that the relative rates of DNA repair and DNA replication are altered. This may be very important because there is evidence that the existing DNA repair systems may fail to recognize DNA alterations *after* replication. Consequently, repair becomes less effective in protecting cells from the consequences of endogenous genetic damage. This is shown by the work of Berman et al. (1978) and Weymouth and Loeb (1978) who found that increased division of normal cells increases their susceptibility to mutagenic events. This is also demonstrated by the studies of McCormick (1979) who found that momentarily arresting cell division to give added time for DNA repair greatly diminished the mutational action of uv light.

Further indication that increased cell division can be significant comes from the observations of Laroye (1974) who found that many cancers develop in chronically inflamed or scarred tissue (colonic cancer in patients with ulcerative colitis or squamous cell carcinomas in ulcers of burn scars). Berenblum (1929) observed that repeated freezing of the skin with dry-ice induced tumors, and Peraino et al. (1973) reported the enhancement of "spontaneous" tumors in mice by dietary phenobarbital, a treatment also known to stimulate DNA synthesis. Similarly, the tumor yield from a known genetic carcinogen is enhanced greatly after partial hepatectomy (Date et al., 1976) or by physical trauma at the site of initiation (Berenblum, 1944).

Although the tumors that develop from each type of mechanism cannot be distinguished, there are fundamental differences in the type of safety precautions appropriate to each type of agent. Consequently, these will be discussed separately in the sections to follow.

II. Factors Influencing Genetic Events

Some individuals have invoked the somatic mutation theory as grounds for postulating that since a single molecule of a chemical could conceivably react with DNA to cause a

mutation, there can be no threshold in carcinogenesis. Although the subject of thresholds can never be experimentally resolved, it is clear that there are a number of factors which can dramatically affect the rate of tumor production.

To illustrate why this is true, refer to Figure 1 which shows the typical chemical processes that determine the fate of a chemical (C) in the body. Each arrow represents a first-order rate process. The dominant process governing the fate of the chemical may be different for different species, different chemicals, and frequently, for different doses of the same chemical. Interferring with excretion or detoxification will enhance covalent binding of a reactive, electrophilic ("electron-seeking") metabolite (RM) to macromolecules, both genetic (CBG) and nongenetic (CBN).

For example, furosemide, a diuretic, is excreted predominantly intact in the urine when low doses are given to patients. When high doses are administered, renal clearance is overwhelmed causing a disproportionate increase in the formation of toxic metabolites which react covalently with macromolecules. As another example, bromobenzene is transformed in the liver to the chemically reactive bromobenzene-3,4-epoxide. This molecule is detoxified enzymatically by conjugation with glutathione. However, if large doses are administered, glutathione is depleted and the reactive metabolite reacts instead with macromolecules.

Actual metabolic reactions are often more complex than the foregoing illustrations. For many chemicals, absorption, distribution, biotransformation, and excretion occur via active transport or enzymatic reactions.

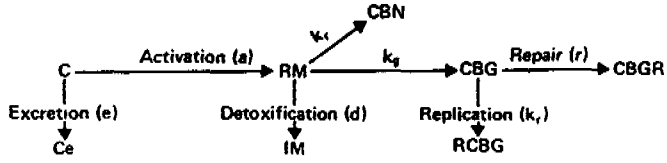
Rather than being best portrayed by first-order or linear rate processes in accordance with the equation

$$\frac{\partial C}{\partial t} = -KC,$$

these reactions are described more accurately by Michaelis-Menten kinetics or dose-dependent kinetics in accordance with the equation

$$\frac{\partial C}{\partial t} = \frac{V_m C}{K_m + C}.$$

This suggests that at some-level these reactions may "saturate".



C = Chemical
RM = Reactive Metabolite
Ce = Excreted Chemical
IM = Inactive Metabolite
CBN = Covalent Binding, Nongenetic
CBG = Covalent Binding, Genetic
CBGR = Repaired Covalently Bound Genetic Material
RCBG = Retained Genetic Program, Critical & Noncritical

Fig. 1. Chemical processes determining the fate of a chemical (C) in the body

It is likely that saturation of detoxification is the rule rather than the exception for chemicals possessing a low order of toxicity, because high doses can be administered without killing the animal. The implication of saturating protective mechanisms such as DNA repair can be illustrated by modelling the processes shown in Figure 1. In this figure the processes referred to previously (absorption, excretion, metabolic activation) are considered to occur in accordance with Michaelis-Menten kinetics. Reaction of the reactive metabolite (RM) with macromolecules some of which are nongenetic (CBN) and genetic (CBG) is perceived to occur as an apparent first-order rate process. Replication of genetic material (CBG) having bound covalently to it the reactive metabolite is represented, naively, as an apparent first-order rate process. This process gives rise to replicated genetic material possessing a finite probability, albeit very small, of programming the cell to become cancerous.

Repair of genetic material having reactive metabolite bound covalently to it does occur and there exists evidence that the repair process can be overwhelmed (Pegg et al., 1976). Hence this process is envisioned to occur in accordance with Michaelis-Menten kinetics.

Using the model in Figure 1 as discussed above, a series of differential equations were ascribed to the model and realistic values for the various constants selected (Gehring and Blau, 1977). Using different doses (C_0), the equations were numerically integrated using a computer. As a function of dose, values were determined for nongenetic material bound covalently with reactive metabolite (CBN/C_0), genetic material bound covalently with reactive metabolite and repaired ($CBGR/C_0$), and genetic material bound covalently with reactive metabolite but not repaired ($RCBG/C_0$). The values were normalized for dose. The results are shown, in Figure 2.

Figure 2 shows that with increasing doses there occurs a disproportionate increase in the amount of reactive metabolite bound to genetic material which escapes repair ($RCBG/C_0$). Since formation of such material has been associated with chemical carcinogenesis (Goth and Rajewsky, 1974), this highly conservative model illustrates why excessive doses of some chemicals cause discernible increases in cancer while smaller doses may be without effect. It also illustrates clearly why data generated from such studies cannot be extrapolated as a simple linear function of dose to predict the incidence of cancer which may be incurred by exposure to smaller amounts of a chemical.

Data developed for two potent carcinogens, N-ethyl-N-nitrosourea (ENU) and dimethylnitrosamine (DMN) demonstrate the appropriateness of certain aspects of the model, specifically the existence of a saturable repair mechanism. Administration of rather high doses of ENU to newborn rats results in a high incidence of malignant tumors of the central and the peripheral nervous systems. At such dose levels, liver tumors do not develop. Goth and Rajewsky (1974) correlated these events with ethylation of the 0--6 position of guanine in DNA, (i.e., alkylation of DNA bases, as discussed earlier). Excision repair by enzymatic removal of the ethylated guanine from DNA occurs readily in liver but only poorly in nervous tissue. It has been shown by Gerchman and Ludlum (1973) that 0--6 alkylation of guanine results in misincorporation of bases in a bacterial nucleic acid polymerase system. Thus, in nervous tissue the 0--6-ethylguanine in DNA has a greater opportunity to miscode DNA replicated for daughter cells. The ethylated guanine may pair with thymine rather than cytosine. As a consequence, cancer occurs in nervous tissue but not in liver.

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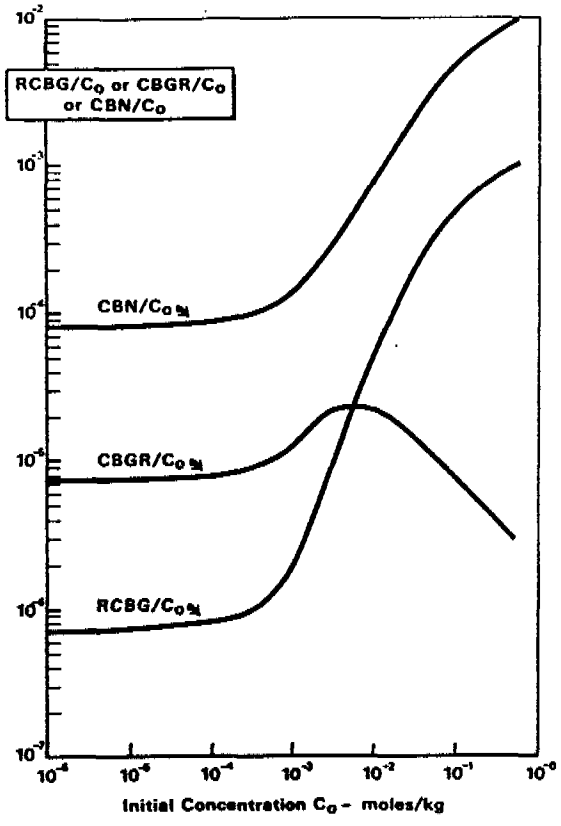

Fig. 2. Effect of increasing initial dose (C_0) upon fraction of chemical bound to nongenetic macromolecules (CBN), fraction bound to genetic macromolecules and subsequently repaired (CBGR), and fraction bound to genetic macromolecules and fixed by replication (RCBG)

DMN given as a large single dose produces kidney tumors in rats, but does not produce liver cancer. Recently, Pegg et al. (1976, 1977) have demonstrated that excision repair of DNA having guanine methylated in the 0—6 position occurs readily in the liver of rats given both a high and low dose of DMN. In the kidney; however, repair of DNA having 0—6 methylated guanine occurs readily only after administration of low, noncarcinogenic doses, Figure 3.

Thus, we must conclude that alkylation of DNA may lead to cancer induction. Further, we must conclude that administration of high doses inhibits or exceeds the capacity of the repair mechanism. Indeed, the data presented on ENU and DMN indicate that cancer occurs if the target tissue has a poor capacity for repair of DNA of if the doses are of sufficient magnitude to inhibit repair or exceed the capacity of the repair process.

Another important consideration is the role of the various biotransformation reactions in the expression of the toxicity of any given chemical.

For example, it is clear that for many of the small halogenated hydrocarbons, metabolic activation is required for toxicity. This may be indicated in a variety of ways; for example, by finding increased toxicity after induction of mixed function oxidases (MFO) or decreased toxicity after inhibition of MFO (Ilett et al., 1973). Alternatively, a requirement for S-9 liver extract in the Ames bacterial mutagenicity test indicates that metabolic activation is occurring.

This information is very important when attempting extrapolation of results from animal tests to man. As pointed out by Rall (1969), it is often possible to estimate roughly the relative sensitivity of several species to a material by using the approximation that the basal metabolic rate is roughly proportional to the body surface area. What this means is that, if we neglect factors other than metabolism, a large animal species is more sensitive than a small one to a directly toxic agent. Conversely, a large animal species is less sensitive than a small one to toxicity mediated through a reactive metabolite (Reitz et al., 1978). The contribution from oxidative metabolism to relative risk factors in various species is summarized in Table 1.

Application of this rule to a series of six halogenated hydrocarbons giving a positive bioassay in at least one species at the National Cancer Institute of the United States correctly predicts the most sensitive species in every case (Table 2). However, it must be emphasized that for some types of metabolic activation the relative rates may

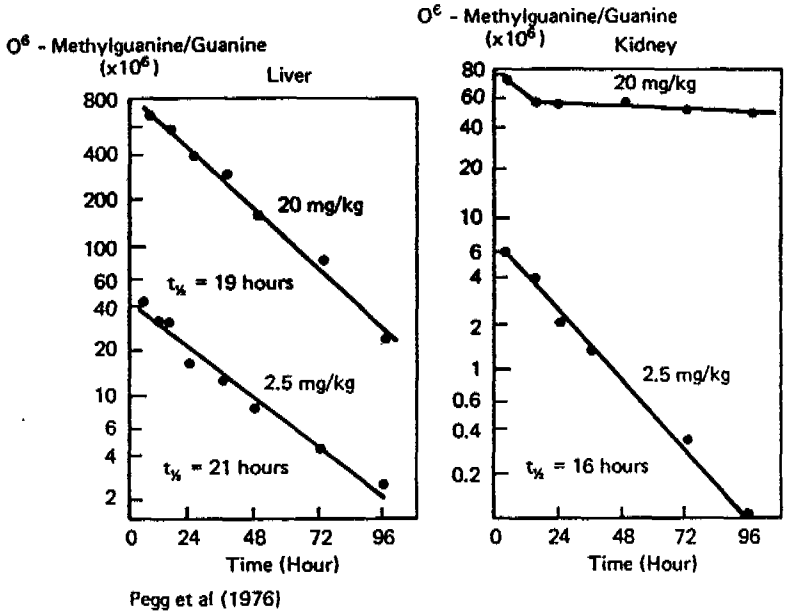

Fig. 3. DNA repair in liver and kidney of rats after 20 mg/kg or 2.5 mg/kg of DMN

Table 1. Predicted relative cancer risk from equivalent doses (mg/kg) calculated on the basis of (body weight)$^{0.5}$*

	A Directly toxic agents	B Metabolically activated agents
Man (70 kg)	1.00	1.00
Dog (20 kg)	0.66	1.52
Rabbit (3 kg)	0.35	2.85
Rat (0.5 kg)	0.18	5.58
Mouse (0.03 kg)	0.08	13.2

* After Rall (1969)

not be a simple function of body size. For example, the metabolic activation of 2-acetaminofluorene involves formation of an active sulfate (Miller, 1978), and the species and organ sensitivity of the tumorigenicity of the agent correlate well with the level of sulfatransferase enzyme. However, in this case, the rat has a higher level of sulfatransferase than the mouse and also develops more tumors when exposed to an equivalent dose of 2-acetaminofluorene. Hence the reliability of interspecies extrapolation depends to a large degree upon how much is known of the details of metabolism.

III. Integration of Concepts for Risk Extrapolation

As a practical example of the application of these concepts, a risk extrapolation will be performed for vinyl chloride (VC). As with many other chemicals, the extrapolation will start with the results of a long-term animal bioassay; in this case the study of Maltoni and Lefemine (1975). In this experiment, rats were exposed to different concentrations of VC (50 to 10,000 ppm) 4 h/day, 5 days/week, for 12 months and subsequently held for observation until death. The tumors observed in these animals are reported in Table 3, column 3.

Before analysis of these data, two important points must be considered:

1) Numerous studies in rats indicate that it is not VC per se which is responsible for production of angiosarcoma but rather a reactive metabolite formed from it in the body (Bartsch et al., 1975; Barbin et al., 1975; Kappus et al., 1976; Malavielle et al., 1975; Bolt et al., 1975; Watanabe et al., 1978). Consequently, the risk of developing a tumor from VC will not be related to the exposure concentration directly, but rather to the rate of metabolism of VC.

Table 2. Carcinogen bioassay results (National Cancer Institute, USA) for a series of materials in which metabolic activation is thought to precede toxicity

	Mice	Rats
Perchloroethylene	+	–
Trichloroethylene	+	–
Allyl chloride	+	–
Trichloroethane (1,1,2)	+	–
Carbon tetrachloride	+	±
Chloroform	++	+

Table 3. Predicted incidence of angiosarcoma in rats exposed to vinyl chloride using various models versus dose (v, μg vc metabolized per day) compared to experimental results

Exposure ppm	Dose v, μg/day	Experimental %	Predicted %			
			Model A	Model B	Model C	Model D
10,000	5,521	14.8 (9/61)	19.8	20.0	19.7	18.9
6,000	5,403	21.7 (13/60)	19.3	20.5	19.3	18.6
2,500	5,030	22.0 (13/59)	17.9	18.1	17.9	17.4
500	3,413	11.9 (7/59)	12.1	11.8	12.2	12.2
250	2,435	6.8 (4/59)	8.1	8.0	8.7	8.8
50	739	1.7 (1/59)	1.4	1.4	2.6	2.8

2) Biotransformation of VC in rats is a nonlinear process occurring in accordance with Michaelis-Menten kinetics:

$$v = \frac{V_m S}{K_m + S}$$

(Watanabe et al., 1976a–c; Bolt et al., 1976). In this equation v and V_m are velocity and maximum velocity, respectively, for the biotransformation of VC expressed as μg equivalents VC metabolized daily. S and K_m are the concentration of VC being inhaled and the Michaelis constant expressed as μg VC/l air, respectively.

To extrapolate the risk to man, the first step is to empirically fit a model relating the quantity of VC metabolized and the observed tumor incidence. There are a number of possible models which could be used for this. Four of the most commonly employed were selected for evaluation. These are:

A) Probit % $= a + b(\log v)$
$= -1.625 + 1.543(\log v)$.

B) Linear % $= a + b(v)$
$= -1.48 + 0.389 \times 10^{-2}(V)$.

C) Linear Forced through Origin
% $= bv$
$= 0.3565 \times 10^{-2}(v)$.

D) One-hit Model (CAG)*
% $= [1 - \exp^{-\beta(v)}] \times 100$
$= [1 - \exp(-0.38 \times 10^{-4} \times v)] \times 100$.

The constants given for the preceding models were determined by linear regression analysis and represent the best fit of the data for the incidence of hepatic angiosarcoma versus the amount of VC metabolized daily in rats exposed to different concentrations of VC (Gehring et al., 1978).

The data used to derive the constants are given in Table 3. Using the equations derived for the models, the predicted incidence of angiosarcoma for the groups of rats exposed to different concentrations of VC were calculated. These results are shown also in Table 3. All four models overpredict the incidence of hepatic angiosarcoma in rats exposed to 10,000 ppm VC. This is as expected, because this exposure resulted in excessive mortality unrelated to development of angiosarcoma.

With respect to the predicted versus experimental incidence of hepatic angiosarcoma experienced by groups of rats exposed to the remaining concentrations of VC, none of the four models is unequivocally more reliable than any other. Thus, any one of the four models represents adequately the experimental data. Since no clear preference is discernible, all four models will be used to predict the incidence of hepatic angiosarcoma experienced or to be experienced by workers exposed to VC.

In order to calculate the equivalent dose of VC metabolite in exposed workers, the following assumptions were made:

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* CAG = Cancer Assessment Group, National Cancer Institute

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1) The exposure period for rats (1 year = one-half lifetime) is equivalent to a 35-year exposure in man (also one half lifetime).

2) The rate of metabolism of VC in both species is low relative to the rate of equilibration between lungs and plasma. This means that inhalation of equivalent vapor concentrations of VC would result in equal plasma concentrations of VC at steady state.

3) The K_m of the enzyme which activates VC is identical in the two species, but V_{max} differs in the two species according to the body surface area formula. That is, the V_{max} (man) in μg VC metabolized per kg body weight $= \sqrt[3]{250/70{,}000}\ V_{max}$ (rat) or V_{max} (man) $= 0.153\ V_{max}$ (rat), where 250 g and 70 kg are assumed to be the body weights of rat and man, respectively.

After correction for the difference in hours of exposure (6 h for rat versus 8 h for man) the μg of VC metabolite produced per day in workers is given by the equation:

$$v = \frac{1675\ \mu g/h \cdot S\ \mu g/l}{860\ \mu g/l + S\ \mu g/l}.$$

This equation may be used in conjunction with the four models and the rat data to predict the incidence of angiosarcoma in workers exposed to any concentration of VC for a given fraction of the working life (35 years). Since a comprehensive survey of VC workers has been published (Equitable Environmental Health, Inc. 1978), an excellent opportunity existed to assess the validity of the various models. The incidence of hepatic angiosarcoma in subgroups of 9677 workers with respect to their duration of exposure are given in Table 4. These workers include 95.1% of the 10,173 in the cohort study; the remainder could not be traced.

The atmospheric concentrations of vinyl chloride to which these workers had been exposed were not quantitated, except subjectively into high, medium, and low categories. Since the time-weighted-average (TWA) exposure recommended by the American Conference of Governmental Industrial Hygienists (ACGIH) prior to 1972 was 500 ppm and subsequently 200 ppm until adoption of the Occupational Safety and Health Act standard of less than 1 ppm in 1974, it is assumed that even those exposures subjectively deemed low were high. Indeed, it is reasonable to expect the TWA exposures of 200 ppm and greater for 8 h were common rather than the exceptions. The actual incidence of angiosarcoma, as well as the predictions from each of the four models is summarized in Table 5 for workers exposed to 200 and 500 ppm VC. Ex-

Table 4. Incidence of angiosarcoma in 9,677 workmen exposed to vinyl chloride

Population	Duration of exposure, years	Mean exposure duration, years	Fraction of working life	Observed angiosarcomas
4,384 (0.31)	<4	2	0.06	1 (18 years)
2,339 (0.27)	5–9	7	0.20	0
946 (0.58)	10–14	12	0.34	2 (15 and 23 years)
1,007 (1.0)	15–19	17	0.49	2 (18 and 19 years)
677 (1.0)	20–24	22	0.63	0
324 (1.0)	25+	27	0.77	0
9,677				

Table 5. Predicted number of angiosarcomas in workmen exposed to 500 or 200 ppm vinyl chloride 8 h/day, 5 day/week for various fractions of a 35-year working life versus the numbers observed using biotransformation and angiosarcoma incidence data from rats and four models for extrapolation

Population	Angiosarcomas observed	Model A		Model B		Model C		Model D	
		500	200	500	200	500	200	500	200
4,384	1	0.2	0.0	0	0	9.4	5.9	10.0	6.3
2,339	0	2.4	0.8	0	0	16.7	10.4	17.7	11.1
946	2	3.0	1.1	0	0	11.5	7.2	12.1	7.6
1,007	2	6.8	2.7	4.0	0	17.6	11.0	18.6	11.7
677	0	7.3	3.0	6.6	0.3	15.2	9.5	16.0	10.1
324	0	4.9	2.1	4.9	1.3	8.9	5.6	9.4	5.9
9,677	5	25	10	16	2	79	50	84	53

amination of the data in this table indicates that Models C and D overestimate the incidence of hepatic angiosarcoma experienced by these workers while Model B underestimates the incidences.

When a TWA of 200 ppm is utilized to represent the atmospheric concentration to which these workers were exposed, the predicted incidence of angiosarcoma of 10 cases using Model A compares favorably with that experienced; five cases. For a significant number of workers the time elapsed since initial exposure has yet to reach 15 years, the shortest time needed for development of angiosarcoma in any of the afflicted workers, Table 4. Thus, it may be anticipated that 3—4 additional cases might occur in this population. It is comforting that an epidemic of cancer cases, as has been envisioned by some, does not seem likely.

The foregoing analysis using a probit percentage incidence model (Model A) has revealed that data collected in experiments on rats may be used to predict the incidence of hepatic angiosarcoma in humans with reasonable accuracy. It is of interest to calculate that, using the model which appears to be most reliable (Model A, Probit %) exposure of workers to 1 ppm VC for 35 years would be expected to result in 0.015 tumors/million exposed population, clearly a rather minor risk.

C. Nongenetic Events in Tumorigenesis

As mentioned earlier, there appears to be at least two mechanisms which can result in tumor induction in animals: Genetic (involving direct DNA damage) and Nongenetic or Cytotoxic, involving prolonged stimulation of DNA replication.

I. Implications for Risk Assessment

Although tumors can be produced by either genetic or epigenetic mechanisms, there are fundamental differences in these mechanisms with important implications for risk extrapolation. Genetic damage may be latent for years and only be detectable with the actual appearance of tumors. Genetic damage may also be irreversible if the lesion escapes DNA repair. In contrast, tissue damage associated with epigenetic mechanisms

)0 or 200 ppm vinyl chloride
ıe numbers observed using bio-
nodels for extrapolation

ıdel C		Model D	
)	200	500	200
4	5.9	10.0	6.3
7	10.4	17.7	11.1
5	7.2	12.1	7.6
6	11.0	18.6	11.7
2	9.5	16.0	10.1
9	5.6	9.4	5.9
	50	84	53

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should be clinically "visible" and is likely to be reversible. More importantly, although there may be some theoretical basis for arguing that genetic carcinogens may not have a threshold, all the cumulative experience of the science of toxicology suggests that there is a threshold for tissue damage and hence for nongenetic mechanisms of carcinogenesis. Thus, although we must continue to exercise great caution when dealing with genetic carcinogens, conventional techniques of industrial hygiene and toxicology can identify exposure levels which do not produce tissue damage and which will preclude the induction of any tumors through nongenetic mechanisms.

II. Experimental Procedures for Assessing the Mechanism of Tumorigenesis

In order to determine what types of safety precaution are appropriate for dealing with a carcinogen whose mechanism is not known, objective laboratory tests must be developed. Several procedures are under investigation currently at Dow's Toxicology Research Laboratory in order to determine their utility in indicating the mechanism of tumorigenesis. Primary attention has been focused on in vivo procedures since these appear to have the greatest potential for understanding the tumorigenic process in the intact animals. Four such indicators will be described, and then experimental results will be employed to classify three chemical agents, dimethylnitrosamine (DMN), vinylidene chloride (1,1-dichloroethylene, VDC) and perchloroethylene (1,1,2,2-tetrachloroethylene, PERC), as genotoxic or cytotoxic.

1. Genetic Indicators

a) *DNA Alkylation.* In this procedure animals are exposed to a radioactive form of the chemical to be tested. Next, tissues are excised and DNA is isolated and purified by a modification of the method of Marmur (1961). A stringent purification of DNA is necessary for these experiments because large amounts of radioactivity become associated with macromolecules other than DNA. Consequently, contamination with even small amounts of these highly labelled materials would affect the estimation of the low levels of DNA bound radioactivity. For this reason, DNA isolated by the procedure of Marmur has been further purified by digestion with specific enzymes to remove protein, glycogen, and RNA (Reitz et al., 1980).

The degree of alkylation (and hence damage) is determined by quantitating the DNA colorimetrically and measuring radioactivity by scintillation counting. For convenience, all alkylations are reported in the units of alkylations/10^6 nucleotide residues.

b) *DNA Repair* is an indicator of preceding DNA damage. This can be estimated in vivo by measuring the rate of incorporation of ^{3}H-thymidine into DNA in the presence of hydroxyurea (Cleaver, 1969; Craddock et al., 1976). While this procedure does not have the sensitivity of the DNA alkylation method, it can be performed with a nonradioactive form of the chemical to be tested. In utilizing this method, it is essential that correction for the effect of the test chemical on the amount of normal replication escaping hydroxyurea be performed as suggested by Arfellini et al. (1978).

2. Nongenetic (Cytotoxic) Indicators

a) *DNA Synthesis* — A common characteristic of the treatments which increase spontaneous or chemically-initiated tumor incidence is that they stimulate cell division

(Marx, 1978). This can be measured directly by administration of ^{3}H-thymidine to animals at a time when regeneration is occurring (48–96 h post exposure). DNA is isolated from the tissues of treated or control animals by the procedures already described (DNA Alkylation).

Since DNA synthesis may vary with age, time of day, nutritional status, etc., a matched control group is always surveyed at the same time as the treated group, and the ratio of treated to control is determined. (A ratio of 1.00 indicates that treatment had no effect, while ratios greater than 1.00 reflect a stimulation of DNA synthesis.)

b) *Histopathology* — The microscopic examination of tissue sections from treated animals has been one of the most useful techniques of toxicology. This technique yields information regarding the sensitivity of various organs, the persistence of the toxic effect, and a rough indication of the severity of cytotoxic lesions.

III. Experimental Classification of Mechanism of Carcinogenesis

The results of studies of DNA alkylation with the three chemicals are shown in Table 6. In each case, the doses chosen for this study are nearly the maximum that can be given to these animals without causing some delayed toxicity, and consequently, approximate a "maximum tolerated dose" (MTD). In each case, the dose tested has been reported also to induce tumors in the indicated species.

Clearly there is a major difference between the ability of these agents to produce DNA alkylation. DMN produces over 3,000 alkylations per 10^6 nucleotides in the sensitive tissue (liver). However, the level of DNA damage induced by the MTD for PERC was not detectable (with a detection limit of 10 alkylations/10^6 nucleotides). DNA damage following exposure to VDC was detected (Table 6) but was very low. In the kidney of mice (which is the site of VDC-related tumors reported by Maltoni, 1977) the alkylation of DNA was more than one-hundred fold less than that observed with DMN. These studies thus classify DMN as an agent with considerable potential to in-

Table 6. DNA alkylation after exposure to VDC, PERC, and DMN

Treatment/tissue	Alkylations/nucleotide × 10^6
VDC-50 ppm (CD-1 mice, inhalation)	
Kidney	30
Liver	6.1
PERC-500 mg/kg (B6C3-F1 mice, p.o.)	
Liver	0[a]
DMN-10 mg/kg (rat, i.p.)	
Liver	3.500[b]
DMN-3 mg/kg (rat, i.p.)	
Liver	1.000[b]

[a] Detection limit 10 alkylations/10^6 nucleotides
[b] Data from Pegg and Hui (1978)

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duce in vivo genetic damage, but do not indicate this potential for either VDC or PERC.

Another indication of DNA damage is the presence of DNA repair following chemical exposure. In Figure 4, the relative amounts of DNA repair occurring after treatment with DMN or VDC are shown. The amount of hydroxyurea-resistant DNA synthesis gives a good dose-response curve in the range of 3–20 mg/kg DMN, increasing 637% over the control value after 20 mg/kg DMN. In contrast, the DNA repair after 50 ppm VDC was increased only 38%, barely above the experimental "noise" in the system. DNA repair was not studied after PERC administration, but the results for DMN and VDC again show a major difference; DMN clearly induced considerable genetic damage while VDC did not.

Indicators of epigenetic activity (DNA synthesis, histopathology) were studied also for these three chemicals. DNA replication, measured 48 h after exposure, was elevated 25-fold over controls in the kidneys of mice exposed to 50 ppm of VDC. The massive increase in DNA synthesis observed in the kidney was not seen in livers from VDC-exposed mice (Table 7). This observation is consistent with the results of Maltoni who reported tumors in the kidney, but not the liver, of VDC-exposed mice.

Small increases in DNA synthesis were also seen in the target site (liver) of mice treated with 500 mg/kg of PERC and after 10 mg/kg DMN. These are consistent with the presence of some cytotoxic activity for these agents also. It is significant, however that if the dose of DMN is reduced to 3 mg/kg, considerable genotoxic activity is still present (Table 6, DNA alkylation, Figure 4, DNA Repair), but cytotoxic activity has disappeared (Table 7). The histopathology of tissues from animals receiving DMN, VDC, and PERC confirms the cytotoxicity predicted by increased DNA synthesis (Table 8). Severe kidney damage, leading to nephrosis and subsequent regeneration,

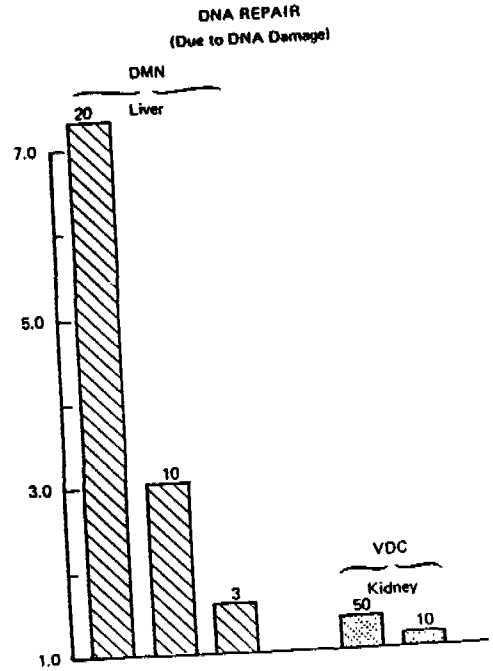

Fig. 4. DNA repair in vivo after administration of DMN (mg/kg, i.p.) or VDC (ppm, 6 h inhalation) to CD-1 mice. Repair is calculated as hydroxyurea-resistant ^{3}H thymidine uptake relative to control

was apparent in the mice exposed to 50 ppm VDC for 6 hours, while VDC effects on the liver were minimal. Similarly, PERC produced moderate cellular damage in the liver of mice receiving 500 mg/kg by gavage. Since the administration of even the highest doses of VDC or PERC fails to give evidence for genotoxicity, but cytotoxicity is evident at

Table 7. Percentage increase in DNA synthesis[a] after exposure to VDC, PERC, or DMN

Treatment/tissue	
VDC-50 ppm (CD-1 mice, inhalation)	
Kidney	2,370[a]
Liver	145
VDC-10 ppm (CD-1 mice, inhalation)	
Kidney	672[a]
Liver	20
PERC-500 mg/kg (B6C3F1 mice, 12 doses, p.o.)	
Liver	82[a]
DMN-10 mg/kg (CD-1 mice, i.p.)	
Liver	97
DMN-3 mg/kg (CD-1 mice, i.p.)	
Liver	14

[a] Significantly different than control, $p < 0.05$, t-test
DNA synthesis is estimated by determining the specific radioactivity of DNA preparations after injection of ^3H thymidine i.p.

Table 8. Summary of histopathology findings in tissues from mice treated with VDC, DMN or PERC

Exposure	Hours post-exposure	Histologic findings	
		Kidneys	Liver
VDC, 50 ppm, 6 h	0	Toxic nephrosis	Slight centribular swelling
	8	Progressing nephrosis	
	24	Progressing nephrosis	
	48	Increased mitotic figures	No effect
	96	Regeneration apparent	
	192	Regeneration continuing	
VDC, 10 ppm, 6 h	0	Slight dilation, swelling	
	96	Nephrosis-variable 0–20% affected	
DMN, 10 mg/kg i.p.	4		Centrilobular swelling Hyaline degeneration
	52		Accentuated lobular pattern, necrosis minimal or absent
DMN, 3 mg/kg i.p.	4		Centrilobular swelling
	52		No effect
PERC, 500 mg/kg p.o.[a]	18	No effect	Moderate hepatic damage

[a] Sacrificed 18 h after the last in a series of 12 daily doses

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Slight centrilobular swelling

No effect

Centrilobular swelling
Hyaline degeneration
Accentuated lobular pattern,
necrosis minimal or absent
Centrilobular swelling
No effect
Moderate hepatic damage

the site(s) where tumors have been reported, these data provide a rational and objective basis for concluding that tumors related to VDC and PERC develop through primarily cytotoxic mechanisms. However, DMN clearly possesses a genotoxic mechanism, and may have a mild cytotoxic mechanism as well.

This implies that although single exposures to DMN may be associated with some degree of excess cancer risk, exposures to levels of VDC or PERC which do not produce chronic tissue damage will not produce excess cancer risk.

D. Summary

It is impossible to prove that any chemical, natural or man-made, cannot cause cancer in man. However, it is possible to estimate the relative degrees of risk associated with various agents. The precision of these estimations increases as experimental procedures elucidate the basic type of mechanism associated with carcinogenesis, the role of absorption, metabolism and distribution, and excretion in increasing or decreasing activity, and the dose dependency of metabolic pathways. We must constantly strive to make the most accurate risk estimations possible so that the complex issues of risk/benefit may be properly considered.

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