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Resolution of Dose-Response Toxicity Data for Chemicals Requiring Metabolic Activation: Example—Vinyl Chloride[1]

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Resolution of Dose-Response Toxicity Data for Chemicals Requiring Metabolic Activation: Example—Vinyl Chloride. GEHRING, P. J., WATANABE, P. G., AND PARK, C. N. (1978). *Toxicol. Appl. Pharmacol.* 44, 581–591. The toxicity of many chemicals results from biotransformation products formed from the chemical rather than from the chemical per se. In such cases, the incremental response may become diminishingly smaller with increasing dose or exposure because activation of the chemical to the toxic form follows apparent Michaelis–Menten rather than apparent first-order kinetics. To illustrate this concept, rats were exposed to concentrations ranging from 1.4 to 4600 ppm of vinyl chloride for 6 hr, and the total amount metabolized was determined. The amount metabolized followed apparent Michaelis–Menten kinetics. For rats, the logarithmic probability incidence of angiosarcoma versus the amount of vinyl chloride metabolized rather than the exposure concentration of vinyl chloride is linear. Assuming no threshold in spite of evidence to the contrary, extrapolation of the data below the range of doses causing experimentally observable responses predicted an incidence of 0.01% hepatic angiosarcoma in rats exposed to 4.6 ppm of vinyl chloride. Theoretical extension of the extrapolation to humans after adjusting for metabolic and body mass differences was undertaken. The theoretical extrapolation for man exposed daily for 8 hr to 1 ppm suggests an incidence of 1.5 per 100,000,000. This theoretical incidence, although a likely overestimate because of a less than predicted incidence in men exposed to 200 ppm and greater, as well as evidence for a threshold in rats, is less than that expected to occur spontaneously. The concepts evolved from this analysis reveals why pharmacokinetics must be considered in designing toxicology experiments as well as in interpretation of the resulting data.

There exists a great deal of uncertainty in predicting the potential response of exposure to chemicals at concentrations below those producing an experimentally discernible response. This is particularly true when the response to the chemical in question is oncogenesis. Statistical projections recommended for assessing the risk of exposure to doses of oncogenic chemicals lower than those producing an observable response include those based on logarithm probability curves (probit curves), logistic curves, or linear curves (one-hit curves) (FDA Advisory Committee on Protocols for Safety Evaluation, 1971). One of the most commonly used statistical projections for risk assessment has been that promoted by Mantel and Bryan (1961) in which a logarithm probability pro-

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jection with a slope of 1 is utilized. A flaw innate to all of these methods is that the dose-response information used to make the projection is based on the dose of chemical administered to the animal rather than the quantity of the administered dose giving rise to the response; the latter may either increase or decrease disproportionately as the administered dose is increased.

The use of high doses to reveal the chronic toxicity incurred with exposure to a chemical is a common, scientifically defensible practice if judgment and scientific rationale are used in designing the experiments and in assessing the resulting data. However, such doses frequently overwhelm the enzymatic processes for activation of the chemical to the toxic form or for deactivation of the toxic form to an innocuous form. In this paper, it is demonstrated how the dose-dependent activation of vinyl chloride to an oncogenic product must be considered in resolving the dose-response of rats exposed via inhalation to vinyl chloride.

METHODS

Material. Vinyl chloride (^{14}C-labeled) was synthesized from 1,2-dichloro[1,2-^{14}C]ethane (New England Nuclear, Lots, No. 819-221 and 819-292, 5.0 and 4.8 mCi/mmol, respectively) directly prior to use (Wagner *et al.*, 1975). Nonlabeled VC (Matheson Gas Products) of 99.9% purity was mixed with the ^{14}C-labeled material to obtain the desired specific activity.

Animals. Male Sprague–Dawley rats (Spartan Research Laboratory) weighing 200–250 g were used throughout the study. Food and water were provided *ad libitum* except during the exposure. Exposures were conducted between 9:00 AM and 3:00 PM (EST). Groups of three to six rats were exposed to various concentrations of ^{14}C-VC for 6 hr.

Exposure and procedure. The rats were exposed by inhalation under dynamic conditions in a 30-liter glass inhalation chamber. The mean analytical concentrations of VC measured by gas chromatography were 1.4 ± 0.3 (±SD), 9.3 ± 0.2, 24.7 ± 1.4, 51 ± 2, 109 ± 23, 250 ± 2, 511 ± 11, 1020 ± 13, and 4600 ± 311 ppm. Details of this exposure and the method of analytical determinations have been reported previously (Watanabe *et al.*, 1976a). Immediately following the 6-hr exposure to various concentrations of ^{14}C-VC (1.4–4600 ppm), the rats were killed by a blow to the head, and the carcass was analyzed for total radioactivity (Watanabe *et al.*, 1976b). Previous studies have shown that only a small percentage of radioactivity (<12%) is excreted as metabolites other than ^{14}C-VC per se during 72 hr following a 6-hr inhalation exposure (Watanabe *et al.*, 1976a). A large proportion of the 12% is comprised of $^{14}CO_2$ excretion 72 hr after exposure. Furthermore, very little urine is excreted during the exposure period. Thus, the nonvolatile radioactivity determined immediately after exposure in the tissue and carcass is a good estimate of the total amount of metabolized VC.

RESULTS

Consistent with the results of previous studies (Watanabe *et al.*, 1976a,b), the metabolism of VC by rats does not increase proportionately with increasing concentrations of VC being inhaled (Table 1). The nonlinearity of the amount of VC

metabolized during 6 hr of exposure to various concentrations of VC appeared to be in accordance with Michaelis–Menten kinetics as described by the equation:

$$v = \frac{V_m S}{K_m + S}. \quad (1)$$

In this equation, v and V_m, are the velocity and maximum velocity, respectively, for the biotransformation of VC expressed as microgram equivalents of VC metabolized per 6 hr. S and K_m are the concentration of VC being inhaled and the Michaelis constant expressed as micrograms of VC per liter of air, respectively.

TABLE 1

PARAMETERS FOR DESCRIBING THE METABOLISM OF INHALED VINYL CHLORIDE (VC) USING MICHAELIS–MENTEN KINETICS

Exposure concentration S (ppm of VC)	S(μg of VC/liter of air)[a]	v(μg of VC metabolized[b]/6 hr)	v/S
1.4	3.6	30 ± 3[c]	8.33
9	23.0	242 ± 26	10.52
25	64.0	557 ± 42	8.70
51	130.6	1181 ± 93	9.04
109	279.0	2406 ± 173	8.62
250	640.0	3826 ± 345	5.98
511	1308.2	6263 ± 355	4.79
1020	2611.2	4257 ± 765	1.63
4600	11776.0	9255 ± 1467	0.79

[a] 1 ppm of VC = 2.56 μg of VC/liter of air.
[b] Determined from the total radioactivity in the carcass.
[c] Mean ± SD.

To ascertain whether Michaelis–Menten kinetics were applicable, the data in Table 1 were analyzed in accordance with the linear Woolf–Augustinson–Hofstee transformation of the Michaelis–Menten equation (Segel, 1976),

$$v = -K_m \frac{v}{S} + V_m. \quad (2)$$

It can be seen from the plot (Fig. 1) that the data appear to lie along a straight line thus verifying, at least visually, the Michaelis–Menten model. V_m and K_m can be estimated by the ordinate intercept and the slope of the line or they can be estimated directly by fitting the nonlinear Michaelis–Menten model. Both procedures yield similar parameter estimates. The estimates derived by fitting the model directly are 8558 ± 1147 (SD) μg of VC metabolized and 860 ± 159 (SD) μg of VC/liter of air for V_m and K_m respectively.

Once a means is obtained to calculate the amount of VC metabolized by rats as a function of exposure, it is then possible to relate the untoward effects associated with VC exposure to the amount biotransformed rather than the exposure concentration of VC per se incurred by rats exposed to VC.

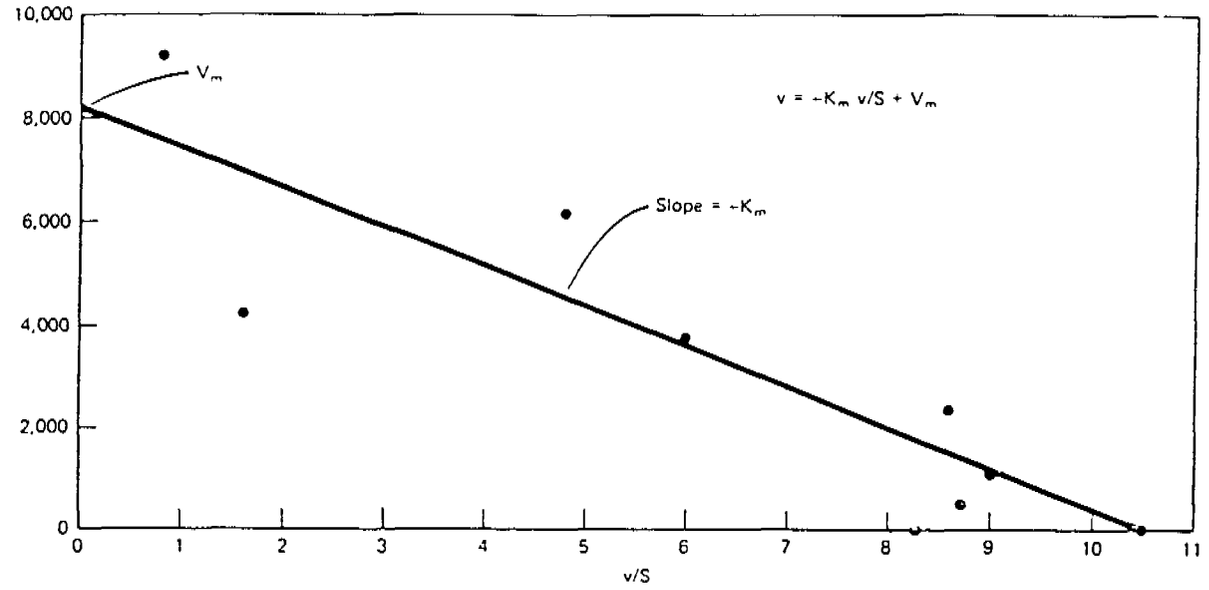

FIG. 1. Metabolism of vinyl chloride analyzed in accordance with the Woolf–Augustinson–Hofstee linearized form of the Michaelis–Menten equation. Values of v and v/S were taken from Table 1. The line was fit by linear regression analysis. The correlation coefficient, R, was 0.88.

Maltoni and Lefemine (1975) reported the incidence of hepatic angiosarcoma in rats exposed to different concentrations of VC, 4 hr/day, 5 days/week for 12 months and subsequently held for observation until death (Table 2). Before attempting to relate these data to the amount of VC biotransformed in accordance with the Michaelis–Menten equation using the previously determined values of V_m and K_m, the value for V_m

TABLE 2

CORRELATION BETWEEN EXPOSURE CONCENTRATION OF VINYL CHLORIDE, METABOLISM AND INDUCTION OF HEPATIC ANGIOSARCOMA IN RATS

Exposure concentration S(ppm of VC)	S(μg of VC/liter of air)[a]	v(μg of VC metabolized[b]/4 hr)	log v	Percentage incidence of hepatic angiosarcoma[c]
10,000	25,600	5,521	3.742	15
6,000	15,360	5,403	3.733	22
2,500	6,400	5,030	3.702	22
500	1,280	3,413	3.533	12
250	640	2,435	3.386	7
50	128	739	2.869	2

[a] 1 ppm of VC = 2.56 $\left(\frac{\mu g \text{ of VC}}{\text{liter of air}}\right)$.

[b] V_m corrected for 4-hr exposure, 8558 $\left(\frac{\mu g \text{ of VC metabolized}}{6 \text{ hr}}\right) \cdot 4/6 = 5706$

$$\left(\frac{\mu g \text{ of VC metabolized}}{4 \text{ hr}}\right) : v = \left[5706 \left(\frac{\mu g \text{ of VC}}{4 \text{ hr}}\right) \cdot S(\mu g/\text{liter})\right] \Big/ [860(\mu g/\text{liter}) + S(\mu g/\text{liter})].$$

[c] From Maltoni and Lefemine (1975).

must be adjusted for the shorter exposure duration used by Maltoni and Lefemine, 4 versus 6 hr. This adjustment is accomplished by multiplying V_m by 4/6. Thus, the amount of VC biotransformed daily by rats exposed to the various concentrations used in the experiment of Maltoni and Lefemine can be calculated from the equation:

$$v = \frac{5706\left(\frac{\mu g \text{ of VC}}{4 \text{ hr}}\right) \cdot S\left(\frac{\mu g}{\text{liter}}\right)}{860\left(\frac{\mu g}{\text{liter}}\right) + S\left(\frac{\mu g}{\text{liter}}\right)} \tag{3}$$

The resulting values for v are given in Table 2.

Figure 2A, depicts a logarithm probability plot (probit plot) of the incidence of hepatic angiosarcoma observed in rats by Maltoni and Lefemine (1975) versus the amount of VC biotransformed for 4 hr of exposure, v, or the exposure concentration, S. The incidence of hepatic angiosarcoma in rats is linear with respect to log v but not log S. The line drawn for log v versus tumor incidence (Fig. 2) was determined by using a probit regression analysis program, and the equation relating the incidence of hepatic angiosarcoma to log v was:

$$\text{probit response} = -1.625 + 1.543 \log v \tag{4}$$

Using the foregoing equation, a projection below the levels of exposure producing an experimentally discernible response has been made (dashed line). Assuming no

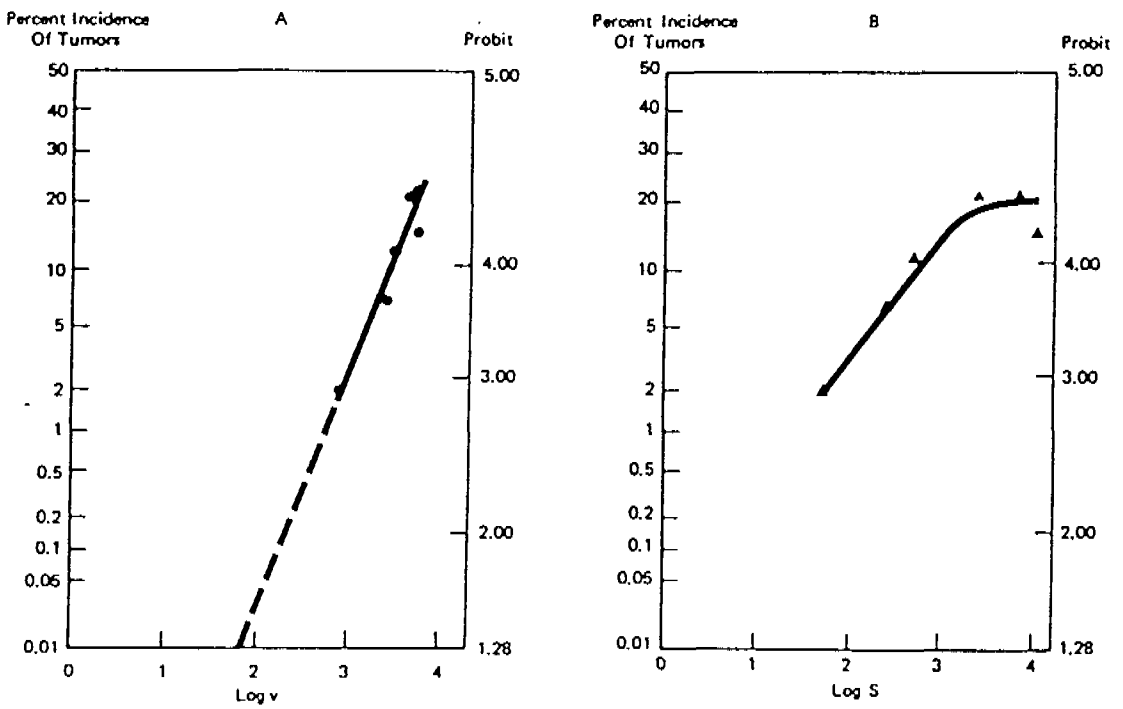

FIG. 2. (A) Metabolism of vinyl chloride expressed as log v (Micrograms of VC metabolized/4 hr) versus percentage incidence of hepatic angiosarcoma (probability scale). (B) Exposure concentration expressed as log S (parts per million) versus the percentage incidence of hepatic angiosarcoma. The probit equivalents of the percentage incidence are shown on the right-hand ordinate. The solid line is the best fit for experimentally observed responses while the dashed line represents extrapolation below those doses producing an observable response assuming no threshold.

threshold for the induction of angiosarcoma in rats exposed to VC, the exposure concentration producing one angiosarcoma in 10,000 rats can be calculated. The probit percentage representing an incidence of 0.01% is 1.28. Substitution of this value into the Eq. (4) yields:

$$\text{Log } v = 1.8827,$$
$$v = 76.33\ \mu\text{g of VC metabolized/4 hr}$$

Using Eq. (3), the concentration of exposure to VC needed to give this value for v is 11.66 μg/liter or 4.6 ppm [approximate 95% confidence limits obtained by substituting the upper and lower 95% confidence limits for v in Eq. (3) and solving for S are 0.03–8.7 ppm]. Hence, exposure of rats to 4.6 ppm of VC for 4 hr daily, 5 days/week for 1 year can be expected to produce one angiosarcoma per 10,000 rats if the dose-response curve remains valid at exposures less than those producing a discernible experimental response.

DISCUSSION

For many chemicals, toxicity may not be a function of exposure to the chemical per se, but rather to a biotransformation product of the chemical. Frequently, production of a toxic metabolite is dependent upon enyzmatically mediated reactions which are classically described by Michaelis–Menten kinetics. Since enzymatically mediated reactions are concentration dependent and saturable, toxicity resulting from exposures to chemicals requiring activation to a toxic form cannot be related directly to the magnitude of exposure or dose. In such a case, it is necessary to determine the amount of the chemical undergoing biotransformation as a function of dose or exposure before a meaningful dose-response relationship can be established.

There is considerable evidence that vinyl chloride requires bioactivation to produce tumors. Metabolic activation is required to induce mutations in bacteria exposed to VC (Bartsch *et al.*, 1975; Malavielle *et al.*, 1975; Rannug *et al.*, 1974). Covalent binding of ^{14}C to hepatic macromolecules in rats (Watanabe *et al.*, 1978) exposed to ^{14}C-VC also requires bioactivation. Covalent binding of electrophiles to DNA has been associated with tumorigenesis.

For vinyl chloride-induced hepatic angiosarcoma in rats, a logarithmic probability plot (probit plot) of the incidence versus the amount of vinyl chloride metabolized, v, over a range of exposures from 50 to 10,000 ppm of VC gives a classical straight line (Fig. 2A). The dose-response relationship is not a straight line when plotted as a function of the exposure concentration, S (Fig. 2B). These results support further the conclusion that VC requires biotransformation to an active metabolite for tumorigenesis. Furthermore, a more reasonable evaluation of the dose-response data for vinyl chloride-induced tumorigenesis requires knowledge of the amount of VC activated as a function of exposure. After arbitrarily excluding data acquired from rats exposed to concentrations of VC exceeding 500 ppm in the experiment of Maltoni and Lefemine (1975), Schneiderman *et al.* (1975) extrapolated the remaining data to predict an incidence of 0.01% hepatic angiosarcoma in rats exposed to 1 ppm of VC. This number is reasonably close to our prediction of 4.6 ppm of VC for the same incidence. If Schneiderman *et al.* (1975) had used all of the data, a dose-response curve with an

unrealistically shallow slope would have resulted and the predicted level causing 0.01% hepatic angiosarcoma would have been much smaller, on the order of 0.00001 ppm.

The concepts developed herein allow use of all of the data presented by Maltoni and Lefemine (1975) to construct a dose-response curve on a scientifically defensible basis. Assuming that the resulting dose-response curve can be projected beyond the range of the experimentally discernible responses, the exposure concentration required to produce an incidence of 0.01% hepatic angiosarcoma in rats is 4.6 ppm.

Aside from interpreting toxicity data for chemicals requiring activation to a toxic form, there are some practical implications of the concepts presented herein for designing experiments to assess the toxocity, including carcinogenicity, of such chemicals. For these chemicals, increasing the concentration above the apparent K_m will produce diminishingly smaller increments in the response; no increase in the response is to be expected when the exposure concentration is two or three times K_m. Since total dose is a function of exposure time as well as concentration, it is important to determine the effect of exposure time on the response. As shown in Eq. (3), the only parameter influenced by exposure time is V_m, which is increased linearly with time. Therefore, after the concentration to which the animals are exposed becomes two to three times K_m, the amount metabolized, v, will increase linearly with increasing exposure time. For this reason, the gradation of incidence of angiosarcoma in rats exposed to high concentrations of VC will become a function of exposure time rather than concentration. This reasoning makes it imperative that the duration of exposure as well as exposure concentration be considered in evaluating the results of epidemiological studies of people exposed to high concentrations of VC in the work environment.

Unless the dose-dependent, Michaelis–Menten type pharmacokinetic parameters are resolved prior to designing the experiment, the results may be robbed of much of their value for characterizing the dose-response function for the untoward effects observed. Thus, the current approach using the maximum tolerated dose as defined presently and fractions thereof may be scientifically unsound if the objective is to assess the potential toxicity of exposure to much lower doses or exposures. For some chemicals detoxification of the chemical per se or reactive metabolites formed from the chemical may also be dose-dependent and saturable leading to a build-up of toxic materials. In such cases, the incremental responses to increasing doses or exposures will become disproportionately larger rather than smaller (Gehring and Blau, 1977).

In the foregoing analysis of the dose-response data for the induction of angiosarcoma in the rat, no threshold for the response was assumed. As indicated, extrapolation below the range of doses resulting in an observable response may overestimate the response in rats because there is evidence that detoxification of reactive metabolites of VC may occur more efficiently in rats exposed to concentrations of VC below 50 ppm (Watanabe *et al.*, 1976c). Indeed, further analysis of the data reported by Maltoni and Lefemine (1975) also provides an indication of a practical threshold. In a subsequent presentation of these data, it was revealed that the latencies for the development of hepatic angiosarcoma were, respectively, 64, 70, 78, 81, 79, and 135 weeks for rats exposed to 10,000, 6000, 2500, 500, 250, and 50 ppm of VC (Maltoni, 1975). These results indicate that at some low levels of exposure the time required for induction may exceed considerably the life expectancy for rats. This is consistent with the work of others suggesting that multiples of a lifetime may be required for expression of cancer in

response to low doses of a carcinogen (Druckrey, 1967; Albert and Altshuler, 1973). Thus, extrapolation of the data obtained for rats below the range of exposures causing a discernible response may be expected to overestimate the projected incidence.

The ultimate objective of a toxicological study is to develop data which can be used to assess the potential risk for man. It is worthwhile to utilize the concepts presented herein to achieve this objective realizing fully that such extrapolation is fraught with uncertainties. The basic assumptions made are:

(i) Induction of angiosarcoma is related to the amount of reactive metabolite of VC per unit of mass.
(ii) Exposure of rats for 12 months approximates exposure of workers for their working life.
(iii) There is no threshold for the induction of angiosarcoma in either rats or man which likely overestimates the assumption of risk as discussed above.
(iv) The efficiency of the metabolic processes involved in the conversion of VC to the reactive form is proportional to the body surface area. Since data for the biotransformation of VC by man are not available, the most logical basis for translation of the animal data to man would seem to be on the basis of body surface area.

The last assumption deserves comment. There are considerable data in the literature showing that metabolism in general and other physiological parameters as well are relatable directly to the surface area of the body (Schmidt-Nielsen, 1970; Pinkel, 1958). For this reason, administration of biologically active chemicals to various species frequently gives an equivalent response when the dose is administered in proportion to the surface area of the body, that is dose per square meter of body surface (Pinkel, 1958). This relationship is gaining recognition in estimating the risk incurred by man from exposure to chemicals in the environment (Committee on Safe Drinking Water, National Research Council, 1977). In utilizing this relationship it must, however, be recognized that the original relationship was developed for biologically active agents. Since metabolism and other physiological processes involved in detoxification are more active in smaller animals, the dose of a biologically active chemical per unit of mass required to produce a given effect increases as the body decreases, while the dose per unit surface area remains relatively constant. However, for a chemical requiring activation to the biologically active toxic form, the total amount transformed will be roughly proportional to the body surface area. Since toxicity is a function of the concentration of the active form in tissue, this total amount transformed must then be normalized for mass to estimate an equivalent response.

Using aforementioned rationale, the maximum velocity, V_m, for a 70 kg man can be estimated by calculation using the V_m value obtained for a 0.250-kg rat. The V_m of man for VC will be:

$$V_m\,(\text{man}) = V_m\,(\text{rat})\left(\frac{1.85\ \text{m}^2}{0.045\ \text{m}^2}\right)$$

or

$$V_m\,(\text{man}) = (8558\ \mu\text{g/6 hr})\left(\frac{1.85\ \text{m}^2}{0.045\ \text{m}^2}\right) = 351829\ \mu\text{g/6 hr},$$

where the values 1.85 and 0.045 m^2 are the body surface areas of a 70-kg man and a 0.250-kg rat, respectively (Pinkel, 1958). For an 8-hr exposure, the value is 469,105 μg/8 hr. In order to use this number to theoretically estimate the response in man using data collected in rats, the V_m for man must be adjusted to a mass equivalent to that of rats since toxicity is a function of concentration in tissue. To do this the number is divided by 70 kg/0.25 kg or 280. The resulting V_m for man on a mass equivalent basis to that of rats is 1675 μg/8 hr. Using this value, the amount of VC transformed to a reactive form by man on a mass equivalent basis to rat is given by the equation:

$$v(\mu g/8\text{ hr}) = \frac{1675\ \mu g/8\text{ hr} \cdot S(\mu g/\text{liter})}{860\ \mu g/\text{liter} + S(\mu g/\text{liter})} \tag{5}$$

Using this equation, the amount of VC transformed by man on a mass equivalent basis to rats was calculated as a function of exposure concentration and the expected incidence of angiosarcoma estimated from Eq. (4) (Table 3).

For men exposed to greater than 200 ppm of VC, the incidence of angiosarcoma has been reported to be 0.02% (Fox and Collier, 1977). Hence, the theoretical calculated incidence using data from rats exceeds that currently detected by approximately 50-fold. This may indicate that people are less sensitive than rats to the induction of angiosarcoma, or it may indicate that a practical threshold for the induction of angiosarcoma had been attained. Consistent with this latter possibility is that v on a mass equivalent basis to rats for men exposed to 200 ppm is 625. This number lies below that for rats exposed to 50 ppm. As indicated previously, angiosarcoma observed in rats exposed to 50 ppm occurred only in a rat that lived 135 weeks (Maltoni, 1975). Further, this rat did not die as a result of the angiosarcoma but was killed. Thus, as indicated previously, projection of the data collected in rats below the range of exposures producing an observable response may overestimate the incidence.

TABLE 3

THEORETICAL AMOUNTS OF REACTIVE PRODUCT FORMED FROM VC BY A 70-KG MAN EXPOSED CONTINUOUSLY FOR 8 HR AND THE CORRESPONDING EXPECTED INCIDENCE OF ANGIOSARCOMA AS PREDICTED FROM DATA COLLECTED IN RATS ASSUMING NO THRESHOLD

Exposure concentration		v(μg of VC metabolized[b]/8 hr)	log v	Probit response[c]	Theoretical percentage incidence of angiosarcoma[c]
(ppm)	(μg/liter)[a]				
200	512	625	2.79	2.68	1.02
50	128	217	2.34	1.98	0.11
5	12.8	24.6	1.39	0.52	3.74×10^{-4}
1	2.56	4.97	0.70	−0.54	1.5×10^{-6}

[a] 1 ppm = 2.56 μg/liter.

[b] v has been calculated using the Michaelis–Menten equation after calculating the V'_m for man from the V'_m determined for rats. This calculation was made by assuming V'_m is proportional to body surface area and subsequently adjusting it to a mass equivalent to that of rats. See text.

[c] The expected probit response was calculated from probit Eq. (3) (see text). Subsequently the theoretical percentage incidence of angiosarcoma was determined from the respective probit.

In spite of the likelihood of overestimating the incidence of angiosarcoma in rats or man, the incidence predicted for people exposed to 1 ppm, the current OSHA (Occupational Safety and Health Act) standard, is very small (1.5 per 100 million). This value which is likely an overestimate is below the expected incidence of spontaneous angiosarcoma reported to be 20 to 25 cases in the U.S. annually (Makk *et al.*, 1976).

In summary, it has been demonstrated that the incidence of VC induced angiosarcoma in rats is related not to the concentration of exposure but rather to the amount of VC biotransformed. Biotransformation of VC by rats is a dose-dependent process characterized by Michaelis–Menten type kinetics. The concepts evolved from this analysis reveal why pharmacokinetics must be considered in designing toxicology experiments as well as in interpretation of the resulting data. Having characterized the dose-response for induction of angiosarcoma in rats as a function of the amount of VC biotransformed, a theoretical estimate of the incidence expected to occur in exposed men was undertaken using the data collected in rats. For daily 8-hr exposures to 1 ppm, the predicted incidence is 1.5 in 100,000,000 which is less than that expected to occur spontaneously. This predicted incidence is likely an onverestimate of that which will occur as a result of exposure to 1 ppm because there is some evidence for at least a practical threshold in both rats and man. There are no illusions that this estimate by extrapolation of data outside the range of doses causing experimentally observable responses and subsequently to man is without flaws. However, the rationale used represents a new approach which utilizes more logic than methods employed currently for such extrapolation.

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