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CHALLENGING DOGMA: Thresholds for Genotoxic Carcinogens? The Case of Vinyl Acetate

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■ **Abstract** Although many questions remain unanswered, the general principle of the sequence of events leading to cancer after exposure to genotoxic carcinogens has become increasingly clear. This helps to understand the parameters that influence the shape of the dose-effect curve for carcinogenesis, including metabolic activation and inactivation of carcinogens, DNA repair, cell cycle control, apoptosis, and control by the immune system. A linear dose-response relationship with no observable threshold seems to be a conservative but adequate description for the carcinogenic activity of many genotoxic carcinogens, such as aflatoxin B1, the tobacco-specific nitrosoketone NNK, and probably N,N-diethylnitrosamine. However, extrapolation models connecting the high-level risk to the zero intercept have clearly resulted in overestimations of risk. Vinyl acetate is an example that is discussed extensively in this review. At extremely high and toxic doses, vinyl acetate is carcinogenic in rats and mice and causes chromosomal aberrations. In tissues of contact, vinyl acetate is converted to acetic acid and acetaldehyde. Only when threshold levels are achieved do critical steps in the mechanism ultimately leading to cancer become active, namely pH reduction in exposed cells of more than 0.15 units leading to cytotoxicity, damage to DNA, and regenerative proliferation. Consistent with the known exposure to endogenous acetic acid and acetaldehyde, tissues sustain a certain level of exposure without adverse effects. Physiological modeling shows that the conditions necessary for carcinogenesis are in place only when threshold levels of vinyl acetate are exceeded. The example of vinyl acetate underlines the importance of toxicological research that unequivocally identifies genotoxic carcinogens acting through a threshold process.

INTRODUCTION

A conservative strategy of carcinogenic risk evaluation is to connect the high-level risk to the zero intercept and define the slope of the line as a risk coefficient for carcinogens per unit of dose (1). However, in some cases this type of calculation may result in unreasonable conclusions. One of the most provocative examples is presented by Goldman (1): Assume that every human being on Earth adds a 3-cm lift to his shoes for 20 years. The resulting increase in cosmic ray dose, which doubles for every 2000 m in altitude, is extremely small, but multiplied by the population of Earth results in a dose that causes cancer in approximately 30,000 individuals in 50 years. Although the mathematics in this example are correct, it would rightfully never be accepted as a scientific basis to ban high shoes.

The (unacceptable) linear extrapolation model of radiation risk represents only one of several examples showing that the old dogma based on the assumption of "no threshold" for genotoxic carcinogens does not hold true in all cases. Such considerations stimulated opposition against the no threshold assumption inherent in extrapolating risk linearly to the zero dose intercept. Several examples have been published in *Science*:

- "The current mode of extrapolating high-dose to low-dose effects is erroneous for both chemicals and radiation. Safe levels of exposure exist. The public has been needlessly frightened and deceived, and hundreds of billion of dollars wasted" (2).
- ". . . it is time to seriously consider the utility of implementing a concept of an effective or practical threshold for risk, that is, negligible risk" (1).
- "Toxic effects of high risk doses often do not occur at low doses . . . the last thing we want to do . . . is to put our limited resources into protecting people from things that are harmless" (3).

Without any doubt, these and other provocative statements have initiated a fruitful discussion about risk assessments of low-level exposures. However, it is dangerous to generalize that safe levels of exposure exist. For instance, there is no convincing evidence showing that aflatoxin B1 (AFB1) acts by a threshold mechanism. This leads to the question of whether modern toxicology should differentiate between the two types of genotoxic carcinogens, those acting by a practical threshold and those acting by a nonthreshold mechanism (or at least by a mechanism for which a threshold has not yet been shown). In this article, the molecular mechanisms are reviewed, explaining the difference between both types of genotoxic carcinogens. Special attention is given to the case of vinyl acetate. Vinyl acetate serves as an example for a number of high-volume industrial chemicals, including trichloroethylene (4), acrylonitrile (5), and 1,3-butadiene (6), for which threshold or practical threshold mechanisms of carcinogenesis are currently being debated.

THRESHOLD MECHANISMS

Although many questions remain unanswered, the general principle of the sequence of events leading to cancer after exposure to genotoxic carcinogens has become increasingly clear (Figure 1). This helps to understand the parameters that influence the shape of the dose-effect curve for carcinogenesis. Lack of metabolic activation of a precarcinogen in a certain animal species (Figure 1) would result in a mechanism that precludes its carcinogenic action in that species. This is obvious because, per definition, a substance is not a genotoxic carcinogen if it cannot be activated to damage DNA in that species. Nevertheless, the capacity for metabolic

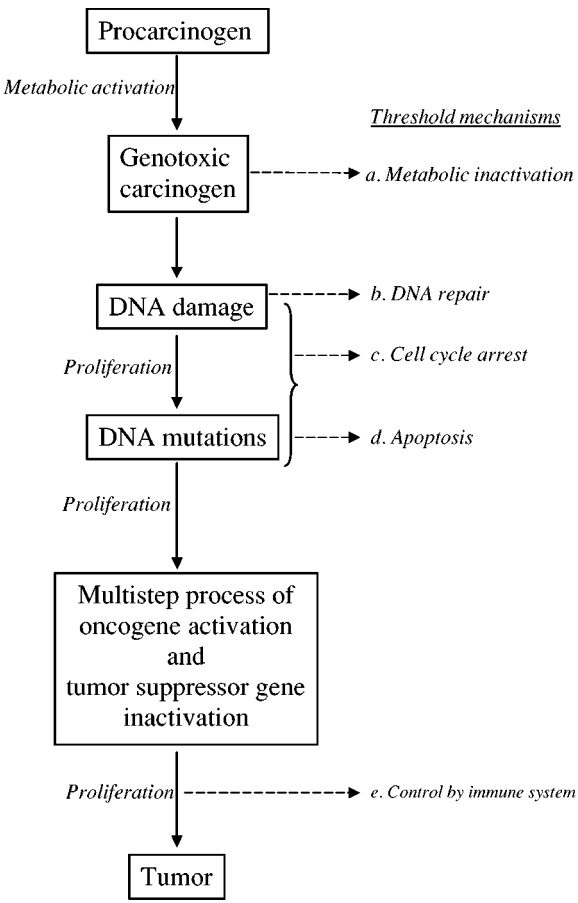

Figure 1 Possible threshold mechanisms of genotoxic carcinogens.

activation influences the shape of the dose-effect curve. For instance, the slope of the dose-effect curve of N,N-diethylnitrosamine (DEN)- or the tobacco-specific nitrosoketone NNK-induced liver tumor incidence in rats decreases at high doses due to saturation of the metabolic activation by CYP2E1 (Figures 6 and 9).

The next step on the path to cancer that influences the shape of the dose-effect curve is the metabolic inactivation of genotoxic carcinogens (Figure 1*a*). As a typical example, we present the influence of microsomal epoxide hydrolase (mEH), which influences the dose-effect curve in a way characteristic for the influence of metabolic inactivation. mEH inactivates styrene oxide by hydrolysis. Styrene oxide binds to DNA, predominantly to the N7-positon of guanine, which finally leads to DNA strand breaks. Human mEH was transfected into a Chinese hamster cell line (V79 cells) that, before transfection, expressed only very low activities of this enzyme [Figure 2*A*,*B*; (7)]. In V79 cells that express only very low mEH, styrene oxide leads to the formation of DNA strand breaks in a dose-dependent manner with no observable threshold [Figure 2*C*; (7–9)]. Chinese hamster cells genetically engineered to express human mEH at levels giving rise to similar activities as observed in human liver are protected from measurable genotoxic effects of styrene oxide up to 100 μM. Due to the detection limit of the assay, it is not possible to differentiate whether transfection of mEH introduced a "practical threshold," characterized by a very low level of DNA damage (not measurably exceeding the background in absence of styrene oxide), or a "real threshold," characterized by a dose-effect curve crossing the abscissa at 100-μM styrene oxide, i.e., representing truly zero effect up to a concentration of 100-μM styrene oxide. Although a conclusion cannot be obtained experimentally, we favor the model of a practical threshold because it seems improbable that a detoxifying enzyme can completely exclude small numbers of genotoxic molecules from reaching the DNA. If the assumption that detoxification does not guarantee completeness is accepted, it is possible to conclude that metabolic inactivation results in practical, albeit not perfect, thresholds. Whether a detoxifying enzyme qualifies as a basis for a practical threshold depends on the speed and capacity of removal of the reactive species from the system compared with the speed of the translocation of the reactive species from the site of its generation to the nucleus and reaction with the DNA. The recently discovered two-step mechanism of the xenobiotic metabolizing mEH leads to the observed practical threshold (Figure 2*C*). This mechanism consists of a very fast first step of removal of the epoxide by covalent interaction with the enzyme followed by the much slower hydrolysis of the complex. The high capacity of this mechanism is provided by an unusually high amount of enzyme. Thus, mEH soaks up the genotoxic epoxide like a sponge up to a concentration of epoxide that has titrated out the high amount of enzyme (7, 8). Such mechanisms are also being discussed for trichloroethylene (4).

DNA repair is known to protect cells from fixation of DNA damage in the newly synthesized DNA strand as heritable mutations, thereby generating the situation of a race between repair and proliferation-dependent DNA synthesis (Figure 1*b*). It is not a matter of debate that DNA repair can effectively reduce tumor incidence.

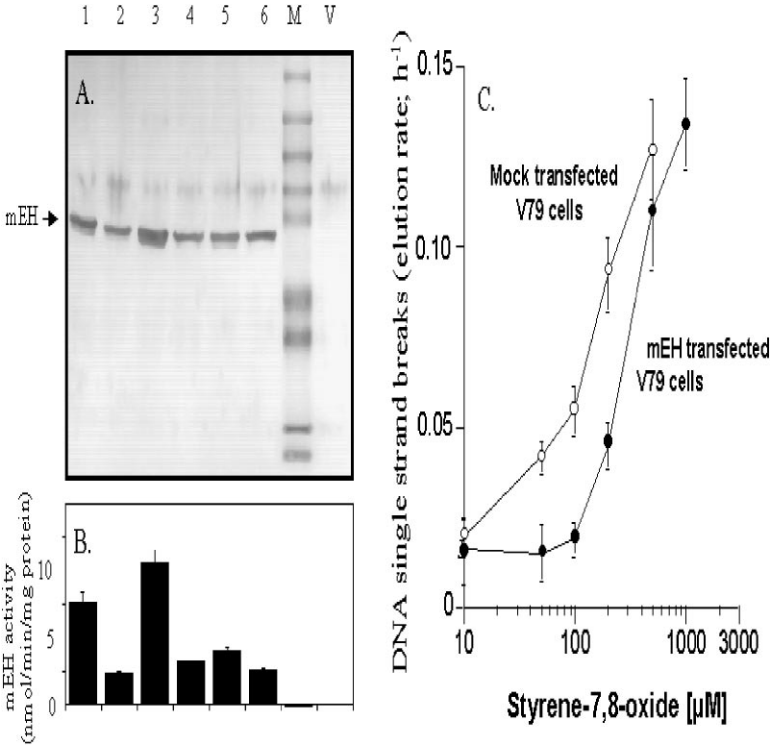

Figure 2 Recombinant expression of human mEH protects V79 Chinese hamster cells (V79 cells) from styrene-7,8-oxide-induced DNA single-strand breaks. (*A*) Western blot analysis of mEH transfected (*lanes 1–6*) and mock transfected (*lane V*) V79 cells. S9 fractions of the different clones were analyzed with a polyclonal antibody against purified human mEH raised in rabbits. Lane M: prestained molecular weight markers (195, 112, 84, 63, 52, 35, and 32 kd). (*B*) mEH activity of the same clones using styrene-7,8-oxide as a substrate. Values are expressed as nanomoles of styrene glycol formed per minute and milligrams of protein. (*C*) Effect of styrene-7,8-oxide on DNA single-strand breaks using mEH transfected clone No. 3 [from (*A*) and (*B*)] and mock transfected V79 cells (7).

However, it seems unlikely that DNA repair mechanisms are perfect. If an extremely low dose of a carcinogen induces only a single DNA adduct in a cell, this adduct has a probability to persist unrepaired until fixation as a mutation occurs. Although this probability may be low, it is unlikely zero. If this assumption is accepted, the influence of DNA repair may be regarded analogously to the discussions above concerning metabolic inactivation: Practical, but not perfect, thresholds may be introduced. A carcinogen may induce DNA repair enzymes. For instance, the DNA repair protein O6-methylguanine-DNA-methyltransferase (MGMT) that is

responsible for direct reversal of the miscoding DNA lesion O6-methylguanine has been reported to be inducible by diethylnitrosamine and ethylnitrosourea in mammalian tissues (9a). One might expect that the increased capacity for repair may reduce levels of DNA damage below baseline levels (i.e., below the levels of "spontaneous" DNA lesions), leading to a tumor incidence lower than in the control group. Although treatment with low doses of some carcinogens has led to a lower yield of some tumors than in the control group, to our knowledge, this theoretical possibility with respect to induction of DNA repair enzymes has not yet been supported by convincing overall tumor incidence data.

Cell cycle arrest can be induced as a consequence of DNA damage or interference with signal transduction in target cells (Figure 1*c*). Low levels of a carcinogen may even decrease cell cycle progression below baseline rates (10, 11). Because under specific circumstances the protective influence of decreased cell division can be stronger than the deleterious influence of increased DNA damage, the combination of both effects may result in a decreased tumor incidence. Higher levels of the same substance increase cell cycle progression due to cytotoxicity and regenerative cell proliferation, resulting in an increased tumor incidence. As a consequence, a J-shaped dose-effect curve results. This mechanism has been observed for nongenotoxic carcinogens, such as TCDD (12) or caffeic acid [(13), reviewed in (10)], but has also been postulated for genotoxic carcinogens, such as 2-acetylaminofluorene and ionizing radiation. The benefit of the first, decreasing part of a J-shaped dose-effect curve must be interpreted with caution because a decrease in cell proliferation below baseline may interfere with normal tissue regeneration. In addition, the protective influence may be tissue or cell-type specific. Cell cycle delay may be induced in one cell type, whereas other, more sensitive cell types may respond with regenerative proliferation. Nevertheless, cell cycle progression and regenerative proliferation probably represent the most relevant key parameters concerning threshold mechanisms. Due to the lack of fixation of DNA damage as a stable mutation in a newly synthesized daughter strand of DNA, a genotoxic substance will not be able to induce tumors in tissues that do not proliferate. For instance, the extremely low proliferative capacity of cardiomyocytes protects this cell type from carcinogenesis even if genotoxic substances induce DNA damage in cardiomyocytes. The latter has been shown for heterocyclic amines as assessed by ^{32}P-postlabelling analysis (13a), suggesting that cardiomyocytes are not more resistant to primary DNA damage than cells of organs that are susceptible to carcinogenesis. On the other hand, a genotoxic substance causes tumors with an extremely high probability when both DNA damage and cell proliferation are induced in target tissues. The situation becomes complex when a given dose of a substance induces DNA damage but not cytotoxicity and proliferative cell regeneration. In this circumstance, the result may depend on baseline proliferation of the relevant tissue. Rapidly proliferating cells in bone marrow or the crypt cells of the colon are at high risk for neoplastic transformation. On the other hand, for cells with a relatively low baseline proliferation, such as the olfactory epithelium, the latency period for carcinogenesis may exceed life expectancy (14, 15). Of high interest

are genotoxic substances for which both induction of regenerative proliferation and genotoxicity act through a threshold process. For instance, paracetamol and vinyl acetate belong to this class of substances (see below).

Apoptosis (Figure 1*d*) and the control of neoplastically transformed cells by the immune system (Figure 1*e*) are additional mechanisms influencing the shape of the dose-effect curve. Cells may undergo apoptosis as a consequence of DNA damage induced by relatively high doses. There is no doubt that this process can reduce tumor rates. However, little is known about the efficiency of apoptotic mechanisms at low doses and whether such mechanisms can lead to thresholds for carcinogenesis.

Considering the complexity of mechanisms that may introduce practical or perfect thresholds, it becomes clear that evaluation of the risk of genotoxic substances is not easy, but also not impossible. Selected examples of genotoxic carcinogens with and without practical threshold mechanisms are reviewed in the next section.

EXAMPLES OF CARCINOGENS WITH AND WITHOUT PRACTICAL THRESHOLDS

Aflatoxin B1

Aflatoxin B1 (AFB1) is one of the most potent human hepatocarcinogens known. It represents a liver cell type–specific toxin because it induces the formation of tumors developing from parenchymal and bile duct epithelial liver cells, but not from other cell types present in the liver, such as Kupffer or endothelial cells (16). AFB1 is a typical representative of the class of carcinogens showing a linear dose-response relationship in the low dose range. For instance, a 24-month study with male Fischer rats exposed to five doses between 1 and 50 ppb in the drinking water resulted in a linear dose-response curve, with the liver tumor incidence being 80% at the highest dose [Figure 3; (17), reviewed in (18)]. The linear increase in liver tumors corresponds well to the also linear induction of the main DNA adduct (dG-N7-AFB1) formed by AFB1 [Figure 3; (19)]. DNA adducts were measured after eight weeks of continuous administration of AFB1 in the drinking water. After this period, a steady state is achieved between adduct formation and removal. A linear low dose-response to AFB1 has also been shown in other species. For instance, tumor incidence in rainbow trout exposed to 50–250-ppb AFB1 as embryos increased without an obvious threshold [Figure 4; (20)]. Large species differences in AFB1 susceptiblilty are known (21). Rats represent a very sensitive species, whereas mice are much more resistant. It is important to use the rat model when extrapolating to human carcinogenicity because both rats and man—compared to mice—are relatively poor conjugators of activated AFB1. The molecular mechanisms of these interspecies differences have been reviewed (21).

AFB1 requires metabolic conversion to AFB1 exo-8,9-epoxide in order to cause DNA damage (22–24). In humans, AFB1 is activated primarily by CYP3A4 and 1A2. The AFB1 epoxide reacts with guanine, resulting in 8,9-dihydro-8-(N^7guanyl)-9-hydroxyaflatoxin B1 (AFB1-N7-Gua) as a main DNA adduct. The

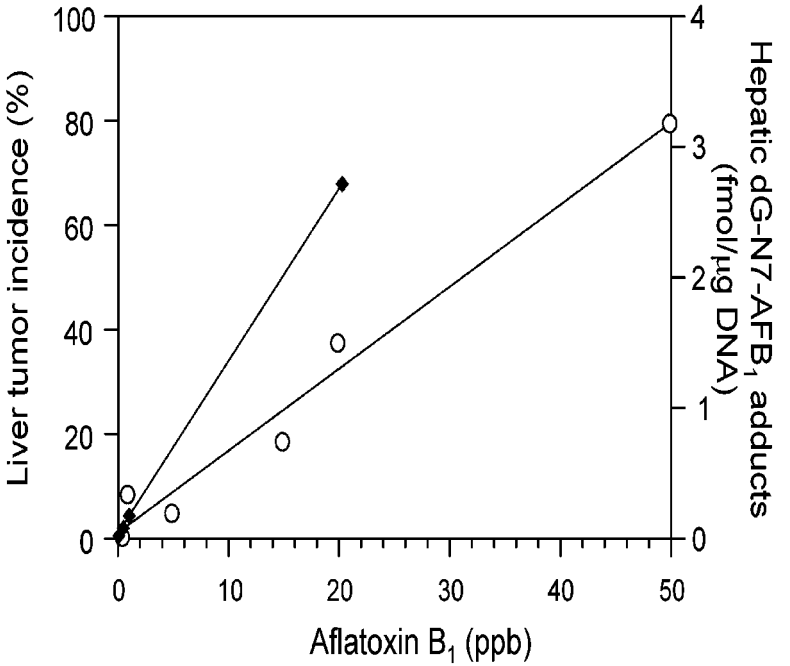

Figure 3 Liver tumors (○) and DNA adducts (♦) induced by AFB1. Male Fischer rats were exposed to five doses of AFB1 between 1 and 50 ppb in the drinking water for 24 months for analysis of tumor incidence. DNA adducts were measured after eight weeks of continuous administration of AFB1 in the drinking water (17–19).

positively charged imidazole ring of the resulting molecule facilitates depurination, leading to an apurinic site. Alternatively, the imidazole ring of AFB1-N7-Gua opens to form the stable AFB1 formamidopyrimidine (AFB1-FAPY). The initial AFB1-N7-Gua, AFB1-FAPY, and the apurinic site represent likely precursors to the mutagenic effects of AFB1. Bailey et al. presented strong evidence that the initial AFB1-N7-Gua adduct is extremely efficient in inducing mutations (25). Two main mutations are induced (Figure 5): (*a*) G→T transversions are targeted to the original site of the adduct (Figure 5, *upper panel*). Such G→T transversions have been identified in the p53 tumor suppressor gene in the third position of codon 249 (AGG) in approximately half of all examined hepatocellular carcinomas of humans exposed to AFB1 (26, 27). (*b*) The C→T transition occurs on the 3′ face of the modified guanine (Figure 5, *lower panel*). The AFB1 moiety of the AFB1-N7-Gua adduct intercalates on the 3′ face of guanine. As a consequence, the base 3′ of the adduct (a cytosine in Figure 5) may rotate out of the helix, leading to the insertion of adenine across from the AFB1 adduct, finally resulting in a C→T transition (25). Such C→T transitions have been identified in codon 12 of the c-ki-ras oncogene of rat hepatocellular carcinomas (28, 29). It can be assumed that the linear relationship between AFB1 exposure and tumor incidence at low doses as well as

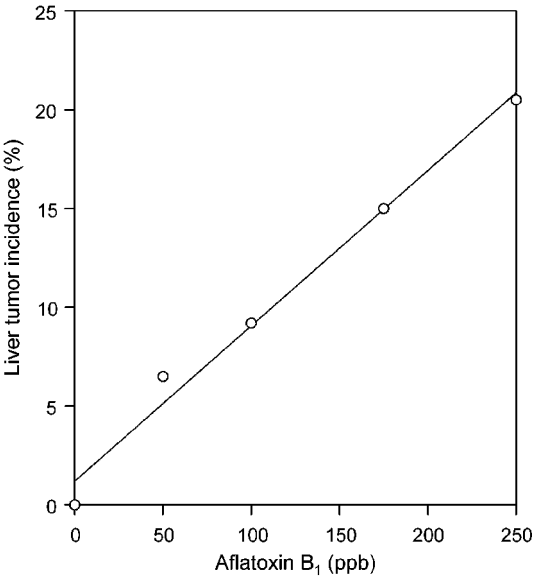

Figure 4 AFB1 liver carcinogenicity in rainbow trout. Rainbow trout embryos were exposed to AFB1 for 30 min. At 11 months of age, trouts were sacrificed and analyzed for liver tumors. The number of fish were 200, 370, 249, 400, and 400 in the 0, 50, 100, 175, and 250 ppb exposure groups, respectively (20).

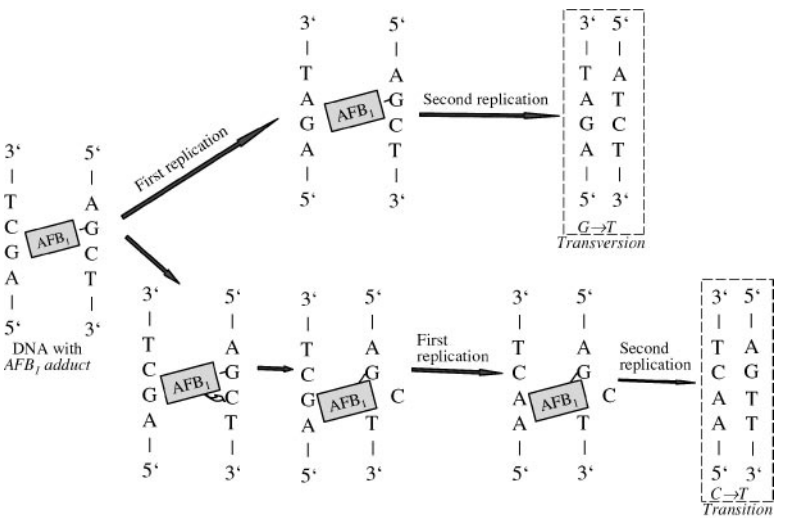

Figure 5 Two types of mutations (G→T transversion and C→T transition) resulting from the AFB1-N^7-guanine adduct [8,9-dihydro-8-(N^7 guanyl)-9-hydroxyaflatoxin] B1. Newly synthesized DNA strand: dashed boxes.

the direct relationship between AFB1-DNA adducts and tumor incidence require several preconditions. In the case of AFB1, none of the possible mechanisms (*a–e* in Figure 1) seem to be effective enough to introduce a measurable threshold. The possibility that a threshold could be observed for doses lower than those used in the experiments shown in Figures 3 and 4 cannot be excluded. Such experiments would be difficult because extremely large numbers of animals would be required. However, unless data are available, the most conservative model, which is a linear dose-response extrapolation with no assumed threshold, should be used for AFB1 risk evaluation.

N,N-Diethylnitrosamine

Similar to AFB1, N,N-diethylnitrosamine (DEN) is a rodent liver carcinogen when administered continuously in low doses. In a large lifetime tumorigenesis study involving 1140 male Wistar rats, 15 different doses ranging between 0.033 and 16.9 ppm were given in the drinking water (30). No threshold was observed in the low dose range, although the dose-response curve was not linear but approached a plateau at higher doses (Figure 6). O^4-ethylthymidine (O^4-Et-dT) was considered

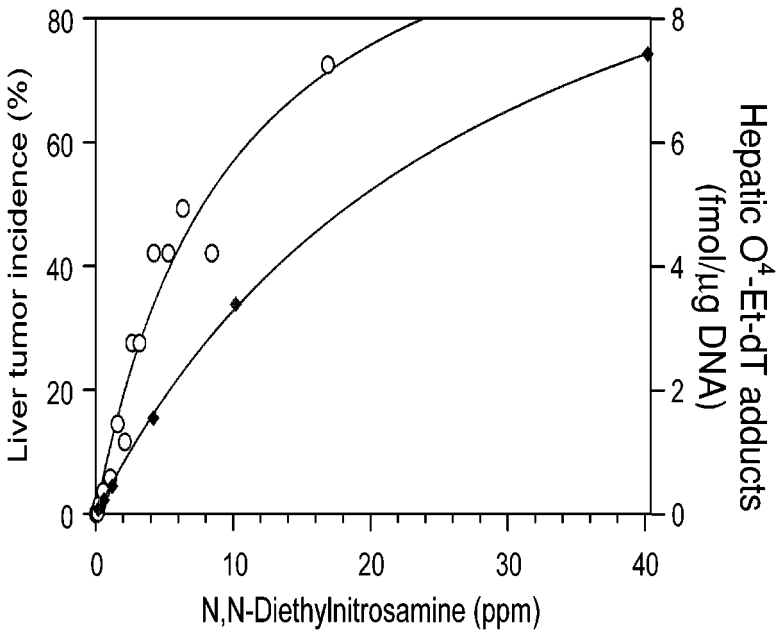

Figure 6 Liver tumor incidence (○) in a large lifetime study involving 1140 male Wistar rats. Fifteen different doses of DEN ranging between 0.033 and 16.9 ppm were given in the drinking water (18, 30). Data for O^4-ethylthymidine (♦), the major promutagenic DNA adduct responsible for induction of liver tumors, were obtained from another study with male Fischer rats (31) because adduct data from Wistar rats are not available.

to represent the major promutagenic DNA adduct responsible for induction of liver tumors (31). When DNA adducts were analyzed in another rat strain (male Fischer rats), a similar shape of the dose-effect curve was obtained as in liver tumor incidence (Figure 6). Thus, DEN represents a carcinogen showing a nonlinear dose-response without a threshold and with a very good correlation between DNA adduct induction and tumor incidence when administered continuously in the drinking water. However, different results were obtained in studies using short-term or intermittent instead of chronic lifetime exposures. Seven hundred fifty 14-day post-hatch medaka were divided into 10 groups of 75 fish each, and replicate groups were exposed to 0, 10, 25, 50, and 100 ppm DEN for 48 h (32). No increase in hepatic adenomas or carcinomas was observed for the two lowest doses of 10 and 25 ppm DEN, whereas an increase was reported for 50 and 100 ppm (Figure 7). The different dose-response curves for short- versus long-term administration may be due to interspecies differences. Williams et al. also observed nonlinearities in several studies with male Fischer 344 rats (33–36). The data shown in Figure 8 were obtained with five doses ranging from a cumulative total of 0.5 to 4 mmol DEN per kg body weight given intermittently as weekly i.p. injections for 10 weeks. No liver carcinomas were observed up to an exposure of 1 mmol/kg. Exposure to 2 mmol/kg and greater caused liver carcinomas in almost all of the 12 exposed rats [Figure 8; (35)]. Whereas these data were obtained by the i.p. administration route, the authors later obtained similar results by once weekly intragastric instillation

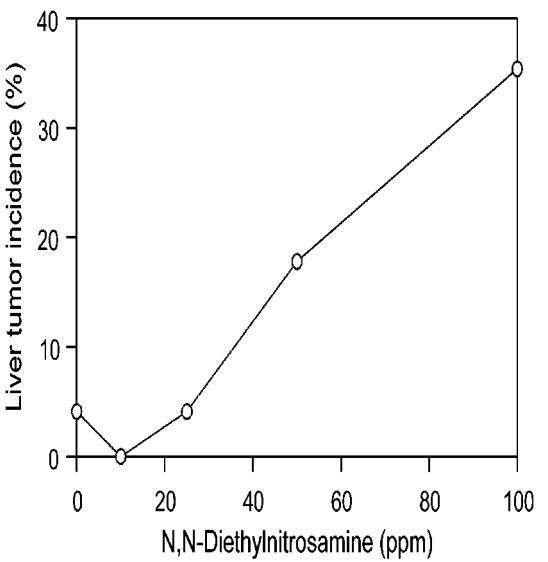

Figure 7 Liver tumor induction by DEN in fish. Japanese medaka were exposed to DEN for 48 h at 14 days post hatch. At 6 months of age, fish were sacrificed and analyzed for liver tumors. The number of fish were 49, 44, 49, 45, and 48 in the 0, 10, 25, 50, and 100 ppm exposure groups, respectively (32).

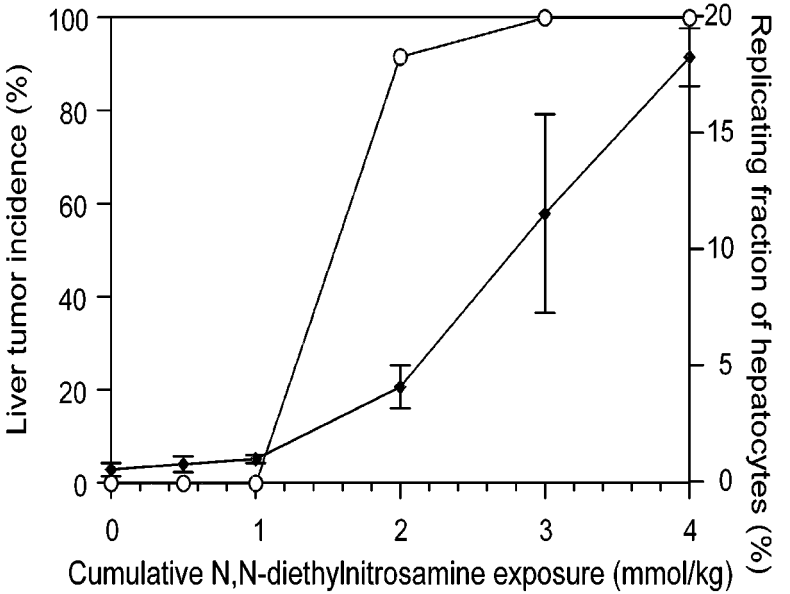

Figure 8 Incidence of liver carcinomas (○) in male Fischer 344 rats (35). Ten doses ranging from a cumulative total of 0.5 to 4 mmol DEN per kg body weight were given as weekly i.p. injections for 10 weeks. After a period of 38 weeks 12 rats were examined per dose group. For determination of the replicating fraction (♦) of hepatocytes, the same administration schedule was applied to male Fischer 344 rats. Values are means and standard deviations of five rats. The replicating fraction was determined by immunohistochemical analysis of bromodeoxyuridine incorporation.

(34). Thus, the type of exposure, continuous for lifetime versus intermittent or short-term exposure, appears to strongly influence the low dose–response curve. The cumulative exposure of 2 mmol/kg yielded a 92% liver cancer incidence (35). This effect can be best compared to that of the highest cumulative exposure achieved in the continuous lifetime study of Peto et al. (30) that was calculated to be approximately 10-mmol/kg cumulative exposure resulting in a 78% incidence of liver tumors. Thus, the cumulative exposure in the study of Williams et al. was even smaller compared to the study of Peto et al. But because DEN was administered in only ten (individual) doses, the single doses in the Williams et al. study were much higher. The mechanisms responsible for the observed nonlinearity were also examined by Williams et al. (34, 35). Interestingly, induction of DNA adducts does not explain the observed nonlinearity because the lowest exposures also produced a clear level of DNA ethylation in the liver, even when given as only a single dose (data not shown). However, cytotoxicity and cell proliferation in rat liver correlated well with tumor incidence. Whereas the nontumorigenic cumulative doses of 0.5 and 1 mmol/kg did not cause a significant increase in the replicating fraction

of hepatocytes, a strong increase was observed for higher doses (Figure 8). It is tempting to speculate that the differences between the studies of Peto et al. and Williams et al. are due to differences in toxicity in the low-dose range (with an expected linear toxicity dose response in the Peto study), but the relevant data are not presently available.

In conclusion, the dose-response relationship for DEN in the low-dose range appears to depend on the schedule of administration of the test substance, resulting in a linear relationship for continuous lifetime exposure but a nonlinear relationship with a practical threshold at 1-mmol DEN/kg for intermittent weekly administration. We believe that the difference between lifetime exposure to very low daily doses (30) and intermittent weekly administration of relatively high doses (35) can be explained by the difference in the period between administration of DEN and analysis of tumors, and in differences of cytotoxicity. Although the low daily doses of DEN in the study of Peto et al. (30) induced DNA adducts, they were so low that probably no significant toxicity was induced. Nevertheless, the period of more than two years in this lifetime study was long enough to allow formation of mutations and later carcinomas due to the low baseline proliferation of hepatocytes. Thus, in absence of a DEN-induced influence on hepatocyte proliferation, the linear induction of DNA adducts explains the also linear dose-response relationship of liver tumors. In contrast to the study of Peto et al. (30), a shorter period between administration of DEN and analysis of carcinomas was chosen in the study of Williams [only 38 weeks; (35)]. This time period was probably not long enough to allow formation of tumors at normal (low) baseline proliferation of hepatocytes. However, when proliferation was increased by administration of cytotoxic doses of DEN, carcinomas could be induced also during the relatively short latency period of 38 weeks. Thus, cytotoxicity-induced proliferation seems to be a necessary prerequisite to cause carcinomas after relatively short latency periods. Because cytotoxicity and the induced proliferation were not linear with dose in William's study (Figure 8), a nonlinear dose response for carcinomas is a plausible consequence. Based on the assumption that human exposure to environmental carcinogens is intermittent (35), it has been suggested that low-level exposures to DEN may represent no cancer risk to humans (34). However, due to the remaining uncertainties (such as the effect of intermittent low-dose DEN on human hepatocyte proliferation and the question of whether individuals may also be exposed rather continuously), the conservative linear model with no threshold assumption may be most appropriate for extrapolation of human cancer risk due to DEN until additional clarifying data become available.

NNK [4-(N-Methyl-N-nitrosoamino)-1-(3-pyridyl)-1-butanone]

In the previous sections, AFB1 and DEN are discussed as nonthreshold carcinogens of the liver. Similarly, the linear dose-response relationship with no observable threshold seems to be an adequate description for the carcinogenic activity of several genotoxic carcinogens in lung. One of the best documented examples

is the tabacco-specific nitrosoketone NNK, which is a nicotine derivative forming the promutagenic O^6-methylguanine and pyridyloxobutyl DNA adducts (37–39) that are responsible for lung cancer in rats. NNK is also relevant for nonsmokers because nonsmoking women exposed to environmental tobacco smoke have been reported to take up and metabolize NNK, which could increase their risk of lung cancer [(40); review in (18)]. Analogous to DEN, a good correlation between DNA adducts (O^6-methylguanine) and tumor incidence has been observed (Figure 9). Both tumor incidence and DNA adducts show a nonlinear dose-response curve without an obvious threshold.

2-Acetylaminofluorene (2-AAF)

Probably the largest tumorigenicity study ever conducted was with the polycyclic aromatic amide 2-AAF published in 1979 (41). Approximately 24,000 female

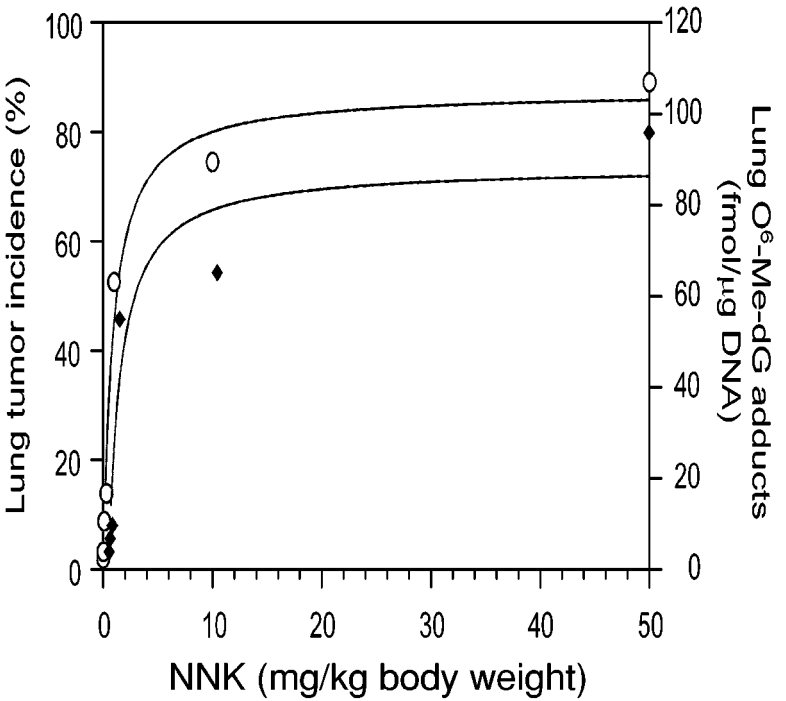

Figure 9 Lung tumor incidence (○) was determined in male Fischer rats (experimental data and modeling of dose dependence). The N-nitrosoketone was administered as subcutaneous injections three times a week for 20 weeks. Rats were sacrificed and analyzed for lung tumors after 31 months. O^6-Methyldeoxyguanosine (O^6-Me-dG) (♦) was measured in lung Clara cells at four weeks in male Fischer rats given subcutaneous injections three times a week (18, 38, 103).

BALB/c mice were given continuous administration of seven concentrations of 2-AAF in the diet, ranging from 30–150 ppm. Tumor incidence after 24 months is shown in Figure 10. In the bladder, tumor incidence was not increased for 30, 34, and 45 ppm, but increased weakly for 75 ppm, and a steep increase followed at doses of 100 and 150 ppm (Figure 10*A*). In contrast to bladder tumor incidence, the level of DNA adducts in bladder tissue increased linearly in the dose range between 15 and 150 ppm (Figure 10*A*). The tumorigenic effects of 2-AAF in mouse target organs have been reported to be associated with the formation of only one DNA adduct, N-(deoxyguanosine-8-yl)-2-aminofluorene (dG-C8-AF) (18, 42). However, a different scenario was observed in the liver (Figure 10*B*). Liver incidence and DNA adducts increased linearly with dose. Thus, the scenario observed with 2-AAF in liver is similar to that of AFB1. The example of 2-AAF shows that dose-effect relationships at low doses do not only depend on the nature of the substance tested, but may also be tissue specific.

4-Aminobiphenyl

Exposure of mice to 4-aminobiphenyl (4-ABP) has been shown to result primarily in the formation of one adduct, N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP) (43). DNA adducts resulted in a linear dose-response relationship in liver and bladder tissue of male BALB/c mice 28 days after administration of six doses between 7 and 220 ppm in the drinking water [Figure 11*A*,*B*; (43, 44)]. In contrast, tumor incidence was not increased for 7, 14, and 28 ppm in the bladder, and no increase in liver tumors was observed in male BALB/c mice. The situation is complicated by a gender difference in the susceptibility to 4-ABP. In contrast to male BALB/c mice (Figure11*A*), a linear low-dose-effect relationship for hepatic DNA adducts and liver tumor incidence was observed for female mice (Figure 11*C*). In contrast to male mice, only relatively low bladder tumor incidences could be induced in female animals (Figure 11*D*). This example shows that in rodents, occurrence of thresholds can be sex and tissue dependent.

THE CASE OF VINYL ACETATE

The genotoxic carcinogens discussed thus far did not exhibit thresholds in all organs or under all treatment modes. Because the same seems to be the case for the majority of genotoxic carcinogens, it has been proposed that a nonthreshold dose-response relationship should always be assumed. However, this assumption is no longer acceptable. At least some genotoxic carcinogens induce tumors via mechanisms that have highly nonlinear dose-response curves in which practical, if not true, thresholds exist. A well-studied example to illustrate this point is vinyl acetate. Vinyl acetate is genotoxic because it induces chromosomal aberrations, DNA protein cross-links, and sister chromatid exchanges. Bioassay data show that vinyl acetate is carcinogenic in rats and mice by the oral route and in rats by the inhalation route. However, all carcinogenic responses are expressed at very high

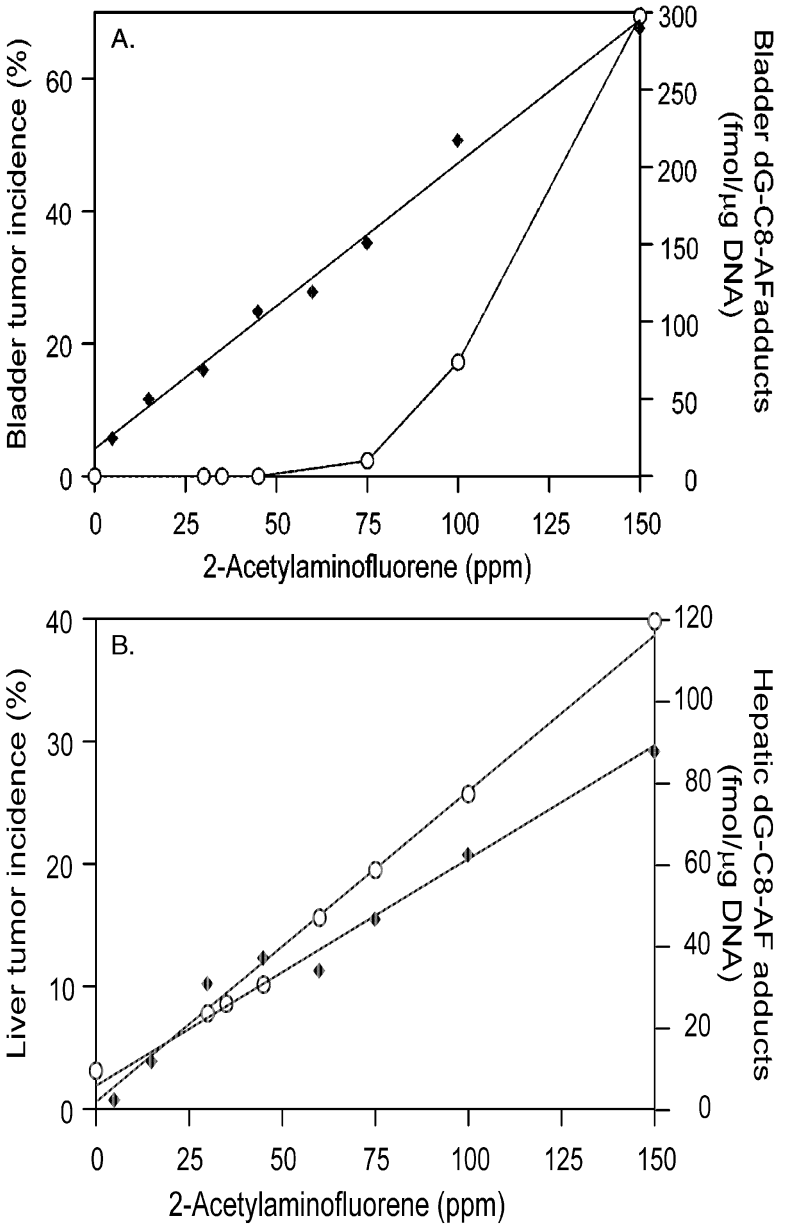

Figure 10 (*A*) Bladder tumor incidence (○) in female BALB/c mice after administration of 2-AAF in the diet (41). Mice were analyzed for tumors after 24 months. In the similarly conducted DNA adduct study, N-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) (♦) was measured in bladder tissue of mice (18). (*B*) Liver tumor incidence (○) in female BALB/c mice after administration of 2-AAF in the diet (41). Mice were analyzed for tumors after 24 months. In the similarly conducted DNA adduct study, N-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) (♦) was measured in liver tissue of mice (18).

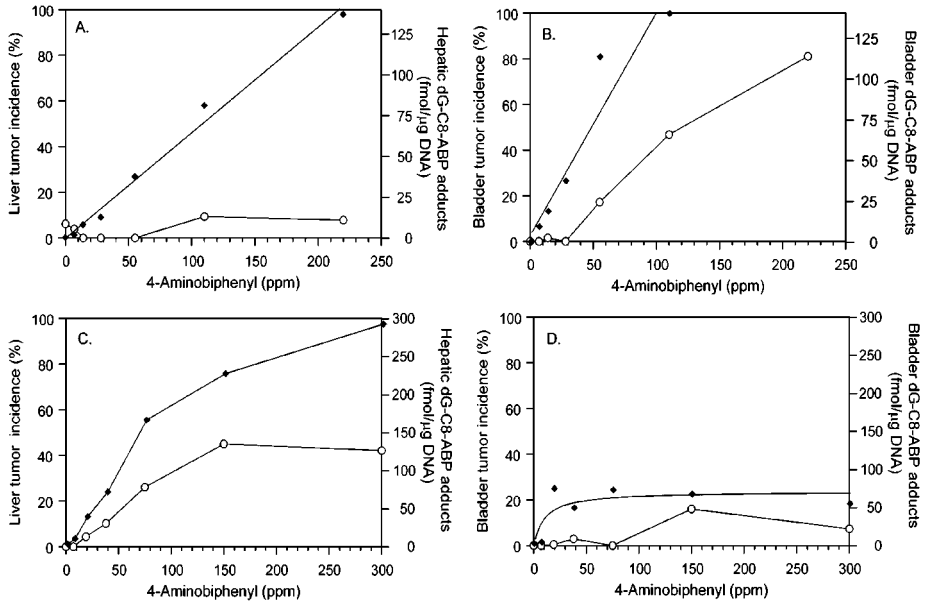

Figure 11 4-ABP induced liver (*A* and *C*) and bladder (*B* and *D*) tumors (○) in relation to the DNA adduct N-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP) in male (*A* and *B*) and female (*C* and *D*) BALB/c mice. ABP was administered chronically in the drinking water. Tumor incidence was examined after 24 months, whereas DNA adducts (♦) were measured after 28 days of exposure (18, 42–44).

dose levels that exceed standard definitions of maximum tolerated dose (MTD). In this section, we show that the dose-response curve of vinyl acetate has a practical, if not a perfect, threshold.

Carcinogenicity in Rats and Mice

ORAL EXPOSURE Vinyl acetate has been tested extensively for carcinogenic activity after oral exposure. Four studies have been conducted, all with administration via the drinking water.

1. Data from a study conducted by the Japanese Bioassay Research Center (JBRC) (45) are reviewed here. Data summary tables and summarized methodological details are available. Groups of 50 male and female rats and mice were administered 0, 400, 2000, or 10,000 ppm vinyl acetate for up to 104 weeks. The stock solution (98% pure vinyl acetate) contained impurities of hydroquinone (5 ppm), acetic acid, and water. Except for hydroquinone, the concentrations of the impurities were not specified. Vinyl acetate drinking water solutions were prepared twice a week. The solutions were kept in airtight containers and analyzed for vinyl acetate content before and after

administration. Body weight gain was significantly reduced in male and female rats and mice in the 10,000-ppm group. The only tumors seen in the JBRC study that were clearly attributable to vinyl acetate were of the upper digestive tract. In rats, oral cavity squamous cell carcinomas were the only tumors observed to be statistically significant. These were observed in the 10,000-ppm group. In mice, tumors of the oral cavity, esophagus, and stomach were observed. With the exception of one squamous cell papilloma, there were no tumors of the upper digestive tract observed in mice of the 0, 400, or 2000 ppm groups. The single squamous cell papilloma was seen in the esophagus of a 2000-ppm female mouse. Tumor incidence was higher in mice than rats. This difference is likely to be related to the higher mg/kg/day intake in mice relative to rats. The data suggest that the mice received approximately twice the dose received by the rats, on a mg/kg/day basis.

2. Maltoni et al. (46) exposed mice to concentrations of 0, 1000, or 5000 ppm vinyl acetate in drinking water. A number of contaminants in the stock test sample (benzene, 30–45 ppm; methyl and ethyl acetate, 50 ppm; crotonaldehyde, 6–16 ppm; acetaldehyde, 2–11 ppm; and acetone, 330–500 ppm) were identified. Although the presence of benzene in the sample is a concern, the levels of all of these contaminants are too low to be definitively confounding factors. This study appears to have taken appropriate precautions against vinyl acetate degradation. Drinking water solutions were prepared daily. Mice were exposed in utero, from the twelfth day of pregnancy. Both breeders and offspring were exposed throughout their lifespan, i.e., approximately 136 weeks. Group sizes of the male and female breeders were 13 and 37, respectively. Group sizes of the offspring ranged from 37 females in the 1000 ppm group to 49 males in the 5000 ppm group. A variety of tumors were observed in this study, many of which can be clearly attributed to vinyl acetate. The compound-related tumors are largely of the upper digestive tract. A number of nondigestive tract tumors (e.g., zymbal gland, lung, liver, uterine, and mammary gland) were observed, but these all occurred with high background incidence. Therefore, without adjusting for age, these tumor data cannot be evaluated with certainty. Squamous cell carcinoma of the oral cavity, tongue, esophagus, and forestomach were all treatment related at 5000 ppm. There were no tumors among mice administered 1000 ppm. Because drinking water consumption data were not collected, the concentrations used cannot be precisely converted to mg/kg/day dose rates. However, the dose received by the mice can be estimated from a recent 90-day drinking water study in mice conducted at 1000 and 5000 ppm (47). Male mice administered 1000 ppm or 5000 ppm consumed 254 mg/kg/day or 1185 mg/kg/day, respectively.
3. Bogdanffy et al. (48) administered vinyl acetate in drinking water at concentrations of 0, 200, 1000, or 5000 ppm for 10 weeks to male and female rats that were subsequently mated. The offspring were then culled into two

groups of 60 for the main study and 30 for satellite groups. Exposure to the drinking water then continued to 104 weeks. Drinking water solutions used in this study have been carefully controlled for both contaminants and spontaneous breakdown of vinyl acetate. A number of contaminants in the stock test sample (hydroquinone $\leq$1 ppm, acetic acid $\leq$11.5 ppm, acetaldehyde $\leq$71 ppm, and water $\leq$389 ppm) were identified but were too low to be of concern as confounding factors. The drinking water was formulated daily (and slightly overformulated to account for decomposition), and therefore exposure of rats to acetaldehyde or acetic acid was minimal. Other than decreased water and food consumption, there were no effects reported on standard parameters measuring systemic toxicity such as body weight gain or clinical parameters (hematology, chemistry, urinalysis). The dose rates for the 200, 1000, and 5000 ppm groups were approximately 13, 62, and 252 mg/kg/day, respectively. The no observed adverse effect level (NOAEL) of 200 ppm was based on the effects on food and water consumption. The authors conclude that there were no non-neoplastic or neoplastic lesions observed that were compound related. Two squamous carcinomas were observed in the oral cavity of males exposed to 5000 ppm. Because the incidence of these tumors was within historical control ranges, they cannot be considered compound related. The MTD was not exceeded, but effects on the animals (e.g., water and food consumption) clearly demonstrate adequate exposure. It must be concluded, therefore, that based on this study alone vinyl acetate would not be considered carcinogenic in rats via the oral route. Conversion of doses to mg/kg/day shows that the dose rate of 252 mg/kg/day (5000 ppm) in the rat study of Bogdanffy et al. (48) is approximately equivalent to the dose rate at the NOAEL of approximately 254 mg/kg/day (1000 ppm) in the mouse study of Maltoni et al. (46). From these calculations, it can be seen that, when adjusted for dose, the rat and the mouse studies appear to be similar. These estimations would also suggest the high dose of the Maltoni study exceeded the limit dose of 1000 mg/kg/day according to OECD (Organization for Economic Co-Operation and Development) (49, 50).

4. In the oldest published oral carcinogenicity study, Lijinksy & Reuber (51) administered vinyl acetate in the drinking water to male and female rats for two years at concentrations of 1000 or 2500 ppm. However, this study is deficient in several aspects, most notably that the drinking water solutions were prepared only once per week. The authors recognized a decomposition rate of approximately 8.5% per day. Therefore, by the end of the week the animals in the 2500 group, for example, were exposed to approximately 1300 ppm vinyl acetate and significant quantities of breakdown products, including acetaldehyde and acetic acid. The authors also did not purify the vinyl acetate prior to preparation of the drinking water solutions. Thus, the rats were also exposed to unspecified impurities. These problems have been discussed by the authors. In addition, only 20 rats were in each group, whereas the standard for such a study is generally approximately

50. Thus, the statistical power for detecting true positive responses and for discriminating against false positive and false negative outcomes is compromised. Therefore, we do not consider the data of this study in the present review.

Summary of carcinogenicity studies with oral exposure Vinyl acetate is clearly carcinogenic in mice and rats by oral ingestion. When the data are plotted collectively, there is a clear break in the dose-response curve (Figure 12). Responsiveness of rats and mice is similar when the dose is expressed in a mg/kg/day format. Because carcinogenicity only occurs at dose levels that exceed an MTD and are beyond what is expected of a limit test of 1000 mg/kg/day according to OECD guidelines (49, 50), the respective studies must be considered excessive. Regarding multiple sites of carcinogenicity, the only tumors clearly associated with vinyl acetate exposure are of the upper digestive tract. Tumors are located in the oral cavity, esophagus, and forestomach. All of these tissues are lined with squamous epithelium and do not display marked differences in histological makeup of the lining epithelium. Not surprisingly, all tumors are of the same histiogenic origin. Generally, multiple-site carcinogenesis is considered for agents, such as nitrosamines, that affect a variety of systemic organs and different cell types. Nitrosamines are carcinogenic to the esophagus and the brain, tissues of entirely

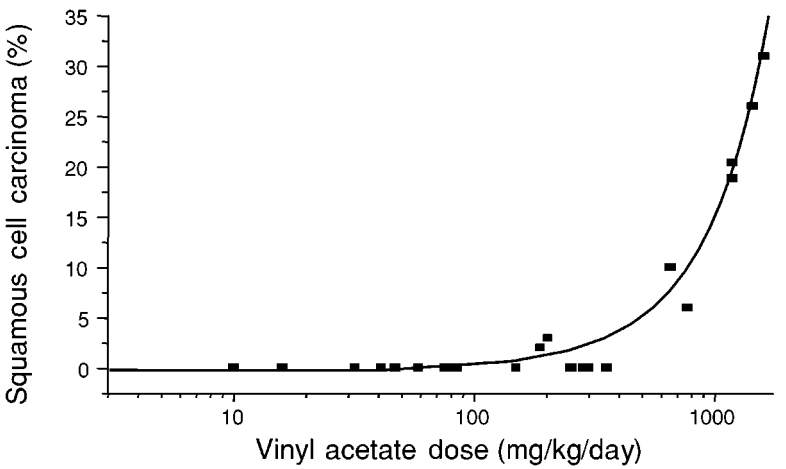

Figure 12 Composite dose-response data for rat and mouse drinking water bioassays. Vinyl acetate induced squamous cell carcinoma of the oral cavity, esophagus, and forestomach. The tumor incidence was greatest for the oral cavity. These data, which include male and female rats and mice, illustrate the sharp break in the dose-response curve with clear evidence for a practical threshold. Data are from Bogdanffy et al. (65), Maltoni et al. (46), and JBRC (45).

different histology (52). It is not appropriate to consider the oral cavity, esophagus, and forestomach as multiple organs when the tumors all appear to be derived from squamous epithelial lining and all of the tumors are histologically similar. This argument is consistent with published guidelines for combining neoplasms for evaluation in carcinogenesis studies (53). There is, therefore, no evidence for multiplicity of tumor sites. Furthermore, the tumor incidence decreases from the oral cavity to the forestomach. This is a characteristic that seems to be more appropriately associated with a site-of-contact carcinogen.

INHALATION EXPOSURE There are two studies that have tested the carcinogenic potential of vinyl acetate by the inhalation route.

1. Bogdanffy et al. (54) exposed rats and mice (60 per sex per group) to 0, 50, 200, or 600 ppm vinyl acetate for 104 weeks. The test material purity was characterized and found to be >99% pure. Impurities in the stock material included acetic acid (≤10 ppm), acetaldehyde (≤65 ppm), water (≤472 ppm), and hydroquinone (<1 ppm). None of these impurities are likely to have confounded the results. Body weight gain in rats and mice was reduced in the 600 ppm groups, and in mice in the 200 ppm groups. At the end of the study, body weight gain was 10% and 15% below controls for rats and mice, respectively. There was no negative effect on survival. Vinyl acetate induced nasal tumors in both male and female rats. No nasal tumors, or other tumors, were noted in mice. The nasal cavity tumors were all discovered at the terminal kill, with the exception of one benign tumor found in an animal that died two weeks before termination of the study. Of the 12 tumors diagnosed, 5 were benign and 7 were malignant. Most of the malignant tumors were squamous cell carcinomas; 1 tumor was a carcinoma in situ. The tumors were localized to regions lined with olfactory epithelium (n = 5), respiratory epithelium (n = 2), cuboidal epithelium (n = 2), and three were of unknown origin. Published historical control data for rat nasal tumors show that nasal tumors in unexposed controls are rare (55).
2. Maltoni et al. (56) performed an inhalation bioassay primarily to study vinyl chloride. Groups of rats were exposed to 0 ppm (68 male and female combined) or 2500 ppm (96 male and female combined) vinyl acetate for 4 hours per day, 5 days per week, for 52 weeks. After the exposure period, all animals were kept under observation until spontaneous death (135 weeks total). There was significant mortality among the rats. Fifty-eight and 49 rats (male and female combined) remained after the treatment period in the 0 ppm and 2500 ppm groups, respectively. The authors did not observe tumor induction in rats exposed to vinyl acetate. However, the authors conclude that exposure to vinyl acetate caused death of the animals within a period that did not allow a correct carcinogenicity test. However, it is worth noting that even at such high exposure concentrations, there were no tumors reported during the first 12 months of testing or during the exposure-free phase.

Summary of carcinogenicity studies with inhalation exposure Vinyl acetate is clearly carcinogenic by the inhalation route in rats, but not mice. Whereas the Maltoni et al. (56) study is inadequate for assessing carcinogenicity, the Bogdanffy et al. (54) study is sufficient. Although vinyl acetate was clearly carcinogenic in rats, carcinogenicity was only expressed at high exposure levels (600 ppm). The MTD (defined as 10% retardation in weight gain) was exceeded in rats exposed to 600 ppm. There was no evidence for systemic carcinogenicity.

Other In Vivo Studies in Experimental Animals

Induction of hepatic enzyme-altered foci (ATPase, GGTase) was investigated after administration of vinyl acetate (200 and 400 mg/kg/day, orally) to newborn rats for 3 weeks, with or without subsequent promotion by phenobarbital (57). No foci were observed in vinyl acetate–treated animals at the age of 14 weeks, whereas the structurally related compounds vinyl carbamate and vinyl chloride induced enzyme-altered foci under comparable experimental conditions. The negative result was not surprising considering the short exposure period and relatively low doses compared to the carcinogenicity studies discussed above.

Another in vivo study with a negative result was performed by Simon et al. (58). After administration of (^{14}C) vinyl acetate to male and female rats, either orally or by inhalation, no specific hepatic DNA adducts, known to occur after administration of labelled vinyl halides or vinyl carbamates, could be observed in liver.

Epidemiological Studies of Vinyl Acetate Carcinogenicity

A cohort study including 4806 individuals employed at a plant for the manufacture of synthetic chemicals in the United States was performed between 1942 and 1973 by Waxweiler et al. (59). The cohort had an excess risk for cancer of the respiratory system [resulting in a standardized mortality ratio of 1.5 (95 % confidence interval: 1.1–2.0)]. Thus, exposure of these cancer patients to 19 chemicals, including vinyl acetate, was examined. Exposure of the patients with cancer of the respiratory system to vinyl acetate was below the mean exposure expected for the members of the cohort with the same year of birth and age at commencement of work in the plant. A subgroup of employees with undifferentiated non-small-cell lung cancer had a slight, but statistically nonsignificant, cumulative exposure to vinyl acetate. Thus, this (59) does not provide evidence for a carcinogenic effect of vinyl acetate in humans.

A case-control study was performed in a cohort of 29,139 men employed in a chemical manufacturing environment (60). Nested case-control studies of non-Hodgkin's lymphoma, multiple myeloma, nonlymphocytic leukemia, and lymphocytic leukemia were conducted in men from two chemical facilities and a research center. Exposure odds ratios were examined in relation to 21 specific chemicals. The results are difficult to interpret because exposure to vinyl acetate was associated with a decreased risk for nonlymphocytic leukemia (odds ratio:

0.5), but slightly increased odds ratios for non-Hodgkin's lymphoma (odds ratio: 1.2) or multiple myeloma (odds ratio: 1.6).

In conclusion, evaluation of epidemiologic data on a possible carcinogenic effect of vinyl acetate is difficult because most individuals in the existing epidemiological studies were exposed to several chemicals. Nevertheless, the existing data do not support a carcinogenic effect of vinyl acetate in humans.

Metabolism and Genotoxicity of Vinyl Acetate

Exposure of tissues to vinyl acetate results in metabolic conversion to acetic acid and acetaldehyde at the site of contact. The histochemical localization of carboxylesterase and aldehyde dehydrogenase in nasal tissue have been described in detail (61–65). These enzymes rapidly and almost completely convert vinyl acetate to acetic acid and acetaldehyde in nasal tissue. At high concentrations, acetaldehyde induces DNA-protein cross-links that lead to chromosomal aberrations. Formation of DNA-protein cross-links is facilitated by low intracellular pH (pH_i). A low pH microenvironment is caused by acetic acid formation from both vinyl acetate hydrolysis and acetaldehyde oxidation to acetic acid and liberation of protons (66). Acetaldehyde, as discussed above, is a known clastogen but does not appear to induce point mutations. In fact, the profiles of genotoxic activity for acetaldehyde and vinyl acetate are almost identical and vinyl acetate is not active as a clastogen without a source of carboxylesterase added. Thus, the clastogenic activity of vinyl acetate must be attributed to metabolic formation of acetaldehyde. It has been reported that acetic acid, formed intracellularly from vinyl acetate hydrolysis, contributes to the genotoxic activity (67, 68) and tumor progression (69). It is well known that a low pH can have a confounding effect on genetic toxicity tests using mammalian cells (70). Low pH has also been shown to induce cellular transformation of Syrian hamster embryo cells (71, 72). In fact, acetic acid induces chromosomal aberrations in Chinese hamster ovary (CHO) cells. It is likely that the genotoxic activity of acetaldehyde is attributable, at least in part, to intracellular acidification because two protons are released when acetaldehyde is oxidized to acetic acid in the presence of aldehyde dehydrogenase and NAD^+. Intracellular acidification has been reported to facilitate acetaldehyde-induced genotoxicity (73). The authors used a model system for measuring DNA-protein cross-links (the initial event finally leading to chromosomal breaks) involving incubations of calf thymus histone protein with plasmid DNA and measurement of covalently bound DNA-histone protein complexes. Cross-links appeared to be between DNA and amino acid residues, guanosine and lysine, respectively. In their studies, it was first shown that DNA-protein complex formation requires carboxylesterase-dependent metabolism of vinyl acetate to acetaldehyde and acetic acid. Next, it was shown that the formation of acetaldehyde-induced DNA-protein cross-links is increased in the presence of increasing concentrations of acetic acid. Finally, they demonstrated that acetaldehyde-induced DNA-protein cross-links are increased with simple reduction in pH. The proposed mechanism for this increase is ionization to positively

charged amino acid groups of histone proteins, resulting in a higher affinity for the negatively charged DNA. The resulting tight association of histone protein with DNA may be a prerequisite for the formation of DNA-protein cross-links by acetaldehyde, which, in addition, is increased by the higher electrophilicity of the carbonyl carbon upon protonation of acetaldehyde. In conclusion, although further studies are required in this field, strong evidence has been presented showing that intracellular acidification is a prerequisite for the genotoxic activity of vinyl acetate and acetaldehyde. In addition, the acetaldehyde-DNA-protein cross-link was found to be very unstable, with a half-life of approximately six hours (74, 75).

Mechanistic Data Support a Practical Threshold for Vinyl Acetate

Tissues can accommodate exposure to acetic acid and acetaldehyde without adverse effects up to a certain level of exposure (76). This is consistent with the known exposure of tissues to endogenous acetic acid and acetaldehyde. It is known that acetaldehyde is a natural constituent in the body and a metabolic by-product of threonine metabolism (77, 78). Background levels of approximately 0.3-μg/mL acetaldehyde exist in blood. Therefore, it would seem reasonable that exposures to vinyl acetate that do not raise tissue acetaldehyde levels beyond the range of natural background levels in blood or tissues would also be below biological thresholds. Using a physiologically based pharmacokinetic (PBPK) model, it is possible to predict tissue exposure to acetaldehyde resulting from inhalation exposure to vinyl acetate. Figure 13 shows the predicted basal cell acetaldehyde levels in humans during exposure to 1-ppm vinyl acetate. When critical levels of vinyl acetate are achieved, thresholds are exceeded and five critical steps in the mechanism ultimately leading to cancer become active (Figure 14). The threshold for pH_i reduction in neuronal cells that does not induce cytotoxicity in vitro is 0.15 pH unit (79). The lowest concentration of acetaldehyde that has induced sister chromatid exchanges (SCEs) in vitro in CHO cells is 3.9 μg/mL (80). SCEs are not considered to be a valid marker of mutagenic damage and are generally overly sensitive. A more appropriate and more widely accepted benchmark genetic toxicity endpoint would be chromosomal aberrations. Chromosomal aberrations are also mechanistically consistent with the data, suggesting that acetaldehyde induces DNA-protein cross-links. However, because SCEs already occur at lower concentrations than chromosomal aberrations, they were chosen as an endpoint in order to overestimate, rather than underestimate, the risk due to vinyl acetate exposure.

When critical levels of exposure to the ensuing acetaldehyde and acetic acid are achieved, thresholds are exceeded and further critical steps in the mechanism ultimately leading to cancer become active. These steps are illustrated for olfactory epithelium in Figure 14. The PBPK model predicts that in rat nasal olfactory tissue, exposure to 50-ppm vinyl acetate causes a 0.08 unit reduction in pH_i and a basal cell acetaldehyde concentration of 1.7 μg/mL. Fifty-ppm vinyl acetate is a NOAEL,

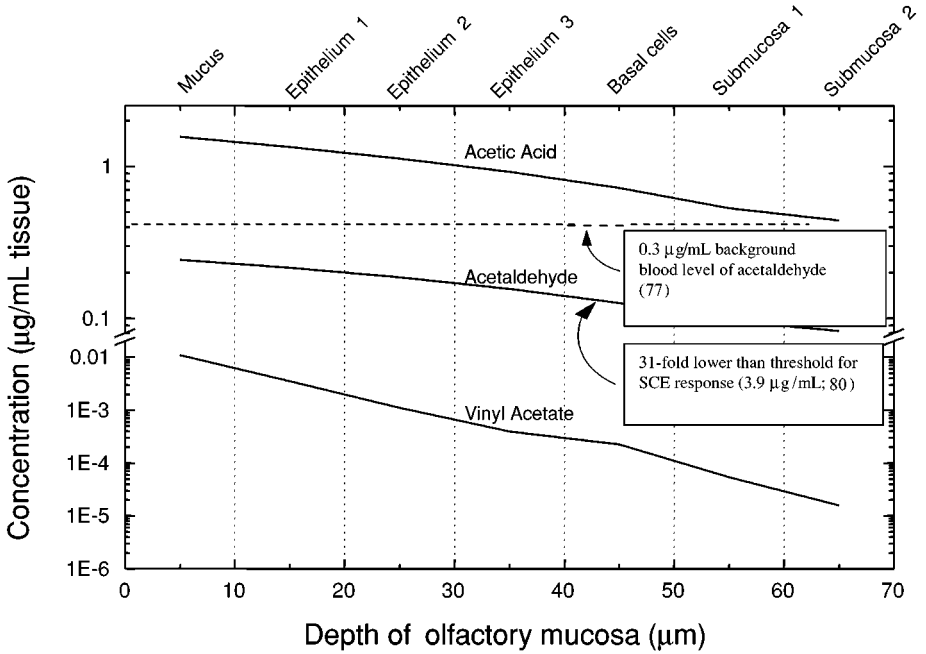

Figure 13 Dosimetry in human epithelium at an exposure to 1-ppm vinyl acetate (104). Predicted steady state concentrations of acetaldehyde, acetic acid, and vinyl acetate throughout the olfactory nasal mucosa of humans exposed continuously to 1 ppm vinyl acetate. Concentrations of acetaldehyde at the basal cell layer are critical for consideration because basal cells are the progenitor cells for the epithelium and are the target cell for carcinogenesis. The figure illustrates that at 1 ppm vinyl acetate, basal cell acetaldehyde concentrations are predicted to be approximately 3 times lower than background blood acetaldehyde levels and more than 31 times lower than the lowest concentration shown to induce SCE. SCE are sensitive markers of genetic damage and of questionable relevance. The margin of safety relative to the more appropriate endpoint of chromosomal aberrations is much greater. Obe et al. (80) reported the lowest level to induce SCE in normal human lymphocytes or lymphocytes from Fanconi's anemia patients to be 15.6 μg/mL or 7.8 μg/mL acetaldehyde, respectively. The margin of safety below the chromosomal aberrations endpoint is 124-fold.

and the pH_i reduction and basal cell acetaldehyde levels are below the thresholds of their effects. As the dose level increases to 200 ppm, pH_i is predicted to be reduced by 0.25-pH units, a value slightly above the threshold, and cytotoxicity, such as olfactory degeneration, occurs at an incidence between 8% and 10% (Figure 14, *step 3*). However, the cell proliferation response at 200 ppm is weak (*step 4*). Levels of acetaldehyde of 5.4 μg/mL are slightly in excess of the threshold for genotoxicity. Thus, at 200 ppm there is minimal exposure above threshold levels of acetaldehyde (*step 1*), minimal pH_i reduction above the threshold (*step 2*), enhanced olfactory

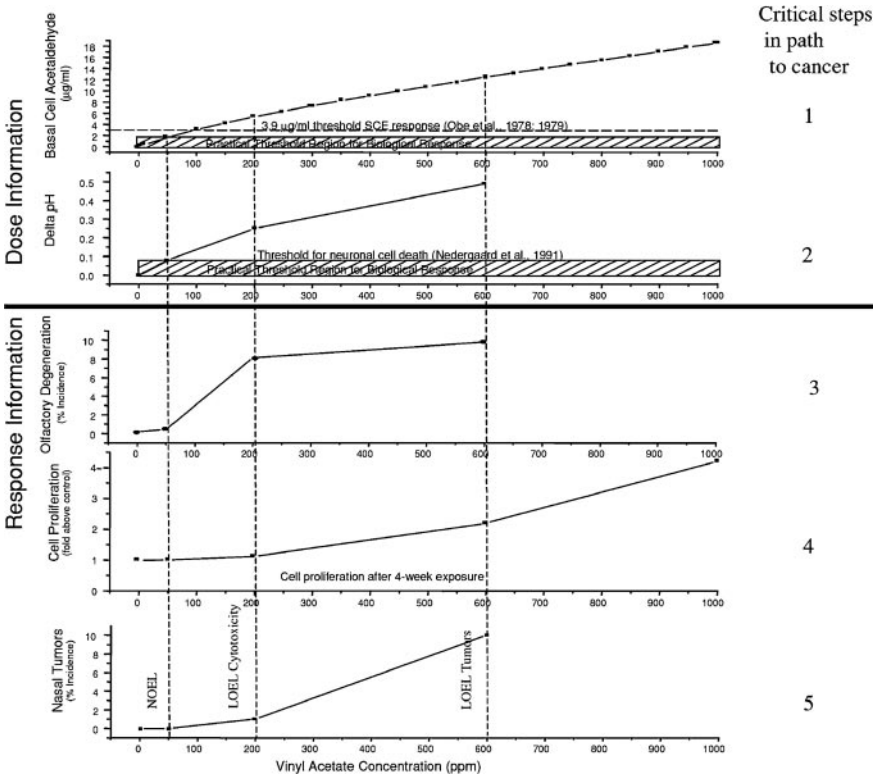

Figure 14 Composite presentation of dose and response data for the five critical steps on the pathway to carcinogenesis in nasal olfactory epithelium. Panel 1 shows the predicted steady state concentration of acetaldehyde at the basal cells, the progenitor cells of nasal cancer, in relation to in vitro doses that produce sister chromatide exchanges. Panel 2 shows predicted pH_i changes in olfactory epithelium of the rat in relation to changes in pH_i that are cytotoxic to neuronal cells in vitro. pH_i reduction is proposed to be the critical step leading to cytotoxicity. Panel 3 shows olfactory degeneration in rats as a cytotoxic endpoint. Basal cell proliferation and the incidence of nasal tumors in rats is presented in panels 4 and 5. Olfactory degeneration (cytotoxicity) is observed at 200 ppm. Because acetaldehyde levels are only slightly above thresholds, there is no significant tumor response. At 600 ppm, all thresholds are exceeded, cell proliferation is significantly enhanced, and a significant incidence of nasal tumors is observed. The mechanism of action for nasal respiratory and oral cavity tumors is similar, with the exception that the cause of the proliferative response in oral cavity may be only subtly related to cytotoxicity and is more likely the result of known mitogenic effects due to reduced pH_i.

degeneration (*step 3*), and slightly enhanced cell proliferation (*step 4*). At 200 ppm, one nasal tumor was observed that was not statistically significant (*step 5*). At 600 ppm, acetaldehyde levels are predicted to be markedly above threshold at 12.4 μg/mL (*step 1*), pH_i is predicted to be markedly reduced by 0.49-pH units (*step 2*), olfactory degeneration is strongly enhanced (*step 3*), and basal cell

proliferation is more than twofold above control (*step 4*). At 600 ppm, all of the critical steps in the mechanism of carcinogenesis are active and tumors now appear at a statistically significantly increased rate (*step 5*). This sequence of events and the physiological modeling suggest that only when critical exposure concentrations, threshold levels for pH_i reduction, and cellular proliferation (induced by cytotoxicity in olfactory epithelium) are achieved, all the conditions necessary for a complete carcinogenic mechanism in place.

There is another area of mechanistic work that deserves discussion regarding the generality of the mechanism discussed above for both olfactory and respiratory tissue as well as the upper digestive tract. The mechanism of action described above for olfactory epithelium suggests that cytotoxicity is the first adverse cellular response to vinyl acetate exposure of both respiratory and olfactory tissue. Events such as cell proliferation and tumor formation become significant only at higher concentrations. The data to support this mechanism are clear for olfactory tissue but require careful analysis for respiratory tissue. In respiratory tissue, there are several pieces of information that support cytotoxicity as the first step in carcinogenesis; for example, the observation of respiratory epithelial degeneration and cell proliferation in rats exposed for one or five days to 1000-ppm vinyl acetate (81) and the in vitro cytotoxicity studies that show acid phosphatase release from nasal turbinates in culture (74). The cell proliferation responses, which were also significant at 600 ppm, most likely represent subtle cytotoxic responses that are repaired or not evident microscopically. The lack of a more pronounced response in respiratory tissue has been recognized as an unresolved question (54, 82).

An alternative hypothesis for the cell proliferation stimulus in respiratory epithelium has recently emerged (66) that may also clarify the mode of action in the upper gastrointestinal tract. Literature reports suggest that reductions in pH_i can also induce mitogenesis. Alterations in pH_i are involved in stimulation of cell growth and transformation. For instance, Syrian hamster embryo cells cultured at pH 6.7 show a marked increase in life span compared to those cultured at pH 7.3, as measured by the number of population doublings that occur before cellular senescence (72). The higher proton burden of the intracellular environment has been shown to displace $Ca2^+$ from intracellular binding sites (83). $Ca2^+$ displaced from the growth and differentiation factor (GDF) protein blocks the intracellular signaling that leads to differentiation (84). Blockage of the differentiation pathway could promote sustained proliferation, expansion of the undifferentiated cell population, and clonal expansion of spontaneous or chemical-induced mutants. Although substantiation of the hypothesis that intracellular acidification is mitogenic in nasal or oral cavity mucosal cells suggests further experimentation, the proposal is supported by the literature and could provide a fundamental linkage to many tumor promotion mechanisms. Regardless of the mechanism for induction, it is clear that cell proliferation is induced in nasal respiratory and upper gastrointestinal tract epithelial tissues and that this step is critical to the complete expression of the carcinogenic potential of vinyl acetate (85).

It has been argued that respiratory and olfactory tissue are not likely to respond similarly to the same exposure to acetic acid or reduced pH_i and that respiratory and olfactory tissues may have different biochemical capacities for responding to alterations in pH_i. There is precedent in the literature to support this position (86–88). Similar to the nasal cavity, the oral cavity possesses carboxylesterase that has been localized to squamous epithelium (89, 90). The activity in oral mucosa of rats and mice was similar and was correlated to regions shown to be active by histochemistry (91). However, the carboxylesterase activity of the rat oral mucosa was approximately 100 times lower than that of nasal tissue. Recently, cell proliferation has been measured in the oral mucosa of rats and mice administered vinyl acetate in drinking water (92). The oral cavity is lined with squamous epithelium. Rats and mice were exposed to concentrations of up to 24,000 ppm for 92 days. Less than twofold, but significant, increases in oral mucosal basal cell proliferation were observed in rats evaluated on days 29 and 92. In mice, the responses were more pronounced, with approximately 2.4- and 3.4-fold increases being observed at day 92 in the 10,000 and 24,000 ppm groups, respectively. The greatest proliferative response observed in mice was in the lower jaw, which was also the region of greatest tumor formation observed in the JBRC study. In conclusion, although the support for the proposed mechanism of action of vinyl acetate on oral cavity mucosa is not as robust as for the nasal cavity, the research to date provides a parallel picture in which enhanced epithelial cell proliferation is induced when critical thresholds are exceeded. The data further suggest an even higher level of a practical threshold in these tissues for which the formation of some tumors have also been reported after exposure to very high doses of vinyl acetate. The implication of the discussed five-step mechanism is that practical thresholds of exposure exist below which there is no substance-related increased risk for cancer.

Values of Practical Thresholds in Relation to Human Vinyl Acetate Exposure

As shown above, there is clear evidence for a practical threshold for vinyl acetate–induced carcinogenesis; an exposure ranging between 50 and 200 ppm vinyl acetate was shown to cause cytotoxicity, but the proliferation response was only weak. In this concentration range, no significant increase in carcinogenicity was observed in experimental animals, but it began at 600 ppm. An even more conservative practical threshold is 50 ppm. Below 50 ppm, no cytotoxicity and no cell proliferation could be induced by vinyl acetate. Consequently, no carcinogenesis was observed in experimental animals in this dose range. In addition to these studies based on experimental animals, PBPK modeling predicts that exposure to 1-ppm vinyl acetate leads to a basal cell acetaldehyde level that is approximately three times lower than the endogenous concentration of acetaldehyde in vivo. Thus, a concentration of 1 ppm can be expected to be far below concentrations for which our organism would not have established compensatory mechanisms, such as pH-buffering of

the ensuing acetic acid to prevent significant damage. Based on limited data on the likely irritation threshold in humans, current occupational exposure limits have been set to 10 ppm in some countries (93, 94).

These practical thresholds can be compared to occupational vinyl acetate exposures. Concentrations of 0.07–0.57-ppm vinyl acetate were reported in ambient air in an area where several vinyl acetate manufacturers were located (95). An ambient air concentration of 0.14×10^{-3} ppm was detected near a chemical waste disposal site (96). Although most studies published in 1990 or later report relatively low vinyl acetate exposures, for instance <0.22 ppm for polyvinyl acetate painters (97) or <9.9 ppm in various Finnish industries (98), earlier studies report much higher occupational exposures. For instance, in 1969, a maximal exposure of 49 ppm was reported in vinyl acetate production and polymerization industries in the United States (99). As a consequence of the practical threshold for vinyl acetate, it seems to be very important to avoid extreme exposures because at concentrations of 600 ppm, albeit for a lifetime exposure, carcinogenesis was observed. On the other hand, to our knowledge, almost all occupational exposures reported in the past decade were much lower. Exposure limits for vinyl acetate are different in several countries. Examples for current national occupational exposure limits (time-weighted average) are 10 ppm in the United States and Germany, 8.5 ppm in France, and 2.8 ppm in Poland and the Russian Federation (93, 94, 100). Regarding ambient lifetime exposures, a limit of 0.4–1.0 ppm has been recommended (101). Lower concentrations are below a practical threshold, where the prerequisites for vinyl acetate carcinogenesis, namely cytotoxicity and regenerative cell proliferation, are not observed, whereas significant vinyl acetate–induced carcinogenesis itself is not observed below 600 ppm.

CONCLUSIONS

A linear low dose–response relationship with no observable threshold seems to be a conservative but adequate description for the carcinogenic activity of many potent genotoxic chemicals, such as AFB1 or NNK. However, for several genotoxic carcinogens, sufficient data provide an adequate base for the judgement that they operate by mechanisms that establish practical thresholds. For some, a nonlinear dose–response relationship at low doses has been observed for certain tissues and cell types, whereas other organs showed a linear dose–response relationship at low doses. 2-AAF and 4-ABP are examples of this class of carcinogens. On the other hand, vinyl acetate and formaldehyde belong to a class of carcinogens showing clear “practical” or possibly even “real” thresholds.

We finish with a question concerning an old but still controversially debated substance: Would you use a genotoxic carcinogen for alleviation of a harmless headache? Certainly the majority, including physicians and pharmacologists, would say no. However, reality is different. Although controversial, paracetamol (acetaminophen), one of the world’s most popular over-the-counter drugs may be considered as a genotoxic carcinogen because it causes liver and bladder tumors in

certain rat and mouse strains, covalently binds to DNA, and induces chromosomal aberrations (102). On the other hand, some negative carcinogenicity studies have been published. When carcinogenicity was observed, this occurred only at extremely high cytotoxic doses. The carcinogenicity studies and the experience with patients taking paracetamol taken together do not suggest a problem with carcinogenicity at therapeutic doses. Again, the reason for the lack of carcinogenicity seems to be the existence of a practical threshold concentration for carcinogenicity that is not reached at therapeutic doses. There are some solid biochemical findings inferring mechanistic reasons for the existence of a practical threshold dose of paracetamol, below which it may reasonably be expected that a significant increase in the cancer rate does not occur; paracetamol at therapeutic doses is almost completely conjugated to glucuronide and sulfate with very low amounts of electrophilic quinone imine formed, which is detoxified by conjugation with glutathione. The systems for conjugation to glucuronide and sulfate are overwhelmed, and consequently the formation of quinone imine increases only at higher doses. At much higher doses, glutathione is depleted, and then extensive covalent modification of cellular macromolecules starts. The example of paracetamol underlines the importance of toxicological research that unequivocally identifies genotoxic carcinogens acting through a practical threshold process.

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