

Results of Animal Studies Suggest a Nonlinear Dose-Response Relationship for Benzene Effects

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Considering the very large industrial usage of benzene, studies in risk assessment aimed at the evaluation of carcinogenic risk at low levels of exposure are important. Animal data can offer indications about what could happen in humans and provide more diverse information than epidemiological data with respect to dose-response consideration. We have considered experiments investigating metabolism, short-term genotoxicity tests, DNA adduct formation, and carcinogenicity long-term tests. According to the different experiments, a saturation of benzene metabolism and benzene effects in terms of genotoxicity seems evident above 30 to 100 ppm. Below 30 to 60 ppm the initiating effect of benzene seems to be linear for a large interval of dosages, at least judging from DNA adduct formation. Potential lack of a promoting effect of benzene (below 10 ppm) could generate a sublinear response at nontoxic levels of exposure. This possibility was suggested by epidemiological data in humans and is not confirmed or excluded by our observations with animals.

Introduction

Benzene is one of the chemicals with the largest industrial production in the world. Production of benzene of all grades in 1980 in the U.S. totaled more than 5 million tons (1). Millions of tons of benzene were also produced in Europe, the USSR, and Japan. Because of its presence in many fuels and as a contaminant in many organic chemicals and solvents, it has been estimated that in 1980 about 2 million workers were potentially exposed by benzene in the U.S. alone (1).

As is reasonable to expect, epidemiological data show very small tumor incidences at low levels of exposure (for instance clearly below 30 ppm of average exposure during a working day). Most epidemiological evidence seems to concern levels of exposure roughly in the order of 30 ppm and higher (2). In a very recent paper by Rinsky et al. (3), an attempt was made to investigate the relative risk in humans for levels of exposure lower than 10 ppm

over a 40-year working lifetime. Only three to four cases in excess of the number expected were observed in this low range. In this situation it is difficult to establish, on the basis of epidemiological data alone, a type of dose-response relationship allowing extrapolations of tumor incidences at 10 ppm or 1 ppm (average exposure during a work day).

We were interested in investigating the situation in small rodents. For small rodents, data are available that can allow the construction of dose-response relationships for several types of parameters: production of metabolites, formation of DNA adducts, response to tests of genotoxicity, [chromosomal damage, micronuclei induction, induction of sister chromatid exchanges (SCEs)], and also long-term experiments of carcinogenicity.

Formation of metabolites, formation of DNA adducts, and response to tests of genotoxicity give information mainly about the initiating potential of benzene. When tumor formation in long-term experiments is examined, we see the effects of benzene both on initiation and promotion. Toxicity (for instance, bone marrow toxicity) and compensatory cell regeneration could have a significant promoting effect. Unfortunately, we were unable to find studies that investigated the pure promoting activity of benzene.

In spite of the lack of information about the promoting activity of benzene, the animal data about benzene remain much richer than epidemiological data; in particular, many more observations are possible about dose-

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response relationships. In this paper we have tried to investigate at different levels of exposure animal data about benzene, specifically in the perspective of what these data can suggest in terms of dose-response relationships. As a general trend, the results seem to point in the direction of a saturation of metabolism and probably of the initiating potential of benzene above 30 to 50 ppm. This could confirm some epidemiological data concerning high levels of exposure.

Epidemiological Data

It is difficult to establish a dose-response relationship between level, frequency, and duration of benzene exposure and increased risk of leukemia. Three papers are available, dealing with similar levels of exposure and similar levels of increased risk. In a paper by Ott et al. (4) workers were exposed to ranges between 1 and 30 ppm of benzene (per work day) for approximately 8 to 9 years. This gave a relative risk of myelogenous leukemia of 3.75.

In a paper by Rinsky et al. (3) cases were exposed to an average of 24 ppm of benzene per work day for an average duration of 8.7 years. The standardized mortality ratio for leukemia was 3.37. In a paper by Yin et al. (5) cases were exposed to a median value of 28.4 ppm per work day for an average time that can be roughly estimated around 9 years. The standardized mortality ratio for leukemia was 5.74.

It is difficult to normalize completely data from different papers, and the concordance among the data from the three different papers that we have previously mentioned can already be considered rather good.

In a paper by Vigliani (6), higher levels of exposure were considered. Cases were exposed to 200 to 500 ppm of benzene per work day for an average length of exposure of about 16 years. According to the author, the risk of leukemia was at least 20 times higher than for the general population. Because in this case the level of exposure was at least 10 times the level of exposure reported in the three previous works and the length of exposure was about twice, we could expect a relative risk much higher than 20 (perhaps around 50-90). It is difficult to say if this nonlinear relationship reflects a flattening out of the response above 30 ppm and 8 years of average length of exposure, or if it is simply related to an imperfect compatibility of the data.

What about levels of exposure below 30 ppm per working day? Rinsky et al. (3) have suggested that below the equivalent of 10 ppm over a 40-year working lifetime, the dose-response relationship could be sublinear. The number of cases on which this suggestion is based is too small to reach a clear statistical significance; however, in our opinion, this suggestion is intriguing for the following reason. It has been observed by Yin et al. (5) that leukemia mortality among benzene poisoning cases was 49 times that in benzene workers.

It is quite possible that cell death and compensatory cell regeneration in bone marrow cells could have a promoting effect in benzene leukemogenesis. If benzene is a com-

plete carcinogen because it is endowed with both initiating and promoting activity, the combination of the two effects could generate the type of curve hypothesized by Rinsky et al. (3) in the low range of benzene exposure.

In summary, the dose-response of the leukemia incidence curve for benzene could be a sigmoid curve that flattens out at levels of exposure above 50 to 100 ppm per work day and is perhaps sublinear at levels of exposure below 10 to 30 ppm (Table 1).

Metabolism of Benzene in Rodents

The dose-response relationship for benzene metabolism seems to depend, as is reasonable to expect, on the level of exposure. Jongeneelen et al. (7) have exposed male Wistar rats for 8 hr to inhalation of 0, 7.5, 10, 13, 21, and 33 ppm of benzene. S-Phenyl-N-acetyl-cysteine and phenol were determined in the 24-hr urine of the exposed rats. The dose-response relationship was linear for both metabolites, suggesting that in rats, nonsignificant saturation of benzene metabolism is present for a level of inhalation exposure lower than 30 ppm.

The situation seems to be different for higher levels of exposure. Ulanova and Avilova (8) have shown that the content of phenol in the urine of rats after a single 4-hr inhalation exposure increases much more slowly than the exposure level for levels of exposure above 100 mg/m³ (~30 ppm), suggesting a progressive saturation of metabolism above this concentration. Similarly, Grilli et al. (9) have found, using a closed inhalation chamber, that metabolism of benzene in the chamber followed essentially zero-order kinetics above 50 ppm (at equilibrium between rat and chamber), and first-order kinetics below 50 to 30 ppm. Zero-order kinetics is equivalent to saturation of metabolism.

In a different experiment, rats exposed for 6 hr to benzene concentrations of 125, 250, 625, or 1250 ppm excreted similar amounts of phenol in urine (10). Even in this experiment benzene metabolism was already saturated above 100 ppm.

Sabourin et al. (11) showed an increased amount of unmetabolized benzene in air above 130 ppm in B6C3F₁ mice and F344/N rats after 6-hr inhalation exposure. Total production of metabolites in urine and feces was also evaluated. Saturation of metabolism was more marked in mice than in rats, mainly because absorption was higher in mice than in rats because of increased ventilation. The

Table 1. Summary of quantitative epidemiological data available for human occupational exposure to benzene.

Level of exposure	Relative risk	Dose-response relationship
Around 3 ppm, about 8 years of exposure (3)	1.4	Linear or sublinear
Around 30 ppm, 8-9 years of exposure (3-5)	3-6	Linear
Around 200-500 ppm, about 16 years of exposure (6)	20	Supralinear?

Table 2. Summary of data concerning benzene metabolism in rodents.

Level of exposure	Dose-response relationship	Reference
Up to 30 ppm	Linear	(7)
30 ppm and above	Supralinear	(8)
30-50 ppm and above	Supralinear	(9)
Above 100 ppm	Supralinear	(10)
Above 130 ppm	Supralinear	(11)

same authors (11) reported a saturation of benzene metabolism after administration by gavage in both rats and mice for dosages above 50 mg/kg.

In conclusion, benzene metabolism seems to be saturated in rodents at the highest concentrations. Saturation seems to start most probably at 30 to 50 ppm (Table 2).

Short-Term Tests

Mazzullo et al. (12) have investigated the dose-response relationship for DNA adducts in rats after IP acute administration. Liver, kidney, lung, stomach, and spleen were investigated. The range of dosages explored was between 1.04×10^{-6} and 6.23 mmole/kg. The dose-response relationship in liver was strikingly linear from the lowest dosage up to 0.623 mmole/kg. A flattening out of the response appeared at the highest dosage.

Grilli et al. (9) have examined the response to benzene in 180 different experiments investigating the genotoxicity of benzene. The main categories of these tests of genotoxicity were DNA damage and repair, mutations, and chromosomal anomalies, including SCEs.

While tests of mutagenicity were positive in only 24% of the cases, tests related to chromosomal alterations were positive in 76% of the cases. It has been suggested by Ashby et al. (13) that when large numbers of tests are considered for a given chemical, a 20% of positive results is more typical of a negative compound and 80% of positive results is more typical of a clearly positive compound. Therefore we have assumed that most typically benzene clearly shows its genotoxicity at the chromosomal level. Especially, it is very common to find positive results for the induction of micronuclei, chromosome aberrations, and SCEs in rodents after treatment with benzene.

For several experiments concerning micronuclei, chromosome aberrations, and SCEs, we were able to investigate the dose-response relationship. The results are reported in Table 3. While the experiments considered all seem to suggest a flattening out of the response to benzene between 10 and 100 ppm, both for micronuclei induction, chromosomal anomalies, and SCEs, a paper which, to some extent, disagreed with such findings was recently published (14). In that paper, the induction of chromatid breaks in lymphocytes of mice after subchronic exposure to benzene was investigated. Mice were treated daily by gavage with 0, 36.6, 73.2, and 146.4 mg/kg of benzene for 2 weeks. Judging from the graph shown by the authors, the response was essentially linear. According to EPA computations (15,16), 146.4 mg/kg corresponds roughly to 42 ppm/day. The animals used were Swiss ICR male mice. It is difficult to say if the linear response was specifically related to the strain used or if a flattening out of the response would have appeared above 40 ppm.

If we accept the idea that the majority of the results

Table 3. Dose-response relationship for the clastogenic activity of benzene in rodents.

Species	Route of administration	End point and dose unit	Effect ^a /dosage ratio at different dosage		
			Low	Medium	High
Male Swiss mice	Oral ^b	Micronuclei/1000 PCE, ^c mg/kg	4-2.4/8.8 ^d = 0.18	6-2.4/88 = 0.04	28.7-2.4/880 = 0.03
	Oral ^b	Chromosome aberrations per metaphase, mg/kg	0.06-0.03/8.8 ^d = 0.0034	0.16-0.03/88 = 0.0015	1.1-0.03/880 = 0.0012
Male B6C3F ₁ mice	Oral ^c	Micronuclei/1000 NCE, ^e mg/kg	2.5-1.2/25 ^f = 0.052	5.8-1.2/100 = 0.046	9.3-1.2/600 = 0.013
Male Wistar rats	Inhalation ^h	% Cells with chromosome anomalies, ppm 6 hr	3.03-1.54/10 = 0.15	4.45-1.54/100 = 0.030	7.81-1.54/1000 = 0.0063
Male DBA/2 mice	Inhalation ⁱ	SCE/metaphase, ppm 6 hr	7.6-5.9/10 = 0.17	9.5-5.9/100 = 0.036	13.8-5.9/1000 = 0.0079
	Inhalation ⁱ	Micronuclei/1000 PCE, ppm 6 hr	9-2.1/10 = 0.67	20.3-2.1/100 = 0.18	28.1-2.1/1000 = 0.026
Sprague-Dawley rats	Inhalation ⁱ	SCE/metaphase, ppm 6 hr	10.4-8.6/10 = 0.18	11.1-8.6/30 = 0.083	
Sprague-Dawley rats	Inhalation ⁱ	Micronuclei/1000 PCE, ppm 6 hr	6.2-2.0/10 = 0.42	7.6-2.0/30 = 0.19	

^aTreated values minus control values.

^bTwo doses were administered 24 hr apart and animals were sacrificed 30 hr after the first dose (30).

^cPCE, polychromatic erythrocytes.

^dCorresponding to 3.76 ppm according to EPA computations (15) assuming a respiratory absorption coefficient of 0.5 and a ventilation capacity of 0.03 L/min for the mouse (16).

^eSubchronic treatment within NTP study (25). Micronuclei were measured after exposure for 5 days/week for 4 months (31).

^fNCE, normochromatic erythrocytes.

^gCorresponding to 10.7 ppm according to EPA computations (15), assuming a respiratory absorption coefficient of 0.5 and a ventilation capacity of 0.03 L/min for the mouse (16).

^hExposure of 6 hr (32).

ⁱExposure of 6 hr (33).

at the level of chromosomal damage seem to suggest, in the case of benzene, a flattening out of the response above 30 ppm, we have to show that there are other direct-acting compounds that generate a linear response over a larger interval than the interval of response observed here. This control is needed to rule out the possibility that the responses observed are intrinsically nonlinear in the range under scrutiny even for direct-acting compounds.

Neal and Probst (17) have investigated the induction of SCEs *in vivo* in hamster bone marrow cells. A very good linear response was observed for mitomycin C (MMC) over the whole range of dosages examined and up to 37 SCEs per metaphase. Linear responses were also observed for several other compounds like *N,N',N''*-triethylenethiophosphoramidate, hycanthone, busulfan, and triethylene melamine with up to 31 SCEs per metaphase. Only cyclophosphamide, which is known to require metabolic activation, showed a nonlinear dose response. Carrano et al. (18) have shown *in vitro* in CHO cells a very good linear response for MMC and ethyl methanesulfonate (EMS) up to more than 50 SCEs per cell. Because in our results (Table 3) the number of SCEs per metaphase never exceeded 15, the flattening out of the response cannot be considered intrinsic to SCE behavior, but can be reasonably attributed to the saturation of benzene metabolism.

Kliesch et al. (19) have examined the induction of micronuclei *in vivo* in bone marrow cells after treatment with doubling dosages of methyl methanesulfonate (MMS), MMC, and procarbazine (PC). The response was clearly linear for both MMS and MMC with up to 70 to 80 micronuclei per 1000 polychromatic erythrocytes (PCEs). Only for PC, which required metabolic activation, was a flattening out of the response observed.

Similar results have been reported by Aeschbacher (20). The number of micronuclei per 1000 PCEs was studied in several inbred strains of mice. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), MMC, and MMS were investigated. For all three compounds tested, and essentially in all the strains examined, micronuclei gave a linear response with up to 20 micronuclei for MNNG, up to 100 micronuclei for MMC, and up to 50 micronuclei for MMS.

Similar linear responses were also obtained for the induction of micronuclei *in vitro*. Bonatti et al. (21) examined the induction of micronuclei in V79 cells. Both ethyl nitrosourea (ENU) and EMS induced a linear response up to 100 micronuclei per 1000 cells.

Lasne et al. (22) also examined the induction of micronuclei in V79 cells. MNNG, MMS, and EMS were examined only at low concentrations. The response was perfectly linear for MNNG and EMS and almost linear for MMS. However, only up to 10 to 15 micronuclei per 1000 cells were induced at the low concentrations tested.

If we consider that we observed a flattening out of the induction of micronuclei in response to benzene for number of micronuclei below 10/1000 cells, as reported in Table 3, it appears that this saturation of the response is not intrinsic to the micronuclei indication but rather because of the metabolic activation of benzene. We have to under-

line that to show the intrinsically linear response of SCE and micronucleus induction over a sufficiently large range we did not perform any selection whatsoever of the available literature, but just used the first six suitable papers available in our files.

Carcinogenicity in Animals

Several studies have shown that benzene is a carcinogen in mice and rats (23-27). Different organs are affected but leukemias are not particularly prominent and are not usually of the myeloid type. While the main target organ seems to be different in humans and rodents, oncogenic potency seems to be similar in both species.

Grilli et al. (9) have discussed in detail the notion that the ratio of tumor latency to life-span is as a first approximation similar in humans and rodents. Assuming that this is so, from the data of Maltoni et al. (26) we can make the extrapolation that 100 ppm, 8 hr/day, 5 days a week, for 2 years, will induce an overall tumor incidence of 111/1000 rats over controls.

By applying a similar extrapolation to the data of Snyder et al. (24), we obtain an overall tumor incidence of 67/1000 mice over controls (9). It has been estimated by the IARC Working Group (2) that in humans, 100 ppm of benzene over a 45-year working lifetime would include 140 to 170 cases of leukemia per 1000 exposed workers. The order of magnitude of risk estimations in rodents and humans appears to be very similar, especially considering that carcinogenic potencies can span a range of more than 10^7 times (28,29).

If the quantitative dimension of the phenomenon of tumor induction is similar in humans and rodents, it can be interesting to look at the dose-response relationship of cancer incidence in rodents. This dose-response relationship is analyzed in Table 4. This table seems to suggest that above 25 ppm [approximate transformation in parts per million for rats according to (15,16)] cancer incidence increases less than proportionally to the dosage. Oncogenic potency index (OPI), which is essentially incidence divided by dosage, seems to decrease by about five times when the dosage is increased from about 25 to about 250 ppm.

In conclusion, at least the gavage experiments reported in Table 4, for which several dosages are available, seem to suggest a possible saturation of benzene metabolism for the highest dosages. No dose-response relationships were available for inhalation experiments.

Discussion

We have seen that several data concerning the production and excretion of benzene metabolites in rodents seem to suggest some saturation of metabolism for levels of exposure above 30 to 50 ppm. Except for one paper showing linearity up to the equivalent of 40 ppm, all the papers concerning the genotoxicity of benzene (chromosome anomalies, SCEs, and micronuclei) showed a flattening out of the response starting at levels of exposure between 30 and 100 ppm.

Table 4. Oncogenic potency in rodents for different dosages of benzene.

Dosage, mg/kg <i>pro die</i> , gavage	Equivalent dosage, ppm <i>pro die</i> , 24-hr inhalation ^a		Oncogenic potency index ^b		
	Rats	Mice	F344 rats (25) ^c	B6C3F ₁ mice (25)	S-D rats (26)
25	27	7	1.14	0.88	
50	53	14	0.82	0.56	
100	107	29	0.59	0.53	
200	214	57	0.49		
250	267	71			0.23
500	534	143			0.16

^aAccording to Lee et al. (15) and Gold et al. (16).

^bOncogenic potency index as a function of dosage of benzene in mice and rats = $-\ln(1 - I)/Dt$, where I is the excess incidence over controls (proportion of animals with at least one malignant tumor for all tumors), t is the length of observation (time unit = 2 years), and D is the dosage in millimole per kilogram per day equivalent to the total dose divided by a 2-year exposure (28). Only dosages which produced statistically significant effects ($p < 0.05$) were considered.

^cNumbers in parentheses are references.

In a paper by Mazzullo et al. (12), DNA adduct formation was explored for a very large range of dosages, from 6.23×10^{-6} to 6.23 mmole/kg. Saturation of metabolism started at the equivalent of 60 ppm, and it was evident only in some tissues and macromolecules.

Below 60 ppm the data were remarkable for the linearity of the dose-response relationship. In three different experiments in rats and mice, in which benzene was administered chronically by gavage and long-term carcinogenicity was investigated, a flattening out of the dose-response relationship was apparent at dosages equivalent to 30 to 50 ppm and above.

Three different epidemiological investigations concerning levels of exposure around 30 ppm per work day, for an average length of exposure of about 8 to 9 years, are quite consistent in suggesting a range of relative risk between three and six. In a paper by Vigliani (6), higher levels and durations of exposure are considered and some flattening out of the dose-response relationship seems observable.

In a paper by Rinsky et al. (3), levels of exposure below 10 ppm per working day are also considered, and the few data available suggest an interesting sublinear response. As shown by Yin et al. (5), the risk of leukemia is very much increased when benzene toxicity is present. This observation suggests a promoting effect of benzene that could also explain the sublinear dose-response reported by Rinsky et al. (3) for levels of exposure below 10 ppm. Indeed, below 10 ppm the promoting effect related to benzene toxicity and compensatory cell regeneration of bone marrow cells could presumably be almost negligible.

In summary, from DNA adduct data, we have evidence that the preliminary step of initiation probably displays a linear dose-response relationship even for very low benzene dosages. Above 30 to 50 ppm, metabolism and genotoxicity data suggest a flattening out of the initiation response. This flattening out of the initiation response is also probably detectable in long-term carcinogenicity experiments in rodents and perhaps also in the epidemiological data of Vigliani (6).

Below 10 ppm it could be reasonable to assume a negligible promoting effect of benzene (especially a negligible clonal expansion of initiated clones). This could be in agreement with the recent epidemiological data of

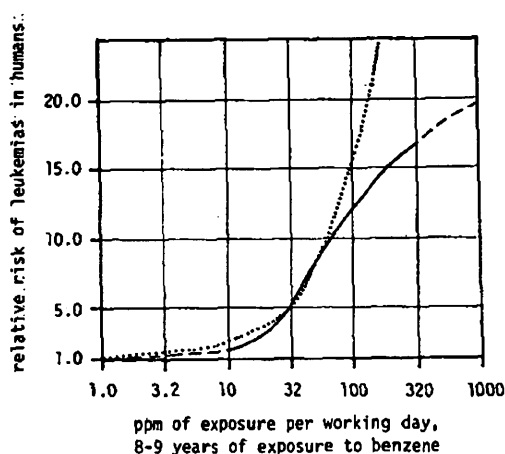


FIGURE 1. Possible dose-response relationship for benzene at different levels of exposure in humans. Solid and dashed line: assumed behaviour of benzene. Dotted line: linear dose-response relationship on semilog paper.

Rinsky et al. (3). A possible final synthesis of the situation is shown in Figure 1. The effect of benzene is represented by a sigmoid curve, sublinear below 10 to 30 ppm and flattened above 50 to 100 ppm.

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