

IN VIVO AND IN VITRO BINDING OF BENZENE TO NUCLEIC ACIDS AND PROTEINS OF VARIOUS RAT AND MOUSE ORGANS

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SUMMARY

Benzene binds to macromolecules of various organs in the rat and mouse in vivo. Labelling of RNA and proteins is higher (1 order of magnitude) than DNA labelling, which is low in many organs (liver, spleen, bone marrow and kidney), and negligible in lung; no difference between labelling of rat and mouse organs was found. The covalent binding index (CBI) value was about 10, i.e. typical of genotoxic carcinogens classified as weak initiators.

In vitro binding of benzene to nucleic acids and proteins is mediated by hepatic microsomes, but not by microsomes from kidney, spleen and lung, or by cytosol from whatever organ. Nucleic acid binding can be induced by pretreatment with phenobarbitone (PB) and suppressed in the presence of SKF 525-A, of cytosol and/or GSH or of heat-inactivated microsomes. Labelling of exogenous DNA is low and is similar in the presence of rat or mouse microsomes in agreement with the low interaction with DNA measured in vivo.

INTRODUCTION

Benzene is extensively produced by the chemical industry (over 15 million tons per year) and widely used as a chemical intermediate for producing pesticides, dyes, plastic resins and commercial gasoline [14]. Nowadays benzene is an environmental pollutant (air, water, food, feed, tobacco, pyrolysis products): the daily inhalation for man in the general urban atmosphere is about 0.6 mg. Its discharge into the atmosphere is regulated in some countries [33]; the permissible level of occupational exposure has been established in most countries as the time-weighted average concentration of 10 ppm [14]. Benzene is myelotoxic and metabolized by microsomes to epoxide and phenol derivatives [18,19,31] including free radical

derivatives [16] which are all able to interact with nucleophilic centers of proteins [16,18,31]. The evidence for benzene genotoxicity in various experimental systems is contradictory. Benzene induces neither mutagenic effects nor unscheduled DNA synthesis but gives rise to chromosomal aberrations and increases the frequency of sister-chromatid-exchanges and of micronuclei. The agent is embryotoxic but is neither embryo-lethal nor teratogenic. Myelotoxicity and chromosomal aberrations have also been demonstrated in man [14].

Benzene induces carcinomas of the Zymbal gland and oral cavity in rats after intragastric administration, and, after inhalation, a very low occurrence of hepatocarcinomas, along with liver hyperplasia and dysplasia [23]. Nevertheless, evidence for carcinogenicity in rodents is limited when the incompleteness of experimental data as regards different species, sexes, routes of administration and the conflicting findings on leukemia induction in mice [14] are considered. On the contrary, there is sufficient evidence for leukemia induction in man.

The covalent binding index (CBI) could be used as a short-term assay of carcinogenicity for suspected initiators or to compare the carcinogenic potency of different chemicals proven to exert an oncogenic effect [20], thus providing information about the mechanism of action (initiation and promotion). Here, we aimed at obtaining such information by measuring *in vivo* and *in vitro* binding of benzene to nucleic acids of various murine organs.

MATERIALS AND METHODS

Radiochemicals and chemicals

[U-¹⁴C] Benzene (99.8 mCi/mmol; radiochemical purity 99.5%; chemical purity 99%) was purchased from The Radiochemical Centre, Amersham, U.K. and its specific activity was made 20 mCi/mmol by adding cold benzene to the tracer.

DNA, polynucleotides, β -nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), and glutathione, reduced form (GSH), were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; SKF 525-A was a gift from Smith, Kline and French, Welwyn, U.K.; PB was obtained from Carlo Erba, Milan, Italy; lumasolve from Lumac, Basel, Switzerland; ready-solve MP from Beckman, Milan, Italy. Other chemicals, all of analytical grade, were obtained from Merck, Darmstadt, F.R.G.

In vivo binding to DNA, RNA and proteins

Four male Wistar rats (~250 g) and 12 male BALB/c mice (25 g) received *i.p.* 127 μ Ci (6.35 μ mol)/kg body wt [¹⁴C]benzene dissolved in 0.5 ml (for rats) and 0.1 ml (for mice) sterile 0.9% NaCl solution containing ethanol which was administered at the dosage of 105 mg/kg body wt. Animals were kept without food and killed 22 h after injection. In order to obtain DNA, RNA and proteins, kidneys, lungs and representative aliquots of liver homo-

genates were pooled and processed according to a phenol method [24]. The same technique was performed on either single rat liver homogenates or on 6 different mouse liver homogenates (each pooling aliquots of homogenates from 2 individual organs) to obtain an estimation of individual variability. Fractions were exhaustively washed with organic solvents until no radioactivity was extracted from macromolecules whose recovery, purity and labelling was measured as described elsewhere [24]. In the case of bone marrow, obtained by the method of Gill et al. [7], and mouse spleen, due to limited tissue availability, only DNA labelling was measured. Cells were lysed with 4.4 ml 0.1 M Tris—0.1 M KCl—1 mM EDTA (pH 8)—1% SDS at 0°C for 15 min and subjected to CsCl sedimentation as reported elsewhere [2]: the same procedure was performed on aliquots of pooled rat spleen to compare DNA labellings obtained with the two methods described. Organs were processed immediately or stored at -30°C for a maximum of 1 month.

In vitro interaction with nucleic acids and proteins

Microsomes and cytosol were obtained according to Ref. 24 from the pooled liver, lungs, kidneys and spleen of animals which received [12]-phenobarbitone (PB) (100 mg/kg body wt per day, i.p., dissolved in sterile 0.9% NaCl solution, 0.5 ml for rat (11 animals, ~300 g) and 0.2 ml for mouse (67 animals, ~25 g), during the 2 days prior to killing in order to induce enzymatic activity [29]; 4 rats and 15 mice received no PB. Enzymatic fractions were stored at -35°C for no more than 1 month. Standard incubation mixture consisted of 2.5 μ Ci [14 C]benzene in 6.25 μ l ethanol, 1.5 mg calf thymus DNA or polynucleotide, 2 mg PB-induced microsomal protein + NADPH (0.8 mM) or 6 mg PB-induced cytosolic protein + GSH (10 mM) to a final volume of 3 ml 0.08 M potassium phosphate—5 mM MgCl₂ buffer (pH 7.7). Incubation was carried out in triplicate at 37°C in air for 60 min under shaking in the dark. Blanks were performed in the absence of enzymes. The influence on microsome-mediated binding extent of various parameters including non-induced enzymes, time-course (5-, 10-, 30-min incubation), concentration of benzene (1.25 μ Ci and 5 μ Ci), addition of 6 mg cytosolic protein and/or GSH (10 mM) to microsomal system, were also tested. In some trials, further blanks (zero time incubation, enzymes but no cofactors, enzymes inactivated with heat at 100°C for 10 min) were also carried out. Reaction was stopped by chilling at -20°C, microsomes (when present) were separated by ultracentrifugation at 105,000 $\times g$ for 1 h at 0°C and proteins were removed from the aqueous layer by phenol extraction. For re-isolation of macromolecules (DNA or polynucleotides, microsomal RNA and proteins, cytosolic proteins) see Ref. 24. The statistical evaluation was performed with Student's *t*-test.

RESULTS

Benzene binds to DNA, RNA and proteins of rat and mouse organs *in vivo*, after administration of a dosage which is nearly 6000-fold lower than the LD₅₀ for rat (Table 1). Labelling of RNA and proteins is higher than

TABLE 1
IN VIVO AND IN VITRO BINDING (pmol/mg) OF BENZENE TO MACROMOLECULES

Organs	In vivo binding			In vitro binding		
	DNA	RNA	Proteins	Calf thymus DNA ^a	Microsomal RNA ^b	Microsomal proteins ^b
<i>Rat</i>						
Liver	0.14 ± 0.05 ^c (7) ^e	2.29 ± 0.27	5.43 ± 0.10	13.06 ± 1.18 ^{b,d,*}	29.55 ± 2.23	226.62 ± 38.57
Kidney	0.56	7.55	3.87	3.90 ± 0.76	3.60 ± 0.42	55.34 ± 6.40
Lung	0.14	1.85	5.41	2.56 ± 0.35	3.06 ± 0.33	55.41 ± 14.51
Spleen	0.65 ± 0.68 ^f	4.59	6.49	3.38 ± 0.69	0.43 ± 0.02	24.57 ± 5.39
Bone marrow	0.70 ^f	N.D. ^g	N.D. ^g	N.D. ^g	N.D. ^g	N.D. ^g
<i>Mouse</i>						
Liver	0.30 ± 0.08 ^c (15) ^e	2.03 ± 0.13	5.76 ± 1.06	16.08 ± 2.16 ^{h,*}	46.65 ± 2.89	591.05 ± 38.17
Kidney	0.27	4.80	4.17	3.96 ± 1.00	5.63 ± 0.40	54.31 ± 5.28
Lung	0.04	0.86	5.32	2.70 ± 0.32	6.37 ± 0.53	71.89 ± 2.97
Spleen	0.63 ^f	N.D. ^g	N.D. ^g	2.14 ± 0.10	1.31 ± 0.09	33.99 ± 8.26
Bone marrow	0.36 ^f	N.D. ^g	N.D. ^g	N.D. ^g	N.D. ^g	N.D. ^g

In vivo data were obtained from pooled organs of 4 male Wistar rats and 12 male BALB/c mice 22 h after i.p. administration by a phenol method. In the case of liver, labelling of each individual organ (rat) and of 6 different pools (each of 2 organs of mice) were also evaluated: such values are shown as mean ± S.E. The binding data collected in vitro are mediated by PB-induced microsomes from the same organs of the 2 species, except for bone marrow, under standard procedure (60-min incubation at 37°C in air, in the dark). Generally, they are reported as mean ± S.E. of 3 values (8 in the case of rat liver, 5 in the case of mouse liver) of total binding: control experiments (blanks) have been systematically performed in the absence of enzymes.

- ^aBlank value of DNA labelling (mean of 6 values $\pm$ S.E.): 2.59 ± 0.20 pmol/mg. Further blanks performed in duplicate with hepatic rat microsomes: in the absence of coenzymes 2.09, 1.69 and 18.29 pmol/mg; in the presence of 1.5 mM SKF 525-A 2.36, 5.74 and 60.05 pmol/mg; with heat-inactivated microsomes (at 100°C for 10 min) 1.55, 1.98 and 58.76 pmol/mg for DNA, RNA and proteins, respectively.
- ^bBinding to DNA and RNA mediated by PB-induced microsomes is strongly inhibited to background values in the presence of cytosol and/or GSH; labellings of microsomal proteins (pmol/mg, mean of 2 values) are the following: 101.51, 71.51 and 18.90 in the presence of cytosol, GSH and cytosol + GSH, respectively.
- ^cLabelling of macromolecules from pooled livers: rat 0.16, 2.43, 6.04 pmol/mg; mouse 0.32, 1.98, 5.36 pmol/mg for DNA, RNA and proteins, respectively.
- ^dWith non-induced microsomes DNA labelling was 8.09 pmol/mg (mean of 2 values).
- ^eCBI values, calculated according to Ref. 20, corresponding to 0.044 and 0.097 μ mol/mol DNA P (according to Ref. 30) for rat and mouse liver, respectively.
- ^fLabelling from CsCl sedimentation.
- ^gN.D. = not determined.
- ^hCorresponding to 714 ± 95.7 dpm/mg (total binding).
- * Statistically significant ($P < 0.01$) differences at Student's *t*-test between total binding and blanks (net labellings, 10.47 and 13.49 pmol/mg DNA for rat and mouse liver, respectively).

DNA labelling and similar in the different organs of both species. DNA labelling is low and comparable in liver, spleen and kidney (1 adduct/ 10^7 nucleotides is formed in mouse liver DNA) and negligible in mouse lung. Moreover, there is agreement either between phenol extraction and CsCl sedimentation in measuring DNA labelling of rat spleen and between labelling of liver DNA from pooled organs and the mean value of single labelling determinations.

Liver microsomes bioactivate benzene to forms capable of interacting with exogenous DNA and microsomal macromolecular constituents. Binding increases linearly with time up to 30 min, then the increase is sublinear up to 60 min, the incubation time chosen as the standard procedure. Binding extent is directly related to the concentration of tracer (data not shown). When using PB-induced microsomes from various organs (the same as those examined *in vivo* except for bone marrow), a statistically significant binding to DNA (total binding-blanks) is mediated by liver microsomes alone: no difference between the activity of rat and mouse enzymes is evidenced. Such binding, as well as that to microsomal RNA and proteins, is practically suppressed by SKF 525-A, a potent inhibitor of hepatic microsomal oxidation [8], by pre-heating of microsomal fractions or when cofactors are omitted. A very low chemical reactivity *per se* of benzene towards macromolecules is, however, observable in blank experiments. Furthermore, PB

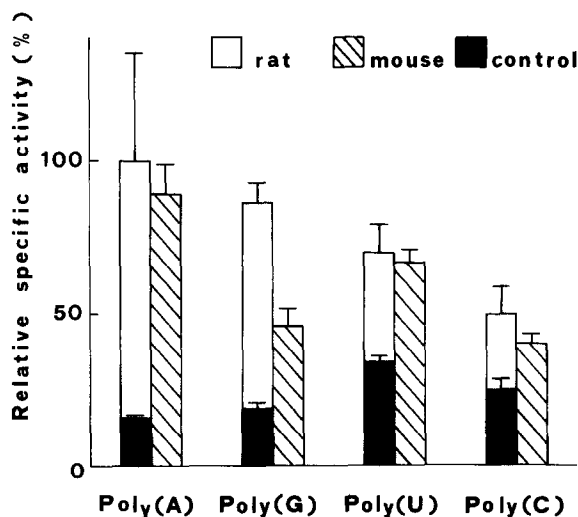


Fig. 1. *In vitro* binding of benzene to synthetic polynucleotides mediated by PB-induced hepatic microsomes of rat and mouse under standard incubation conditions (see Table 1). Data from triplicate experiments are reported as percentage (mean $\pm$ S.E.) of the binding to poly(A) mediated by rat microsomes taken as 100 (5.17 ± 1.84 pmol/mg). ■, blank values obtained in the absence of enzymes. Labelling of rat liver microsomal RNA and proteins, given for example in the case of interaction with poly(A), is: 37.93 ± 7.52 and 464.36 ± 118.91 pmol/mg, respectively.

induced-microsomes are twofold more active than normal microsomes in mediating an interaction between benzene and DNA, whereas the addition of cytosol and/or GSH to the microsomal system strongly inhibits the extent of binding. Cytosol is not capable of mediating a statistically significant interaction with macromolecules whatever the organ tested (data not shown). The pattern of interaction with polyribonucleotides mediated by liver microsomes is shown in Fig. 1. Labelling decreases in the following order: poly(A) > poly(G) > poly(U) > poly(C) and is lower than DNA labelling measured under identical conditions: nevertheless, the opposite situation is observed, regardless of polynucleotide considered, with labelling of microsomal RNA and proteins, an example of which is given in the legend of Fig. 1.

DISCUSSION

The covalent binding index (CBI) of benzene is of the same order as that found with other chemicals defined as weak carcinogens (Table 2) and lower

TABLE 2

BINDING OF SOME HEPATOCARCINOGENS TO RAT LIVER DNA IN TERMS OF CBI

Oncogen	Reference	CBI ^a	Oncogenic potency ^a
Aflatoxin B ₁	[5]	17,000	Strong
Dimethylnitrosamine	[9]	2310	
Aflatoxin M ₁	[20]	1600	
2-Acetylaminofluorene	[10]	560	Moderate
Vinyl chloride	[3]	525	
1,2-Dibromoethane ^b	[1]	515	
o-Aminoazotoluene	[17]	230	
1,2-Dichloroethane	[1]	47	Weak
Carbon tetrachloride	[27]	46 ^c	
Urethan	[26]	34	
Epichlorohydrin ^b	[24]	23	
α-Hexachlorocyclohexane	[28]	<0.1	Cocarcinogen or promoter
Saccharin ^d	[22]	<0.005	

^aClassification of CBI values with respect to oncogenic potency [20]: in the thousands, strong; in the hundreds, moderate; in the tens, weak for initiators; <1 non-genotoxic oncogens (promoters and cocarcinogens).

^bThe hepatocarcinogenic effect of such oncogenic chemicals has not been proven up to now.

^cMouse liver instead of rat liver.

^dNon-hepatocarcinogen capable of inducing bladder tumors (CBI value in bladder DNA < 0.05).

than that found with its halogenated derivatives bromobenzene (CBI = 255 and 225 for rat and mouse liver, respectively) (unpublished data) and chlorobenzene (CBI = 38 and 36 for rat and mouse liver, respectively) (unpublished data). The CBI value reported here for benzene agrees with the previous report of Lutz and Schlatter [21] concerning only liver DNA from rats exposed to the chemical by inhalation; it is higher than that typical of promoters but many times lower than that of such potent carcinogens as aflatoxin B₁ (Table 2). We are aware of another study on benzene interaction with nucleic acids in vivo [6] which is, however, not comparable to the present study since no attempt to separate DNA from RNA in the nucleic acid fraction and purify them from protein contamination was performed. The enzyme-mediated binding of benzene to nucleic acids in vitro is performed by liver microsomes alone: binding extent is low and inferior to that found with chlorobenzene (2-fold) and bromobenzene (4-fold) (unpublished data) under identical experimental conditions. On the other hand, the microsome-mediated binding we found is similar to that previously reported [16,31], measuring a 10-fold lower binding to microsomal proteins alone by incubating a 7-fold inferior concentration of benzene. Purine polynucleotides are better binding acceptors than pyrimidine polynucleotides: labelling is unexpectedly lower than DNA labelling, unlike previous reports for different chemicals [1,24]. Benzene is bioactivated by microsomal mixed function oxidase system; the activation of benzene occurs mainly in liver and, possibly, in bone marrow [15]. The active intermediate formed in liver should be stable enough to exert its effect at a distance as shown by labelling of proteins in various organs. Similar to the metabolic activation of bromobenzene and chlorobenzene [32, unpublished data], the phenolic derivative and *p*-benzoquinone are the benzene intermediates most probably involved in the interaction with DNA which is suppressed by GSH [11]. Phenol is originated either from benzene oxide [18] or from phenolic radical [16] whereas *p*-benzoquinone is generated from phenol [19]. Both metabolites covalently bind to cellular macromolecules: the conjugation of *p*-benzoquinone with GSH is predominantly a non-enzymatic process [19].

On the whole, both in vivo and in vitro interactions give evidence for a weak initiating activity of benzene: this agrees with results from other short-term assays of genotoxicity [14] which are partly contradictory. It should be pointed out that, in order to evaluate the carcinogenicity of chemicals, all short-term data, chemical properties, metabolism and mechanism of action should be considered along with data from long-term assay [13], collected in both sexes of different species, in different experiments by various routes of administration. Moreover, the statistical evaluation we performed with Fisher exact and Cochran-Armitage tests on data from a recent long-term assay [23] showed that the dosage inducing a significant occurrence of carcinomas of Zymbal gland and oral cavity in both female and male rats (500 mg/kg per day) is many fold higher than that administered when dealing with such potent carcinogens as aflatoxins, dimethyl-

nitrosamine, 7,12-dimethylbenz[a]anthracene and 4-nitroquinoline-1-oxide. Therefore, the results of long-term assays performed with benzene [14,23] are not yet satisfactory. Nevertheless, benzene carcinogenicity in humans is sufficiently proven and differences in organ susceptibility between rodents and man seem to exist. In our opinion, what needs to be done in the future is: (a) to test the initiating and/or promoting effects of benzene in liver model systems of Farber [4] and Peraino [25] on female rats which develop liver hyperplasia and dysplasia and present a very low incidence of liver tumors [23]; (b) to collect further information on the adducts with DNA: however, strong limitations in performing such a study are due to the extremely low specific activities of nucleic acids achieved in *in vitro* systems.

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