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Covalent binding of styrene to DNA in rat and mouse

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Covalent binding of [7-^{3}H]styrene (S) to DNA *in vivo* was measured and evaluated in a quantitative manner in order to investigate whether DNA adduct formation could form a mechanistic basis for tumor induction in a carcinogenicity bioassay. [7-^{3}H]S was administered by inhalation in a closed chamber to male and female CD rats and B6C3F1 mice. After 4.5–6 h (rats) and 6–9 h (pools of four mice), S doses of 23–39 and 85–110 mg/kg respectively had been metabolized. DNA was purified to constant specific radioactivity which was measurable in all samples. DNA was enzymatically degraded to the 3′-nucleotides which were separated by HPLC for the detection of radiolabeled nucleotide-S adducts. The fractions with the normal nucleotides contained most of the radioactivity. In mouse liver DNA, a minute but significant level of adduct radioactivity was also detected. In the units of the Covalent Binding Index CBI = (μmol adduct/mol DNA nucleotide)/(mmol chemical/kg body wt), values of 0.05–0.09 and 0.07–0.18 were calculated for males and females respectively. In the rat, no DNA adducts were detectable in the liver at a limit of detection of 0.1 CBI units. Two of the four lung samples of the female rats showed adduct-related radioactivity corresponding to 0.07 CBI units. The CBI values are compatible with styrene 7,8-oxide as the reactive intermediate. The data are compared with CBI values and carcinogenic potencies of established genotoxic carcinogens. It is concluded that the DNA-binding potency of S is so low that significant tumor induction in a standard bioassay for carcinogenicity is unlikely to be due to DNA adduct formation alone. Consequences for a human risk estimation are discussed.

Introduction

Styrene (S*; vinyl benzene; $C_6H_5-CH=CH_2$) is widely used for the manufacture of polymers and reinforced plastics. High-level exposure of humans to S can occur by inhalation at the workplace; low-level exposure is due to smoking and air pollution (1). Exposure to S migrating from packaging into food appears to be negligible (2).

Epidemiological studies investigated the carcinogenic potential of S in humans (reviewed in 3). An insignificantly elevated level of lymphatic and hematopoietic cancer was reported in some of the older studies which were based on mixed chemical exposures, including benzene. The two most recent studies, on the other hand, showed a decrease in these tumor types. Carcinogenicity of S was investigated in nine long-term animal studies. At very high dose levels, S appeared to affect the lung and the liver in the mouse and the mammary gland in the rat, i.e. organs with high spontaneous tumor incidence. However, all these studies had some deficiencies or limitations (e.g. toxicity), so that the International Agency for the Research on Cancer (IARC) considered the evidence of carcinogenicity in animals to be limited (4).

Mutagenicity of S has been reviewed by Barale (5). *In vitro*, S was occasionally found to be mutagenic, exclusively with metabolic activation. In animals, S was very weakly genotoxic (6). In humans, positive and negative cytogenetic studies were reported (5). Detectable effects appeared to be limited to workplace exposure levels >50 p.p.m. (7).

Covalent binding of S to DNA was investigated in the mouse after i.p. injection (8). The DNA-binding potency expressed in the units of the Covalent Binding Index (CBI) for liver DNA was between 2 and 5; this is in the potency range of weakly genotoxic carcinogens (9). It was therefore considered important to reinvestigate the DNA-binding potency of S.

In this study, covalent binding of tritiated S to DNA was investigated in rat and mouse by inhalation, and the data were analyzed in terms of a DNA-binding potency. The results were compared with other carcinogens (9) in order to answer the question whether DNA adduct formation could form the mechanistic basis for a putative tumor induction in a carcinogenicity bioassay.

Materials and methods

Chemicals

Twice 20 mCi [7-^{3}H]S (mol. wt 104.15), containing 10–15 p.p.m. 4-tert-butylcatechol as stabilizer, were purchased in 2 mCi ampoules from The Radiochemical Centre, Amersham, UK. The two batches had specific activities of 21 mCi/mmol (0.2 mCi/mg) and 20 mCi/mmol (0.19 mCi/mg) and were used for the experiments with the rats and mice respectively. The ampoules were stored at −20°C in the dark and used within 1 month after delivery. The radiochemical purity determined by the supplier immediately prior to shipment was indicated to be 92.4 and 89.5% respectively. In order to check whether the [7-^{3}H]S was in monomeric form when used, the specific activity was checked during the inhalation exposures by radio-GC, on a 20 m×0.3 mm column filled with PS 089 (95% dimethyl/5% diphenylpolysiloxan from Hüls America, Inc.) and run with hydrogen at 50°C. The specific activity indicated by the supplier could be confirmed.

Animals

Six male and six female CrI:CD® BR rats and 24 male and 24 female B6C3F1/CrlBR mice from Charles River Wiga, Sulzfeld, Germany were used. They were acclimatized in Macrolon cages for at least 1 week after delivery and were fed *ad libitum* with tap water and maintenance diet NAFAG 890, purchased from Nafag AG, Gossau SG, Switzerland.

Inhalation exposure

Single rats or groups of four mice (weights given in the tables) were exposed to tritiated S by inhalation in a closed chamber (10). The chamber consists of a 2 l desiccator with an inlet for oxygen and a septum for air sampling. Expired carbon dioxide is adsorbed on soda lime placed into the chamber. The resulting atmospheric pressure reduction is compensated by an influx of oxygen with which tritiated S is carried along.

The ampoule containing 2 mCi tritiated S (10 mg for rats, 10.4 mg for the pools of four mice) was centrifuged at 2000 *g* for 5 min, cooled at the bottom with liquid nitrogen, cut open and immediately put into the oxygen flow system of the inhalation apparatus. Air samples taken from the chamber were analysed by GC for the time course of the S concentration. The exposure was terminated

*Abbreviations: S, styrene; CBI, covalent binding index = DNA adduct level/dose = (μmol adduct/mol DNA nucleotide)/(mmol chemical/kg body wt); SO, styrene 7,8-oxide.

after at least four metabolism half-lives. The animals were killed with ether; liver and lung of rats and liver of mice were excised, pooled within the inhalation groups (mice) and kept frozen at −20°C. The mouse lung was not investigated because the yield of DNA would not have resulted in a satisfactory detection limit.

Isolation of DNA

DNA was isolated via chromatin precipitation according to Sagelsdorff *et al.* (11). Repurification to constant specific activity was carried out as previously reported (12).

Control experiments were performed using DNA isolated from untreated animals. (i) Radioactivity controls were compared with historical values to show that the work-up of the DNA samples had been performed without external contamination with radiolabel. (ii) An *in vitro* incubation of a chromatin supernatant from a treated animal with a chromatin pellet from a control (13) was done to investigate whether radioactive chemicals present in the tissue when the animals were killed could irreversibly associate with the DNA during work-up.

HPLC analysis of DNA nucleotides

DNA was digested to constituent nucleotides according to Sagelsdorff *et al.* (13). The resulting deoxynucleotides were separated by HPLC on an Eurosphere RP18 5μ column (250×4 mm) with 2 ml/min 30 mM ammonium formate buffer, pH 3.8/2−60% methanol gradient system according to Cantoreggi and Lutz (12). The fractions were dried down on a rotatory evaporator and dissolved in 1 ml water.

Scintillation counting

DNA samples and nucleotide fractions were counted in 10 ml Ultima Gold® (Packard) in plastic vials for 100 min in a liquid scintillation counter LS 6000 LL (Beckman, Fullerton CA) equipped with the LowLevel® option (rat liver DNA and nucleotide fractions). The significance level at two standard deviations, given by the Poisson distribution of the radioactive decay plus vial-to-vial differences, had been determined to be 1.1 c.p.m. in an optimized tritium channel. The counting efficiency was between 47 and 52%.

The net radioactivity was calculated by subtracting the radioactivity of an equivalent sample isolated from animals treated with unlabeled S, both for DNA samples and for fractions of the nucleotide analyses.

Calculations

The concentration of a DNA solution was calculated from the optical density of a diluted aliquot at 260 nm using an absorbance of 20 for 1 mg DNA/ml. Depending on the amount of DNA available, an aliquot of 0.25−1 mg DNA was diluted with calcium succinate buffer to a standardized concentration (usually the concentration of the most dilute sample of the ongoing experiment).

The concentration of the nucleotide solutions was calculated from the optical density of a diluted aliquot at 260 nm assuming an absorbance of 35 for a nucleotide mixture equivalent to 1 mg digested DNA/ml.

The specific DNA radioactivity was expressed as DNA adduct level per dose administered and converted to the units of the CBI (14), CBI = (μmol adduct/mol DNA nucleotide)/(mmol chemical/kg body wt). Doses are given in the tables.

Results

Kinetics of S in the inhalation chamber

The concentration−time course of S in the chamber was characterized by three phases. The first phase was dominated by an influx of S into the chamber with maximum concentrations of 274−300 and 385−464 p.p.m. registered after 1−2 h in the experiments with rats and pools of mice respectively. Equilibrium

Table I. Investigation of the covalent binding of [7-^{3}H]styrene to DNA isolated from liver and lung of male CD rats after inhalation exposure

Rat no./weight (g)	1/452	2/440	3/347	4/437	5/411	6/353
Dose (mg/kg)	20.1	20.7	28.8	22.9	24.3	28.3
(10^{10} d.p.m./kg)	*in vitro* incubation	control	1.27	1.0	1.07	1.25
Liver DNA						
First purification (radioactivity in 1 mg DNA)						
Total counts (c.p.m.)	21.1	6.8	135.6	251.8	127.2	62.3
Specific activity (d.p.m./mg)	27.5	–	219	442	204	79
Second purification						
Specific activity (d.p.m./mg)		–	128	43	22	47
Third purification (radioactivity in 0.5 mg DNA)						
Total counts (c.p.m.)	5.7	5.5	35.9	12.7	10.7	17.4
Specific activity						
(d.p.m./mg)		–	129	30	22	50
(CBI units)		–	3.1	0.9	0.6	1.2
Nucleotide analysis						
Radioactivity eluting without optical density (%)			<3%	<13%	<19%	<8%
Covalent DNA binding (CBI units)	–	–	<0.1	<0.2	<0.2	<0.1
Lung DNA						
First purification (radioactivity in 0.2 mg DNA)						
Total counts (c.p.m.)	9.5	9.6	16.6	23.1	14.5	12.6
Specific activity (d.p.m./mg)		–	64	135	49	30
Second purification (radioactivity in 0.15−0.2 mg DNA)						
Total counts (c.p.m.)	9.7	9.8	15.2	19.1	13.1	13.6
Specific activity						
(d.p.m./mg)		–	74	123	34	39
(CBI units)		–	1.8	3.8	1.0	1.0
Nucleotide analysis						
Radioactivity eluting without optical density (%)			ND	<5%	ND	<19%
Covalent DNA binding (CBI units)	–	–	–	<0.3	–	<0.1

ND, not done (no DNA left).

for S between the atmosphere and the animal was reached after another 0.5–1 h. Thereafter, the concentration of S in the chamber decreased as a result of metabolism. The experiment was terminated when the concentration of S in the chamber atmosphere had dropped to between 18 and <5 p.p.m. (42 p.p.m. for the female mouse pool no. 3). This was after 4.5–6 h with the rats and 6.25–9 h with the mice. Taking into account the metabolism half-life deduced from the third phase, >95%

Table II. Investigation of the covalent binding of [7-^{3}H]styrene to DNA isolated from liver and lung of female CD rats after inhalation exposure

Rat no./weight (g)	1/282	2/281	3/284	4/267	5/280	6/259
Dose (mg/kg)	32.3	32.4	35.2	37.4	35.7	38.6
(10^{10} d.p.m./kg)	*in vitro* incubation	control	1.55	1.65	1.57	1.70
Liver DNA						
First purification (radioactivity in 1 mg DNA)						
Total counts (c.p.m.)	10.5	6.7	219	1021	230	116
Specific activity (d.p.m./mg)		–	401	1943	422	203
Second purification						
Specific activity (d.p.m./mg)		–	53	121	61	59
Third purification (radioactivity in 0.5 mg DNA)						
Total counts (c.p.m.)	5.9	5.7	14.8	18.4	27.3	17.2
Specific activity (d.p.m./mg)		–	38	54	91	48
(CBI units)		–	0.8	1.0	1.8	0.9
Nucleotide analysis						
Radioactivity eluting without optical density (%)			<11%	<8%	<8%	<7%
Covalent DNA binding (CBI units)	–	–	<0.1	<0.1	<0.2	<0.1
Lung DNA						
First purification (radioactivity in 0.2 mg DNA)						
Total counts (c.p.m.)	10.3	10.6	18.8	23.0	20.9	36.0
Specific activity (d.p.m./mg)		–	84	124	104	251
Second purification (radioactivity in 0.2 mg DNA)						
Total counts (c.p.m.)	9.5	10.0	18.3	23.3	19.5	37.1
Specific activity (d.p.m./mg)		–	85	131	96	272
(CBI units)		–	1.7	2.5	1.9	4.9
Nucleotide analysis						
Radioactivity eluting without optical density (%)			3%	3%	<4%	<5%
Specific activity (d.p.m./mg)			3.8	3.6	<4.3	<12.9
Covalent DNA binding (CBI units)	–	–	0.07	0.07	<0.08	<0.23

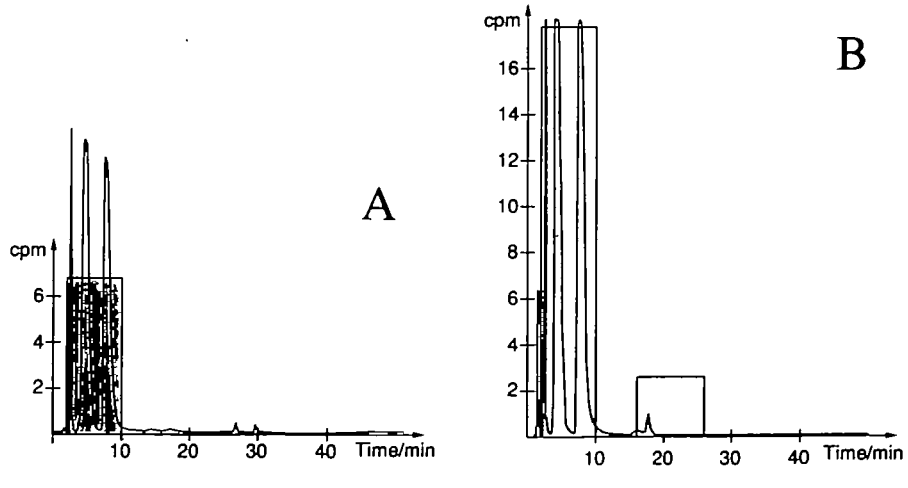

Fig. 1. Reverse-phase HPLC profiles of optical density (solid line) and tritium radioactivity (bars; c.p.m.) of deoxyribonucleotides obtained by enzymatic hydrolysis of DNA. (A) From the liver of a male Crl:CD® BR rat (no. 4), 6 h after inhalation of 1.0×10^{10} d.p.m./kg (22.9 mg/kg) [7-^{3}H]styrene. (B) From the pooled livers of four male B6C3F1 mice (pool no. 4), 8 h after inhalation of 3.9×10^{10} d.p.m./kg (92.4 mg/kg) [7-^{3}H]styrene.

of the amount of S put into the system had been metabolized within the exposure period.

DNA binding of [^{3}H]S in the rat

DNA isolated from the liver and the lung of the rats was analyzed for radioactivity and nucleotide-S adducts. The results are summarized in Tables I and II, for males and females respectively.

In the liver, two rounds of DNA repurification were required to remove all reversibly bound radioactivity and obtain constant specific activities. All DNA samples were radiolabeled at levels of 22–129 d.p.m./mg.

Radioactivity irreversibly associated with the DNA is not necessarily due to nucleotide–carcinogen adduct formation, but can be the result of biosynthetic incorporation of radiolabel via DNA biosynthesis. Tritiated water is formed from the 7-^{3}H label of S during metabolism from mandelic acid to phenylglyoxylic acid. Tritium from water can then be incorporated into the 2′-position of the deoxyribose in the ribonucleotide reductase step.

In order to distinguish adduct formation from radiolabel incorporation, nucleotides were analyzed for radioactivity. HPLC profiles with rat liver DNA showed that all radioactivity eluted with normal nucleotides (Figure 1A). At elution times known for S-nucleotide adducts (12), no radioactivity was measured at a detection limit of 1.1 c.p.m. Conversion of this detection limit to the units of the CBI showed a maximum possible DNA-binding potency of CBI <0.1 for both males (Table I) and females (Table II).

In the rat lung, only one repurification round was necessary to show constant specific DNA radioactivity for all samples. Not all samples could be subjected to nucleotide analysis because of low DNA yields. For the males (Table I), all detectable DNA radioactivity eluted with the natural nucleotides. The limit of

Table III. Investigation of the covalent binding of [7-^{3}H]styrene to DNA isolated from the liver of male B6C3F1 mice after inhalation exposure

Mice no./weight (g)	1/117	2/120	3/121	4/112	5/118	6/123
Dose (mg/kg)	77.6	76.0	86.0	92.4	87.8	84.7
(10^{10} d.p.m./kg)	*in vitro* incubation	control	3.6	3.9	3.7	3.6
First purification (radioactivity in 0.35–0.87 mg DNA)						
Total counts (c.p.m.)	10.3	9.6	59.6	82.6	30.1	50.7
Specific activity (d.p.m./mg)		–	115	177	116	123
Second purification (radioactivity in 0.5 mg DNA)						
Total counts (c.p.m.)	10.0	9.6	22.7	30.3	32.5	31.5
Specific activity (d.p.m./mg)		–	51	80	89	85
(CBI units)		–	0.4	0.6	0.7	0.7
Nucleotide analysis						
Radioactivity eluting with optical density (c.p.m.)			8.6	17.8	18.6	20.1
Radioactivity eluting without optical density (c.p.m.)			2.1	2.6	2.2	1.2
Specific activity (d.p.m./mg)			9.6	11.6	9.8	5.4
Covalent DNA binding (CBI units)	–	–	0.08	0.09	0.08	0.05

Table IV. Investigation of the covalent binding of [7-^{3}H]styrene to DNA isolated from the liver of female B6C3F1 mice after inhalation exposure

Mice no./weight (g)	1/88	2/94	3/95	4/95	5/102	6/97
Dose (mg/kg)	103.3	96.9	109.9	109.0	102.1	107.7
(10^{10} d.p.m./kg)	*in vitro* incubation	control	4.6	4.6	4.3	4.5
First purification (radioactivity in 0.40–0.58 mg DNA)						
Total counts (c.p.m.)	9.3	9.0	68.0	84.3	29.7	75.3
Specific activity (d.p.m./mg)		–	295	377	101	332
Second purification (radioactivity in 0.5 mg DNA)						
Total counts (c.p.m.)	9.6	9.4	60.6	49.4	36.8	49.6
Specific activity (d.p.m./mg)		–	206	156	107	157
(CBI units)		–	1.4	1.0	0.8	1.1
Nucleotide analysis						
Radioactivity eluting with optical density (c.p.m.)			22.9	33.3	23.4	40.8
Radioactivity eluting without optical density (c.p.m.)			2.5	4.0	2.1	3.0
Specific activity (d.p.m./mg)			26.6	18.1	9.6	13.6
Covalent DNA binding (CBI units)	–	–	0.18	0.12	0.07	0.09

detection of adducts was at CBI $<0.1-<0.3$. With the females (Table II), the yield of DNA was higher. In two samples, the radioactivity measured in the fractions known to contain adducts (12) was just detectable (CBI = 0.07).

DNA-binding of [^{3}H]S in the mouse

DNA was isolated from the pools of four livers. After the first repurification, one sample already showed constant specific activity and the remaining had only slightly been reduced (Tables III and IV). A second repurification was not necessary. HPLC analysis of the nucleotides revealed, in all eight samples, a small but significant amount of radioactivity in fractions eluting after the natural nucleotides (Figure 1B). The elution time was similar to the early-eluting S-nucleotide adduct(s) generated from DNA plus SO *in vitro* (12). Expressed in the units of the CBI, the DNA-binding potency was at the extremely low level of $0.05-0.09$ and $0.07-0.18$ for males and females respectively. The average was 0.10 ± 0.04 CBI units.

Discussion

S was shown to have a very low DNA-binding potency *in vivo* after inhalation exposure of rodents. Using high levels of radiolabeled S, the low limit of detection of DNA adducts allowed to establish a DNA-binding potency of CBI ~ 0.1 for mouse liver DNA. This very low genotoxic potency is in good quantitative agreement with the borderline mutagenicity of S *in vivo*.

What is the reactive intermediate? Metabolism of S involves, to almost 100%, the intermediate formation of the 7,8-epoxide (SO). The question is whether this epoxide could be responsible for the DNA adduct formation seen with S. SO is a relatively inert epoxide (15). SO is also weakly mutagenic in a number of test systems without metabolic activation (5). DNA binding by SO was not detectable *in vivo* but the limit of detection was not as good as in the present experiments with S: a CBI <0.6 was found for mouse liver after i.p. injection (12). The CBI = 0.1 measured here for S is therefore not in contradiction to the negative findings with SO. The data are consistent with the idea that SO was responsible for the DNA adduct formation seen here with S.

A minor pathway in S metabolism ($<1\%$) results in the formation of 4-vinylphenol (16,17), probably via the styrene 3,4-epoxide. The low CBI value of 0.1 does not allow for the possibility that this or another metabolic pathway could result in high levels of DNA adducts.

The present CBI values for S in mouse liver DNA are $20-50$ times lower than the values that could be derived from another report (8). A similar discrepancy was noted in the experiments with SO: a CBI value of 2.2 was deduced from Byfält Nordqvist *et al.* (8), while our values were below a limit of detection of CBI <0.6 under identical experimental conditions (12). Byfält Nordqvist *et al.* apparently did not purify the DNA to constant specific activity. It cannot be excluded, therefore, that their DNA had not completely been freed from non-covalently bound radiolabeled S metabolites.

Chemical stability of DNA adducts is a prerequisite for a correct interpretation of DNA-binding experiments. Evidence that this requirement is met comes from Vodicka and Hemminki (18) who showed a half-life of 10 days for SO–DNA adducts at pH 4.2 and room temperature in double-stranded DNA. Under our experimental conditions for the DNA purification (neutral pH, temperature mostly 4°C), the half-life could be even longer. Furthermore, in the present experiments, DNA could be repurified to constant specific activity. This would not be possible with labile adducts. It can therefore be assumed that the adduct concentrations measured after DNA purification reflected the situation at the time the animals were killed.

No significant difference was seen between mice and rats or between sexes. In the rat liver, the limit of detection was at CBI <0.1 in both males and females (Tables I and II), while the highest value seen in the mouse liver was at CBI = 0.09 (male, Table III) and CBI = 0.18 (female, Table IV). Therefore, DNA-binding potency data would not help explain species or sex differences tentatively seen in tumor induction (4).

It has been shown that potencies of genotoxic carcinogens correlate quite well with CBI values for liver DNA [$r = 0.81$ for 29 activation-dependent mutagenic carcinogens (9)]. The range of CBI values spanned from 2 to 10 000, carcinogenic potencies, expressed as TD_{50} values, from 8 to 0.000001 mmol/kg/day. Vinyl chloride, to mention one of the data points, had a CBI = 8 for mouse liver DNA (19) and a TD_{50} value for hemangiosarcomas in the liver of 1 mmol/kg/day (20).

Using the above correlation of CBI versus TD_{50}, the DNA binding potency of S as determined here (CBI = 0.1) extrapolates to a TD_{50} value of ~1000 mmol S/kg/day. This is equivalent to a dose of ~100 g S/kg/day, required theoretically to induce a 50% tumor incidence in a standard lifespan if DNA adduct formation is the mechanism of carcinogenic action. This correlation could very tentatively be used to estimate a cancer risk in humans, under the assumptions of a linear dose–response relationship, the absence of species differences, and proportionality with duration of exposure. Exposure of a worker for 15 years to a daily dose of 10 mg/kg (resulting from an 8 h exposure at 20 p.p.m. for instance) would result in a theoretical cancer risk of 1 in 10^5 lives (1 in 2 at 100 g/kg/day for life; 1 in 20 000 at 10 mg/kg for life; 1 in 100 000 if exposed only for one-fifth of the standard human lifespan).

In conclusion, the level of DNA adduct formation by S is highly unlikely to become responsible for a significant increase in tumor incidence in a bioassay. Changes observed in the spontaneous tumor pattern in standard bioassays with S are more likely due to unspecific effects at maximum tolerated dose levels.

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