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METABOLISM *IN VITRO* OF TRIS(2,3-DIBROMOPROPYL)-PHOSPHATE: OXIDATIVE DEBROMINATION AND BIS(2,3-DIBROMOPROPYL)PHOSPHATE FORMATION AS CORRELATES OF MUTAGENICITY AND COVALENT PROTEIN BINDING

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Abstract—Tris(2,3-dibromopropyl)phosphate (Tris-BP) was found to be metabolized by liver microsomes obtained from untreated and phenobarbital-pretreated rats. Metabolites of Tris-BP, whose formation was dependent on NADPH and oxygen, included bromide ion and bis(2,3-dibromopropyl)-phosphate (Bis-BP). The rates of formation of these metabolites were markedly increased in liver microsomes isolated from phenobarbital-pretreated rats compared to microsomes from untreated rats. In the presence of either SKF 525-A or metyrapone, the formation rates of bromide ion and Bis-BP were decreased, whereas α-naphthoflavone had no effect. The effects of the various treatments on bromide release and Bis-BP formation paralleled those that have been previously observed with respect to the activation of Tris-BP to mutagenic and covalently protein bound metabolites. Furthermore, rates of oxidative debromination of several Tris-BP analogs directly correlated with their respective mutagenicities. Addition of glutathione (GSH) to microsomal incubations of Tris-BP increased bromide release substantially over control, values but had no effect on Bis-BP formation. On the other hand, the addition of GSH to microsomes decreased covalent binding and mutagenicity of Tris-BP with increased formation of water soluble metabolites. GC/MS analysis of ethyl acetate extracts from incubations of rat liver microsomes with Tris-BP identified 2-bromoacrolein (2-BA) as a metabolite. Introducing deuterium at the carbon atom number 1 of the propyl moiety of Tris-BP had no effect on either bromide release or mutagenicity, whereas the analog labelled at carbon atom 3 showed significant isotope effects on both activities. In contrast, deuterium substitution at carbon atom 2 gave a significant isotope effect on bromide release, but not on mutagenicity. The data indicate that Tris-BP can be metabolized by rat liver microsomes to Bis-BP and 2-bromoacrolein catalyzed by cytochrome P-450 in a process liberating bromide ions. Further, the results are consistent with oxidation at the terminal carbon atom of Tris-BP thereby forming 2-bromoacrolein, which is postulated to be the metabolite mainly responsible for Tris-BP mutagenicity.

The flame retardant tris(2,3-dibromopropyl)phosphate (Tris-BP)‡ has been shown to be mutagenic [1, 2] and carcinogenic [3, 4] as well as to cause acute renal tubular necrosis [5]. Microsomal, NADPH-dependent oxidative metabolism is necessary for converting Tris-BP to mutagenic [6] and covalently protein bound [7] intermediates. Studies by Lynn *et al.* [8] have shown that Bis-BP is an urinary metabolite of Tris-BP. Whereas Bis-BP is inheritantly less mutagenic than Tris-BP [9], Bis-BP is at least as potent as Tris-BP in causing nephrotoxicity [10, 11].

In order to gain insight into the pathways for metabolic activation of Tris-BP, *in vitro* conditions for bromide release and Bis-BP formation were studied.

‡ Abbreviations used: Tris-BP, tris(2,3-dibromopropyl)phosphate; Bis-BP, bis(2,3-dibromopropyl)phosphate; 2-BA, 2-bromoacrolein; PB, phenobarbital; GSH, glutathione; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Chemicals

Synthesis of ^{3}H-Tris-BP, tris(3-bromopropyl)-phosphate and tris(2-bromopropyl)phosphate: ^{3}H-Tris-BP was prepared by acylation of 1-^{3}H-2,3-dibromopropanol as previously described [7], and tris(2-bromopropyl)phosphate and tris(3-bromopropyl)-phosphate from the corresponding monobrominated propanols as described [6]. 2-BA was prepared by dehydrobromination of 2,3-dibromopropanal with triethylamine. Sodium hypochlorite was prepared by the method of Wright *et al.* [12]. Bis-BP and 2,3-dibromopropylphosphate were prepared by reacting either 2 or 1 equivalents, respectively, of 2,3-dibromopropanol with phosphorus oxychloride in pyridine at room temperature. Dropwise addition of the alcohol over a 30 min period, to phosphorus oxychloride in pyridine largely prevented the formation of mixtures of Tris-BP, Bis-BP, and 2,3-dibromopropyl-phosphate. After reactions were allowed to stir

an additional hour at room temperature pyridine was removed by partitioning the reaction mixture between chloroform and 10% sulfuric acid. The alkylated phosphates were then extracted into 5% sodium bicarbonate solution and finally back-extracted into chloroform after acidification with sulfuric acid. After drying over anhydrous sodium sulfate and removal of chloroform, Bis-BP and 2,3-dibromopropylphosphate were found to be approximately 95% pure by TLC as determined by using tracer amounts of ^{3}H-2,3-dibromopropanol in reaction mixtures. TLC was performed on 5 cm × 20 cm 250 μm silica gel plates (Analtech, Newark, DE) using two solvent systems, hexane: diethyl ether (1:4 v/v) and ethyl acetate: ethanol:water (10:2:1 by vol.); R_F of Bis-BP was 0.31 and 0.70 respectively in the two systems; R_F of 2,3-dibromopropylphosphate was 0.08 and 0.38. ^{1}H-NMR were recorded on a Varian EM360A instrument at 60 MHz using TMS as the lock signal: (ppm) 3.79 (skewed doublet of C-3 methylene hydrogens), 4.35 (skewed doublet of C-1 methylene hydrogens plus multiplet of C-2 hydrogen), 10.42 (broad singlet of O—H-proton). The specifically deuterated 2,3-dibromopropanols used for preparation of the deuterium labelled Tris-BP analogs were obtained by bromination of specifically deuterated allyl alcohols synthesized by published methods [13, 14]. Mass spectra of Bis-BP and 2,3-dibromopropylphosphate were recorded on a VG MicroMass 7070H instrument using a VG-2000 data system. Samples were analyzed by direct probe insertion using chemical ionization with methane as the reagent gas. Spectra were recorded at a nominal resolution of 1000 (10% valley) using an accelerating voltage of 4 kV, an electron energy of 100 eV and an emission current of 500 μA. Greater than 90% of the total ion current was carried by the MH^+ ion cluster centered at m/z 499 for Bis-BP and m/z 299 for 2,3-dibromopropylphosphate. Other chemicals were obtained from the following sources: Tris-BP, 2,3-dibromopropanol, α-naphthoflavone from Aldrich Europe (Beerse, Belgium); NADP, glucose 6-phosphate, yeast glucose 6-phosphate dehydrogenase, GSH, sodium tungstate, rosaniline-HCl from Sigma Chemical Company (St. Louis, MO, U.S.A.); ammonium molybdate from May and Baker Ltd. (Dagenham, U.K.); DMSO, cobaltous chloride from Merck-Schuchardt (Darmstadt, F.R.G.); PB from The Norwegian Medicinal Depot (Oslo, Norway); SKF 525-A from Smith, Kline & French Laboratories (Philadelphia. PA, U.S.A.); metyrapone from Ciba-Geigy AG (Basle, Switzerland); Sephadex G-10 from Pharmacia Fine Chemicals (Uppsala, Sweden).

Treatment of animals

Male Wistar rats (200–250 g, Bomholtgård Breeding and Research Centre, Ejby, Denmark) were pretreated with either PB (75 mg/kg in saline i.p. 72 hr, 48 hr and 24 hr before death), or cobaltous chloride (60 mg/kg in saline s.c. 48 hr and 24 hr before death). Controls received vehicle alone. All animals were allowed a pelleted feed (Norwegian Standard No. 3155, Møllecentralen, Oslo, Norway) and water *ad libitum*, and kept in plastic cages on hardwood bedding.

Preparation of liver subfractions

If not otherwise stated, pooled, washed hepatic microsomes were prepared from 3 to 4 rats as described [15]. The microsomal pellet was resuspended in sterile 30% glycerol–1.15% KCl containing 20 mM Tris-buffer, pH 7.4 and was kept at −70° until use. In the bromide release experiments, due to interference of chloride ions, GSH as well as glycerol with the measurement of bromide ion concentrations, microsomes were prepared as described above, except the Tris–KCl buffer was substituted with 0.1 M Na-phosphate buffer pH 7.4 (bromide sensitive electrode) or microsomes were used freshly prepared without glycerol (rosaniline method). Protein concentrations were determined according to Lowry *et al.* [16] using bovine serum albumin as standard.

Incubation assays

Mutagenicity assay. Mutagenic activity was assayed in a quantitative modification of the *Salmonella* mutagenicity test of Ames *et al.* [17] using the TA 100 tester strain, 2 mg/ml microsomal protein, NADPH-cofactors and test substance in DMSO (1% final cons.) as previously described [6].

Bromide release, Bis-BP formation and covalent protein binding assays. Bromide release was determined either using a bromide sensitive electrode or a rosaniline method. Microsomal incubations containing (3 ml, final concentrations) 0.5 mg/ml protein, 0.5 mM ^{3}H-Tris-BP (1,500 dpm/nmol) and a NADPH-generating system [16] in 0.1 M Na-phosphate buffer, pH 7.4 were carried out at 37° for 10 min. In time-course and protein concentration studies incubation times up to 30 min and 0.5–4.0 mg/ml protein respectively were used. In some experiments GSH was added to the incubations. Each incubation was split into two equal aliquots. One fraction was used for the determination of the bromide release whereas the other fraction determined Bis-BP formation as well as water soluble metabolites. The protein from the combined fractions was used for determination of the amount of ^{3}H-Tris-BP covalently bound to microsomal protein.

Analytical procedures

Bromide release. (A) Bromide determination with the bromide ion sensitive electrode: Microsomal incubations were stopped on ice, protein precipitated by addition of 30% TCA and the incubations were centrifuged at 3200 rpm for 5 min in a Sorvall GLC-3 centrifuge. The precipitated protein was used for the covalent protein binding assay. To 1.0 ml supernatant was added 0.5 ml of 3 M KNO_3 and after vortexing 1.0 ml was used for the bromide ion determination using a HNU bromide ion sensitive and a double junction reference electrode (HNU Systems Inc., Newton, MA, U.S.A.). Addition of 100 μl of ascorbate (100 mg/ml) to 1.0 ml supernatant eliminated any interference due to GSH when determining bromide release. Bromide concentrations were calculated from a bromide ion standard curve (10^{-6}–10^{-2} M Br ion concentration with 0.5 mg/ml microsomal protein) and corrected for non-specific bromide ion release. The measurements were con-

sidered stable when the meter fluctuations were less than 1 mV/min. (B) Bromide determination with the rosaniline method: Microsomal incubations were stopped on ice and to 0.5 ml was added 9.5 ml of a protein precipitant solution [19]. Bromide ion was measured spectrophotometrically at 570 nm and calculated from a standard curve according to Goodwin [19]. Values were corrected for non-specific bromide ion release. Both methods (A and B) gave similar results. Since both methods showed similar sensitivity the bromide ion sensitive electrode method being less laborious was selected. The detection limit for bromide ion in this study was in the order of 5 nmole/ml.

Water-soluble metabolites. To 1.25 ml of microsomal incubate was added 5.0 ml ice-cold ethyl acetate (saturated with H_2O) to terminate the reactions and to extract unmetabolized Tris-BP and non-polar products. Extractions were repeated thrice resulting in a 98–100 per cent extraction of unmetabolized Tris-BP (data not shown). The protein fraction obtained from the ethyl acetate extraction was combined with the protein isolated from the bromide ion procedure. The remaining aqueous layer was termed the "total water soluble fraction". After acidification (to pH 1), Bis-BP was quantitatively extracted from the aqueous layer with diethylether (3 × 5 ml). The resulting aqueous fraction was termed "pH 1 residue". Preliminary experiments with the ^{3}H-Bis-BP fractions (prepared from microsomal incubation of ^{3}H-Tris-BP and fractionated on a Sephadex G-10 column, data not shown) showed that >94 per cent of the radiolabel could be extracted into the ether layer after adjusting pH to 1. At pH 7.4, only 2–3 per cent of the radiolabel could be extracted into ether.

Identification of Bis-BP. Chromatography of the total water-soluble fraction from microsomal incubations with ^{3}H-Tris-BP on Sephadex G-10 was found to give a large radioactive peak eluting approximately after 2 bed volumes. In addition, ether extracts of the acidified total water-soluble fraction from microsomal incubations with Tris-BP chromatographed exclusively in this region on Sephadex G-10. Greater than 94% of the radioactivity that eluted in this peak was Bis-BP as determined by HPLC isolation and scintillation counting of the radioactivity in a cold carrier pool of synthetic Bis-BP. The Bis-BP eluted on a Dupont Zorbax C-8 column (4.6 mm × 25 cm) using a mobile phase of 70% water, adjusted to pH 2.3 with HCl, and 30% acetonitrile. Retention time of Bis-BP was approximately 6 min at a flow rate of 1 ml/min on a Dupont model 850 liquid chromatograph. Samples of the radioactive peak that eluted from the Sephadex G-10 column from microsomal incubations of ^{3}H-Tris-BP, were also subjected to mass spectral analysis on a VG 7070H instrument using direct probe insertion and chemical ionization with methane as reagent gas. Samples gave spectra identical to synthetic Bis-BP.

Identification of 2-bromoacrolein. Assays were performed by incubating for 20 min at 37° Tris-BP with 2 mg/ml liver microsomal protein from untreated rats with and without a NADPH-generating system. Equal volumes of cold methanol were added to terminate reactions and the metabolites were extracted with 3 vol. of ethyl acetate. The ethyl acetate extracts were then concentrated and analyzed by gas chromatography mass spectrometry (GC-MS) on a VG 7070 H instrument, equipped with a Hewlett-Packard 5710A GC and on-line to a VG Model 2035 data system. Analyses were performed in the EI7070H mode using an accelerating voltage of 4 kV, an electron energy of 70 eV and a trap current of 100 μA. The GC was equipped with a 30 m × 0.32 mm i.d. fused silica column coated with DB-5 as a stationary phase (J & W Scientific). Analysis of 2-bromoacrolein was performed using the following conditions: carrier gas, He (head pressure, 15 psi); injector temp. 250°; temp. program, splitless injection at oven temp. of 50°, then temp. raised after 5 min by 5°/min to 150°. GC interface temp., 200°; ion source temp. 200°. Retention time of 2-bromoacrolein was approximately 7.5 min or 10.5 methylene units (MU) when measured relative to a homologous series of n-alkanes.

Covalent protein binding

The combined protein was assayed according to Søderlund *et al.* [7].

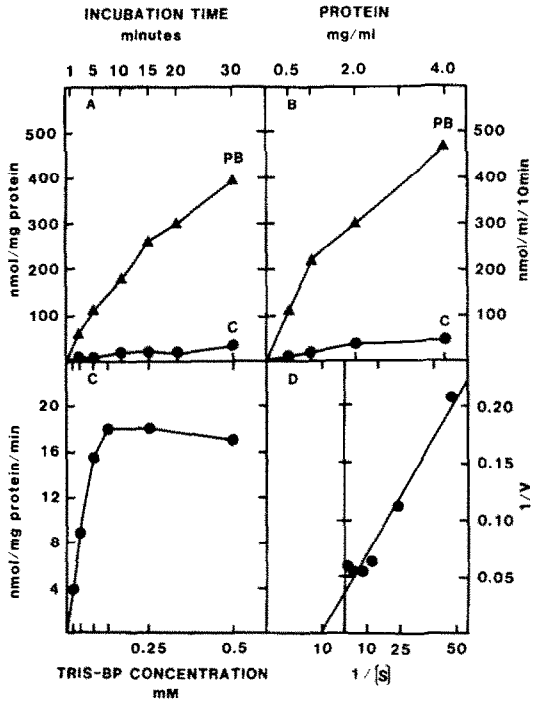


Fig. 1. Time-course (A), protein-dependency (B), substrate-dependency (C) and Lineweaver–Burk plot (D) of bromide release from Tris-BP with liver microsomes from control (C) or phenobarbital (PB)-pretreated rats. 0.5 mM Tris-BP was incubated in the presence of NADPH, with 0.5 mg/ml protein (A) or for 10 min (B) and bromide release was measured with a bromide selective electrode, Substrate-dependency (C) and Lineweaver–Burk plot of PB-pretreated microsomes (0.5 mg/ml) were assayed after 10 min incubation (C, D). Each point is the mean of duplicate estimations with pooled microsomes from 3–4 rats, values are typical of several experiments. K_m and V_{max} values were determined after regression analysis.

Table 1. Effect of pretreatments on Tris-BP bromide release, Bis-BP and pH 1 residue formation, Tris-BP mutagenicity and Tris-BP covalent protein binding

Pretreatment	Bromide release (nmole/mg protein/min*,†)	Bis-BP formation % activity in incubation	pH 1 residue % activity in incubation	Mutagenicity His^+ Rev./10^6 bact,‡§	Covalent protein binding (pmole/mg protein/min*)
Control	1.93 ± 0.84	1.41 ± 0.46	0.41 ± 0.07	39.8 ± 6.7	303 ± 117
	(100 ± 43)	(100 ± 33)	(100 ± 17)	(100 ± 17)	(100 ± 39)
Phenobarbital	24.73 ± 0.58	5.94 ± 0.36	2.24 ± 0.14	292.2 ± 16.4	1220 ± 201
	(1275 ± 30)	(421 ± 26)	(546 ± 34)	(734 ± 41)	(403 ± 66)
Cobaltous chloride	0.27 ± 0.05	0.33 ± 0.37	0.09 ± 0.16	1.50 ± 0.3	61 ± 60
	(14 ± 3)	(23 ± 26)	(23 ± 39)	(4 ± 1)	(20 ± 19)

Assays performed as described in Materials and Methods. Values are means ± S.D. of 3–4 determinations and have been corrected for non-specific activities.

* 0.5 mM Tris-BP.

‡ 0.10 mM Tris-BP.

† Values in parenthesis are per cent of controls.

§ From Søderlund *et al.* [6].

RESULTS

Microsomal metabolism of Tris-BP

Incubation of Tris-BP with liver microsomes in the presence of NADPH resulted in the release of bromide ion into the incubation medium, this reaction was time- and protein-dependent (Fig. 1A, B). Bromide release increased with increasing substrate concentration up to 0.125 mM Tris-BP (Fig. 1C), with an apparent $V_{max} = 27.9$ nmole/mg protein × min and $K_m = 96.6\ \mu M$ using microsomes from PB-treated rats (Fig. 1D). Formation of water soluble metabolites and Bis-BP paralleled bromide release. Virtually no bromide could be detected in the absence of NADPH. With 1.5 mg PB-pretreated microsomal protein 13 per cent of the substrate was converted to water-soluble products after 30 min. Of this, approximately 50–60% was shown to be in the Bis-BP fraction. NADPH-dependent microsomal bromide release, Bis-BP formation, mutagenicity and covalent protein binding all were considerably increased after PB-pretreatment (Table 1). In contrast, the cytochrome P-450 inhibitor cobaltous chloride [20], markedly decreased bromide release, Bis-BP formation, mutagenicity and covalent protein binding.

Several inhibitors of cytochrome P-450 metabolism were tested for their effects on oxidative debromination of Tris-BP and were compared to Tris-BP mutagenicity and microsomal covalent protein binding using microsomes from PB-pretreated animals (Table 2). A nitrogen atmosphere markedly inhibited all reactions. Both bromide release and Bis-BP formation were inhibited more than 75 per cent by carbon monoxide. However, the effect of carbon monoxide on Tris-BP mutagenicity and covalent protein binding were not decreased to the same extent. SKF 525-A, a competitive inhibitor of cytochrome P-450, decreased metabolism of Tris-BP as well as mutagenicity and covalent protein binding, Metyrapone, which selectively inhibits the phenobarbital-inducible forms of the cytochrome [21], almost totally blocked bromide release and Bis-BP formation and markedly inhibited mutagenicity and covalent protein binding. On the other hand, α-naphthoflavone, which selectively inhibits the

Table 2. Conditions for Tris-BP bromide release, Bis-BP and pH 1 residue formation, Tris-BP mutagenicity, and Tris-BP covalent protein binding with liver microsomes from phenobarbital-pretreated rats

Conditions	% of Control*				
	Bromide† release	Bis-BP† formation	pH 1† residue	Mutagenicity‡	Covalent† protein binding
CO/O_2 (4:1)	16.8	18.1	32.8	59.6	34.0
N_2/O_2 (4:1)	98.0	104.6	102.8	79.2	104.0
N_2	36.1	30.1	40.1	26.5	51.0
SKF 525-A, 0.2 mM	16.3	10.9	28.8	51.3	47.8
Metyrapone, 1.0 mM	7.5	7.7	17.4	36.7	25.5
α-Naphthoflavone, 0.1 mM	97.8	88.3	91.0	87.3	99.3
Glutathione, 1.0 mM	177.0	89.1	235.0	4.5	4.8

* Assay performed as described in Materials and Methods. Values are means of 2–3 estimations.

† 0.5 mM Tris-BP.

‡ 0.05 mM Tris-BP.

Table 3. Bromide release and mutagenicity of Tris-BP and analogs with liver microsomes from phenobarbital-pretreated rats

Test compound	Bromide release (nmole/mg protein/min)	Mutagenicity His$^+$ Rev./10^6 Bacteria
Tris-BP*	17.3†	84.2†
Tris(3-bromopropyl)phosphate	1.9	0.5
Tris(2-bromopropyl)phosphate	1.9	2.7
Bis(2,3-dibromopropyl)phosphate	7.4	12.3
2,3-Dibromopropylphosphate	2.7	3.5
2,3-Dibromopropanol	1.3	5.9

Assays performed as described in Materials and Methods.
* Substrate concentrations were 0.5 mM for bromide release and 0.05 mM for mutagenicity.
† Values are means of duplicate incubations.

methylcholanthrene-inducible forms of the cytochrome [21], showed very little effect on Tris-BP metabolism. Addition of 1.0 mM GSH to the incubate, earlier shown to totally inhibit Tris-BP mutagenicity and covalent binding, resulted in an increase in the overall formation of water-soluble metabolites, as well as bromide release with no increase of the Bis-BP fraction (Table 2). The GSH dependent increase in total water soluble metabolites could be fully accounted for by an increase in non-ether extractable metabolites (pH 1 residue). GSH interference was overcome by oxidizing GSH with ascorbate when using the bromide selective electrode.

Analogs of Tris-BP were studied to correlate bromide release with metabolic activation to mutagens (Table 3). A concentration of 0.05 mM Tris-BP was chosen so as to not elicit bacterial cytotoxic effects [6]. Both the number of bromines in the propyl moiety and the number of ester groups in the parent molecule of Tris-BP affected bromide release and mutagenicity in a parallel fashion. Maximal activities of bromide release and mutagenicity were achieved with the Tris-phosphoric acid ester of the dibrominated propanol. The di-ester (Bis-BP) showed slightly higher activities than the mono-ester with respect to bromide release and mutagenicity.

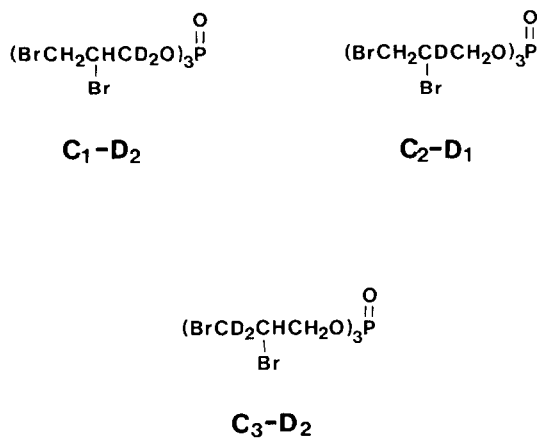


Fig. 2. Structures of specifically deuterated Tris-BP analogs.

Bromide release and mutagenicity of specifically deuterated Tris-BP analogs (Fig. 2) were tested to gain information about the regiospecificity of Tris-BP metabolism. The relative effects of deuterium labelling of Tris-BP on bromide release and mutagenicity with microsomes from untreated rats are shown in Fig. 3. With respect to mutagenicity an 80 per cent decrease was observed with the C_3–D_2 compound, whereas deuterium substitution at the C_1 or C_2 position had no effect on Tris-BP mutagenicity.

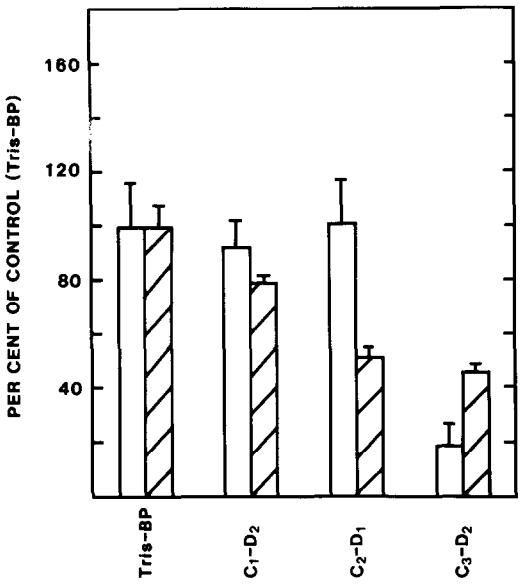
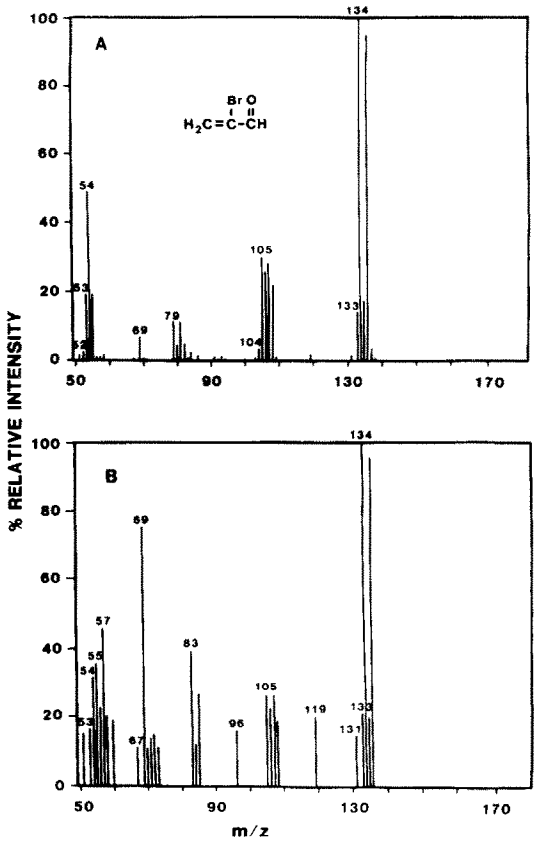


Fig. 3. Relative effects of deuterium labelling of Tris-BP on mutagenicity and bromide release with microsomes prepared from untreated rats. Mutagenicity (open bars) was determined after incubating 0.01 mM test substance with 2 mg/ml of hepatic microsomal protein from control rats, NADPH-cofactors and Salmonella TA 100 for 30 min at 37°. Mutagenicity of the various deuterium analogs is expressed in per cent of Tris-BP and are means of 4 plates from 2 separate experiments. Bromide release (hatched bars) was assayed according to Materials and Methods using a bromide selective electrode. 0.5 mM test substance was incubated for 10 min at 37° with 0.5 mg/ml microsomal protein from control rats and NADPH-cofactors. Values are means ± S.D. of 3 incubations.



Deuterium at C_2 or C_3 decreased bromide release with control microsomes to approximately 50 and 40 per cent of Tris-BP, respectively, whereas an insignificant decrease was found with the C_1–D_2 compound.

Incubations of Tris-BP with rat liver microsomes were analyzed for the presence of 2-bromoacrolein by GC-MS. Results of these studies (Fig. 4B) clearly demonstrated that 2-bromoacrolein was formed in a NADPH-dependent reaction. Analysis of the spectrum shows parent ions at *m/z* 134/136 as well as fragment ions at *m/z* 133/135 (loss of H), *m/z* 106/108 (loss of CO) and *m/z* 105/107 (loss of CHO). The spectrum is identical to the spectrum observed with a 2-bromoacrolein synthetic standard (Fig. 4A). Incubations without the NADPH-regenerating system failed to yield any significant levels of 2-bromoacrolein.

DISCUSSION

We have previously shown that mutagenicity caused by Tris-BP in *Salmonella typhimurium* required oxi-

Fig. 4. Electron impact mass spectrum of synthetic 2-bromoacrolein (A) and metabolite (B) isolated from incubations of rat liver microsomes. Tris-BP was incubated in the presence of NADPH and 2 mg/ml of liver microsomal protein from untreated rats for 20 min. Ethyl acetate extracts were analyzed by GC-MS described in Materials and Methods and compared with a synthetic 2-bromoacrolein standard.

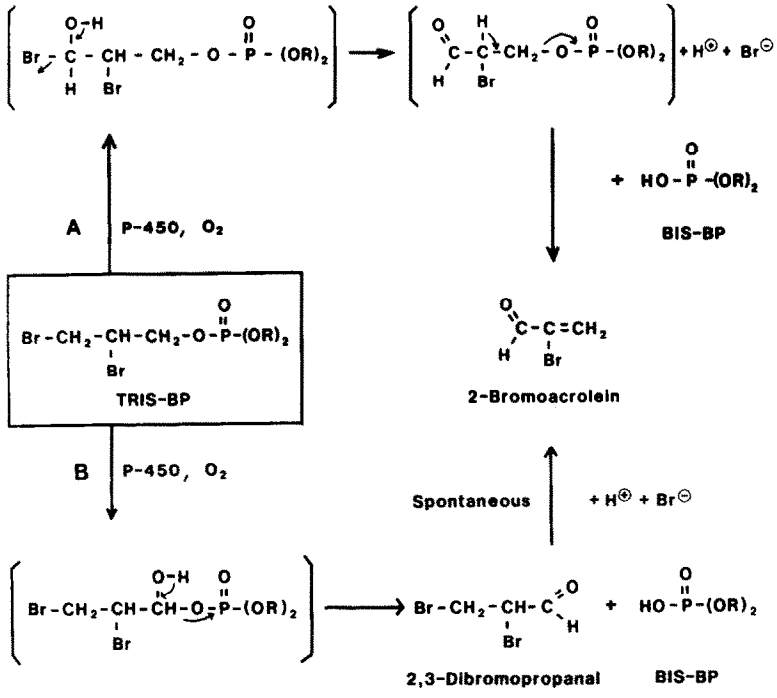


Fig. 5. Proposed pathway for Tris-BP oxidation leading to bromide ion and Bis-BP formation. R = 2,3-dibromopropyl group.

dative metabolism by cytochrome P-450 [6]. We have now found that bromide release from Tris-BP and the formation of Bis-BP also are formed in cytochrome P-450 dependent pathways which require oxygen and NADPH (Tables 1 and 2). Bromide release and Bis-BP formation are likely formed by additional pathways as well. The amounts of bromide detected in an atmosphere of nitrogen may be indicating that reductive metabolism yields bromide from Tris-BP, and large amounts of bromide are formed in the presence of glutathione (Table 2). Significant amounts of Bis-BP also are formed in the absence of oxygen indicating that hydrolytic pathways may be involved (Table 2). However, the effects of all treatments and conditions on bromide release and Bis-BP formation except the addition of glutathione paralleled the effects of mutagenicity. Moreover, bromide release paralleled mutagenicity in a series of Tris-BP analogs (Table 3).

Furthermore, isotope effects on bromide release from two specifically deuterated analogs of Tris-BP paralleled the isotope effects on mutagenicity (Fig. 3). Thus, the analog labelled with deuterium at C_1 showed no isotope effect on either bromide release or mutagenicity whereas the analog labelled at C_3 showed significant isotope effects on both. In contrast, the analog labelled with deuterium at C_2 showed a significant isotope effect on bromide release, but not on mutagenicity. One explanation for this apparent anomaly is that oxidative debromination does occur at C_2, thereby producing a deuterium isotope effect on bromide release, but that the resulting product of this reaction is not mutagenic.

Consistent with these results is the detection of 2-bromoacrolein as an oxidative metabolite of Tris-BP (Fig. 4). Based on the deuterium isotope effects on mutagenicity and bromide release and on the strong, direct mutagenic effect of 2-bromoacrolein (Gordon *et al.*, in preparation) we would postulate that this is the major metabolite which is responsible for mutagenicity caused by Tris-BP. Furthermore, the data suggest that this metabolite most likely arises by initial oxidation at C_3 to yield a gem-bromohydrin (Fig. 5, Pathway A). Bromide release then occurs by dehydrobromination of the gem-bromohydrin and both 2-bromoacrolein and Bis-BP formation arise from a β-elimination (dehydrophosphorylation) reaction. Thus, the bulk of our data, which correlates mutagenicity with oxidation by cytochrome P-450 to yield both bromide and Bis-BP, can be explained by this mechanism. Additional routes of metabolism yield bromide and Bis-BP, but these pathways are apparently not involved in the generation of metabolites which are directly mutagenic. Elucidation of these pathways and their possible role in nephrotoxicity and carcinogenicity will require additional studies.

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