

Covalent Interaction of Metabolites of the Carcinogen Trichloroethylene in Rat Hepatic Microsomes¹

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SUMMARY

Trichloroethylene (TCE), a structural analog of vinyl chloride, is known to induce hepatocellular carcinoma and other tumors in C57BL/6 × C3H/He F₁ (hereafter known as B6C3F₁) hybrid mice. TCE epoxide, a possible metabolite, is expected to be highly reactive toward cellular nucleophiles, e.g., proteins and nucleic acids. Hence, the microsomal metabolism of TCE and its covalent binding to microsomal protein were examined. Rat liver microsomes were incubated *in vitro* with [¹⁴C]TCE. The results showed that TCE binds covalently to microsomal protein since extensive organic extractions and Pronase digestion do not dissociate the TCE-protein complex. The binding was decreased by 7,8-benzoflavone, blocked by SKF-525A, and enhanced by i.p. administration of phenobarbital. The possibility that TCE epoxide, once formed, could be converted to water-soluble products through enzymatic hydrolysis by epoxide hydrolase was also investigated. Addition of 3,3,3-trichloropropene oxide, a potent inhibitor of epoxide hydrolase, to the incubation system markedly enhanced the binding of TCE. These observations support the view that, in order to bind to protein, it is necessary for TCE to be metabolized to its epoxide, a reactive intermediate that is most likely involved in TCE carcinogenesis and toxicity.

INTRODUCTION

TCE² has been used for many years as an anesthetic (5). It is also widely used as a degreasing agent for metals and as an extractant in the foodstuff industry (18). Several metabolites of TCE in humans and animals have been characterized (4, 10, 11) and it has been suggested that TCE is metabolized via an epoxide (16). On the basis of its known metabolic products and the possibility of an epoxide intermediate, it has also been predicted that TCE is probably carcinogenic, particularly to the liver (20). This suggestion was made, in part, because of its structural similarity to vinyl chloride, a known liver carcinogen in animals and man (6, 20). Recently, TCE was shown to be carcinogenic by feeding to B6C3F₁ hybrid mice, but not in Osborne-Mendel rats (19).

Trichloroethylene epoxide has not been characterized as a metabolite of TCE, but the synthetic compound is known (B. L. Van Duuren and S. Kline, unpublished data).

The present study was undertaken in order to obtain evidence for the covalent interaction of TCE metabolites with rat liver microsomes *in vitro*.

MATERIALS AND METHODS

Chemicals. [1,2-¹⁴C]TCE (specific activity, 1 mCi/mmol) was custom-labeled by New England Nuclear, Boston, Mass. PB was obtained from Elkins-Sinn, Inc., Cherry Hill, N. J. 7,8-BF was purchased from Aldrich Chemical Co., Milwaukee, Wis. SKF-525A and TCPO were provided by Dr. A. Lu of Hoffmann-La Roche, Nutley, N. J. G-6-P, G-6-P dehydrogenase, GSH, mercaptoethanol, urea, and Pronase were all purchased from Sigma Chemical Co., St. Louis, Mo.

Preparation of Microsomes. Male Sprague-Dawley rats (A. R. Schmidt, Madison, Wis.), 5 to 9 weeks of age, were maintained on a commercial diet and water *ad libitum*. Liver microsomes were prepared according to the method of Levin *et al.* (12). A 25% liver homogenate was prepared in 0.05 M Tris-HCl buffer, pH 7.5, containing 1.15% KCl. It was then centrifuged at 12,000 × *g* for 15 min at 4°. The microsomal pellet was collected from the supernatant obtained from the above centrifugation by spinning at 105,000 × *g* for 60 min. The microsomal pellet was washed twice with 1.15% KCl containing 10 mM EDTA. The washed pellet was suspended in 0.05 M potassium phosphate buffer, pH 7.5, at a protein concentration of 8 to 9 mg/ml. The microsomal pellet was at times stored at -20° under nitrogen and a layer of 0.25 M sucrose. Such samples retained enzymatic activity unchanged up to 2 weeks.

Induction of Mixed-Function Oxidases. Animals weighing 40 to 45 g were given PB i.p. in 0.9% NaCl solution at a dose of 100 mg/kg of body weight, once daily, for 3 days. The animals were fasted for the last 18 hr and killed 24 hr after the last injection. The control animals received injections of 0.9% aqueous NaCl solution. Microsomal preparations were then made as described above.

Binding of TCE to Microsomes. Microsome preparations were incubated with [¹⁴C]TCE in the presence of a NADPH-regenerating system in a closed vial with shaking at 37° for 60 min. The complete incubation mixture consisted of a microsomal preparation containing 500 µg of microsomal protein, 10.0 µmoles of MgCl₂, 2.5 µmoles of G-6-P, 1.3 units of G-6-P dehydrogenase, 1.0 µmole of NADP, 0.83

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² The abbreviations used are: TCE, 1,2,2-trichloroethylene; PB, sodium phenobarbital; 7,8-BF, 7,8-benzoflavone; SKF-525A, β-diethylaminoethyl diphenylpropyl acetate; TCPO, 1,2-epoxy-3,3,3-trichloropropane; G-6-P, glucose 6-phosphate; GSH, reduced glutathione; TCA, trichloroacetic acid.

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μ mole of TCE (specific activity, $1 \mu\text{Ci}/\mu\text{mole}$) in 1% acetone and 0.88 ml of 0.05 M potassium phosphate buffer, pH 7.4, in a total volume of 1.0 ml. The reaction was terminated by the addition of TCA to a final concentration of 5%. The mixture was then centrifuged at low speed for 15 min and the supernatant was discarded. The protein pellet was heated at 90° for 15 min in the presence of 5 ml of 5% TCA for hydrolysis of RNA. The protein residue obtained by low-speed centrifugation was suspended in 5 ml of methanol:ether (3:1) and heated at 60° for 15 min. After the supernatant was discarded, the protein was extracted 10 times with the same solvent mixture (17) and, finally, washed once with ether. The residue was air dried and dissolved in 0.2 ml of 0.5 N NaOH. Aliquots were then taken for liquid scintillation counting, using Aquasol (New England Nuclear, Boston, Mass.) as counting solution, and also for the determination of protein content. The protein was assayed by the method of Lowry *et al.* (13), with crystalline bovine serum albumin as standard. For determination of the 0-hr value of the binding, microsomes were denatured with TCA prior to the substrate addition and incubation.

In order to determine whether TCE itself binds covalently with microsomal protein, the washed protein residue obtained as described above was suspended in buffer and then digested with 100 μg of Pronase at 37° for 4 hr. This digest was then extracted with 4 volumes of ether to remove any TCE that was not covalently bound. Radioactivities were determined before and after hydrolysis.

RESULTS

Binding of TCE to Microsomal Protein. From measurements of the amount of TCE bound per mg of protein with time, it was determined that maximum binding is reached within 60 min and then remains at a plateau. The binding between 0 and 60 min was linear. In subsequent experiments, the microsomes were incubated for 60 min only.

The purified microsomal protein was hydrolyzed with Pronase. The hydrolysate, after extraction with ether, was found to retain all of its radioactivity. This result indicated that the binding of TCE occurs with the amino acid residues of microsomal protein.

Effect of SKF-525A and 7,8-BF on the Binding of TCE to Microsomal Protein. Table 1 shows the effect of 2 inhibitors of mixed-function oxidase on the binding of TCE to microsomal protein. When microsomes were incubated with [^{14}C]TCE in the presence of either SKF-525A or 7,8-BF, a diminution in the amount of TCE bound to protein was observed. At a concentration of 2.5×10^{-3} M, SKF-525A inhibited the binding by 25% compared with the control. When this concentration was increased to 3.75×10^{-3} M, SKF-525A almost totally blocked TCE from binding to protein (95%). 7,8-BF had a similar effect on TCE binding to microsomal protein, as shown in Table 1.

Acceleration of the Binding of TCE to Microsomes by *in Vivo* Treatment With PB. Table 2 shows the effect of PB on the TCE binding to protein when administered *in vivo*. PB at a dose of 100 mg/kg of body weight administered for 3 days increased the binding of TCE to microsomal protein by 64%.

Table 1

The effect of SKF-525A and 7,8-BF on the binding of [^{14}C]TCE to hepatic microsomal protein

Microsomes were incubated with [^{14}C]TCE for 60 min, as described in Materials and Methods

Addition to the incubation system (M)	[^{14}C]TCE binding to protein		% change over the control
	dpm/mg protein	nmoles/mg protein	
None	5726 $\pm$ 113	2.59	
SKF-525A			
2.5×10^{-3}	4289 $\pm$ 243	1.92	-25
3.75×10^{-3}	291 $\pm$ 67	0.13	-95
7,8-BF			
1.25×10^{-3}	4188 $\pm$ 128	1.89	-27
2.5×10^{-3}	4028 $\pm$ 85	1.81	-30

* Results are the average of 4 analyses in each group mean S.D.

Table 2

The effect of *in vivo* treatment of PB on [^{14}C]TCE-protein binding

The animals were treated either with 0.9% NaCl solution or PB *vivo*. Hepatic microsomes were isolated and incubated with [^{14}C]TCE *in vitro* as described in "Materials and Methods." Results are the average of 4 animals in each group.

<i>In vivo</i> treatment	[^{14}C]TCE binding to protein		% change over the control
	dpm/mg protein	nmoles/mg protein	
0.9% NaCl solution	4348 $\pm$ 631*	1.96	
PB	7121 $\pm$ 388	3.21	+64

* Mean $\pm$ S.D.

compared with control animals, when microsomes were incubated for 60 min.

Effect of Various Agents on the Binding of TCE to Microsomal Protein. In order to obtain further information concerning the nature of the binding, several agents were incubated individually with microsomes (Table 3). When microsomes were incubated with different concentrations of urea, GSH, 1-methy-2-mercaptoimidazole, and mercaptoethanol, they all decreased the extent of binding of TCE to microsomal protein.

Effect of TCPO on TCE-Protein Binding. Table 4 demonstrates the enhancing effect of TCPO, a potent inhibitor of epoxide hydrolase (15), on the TCE binding to microsomal protein. TCPO stimulates TCE binding, which is concentration dependent. At a concentration of 1.25×10^{-2} M or more, it caused an augmentation of the binding of 76 to 91%.

DISCUSSION

A number of experimental studies have provided evidence that some indirect-acting carcinogens are metabolized to their activated carcinogenic intermediates by means of a cytochrome P-450-dependent mixed-function oxidase (2, 14). The intermediate epoxides from such indirect-acting carcinogens may then interact covalently at nucleophilic sites in nucleic acids and proteins, and one or more of these processes are probably responsible for their carcinogenic activity.

It has been suggested that an epoxide intermediate (Chart 1, Structure 1) is the activated carcinogenic intermediate of vinyl chloride (20). This suggestion was based on its similarity in structure to epoxide carcinogens such as glycidaldehyde, Structure 2, and *N*-chloro-ether carcinogens such as bis(chloromethyl)ether, Structure 3. Chloroethylene oxide, Structure 1, contains both the epoxide and *N*-chloroether moieties of Structures 2 and 3. In a recent report it was shown that rat liver microsomes catalyze the covalent binding of [¹⁴C]vinyl chloride to macromolecules (7). In another study (1), evidence was obtained for the formation of epoxides from vinyl chloride and vinyl bromide by rat liver microsomes. These findings are consistent with the proposal (20) that an epoxide intermediate is involved in the metabolism of vinyl chloride.

On the basis of these considerations, it is likely that TCE, Structure 4, is also metabolized via an epoxide (Structure 5) (20). This was suggested earlier by Powell (16) on the basis of metabolic studies.

SKF-525A is a known inhibitor of the metabolism of various substrates of cytochrome P-450 (3). This agent is shown in the present study to inhibit the covalent binding of TCE to rat liver microsomal proteins.

7,8-BF, an inhibitor of 7,12-dimethylbenz(a)anthracene-induced mouse skin carcinogenesis (8), is known to decrease the covalent binding of 7,12-dimethylbenz(a)anthracene to DNA, RNA, and protein in mouse skin (9). In line with these earlier findings, the present work showed that 7,8-BF inhibited the covalent binding of TCE to rat liver microsomal proteins.

The experiments with TCPO, a potent inhibitor of epoxide hydrazide (15), showed that this agent causes an enhancement of TCE binding to microsomal protein. This observation is also consistent with the formation of an epoxide from when then binds to microsomal protein. PB injected

Table 4

The effect of TCPO on [¹⁴C]TCE binding to protein

Hepatic microsomes were isolated from animals weighing 40 to 45 g. as in Table 2, and as described in Materials and Methods

Addition to the incubation system (M)	[¹⁴ C]TCE binding to protein		% change over the control
	dpm/mg protein	nmoles/mg protein	
None	4460 ± 360	2.0	
TCPO			
2.5 × 10 ⁻⁴	5058 ± 427	2.28	+13
5.0 × 10 ⁻⁴	5708 ± 957	2.57	+28
2.5 × 10 ⁻³	5547 ± 123	2.52	+11
5.0 × 10 ⁻³	7539 ± 381	3.03	+50
2.5 × 10 ⁻²	8518 ± 162	3.84	+91

Results are the average of 4 analyses in each group, mean ± S.D.

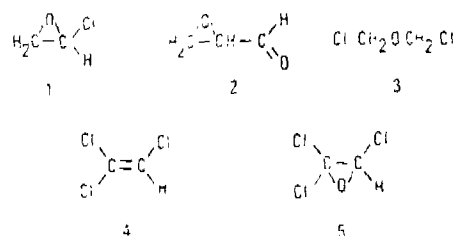


Chart 1. 1, chloroethylene oxide; 2, glycidaldehyde; 3, bis(chloromethyl) ether; 4, trichloroethylene; 5, trichloroethylene epoxide

into the animals prior to the examination of TCE binding also enhanced the binding of TCE to microsomal proteins. The other agents, listed in Table 3, all decreased the binding of TCE to hepatic microsomal proteins as expected.

The present report provides evidence for the covalent binding of TCE to rat liver microsomal proteins from *in vitro* experiments. These results suggest that the binding is via an epoxide or other related electrophilic species.

Table 3

The effect of various agents on the binding of [¹⁴C]TCE to hepatic microsomal protein

Microsomes were incubated with [¹⁴C]TCE for 60 min as described in Materials and Methods.

Addition to the incubation system (M)	[¹⁴ C]TCE binding to protein		% change over the control
	dpm/mg protein	nmoles/mg protein	
None	5756 ± 113	2.59	
GSH			
5 × 10 ⁻⁵	3119 ± 102	1.4	-46
5 × 10 ⁻⁴	1954 ± 67	0.88	-66
1-Methyl-2-mercaptoimidazole			
5 × 10 ⁻⁵	2510 ± 299	1.13	-56
5 × 10 ⁻⁴	990 ± 28	0.45	-83
Mercaptoethanol			
5 × 10 ⁻⁵	4533 ± 174	2.04	-21
Urea			
0.05	5566 ± 248	2.51	-3
0.1	4434 ± 141	2.0	-23
0.5	3317 ± 124	1.49	-42

Results are the average of 4 analyses in each group, mean ± S.D.

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