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Bioactivation and Covalent Binding of Halothane to Liver Macromolecules

by I. Glenn Sipes,* Thomas L. Podolsky,* and Burnell R. Brown, Jr.*

In this manuscript we report our attempts to determine if ^{14}C-halothane or its metabolites interact with DNA. Three bioactivation systems were used: *in vitro* microsomal incubations, isolated hepatocytes, and *in vivo* administration. Even though we used optimal conditions for bioactivation, no significant covalent binding of ^{14}C to DNA was observed. Slight ^{14}C activity above background (6 dpm/0.1 mg DNA) was observed in the microsomal incubations but is considered insignificant because it was not reduced when NADPH was omitted from the incubations. We were able to demonstrate covalent binding to nuclear lipids and proteins when rats were pretreated with phenobarbital and maintained in a hypoxic environment (14% O_2). Similarly, these conditions markedly increased covalent binding of ^{14}C from ^{14}C-halothane to microsomal proteins and lipids. Isolated rat hepatocytes proved to be a viable system for studying the bioactivation of halothane. In this system it was also possible to demonstrate increased binding under N_2 and/or phenobarbital pretreatment.

Introduction

In recent years, attention has been focused on the activation of relatively inert chemicals by liver microsomal enzymes during the process of biotransformation or drug metabolism (*1, 2*). The products of this "bioactivation" of drugs and other xenobiotics are reactive intermediates, species possessing a chemical reactivity that is greater than that of the parent compound or its urinary, biliary, or fecal metabolites. These reactive intermediates can covalently interact with tissue macromolecules such as proteins, lipids and nucleic acids and thus alter cellular integrity (*1, 2*). Reactive intermediates from such diverse compounds as carbon tetrachloride (*3*), chloroform (*4*), bromobenzene (*5, 6*), acetaminophen (*7, 8*), and furosemide (*9*) as well as others have been implicated as the causative agents in the hepatotoxicity associated with administration of these compounds. Similarly, the production of hepatic neoplasms by chronic exposure to dimethylnitrosamine (*10*), *N*-acetylaminofluorene (*11*), vinyl chloride (*12*) and trichloroethylene (*13*) is thought to result from endogenously produced reactive intermediates.

Halothane (1,1,1-trifluoro-2-bromo-2-chloroethane) undergoes biotransformation in man (*14, 15*) and laboratory animals (*16*) and metabolites (reactive intermediates) of halothane have been shown to covalently bind to liver proteins and lipids in the rat (*17–20*). The isolation from human urine of *N*-trifluoroacetyl-2-aminoethanol and *N*-acetyl-*S*-(2, bromo-2-chloro-1, 1-difluoroethyl-L-cysteine) by Cohen et al. (*15*) strongly suggests that halothane is also bioactivated by man. The production of reactive intermediates may explain the rare, unpredictable and sporadic cases of hepatic failure following anesthesia with halothane (*21*). Certain individuals may produce an abnormally high amount of reactive intermediates that may overwhelm detoxification mechanisms.

The production of reactive intermediates by man also raises concern about the chronic exposure of anesthesiologists and other operating room personnel to trace concentrations of halothane and other anesthetic gases. Indeed, numerous epidemiological studies have reported a higher incidence of spontaneous abortions, congenital abnormalities, liver disease and various malignancies in anesthesiologists and operating room personnel as com-

*Toxicology Program and the Department of Anesthesiology, Health Sciences Center, University of Arizona, Tucson, Arizona 85724.

pared to the general population or unexposed medical and nursing professionals (*22*). The structural similarity of halothane and some of its known metabolites to such carcinogens as vinyl chloride, vinylidene chloride, and trichloroethylene underscores the concern about anesthetics and cancer (*13*).

One of the suggested mechanisms of chemical carcinogenesis is the somatic mutation theory whereby a heritable change in nucleotide sequence results from an alteration in the primary structure of DNA (*24*). Nearly all known chemical carcinogens covalently bind to DNA. If this altered DNA is not repaired or is subject to error prone repair, then a heritable change in nucleotide sequence can occur (*24*). Repeated insult to DNA by chronic exposure to carcinogens may result in a number of mutations that ultimately leads to invasive cells.

The reported cases of halothane-induced hepatitis following anesthesia and the epidemiological studies correlating an increased cancer risk with chronic exposure to inhalation anesthetics prompted us to study the covalent binding of halothane to liver macromolecules, with particular emphasis on binding to DNA. We report significant binding of ^{14}C from ^{14}C-halothane to liver proteins and phospholipids, particularly under hypoxic conditions. However, no binding to DNA or RNA was observed when halothane was administered *in vivo* or added to microsomal incubations or isolated liver cells.

Materials and Methods

Animals and Pretreatments

Male Sprague-Dawley rats, 150–250 g, were obtained from Hilltop Laboratories and fed Purina rat chow and water *ad libitum*. For induction of the microsomal enzymes animals were pretreated intraperitoneally with either Aroclor 1254 (a polychlorinated biphenyl containing 54% Cl) at a single dose of 500 mg/kg in sesame oil 120 hr prior to sacrifice or phenobarbital (80 mg/kg) in normal saline for four consecutive daily doses beginning 5 days before sacrifice. Control rats received an appropriate volume of solvent (sesame oil or normal saline).

Covalent Binding Studies

Microsomal incubations were performed as previously described (*19, 25*). Briefly, microsomes were isolated by differential centrifugation (9000 *g* for 20 min; 105,000 g for 60 min) from liver homogenized in cold Tris–KCl buffer (0.05*M* Tris, 1.15% KCl, pH 7.4). After the first sedimentation of the microsomal pellet, the microsomes were washed with Tris–KCl buffer by rehomogenization followed by recentrifugation at 105,000 *g* and then frozen overnight at −70°C.

For incubation with ^{14}C-halothane the microsomes were resuspended in Tris–KCl buffer and adjusted to the desired protein concentration as determined by the Biuret procedure (*26*). Each incubation vial contained the following in a final volume of 2.5 ml: 2–8 mg/ml microsomal protein; ^{14}C-halothane dissolved in ethanol (0.125–1.0*mM*); specific activity 1.5–3.7 mCi/mmole); 0.13*mM* NADPH; 0.07 *mM* NADP; 1.16*mM* glucose-6-phosphate; 2 I.E.U. glucose-6-phosphate dehydrogenase, 50*mM* Tris; 2.3*mM* $MgCl_2$ and 2.9 mg KCl at a final pH of 7.4. Certain incubation vials contained 2 mg/2.5 ml calf thymus DNA (Calbiochem).

Incubations were performed in closed 20-ml flasks at 37°C for the desired time (usually 30 min) under air or N_2. Samples containing heat denatured microsomes or no NADPH were run concurrently to determine nonenzymatically bound radiolabel. At the end of incubation, a 1.0 ml aliquot was removed and added to 5 ml of $CHCl_3$: methanol (3:1) to extract phospholipids.

To remove noncovalently bound radiolabel, the $CHCl_3$–methanol extracts were washed three times with equal volumes of Tris–KCl buffer and then with water to extract polar metabolites. The $CHCl_3$× methanol was then evaporated at 60°C with N_2. This evaporation procedure was repeated with $CHCl_3$ (5 ml × 3) and with halothane (2 ml × 2) to remove any ^{14}C-halothane or volatile metabolites. Since Van Dyke (*17*) reported the lipid binding to be associated primarily with phospholipids, binding to the lipid fraction is expressed in terms of phosphorus as determined by the procedure of Dittmer and Wells (*29*). The radioactivity in the lipid fraction as well as in the protein, RNA and DNA was determined by liquid scintillation counting, Aquasol being used as the scintillation cocktail.

To the remaining incubate was added 4 ml of 6% *p*-aminosalicylic acid–1% NaCl to aid in the separation of nucleic acids from protein. Three phenol extractions [procedure of Kirby (*27*)] were used to separate RNA and DNA from protein. Protein was precipitated from phenol extracts by using two volumes of methanol. The isolated proteins were subjected to numerous solvent extractions (*25*) to remove noncovalently bound radiolabel. The repeated ethanol, ethoxyethanol, and ethanol–cresol precipitations of nucleic acids were sufficient to remove any radiolabel. RNA and DNA were separated by the selective alkaline hydrolysis of RNA followed by

perchloric acid precipitation of DNA. For quantitation of DNA, RNA, and protein, the procedures of Burton (*30*). Ceriotti (*31*), and Lowry (*32*) were used respectively.

Isolated rat hepatocytes were obtained by the collagenase procedure of Hayes and Brendel which is described in detail elsewhere (*33*). The twice-washed cells were resuspended in Waymouth's Hepes medium at a concentration of 40 mg/ml (wet weight), and then 5-ml aliquots were added to 50-ml incubation flasks with raised conical bottoms. ^{14}C-Halothane was added to each incubation flask, which was then capped with a ground glass stopper and the reactions performed at 37°C in a gyrating incubator. At the end of incubation, the cells were pooled, homogenized with a glass on glass homogenizer, and subjected to differential centrifugation for isolation of the microsomal fraction. From this fraction microsomal phospholipids were isolated as described above, while proteins were precipitated from the Tris–KCl resuspended microsomes by 10% trichloroacetic acid (TCA). Both fractions were extensively extracted with various solvents as for microsomal incubations.

In the *in vivo* experiments, phenobarbital-pretreated and control rats were placed in a 25-liter Plexiglas chamber and exposed to a hypoxic environment (86% N_2, 14% O_2) for 60 min prior to the intraperitoneal injection of ^{14}C-halothane (88 μCi/kg, 3.7mCi/mmole), and then immediately returned to the hypoxic environment for 2 hr. Similarly, induced and control rats were injected with ^{14}C-halothane but maintained in room air. At the end of 2 hr the animals were sacrificed and the livers removed and divided into three sections of 2 g each. These sections were used for (*1*) isolation of DNA and RNA by the Kirby procedure; (*2*) isolation of microsomal proteins and lipids, and (3) isolation of intact nuclei (*34*). From the intact nuclei phospholipids were extracted as above and proteins were isolated by precipitation with 10% TCA.

Results

Figures 1–4 summarize the results of our initial *in vitro* microsomal studies that were conducted to establish the effects of time of incubation, microsomal protein concentration, and halothane concentration on the covalent binding of halothane to microsomal protein and lipids. Since we had previously reported that pretreatment of rats with Aroclor 1254 greatly enhanced covalent binding (*19*), our plans were to use microsomes from Aroclor 1254-pretreated rats in investigating the possible interaction of reactive intermediates of halothane with DNA. However, subsequent studies revealed

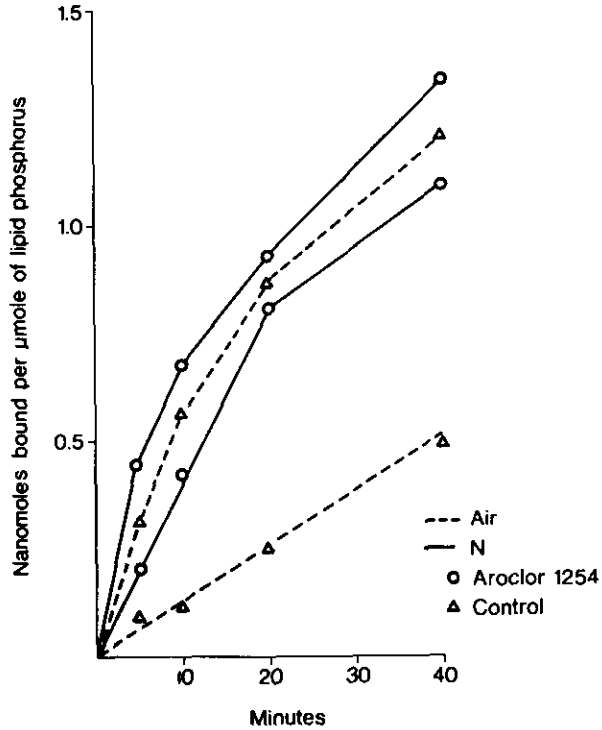

FIGURE 1. Time curve for the *in vitro* covalent binding of ^{14}C-halothane to microsomal phospholipids. Incubations contained 0.3*mM* ^{14}C-halothane, 4 mg/ml microsomal protein and a NADPH generating system. N_2 indicates vials were flushed for 5 min with 100% N_2 to approach anaerobic conditions. Aroclor 1254 indicates microsomes were obtained from rats pretreated with 500 mg/kg Aroclor 1254 (IP).

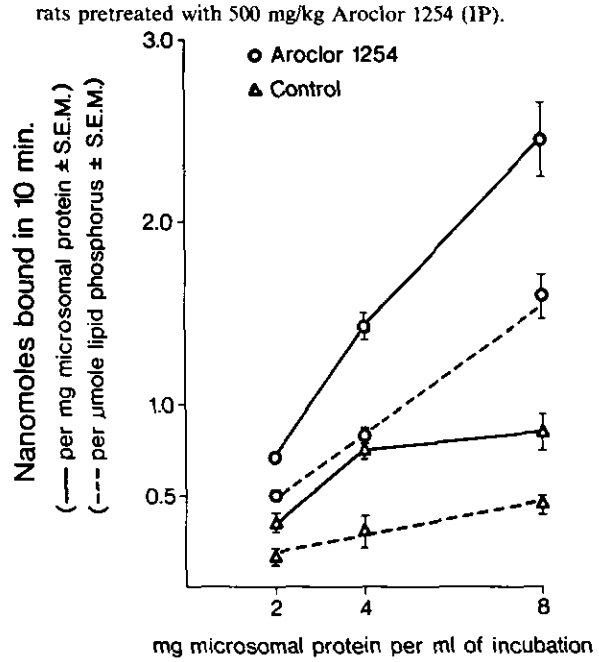

FIGURE 2. Effects of microsomal protein concentration on covalent binding of ^{14}C-halothane to microsomal lipids and protein. Incubations performed in air. Other conditions as in Figure 1.

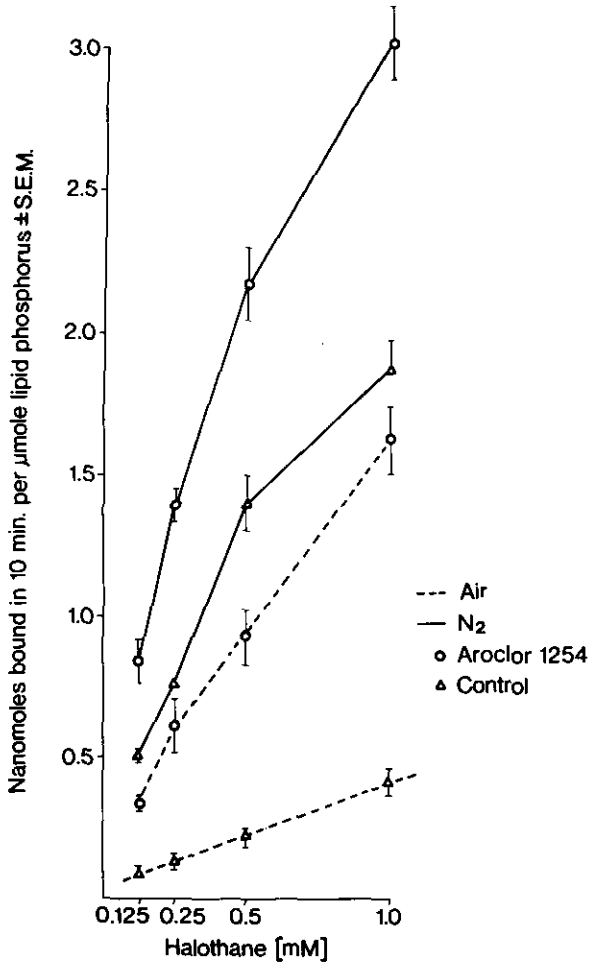

FIGURE 3. Effect of halothane concentration on the *in vitro* covalent binding of ^{14}C-halothane to microsomal lipids. Conditions as in Figure 1.

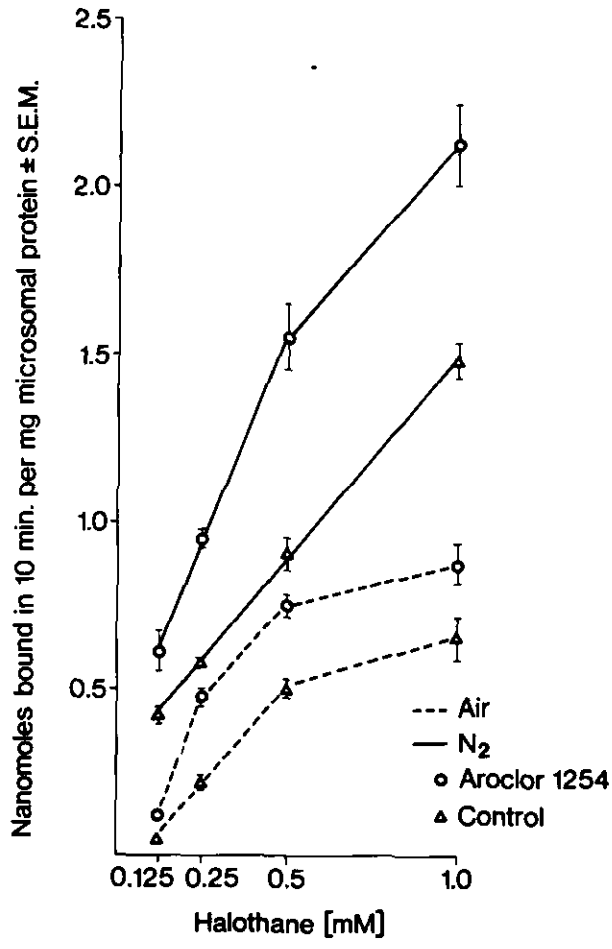

FIGURE 4. Effect of halothane concentration on the *in vitro* covalent binding of ^{14}C-halothane to microsomal protein. Conditions as in Figure 1.

that Aroclor 1254 pretreatment greatly reduced the yield of nuclei from whole liver and from isolated hepatocytes. Since this was not the case with phenobarbital pretreatment, the *in vivo* and isolated hepatocytes studies were conducted in phenobarbital and control rats. Both inducing agents were used for the microsomal incubations.

The two most important points to be gained from the data in Figures 1–4 are that microsomal induction increases covalent binding of halothane metabolites to both lipids and proteins and that a reduced O_2 tension, incubation under N_2, greatly enhances this binding to induced as well as control microsomes. Although only the data for Aroclor 1254 pretreatment is presented, microsomes from phenobarbital treated rats displayed an enhanced binding of similar magnitude. We are confident that covalent binding is related to metabolism, since omission of NADPH or heat denaturation of microsomes severely reduces binding. However, it should be emphasized that there was always a low level of binding in these blanks which was routinely subtracted. It can become significant at low concentrations of microsomal protein and short incubation periods (1–5 min).

From these initial studies, it was decided to incubate microsomes with calf thymus DNA for 30 min at a protein concentration of 4 mg/ml and a final halothane concentration of 0.1*mM*. This low concentration of halothane was chosen to avoid diluting the specific activity of the ^{14}C-halothane. At low specific activities any spurious radioactivity associated with DNA calculates as a high and misleading number of picomoles of ^{14}C bound to DNA.

The data in Table 1 summarize the results of four separate microsomal incubations that were performed to determine whether or not metabolites of halothane were covalently bound to calf thymus DNA. The ^{14}C activity associated with the DNA fraction was always less than 6 dpm/0.1 mg DNA.

Table 1. *In vitro* covalent binding of ^{14}C from ^{14}C-halothane to microsomal proteins and lipids.[a]

Pretreatment	^{14}C from microsomal proteins, pmole/mg		^{14}C from microsomal lipids, pmole/μmole		^{14}C from calf thymus DNA, pmole/mg DNA[b]	
	N_2	Air	N_2	Air	N_2	Air
Phenobarbital	346 ± 19	115 ± 25	1035 ± 19	586 ± 48	7 ± 4	5 ± 1
Aroclor 1254	320 ± 38	75 ± 16	1040 ± 40	290 ± 34	10 ± 2	5 ± 1
Control	65 ± 6	28 ± 3	272 ± 46	75 ± 25	9 ± 3	8 ± 3

[a]Incubations contained 0.1*mM* halothane. For the protein and lipids, background binding was determined by omission of NADPH and has been subtracted from the above values.
[b]The DNA values represent radioactivity associated with the isolated and extracted DNA and represent calculations from 6 dpm above background or less. The level of radioactivity associated with the DNA was not reduced by omission of NADPH and probably represents trace contamination rather than covalent binding.

There was no difference in DNA associated radioactivity when incubations were performed with or without the addition of NADPH or when induced microsomes were used. The binding to DNA as opposed to that of microsomal lipids or protein, is presented without subtraction of background counts (i.e., samples without NADPH) for comparison of DNA with lipids and proteins. The binding to protein and lipids was always studied to insure that the microsomal system was active and induced. These studies complement those presented in Figures 1–4, in that microsomal induction and incubation under N_2 enhance the binding to microsomal proteins and lipids. The quantity of radioactivity associated with microsomal RNA was similar to that associated with the DNA. Very few counts (< 10 dpm above background) were observed in the RNA fraction obtained by alkaline hydrolysis of the precipitated nucleic acids.

After some experimentation, the isolated hepatocytes proved to be a viable system for studying the bioactivation of halothane. In fact, for a 30 min incubation, it was possible to demonstrate enhanced covalent binding of halothane metabolites to both microsomal and nonmicrosomal lipids and proteins when the cells and ^{14}C-halothane were incubated under N_2 (Table 2). The covalent binding to proteins and lipids was greater in the cells obtained from phenobarbital induced animals. This enhancement was particularly evident in the microsomal fraction when the cells were incubated in air. In the study presented in Table 2. NADPH was added to the incubating cells to aid biotransformation since it may leak out of freshly isolated cells. Also Weddle et al. (*35*) reported that addition of NADPH enhanced CCl_4-induced lipoperoxidation in isolated hepatocytes.

We have only been partially successful in extracting DNA from isolated cells. The yield of DNA that is recovered is only about one-tenth the yield that would be expected on the basis of weight of liver. However, no radioactivity was present in the DNA or RNA that was obtained from the cells incubated with ^{14}C-halothane.

Table 3 summarizes our results to date on the *in vivo* covalent binding of metabolites of halothane to liver microsomal and nuclear macromolecules. Phenobarbital pretreatment resulted in a marked increase in covalent binding to microsomal proteins and lipids. This enhanced binding was most evident when the rats were maintained at 14% O_2 rather than atmospheric conditions (21% O_2).

Perhaps the most interesting observation was the tremendous increase in the binding to nuclear lipids in phenobarbital-pretreated animals maintained under hypoxic conditions. Although the radioactivity associated with this fraction was 80 dpm above background, the low level of nuclear phospholipids as compared to microsomal phospholipids inflates the value when binding is expressed as picomoles/μmole. The binding to nuclear protein in phenobarbital-pretreated hypoxic rats represents

Table 2. *In vitro* covalent binding of ^{14}C from ^{14}C-halothane to isolated rat liver cells.[a]

	^{14}C from protein, pmole/mg protein		^{14}C from lipids, pmole/μmole lipid P	
	Air	N_2	Air	N_2
Phenobarbital-treated				
Microsomal	96	182	379	817
Nonmicrosomal	12	75	77	678
Control cells				
Microsomal	13	60	43	446
Nonmicrosomal	5	23	32	281

[a]Incubations were for 30 min under air or N_2, at a final concentration of 0.06*mM* halothane. Each value represents the mean of two separate incubations.

Table 3. *In vivo* covalent binding of ^{14}C from ^{14}C-halothane to rat liver macromolecules.[a]

	Phenobarbital-pretreated rats		Control rats	
	Air	14% O_2[b]	Air	14% O_2[b]
^{14}C from microsomal protein, pmole/mg protein	20	80	8	22
^{14}C from microsomal lipids, pmole/μmole lipid P	32	180	7	20
^{14}C from nuclear proteins, pmole/mg protein	3[c]	12	3[c]	3[c]
^{14}C from nuclear lipids, pmole/μmole lipid P	12[c]	229	8[c]	9[c]
^{14}C from nucleic acids, pmole/mg	None		None	

[a]Each value represents the mean of two animals. Rats received 88 μCi/kg of ^{14}C-halothane intraperitoneally and were sacrificed after 2 hr.

[b]These animals were maintained at 14% O_2/86% N_2 for 1 hr prior to and after administration of halothane.

[c]These values were calculated from less than 10 dpm above background. No radioactivity was detected in RNA or DNA as isolated by the Kirby procedure.

about 50 dpm above background, but the binding appears much lower than to lipids due to the higher yield of nuclear protein.

No covalent binding of halothane or its metabolites to RNA or DNA was observed. In fact, no radioactivity was recovered in the purified DNA or RNA fractions.

Discussion

The primary objective of this study was to determine whether halothane or its metabolites covalently interact with DNA. Three separate bioactivation systems were used to answer this question: liver microsomal incubations, incubation with isolated hepatocytes, and *in vivo* administration. Procedures known to enhance the biotransformation and covalent binding of halothane to lipids and protein, microsomal enzyme induction, and hypoxia, were also used to optimize favorable conditions for bioactivation.

In only one system, microsomal incubation, was radioactivity observed in the isolated and purified DNA. However, these data are highly suspect because the level of radioactivity was only 6 dpm or less above background and omission of NADPH from the incubations did not decrease binding. Therefore, it is unlikely that the radioactivity associated with calf thymus DNA represents true covalent binding of a reactive intermediate of halothane to DNA. This radioactivity may represent a failure to extract halothane, one of its metabolites, or a contaminant in the ^{14}C-halothane completely from the DNA fraction, or alternately, contamination of DNA with traces of protein. However, it should also be emphasized that if binding does occur to DNA, one would expect it to be extremely low. Also, it is possible that a relatively minor NADPH-independent pathway for bioactivation of halothane may be present in the microsomal fraction.

As stated, the microsomal incubations were performed to optimize bioactivation and binding conditions. This is a highly nonphysiological system, since the endoplasmic reticulum and DNA are never in such intimate contact. Therefore, when no significant binding was found in this system, it is not surprising that binding to DNA was not observed in isolated hepatocytes or whole animal studies. Rao (*20*) also failed to observe covalent binding of halothane to DNA when administered intraperitoneally to phenobarbital treated rats. In addition, Baden et al. (*36*) reported that halothane was not mutagenic to *Salmonella typhimurium* when the bacteria and halothane were incubated with liver homogenates (900 *g* supernatant).

Of particular interest in the binding of halothane metabolite(s) to nuclear proteins and lipids, which became evident in the phenobarbital pretreated rats subjected to hypoxia. Since certain nuclear proteins are involved in DNA synthesis and repair, any alteration in their structure or activity may reduce the fidelity of DNA replication or alter the normal transcription process. Additional studies are being done to confirm these preliminary results. Also, studies with isolated nuclei should be performed, since it is known that nuclei can bioactivate certain xenobiotics (*37*).

We were able to confirm previous reports that microsomal enzyme induction and/or incubation under reduced oxygen tension enhances the *in vitro* covalent binding of halothane metabolites to microsomal protein and lipids (*17, 18, 38, 39*). In addition we were able to demonstrate enhanced covalent binding in isolated hepatocytes when the cells were isolated from phenobarbital-pretreated rats or incubated under N_2.

In 1976 Widger et al. (*18*) reported that there was

a significant increase in the *in vivo* covalent binding of halothane metabolites to microsomal protein and lipids when phenobarbital treated rats were subjected to reduced oxygen tension and exposed to ^{14}C-halothane via inhalation. Similar results were obtained in this study when the ^{14}C-halothane was administered intraperitoneally. These findings are of toxicological significance since it is now possible to consistently induce extensive centrilobular liver necrosis by halothane anesthesia (1%) in phenobarbital pretreated rats maintained at 14% O_2 and 85% N_2O or N_2 (*40, 41*).

In conclusion it is suggested that a continued effort be directed towards firmly establishing whether or not halothane or certain of its metabolites can interact with DNA. The only rational approach is to conduct similar studies with ^{14}C-halothane of much higher specific activity than currently available. This will permit the administration of higher doses as well as more radioactivity. In addition, attention should also be directed to the nature of halothane metabolites with nuclear proteins and lipids.

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