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Subcellular Binding of Halothane-1-^{14}C in Mouse Liver and Brain

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Abstract □ The extent of binding of halothane-1-^{14}C to mouse liver and brain 24 hr. after injection of a subanesthetic dose was measured utilizing several techniques: (*a*) exhaustive dialysis with phosphate buffer, (*b*) solvent extraction with 0.25 *M* sucrose and toluene, (*c*) protein precipitation with trichloroacetic acid, and (*d*) soxhlet extraction with methanol and benzene. A substantial portion of the radioactivity was not removed by these procedures and thus was bound both in liver and brain, the liver containing a greater amount of bound radioactivity on both an absolute basis and a protein content basis. The liver microsomal fraction, subjected to exhaustive dialysis, contained a higher specific activity (disintegrations per minute per milligram protein) than the whole homogenate, 9000×*g* pellet, or cytosol. The combination of trichloroacetic acid precipitation and soxhlet extraction with methanol and benzene (1:1) removed virtually all radioactivity from the brain, whereas 13% of the initial radioactivity remained in the liver as tightly bound material. Thus, halothane or a metabolite is tightly and probably covalently bound to a protein or other high molecular weight component in mouse liver but not in the brain.

Keyphrases □ Halothane, radiolabeled—*toxicity*, subcellular binding in mouse liver and brain □ Toxicity, halothane—subcellular binding in mouse liver and brain, radiolabeled, type of bonding □ Hepatitis—subcellular binding of halothane-1-^{14}C to mouse liver and brain, type of bonding, distribution of radioactivity

Halothane (1,1,1-trifluoro-2-bromochloroethane) possesses two properties of the ideal anesthetic: it is potent and nonexplosive. However, fatal hepatic necrosis has been reported in a small portion of exposed human subjects (1–4). While the incidence of hepatitis after halothane reportedly represents no greater a risk than surgical anesthesia itself (5), it is nevertheless a well-established untoward reaction that deserves investigation. The recurrence of hepatitis in sensitized anesthetists after exposure to only traces of halothane (6, 7) and the overall low incidence which increases with multiple exposures (5) suggest that halothane-induced hepatic damage is the result of an allergic reaction.

If halothane hepatitis is caused by a sensitization reaction, then halothane or a metabolite must form a hapten of an antigen by binding covalently to a macromolecule (8). Nonvolatile metabolites of halothane have been found to persist in animal liver (9) as well as human urine (10, 11). The presence of high molecular weight metabolites of halothane in liver and urine (12) indicates that a metabolite of halothane may be associated with a macromolecule; however, no direct evidence exists as to whether these metabolites are, in fact, covalently bound. In support of this suggestion, other halogenated hydrocarbons, such as carbon tetrachloride (13) and bromobenzene (14), do form covalent bonds with endogenous substances. Therefore, the binding character of halothane-1-^{14}C *in vivo* in mice was investigated.

EXPERIMENTAL

Dosage and Tissue Preparation—Female Swiss Webster albino mice[1] (28–30 g.) were fasted for 24 hr., and 0.5 mg. of halothane-1-^{14}C in saline[2], containing 4 μc. of radioactivity was administered by intraperitoneal injection. Twenty-four hours later, the mice were sacrificed, the livers were perfused with cold 0.25 *M* sucrose, and both the livers and brains were removed and placed in ice-cold 0.25 *M* sucrose. Ten percent homogenates of these organs were prepared in cold 0.25 *M* sucrose, using a Potter–Elvehjem tissue grinder[3]. An aliquot of these homogenates was removed for analysis of bound radioactivity and protein, and the remainder was centrifuged at 9000×*g* (average) in a refrigerated centrifuge[4] for 20 min. The pellet was removed for analysis, and the supernate was recentrifuged at

[1] Flow Research Laboratories, Dublin, Va.
[2] New England Nuclear, Boston, Mass.
[3] No. 62400, VWR Scientific Co., Baltimore, Md.
[4] Model HR-1, International Equipment Co., Boston, Mass.

Table I—Effect of Trichloroacetic Acid Precipitation and 24-hr. Soxhlet Extraction with Methanol–Benzene (1:1) on the Radioactivity Found in the Livers and Brains of Mice Pretreated with Halothane-1-^{14}C (0.5 mg.) 24 hr. before Sacrifice

Organ	Trichloroacetic Acid Precipitate, Percent Total Organ Disintegrations per Minute Remaining, Mean ± *SE*[a]	Liver–Brain Ratio	Extracted Protein, Percent Total Organ Disintegrations per Minute Remaining, Mean ± *SE*[a]	Liver–Brain Ratio
Liver[b]	25.3 ± 1.6	7.4	13.1 ± 0.7	21.8
Brain[b]	3.4 ± 0.4		0.6 ± 0.04	

[a] Standard error of the mean. [b] $n = 6$.

78,480×g (average) in an ultracentrifuge[5] for 160 min. to prepare microsomes and cytosol, which were also analyzed for bound radioactivity and protein.

Exhaustive Dialysis—The livers and brains from six mice were combined and separated into the fractions as previously described. Each fraction was dialyzed in a visking tubing[6] against 4000 volumes of 0.01 *M* phosphate buffer (pH 7.2), which flowed continuously for 18 hr. at 4°[7]. Protein and radioactivity contents were determined both before and after dialysis. Protein was determined using the Lowry *et al.* (15) method with bovine serum albumin as the standard. The radioactivity was measured in a liquid scintillation spectrometer[8], and disintegrations per minute were calculated using toluene-^{14}C as an internal standard.

Washing, Extraction, and Centrifugation—The 9000×g pellet and microsomal fractions were separated from the 9000×g supernate and cytosol, respectively, resuspended three times in an equivalent volume of cold 0.25 *M* sucrose, and recentrifuged to remove residual water-soluble radioactivity. The washed 9000×g and microsomal pellets were then suspended in 10 times their volume of toluene, shaken manually, and recentrifuged to extract soluble nonpolar radioactivity.

Soxhlet Extraction—Protein was precipitated by the addition of an equal volume of 1 *M* trichloroacetic acid to a homogenate and removed by centrifugation at 2000 r.p.m. for 10 min. in a refrigerated centrifuge[9]. The denatured protein was washed three times with an aqueous 5% trichloroacetic acid solution and recentrifuged. An aliquot of the washed protein precipitate was taken for the determination of bound radioactivity. The remaining protein precipitate was placed in a thimble and extracted with methanol–benzene (1:1) for 24 hr.[10] in a microsoxhlet apparatus[11]. The final extracted protein was solubilized[12] and then analyzed for radioactivity.

RESULTS AND DISCUSSION

If halothane or a metabolite binds covalently with a hepatic macromolecule *in vivo*, then a portion of the liver radioactivity would not be dialyzable. To test this hypothesis, halothane-1-^{14}C was administered to mice. Then the brains and livers were removed, homogenized, fractionated, and subjected to exhaustive dialysis. The amount of nondialyzable radioactivity was determined. The results of this experiment (Figs. 1 and 2) indicate that a significant amount of nondialyzable radioactivity remains after 24 hr. in all liver fractions. Trifluoroacetate, a known metabolite of halothane (16, 17) probably constitutes most of the dialyzable fraction. The distribution of halothane-1-^{14}C-derived radioactivity in the liver was shown to predominate in the microsomal fraction, which had the highest amount of protein-associated counts. These results show that binding occurs in the liver, the location of halothane metabolism (18), and suggest that a metabolite, possibly formed in the endoplasmic reticulum, is the moiety bound.

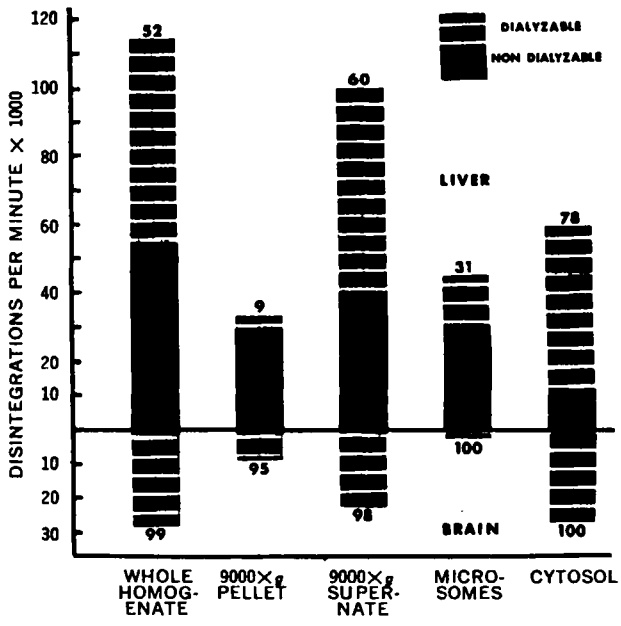

Figure 1—*Relationship between dialyzable and nondialyzable radioactivity in liver and brain subcellular fractions pooled from six mice pretreated with 0.5 mg. of halothane-1-^{14}C 24 hr. before sacrifice. (Numbers represent percentage of initial radioactivity that was dialyzable.)*

It is possible that either nonpolar halothane or a lipid-soluble metabolite is not removed by exhaustive dialysis against phosphate buffer. To test this alternative, the microsomal fraction was washed three times with 0.25 *M* sucrose and extracted with 10 volumes of toluene to remove lipid. This procedure removed 60% of the radioactivity from the microsomes, while only 31% was removed by dialysis. These results suggest that while halothane or a metabolite may be sequestered in lipid, at least 40% is bound to other tissue components. The data would also support the possibility that a portion of the radioactivity was not dialyzable against an aqueous buffer, because it was unmetabolized halothane. However, this is unlikely since dialysis did remove virtually all of the radioactivity from the brain, where small amounts of unchanged halothane would also be expected to reside under the conditions of the experiment. A more likely explanation is that part of the binding occurs on lipoprotein which, while not dialyzable, is nevertheless soluble in toluene.

To measure only protein-bound radioactivity, the liver and brain homogenates were treated with trichloroacetic acid followed by exhaustive extraction with solvent. Preliminary results of this trichloroacetic acid–soxhlet method showed that maximal extraction of radioactivity occurred at 24 hr., using a solvent system of equal amounts of methanol and benzene. Therefore, these conditions were used in all extractions. The results (Table I) show that 25.3% of the liver and 3.4% of the brain initial radioactivity were precipitated by trichloroacetic acid. The soxhlet technique extracted an additional 12.2% from the trichloroacetic acid precipitate of liver, leaving 13.1% of the initial radioactivity as tightly bound material. In contrast, soxhlet extraction of trichloroacetic acid precipitates from brain removed virtually all of the radioactivity in this tissue. The liver-to-brain ratio of radioactivity remaining after trichloroacetic acid precipitation increased from 7.4 to 21.8 after soxhlet extraction. This 22-fold higher amount of nonextractable radioactivity in the liver relative to the brain demonstrates that factors other than the initial concentration of halothane in a tissue determine the extent of binding.

[5] Model L-2, Rotor 30, Beckman Instruments, Palo Alto, Calif.
[6] No. 077-024, W. H. Curtin & Co., Rockville, Md.
[7] Dialysis for 24 additional hr. did not significantly reduce the amount of nondialyzable radioactivity, so this technique was considered to be exhaustive.
[8] Tri-Carb model 3003, Packard Instrument Co., Downers Grove, Ill. The scintillation cocktail consisted of: 2,5-diphenyloxazole, 0.55% w/v; 1,4-bis[2-(5-phenyloxazolyl)]benzene, 0.01% w/v; Triton X-100, 25% v/v; and toluene, 75% v/v.
[9] Model PR-2, International Equipment Co., Boston, Mass.
[10] These parameters were initially examined using various solvent systems (methanol, methanol–benzene (1:1), or benzene) for 6, 12, 24, or 48 hr.
[11] VWR Scientific Co., Baltimore, Md.
[12] Using hyamine hydroxide, Packard Instrument Co., Downers Grove, Ill.

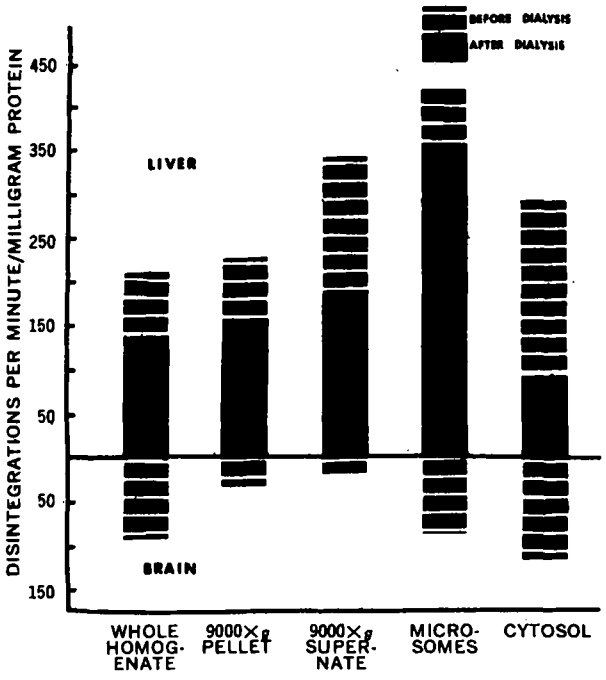

Figure 2—*Effect of dialysis on the specific activity (disintegrations per minute per milligram protein) of pooled liver and brain subcellular fractions from six mice pretreated with 0.5 mg. of halothane-1-^{14}C 24 hr. before sacrifice.*

Since exhaustive dialysis and centrifugal washing do not distinguish between reversible and irreversible binding, the method of solvent (soxhlet) extraction was employed. A similar technique has been used to demonstrate the covalent binding of bromobenzene in the liver (14). The trichloroacetic acid–soxhlet method extracted more halothane-derived radioactivity from whole homogenates of liver than either dialysis or washing, indicating that some of the radioactivity remaining after the use of the latter methods was possibly due to reversibly bound metabolites or that the labeled material may have been covalently bound to lipoprotein. Although trichloroacetic acid precipitation and solvent extraction will remove all of the radioactivity except that which is very tightly bound, it in itself does not constitute a completely rigorous proof of covalent binding. Therefore, protein digestion and the identification of amino acid-bound activity are currently being investigated.

If one proposes a hapten–antigen model for the hepatotoxicity of halothane, then the initial component of this hypothesis would require the covalent attachment of a small molecular weight moiety to some endogenous macromolecule. The altered molecule thus produced could elicit a delayed hypersensitivity *via* sensitized lymphocytes. The appearance of high levels of bound radioactivity in the membraneous fractions of the liver (*i.e.*, 9000×*g* pellet and microsomes) and the association of irreversibly and probably covalently bound radioactivity with protein in the whole liver homogenates subjected to soxhlet extraction are consistent with this suggestion that the altered macromolecule is a membrane protein. Furthermore, the protein-bound radioactivity found in the cytosol of the liver could possibly be the basis for transferral of antigenic material to sites of antibody formation.

From the results of other investigators (9, 10, 12, 17, 18), it would appear that halothane undergoes similar metabolic pathways in humans, mice, and rats. The finding of covalently bound radioactivity in hepatic tissue is consistent with the sensitivity theory and would also explain the low incidence of halothane-induced hepatitis in humans and the relative failure of investigators to reproduce hepatic injury in animals. Variability in both the immunologic and metabolic responses between and among various species may further explain these findings, and it is also possible that the binding phenomenon itself may be highly variable.

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