Biochemical Pharmacology, Vol. 23, pp. 189–195. Pergamon Press, 1974. Printed in Great Britain.

LIVER INJURY FOLLOWING HALOTHANE ANESTHESIA IN PHENOBARBITAL-PRETREATED RATS*

EDWARD S. REYNOLDS† and MARY TREINEN MOSLEN

Departments of Pathology, Peter Bent Brigham Hospital and Harvard Medical School, Boston, Mass. 02115, U.S.A.

(*Received* 29 *March* 1973; *accepted* 29 *June* 1973)

Abstract—Linear foci of centrolobular necrosis on the posterior aspect of the liver were consistently produced by 5 hr of anesthesia with 0·85% halothane in young male rats pretreated with phenobarbital (PBT: 1 g/l. drinking water) for 30 days. Microsomes isolated from livers of these animals immediately upon completion of anesthesia had elevated lipid-conjugated diene and decreased cytochrome P-450 content and ^{14}C-glycine incorporation into protein *in vivo*. Lipid-conjugated dienes and glycine incorporation, but not P-450, returned to pre-anesthesia levels within 2 hr. None of these alterations were evident after anesthesia in livers of saline-pretreated rats (0·23 g NaCl/l. drinking water). Aside from the persistence of decreased P-450 in PBT-pretreated animals, alterations in lipid-conjugated diene and glycine incorporation did not occur after repeated anesthesia at 48-hr intervals, nor was hepatic necrosis as evident, although anesthesia recovery times increased significantly. These findings indicate that metabolites of halothane are directly hepatotoxic and that repeated anesthesia with halothane at 2-day intervals decreases its hepatotoxic effect—presumably by interfering with its metabolism to toxic metabolites by the endoplasmic reticulum. Similar morphologic lesions can also be produced in PBT-pretreated animals by minute doses of CCl_4.

HALOTHANE ($CF_3CHBrCl$) anesthesia has occasionally been associated with acute hepatic injury in man especially after multiple exposures.[1–3] Reproduction of the lesion in animals with consistency has met with little if any success despite multiple experimental manipulations including length or frequency of exposure and drug or surgical pretreatment in the varied species tested (horse, guinea pig, sheep, rat and mouse).[4–12] In several studies where halothane was administered intraperitoneally, morphologic and biochemical alterations resulted;[5,6] however, results following this method of administration must be examined with caution, for Topham and Tucker[13] described fibrosis, gross peritonitis and subcapsular hepatic inflammation after intraperitoneal injections of halothane.

The hepatotoxicity of halothane has been attributed to its transformation within the endoplasmic reticulum (ER) of the liver cell into a reactive metabolite, specifically a free radical.[2,14] Numerous investigators[4–7] have, therefore, sought parallels between the morphologic and biochemical alterations induced by halothane and carbon tetrachloride, whose activation to a toxin is generally considered to occur through

* This work was supported by U.S. Public Health Service Grants HL-06370 and AM-16183. Halothane was generously supplied by Ayerst Laboratories.

† E.S.R. is a Research Career Development Awardee (6-K-3-GM-7309) of the National Institute of General Medical Sciences, National Institutes of Health, U.S. Public Health Service.

formation of the trichloromethyl radical ($\cdot CCl_3$).[15–17] Production of the toxic metabolites of carbon tetrachloride and other halocarbons is enhanced by pretreatment with phenobarbital (PBT), a potent mixed function oxidase inducer.[18–20]

In this study we found rats pretreated for 30 days with PBT consistently developed focal hepatic necrosis within 24 hr after their first exposure to the anesthetic.

EXPERIMENTAL

Animals. Male Charles River rats (Wilmington, Mass.) of from 50 to 75 g and with free access to Purina Chow were given either sodium phenobarbital (1 g/l.) or an equimolar amount of sodium as NaCl in drinking water for 30 days. Daily consumption of PBT was approximately 10 mg/100 g rat.

Treatment. All animals in each experimental group (saline-control, saline-anesthetized, PBT-control and PBT-anesthetized) were fasted for 16 hr prior to each anesthesia period and if not sacrificed immediately, for 16 hr prior to sacrifice.

PBT and saline-pretreated animals in groups of eight to ten were anesthetized together for 5 hr one to six times at 48-hr intervals in a single 0·025 m^3 plexiglass chamber from a calibrated vaporizer (Vernitrol). Stage III anesthesia was induced with 1% halothane in oxygen for the first 30 min and maintained with 0·85% halothane for the remainder. Temperature of the anesthetic chamber was maintained at 22° ± 2°, and oxygen flow kept at 4 l./min. Total dose of halothane inhaled during this anesthesia period is estimated to range between 50 and 75 m-moles halothane/kg rat based on the respiratory data of Guyton.[21] Control animals were exposed to room air during this period. ^{14}C-glycine (uniformly labeled, 115 mCi/m-mole) (New England Nuclear Corp.) (25 μCi/animal) was administered intraperitoneally 15 min prior to sacrifice. Animals receiving CCl_4 were given 26 or 83 μmoles CCl_4/kg by stomach tube 24 hr prior to sacrifice.

Portions of the right anterior hepatic lobe of animals killed by exsanguination were excised for histologic study and the remaining liver was immediately perfused via the hepatic vein with ice cold (0–4°) 0·25 M sucrose. The tissue was then pulped in a hand press and homogenized in 5 vol. of 0·25 M sucrose. A small fraction of this homogenate was put aside to measure total ^{14}C-glycine incorporation. The remainder was centrifuged at 12,500 *g* for 15 min and its supernatant recentrifuged at 12,500 *g* for 15 min to remove residue and mitochondria. The resultant supernatant was then recentrifuged at 105,000 *g* for 30 min and the pellet, which constituted the microsomal fraction, resuspended by homogenization in 0·25 M sucrose.

Biochemical studies. All chemical and functional parameters of liver microsomes were determined in duplicate on aliquots of microsomes from each experimental animal.

Enzymatic studies were done on the day of sacrifice.

Content of microsomal cytochromes P-450 and b_5 was measured as described by Dallner *et al.*[22], oxidative *N*-demethylation of 4-dimethylaminoantipyrine by the method of Orrenius,[23] and lipid-conjugated diene content and ^{14}C-glycine incorporation into microsomal protein *in vivo* measured according to the methods of Sell and Reynolds.[24] Protein was estimated for the enzyme assays by the method of Lowry *et al.*[25] and lipid phosphorus determined by the Fiske–SubbaRow method after fusion of the sample with ethanolic $Mg(NO_3)_2$.[26,27]

Statistics. Anesthesia recovery time was taken as the interval between removal of the animal from the halothane-containing chamber and return of the "righting" reflex. We used Student's *t*-test as a test of null hypothesis with $P < 0.05$.

RESULTS AND DISCUSSION

Morphologic lesion. Grossly, linear streaks of hemorrhagic necrosis consistently appeared beneath the capsule of the posterior surface of the liver in PBT animals sacrificed within 24 hr after 5 hr of halothane anesthesia (Fig. 1, a and b). A comparable lesion was rarely observed (less than 2 per cent) in saline-pretreated anesthetized animals and never in unanesthetized animals. Gross appearance of the subcapsular lesions was striking: from 10–15 mm in length, 2–3 mm wide, the streaks appeared to radiate from the hilum of the liver where radicles of the portal vein enter. At 24 hr after anesthesia these necrotic regions of hepatic parenchyma were not necessarily distributed about the central veins, in contrast to striking vacuolization of parenchymal cells seen about central veins in adjacent lobules. Identical lesions of similar distribution were also produced in similarly pretreated PBT animals by feeding minute doses of CCl_4 (as low as 4 mg/kg animal) (Fig. 1c). The reasons for the subcapsular localization of the lesions on the posterior surfaces of the liver lobule after both halothane and small doses of CCl_4 in PBT animals are enigmatic. The lesions appear to be related to thin-walled vessels and may form in relationship to branches of the hepatic venous or lymphatic outflow tracts.

To our surprise, repeated exposure of animals to the anesthetic at 48-hr intervals did not induce changes in the control animals, or aggravate the lesion in PBT animals. In fact, incidence of subcapsular streaks of necrosis following halothane in PBT-pretreated animals decreased with repeated anesthesia and was rarely observed in animals sacrificed 24 hr after the fourth and sixth exposures to the anesthetic.

Stenger and Johnson[6] described similar lesions 24 hr after induction of anesthesia by intraperitoneal injection of halothane in PBT-pretreated rats. Hughes and Lang[12] reported areas of focal centrolobular and midzonal necrosis in seven of fifty guinea pigs after repeated 1-hr halothane anesthetics at 2-week intervals. In contrast to our findings, lesions in the guinea pigs increased in frequency and severity with repeated anesthesia.

Biochemical changes. Halothane anesthesia in PBT-pretreated rats was accompanied by a reduction in cytochrome P-450, an integral part of the mixed function oxidase system, apparent immediately upon cessation of anesthesia and at every other interval examined (Table 1) including at 24 hr after the sixth exposure. In contrast, cytochrome b_5 content was not affected by halothane in either PBT- or saline-pretreated animals, and there was no apparent loss of cytochrome P-450 in the non-PBT group. Decreased cytochrome P-450 in the halothane-anesthetized PBT-pretreated animals was not accompanied by comparable decreased oxidative *N*-demethylation of antipyrene which is also controlled by the mixed function oxidase system. Although halothane reversibly decreases the absorption spectrum of the P-450–CO complex formation by 20 per cent *in vitro*,[28] we consider the prolonged drop in P-450 content measured in this study attributable to destruction of the cytochrome as decreased contents are also measured 24 hr after the sixth anesthesia.

Table 1. Effect of halothane anesthesia on liver endoplasmic reticulum in saline- and PBT-pretreated animals*

Halothane anesthesia†		Cytochrome		Oxidative *N*-demethylation	Lipid-conjugated diene	^{14}C-glycine incorporation *in vivo*
No. of anesthesias	Interval following (hr)	b_5 (μmoles/g protein)	P-450	CH_2O (μmoles/g protein/min)	(μmoles/g lipid)	(cpm/mg protein)
Saline pretreatment:						
Control (7)		0·57 ± 0·03	0·68 ± 0·04	2·4 ± 0·2	11·1 ± 1·8	776 ± 91
1 (3)	0	0·53 ± 0·03	0·57 ± 0·05	2·2 ± 0·2	13·5 ± 1·8	527 ± 100
2 (6)	0	0·52 ± 0·02	0·58 ± 0·07	1·9 ± 0·1‡	14·2 ± 1·5	619 ± 363
6 (3)	0	0·58 ± 0·04	0·70 ± 0·04	1·7 ± 0·2‡	9·7 ± 1·3	nd§
Phenobarbital pretreatment:						
Control (7)		0·80 ± 0·04‖	1·67 ± 0·07‖	4·6 ± 0·4‖	14·0 ± 1·0	373 ± 43‖
1 (5)	0	0·77 ± 0·02	1·25 ± 0·03‡	4·6 ± 0·5	18·7 ± 1·4‡	199 ± 23‡
1 (4)	2	0·74 ± 0·02	1·17 ± 0·09‡	3·8 ± 0·4	15·8 ± 0·9	662 ± 196
2 (5)	0	0·78 ± 0·03	0·97 ± 0·11‡	4·2 ± 0·8	16·2 ± 1·6	279 ± 26
6 (3)	0	0·83 ± 0·04	1·26 ± 0·12‡	3·1 ± 0·4	14·4 ± 0·9	nd

* Mean ± S.E.M.
† Five-hr anesthesia (0·85% halothane) repeated at 48-hr intervals. Number in parentheses is number of animals.
‡ Difference between experimentals and respective control $P < 0·05$.
‖ Difference between control groups $P < 0·005$.
§ Not done.

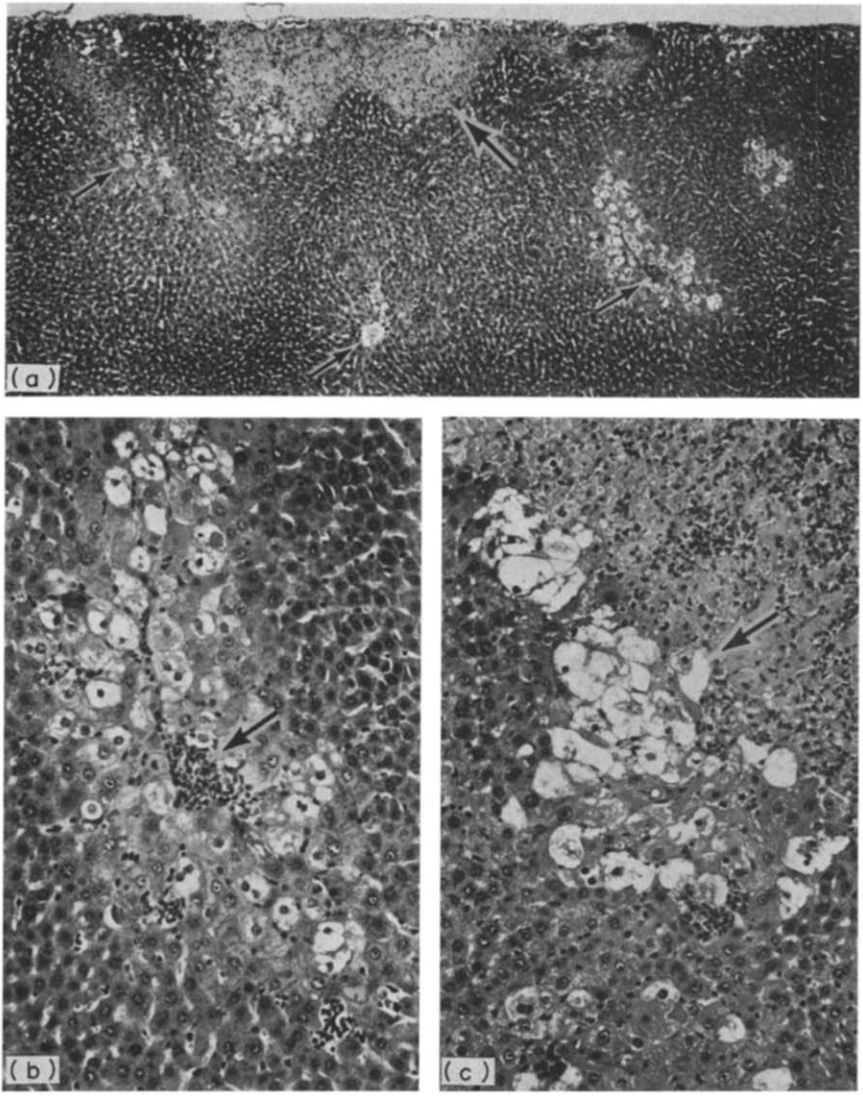

FIG. 1. (a and b). Posterior aspect of right anterior lobe of liver of PBT-pretreated rat 24 hr after a single exposure to halothane. (a) Cross-section through subcapsular necrotic streak (top; big arrow). Vacuolated parenchymal cells are seen about central veins deeper in the hepatic parenchyma (small arrows). Few vacuolated cells are seen about the central vein deepest in the hepatic parenchyma (bottom center) ($\times$ 50). (b) Higher power of a centrolobular region in Fig. 1a. Vacuolated hepatic parenchymal cells ring the central vein (arrow). Many of these cells have pyknotic nuclei ($\times$ 200). (c) Portion of liver parenchyma from posterior aspect of right anterior lobe of liver from a PBT-pretreated rat fed CCl_4 (12·8 mg/kg) 24 hr previously. Necrotic parenchyma—at upper right, vacuolated parenchyma about central vein (arrow) ($\times$ 200).

PBT animals sacrificed within minutes after their first anesthetic also had increased levels of lipid-conjugated diene, a transient indicator of peroxidation of structural lipids of microsomal membranes.[29,30] Concomitantly there was a significant decrease in incorporation of ^{14}C-glycine into proteins of the ER (isolated as microsomes) in spite of normal uptake of ^{14}C-glycine by the liver as determined by measurement of radioactivity of acid soluble extracts of homogenate. Conjugated diene levels returned to normal within 2 hr, while ^{14}C-glycine incorporation rebounded to a level approximately twice that of controls. Similar changes in microsomal composition and function were not seen after the second exposure to halothane, nor observed in the saline-pretreated anesthetized group.

We examined only the ER (as microsomes) for biochemical alterations because halothane is dehalogenated by the mixed function oxidase system of this cellular fraction.[31] This system plays a primary role in the conversion of other groups of halocarbons to toxic metabolites via free radical mechanisms.[32] As is the case with CCl_4,[29] halothane's destruction of cytochrome P-450 accompanied by transient increases in diene conjugation of microsomal lipids and disruption of protein synthesis *in vivo* supports the concept of a free radical mechanism of metabolism and of injury for halothane. Further support is provided by studies of Brown[7] who reported that increases in lipid-conjugated diene of microsomes isolated from livers of PBT-treated rats after halothane anesthesia were inhibited by prior administration of free radical scavengers DPPD (*N,N'*-diphenyl-*p*-phenylene diamine) and SKF 525-A (2-diethylaminoethyl-2,2-diphenyl-1-valerate HCl).

Anesthesia recovery times. Anesthesia recovery times in multiple separate experiments were definitely prolonged in the PBT group after the second, third and fourth exposures, gradually decreasing thereafter (Fig. 2). In contrast, anesthesia recovery times of saline-pretreated animals abruptly decreased after the second exposure to

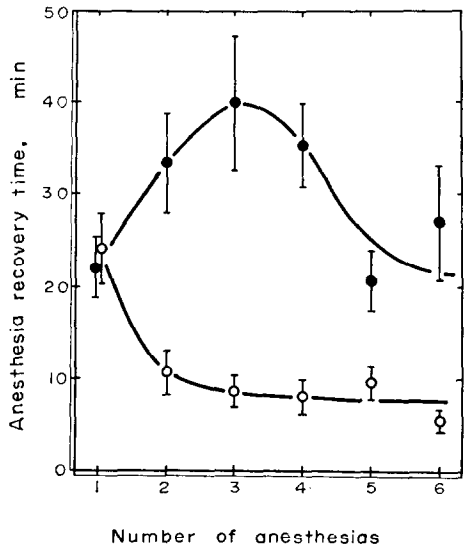

FIG. 2. Anesthesia recovery times after repeated halothane anesthesia in PBT- (●) and saline- (○) pretreated rats. Each point represents data from at least eleven animals; results from three separate experiments were pooled. I is the standard error of the mean.

less than half that of animals anesthetized for the first time. Animals repeatedly anesthetized maintained or gained weight, kept themselves clean and displayed "normal" activity.

As anesthesia recovery time may in some way reflect the ability of the animal to clear blood and brain of halothane, a process in which the liver participates by metabolizing residual anesthetic, the results obtained here represent a paradox. Phenobarbital pretreatment enhances metabolism of relatively small amounts of halothane.[31] Thus PBT-pretreated animals should eliminate halothane more rapidly and awaken faster than animals pretreated with saline.

These seemingly paradoxical findings can be rationalized if the initial 5-hr anesthetization with halothane transiently decreases the ability of the liver to metabolize halothane. Decreased ability to metabolize halothane may upon re-anesthetization at short intervals result in increased recovery times and an apparent protection against liver injury. Thus the protective effect of the initial anesthetization with halothane may operate by a mechanism similar to that by which a small dose of CCl_4 protects against a subsequent normally lethal dose of the same chlorocarbon[33]—that is by partially destroying the mixed function oxidase system of liver endoplasmic reticulum.

Shortening of the anesthesia recovery times in the saline-pretreated group in the absence of demonstrable lesions suggests that these animals adapt to the repeated short-interval exposure by increased rates of metabolism of halothane via alternate pathways.[31,34–36]

This demonstration of an immediate hepatotoxic effect of halothane anesthesia and its relationship to prior conditioning of the animal suggests that similar relationships may be operative in man and account for the seemingly random incidents of "halothane hepatitis."

REFERENCES

1. J. P. BUNKER, W. H. FORREST, JR., F. MOSTELLER and L. D. VANDAM, *The National Halothane Study*. U.S. Govt. Printing Office, Washington, D.C. (1969).
2. F. M. T. CARNEY and R. A. VAN DYKE, *Anesth. Analg.* **51,** 135 (1972).
3. W. D. REED and R. WILLIAMS, *Br. J. Anaesth.* **44,** 935 (1972).
4. O. ALMERSJÖ, *Acta chir. scand.* (suppl.) **51,** 135 (1972).
5. D. C. DAVIS, D. H. SCHROEDER, T. E. GRAM, R. L. REAGAN and J. R. GILLETTE, *J. Pharmac. exp. Ther.* **177,** 556 (1971).
6. R. J. STENGER and E. A. JOHNSON, *Proc. Soc. exp. Biol. Med.* **140,** 1319 (1972).
7. B. R. BROWN, JR., *Anesthesiology* **36,** 458 (1972).
8. M. B. CHENOWETH, B. K. J. LEONG, G. L. SPARSCHU and T. R. TORKELSON, in *Cellular Biology and Toxicity of Anesthetics* (Ed. B. R. FINK), p. 275. Williams & Wilkins, Baltimore (1972).
9. C. GOPINATH, R. S. JONES and E. J. H. FORD, *J. Path. Bact.* **102,** 107 (1970).
10. M. W. HULL and M. G. REILLY, *Am. J. vet Res.* **29,** 1161 (1968).
11. J. RAVENTOS, *Br. J. Pharmac. Chemsther.* **11,** 394 (1956).
12. H. C. HUGHES, JR. and C. M. LANG, *Anesthesiology* **36,** 466 (1972).
13. J. C. TOPHAM and M. J. TUCKER, *Br. J. Anaesth.* **44,** 665 (1972).
14. A. STIER, *Anesthesiology* **29,** 388 (1968).
15. T. F. SLATER, *Nature, Lond.* **209,** 36 (1966).
16. E. GORDIS, *J. clin. Invest.* **48,** 203 (1969).
17. T. C. BUTLER, *J. Pharmac. exp. Ther.* **134,** 311 (1961).
18. A. A. SEAWRIGHT and A. E. M. MCLEAN, *Biochem. J.* **105,** 1055 (1967).
19. A. E. M. MCLEAN, *Br. J. exp. Path.* **51,** 317 (1970).
20. B. B. BRODIE, W. R. REID, A. K. CHO, G. SIPES, G. KRISHNA and J. R. GILLETTE, *Proc. natn. Acad. Sci. U.S.A.* **68,** 160 (1971).
21. A. C. GUYTON, *Am. J. Physiol.* **150,** 70 (1947).
22. G. DALLNER, P. SIEKEVITZ and G. E. PALADE, *J. Cell. Biol.* **30,** 97 (1966).

23. S. ORRENIUS, *J. Cell. Biol.* **26,** 713 (1965).
24. D. A. SELL and E. S. REYNOLDS, *J. Cell. Biol.* **41,** 736 (1969).
25. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
26. C. H. FISKE and Y. SUBBAROW, *J. biol. Chem.* **66,** 375 (1925).
27. B. N. AMES and D. T. DUBIN, *J. biol. Chem.* **235,** 769 (1960).
28. R. A. VAN DYKE and L. RIKANS, in *Cellular Biology and Toxicity of Anesthetics* (Ed. B. R. FINK), p. 193. Williams & Wilkins, Baltimore (1972).
29. R. O. RECKNAGEL and A. K. GHOSHAL, *Lab. Invest.* **15,** 132 (1966).
30. E. S. REYNOLDS and H. J. REE, *Lab. Invest.* **25,** 269 (1971).
31. R. A. VAN DYKE, *J. Pharmac. exp. Ther.* **154,** 364 (1966).
32. E. S. REYNOLDS and M. T. MOSLEN, in *Free Radicals in Biology and Medicine* (Eds. H. DEMOPOULOS and W. PRYOR), Vol. II, Academic Press, New York, in press.
33. E. A. GLENDE, JR., *Biochem. Pharmac.* **21,** 1697 (1972).
34. R. A. VAN DYKE, M. B. CHENOWETH and A. VAN POZNAK, *Biochem. Pharmac.* **13,** 1239 (1964).
35. A. STIER, H. ALTER, O. HESSLER and K. REHDER, *Anesth. Analg.* **43,** 723 (1964).
36. I. C. GEDDES, *Laval med.* **42,** 574 (1971).