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Hepatic Necrosis Caused by Halothane and Hypoxia in Phenobarbital-treated Rats

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Other studies have indicated that hypoxia enhances the binding of halothane metabolites to components of the hepatic microsomal fraction. The authors pretreated Wistar rats with phenobarbital, 75 mg/kg, daily for four days to induce the hepatic drug-metabolizing enzyme system and subsequently made them hypoxic ($F_{I_{O_2}}$ = .08) while they were receiving halothane, 0.6 per cent. Centrilobular hepatic necrosis was well developed by six hours following exposure, and early stages of resolution were evident by 48 hours. Hemorrhage occurred within the necrotic areas, and leukocytosis was not prominent. Normal rats or those depleted of hepatic glutathione did not experience hepatic necrosis when made hypoxic and given halothane. Rats receiving halothane in adequate oxygen ($F_{I_{O_2}}$ = 0.50) after phenobarbital pretreatment showed no hepatic necrosis. Plasma fluoride values were normal (<2 μM) immediately upon completion of halothane exposure when the animals received adequate oxygen, whether or not they had received phenobarbital pretreatment. When halothane was administered to hypoxic animals, plasma fluoride values averaged 19 ± 2 μM (Mean ± SEM), and pretreatment with phenobarbital caused a further increase in plasma fluoride to 24 ± 2 μM. Plasma fluoride values thus increased indicate that the production of defluorinated halothane metabolites is caused primarily by hypoxia. In animals showing hepatic necrosis, cytochrome P-450 was decreased but cytochrome b_5 was not changed. The selective decrease in cytochrome P-450 suggests a specific, persistent involvement of this enzyme rather than a generalized destruction of microsomal enzymes. It is concluded that halothane anesthesia causes hepatic necrosis in rats when combined with phenobarbital stimulation of the hepatic drug-metabolizing enzyme system and hypoxia. (Key words: Anesthetics, volatile: halothane. Biotransformation: enzyme induction; fluorometabolites. Enzymes: cytochrome b_5; cytochrome P-450. Hypnotics: barbiturates, phenobarbital. Hypoxia. Liver: glutathione; microsomes; necrosis. Metabolism: anesthetic; microsomes; enzyme induction.)

FOLLOWING THE INTRODUCTION of halothane in 1956, clinical reports of the sporadic occurrence of massive hepatic necrosis after its use prompted the National Halothane Study[1] in 1965. This large retrospective study of the occurrence of hepatic failure after anesthesia attempted to relate the type of anesthesia and operation to the incidence of massive hepatic necrosis. The study neither proved or disproved a relationship between hepatic necrosis and halothane administration, and reports of hepatic necrosis after administration of halothane have recurred sporadically to the present. Much of the early work on this problem assumed a chemical etiology (*i.e.*, that halothane or one of its metabolites was acting as a hepatotoxin). This approach was strengthened by the demonstration of Van Dyke, Chenoweth and Van Poznack, in 1964, that several volatile anesthetics, including halothane, are metabolized primarily in the liver.[2] The principal metabolic products of halothane metabolism, chloride, bromide and trifluoroacetic acid, have not been shown to be hepatotoxic. Possible intermediates in their production have eluded detailed study until recently.[3]

Other reports[4–6] have suggested that halothane could produce hepatic damage by a hypersensitivity mechanism, perhaps by halothane or a metabolite acting as a hapten. Investigations of such hypersensitivity mechanisms have failed to demonstrate hepatic damage, although studies have shown cutaneous sensitivity[7] and *in-vitro* evidence of antibody formation.[8]

More recently, Cohen *et al.*[9] demonstrated three metabolites in human urine following the administration of halothane. One of these was a difluoroethane conjugate of cysteine, which putatively resulted from the scavenging of a difluoroethylene metabolite of halothane by the tripeptide glutathione. The finding of such a metabolite was novel because of the presumed stability of the carbon–fluorine bonds of the fluorine-bearing carbon atom of halothane. Studies of Widger, Gandolfi, and Van Dyke[10] have shown that under hypoxic conditions, serum fluoride levels increase during halothane anesthesia in rats. They have further shown that an unidentified metabolite of halothane is firmly bound (probably covalently) to phospholipids and proteins of hepatic microsomes, also under hypoxic conditions. Such binding is associated with hepatotoxicity after exposure to bromobenzene, carbon tetrachloride, and acetaminophen.[11] Thus, these three studies suggest that hepatic necrosis after halothane administration is possible *in vivo* when oxygen availability is limited, and that glutathione may play a role in protecting the liver in such circumstances. The present study was undertaken to test this

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hypothesis and to provide a morphologic description of any halothane-induced hepatic damage.

Materials and Methods

Male Wistar rats weighing 150–250 g were fed Purina Rat Chow *ad lib.* for five days prior to study. The animals were housed in metal cages with wire mesh floors in air-conditioned quarters with a regulated diurnal cycle (lighted from 7 A.M. to 7 P.M.). All animals were fasted for the 24 hours immediately prior to sacrifice. The use of insecticides was avoided. A total of 119 rats was used in this study. Individual animals were randomly assigned to the following groups:

Group I (n = 36) was exposed to halothane, 0.5–0.8 per cent, in oxygen, 8 per cent, for one hour. Controls for this group received a one-hour exposure to oxygen, 8 per cent, alone. Following exposure animals were allowed to recover for 24, 36, 72, or 96 hours.

Group II (n = 10) was pretreated with diethyl maleate, 0.7 mg/kg, in an equal volume of corn oil, intraperitoneally (to deplete hepatic glutathione[12]), 30 min prior to beginning a one-hour exposure to halothane, 0.6 per cent, in oxygen, 8 per cent (experimental subgroup) or to oxygen, 8 per cent, alone (control subgroup). These animals were allowed to recover for 48 hours.

Group III (n = 20) was pretreated for four days with phenobarbital, 75 mg/kg, intraperitoneally, daily, to induce the hepatic drug-metabolizing enzyme system.[13] On the fifth day, the animals were treated with halothane, 0.6 per cent, in oxygen, 50 per cent, for one hour (experimental subgroup) or were given oxygen, 50 per cent, alone for one hour (control subgroup). These animals were allowed to recover for 24 to 48 hours.

Group IV (n = 29) was pretreated with phenobarbital, intraperitoneally, for four days in the same manner as Group III. On the fifth day, the animals were exposed to halothane, 0.6 per cent, in oxygen, 8 per cent, for one hour (experimental subgroup) or to oxygen, 8 per cent, alone for one hour (control subgroup). These animals were allowed to recover for 6, 24, or 48 hours.

Exposure to the various gas mixtures was accomplished in a 24-l glass enclosure. Oxygen concentrations were obtained by adding U.S.P. oxygen or nitrogen (chromatography grade) to compressed air (breathing quality), each via a calibrated flowmeter. The desired oxygen concentration was confirmed by a Foregger® oxygen analyzer, which also served continuously to monitor the oxygen concentration in the enclosure. Halothane was added to the gas mixture presented to the enclosure from a calibrated halothane vaporizer.§ Halothane concentration in the chamber was confirmed and monitored by gas chromatography. When the chamber was initially closed, a 10 l/min flow was used until the oxygen and halothane concentrations had stabilized (about 10 min), at which time the flow was decreased to 5 l/min. When halothane was not being used the vaporizer and its tubing were physically removed from the inlet line. Temperature within the enclosure was maintained at 32–34 C to provide a neutral thermal environment for the anesthetized animals. No more than seven rats were placed in the enclosure at any one time.

Upon completion of the recovery intervals, animals from Groups I–IV were sacrificed by cervical dislocation, their livers exposed, and portions of the right lateral lobes removed for microscopic study. The tissue was immediately submerged in glutaraldehyde, 3 per cent, where it was diced into cubes approximately 1 mm on a side. The tissue was postfixed in osmium tetroxide, 1 per cent, dehydrated in graded ethyl alcohols, and embedded in an Epon–Araldite mixture.¶ Sections of each specimen were cut 0.5–1.0 μm thick and stained with toluidine blue, 0.5 per cent, in sodium borate, 0.5 per cent. Several sections from at least two separate tissue blocks from each animal were examined for representative morphologic features by light microscopy. (Results of electron microscopic examination of these tissues will be reported in a subsequent publication.)

In Group III, animals that had recovered for 24 and 48 hours, and in Group IV, animals that had recovered for six and 24 hours had the remaining portions of their livers prepared for assay of two enzymes of the hepatic drug-metabolizing enzyme system. Microsomes were prepared by homogenizing the liver in sucrose, 0.25 M, buffered to *p*H 7.4 with Tris, 0.05 M. The homogenates were centrifuged for 20 min at 10,000 × *g* at 4 C. The supernatant was then centrifuged at 100,000 × *g* for one hour to sediment the microsomal pellet, which was resuspended in Tris, 0.05 M (*p*H 7.4). The resulting microsomal suspensions were then assayed for protein, cytochrome P-450 and cytochrome b_5 as previously described.[14] Wavelength calibration of the spectrophotometer was carefully checked against a holmium oxide filter prior to each cytochrome P-450 determination.

Another series of animals (n = 24) was established to study the effects of the phenobarbital pretreatment

§ Fluotec 3, Cyprane Ltd., Lancaster, New York.

¶ Epon® 812, 29.25 g, Dodecenyl succinic anhydride, 67.70 g, Araldite® 502, 16.70 g, tri-dimethyl amino methyl phenol, 1.96 g, dibutyl phthalate, 4.10 g.

and hypoxia on plasma fluoride levels after a one-hour exposure to halothane, 0.6 per cent. Subgroups of six animals each received: 1) one hour of halothane, 0.6 per cent, in oxygen, 50 per cent; 2) pretreatment with phenobarbital for four days (as in Groups III and IV, above), and subsequently, one hour of halothane, 0.6 per cent, in oxygen, 50 per cent; 3) one hour of halothane, 0.6 per cent, in oxygen, 8 per cent; 4) pretreatment with phenobarbital for four days and then one hour of exposure to halothane, 0.6 per cent, in oxygen, 8 per cent. These animals were sacrificed immediately after the one-hour exposures and blood was collected for assay of plasma fluoride.

The fluoride determinations were carried out using an Orion fluoride ion electrode, reference electrode, and millivoltmeter. Plastic utensils were used exclusively to process the specimens and standards.[15] Standard fluoride solutions from 10^{-6} M to 10^{-2} M were prepared daily using sodium fluoride, 0.10 M standard (Orion), and outdated human plasma. Both samples and standards were diluted 1:1 in Orion Total Ionic Strength Adjustment Buffer–II (without CDTA)** immediately prior to measurement.

Significance of differences in values of cytochrome P-450 and cytochrome b_5 between control subgroup means and experimental subgroup means at each sampling time were tested using the t test for unpaired data. The significance of differences of means of plasma fluoride concentrations was tested for the four groups using Duncan's multiple range test. Differences were designated as significant when $P < 0.05$.

Results

Light microscopic study of livers revealed only minimal morphologic changes in animals that received halothane with oxygen, 8 per cent (Group I); animals pretreated with diethyl maleate that received halothane with oxygen, 8 per cent (Group II); animals pretreated with phenobarbital that received halothane with oxygen, 50 per cent (Group III); and animals pretreated with phenobarbital that received oxygen, 8 per cent, without halothane (Group IV—control subgroup). The changes seen in these animals were typically those of glycogen depletion and mild-to-moderate lipid accumulation scattered throughout the lobules.

The livers from animals that had been pretreated with phenobarbital and subsequently received halothane with oxygen, 8 per cent (Group IV—experimental subgroup) showed markedly different morphologic features. Numerous areas of hepatic necrosis, primarily in the central regions of lobules, with sparing of periportal cells, were found. The necrotic areas were well developed by six hours after exposure to halothane (fig. 1), and showed pyknotic nuclei, loss of nuclei, cloudy swelling, loss of cell margins, foam cells, etc. The necrotic areas showed small hemorrhagic regions and no leukocytic response. By 24 hours after the anesthetic exposure, the necrotic areas were extensive, involving 70–80 per cent of most lobules. Central vein remnants occasionally were found, but it was difficult to identify central veins within the necrotic areas, the peripheries of which typically demonstrated relatively normal cells immediately adjacent to the hepatic portal triads. The necrotic areas were infiltrated to a limited extent by small leukocytes. Polymorphonuclear leukocytes were occasionally seen, but they did not predominate. Hemorrhage was still present at this time, and friability of the tissue was often pronounced. By 48 hours following treatment (fig. 2), the necrotic areas were somewhat more discrete. Leukocytic infiltrates persisted but were never extensive, and hepatic repair was evidenced by frequent mitotic figures, usually in the zone between the frankly necrotic cells and the relatively normal periportal cells.

Microsomal cytochrome P-450 was unchanged after exposure to halothane in the phenobarbital-pretreated rats when adequate oxygen was given (Group III). When halothane was administered with oxygen, 8 per cent (Group IV), there was a 50–60 per cent decrement in microsomal cytochrome P-450 (table 1). Microsomal cytochrome b_5 was found to be unchanged by anesthesia with halothane in the phenobarbital-pretreated rats whether they had received adequate oxygen (Group III) or were hypoxic (Group IV) (table 2). Plasma fluoride was less than 2 μM immediately following halothane exposure when adequate oxygen (50 per cent) was given, whether or not the animals had been pretreated with phenobarbital (fig. 3). When halothane was administered to hypoxic rats not induced with phenobarbital, plasma fluoride averaged 19 ± 2 μM (mean ± SEM). When halothane was administered to hypoxic rats previously induced with phenobarbital, their plasma fluoride was further increased to 24 ± 2 μM.

Discussion

This study demonstrates that under very specific conditions halothane may act as an hepatotoxin in rats. The conditions necessary are a period of modification of the hepatic microsomal drug-metabolizing enzyme system followed by hypoxia during which

** Instruction Manual for Fluoride Electrodes. Orion Research Inc., Cambridge, Massachusetts, 1976, pp 13.

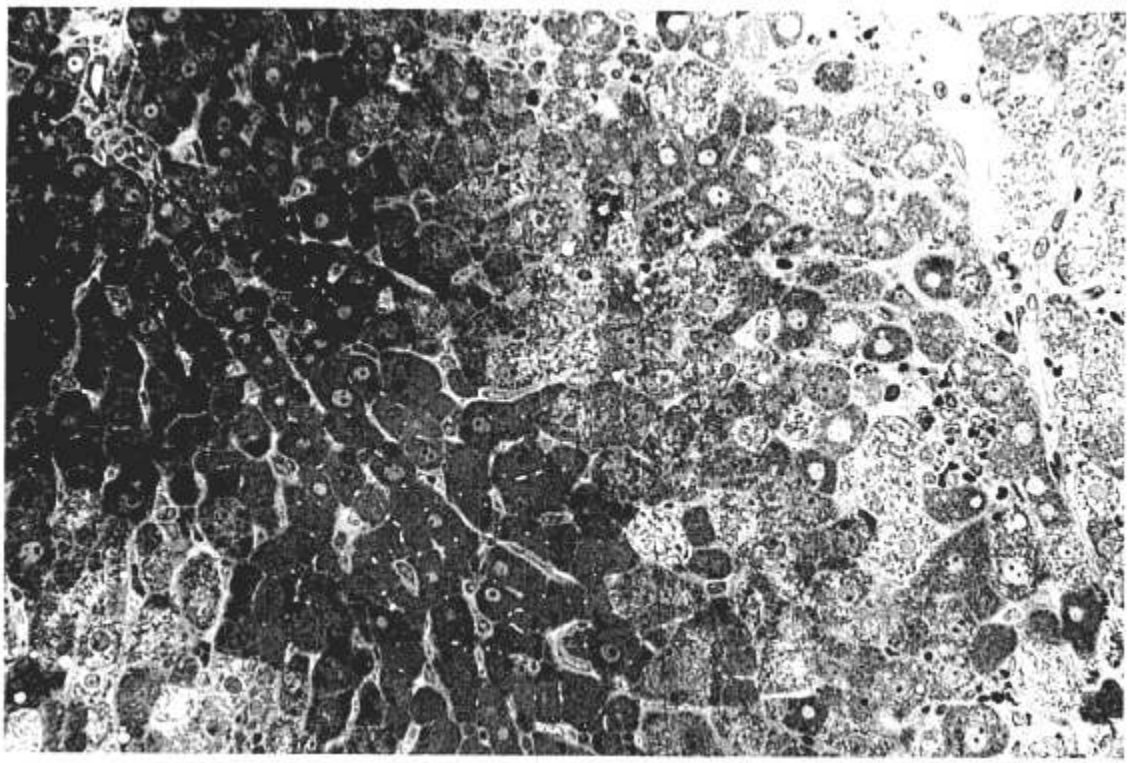

FIG. 1. Hepatic morphology six hours after halothane, 0.6 per cent, and hypoxia ($F_{I_{O_2}} = 0.08$) in a phenobarbital-pretreated rat. A portal triad is in the upper left corner and a central vein extends along the right side of the micrograph. Note focal necrosis sparing the periportal cells. ×150.

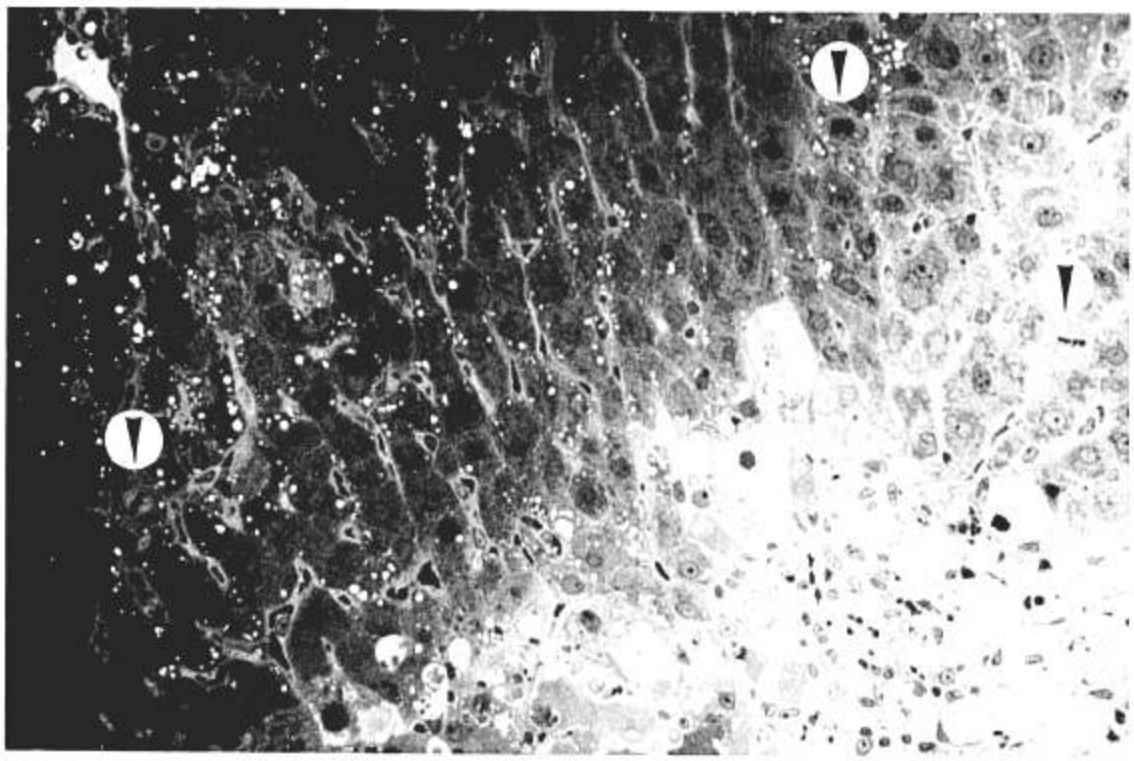

FIG. 2. Hepatic necrosis 48 hours after halothane and hypoxia in a phenobarbital-pretreated animal. Note the occurrence of frequent mitoses (*arrows*). ×150.

halothane is administered. The data presented show that halothane administered after phenobarbital-stimulated enzyme induction alone or during hypoxia alone does not result in hepatic necrosis.

Leinweber *et al.*[16] have studied normal rats made hypoxic during exposure to halothane, and similarly found that hepatic necrosis did not develop. Subsequently, Widger, Gandolfi and Van Dyke[10] provided two important *in-vivo* observations of the effects of halothane when administered under hypoxic conditions to phenobarbital-induced rats. First, they found that a metabolite of halothane was firmly bound to lipids (and to lesser extent to proteins) of the hepatic microsomal fraction. Second, they observed an increase in serum fluoride concentration that did not occur when halothane was administered with adequate oxygen or when the animals were made hypoxic in the absence of halothane. Evidently, hypoxia promotes the reductive defluorination of halothane[17,10] and quite possibly it is the resulting defluorinated metabolite that binds to hepatic microsomes. Widger *et al.*[10] did not study their animals for more than an hour after exposure to halothane, and they did not look for hepatic structural damage. However, their findings suggested that animals whose hepatic enzymes were induced and which survived halothane and hypoxia should be examined for hepatic cellular damage.

In our study, phenobarbital pretreatment under conditions of adequate oxygenation did not increase plasma fluoride values. When halothane was administered to hypoxic animals, plasma fluoride values were increased more than tenfold, and pretreatment with phenobarbital resulted in further increases in plasma fluoride values. We interpret these data to mean that even though the defluorinating activity is inducible by phenobarbital, hypoxia seems to be the predominant factor causing the increased defluorination of halothane. It is, therefore, somewhat surprising to find that halothane administered to hypoxic rats without phenobarbital pretreatment failed to cause hepatic cellular damage.

Since Cohen[9] reported a difluoroethane conjugate of cysteine to be a product of halothane metabolism, we sought to determine whether glutathione was protecting the livers from damage in non-induced animals (Group III). These animals were pretreated with diethyl maleate to deplete hepatic glutathione. Subsequent anesthesia with halothane in oxygen, 8 per cent, did not cause hepatic necrosis. We interpret this to show that even when defluorination of halothane is promoted by hypoxia, glutathione is not likely to be the sole protector of the liver. Brown[12] and Van Dyke[17,18] have concluded that glutathione

TABLE 1. Effects of Halothane and Hypoxia on Microsomal Cytochrome P-450 in Phenobarbital-pretreated Male Wistar Rats

Treatment	Cytochrome P-450 ± SEM (nmol/mg Protein) after Recovery Interval: 6 Hours	24 Hours	48 Hours
Phenobarbital; 50 per cent oxygen	—	1.11 ± .18	.81 ± .21
Phenobarbital; 50 per cent oxygen; 0.6 per cent halothane	—	1.11 ± .23	.93 ± .15
Phenobarbital; 8 per cent oxygen	.95 ± .16	1.52 ± .92	—
Phenobarbital; 8 per cent oxygen; 0.6 per cent halothane	.44 ± .07*	.64 ± .12*	—

* $P < .005$.

TABLE 2. Effects of Halothane and Hypoxia on Microsomal Cytochrome b_5 in Phenobarbital-pretreated Male Wistar Rats

Treatment	Cytochrome b_5 ± SEM (nmol/mg Protein) after Recovery Interval: 6 Hours	24 Hours	48 Hours
Phenobarbital; 50 per cent oxygen	—	.55 ± .05	.49 ± .07
Phenobarbital; 50 per cent oxygen; 0.6 per cent halothane	—	.59 ± .13	.54 ± .02
Phenobarbital; 8 per cent oxygen	.48 ± .05	.51 ± .18	—
Phenobarbital; 8 per cent oxygen; 0.6 per cent halothane	.49 ± .03	.41 ± .10	—

is not of major importance in the metabolism of halothane. Since the cysteine conjugate of the difluoroethane metabolite observed by Cohen was obtained from human material, and since rats were used in the studies by Brown and Van Dyke and in this work, it is possible that an important species variation is present.

At least two other factors should be considered in attempting to explain the failure of hypoxia and halothane to produce hepatic necrosis in non-induced animals. In this study we have investigated only one combination of oxygen and halothane concentrations. Assuming there is a threshold of either which is critical for the production of cellular damage in the absence of phenobarbital pretreatment, this study would not have detected it. Second, the production of hepatic necrosis only in animals pretreated with phenobarbital suggests qualitative differences in the metabolism of halothane produced by the induction process. It is now known that there are

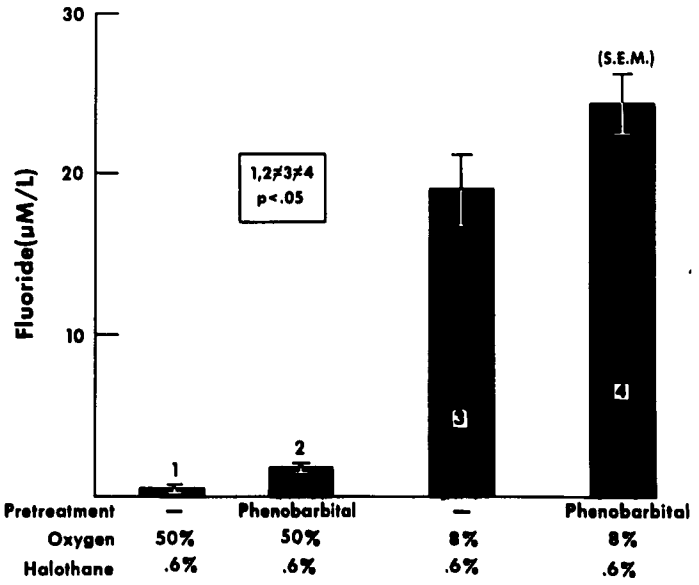

FIG. 3. Effects of phenobarbital and hypoxia on plasma fluoride values after exposure to halothane.

several variants of cytochrome P-450, the terminal oxidase of the hepatic microsomal drug-metabolizing enzyme system. When reduced and complexed with carbon monoxide, these variants have slightly different absorbance maxima, varying between 447 and 451 nm.[19] They are induced in different proportions by different inducers, and they have different substrate specificities. For example, Aroclor 1254 induces cytochrome P-448.[20] Induction with this compound results in hepatic necrosis after exposure to halothane even in the face of adequate oxygenation.[21,22] It is well established that phenobarbital results in the induction of cytochrome P-450.[13,18] We suggest that a minor variant may also be sufficiently induced by phenobarbital and may act with hypoxia to facilitate cellular damage, but not in a quantity sufficient to be detected spectrophotometrically.

The decrease in the measured amount of microsomal cytochrome P-450 in the livers of animals in which necrosis developed is interpreted to indicate a binding of halothane (or one of its metabolites) to cytochrome P-450 which persists for some time after the initial exposure. The fact that another microsomal enzyme, cytochrome b_5, was not decreased suggests a specific, persistent involvement of cytochrome P-450 and not a generalized destruction of microsomal enzymes.

Our data suggest that the development of hepatic necrosis after halothane is a sequential process in which the first step is the production of a reactive intermediate resulting from the reductive metabolism of halothane. A second step, apparently facilitated by the phenobarbital-stimulated induction process, is reaction of the metabolite(s) with hepatic cellular components, which leads to irreversible cellular damage.

It is apparent that extrapolation of these results from rats to the clinical use of halothane in man cannot be made. This study does, however, raise some questions to be asked in the clinical setting. Is the patient whose hepatic enzymes are induced by drugs likely to manifest hepatic dysfunction if hypoxia, shock, or decreased hepatic blood flow occurs during halothane anesthesia? The National Halothane Study[1] specifically excluded from consideration patients who had histories of sepsis or shock. Within such a group, is halothane more likely than other anesthetics to result in hepatic dysfunction?

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