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Toxicity of Halogenated Volatile Anesthetics in Isolated Rat Hepatocytes

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This study was designed to determine whether the isolated rat hepatocyte preparation could be used to assess the comparative toxicities of halothane, methoxyflurane, enflurane and chloroform. Suspensions of rat hepatocytes, prepared by a collagenase-based isolation technique, were incubated for 20 or 60 minutes in the presence of various concentrations of these anesthetics. Toxicity was assessed by measuring the leakage of intracellular potassium ions and alanine aminotransferase, decrease in the rate of ureogenesis, and increase in the lactate: pyruvate ratio. Toxicity measured after 20 minutes of incubation was dose-related, and the magnitudes of the effects of chloroform and methoxyflurane were approximately equal, and significantly ($P < 0.01$) exceeded that of halothane. Enflurane did not affect potassium ion or alanine aminotransferase release, even after 60 minutes of incubation, at which time halothane toxicity was markedly increased ($P < 0.002$). Enflurane did produce effects on ureogenesis and the lactate:pyruvate ratio, although these were less than those of the other agents. These data suggest that the toxic effects of halogenated volatile anesthetics measured in this *in-vitro* preparation are of the same comparative order as their clinical hepatotoxic potentials. (Key words: Anesthetics, volatile, halothane; Anesthetics, volatile, enflurane; Anesthetics, volatile, chloroform; Anesthetics, volatile, methoxyflurane; Liver, hepatotoxicity; Toxicity, hepatic.)

IT NOW SEEMS INDISPUTABLE that some cases of postoperative hepatic dysfunction are causally related to the use of halogenated volatile anesthetics. This was a major factor leading to discontinuation of the use of chloroform. Subsequently there has been concern over the possible hepatotoxicity of the newer halogenated volatile anesthetics, notably halothane and methoxyflurane.[1]

Classification of the nature of the hepatic injury and the mechanisms involved is still a matter of some controversy.[1–6] The clinical syndrome has many of the characteristics of a "hypersensitivity" reaction, implying that the mechanism is immunologically based.[3] Experimental studies both *in vivo* and *in vitro* point to a mechanism of toxicity for volatile halogenated anesthetics in common with other halogenated hydrocarbons, *viz.*, direct hepatotoxicity, probably mediated via a metabolite resulting from the actions of microsomal enzymes.[2,6–10] It seems, therefore, that a better understanding of the experimental conditions that affect the toxicity of halogenated volatile anesthetics could improve our knowledge of the clinical syndrome, and, in particular, enable a more accurate prediction of the patients "at risk."

Freshly isolated liver cells in suspension offer an *in-vitro* system that may be useful for evaluating direct toxic effects of drugs. The technique has been successfully used to demonstrate relative cytotoxicities of erythromycins[11] and tricyclic antidepressants,[12] in good agreement with their relative hepatotoxic potentials in clinical use. Similar types of studies, using monolayer cultures of Chang liver cells or rat hepatoma cells, have been directed at the erythromycins,[13] laxatives,[14] and, more recently, the halogenated volatile anesthetics, halothane, methoxyflurane and isoflurane.[15,16]

A possible disadvantage of cell culture techniques is that the cells tend to become de-differentiated and lose some of their organ-specific functions.[17] On the other hand, Chang cells have the advantage that their origin was from human liver.

The viability of freshly isolated rat hepatocytes has been assessed using various indices of membrane integrity and metabolic function.[18,19] The aim of the present investigation was to study the effects of halothane, methoxyflurane, enflurane, and chloroform on some of these indices of viability in freshly isolated rat hepatocytes with a view to determining their relative hepatotoxicities. Chloroform was included in the study as a compound whose hepatotoxicity is well established.

Methods

Male Wistar rats (200–260 g) bred at the University Central Animal House were used as liver donors. They were allowed food (Charlicks M & V 164 mouse cubes) and water *ad libitum*.

Isolated hepatocytes were prepared by the method of Berry and Friend,[20] with minor modifications. The liver was perfused at 37 C via the portal vein *in situ* with a calcium-free physiologic solution (NaCl 96 mM, KCl 1.4 mM, $MgSO_4$ 0.74 mM, KH_2PO_4 2.5 mM, $NaHCO_3$ 30 mM, sodium gluconate 21.7 mM) equilibrated with oxygen, 95 per cent:carbon dioxide, 5

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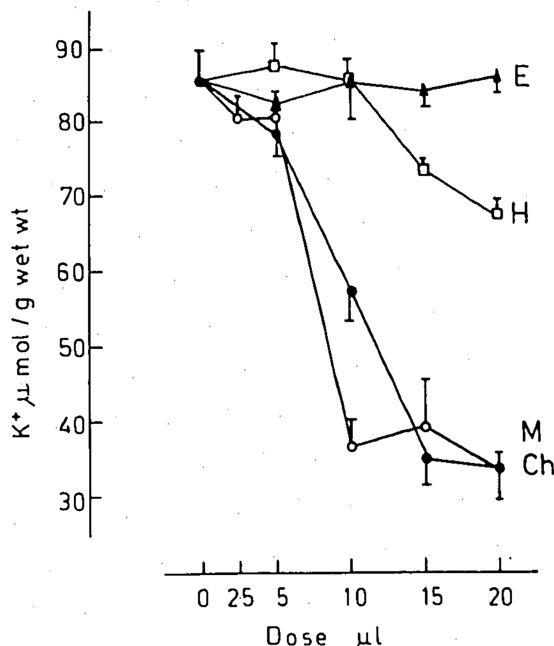

FIG. 1. Intracellular potassium ion contents after incubation of hepatocyte suspensions (20 minutes, 37 C) with various doses of anesthetics. Halothane — □ — H; enflurane — ▲ — E; methoxyflurane — O — M; chloroform — ● — Ch. Each point is the mean of four experiments, and the bar indicates the SEM. Comparative potencies: Ch = M > H = E, $P < 0.01$, n = 15 (doses 5–20 µl). For doses 10–20 µl, H > E, $P < 0.01$, n = 12.

per cent. The liver was then excised and transferred to a temperature-controlled chamber (37 C) and perfusion continued with the medium in a recirculating system, under a constant pressure of approximately 20 cm H_2O (flow rate 30–35 ml/min). Collagenase‡ (Type CLS 11, 140–200 U/mg) was added to give a perfusate activity of approximately 70 U/ml, and perfusion continued for a further 10 min. The liver was then placed in a beaker, the capsule disrupted gently with a spatula, and the dispersed liver incubated for a further 10 min (37 C, 80 oscillations/min) in 2 × 250-ml Erlenmeyer flasks under an atmosphere of oxygen, 95 per cent:carbon dioxide, 5 per cent.

Bovine serum albumin (Sigma, Fraction V), 1.2 per cent w/v, was added, and cells harvested by sieving through nylon mesh (250 and 61 µm). Cells were centrifuged (5 × *g*, 1 minute) and washed twice with this medium. They were washed once with Eagle's Basal Medium§ plus 1.2 per cent bovine serum albumin and then resuspended in this medium at a concentration of 4–5 × 10^6 cells/ml.

Cell yields of 4–6 × 10^8 cells were routinely obtained, with the following criteria of viability: trypan blue exclusion 93–96 per cent; intracellular potassium ion (K^+) content 85.7 ± 3.8 µmol/g wet weight after 20 minutes of incubation.

Volatile anesthetics (2.5–20 µl) were added to 2 ml of cell suspension in pre-gassed (oxygen, 95 per cent:carbon dioxide, 5 per cent) 25-ml Erlenmeyer flasks, which were then immediately sealed. Incubations were carried out at 37 C with shaking (80 oscillations/min) for 20 or 60 minutes.

Portions (1.1 ml) of cell suspensions were precipitated with 1.1 ml perchloric acid (6 per cent, w/v) and the concentrations of lactate,[21] pyruvate,[22] and urea[23] determined in the supernatant. Other portions (0.5 ml) were centrifuged (50 × *g*) for 1 minute. Alanine aminotransferase (ALT) was measured in the supernatant by the method of Reitman and Frankel.[24] The cell pellet was treated with perchloric acid (3 per cent, w/v) and intracellular K^+ concentration determined by flame photometry.

Comparative potencies of the four anesthetics were assessed by tabulating treatment differences in each of the four hepatocyte preparations and determining the statistical significances of these differences using the Wilcoxon matched-pairs signed-rank test.[25]

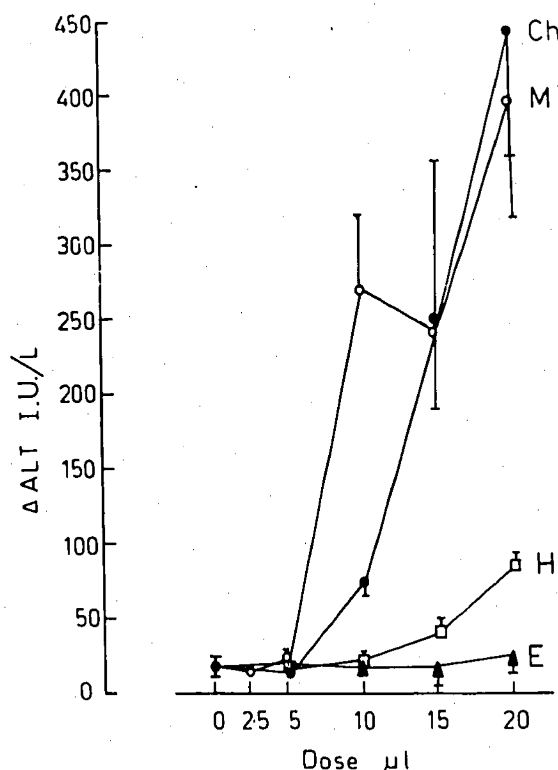

FIG. 2. Alanine aminotransferase activities in the supernatant after incubation of hepatocyte suspensions (20 minutes, 37 C) with various doses of anesthetics. Halothane — □ — H; enflurane — ▲ — E; methoxyflurane — O — M; chloroform — ● — Ch. Each point is the mean of four experiments, and the bar indicates the SEM. Comparative potencies: Ch = M > H > E, $P < 0.01$, n = 15 (doses 5–20 µl).

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Results

Chloroform, methoxyflurane, and halothane produced dose-dependent losses of K^+ from the cell pellet (fig. 1) and release of ALT into the supernatant (fig. 2), compared with control cells. Enflurane did not alter cellular permeability to K^+ or ALT over 20 minutes of incubation, even at the highest dose level (figs 1 and 2), nor were the effects altered when the period of incubation was extended to 60 minutes with the 20-μl dose (fig. 3). In contrast, the effects of halothane were greater with the longer period of incubation (fig. 3) and the amount of damage was similar to that seen after 20 minutes of incubation with the more toxic anesthetics.

Enflurane produced dose-related increases in the concentrations of both lactate and pyruvate (fig. 4). The effects of the other anesthetics were more complex. At low doses, chloroform, methoxyflurane, and halothane stimulated lactate and pyruvate production, while at higher doses this trend was reversed (fig. 4). The lactate:pyruvate (L/P) ratio, a useful index of the redox state of the hepatocytes, was increased by the anesthetics (fig. 5). The effect was dose-dependent and varied among the anesthetics, with enflurane having the least effect, and chloroform the

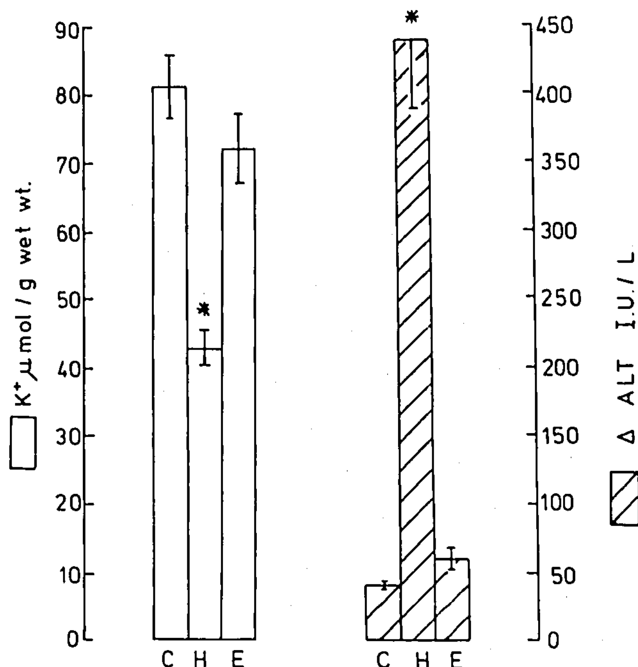

FIG. 3. Intracellular potassium ion contents (*open bars*) and alanine aminotransferase activities in the supernatant (hatched bars) after 60 minutes of incubation (37 C) with 20 μl halothane (H) or enflurane (E), compared with controls (C). * $P < 0.002$, n = 3 (t-test).

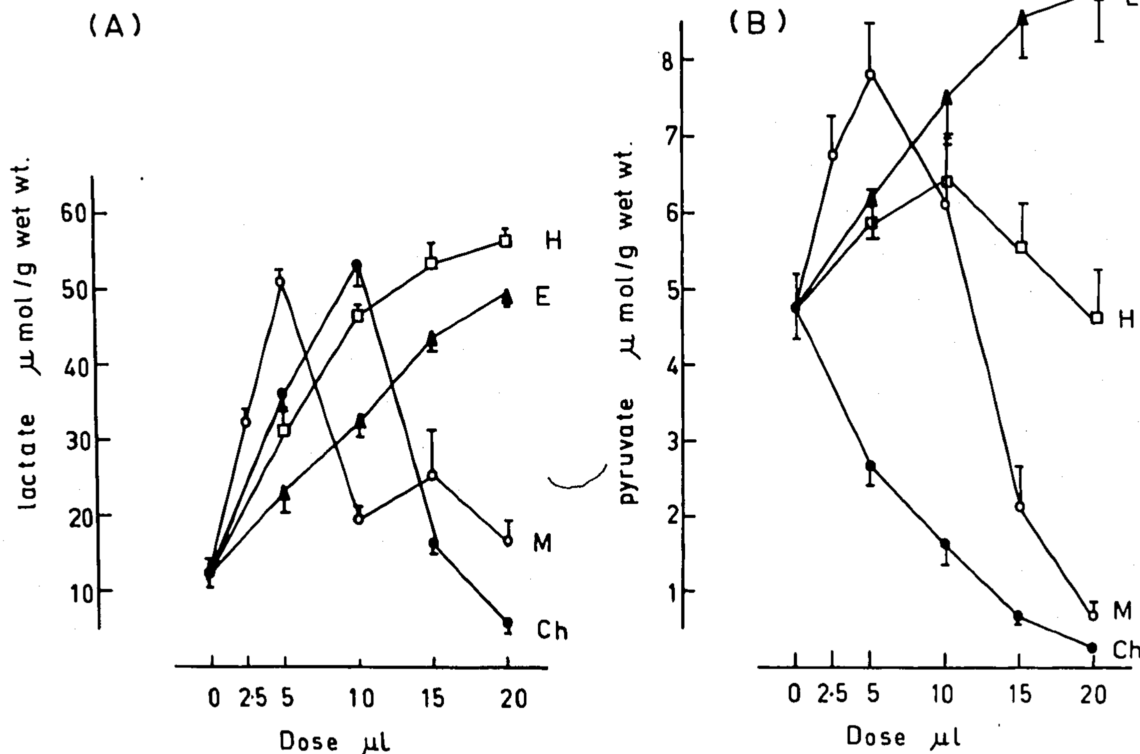

FIG. 4. Lactate (*left figure*) and pyruvate (*right figure*) contents of hepatocytes after incubation (20 minutes, 37 C) with various doses of anesthetics. Halothane —□— H; enflurane —▲— E; methoxyflurane —○— M; chloroform —●— Ch. Each point is the mean of four experiments, and the bar indicates the SEM.

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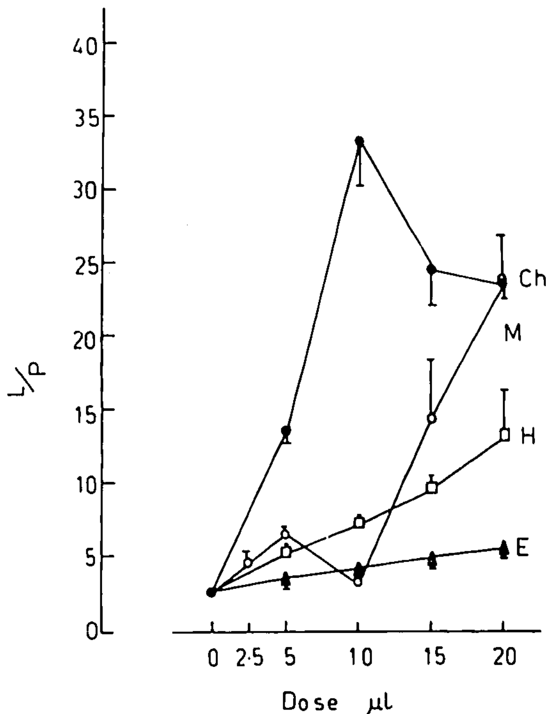

FIG. 5. Lactate:pyruvate ratios (L/P) after incubation of hepatocyte suspensions (20 minutes, 37 C) with various doses of anesthetics. Halothane —□— H; enflurane —▲— E; methoxyflurane —○— M; chloroform —●— Ch. Each point is the mean of four experiments, and the bar indicates the SEM. Comparative potencies: Ch > M = H > E, $P < 0.01$, n = 16 (doses 5–20 μl).

greatest. The rate of urea synthesis was decreased by all of the anesthetics, and enflurane again had the least effect (fig. 6). However, the effect of enflurane on ureogenesis was greater than its effects on the other variables measured. The effects of halothane, methoxyflurane, and chloroform could not be differentiated from one another, and maximal depression of ureogenesis appeared to be achieved with doses of approximately 10 μl of each of these anesthetics.

Discussion

Cytotoxicity of halogenated volatile anesthetics has been clearly demonstrated in freshly isolated rat hepatocytes. The relative order of cytotoxic potencies was found to be: chloroform = methoxyflurane > halothane > enflurane. These results complement the data of Goto *et al.*,[15,16] who studied volatile anesthetic toxicity in hepatocyte cell cultures and reported the following order of cytotoxic potency: Methoxyflurane > halothane > isoflurane > ether.

It is apparent that freshly isolated rat hepatocytes are a useful tool for assessing the hepatotoxic potential of drugs. Studies with erythromycin derivatives and chlorpromazine in isolated hepatocytes[11] have shown toxic effects similar to those seen with other *in-vitro* liver preparations, such as hepatocyte cell cultures[26–28] and the isolated perfused rat liver.[29,30] Furthermore, comparative *in-vitro* toxicities of these drugs are in good agreement with their potentials to produce hepatotoxicity in clinical use.

Extrapolation of our results to the clinical situation infers that enflurane has a lower potential for hepatotoxicity than halothane, a point which at this stage seems justified on the basis of clinical experience.[31] With methoxyflurane, nephrotoxicity is the main cause of concern, but hepatotoxicity is not unknown, and hepatic dysfunction is generally acknowledged to be a contraindication[3] to its use. The hepatotoxic and nephrotoxic effects of chloroform are well known,[1] and are consistent with the high degree of cytotoxicity demonstrated in our experiments.

The doses used in our experiments probably exceed those that might be achieved under clinical conditions. If one assumes equilibration between the medium and gas phase of our 25-ml incubation flasks, the medium concentrations calculated from blood-gas partition coefficients[32] range from two to 20 times the blood concentrations predicted from MAC values. On the other hand, Goto *et al.*,[15,16] in experiments similar to ours, using only a slightly lower dose range, measured anesthetic concentrations in

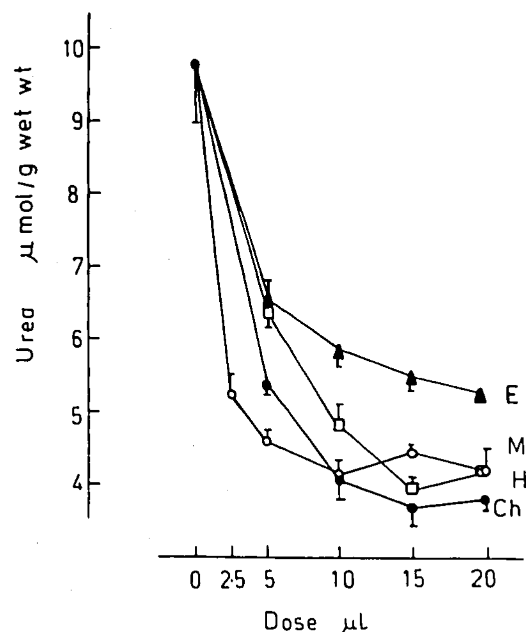

FIG. 6. Urea formed over 20 minutes during incubation (37 C) of hepatocyte suspensions with various doses of anesthetics. Halothane —□— H; enflurane —▲— E; methoxyflurane —○— M; chloroform —●— Ch. Each point is the mean of four experiments, and the bar indicates the SEM. Comparative potencies: Ch > M = H > E, $P < 0.02$, n = 12 (doses 10–20 μl). For doses 5–20 μl Ch = M, $P < 0.05$, n = 16.

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their culture medium which they considered to be in the clinical range.

The methods we chose to evaluate the toxic effects of the anesthetics included tests of cell membrane integrity and of the metabolic status of the cells. Previous *in-vitro* studies of drug toxicity in liver preparations[11–16,26–30] have used relatively crude indices of cellular viability, such as the ability to retain intracellular enzymes (LDH, SGOT, β-glucuronidase).

Increase of plasma ALT is commonly used both clinically and experimentally to assess the extent of hepatocellular damage, since it is relatively specific for liver cells. Release of ALT into the medium from isolated hepatocyte suspensions would indicate disruption of the cell membrane. The ability of the cells to retain K^+ is another index of cell membrane integrity, and it is considered to be more sensitive than many other indices of cellular viability.[19] Both methoxyflurane and chloroform produced marked cellular damage as measured by these criteria, and there was little difference in the dose thresholds or magnitudes of the responses. Halothane was less potent, although an increase in the time factor clearly increased the amount of cellular damage. Enflurane did not alter structural integrity under comparable conditions in our study.

Impairment of the metabolic activity of a cell is a means of assessing its functional integrity, although it need not necessarily be associated with cell death. That both halothane and enflurane produced significant changes in metabolic activity in isolated hepatocytes at doses that did not result in changes in membrane integrity emphasizes this point. Indices of metabolic function that involve cooperative interaction of mitochondrial and cytosolic enzymes and substrates are particularly usedul in assessing hepatocyte function.[33] The lactate:pyruvate ratio reflects the redox state of the cell. The rate of ureogenesis reflects the ability of the cell to maintain adequate oxidative phosphorylation (synthesis of one mole of urea utilizes four moles of ATP).

In our experiments, ureogenesis was the variable that was most sensitive to the effects of the anesthetics tested. Whether this has implications as to the mechanisms whereby these anesthetics result in cell death is a matter for conjecture. Furthermore, it is not known whether the effects of the anesthetics of cellular metabolic function are caused by the parent compounds or their metabolites. Stier *et al.*[34] studied the effects of halothane and its metabolite on hepatic metabolic activity in rats. They concluded that the effects, among which were marked changes in lactate and pyruvate concentrations, were due mainly to the metabolite, trifluoroacetate.

The mechanisms underlying the toxic effects observed and the reasons for the gradation in potencies cannot be determined on the basis of the present study. Possible explanations could take into account the physicochemical properties leading to differences in distributions between cells, medium and gas phases, lipid-solvent effects, or differences in the rates and extents of metabolism.

The relative distribution of the anesthetics in our experiments would be influenced by the temperature, the configuration of the incubation vessels, and the medium/gas partition coefficients. These factors were also recognized by Goto *et al.*,[15] although they found that the concentration of anesthetic agent in the cell and supernatant fractions of the medium varied very little in comparison with the marked differences in partition coefficients. If a difference in the physicochemical properties were the major factor influencing our results, it would be expected that halothane and enflurane, which have similar liquid–gas partition coefficients,[32] would have similar toxic potencies. This was not so, and differences in their cytotoxicities were particularly apparent when a longer period of incubation was used.

The solvent effect of the anesthetics on the cell membrane lipids may be a factor in the response. The relative lipid solubilities of the anesthetics do not correspond exactly to their relative toxicities in this preparation, nor is a lipid-solvent effect consistent with the time-dependency of the halothane response. Although the ability of anesthetics and other lipid solvents to modify cell membrane function reversibly is well recognized, release of ALT in our experiments is not consistent with a reversible change in cell membrane permeability.

The rate or extent of metabolism is probably a more important factor. There is considerable support for the hypothesis that a toxic metabolite formed by splitting a carbon–halogen bond is responsible for the hepatotoxicity (or nephrotoxicity) of halogenated hydrocarbons such as carbon tetrachloride and some of the volatile anesthetics.[7] Microsomal cytochrome P-450 is thought to play a key role in this metabolic activation. Since freshly isolated rat hepatocytes have been shown to retain microsomal drug-metabolizing enzymic activity,[35] it is reasonable to assume that toxic metabolites of halogenated hydrocarbons could be formed under our experimental conditions. This possibility is currently being investigated in our laboratory.

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