

Effects of Halothane on Human and Rat Hepatocyte Cultures

Yannick Mallédant, M.D.,* Laurent Siproudhis, M.D.,† Michèle Tanguy, M.D.,* Catherine Clerc, Ph.D.,‡
Christophe Chesné, Ph.D.,§ Colette Saint-Marc, M.D.,¶ André Guillouzo, Ph.D.**

The aim of this study was to investigate direct cytotoxicity to human and rat hepatocytes in primary culture from halothane and compare it with that of isoflurane, which is known to be minimally metabolized and less toxic *in vivo*. Both human and rat parenchymal cells were isolated by the two-step collagenase perfusion method and after attachment to plastic were incubated with either volatile anesthetic for 24 h. All the cultures were maintained in 20% O₂ condition and were not induced prior to anesthetic treatment. Temperature, atmosphere conditions, and anesthetic concentrations were kept constant during the study period. Evaluation of cytotoxicity was based on morphologic, biologic (determination of both extracellular and intracellular lactate dehydrogenase activity), and metabolic (protein synthesis and secretion) end points. Protein synthesis and secretion rates were found to be the most sensitive parameters in hepatocyte cultures from both species. Protein synthesis was inhibited by 18% and protein secretion by 50% in the presence of 1 and 1.25 mM halothane, respectively, in human cell cultures ($P < 0.05$). With 1.25 mM halothane intracellular lactate dehydrogenase was also decreased; lactate dehydrogenase leakage and morphologic alterations were detected only beyond 5 mM halothane. By contrast, in rat hepatocyte cultures protein secretion was inhibited by 26% and protein synthesis by 20% in the presence of 0.1 and 0.75 mM halothane, respectively, whereas morphologic alterations and a 37% lactate dehydrogenase leakage increase were observed with the concentration of 1 mM ($P < 0.05$). However, isoflurane was found to be less cytotoxic because lactate dehydrogenase leakage and morphology were not altered at concentrations as high as 2.35 mM and 5.5 mM in rat and human hepatocyte cultures, respectively. All of these observations show that halothane can be cytotoxic to adult hepatocytes maintained in standard culture conditions at concentrations much higher than those used in the clinical setting and that the degree of sensitivity of both cells is different. (Key words: Anesthesia, volatile: halothane; isoflurane. Liver, hepatocyte: primary culture. Liver: toxicity.)

LIVER DAMAGE following general anesthesia with halothane can be characterized either by transient liver dysfunction due to a direct toxic effect or by a severe hepatitis, which probably involves an immunologic mechanism. The first form remains controversial because its demon-

stration has been most often based on the study of animal models,¹⁻⁴ the principal one being the male rat pretreated with phenobarbital and exposed to halothane with hypoxia.⁵⁻⁷ These experimental conditions favor the shift of metabolism of halothane to the reductive pathway and, thus, the formation of reactive metabolites.⁸⁻¹⁰ A correlation has been reported between oxygen tension, reductive pathway, and liver injury.¹¹ Moreover, the degree of liver damage is modulated by inhibitors or inducers that affect this metabolic pathway.^{7,12} However, liver lesions induced in rats are difficult to interpret. In addition to the decrease of the inhaled O₂ fraction, halothane has a depressive circulatory and respiratory effect that can be responsible for a major centrilobular hypoxia, which in turn is increased by phenobarbital pretreatment.^{13,14} In addition to the difficulty in defining the respective roles of hypoxia, phenobarbital, and halothane in the occurrence of liver injury, it may be noted that several other studies have led to controversial conclusions.^{4,14-16} The reductive metabolism and appearance of liver lesions are not always correlated, and demonstration of the toxicity of metabolites has not been established.^{17,18} Moreover, interexperiment reproducibility of halothane toxicity under hypoxic conditions is particularly low, and the degree of toxicity depends on the age, sex, and species.^{1,2,18,19} Finally, the same degree of toxicity can be obtained with minimally metabolized halogenated hydrocarbons under conditions similar to those used for halothane.^{13,20}

Such important changes of the environment of hepatocytes cannot be separately investigated *in vivo*. Consequently, to obtain additional information on these critical unexplained issues, some authors have recently turned to simpler models. Thus, the use of isolated hepatocytes either in suspension or in culture allows bypass of respiratory and circulatory effects, permitting study of various parameters on the same cell population in well-defined experimental conditions.²¹⁻²³ For example, using cultured rat hepatocytes, Schieble *et al.* have shown that halothane toxicity was increased in parallel to a decrease of O₂ concentration.²³

However, most *in vitro* studies have been performed on rat hepatocytes, and the results cannot be directly extrapolated to the human situation. For example, it is known that the relative importance of the reductive pathway of halothane could vary from one species to another; the reductive metabolites are formed only at very low O₂ concentrations in the rat,¹ and they are found in the guinea pig as well as in humans in normoxic and hyperoxic

* Assistant Professor of Anesthesiology.

† Research Assistant.

‡ Assistant Professor of Biochemistry.

§ Research Associate.

¶ Professor of Anesthesiology.

** Director of Research.

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Address reprint requests to Dr. Mallédant: Institut National de la Santé et de la Recherche Médicale, Hôpital de Pontchaillou, 35033 Rennes Cedex, France.

atmosphere.^{4,24,25} Therefore, we decided to evaluate the effects of halothane on cultured human hepatocytes and to compare the results with those obtained on male rat hepatocytes. The effects of isoflurane were also investigated as control. Hepatocytes were cultured in a 20% O₂ atmosphere, a condition that allows the cells to maintain their functional capacity for several days.^{26,27}

Materials and Methods

CELL ISOLATION AND CULTURE

Male Sprague-Dawley rats weighing 180–200 g were used. The animals were handled in compliance with French regulations. Hepatocytes were prepared by the *in situ* two-step collagenase perfusion method.²⁸ Briefly, the rats were first anesthetized by intraperitoneal administration of pentobarbital (50 mg/kg). Before opening the peritoneum, 1,000 IU heparin was injected in the femoral vein. The liver was first washed with Hepes buffer, pH 7.65, for 10 min at a flow rate of 30 ml/min, then perfused with the same buffer containing 0.025% collagenase and 0.075% CaCl₂ for 15 min at a flow rate of 20 ml/min.²⁸ Following enzymatic perfusion the liver was excised and the Glisson's capsule was disrupted. The cells were dispersed in L15 Leibovitz medium added with 0.2% bovine serum albumin, then filtered through gauze and washed three times by centrifugation at 50 × g. Cell yield ranged between 400 and 600 × 10⁶ hepatocytes with a viability of 90–97% as determined by the trypan blue exclusion test.

The livers from seven organ donors 19–39 y of age were used in the study. They revealed normal histology. This study has been approved by the INSERM ethical committee.

Human hepatocytes were obtained by perfusing a portion of the left lobe of the livers.²⁹ Hepes buffer was perfused at a flow rate of 75 ml/min for 20 min followed by the 0.05% collagenase solution containing 5 mM CaCl₂ for the same time at 50 ml/min. Cell yield ranged between 6 and 12 × 10⁸ hepatocytes with a viability of 70–85%.

Both rat and human hepatocytes were seeded in polystyrene flasks in Ham F12 medium containing 0.2% bovine serum albumin, 10 µg/ml bovine insulin, 10% fetal calf serum, and antibiotics. Sixty to 80% of the cells attached to plastic within a few hours, aggregated, and formed monolayers. After about 4 h the medium added with 7 × 10⁻⁵ M hydrocortisone hemisuccinate was renewed. In these conditions the cells survived for 1–2 weeks without proliferating.

EXPERIMENTAL DESIGN

The flasks seeded with 2.5 × 10⁶ viable hepatocytes in 4 ml medium were placed in incubator chambers at 37°

C under an atmosphere composed of 20% O₂, 75% N₂, and 5% CO₂. Halothane and isoflurane flowed through 1–3 vaporizer chambers placed on the entrance to the chamber, and a halogen monitor (Servo gas monitor 120, Siemens) determined the concentration of the anesthetic exiting the chamber. The chamber was sealed when the desired concentration was obtained, i.e., 1–11% and 1–12% for halothane and isoflurane, respectively.

After a 24-h incubation, halothane concentration was measured in the culture medium.^{22,30} Ten microliter aliquots were collected in each flask and immediately introduced in extraction tubes containing 0.5 ml distilled water, 5 ml hexane (Merck 4371), and then frozen at –80° C until analysis. Just before determination 0.5 ml of a 0.02% isoflurane solution was added as an internal standard. A standard curve was established with 10, 20, 40, 100, and 200 µl of a 0.25 × 10⁻³ M halothane solution diluted in culture medium. After a 2-min stirring and centrifugation at 2,000 × g for 5 min, 1 µl of the supernatant was injected in a gas/liquid chromatograph (Varian 3400) equipped with an Alltech model R-SL 160 capillary column (30 m × 0.32 mm, 5 µm). Detection was made by electron capture (Varian Ni⁶³, 200° C) with a makeup N₂ flow of 25 ml/min and a Delsi Model Evic 21 integrator. The oven temperature was programmed from 60 to 75° C at 2° C/min. The limit of halothane detection in the medium was 0.025 mM. The linearity of the standard curve was checked in a range of 0.125–7.5 mM. Isoflurane was quantified in the same conditions using halothane as an internal standard. The within-day reproducibility at 0.5 mM was 3.9% for both anesthetics (n = 10).

ASSAYS

Cell monolayers were examined by phase-contrast microscopy, and toxic effects were scored from 0 to +++ according to Ratanasavanh *et al.*³¹: +, appearance of few dense granules or vacuoles and/or slight alterations of the cell shape; ++, presence of several dense granules or vacuoles and shrinkage of some cells; +++, accumulation of a number of intracellular vacuoles or granules and/or disruption of the cell monolayer. A score of ++ was considered to correspond to a toxic effect. Several cell areas were examined in each flask.

Liver cell damage was also evaluated by measuring extracellular and intracellular lactate dehydrogenase (LDH) activity. LDH has been shown to be more sensitive than other cytosolic enzymes to detect unspecific damage. However, particularly in human hepatocyte cultures, intracellular LDH activity is often more accurate than LDH leakage.³² LDH activity was determined using the MA-kit Roche, LDH opt DGKC (reference 14313) and a Cobas Bio apparatus. The values were expressed as IU/ml of

medium and are the mean \pm SD of four experiments in duplicate.

Protein synthesis was measured by the incorporation of ^{14}C -leucine. After exposure to anesthetic the cells were rinsed with phosphate-buffered saline (PBS) and incubated in 2.5 ml of leucine-free medium deprived of fetal calf serum and hydrocortisone and added with 10 μCi ^{14}C -leucine (Amersham, 50 mCi/mmol) for 60 min. After incubation cell monolayers were rinsed with PBS and homogenized. Both media and cells were stored at -80°C until analysis. The samples were thawed at 4°C . Cells were sonicated and a 100 μl aliquot was used for protein determination. Proteins from both samples were precipitated with trichloroacetic acid at the final concentration of 10%. After 120 min they were washed three times by centrifugation, and radioactivity was estimated by liquid scintillation counting. The values were expressed as cpm/ μg intracellular proteins. Total protein concentration of cell homogenates was assayed as described by Bradford.³³ Four different experiments were performed in duplicate on both rat and human hepatocytes.

STATISTICAL ANALYSIS

Values of LDH and intracellular and secreted neosynthesized proteins in cultures treated with halothane or isoflurane were compared with those obtained in control cultures for each experiment by analysis of variance (ANOVA) followed by the Dunnett's test and confirmed by the Newman Keul's test. The values of $P < 0.05$ were regarded as significant.

Results

Anesthetic concentrations were carefully measured in the culture media and found to vary between 0.1 and 7.5 mM for halothane and between 0.3 and 5.5 mM for isoflurane.

When observed, morphologic alterations affected most cells in both rat and human hepatocyte cultures and minimal intraassay differences were obtained. Interassay variations did not exceed 10% for both LDH leakage and protein synthesis and secretion measurements.

At 1 mM halothane induced morphologic alterations and significant changes both in extracellular and intracellular LDH content in rat hepatocyte cultures. Protein synthesis and secretion rates appeared to be slightly more sensitive; indeed, protein synthesis declined 20% and protein secretion declined 26% in the presence of 0.75 mM and 0.1 mM halothane, respectively ($P < 0.05$). Cytotoxicity was dose-dependent at higher concentrations (Figs. 1 and 2).

With human hepatocyte cultures marked differences were obtained according to the end point. Morphologic modifications and an increase of 92% ($P < 0.05$) in extracellular LDH activity were visualized only in the presence of 7.5 mM halothane. By contrast, intracellular LDH and protein secretion were already decreased by 17% and 50%, respectively, in cells treated with a concentration of anesthetic as low as 1.25 mM ($P < 0.05$). Protein synthesis was the most sensitive parameter for the demonstration of an effect of halothane. It was significantly decreased by 18% in the presence of 1 mM anaesthetic ($P < 0.05$) (Figs. 3 and 4).

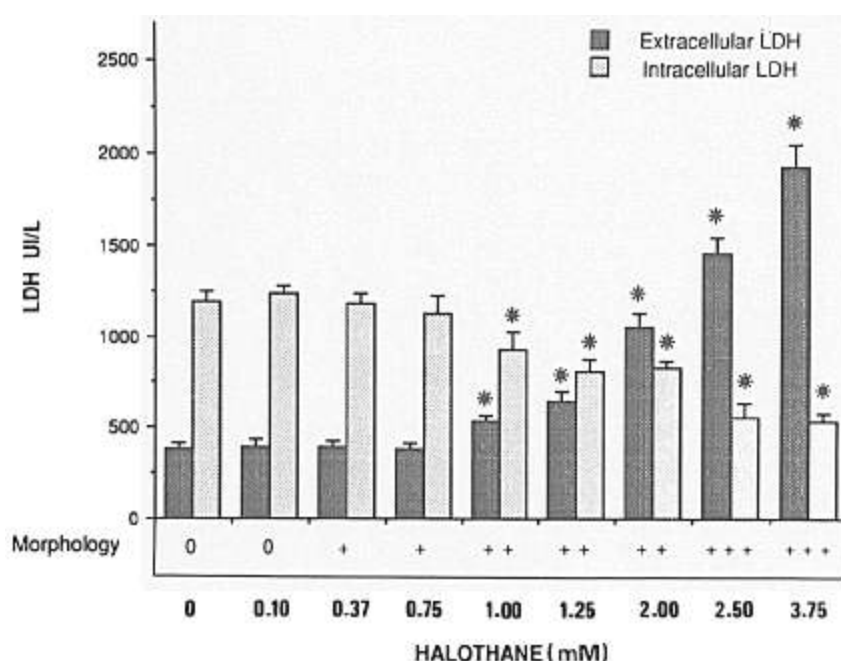
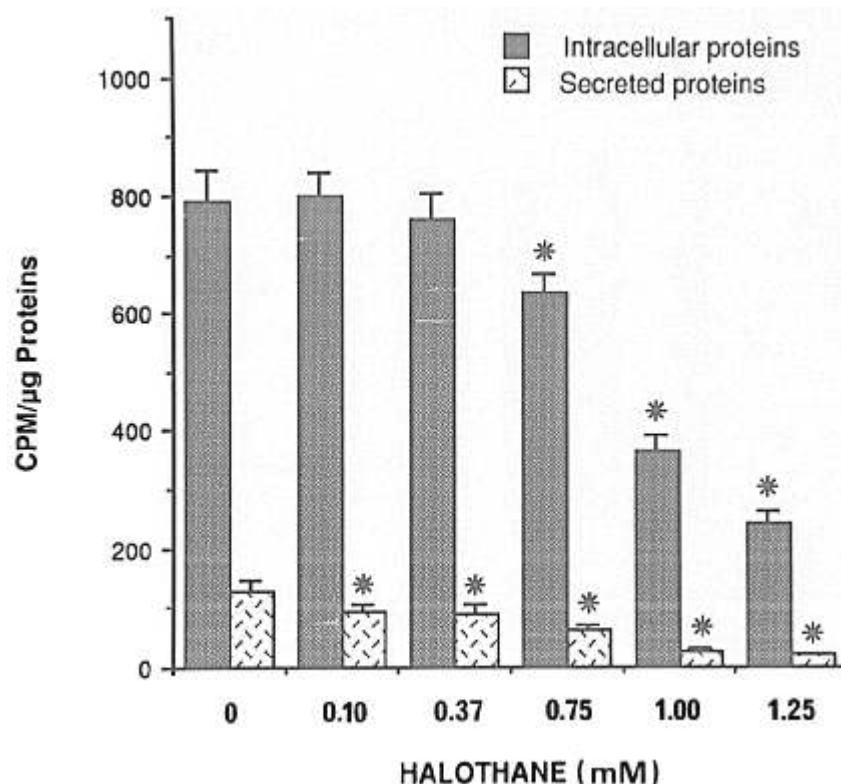


FIG. 1. Morphologic and biologic changes induced by halothane in cultured rat hepatocytes. The degree of morphologic alterations was graded from 0 (controls) to ++++. Values are mean \pm SD of four experiments in duplicate. * $P < 0.05$ versus 0.

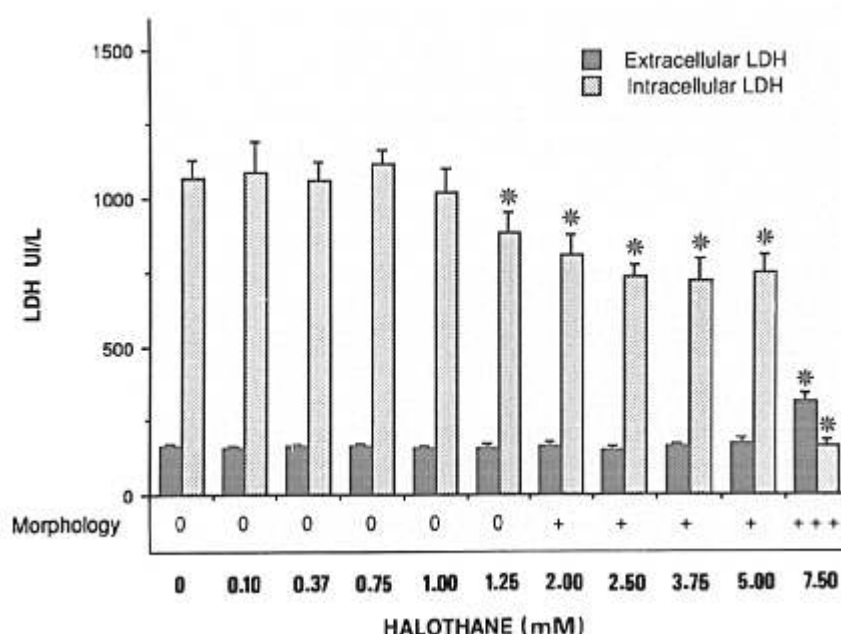
FIG. 2. Effect of halothane on protein metabolism in cultured rat hepatocytes. Values are mean \pm SD of four experiments in duplicate. * $P < 0.05$ versus 0.



When tested in conditions similar to those used for halothane, isoflurane was found to be much less toxic to both rat and human hepatocytes on the basis of morphologic changes and/or LDH leakage. This anesthetic did not clearly affect rat and human hepatocytes at 2.35 mM and 5.5 mM, respectively (Figs. 5 and 6).

By contrast, while they alter the level of secreted proteins at nearly the same concentrations in both species, halothane and isoflurane differently affected neosynthesis of intracellular proteins in human cultures (Figs. 7 and 8). Overall, no significant alteration of protein synthesis was observed in human hepatocytes exposed to isoflurane

FIG. 3. Morphologic and biologic changes induced by halothane in cultured human hepatocytes. The degree of morphologic alterations was graded from 0 (controls) to +++. Values are mean \pm SD of four experiments in duplicate. * $P < 0.05$ versus 0.



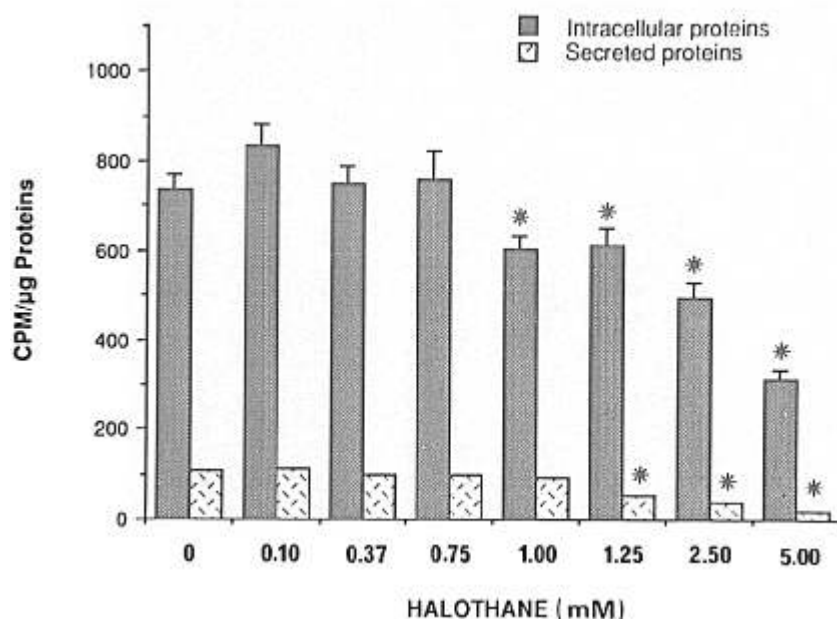


FIG. 4. Effect of halothane on protein metabolism in cultured human hepatocytes. Values are mean \pm SD of four experiments in duplicate. * $P < 0.05$ versus 0.

whatever the concentration used in the range of 0.5–5.5 mM. In addition, in hepatocyte cultures from both species, halothane was found to inhibit secretion of neosynthesized proteins more strongly than isoflurane when increasing anesthetic concentration were used.

Discussion

Both the toxic effect(s) of halothane and the mechanism(s) involved in its toxicity remain a subject of debate. Several studies suggest that hepatotoxicity results from biotransformation of the anesthetic, particularly by the reductive pathway, whereas others do not support such a conclusion.^{8–11,22,34,††} The results reported here clearly show that halothane may be cytotoxic to both rat and human hepatocytes when cultured in a 20% O₂ atmosphere. This condition is that widely used to maintain hepatocytes functional for periods longer than the duration of the reported experiments. Indeed, hepatocytes actively secrete proteins and particularly those of human origin may retain high levels of cytochrome P-450 and various Phase I and Phase II drug metabolizing enzymes for several days, allowing us to investigate the effects of halothane in metabolically active noninduced hepatocytes in the absence of hypoxia.^{35,36} Cytochrome P-450 still represents around 70% and 90% of its initial value in rat and human hepatocytes, respectively, after 24 h of culture. Moreover, the two major isoenzymes, cytochrome P-450-IIC8,10 and cytochrome P-450-IIIA are still well expressed after 1 week or more in human cells.

All these data demonstrate that hepatocyte cultures represent a reproducible and reliable model system for metabolic studies in stable atmosphere and temperature conditions.

Several end points were selected to evaluate anesthetic cytotoxicity and showed different sensitivity. In agreement with the results obtained with many drugs, our observations demonstrate that intracellular LDH activity is a much better marker than extracellular LDH, particularly in human hepatocyte cultures.³² However, in both rat and human hepatocyte cultures, protein synthesis and secretion activities were affected with a concentration of halothane, that had no effect on LDH activity, indicating that these metabolic parameters are reliable and sensitive end points to evaluate a drug effect. Similar conclusions have been previously reported.^{21,22,37,‡‡}

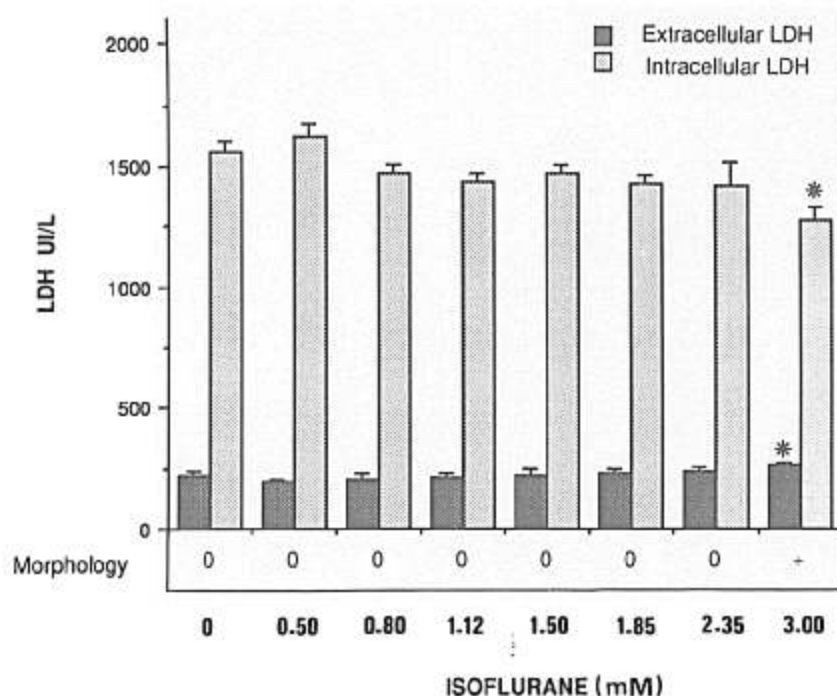
In halothane-treated rat hepatocytes, a decrease in protein secretion was evidenced before any appearance of protein synthesis alterations. Similar observations have been reported with rat hepatocyte suspensions treated with halogenated or nonhalogenated anesthetics, whether the cells were preinduced with phenobarbital or not.^{37,38} However, lower concentrations of anesthetics were required to demonstrate an effect in cultured cells, probably because of the longer exposure time to the anesthetic in our study.

Similarly, evaluation of synthesized intracellular and/or secreted proteins was found to be the most sensitive criterion to reveal effects of isoflurane, the other anes-

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FIG. 5. Morphologic and biologic changes induced by isoflurane in cultured rat hepatocytes. The degree of morphologic alterations was graded from 0 (controls) to +++. Values are mean \pm SD of four experiments in duplicate. * $P < 0.05$ versus 0.

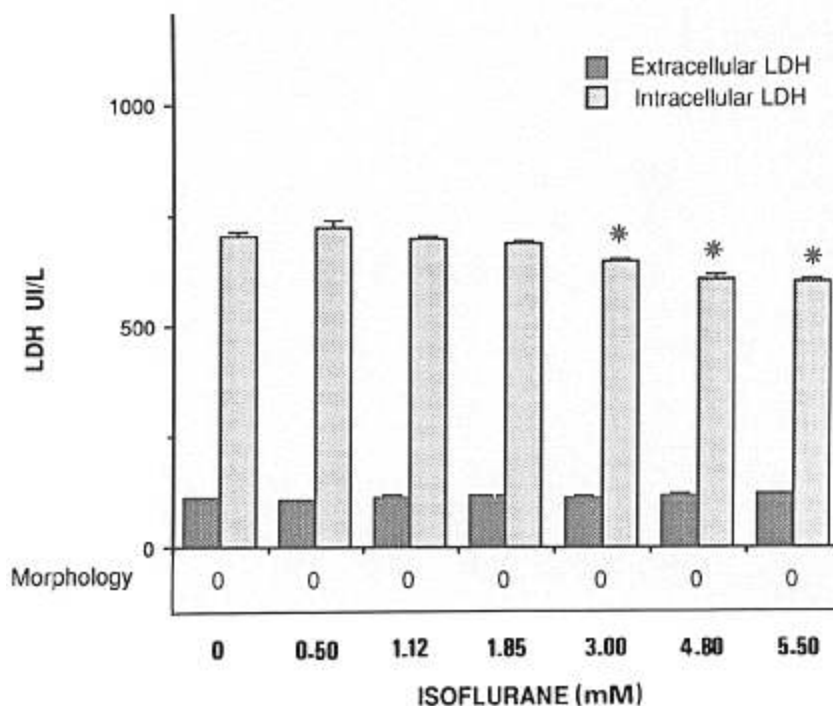


thetic tested in this study. LDH changes, when observed, occurred at much higher concentrations than those found with halothane.

In previous studies the toxic effects of the anesthetics have been related to the occurrence of lipid peroxidation and/or a solvent effect on the cell membrane. The solvent

effect has been suggested by some authors to explain toxicity of volatile compounds at high concentrations over a short incubation period.³⁹ This hypothesis may be questionable because major differences were found between halothane and isoflurane in the occurrence of morphologic changes and membrane permeability; isoflurane did

FIG. 6. Morphologic and biologic changes induced by isoflurane in cultured human hepatocytes. The degree of morphologic alterations was graded from 0 (controls) to +++. Values are mean \pm SD of four experiments in duplicate. * $P < 0.05$ versus 0.



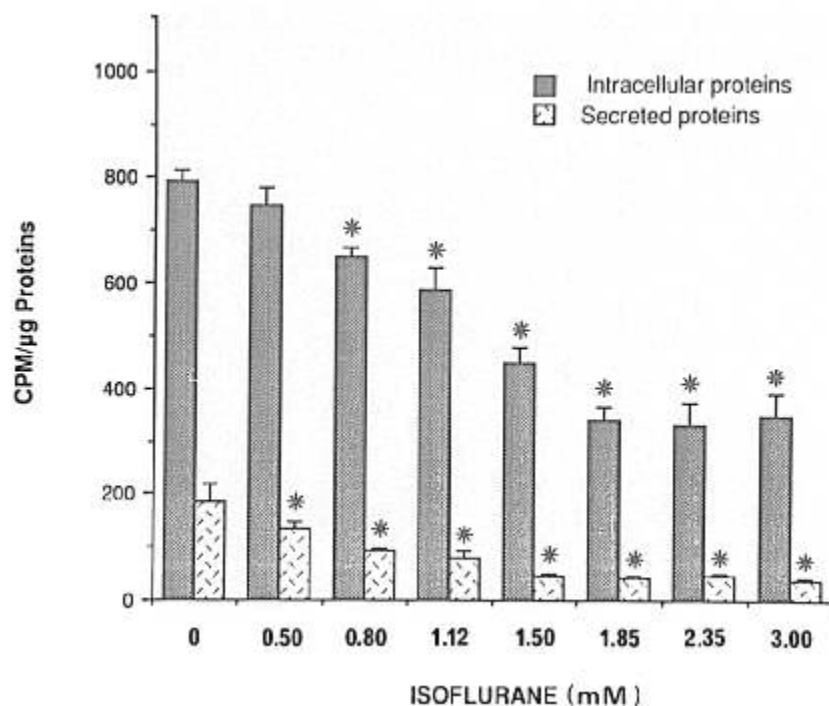


FIG. 7. Effect of isoflurane on protein metabolism in cultured rat hepatocytes. Values are mean \pm SD of four experiments in duplicate. * $P < 0.05$ versus 0.

not affect rat and human hepatocytes at concentrations as high as 2.35 mM and 5.5 mM, respectively. At these concentrations halothane has already induced major effects, suggesting that those could be related to its conversion to reactive metabolites leading to the formation

of hydroperoxides; this phenomenon has already been reported *in vitro* by other authors.^{40,††}

Synthesis and secretion of proteins were affected differently in hepatocytes from the two species by exposure to either halothane or isoflurane, suggesting that the

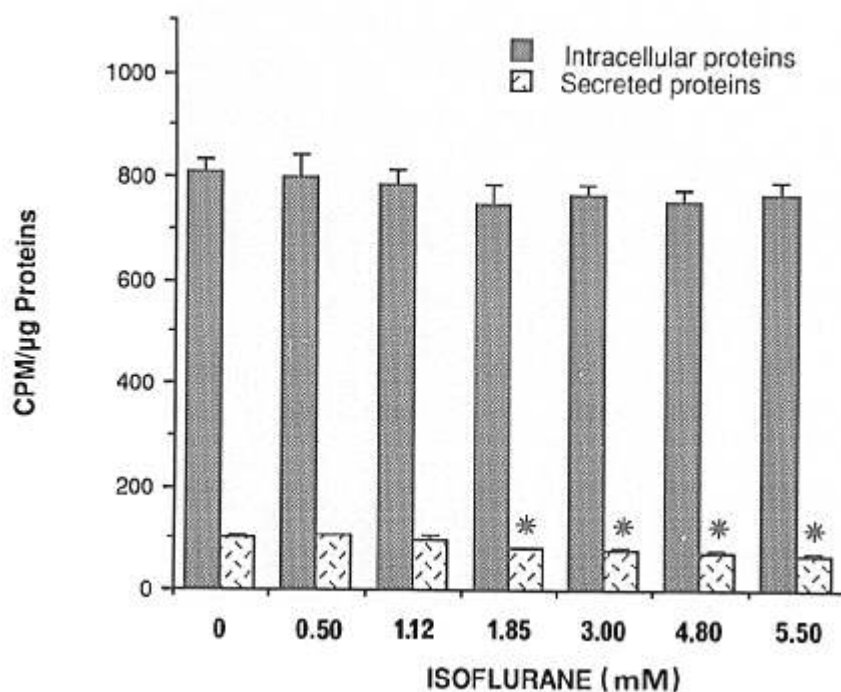


FIG. 8. Effect of isoflurane on protein metabolism in cultured human hepatocytes. Values are mean \pm SD of four experiments in duplicate. * $P < 0.05$ versus 0.

mechanisms of action of the two halogenated anaesthetics are different in both cells. For halothane species differences in the rates and routes of metabolism could be involved. This will merit further investigation.

Although rat hepatocytes were more sensitive than their human counterparts, it may be noted that halothane concentrations much higher than those used in human clinical settings were required to produce a cytotoxic effect. Whether a longer treatment or other culture conditions, particularly hypoxia, would lead to an increased sensitivity of hepatocytes to halothane is presently under study.

Our observations demonstrate a different degree of sensitivity of rat and human hepatocytes to the toxic effects of halothane and suggest that these *in vitro* model systems could represent a suitable tool to investigate the mechanism(s) involved in the toxic effects of anesthetics.

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