Application of Low-temperature Autoradiography to Studies of the Uptake and Metabolism of Volatile Anesthetics in the Mouse

I. Chloroform

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The uptake and metabolism of ^{14}C-labeled chloroform in the mouse were studied with low-temperature whole-body autoradiography. Impulse counting of tissue biopsy specimens established anesthetic concentrations. Body fat was shown to be an important storehouse during the prolonged retention of anesthetic in the body. The concentration of radioactivity in the liver increased during the postanesthetic period, reflecting the accumulation of nonvolatile metabolic products. Two hours later these accounted for slightly more than 4 per cent of the anesthetic administered. Thin-layer radiochromatography of a liver extract established the presence of two nonvolatile chloroform metabolites.

THE TECHNIQUE of whole body autoradiography has had wide application in studies concerned with uptake, distribution, and metabolism of drugs. Introduction of the method by Ullberg [1,2] has made possible the investigation of a number of water-soluble compounds which are subject to loss or translocation with conventional histologic procedures. Unfortunately, this approach is not applicable to drugs which exert significant vapor pressures below −10 C, and, therefore, its use has generally been restricted to nonvolatile compounds. Pellerin [3,4,5] and Chanteur and Pellerin [6] were the first to investigate some general uses of low-temperature autoradiography using the isotopes ^{3}H, ^{35}S, and ^{32}P. Subsequently, Cohen and Wepierre,[7] and Wepierre [8] modified Pellerin's techniques and successfully investigated the distribution and metabolism of ^{14}C-labeled Paracymene, a relatively volatile compound with a boiling point of 176 C.

Although current evidence indicates that the volatile anesthetics are not biologically immune,[9] only limited information is available regarding the metabolism of individual inhalation anesthetic agents. The *in vivo* metabolism of chloroform has been investigated by Paul and Rubinstein [10] following intraduodenal administration of labeled anesthetic to the rat. These workers collected the expired air and demonstrated that 4 per cent of the ^{14}C was recovered as labeled CO_2 within 24 hours, but were unable to find evidence of other chloromethanes. Subsequently, Van Dyke *et al.*[11] confirmed the metabolism of chloroform to CO_2 and, additionally, demonstrated evidence of nonvolatile urinary metabolite(s).

The present investigation represents an attempt to modify the procedures of whole-body autoradiography for use at low temperatures, and to apply the technique to study of the uptake and metabolism of anesthetic agents of marked volatility, *i.e.*, chloroform, diethyl ether, and halothane.

Procedure

^{14}C-labeled chloroform, specific activity 2 mC/mM, was obtained from commercial sources.‡ Radio gas chromatography indicated purity of the material. Twenty μC (0.8 μl) of ^{14}C-labeled anesthetic were diluted with two parts unlabeled chloroform and administered

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by inhalation to each of six male N.M.R.I. strain white mice, averaging 20 gm in weight. A specially constructed anesthesia chamber § permitted continuous administration of the chloroform diluted with oxygen (fig. 1). During the inhalation period, the anesthetic chamber was sealed to prevent loss of radioactive drug. The animals were exposed to the chloroform vapor for ten minutes, then sacrificed at prearranged times of 0, 15, and 120 minutes by immersion in liquid nitrogen. The mice were kept in the liquid nitrogen for at least five minutes, then removed and coated with a detergent solution. The specimens were rapidly embedded in carboxymethylcellulose gel and mounted on a brass block. The entire unit was slowly frozen in liquid nitrogen to prevent uneven cooling and cracking of the carboxymethylcellulose. Each frozen block was divided longitudinally using a rotary saw equipped with a specially constructed carbide blade.¶ This blade produced the smooth cut surface essential for autoradiography. The latter procedure was accomplished by preparing a sandwich of the mounted hemisection, emulsion film,** blotter cushions, and metal cover plate. The sandwich was held together firmly with two angle clamps tightened against the brass surfaces (fig. 2). Exposure of the emulsion film was maintained for a period of nine days in a light-tight insulated box over a deep base of solid carbon dioxide (−78 C). Photographic development was accomplished by standard techniques. After preparation of the autoradiographs, the hemisections were reused as a source of tissue biopsies for impulse counting, for the cutting of thin whole-body sections, and for extraction of radioactive materials present in the liver.

Biopsy specimens were taken from selected tissues in each animal and utilized for impulse counting.†† All biopsies were obtained in the "cold room" (−15 C) and therein transferred to previously-tared vials containing 0.3 ml perchloric acid and 1.0 ml heptane. The sealed vials were removed and reweighed following temperature equilibration in a desiccating chamber. Tissues were digested for 24 to 48 hours at room temperature, then retransferred to the cold room, and 16 ml of scintillation fluid was added.‡‡ During the biopsy

§ Modified from Wepierre.[8]

¶ Haven Saw and Tool Company, Santa Clara, California.

** Structurix D7, Agfa Gevaert.

†† Packard Tri Carb Liquid Scintillation Counter, Downer's Grove, Massachusetts.

‡‡ Scintillation solution consisted of four parts toluene with 0.5 per cent PPO, three parts monoethylene glycol, and 0.1 part of hydrogen peroxide.

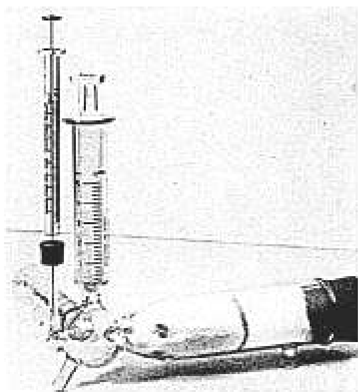

FIG. 1. Apparatus for the administration of volatile anesthetics to the mouse. Hood capacity = 6 ml; reserve oxygen supply contained in 5-ml syringes; liquid anesthetic introduced into chamber with microliter syringe.

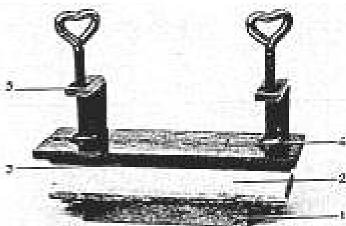

FIG. 2. Sandwich used for low-temperature autoradiography. 1, brass object holder; 2, hemisection of mouse embedded in carboxymethyl cellulose; 3, x-ray emulsion and cushion; 4, brass cover plate; 5, angle clamps.

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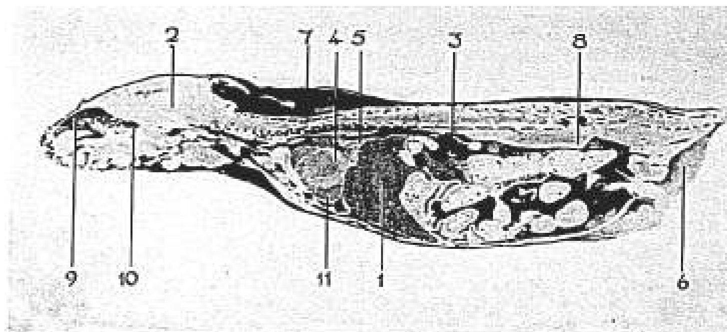

FIG. 3. Autoradiograph prepared from hemisection of mouse sacrificed immediately following inhalation of chloroform. 1, liver; 2, brain; 3, kidney; 4, blood; 5, lung; 6, fat; 7, brown fat; 8, muscle; 9, nasal mucous membrane; 10, Harder's gland; 11, heart muscle.

procedure two additional liver samples were taken from each of the animals. After weighing, these liver biopsies were taken to dryness and subsequently treated in the same fashion as the other specimens.

Following preparation of whole-body autoradiographs from the hemisections, thin (40-μ) sections were cut from alternate blocks and transferred to Scotch tape according to the technique of Ullberg. These thin sections, after drying, served as a framework of anatomic reference for the autoradiographs prepared from the hemisections at −78 C.[7] Prior to autoradiographic exposure, the thin sections were dried in the cold room at −15 C for 48 hours, then heated for an hour at 60 C. The combination of these two efforts removed all volatile radioactivity. Autoradiographs were then prepared from the thin sections and exposed at room temperature according to standard techniques.

In another facet of the study, 20 μC of ^{14}C-labeled anesthetic were diluted with two parts ethyl alcohol and injected into the tail veins of three male mice. The animals remained lightly anesthetized for five to ten minutes. The mice were sacrificed 120 minutes after intravenous injection, and the liver and entire intestinal tract removed. After these organs were weighed, biopsies were taken from the liver and from serial segments of the intestine. The biopsy specimens were allowed to dry and then treated like the other biopsy material.

Finally, the liver tissue from a 120-minute hemisection of mouse was carefully removed, weighed, and the ether-soluble materials extracted. The latter was accomplished by grinding the frozen liver tissue with dry ice in a chilled mortar, then extracting the tissue in a sealed vial to which a small volume of ether had been added. The extracted material was analyzed by thin-layer radiochromatography, employing a solvent system of chloroform-65; methyl alcohol-35 and water-1 (see results).

Results

The autoradiographs in figures 3, 4, and 5 present in pictorial form the distribution and elimination of ^{14}C-chloroform in the mouse.§§ In figure 3 the animal was sacrificed immediately after administration of the anesthetic, and we observe that the highest concentrations of radioactivity are found in the body fat, particularly brown fat. A high concentra-

§§ Concentrations of the anesthetic (plus metabolite) may be determined by comparing relative darkening of the organs or tissues. These relative concentrations, in turn, may be quantified by densitometry, using as reference a film exposed under standardized conditions. More precise concentrations of the anesthetic or metabolite are afforded by biopsy and scintillation-counting techniques (see text).

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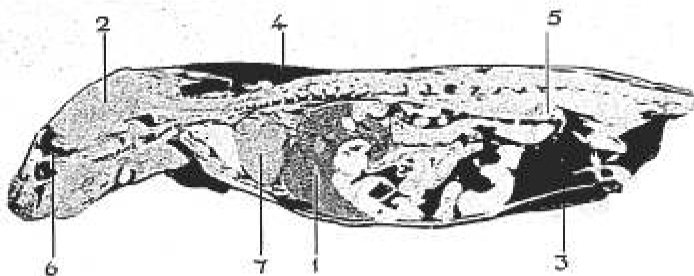

FIG. 4. Autoradiograph prepared from hemisection of mouse sacrificed 15 minutes following inhalation of chloroform. 1, liver; 2, brain; 3, fat; 4, brown fat; 5, muscle; 6, nasal mucous membrane; 7, heart muscle.

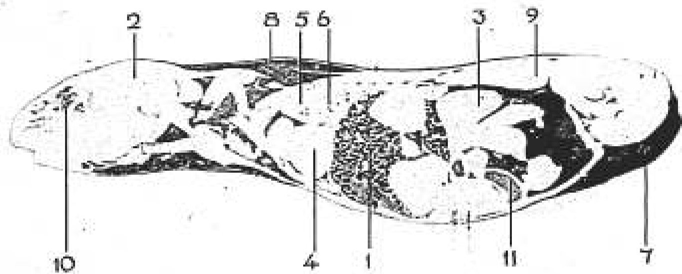

FIG. 5. Autoradiograph prepared from hemisection of mouse sacrificed 120 minutes following inhalation of chloroform. 1, liver; 2, brain; 3, kidney; 4, heart; 5, lung; 6, bronchi; 7, fat; 8, brown fat; 9, muscle; 10, nasal mucous membrane; 11, duodenum.

tion of radioactivity is also present in the liver, while lesser, and relatively uniform, amounts are noted in the blood, brain, lung, kidney, and muscle. In figure 4, the animal was sacrificed 15 minutes after administration of anesthesia, and at this time, there has been a further relative increase of radioactivity in the fat, and peak proportionate concentrations are now present. The relative concentration of radioactivity in the liver has also increased. By 120 minutes (fig. 5), total radioactivity in the animal has decreased considerably, and is essentially confined to the liver, duodenum, and fat. The radioactivity pattern in the first organ has a mottled appearance suggestive of a segmental or localized distribution.

Table 1 provides an analysis of the concentrations of radioactivity present in selected tissues at various times of sacrifice. If we assign a relative value of 1.00 to that concentration of radioactivity present in blood, we can compare the concentrations simultaneously

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present in each tissue. Following sacrifice of the animal after ten minutes' inhalation of anesthetic, most tissues approach a unit concentration with blood. However, in both fat and liver, the concentration exceeds unity. By 15 minutes, the ratio of radioactivity in brown fat has reached its peak and is almost 15 times that found in blood. The relative concentration of radioactivity in the liver continues to increase until the termination of the experiment at 120 minutes, when it reaches a final value 6.7 times in excess of that in the blood.

In table 2 we note the relative concentrations of volatile *vs.* nonvolatile radioactivity in the liver. In the mouse sacrificed immediately following anesthesia, the proportion of the nonvolatile radioactivity in the liver is 13.5 per cent. By 15 minutes, this proportion has increased to 43.7 per cent, and by 120 minutes 85.6 per cent of the liver radioactivity is nonvolatile.

The autoradiographs in figures 6 and 7 provide visual confirmation to the data in table 2. The thin-layer sections (all volatile components removed) show the localization of the nonvolatile metabolites. In figure 6 we note that nonvolatile metabolite begins to appear in the liver immediately following ten minutes' inhalation of chloroform. The concentration of nonvolatile metabolite rapidly increases and reaches its highest level 120 minutes after the inhalation of the anesthetic and at the termination of the experiment (fig. 7). The passage of the metabolites from the liver into the duodenum and intestine may also be noted. Additional traces of metabolites are concentrated in the nasal mucous membranes and in the bronchi.

TABLE 1. Concentrations of Radioactivity (Chloroform Plus Metabolites) in Various Tissues of the Mouse*

Tissue	Total Radioactivity (counts/min/mg)			Tissue/Blood Ratio		
	0 Min	15 Min	120 Min	0 Min	15 Min	120 Min
Blood	260 ± 22.0	103 ± 17.5	37 ± 4.0	1.00	1.00	1.00
Brain	217 ± 16.4	112 ± 9.9	23 ± 2.7	0.84	1.12	0.63
Muscle	288 ± 44.4	110 ± 5.1	26 ± 6.9	0.87	1.07	0.70
Lung	262 ± 24.1	149 ± 12.6	53 ± 8.2	1.01	1.44	1.43
Kidney	284 ± 35.0	145 ± 21.6	56 ± 8.0	1.08	1.41	1.53
Liver	407 ± 36.9	208 ± 9.3	250 ± 17.9	1.56	2.10	6.76
Fat	1,674 ± 201	953 ± 92.7	266 ± 30.1	6.42	9.25	7.18
Brown fat	3,158 ± 384	1,490 ± 98.4	211 ± 40.3	12.12	14.70	5.70

* Animals sacrificed at 0, 15, and 120 minutes following ten-minute inhalation of chloroform. Data represent duplicate determinations in each of two animals at each time sequence (± S.E.).

TABLE 2. Concentrations of Radioactivity in the Livers of Mice Sacrificed 0, 15, and 120 minutes Following Inhalation of Chloroform*

	0 Minutes	15 Minutes	120 Minutes
Total radioactivity (count/min/mg liver tissue)	407 ± 36.9	208 ± 9.3	250 ± 17.9
Volatile radioactivity (derived as the difference between measured total radioactivity and nonvolatile radioactivity)	352 ± 29.9	117 ± 8.8	36 ± 5.4
Nonvolatile radioactivity	55 ± 3.0	91 ± 5.7	214 ± 11.4
Liver/blood ratio (total radioactivity)	1.56	2.10	6.76
Liver/blood ratio (volatile radioactivity)	1.36	1.14	0.97

* Volatile radioactivity removed by drying tissue for 48 hours, followed by heating for an hour at 60 C.

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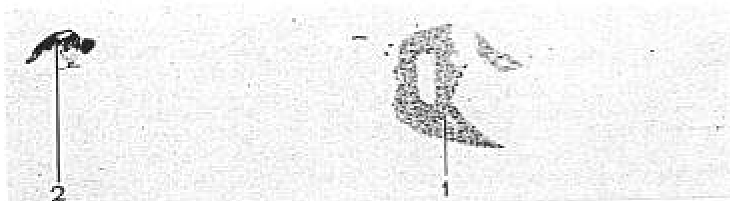

FIG. 6. Autoradiograph of a 40-μ section of mouse sacrificed immediately following inhalation of chloroform. Section dried for 48 hours at −15 C, then heated for an hour at 60 C. 1, liver; 2, nasal mucous membrane.

FIG. 7. Autoradiograph of a 40-μ section of mouse sacrificed 120 minutes following inhalation of chloroform. Sections dried for 48 hours at −15 C, then heated for an hour at 60 C. 1, liver; 2, duodenum; 3, nasal mucous membrane.

Table 3 indicates concentrations of nonvolatile radioactivity present in the livers and intestines of mice sacrificed 120 minutes after an intravenous injection of chloroform. Since in the latter instance the precise dose of the administered anesthetic is known, one can calculate the percentage of injected chloroform converted to nonvolatile metabolite. This represented 4.02 (±0.43) per cent during the two-hour study period, with approximately equal amounts present in the liver and the intestine.

Since all the radioactive material in the liver proved to be ether-soluble, an extract was prepared from the liver of an animal sacrificed 120 minutes after anesthesia. This extract was examined by thin-layer chromatography, and the plates scanned for localization of radioactivity. As noted earlier, impulse counting of liver biopsy material indicated that, at 120 minutes, 85.6 per cent of the radioactivity in the liver was nonvolatile. The radioactivity scan in figure 8 (upper trace) indicates separation of the activity into two peaks. The first peak represents a relatively polar material with an R_f value ¶¶ of 0.08. The second peak has an R_f value of 0.85. Calculation of the percentage of radioactivity for each component indicates that peak 1 comprises 15.4 per cent, and peak 2, 84.6 per cent, of the total nonvolatile radioactivity in the liver.

Unforunately, attempts to determine the characteristics of the major component were unsuccessful. An aliquot of peak 2, taken up in chloroform and adjusted to *p*H 6.2 with phosphate buffer, was treated with 8,000 units bacterial β-glucuronidase at 37 C for 24 hours. As indicated in figure 8 (lower trace), the resultant thin-layer radio chromatogram did not differ from the control. A further attempt was made to alter the characteristics of metabolite 2 by alkaline hydrolysis with 4 N NaOH at 100 C for an hour in a sealed vial. Again, the

¶¶ The R_f value or ratio compares the distance a compound has moved from the application origin with that distance traveled by the solvent front.

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resultant chromatogram did not differ from the control.

Discussion

The volatility of the inhalation anesthetics has presented serious technical difficulties in *in vivo* study of their distribution and metabolism, and only limited data are available. Published tables of partition coefficients [12,13] for chloroform include: blood/gas = 10.3; brain/blood = 1.2; heart/blood = 1.2; liver/blood = 0.9; kidney/blood = 1.1; fat/blood = 26. These values represent a combination of results of *in vivo* and *in vitro* studies, and the fat/blood ratio was determined with olive oil.

Whole-body autoradiography has proved most useful in studying the distribution and metabolism of nonvolatile drugs. As discussed earlier, modifications in autoradiographic methodology have permitted extension of this technique for use with the volatile anesthetic agents. The whole-body approach provides a simultaneous survey of all organ systems, and by adding densitometry or impulse-counting techniques, the concentrations of drug in selected tissues can be estimated with accuracy. Although chloroform exerts a significant vapor pressure at 0 C, it solidifies at −63.5 C. Thus, the combination of freezing in liquid nitrogen (−195 C) and storage in dry ice (−78 C) is sufficient to prevent loss of the volatile anesthetic during autoradiographic study.

Results of the present study with chloroform indicate that high concentrations of anesthetic agent are present in fat and in the liver immediately following a ten-minute period of anesthesia, and that these tissue/blood ratios quickly exceed unity. With fat, this ratio reaches a peak value 15 minutes after anesthesia, but in the liver the tissue/blood ratio continues to rise until termination of the experiment at 120 minutes. The large concentration of radioactivity in the fat * reflects its exceedingly high partition coefficient, which is sufficient to produce a continued transfer of anesthetic to this depot from other tissues even after termination of inhalation of the anesthetic.

The increasing ratio of liver/blood radioactivity represents a continued accumulation of metabolite within this organ. Biopsies of liver taken immediately after anesthetic inhalation contain 13.5 per cent nonvolatile metabolites. By 120 minutes, 85.6 per cent of the radioactivity in the liver consists of nonvolatile metabolites. Table 2 indicates the absolute increases in metabolite at various times of the study. Subtracting the nonvolatile radioactivity from the total radioactivity in the liver, we note that the ratio of volatile radioactivity (liver/blood) remains fairly constant. The autoradiographs in figures 6 and 7 were prepared from thin whole-body sections from which all the volatile radioactivity had been removed prior to autoradiagraphic exposure (see method). From these illustrations it is apparent that the fat does not provide a storehouse for the nonvolatile metabolites, despite a high concentration of volatile radioactivity present in this tissue. The storage of volatile anesthetic in the fat, however, does serve to make it available for hepatic biotransformation over an extended period of time, and

* The early high fat concentration for chloroform in the mouse must be contrasted with a relatively slower uptake of fat-soluble anesthetics in man. This undoubtedly reflects rapid circulation and high metabolism in the mouse, but also may represent species differences in types of fat, etc.

TABLE 3. Concentrations of Radioactivity (Nonvolatile Metabolites) in Liver and Intestine Following Intravenous Injection of 20 μC of ^{14}C-chloroform*

Tissue	Organ Weight (mg)	Radioactivity (counts/min/mg)	Total Radioactivity (counts/min)	Percentage Injected Radioactivity†
Liver	1,119 ± 86	334 ± 41	373,746	2.06
Intestine (plus contents)	2,091 ± 419	171 ± 34	357,561	1.96
				4.02

* Mice sacrificed at 120 minutes. Data represent duplicate determinations in each of three mice (±SE).
† Correction for machine efficiency 41.2 per cent.

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FIG. 8. Upper trace: radiochromatogram of extract of liver from mouse sacrificed 120 minutes after chloroform anesthesia. Solvent system: chloroform-65; methyl alcohol-35; water-1. Peak 1 has $R_f = 0.08$; Peak 2 has $R_f = 0.85$. Lower trace: radiochromatogram following preliminary treatment of peak 2 with 8,000 units β-glucuronidase for 24 hours at 37 C. No change from upper control trace.

thus high fat solubility of the anesthetic may be considered a significant factor in the metabolism of chloroform.

An analysis of the amounts of nonvolatile metabolites present in the intestine and liver (table 3) indicates that 4.02 per cent of the injected anesthetic is metabolized to these products within two hours. Since a considerable amount of volatile radioactivity still remains in body fat at this time (table 1), it is likely that the 24-hour production of nonvolatile metabolite is far in excess of the percentage measured above. Van Dyke *et al.*[11] have further demonstrated that the production of $^{14}CO_2$ continues at a steady rate for at least 12 hours after administration of the anesthetic. We may conclude, therefore, that a significant percentage (far in excess of 4.02 per cent) of the chloroform is degraded by the liver to nonvolatile metabolites. This is, of course, in addition to that carried to CO_2. The possibility that intestinal microorganisms participate in the metabolism of chloroform has been excluded by the studies of Van Dyke *et al.*[11]

The exact mechanism by which chloroform is biologically degraded has not been established. The liver is, as indicated above, the presumed target organ. Heppel and Porterfield[14] demonstrated that halogens (both chlorine and bromine) may be removed enzymatically by a liver halidase present in the microsomal fraction. Recent publications by

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Butler,[15] and by Paul and Rubinstein [10] have shown a similar metabolism for chloroform. Van Dyke *et al.*[11] have further confirmed the metabolism of chloroform to CO_2 (4 to 5 per cent in 24 hours), and in addition demonstrated the presence of ^{36}Cl metabolite(s) in the urine (0 to 2 per cent in 24 hours).

The present study establishes a major role for the liver in the metabolism of chloroform. Nonvolatile metabolic products are found in the liver immediately after the termination of anesthesia, and these continue to increase in amount for the duration of the experiment. Thin-layer chromatography has established the presence of two individual metabolites.

The importance of the metabolism of chloroform in the genesis of hepatocellular toxicity has been suggested previously,[15] and the above evidence is compatible with this possibility. Nonvolatile metabolic byproducts accumulate in the liver for at least two hours (presumably much longer), and whole-body autoradiography suggests a segmental concentration for these metabolites. The toxicity of the metabolic materials formed and the relationship of their localized concentration to hepatocellular dysfunction remain to be established, as does confirmation of the above information in the human species.

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