The Effects of Halothane on Rat Liver Mitochondria*

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ABSTRACT: Halothane ($CF_3CHBrCl$), a widely used anesthetic agent, has been suspected of causing liver necrosis in man. Many halogenated hydrocarbons are recognized hepatotoxins and one of these, CCl_4, is known to bring about striking changes in oxidative phosphorylation of rat liver mitochondria. The effects of halothane on these subcellular organelles were, therefore, investigated. When incubated with normal rat liver mitochondria, halothane uncouples oxidative phosphorylation, induces irreversible swelling, abolishes the 2,4-dinitrophenol-stimulated adenosine triphosphatase (ATPase) activity, and causes a marked decrease in potassium content. Halothane inhibits the oxidation of diphosphopyridine nucleotide linked substrates, yet reduced diphosphopyridine nucleotide (DPNH) is oxidized in the presence of the hydrocarbon. This DPNH oxidation is uncoupled from phosphorylation and is blocked by amytal. Using various substrates, halothane is found to uncouple at all three phosphorylation sites. The ability of halothane, CCl_4, and other hydrocarbons such as chloroform, benzene, and diethyl ether to uncouple oxidative phosphorylation and to cause mitochondrial swelling is closely correlated with their relative oil–water solubilities. After oral administration of halothane to rats, no impairment in mitochondrial function is detected.

Halothane was the first of a series of fluorinated hydrocarbons to be employed as an anesthetic and appeared to be an ideal agent until reports of massive liver necrosis associated with its use began to appear in 1958 (Little and Wetstone, 1964). Halothane ($CF_3CHBrCl$) is liquid at 20°, boils at 50.2°, is very soluble in lipids, having an oil–water solubility ratio of 330 (Raventos, 1956), and is extremely potent as an anesthetic agent. The molecule is quite resistant to biological degradation, only 2.9% of the chlorine atoms and 2.4% of the carbon atoms being excreted as metabolic products (Van Dyke *et al.*, 1964). Liver toxicity studies in animals have demonstrated moderate fat accumulation, nuclear changes, and cloudy swelling without necrosis (Little and Wetstone, 1964). At present, a biochemical rationale for its possible hepatic toxicity does not exist.

CCl_4 has been shown to exert profound effects on mitochondrial function, structure, and composition, uncoupling oxidative phosphorylation (Dianzani, 1954; Calvert and Brody, 1958), causing swelling *in vitro* (Recknagel and Malamed, 1958) and *in vivo* (Oberling and Rouiller, 1956), inhibiting the DNP[1]-stimulated ATPase, and activating a magnesium-dependent ATPase activity (Recknagel and Anthony, 1959). CCl_4 poisoning also leads to a marked accumulation of mitochondrial calcium and a loss of potassium (Thiers *et al.*, 1960). Disintegration of the endoplasmic reticulum (Oberling and Rouiller, 1956), inhibition of protein synthesis (Smuckler *et al.*, 1961), and a decrease of the activities of various enzymes present in microsomes (Recknagel and Lombardi, 1961) are the earliest lesions observed after CCl_4 administration.

In the present investigation, the effects of $CF_3CHBrCl$ on the biochemical functions of mitochondria have been studied and compared with those of CCl_4. While many of the effects are similar, interesting differences between the effects of these compounds have been identified. Both $CF_3CHBrCl$ and CCl_4 are true uncoupling agents, and not simply inhibitors of mitochondrial respiration when employed to study mitochondrial function *in vitro*. Halogenation is not a necessary condition required to obtain such uncoupling; rather, uncoupling potency appears to be a function of the lipid solubility of a series of hydrocarbons, whether halogenated or not.

Experimental Section

Materials and Methods

Mitochondrial Preparations. Healthy young female rats of the Sprague–Dawley strain, weighing between 150 and 200 g, were maintained on a diet of Purina chow and water *ad libitum*. All animals were fasted 16 hr and then sacrificed by decapitation and exsanguination. The livers were immediately perfused with 0.25 M sucrose at 4°, pulped in a stainless steel tissue press (Harvard Apparatus Co.), and homogenized in a loose-fitting, glass Potter–Elvehjem homogenizer. The nuclei and

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[1] Abbreviations used in this work: DNP, 2,4-dinitrophenol; DPN (also known as NAD), diphosphopyridine nucleotide; DPNH (also known as NADH), reduced diphosphopyridine nucleotide; ATP, adenosine triphosphate.

residue were removed by centrifugation twice at 600*g* for 7 min, the mitochondria sedimented at 13,000*g* for 20 min, and then washed once with sucrose. Precautions were taken to avoid metal contamination of reagents and glassware when experiments involved measurements of metal content of mitochondria (Thiers and Vallee, 1957). All preparations were carried out at 4°. The mitochondrial pellet was resuspended in sufficient 0.25 M sucrose to make the volume twice that of the original liver pulp and used immediately. Each milliliter of suspension then contained approximately 10 mg of mitochondria, expressed as dry weight at 100°.

Preincubation with Inhibitors. Halothane and other hydrocarbons were preincubated with normal rat liver mitochondria as described by Reynolds *et al.* (1962) for CCl_4, modified so that 2–5 ml of mitochondrial suspension was mixed with variable volumes of 0.25 M sucrose and with sucrose saturated with the hydrocarbon while maintaining a total volume three times the volume of mitochondrial suspension. The suspension was immediately centrifuged for 20 min at 13,000*g*, the supernatant was decanted, and the pellet was resuspended in 0.25 M sucrose to the original volume. At a concentration of halothane which produced uncoupling, no significant change in respiration occurred within a twofold range of mitochondrial concentration. The dry weight of mitochondria in equivalent experiments was, therefore, kept within these limits. The concentration of a saturated aqueous solution of halothane at 10° is 2.65×10^{-2} M, at 24° is 2.14×10^{-2} M, and at 27° is 2.12×10^{-2} M (Raventos, personal communication, 1965). In some experiments, halothane was added directly to mitochondria in the Warburg flasks.

Manometric Techniques. Standard manometric techniques were employed at 30°. Rates of oxygen uptake (Qo_2) were expressed as O_2 consumed (μl.)/hr per mg of mitochondrial dry weight. Inorganic phosphate was measured by the method of Taussky *et al.* (1953). Each Warburg flask contained 0.5 ml of mitochondrial suspension, 120 μmoles of glycylglycine at pH 7.4, 40 μmoles of potassium phosphate, 20 μmoles of $MgSO_4$, 4 μmoles of ATP (Pabst Laboratories); and sufficient 0.5 M sucrose to bring the measured osmolality of the medium to 0.27. KOH and HCl were used to neutralize all reagents to pH 7.4. The measured potassium content of this reaction mixture was 55 mM when the substrate was glutamate and 67 mM for succinate. The side arm of the flasks contained 100 μmoles of glucose and 0.25 mg of yeast hexokinase, type IV (Sigma Chemical Company). The center well contained 0.10 ml of freshly prepared 10% KOH dispersed by fluted filter paper projecting 5 mm above the well. The total volume in each vessel was 3.10 ml.

The following amounts of various substrates were added to the flasks: 50 μmoles of succinate (Eastman), 50 μmoles of glutamate (Eastman), 30 μmoles of DL-β-hydroxybutyrate (Nutritional Biochemical Corp.), 10 μmoles of ascorbate (Eastman), and 5 μmoles of octanoate (Calbiochem) with 1 μmole of succinate as primer. In some experiments 1 μmoles of DPN (Pabst Laboratories) or 0.02 μmole of cytochrome *c*

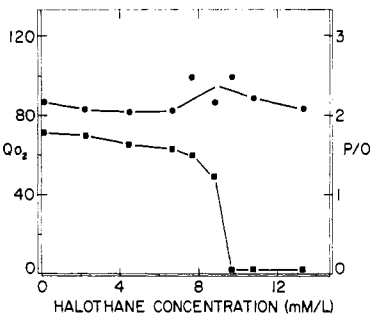

FIGURE 1: Uncoupling of succinate oxidative phosphorylation after preincubation of normal rat liver mitrochondria, with various doses of halothane. Mitochondria suspended in 0.25 M sucrose were covered with variable volumes of 0.25 M sucrose saturated with halothane at 4°, the total volume brought to three times the volume of original mitochondrial suspension, mixed by inversion, and the suspension spun at 13,000*g* for 20 min. The pellet was resuspended in 0.25 M sucrose and assayed immediately at 30° using succinate as substrate. The Qo_2 (●) and P:O ratios (■) shown represent duplicate assays on different mitochondrial preparations.

(Sigma) were added. In experiments utilizing DPNH (Sigma) as substrate, the method of Lehninger (1951) was employed, modified by the addition of 4 μM ATP and 40 μM inorganic phosphate to the reaction mixture. Ascorbate oxidation required 0.15 μM cytochrome *c* for optimal oxidation and phosphorylation, and optimal butyrate oxidation required the addition of 6 mg of defatted bovine serum albumin (Armour)/flask.

The coefficient of variation of replicate determinations for Qo_2 and P:O ratios determined on aliquots of the same preparation of mitochondria was approximately 5%. The Qo_2 for consecutive determinations on 15 normal rats with glutamate as substrate averaged 89 ± 15, and the P:O ratios averaged 2.55 ± 0.12.

Swelling experiments were carried out by the method of Lehninger *et al.* (1959) at 25° in either 0.30 M sucrose or 0.15 M KCl buffered with 0.02 M Tris, at pH 7.4. ATPase activities were measured by the method of Recknagel and Anthony (1959) at 25°; the mitochondria were preincubated with halothane at 4° as described, *vide supra.*

Metal Analyses. Aliquots of mitochondrial suspension were wet-ashed with a mixture of 2:1 redistilled concentrated nitric acid (G. W. Smith Co.) and vacuum distilled 70% perchloric acid (G. W. Smith Co.), taken to dryness at 200°, and the ash dissolved in 4.0 ml of 0.5 M ammonium EDTA. Potassium, calcium, and magnesium were determined simultaneously by a flame spectrophotometric method (Margoshes and Vallee, 1956) in 0.5 M ammonium EDTA (Thiers and Hviid, 1962) to suppress anion (phosphate) effects. Accuracy was established by measurement of National Bureau of Standards samples, and found to be within 3% of the given values for calcium and magnesium. The repro-

TABLE I: Effect of Direct Addition of Halothane to Mitochondria in the Assay Medium.[a]

Substrate	Function	Halothane Concn (mmoles/l.) 0	0.7	3.5	7.0	14
Succinate	Qo_2	93	92	98	97	83
	rcr	2.2	2.1	2.1	2.1	0.7
	P:O	1.62	1.64	1.63	1.85	0
Glutamate	Qo_2	112	101	91	72	0
	rcr	4.7	5.9	2.9	2.5	...
	P:O	2.50	2.52	2.48	2.80	...
β-Hydroxybutyrate	Qo_2	36	34	34	22	5.0
	rcr	1.2	1.0	1.1	1.0	0.5
	P:O	2.6	2.5	2.7	2.7	0

[a] Mitochondria were added to the assay mixture, and immediately thereafter various volumes of 0.25 M sucrose, saturated with halothane, were added to the flasks, the mixture was allowed to equilibrate at 30° for 5 min, the manometers were closed, and oxidation, phosphorylation, and the respiratory control ratio (rcr) were measured.

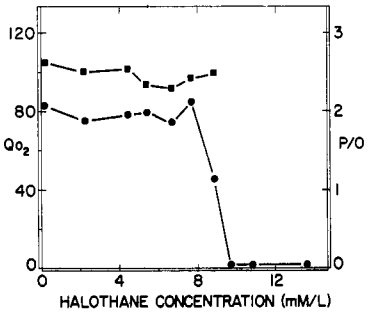

FIGURE 2: The inhibition of glutamate oxidative phosphorylation after preincubation of mitochondria with halothane. Experimental conditions are those described in Figure 1. The failure of oxidation (●) beyond 9 mM halothane precluded further measurements of P:O ratios (■).

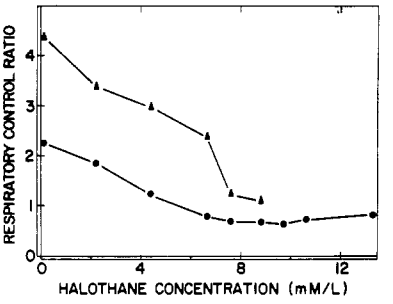

FIGURE 3: The loss of respiratory control after preincubation of mitochondria with halothane. Experimental conditions are those described in Figure 1. The Qo_2 after addition of hexokinase–glucose acceptor system divided by the Qo_2 before adding acceptor is defined as the respiratory control ratio. This ratio for succinate (●) decreases to and remains at 0.6–0.8; the abolition of oxidation of glutamate (▲) beyond 9 mM halothane allows no further ratios to be calculated.

ducibility of the flame method is 3% for these metals at the concentrations measured. Nitrogen was determined on each aliquot of mitochondria by nesslerization.

Oral Administration of Halothane. In vivo experiments with halothane were accomplished by fasting rats for 16 hr and administering a 1:1 mixture of halothane–mineral oil, at a dose of 0.5 ml/100 g of body weight, by polyethylene stomach tube. This dose resulted in the onset of deep anesthesia in 15–30 min, which persisted for 4–6 hr. The rats were fasted 16 hr before sacrifice.

Results

Halothane Effects on Oxidative Phosphorylation. When succinate serves as the substrate, uncoupling of oxidation from phosphorylation occurs rather abruptly after preincubation of the mitochondria in 10 mM halothane (Figure 1). With ascorbate, uncoupling occurs at the same halothane concentration. Oxidation of succinate is not impaired until the concentration of halothane exceeds 14 mM; beyond this point a 10–20% decrease in Qo_2 is noted. When glutamate is the substrate, however, the response to halothane preincubation differs strikingly: oxidation is abolished at 10 mM halothane, phosphorylation being maintained up to the very point where oxidation ceases (Figure 2). Octanoate oxidation is similarly destroyed at this concentration. Direct addition of increasing amounts of halothane to the Warburg flasks, just before assay, inhibits oxidation of glutamate and β-hydroxybutyrate, and uncouples succinate oxidation from phosphorylation at concentrations of 7–14 mM halothane (Table I).

A loss of respiratory control precedes these decreases in oxidation and phosphorylation. As the dose of

halothane is increased, the ratio of glutamate oxidation with and without the hexokinase–glucose acceptor system decreases progressively from the normal range of 4 to 5; no acceptor effect is obtained at 8 mM halothane, just before oxidation ceases (Figure 3). Respiratory control ratios with succinate fall rapidly from the normal range of 2 to 3, such that no acceptor effect is noted at 4–6 mM halothane and the ratio remains at 0.7 until uncoupling is complete at 10 mM halothane (Figure 3). This decrease in respiratory control is due entirely to an increased rate of oxidation without acceptor.

After preincubation with uncoupling concentrations of halothane, the rate of oxidation of succinate is not impaired if exogenous inorganic phosphate is omitted from the media (Table II). A similar phenomenon is observed with uncoupling concentrations of CCl_4 (Table II).

TABLE II: Effect of Inorganic Phosphate Concentration on Ability of Mitochondria Uncoupled by Halothane to Oxidize Succinate.[a]

Uncoupling Agent	P_i Added (μmoles)	P_i Content, before Addition of Acceptor (μmoles)	O Uptake (μatoms/20 min)	P Uptake μmoles/20 min)
Control	41	42.6	16.5	22.5
	0	1.8	5.5	1.4
$CF_3CHBrCl$	41	43.5	16.2	0
(17 mM)	0	2.8	16.0	0
CCl_4	41	42.6	13.5	0
(3.8 mM)	0	2.8	15.9	0

[a] Mitochondria (2-ml aliquots) were preincubated for 20 min at 4° with 0.25 M sucrose containing the uncoupling agents at the concentration shown. Inorganic phosphate (41 μmoles) was added to the first of each pair of Warburg flasks, and none to the second. After 10 min of incubation at 30°, hexokinase–glucose was tipped in and oxidation and phosphorylation measured. Inorganic phosphate contents were measured in a zero-time control before adding the acceptor system. Each flask contained 1 μmole of ATP.

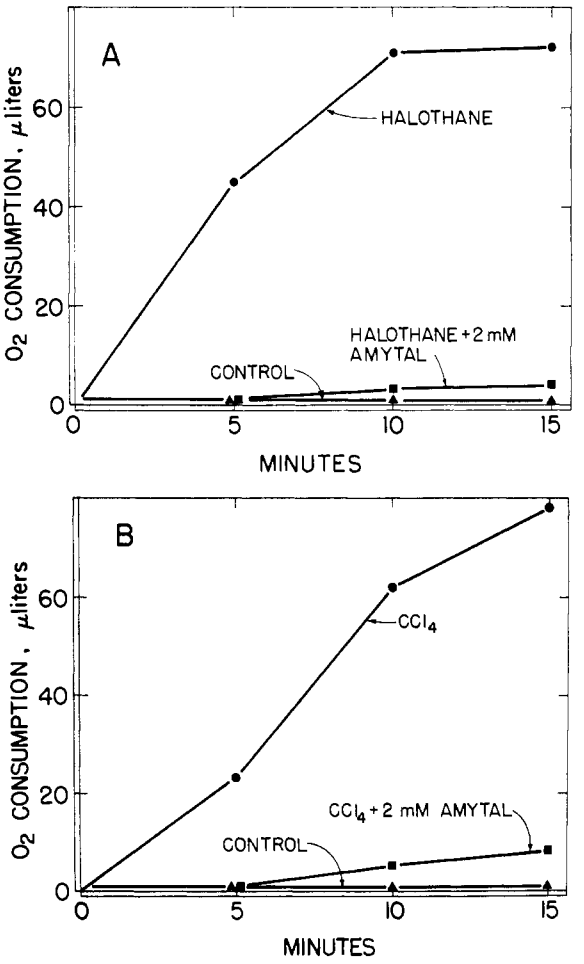

FIGURE 4: The effects of amytal on halothane (A) and carbon tetrachloride (B) induced oxidation of DPNH by rat liver mitochondria. The mitochondria in (A) were preincubated with 17.6 mM halothane, immediately centrifuged at 13,000g for 20 min at 4°, resuspended in 0.25 M sucrose, and assayed at 30° by tipping in 8.8 μmoles of DPNH as substrate. No hexokinase–glucose was used. Each flask contained in addition 40 μM potassium phosphate, 4 μM ATP, 120 μM glycylglycine buffer at pH 7.4, 20 μM $MgSO_4$, and 0.5 ml mitochondrial suspension. Amytal (60 μM) was added to one set of flasks. The same conditions were used in (B), except that the mitochondria were incubated in 4.2 mM CCl_4. Oxidation without hydrocarbon incubation (▲) is compared to the oxygen consumption after halothane or CCl_4 (●), and after addition of 2 mM amytal to the flasks (■).

Attempts were unsuccessful to protect against or reverse this inhibition of glutamate oxidation or the uncoupling of succinate oxidation from phosphorylation. Addition of 1 μM DPN, 0.02 μM cytochrome c, singly or together, or of 2 mg/ml of bovine serum albumin to the Warburg flasks does not restore function when the preincubation concentration of halothane is 10 mM. Nor does 3 mM EDTA added to the sucrose during isolation of mitochondria or during preincubation with halothane protect against the inhibition. Lastly, isolation and incubation of the mitochondria in 0.44 or 0.88 M sucrose does not prevent the halothane uncoupling of succinate oxidation from phosphorylation.

In order to determine whether the loss of DPNH-linked substrate oxidation indicated a block of electron flow from DPNH to cytochrome b, mitochondria were preincubated in halothane, and DPNH was then used as the substrate. Normal mitochondria in isotonic media

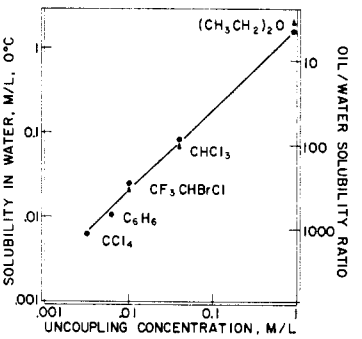

FIGURE 5: The correlation of lipid–water solubility with ability of various agents to uncouple succinate oxidation from phosphorylation. Mitochondria, at a constant concentration, were preincubated with various concentrations of carbon tetrachloride, benzene, halothane, chloroform, and diethyl ether in 0.25 M sucrose at 4°, the total volume being constant at 6 ml. The lowest concentration of hydrocarbon which completely uncoupled succinate oxidative phosphorylation is plotted on the horizontal axis as a function of the solubility of a saturated, aqueous solution of each agent (●) on the left ordinate (International Critical Tables, 1928). The uncoupling concentration is also correlated with the oil–water solubility ratios for halothane, chloroform, and ether, (▲), the right ordinate (Adriani, 1962).

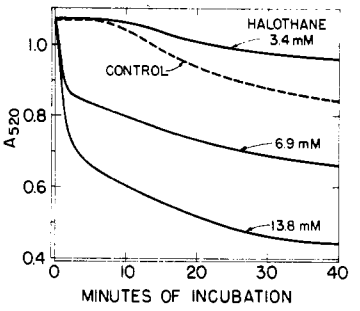

FIGURE 6: Swelling of rat liver mitochondria *in vitro*, upon exposure to halothane. Normal nitochondria isolated on 0.25 M sucrose are added to 3.0 ml of 0.3 M sucrose containing Tris–HCl buffer, 0.02 M, pH 7.4, mixed, and the optical density read at 520 mμ every 2 min at 25°. Halothane was added in the concentrations shown.

do not oxidize exogenous DPNH. In hypotonic media, DPNH is rapidly oxidized if cytochrome *c* is added, as shown by Lehninger (1951), but the maximal P:O ratios are only 0.6. However, 17.6 mM halothane allows rapid oxidation of DPNH without addition of cytochrome *c* (Figure 4A). Although DPNH is rapidly oxidized after preincubation with halothane, no phosphorylation occurs.

Siekevitz (1958) has stated that it is virtually impossible to separate mitochondria completely from contaminating microsomal membranes, and Strittmatter and Velick (1956) have described a DPNH–cytochrome b_5 reductase in the microsomal fraction which can transfer electrons from DPNH to oxygen *via* cytochrome *c* and the terminal part of the mitochondrial respiratory chain. To ensure that electrons were actually passing from DPNH to oxygen through the first part of the mitochondrial electron chain and not through an external, nonphosphorylating chain (Ernster *et al.*, 1963), 2 mM amytal was added to the assay mixture of halothane-treated mitochondria. Amytal almost completely suppresses the oxidation of DPNH (Figure 4A). Analogous results are obtained by preincubating mitochondria with CCl_4. At a concentration of 4.2 mM rapid oxidation of DPNH occurs which can also be blocked with amytal (Figure 4B).

Effects of Other Hydrocarbons on Oxidative Phosphorylation. These effects of halothane and CCl_4 on uncoupling of mitochondria *in vitro* are qualitatively similar but differ quantitatively. This difference was considered to be perhaps a function of their relative water solubilities. Therefore, chloroform and nonhalogenated hydrocarbons such as benzene and diethyl ether were also incubated *in vitro* with mitochondria to find the concentration of hydrocarbon necessary to cause complete uncoupling of oxidation of succinate from phosphorylation. When these critical uncoupling concentrations are plotted *vs.* the relative water solubilities of these hydrocarbons, a linear relationship is apparent over a concentration range of almost three log decades (Figure 5). From the literature only three values were found for olive oil–water solubility ratios of these compounds; *i.e.*, for diethyl ether, chloroform, and halothane. The uncoupling ability of these three agents is directly correlated with their lipid–water solubility ratios (Figure 5).

Halothane Effects on Swelling of Mitochondria. During the experiments in which mitochondria were preincubated with halothane, the pellet was larger, looser, and more translucent than that from normal mitochondria, implying a change in their volume. Therefore, the effects of halothane on swelling were studied over the range of concentration which inhibited oxidation and phosphorylation. A halothane concentration which causes complete abolition of glutamate oxidation, 13.8 mM, rapidly brings about maximal swelling (Figure 6), and at 6.9 mM halothane both the rate and degree of swelling is about half. Phase-contrast microscopy, which showed rapid swelling to diameters 3 to 4 times larger than normal, proved that these changes represented swelling and not disruption of mitochondria. At 3.4 mM halothane, where oxidation and phosphorylation are still normal, but respiratory control ratios are falling, the normal rate of swelling is actually retarded (Figure 6).

At low concentrations of halothane, swelling can be prevented partially on addition of albumin, ATP, or EDTA to the medium. In KCl–Tris buffer, 4 mg/ml of bovine serum albumin prevents 65% of the decrease in optical density which occurs in 6 mM halothane. ATP

(5 mM) prevents 9% and albumin at 2 mg/ml prevents 28% of the swelling at this concentration. The combination of ATP and albumin prevents 52% of the decrease seen at 6.6 mM. At 14 mM halothane neither albumin, ATP, or EDTA nor any combination can prevent swelling. Although 1 mM cyanide prevents spontaneous swelling or that which occurs during oxidation of substrates, it does not prevent swelling in 8.8 mM halothane whether glutamate or succinate is present or not.

In order to assess the generality of the swelling phenomenon seen with halothane and reported for CCl_4 (Recknagel and Malamed, 1958), $CHCl_3$, benzene, and diethyl ether were also examined. The concentration of these agents necessary to cause a maximum rate and degree of swelling was compared with their water solubilities. Similar to their ability to uncouple succinate oxidation, swelling caused by these agents is found to be inversely related to water solubility, reaching a maximum at 67% of saturation on the average (Table III).

TABLE III: Correlation of Water Solubility with Ability to Cause Swelling of Rat Liver Mitochondria.[a]

Agent	Solubility of a Satd Aqueous Solution at 25° (M)	Concn to Achieve Maximum Rate and Degree of Swelling at 25° (M)	% Satn
CCl_4	5.4×10^{-3} [b]	3.6×10^{-3}	67
C_6H_6	1.9×10^{-2} [b]	9.5×10^{-3}	50
$CF_3CHBrCl$	2.1×10^{-2} [c]	1.4×10^{-2}	67
$CHCl_3$	6.5×10^{-2} [b]	4.3×10^{-2}	66
$(CH_3CH_2)_2O$	8.1×10^{-1} [b]	5.4×10^{-1}	67

[a] Swelling was measured in 0.25 M sucrose–0.02 M Tris, pH 7.4, by following the decrease in absorbance at 525 mμ for 30 min. [b] International Critical Tables (1928), interpolated to 25°. [c] Raventos, personal communication, 1965.

Effects of Halothane on ATPase Activity. Halothane produces a striking increase in magnesium-activated ATPase and a corresponding loss of DNP-stimulated ATPase activity (Figure 7), reaching a maximum at a concentration of 17.7 mM halothane. Halothane (4.4 mM) has no effect on magnesium-dependent or DNP-ATPase activity. At 8.8 mM halothane, a concentration which impairs respiratory control but not oxidation or phosphorylation, albumin can restore ATPase activities completely to normal. However, at 17.7 mM halothane, an uncoupling concentration, albumin only maintains DNP-stimulated ATPase at 17% of normal. The halothane concentration which on preincubation at 4° causes a 50% loss of DNP-ATPase activity is found to be approximately 14 mM. This concentration falls in the expected position in the series of DNP-ATPase inhibitions of a group of hydrocarbons studied by Recknagel and Anthony (1959).

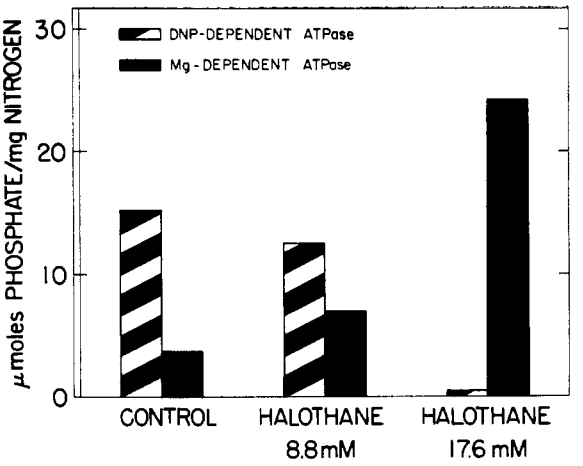

FIGURE 7: Effects of halothane on mitochondrial ATPase activities. The DNP-stimulated ATPase activity (cross-hatched bars) is decreased by halothane, while the magnesium-dependent ATPase activity (black bars) is concomitantly increased. Mitochondria were preincubated at 4° with 8.8 and 17.6 mM halothane as described in Figure 1, and an aliquot assayed for ATPase activities in 0.22 M sucrose, 0.002 M Tris, pH 7.4, at 30°.

Effects of Halothane on Mitochondrial Metal Content. Metal content and respiratory function were compared after exposure to halothane to examine for losses of mitochondrial potassium observed after CCl_4 (Reynolds *et al.*, 1962). Mitochondria isolated in metal-free sucrose contained potassium, calcium, and magnesium in accord with previous results obtained by emission and flame spectrometric analyses (Thiers and Vallee, 1957; Thiers *et al.*, 1960). After exposure to 4.4 mM and 17.6 mM halothane for 10 min at 25°, and then for 20 min at 4° during centrifugation, mitochondrial potassium content decreases proportionately but calcium and magnesium content remains within normal limits (Table IV). Oxidative phosphorylation is normal at 4.4 mM halothane, but is abolished at the higher concentration in these mitochondria.

Preincubation with 1 mM EDTA reduces the magnesium content of mitochondria to a third of normal but oxidative-phosphorylation remains intact. EDTA does not prevent the inhibition of glutamate oxidation or uncoupling of succinate oxidative phosphorylation due to 17.6 mM halothane. Moreover, the combination of EDTA and halothane further reduces the magnesium and calcium content, although it retards potassium loss (Table IV). When mitochondria are incubated with 0.3 mM $CaCl_2$ at 4° without substrate or ATP, they accumulate a sixfold increase in calcium content, while

TABLE IV: Effects of Halothane, EDTA, and Added Calcium on the Potassium, Calcium, and Magnesium Content of Mitochondria.

Conditions	Metal Content (μg/mg of N) K	Ca	Mg
Mean Controls[a]	31.5	1.21	7.72
Range	27.2–39.3	0.81–2.18	5.75–9.60
Halothane[b]	13.3	0.81	6.50
(4.4 mM)	10.6	0.90	7.95
Halothane[b]	6.45	1.07	6.80
(17.6 mM)	6.02	1.24	7.55
EDTA (1 mM)[c]	26.6	1.03	3.10
Halothane (17.6 mM) + EDTA (1 mM)[c]	10.7	0.33	2.50
0.3 mM $CaCl_2$[d]	4.35	6.26	3.64
Halothane (17.6 mM) + 0.3 mM $CaCl_2$[d]	3.83	8.10	5.40

[a] Mitochondrial pellet isolated in metal-free 0.25 M sucrose and glassware, resuspended in sucrose, centrifuged for 20 min at 13,000*g*, and resuspended in sucrose. Control values are the mean and range of determinations on six normal rats. [b] Preincubated with halothane for 20 min at 4° during centrifugation at 13,000*g*, and resuspended in 0.25 M sucrose. [c] Preincubated with or without halothane as in *b*, but in 0.25 M sucrose containing 1 mM EDTA. Qo_2 and P:O ratios were normal for glutamate and succinate in 1 mM EDTA, but 17.6 mM halothane uncoupled these mitochondria in EDTA. [d] Preincubated with or without halothane as in *b*, but in 0.25 M sucrose containing 0.3 mM $CaCl_2$. This concentration of $CaCl_2$ uncouples oxidative phosphorylation.

potassium and magnesium fall to low levels. Adding halothane to the calcium preincubation increases the calcium to a content eight times normal.

Effects of in vivo Administration of Halothane on Oxidation and Phosphorylation. An attempt was made to reproduce these *in vitro* effects by administration of halothane in mineral oil by stomach tube to fasted rats, thereby exposing the liver to a maximal concentration of the agent. The maximum dose which did not produce respiratory arrest was 0.25 ml of halothane/100-g body weight. The P:O ratios of rats sacrificed at 2, 11, 24, 40, and 67 hr after this dose of halothane show no significant change from controls (Table V), and respiratory control ratios are normal in all experiments. Oxidation of glutamate and succinate is uneffected and octanoate oxidation is minimally decreased only at 24 and 40 hr. Three doses given at 24, 96, and 144 hr before sacrifice result in normal P:O ratios and a small decrease in succinate and octanoate oxidation. The calcium content is decreased to 0.4 μg/mg of N, but the potassium and magnesium content of mitochondria is normal 30 hr after halothane *in vivo*. No significant delayed toxicity is observed when 0.18 ml of halothane/100 g is given 18, 15, and 12 days before sacrifice (Table V).

Discussion

Halothane is shown to be a true uncoupling agent of rat liver mitochondria *in vitro*. The inhibition of oxidation of DPN-linked substrates is not due to a block of electron transport in the first part of the respiratory chain from DPNH dehydrogenase to cytochrome *b*, since DPNH itself is oxidized rapidly after preincubation of mitochondria in halothane (Figure 4A). The ability of amytal to block such oxidation indicates that the electrons are flowing through the first part of the normal respiratory chain (Ernster *et al.*, 1963). Since neither DPNH, succinate, nor ascorbate oxidation yields ATP, halothane appears to uncouple at all three phosphorylation sites.

The experiments with CCl_4 also serve to classify the mode of action of this agent on oxidative phosphorylation. Reynolds *et al.* (1962) demonstrated that CCl_4 abolishes octanoate oxidation *in vitro* and Recknagel and Anthony (1959) showed a CCl_4 concentration-dependent inhibition of α-ketoglutarate or glutamate oxidation. The demonstration (Figure 4B) that after CCl_4 incubation DPNH is still oxidized by way of the respiratory chain removes CCl_4 from the list of respiratory inhibitors and places it in the category of true uncoupling agents.

Since DPN-linked substrates are not oxidized after halothane treatment of mitochondria, this hydrocarbon must impair the function of the enzyme systems which generate DPNH within the mitochondrial matrix. DPN is known to leak out of swollen mitochondria, yet the addition of exogenous DPN to halothane-treated mitochondria did not restore their function. Some enzymes of the mitochondrial matrix, such as glutamic dehydrogenase, also leak out of swollen mitochondria (Estrada-O, 1964), and this result could account for the loss of glutamate oxidation after halothane incubation. However, β-hydroxybutyrate dehydrogenase is firmly bound to the mitochondrial membranes and is active after sonic, hypotonic, or chemical disruption of mitochondrial structure, unless its bound DPNH is lost and its essential thiol groups are oxidized (Wise and Lehninger, 1962). The inhibition of β-hydroxybutyrate oxidation after halothane (Table I) may be due to such a loss of bound pyridine nucleotide.

Whatever the mechanism by which halothane uncouples oxidative phosphorylation this effect cannot be prevented or reversed by added DPN, cytochrome *c*, albumin, or hypertonic sucrose. No protection of DPN-linked respiration could be demonstrated with EDTA, in contrast to the report of Reynolds *et al.* (1962) that EDTA protected octanoate oxidation during CCl_4 treatment of mitochondria. Halothane at this

TABLE V: Effect of Halothane *in Vivo* on Oxidative Phosphorylation of Rat Liver Mitochondria.

Hours after Doses of 0.25 ml/100 g	Qo_2			P:O		
	Glutamate	Succinate	Octanoate	Glutamate	Succinate	Octanoate
Normal Values[a]	89 ± 15	107 ± 14	58 ± 6	2.55 ± 0.12	1.68 ± 0.14	1.79 ± 0.18
2	83	104	53	2.76	1.97	2.27
11	90	104	55	2.56	1.89	2.01
24	88	121	45	2.65	1.60	1.80
40	75	95	39	2.52	1.82	1.66
67	71	80	48	2.40	1.82	1.88
24, 96, 144	67	71	45	2.60	1.95	2.17
Days after Doses of 0.18 ml/100 g						
12, 15, 18	77	97	42	2.62	1.86	1.98

[a] Normal values represent the mean and standard deviations of 12 determinations on six normal rats.

juncture appears to be an irreversible uncoupling agent *in vitro*.

A loss of normal respiratory control is the earliest functional impairment detected after halothane exposure (Figure 3). The decrease in respiratory control ratio begins at the lowest concentration of halothane tested (2 mM). No swelling occurs at this concentration; in fact the usual time-dependent swelling is prevented. Oxidation and phosphorylation remain within normal limits until the concentration of halothane reaches 8–10 mM. This phenomenon of loose coupling, preceding and unassociated with uncoupling, is characteristic of other uncoupling agents such as DNP (Lardy and Wellman, 1952) and thyroxine (Niemeyer *et al.*, 1951). The loss of respiratory control after halothane is the result of an increase in State 4 or "acceptor-less" respiration, a result which may occur with other uncoupling agents because of increased ATPase activity. This mechanism cannot be invoked in the case of low doses of halothane since the magnesium-dependent ATPase activity is not increased after incubation in 4 mM halothane. At 8.8 mM halothane, increased magnesium–ATPase could account for this loss of respiratory control (Figure 7).

A significant loss of mitochondrial potassium does occur after preincubation of mitochondria in 4.4 mM halothane (Table IV); this may play a role in the increase of acceptor-less respiration. The loss of respiratory control is not repaired by exogenous potassium, however, since the assay medium contains 55–67 mM potassium. Perhaps it is the firmly bound potassium which halothane releases, a form which cannot be replaced easily by exogenous potassium (Gamble, 1957; Share and Recknagel, 1959). Lehninger (1964) has pointed out that a high acceptor control ratio is a more rigorous criterion of intactness of mitochondrial structure than a high P:O ratio. In this light, 4 mM halothane appears to induce subtle changes in structure which cause a loss of bound potassium and of respiratory control. Seven to eight mM halothane causes frank swelling and loss of DNP–ATPase activity, but complete uncoupling does not occur until 10 mM halothane.

The localization of the halothane uncoupling effect seems to lie between the respiratory chain and the uptake of inorganic phosphate in the coupling process. Electron transport is not impaired by halothane, as noted previously, and oxidation is not retarded by a rate-limiting concentration of inorganic phosphate (Table II). This lack of phosphate requirement is seen with other true uncoupling agents and has led to the hypothesis that they act by causing a breakdown in some high energy intermediate prior to the uptake of inorganic phosphate (Lehninger, 1964, p 92).

Uncoupling of oxidative phosphorylation *in vitro* is a phenomenon not restricted to halogenated hydrocarbons such as halothane, CCl_4, and $CHCl_3$, but also occurs with other nonpolar hydrocarbons such as benzene and diethyl ether. Moreover, the uncoupling potency of these agents is an inverse function of their relative water solubilities and is directly related to their solubilities in lipid (Figure 5). The ability of such compounds to cause mitochondrial swelling is also related to their lipid–water solubilities, the more lipid-soluble agents being the more potent swelling agents (Table III). A similar correlation has been reported for the ability of these hydrocarbons to abolish the DNP-stimulated ATPase activities of mitochondria (Recknagel and Anthony, 1959). Since the electron carriers and coupling enzymes seem to be imbedded in the lipoprotein membranes of the cristae, in three-dimensional recurring assemblies (Lehninger, 1964, p 210), it may be postulated that halothane solubilizes the lipids of the membranes and loosens the physical coupling of electron transport to high energy intermediate formation. Such a hypothesis has been employed to explain the uncoupling action of CCl_4 (Reynolds *et al.*, 1962).

However, nonpolar solvents also exert effects on the aqueous environment, by forming hydrate microcrystals. Pauling (1961, 1964) has postulated that the anesthetic effects of such agents might be due to forma-

tion of clathrate crystals from amino acid side chains of proteins, stabilized at body temperature by incorporating molecules of the anesthetic gases. These microcrystals were then envisioned as interfering with ion movements or masking the active centers of enzymes. In support of his theory, Pauling has shown correlations between the anesthetic potency of a series of nonpolar solvents and the equilibrium partial pressure of their hydrate crystals. When anesthetic potency was compared with ability to stabilize hydrate microcrystals, the same relative positions among CCl_4, halothane, and $CHCl_3$ were found as we obtain here for their uncoupling potentials in mitochondria.

Efforts have been made to decide whether the lipid solubility or the gas hydrate theory best fits the available data, comparing anesthetic potency and various thermodynamic properties (Miller *et al.*, 1965). There is a better correlation between the partial pressure required for anesthesia and solubility in olive oil than there is with solubility in water. Although this analysis favors the lipid phase as the site of action of these compounds in the brain, the imprecision of the data is such that Miller *et al.* (1965) suggest it to be likely "that investigations of a biophysical or biochemical nature in which detailed mechanisms are considered would be a more fruitful approach." The biological effects of nonpolar hydrocarbons in many tissues appear to us to result from their action on lipoprotein-bound subcellular organelles, such as mitochondria.

If hydrate microcrystals were to form inside mitochondria, the activity of internal water molecules would be decreased and water would enter osmotically. Potassium, relatively concentrated within, would tend to move outside the mitochondria. Pauling's theory offers either an alternative or an addition to the lipid solubilization theory usually employed to account for the injurious effects of nonpolar solvents on lipoprotein-bound structures. The present results, however, suggest that it is primarily the solubility of these compounds in the lipid, not the aqueous phase, which best correlates with their uncoupling of oxidative phosphorylation (Figure 5).

The ability of serum albumin to prevent swelling and ATPase changes at 7 mM halothane suggests another effect beyond those of lipid–water interactions. Albumin is thought to protect mitochondrial function and structure because it binds fatty acids which are potent uncoupling and swelling agents. These fatty acids accumulate during ageing or after mitochondrial injury, presumably by activating mitochondrial phospholipase activity (Wojtczak and Lehninger, 1961). Therefore, halothane may activate phospholipases and release free fatty acids.

The remarkable accumulation of mitochondrial calcium which reaches a maximum at 24 hr after CCl_4 poisoning (Thiers *et al.*, 1960) does not occur after halothane administration to rats. This inability of halothane to induce calcium uptake *in vivo* may account for its lesser toxicity compared to CCl_4, even though these agents are both highly lipid soluble. Moreover, no significant changes in respiratory function result from feeding halothane in the manner used to induce CCl_4 poisoning in rats. There are a number of other possibilities which may account for this inability to show any toxic *in vivo* effects with halothane. First, the rat may not be susceptible; species differences have been described for *in vivo vs. in vitro* susceptibility to uncoupling agents (Hoch and Lipmann, 1954). The concentration of halothane in the liver achieved by stomach tube administration may not reach a level which impairs mitochondrial function. Prolonged anesthesia with halothane, however, might achieve saturating and inhibiting concentrations.

Finally, conditioning factors such as potassium or magnesium deficiency, anoxia, or coincident administration of other drugs which affect mitochondria (barbiturates) may play a role *in vivo* and in human halothane toxicity. The presence of such conditioning factors may explain the sporadic nature of liver necrosis after halothane anesthesia, compared to the predictable dose-response toxicity of CCl_4. The present *in vitro* experiments provide a biochemical rationale and hypothesis for halothane toxicity which can be tested by further pharmacologic experiments. The effects of halothane on the functions of microsomes is currently under investigation based on the hypothesis that its effects are a function of its solubility in the lipids of the endoplasmic reticulum.

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The Synthesis of Chitin by Particulate Preparations of *Allomyces macrogynus**

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ABSTRACT: A particulate enzyme from the mycelium of *Allomyces macrogynus* was shown to catalyze the synthesis of chitin from uridine diphospho-*N*-acetylglucosamine (UDP–GlcNAc). The enzyme was associated with both mitochondrial and microsomal fractions but it exhibited higher specific activity and greater stability in the latter. The enzyme was activated by soluble chitodextrin and GlcNAc. Mercaptoethanol increased the activity of the microsomal fraction and maximum activity was observed after storage at −20° for 24 hr; the activity remained unchanged after 5 months in lyophilized preparations held at −20°. The pH optimum for the reaction was 7.8 and the temperature optimum was 30°. The K_m for the reaction with UDP–GlcNAc was 1.2×10^{-3} M, and activation of the enzyme by GlcNAc did not change this value.

Chitin is an important constituent of the cell wall of fungi (Foster, 1949) but little is known about the biosynthesis of this polymeric material. Glaser and Brown (1957) demonstrated the presence of a particulate enzyme in *Neurospora crassa* that catalyzed the synthesis of chitin from uridine diphospho-*N*-acetylglucosamine[1] labeled with carbon-14. A similar synthetic process was shown to occur in cell-free preparations from *Venturia inaequalis* (Jaworski *et al.*, 1965). This paper reports the results of studies on the properties of chitin synthetase obtained from the mycelium of *Allomyces macrogynus*, a fungus whose cell walls contain 60% chitin.

Materials and Methods

UDP, UDP–GlcNAc, GlcNAc, EDTA, Tris, phosphoenolpyruvic acid (tricyclohexylamine salt), and pyruvate kinase (Type II) were obtained from Sigma Chemical Co. Yeast extract and soluble starch were obtained from Fisher Scientific Co. Chitinase was obtained from General Biochemical and mercaptoethanol from Eastman Kodak Co. [^{14}C]GlcNAc was supplied by New England Nuclear Corp. All other chemicals used were AR grade.

UDP-[^{14}C]GlcNAc labeled in the carboxyl carbon of the acetyl group was prepared according to Glaser and

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[1] Abbreviations used in this work: GlcNAc, *N*-acetylglucosamine; UDP–GlcNAc, uridine diphospho-*N*-acetylglucosamine; UDP uridine 5′-diphosphate.