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The Effect of Halothane on Electron Transport, Oxidative Phosphorylation, and Swelling in Rat Liver Mitochondria

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SUMMARY

There appear to be four distinct concentration-dependent effects of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) upon mitochondria. First, at low concentration (0.5–2.0% in the gas phase) there is a rapidly appearing, rapidly reversible effect on electron transport in the region of NADH dehydrogenase. Oxidation with succinate does not appear to be altered in this concentration range. Second, a small but distinct increase in respiration and a significant inhibition of the rate of phosphate-induced swelling with succinate as substrate suggest a limited uncoupling by 0.5–2.0% levels of halothane. The degree of uncoupling appears to be greater at 3–4% halothane. Third, at higher than clinical anesthetic concentrations (3–4%) there is partial inhibition of succinate oxidation. Most of this effect is probably direct, since oxidation is not restored to normal by the addition of 2,4-dinitrophenol and other uncoupling agents. Fourth, halothane in concentrations between 5 and 10% alters the membrane permeability and causes energy-independent swelling of mitochondria, but does not appear to cause complete disintegration of the membrane.

INTRODUCTION

Depression of oxygen uptake during exposure to volatile anesthetics has been reported in rat brain cortex slices, liver slices and heart slices (1), isolated rat liver mitochondria (2), and cell culture (3). The inhibitory effects of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) on glutamate oxidation in isolated rat liver mitochondria were originally suggested to be the result of an oxygen-dependent process (4), while succinate oxidation was not affected (2, 4, 5). Recently rat brain mitochondria were reported to undergo uncoupling of oxidative phosphorylation (6) when the effects of halothane on succinate oxidation were studied. Many drugs, including antimycin A (7), Amytal (8), and rotenone (9), have been used as selective tools to study the complexities of electron transport. The number of studies of the biochemical actions of anesthetic drugs has markedly increased in recent years. Halothane-induced inhibition of electron transport has been studied with several substrates (2, 4, 5). With anesthetic concentrations of halothane, the inhibition is nearly completely localized in the NADH dehydrogenase region.

This paper deals with evidence for four

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separate concentration-dependent actions of halothane on isolated rat liver mitochondria. These include alterations in electron transport, oxidative phosphorylation, and membrane permeability.

MATERIALS AND METHODS

Chemicals

Chemicals were purchased from the following sources: ADP, bovine serum albumin, 3-hydroxybutyrate, Tris, and EDTA, from Sigma; $MgSO_4$, succinic acid, K_2HPO_4, KH_2PO_4, and sucrose, from Mallinckrodt; and 2,4-dinitrophenol, from Fisher Scientific Company. Halothane was donated by Ayerst Laboratories. ADP was standardized by following NADH oxidation in a Zeiss spectrophotometer in the reaction

$$\text{ADP} + \text{P-enolpyruvate} \xrightleftharpoons[\text{EC 2.7.1.40})]{\text{(pyruvate kinase,}} \text{ATP}$$

$$+ \text{NADH} \xrightleftharpoons[\text{EC 1.1.1.27)}]{\text{(lactate dehydrogenase,}} \text{NAD}^+$$

$$+ \text{lactate}$$

Preparation of Mitochondria

Sprague-Dawley rats (Holtzman), weighing 200–300 g, were fed Purina chow and water ad libitum. They were stunned by a blow on the base of the skull and exsanguinated. The livers were removed rapidly, placed in 0.33 M sucrose at 2°, and chilled until the temperature became stabilized at 2–4°. The sample was then weighed and returned to a beaker containing fresh 0.33 M sucrose at 0°. Homogenization was performed with a motor-driven, loosely fitting pestle in an all-glass Potter-Elvehjem homogenizer. The liver was homogenized in a solution of 0.33 M sucrose–0.1 mM EDTA–0.1% bovine serum albumin. The homogenizing solution was diluted 8:1 (v/w) with respect to liver and then centrifuged for 10 min at 1200 × *g* in a Lourdes refrigerated centrifuge, using rotor No. 9RA. The supernatant fluid was decanted into a chilled centrifuge tube and centrifuged for 10 min at 8000 × *g*. The supernatant fluid, including the fluffy layer, was decanted. The pellet was resuspended 2:1 (v/w) with respect to the original weight of liver in a cold room (4°) with fresh homogenizing solution when oxygen electrode tests were to be done, or with 0.33 M sucrose for swelling studies. The mitochondria were again centrifuged at 8000 × *g*, washed once more, and then resuspended. The final suspension for studies on oxidation and phosphorylation was 1:1 (v/w) in a respiratory control medium containing 10 mM $MgSO_4$, 0.2 M sucrose, 0.1 mM EDTA, 0.1% bovine serum albumin, 10 mM KF, and 20 mM potassium phosphate buffer, pH 7.2. The final resuspension for swelling studies was 1:1 (v/w) in 0.33 M sucrose. Mitochondria prepared by this general procedure are morphologically intact upon electron microscopic examination and show good respiratory control.

Oxidative Phosphorylation Assays

A Clark type of electrode was used to measure oxygen uptake. The signal was amplified with a Beckman physiological gas analyzer, model 160, and recorded on a Honeywell model 19 dual-channel recorder. Tests were made in 4-ml cuvettes with a 0.5-inch, Teflon-coated magnetic stirrer rotating continuously at the bottom. Cuvettes were filled with respiratory control medium, stoppered, and placed in a rack suspended in a 26° constant temperature bath. Additions of substrate, ADP, and dinitrophenol were made by means of calibrated microliter syringes with long, stainless steel needles through a 1 mm × 2 cm polyvinyl catheter. The needles were inserted until the tips were near the bottom of the cuvette. Mitochondrial preparations were considered adequate for use if the respiratory control (10) was greater than 5 when 3-hydroxybutyrate was used as substrate and 4 when succinate was used as substrate. Controls were repeated and found to be in the same acceptable limits at the completion of each day of testing. Substrate was added as 40 μl of 0.5 M solutions of 3-hydroxybutyrate or succinate to yield 5 mM concentrations. ADP was added as 7.7 μl of 91.5 mM ADP to yield a 176 μM final concentration. Six microliters of 0.01 M dinitrophenol were added to give a 15 μM concentration. The final mito-

chondrial protein concentration was 1 mg/ml in the cuvette [0.4 ml of a stock suspension containing 10 mg of protein per milliliter as determined by the method of Lowry *et al.* (11)].

Equilibration of Mitochondria and Medium with Halothane

When the mitochondrial suspension or test medium was to be treated with halothane, 4 liters of air per minute were passed through a calibrated halothane vaporizer (Fluotec) and then through a 150-ml suction flask immersed in an ice bath. One milliliter of mitochondrial suspension (10 mg of protein per milliliter) was spread over the bottom of the flask. The flask was agitated and swirled by hand every 5 min during a 20-min equilibration period. The respiratory control medium was equilibrated in a similar flask at 26° for 20 min. The concentrations designated in this paper refer to the halothane vapor concentrations with which mitochondria and/or media were equilibrated.

For the comparison of anaerobic and aerobic equilibration of mitochondria, nitrogen was used instead of air as the carrier gas for the vaporization of halothane during the 20-min prior treatment period. The respiratory control medium to be used for the oxygen consumption test was equilibrated with air containing halothane at the same concentration.

Swelling Studies

For swelling experiments, 0.33 M sucrose–25 mM Tris-HCl, pH 7.35, was equilibrated with halothane for 10 min by bubbling the air-halothane mixture through the solution in a 150-ml suction flask at 25°. When phosphate-induced swelling was studied, 14 μl of 0.5 M succinate or 3-hydroxybutyrate and 17.5 μl of 1 M potassium phosphate buffer, pH 7.4, were added to 3.5 ml of medium to give final concentrations of 2 mM substrate and 5 mM phosphate. For swelling experiments, the swelling medium alone (0.33 M sucrose–25 mM Tris) was equilibrated, because of the 75-fold dilution of the mitochondrial preparation and the low mitochondrial mass (150 μg of protein per milliliter) used in these experiments. Prior equilibration of mitochondria caused no alteration in the results. Temperature was maintained at 26° in a water bath, and optical density readings were made in a Bausch and Lomb Spectronic 20 colorimeter at 520 nm.

RESULTS

Oxidative Phosphorylation

Low concentrations of halothane (0.5–2.0%). In the presence of the NAD^+-dependent substrate 3-hydroxybutyrate the ADP-stimulated rate of oxygen uptake was inhibited in a concentration-dependent manner, with maximal inhibition of 80% (see Fig. 1). This finding is similar to that of Cohen and co-workers, using glutamate at substrate (2, 4, 5). No effect was evidens during the State 2 and 4 rates of respiration, but only in State 3, during phosphorylation of ADP. This inhibition was not altered by the addition of NAD^+. Therefore, the fall in respiratory rate does not appear to have been due to loss of NAD^+ from the mitochondria in this concentration range of halothane. With succinate as substrate, there was a small increase in the State 4 rate, but the State 3 rate with ADP was not decreased, and may even have been slightly increased in this concentration range of halothane (Fig. 2). These are the concentration ranges of halothane used clinically to achieve anesthesia.

There was no difference when nitrogen was used instead of air as the carrier gas during equilibration of mitochondria with halothane (Fig. 3).

Intermediate concentration range of halothane (2–4%). In this concentration range the inhibition of NAD^+-dependent oxidation did not increase over that seen with 2% halothane, in agreement with the report by Cohen and Marshall (2). However, there was a concentration-dependent slowing of succinate oxidation during State 3 and an increase in State 2 (Fig. 4). With 3% halothane the State 2 and State 4 rates were more than doubled, while there was a relatively small but significant inhibition of State 3. With 4% halothane the State 2 rate usually equaled the markedly inhibited State 3

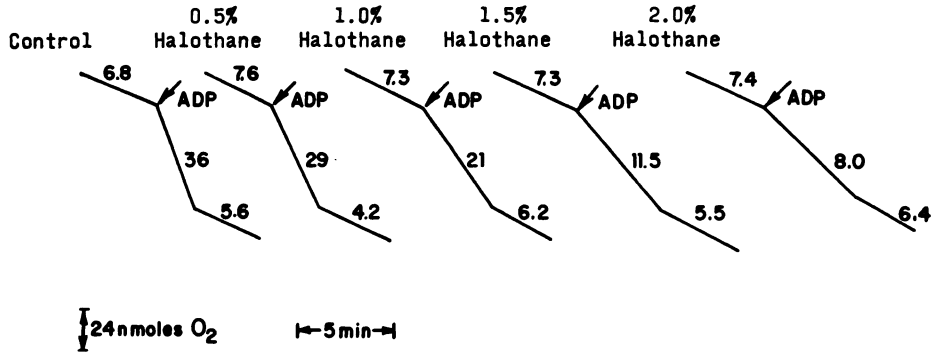

FIG. 1. *Effects of halothane on 3-hydroxybutyrate oxidation*

Oxygen electrode tracings. Each 4-ml test cuvette contained 3.6 ml of respiratory control medium (see MATERIALS AND METHODS) and 0.4 ml of mitochondrial suspension to yield a final protein concentration of 1 mg/ml. Where indicated, 40 μl of 0.5 M 3-hydroxybutyrate (final concentration, 5 mM) and 7.7 μl of 91.5 mM ADP (final concentration, 176 μM) were added. The medium and mitochondria were first treated with the indicated concentrations of halothane in air. Rates entered on each part of the tracing are expressed as nanomoles of O_2 per milligram of protein per minute.

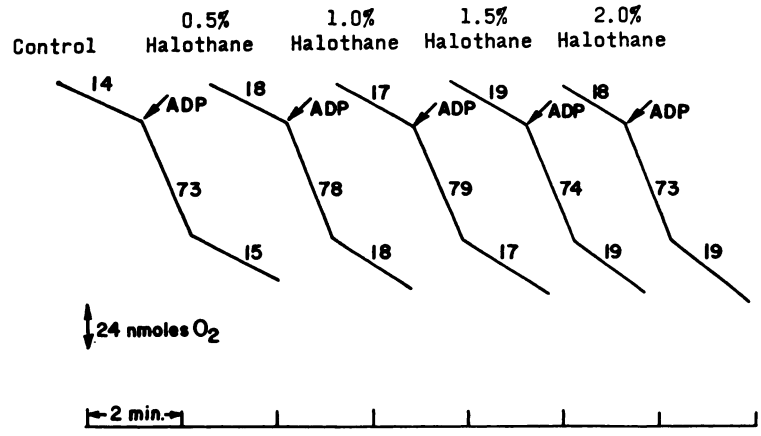

FIG. 2. *Effects of halothane on succinate oxidation*

Oxygen electrode tracings. Conditions were the same as in Fig. 1, except that 40 μl of 0.5 M succinate were added instead of 3-hydroxybutyrate. Rates entered on the tracings are expressed as nanomoles of O_2 per milligram of protein per minute.

rate. This slower than normal rate of State 3 respiration was not accelerated upon addition of dinitrophenol (Fig. 4) in the standard respiratory control medium. Since others have not reported inhibition of succinate oxidation at concentrations of halothane even higher than 4%, tests were carried out in a medium without fluoride, which was present in our standard medium but not used by others. The presence of fluoride in the test medium produces some inhibition of both State 3 and 4 oxidation, without change in the respiratory control, in untreated mitochondria. However, the changes in State 3 and 4 oxidation rates induced by 2–4% halothane treatment were not significantly different in the absence of fluoride. One difference was observed. Some stimulation of the oxidation rate by dinitrophenol did occur in the 4% halothane-treated mitochondria in the absence of fluoride, in contrast to the negative findings in the presence of fluoride. This difference merits further study.

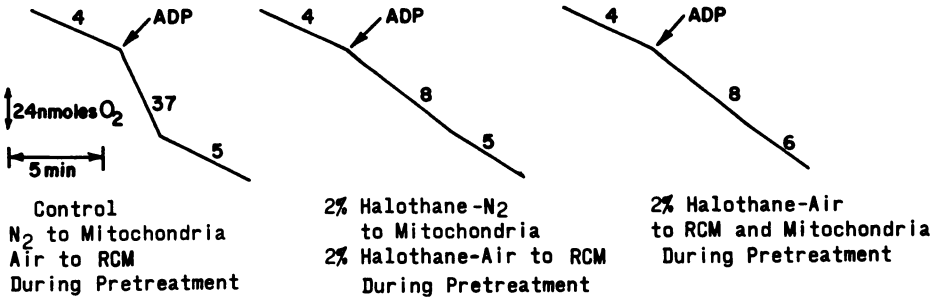

FIG. 3. *Comparison of mitochondria initially treated with halothane, using nitrogen, instead of air, as the carrier gas*

Oxygen electrode tracings. Test conditions were the same as in Fig. 1. The mitochondria were first treated with 2% halothane for 20 min with either air or nitrogen as the carrier gas. The respiratory control medium (RCM) was initially treated with 2% halothane in air. Rates entered on the tracings are expressed as nanomoles of O_2 per milligram of protein per minute.

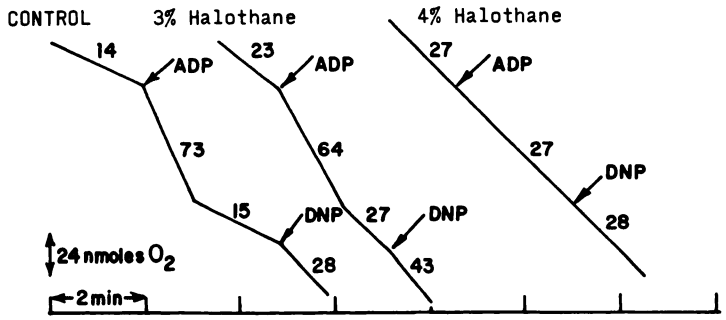

FIG. 4. *Effect of halothane on succinate oxidation; intermediate concentrations (2–4%)*

Oxygen electrode tracings. Test conditions were the same as in Fig. 2, except that 6 μl of 10 mM dinitrophenol (DNP) were added when indicated, to yield a final concentration of 15 μM. Rates entered on the tracings are expressed as nanomoles of O_2 per milligram of protein per minute.

Electron micrographs of mitochondria exposed to 2% halothane show primarily the condensed form described by Hackenbrock (12). After exposure to 4% halothane there are several forms, a few of them condensed, but most being orthodox or swollen.[1]

Rapidity of onset and reversibility. Figure 5 illustrates experiments which indicate the rapid onset and rapid reversibility (in less than 5 min) of the inhibition of NAD^+-dependent electron transport by 0.5–2.0% halothane. For example, prior treatment of mitochondria with 2% halothane for 20 min at 0° and testing in medium that had been equilibrated with halothane gave 80% inhibition of respiration. However, when the mitochondria were first treated with 2% halothane for 20 min and then tested in respiratory control medium which had not been equilibrated with halothane, there was a marked diminution of the degree of inhibition of electron transport with 3-hydroxybutyrate as substrate. This suggests that halothane moved from the mitochondria into the medium, thus reducing the concentration in the mitochondria and decreasing the inhibition of NADH dehydrogenase (EC 1.6.99.3). The new equilibrium of mitochondria with the respiratory control medium appeared to be complete within 3–5 min. When untreated mitochondria were added to medium which had been equilibrated with halothane, the equilibration of the medium and mitochondria again

[1] E. Smith, personal communication.

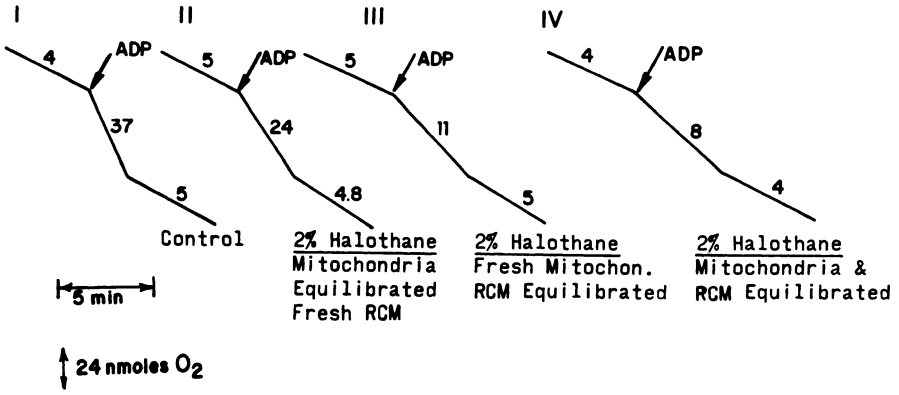

FIG. 5. *Rapid onset and reversibility of the effect of halothane on NAD⁺-dependent electron transport*
Oxygen electrode tracings. Test conditions were the same as in Fig. 1, with 3-hydroxybutyrate. I. Control. II. Mitochondria exposed to 2% halothane and then added to respiratory control medium (RCM) not equilibrated with halothane. III. Respiratory control medium equilibrated with 2% halothane, then fresh untreated mitochondria added to the cuvette. IV. Both mitochondria and medium equilibrated with 2% halothane. Rates entered on the tracings are expressed as nanomoles of O_2 per milligram of protein per minute.

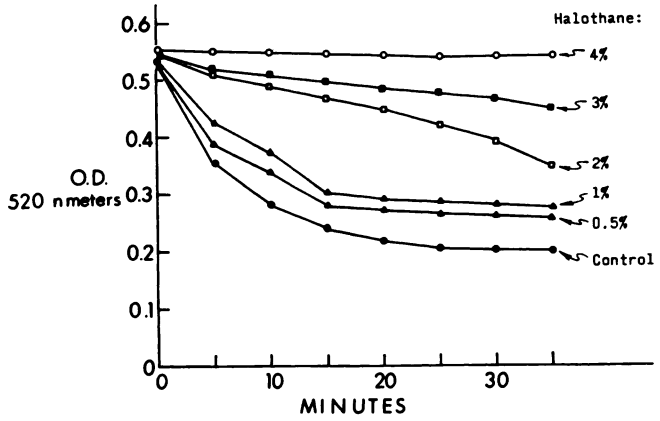

FIG. 6. *Effect of halothane on energy-dependent, phosphate-induced swelling with 3-hydroxybutyrate as substrate*
The test medium consisted of 3.5 ml of 0.33 M sucrose with 25 mM Tris-HCl (pH 7.35, 26°), 17.5 μl of 1 M potassium phosphate buffer, and 14 μl of 0.5 M 3-hydroxybutyrate. The sucrose-Tris buffer solution was equilibrated with halothane in air at the indicated concentrations. Then 46 μl of mitochondrial suspension (protein, 10 mg/ml) were added to give a final protein concentration of 150 μg/ml.

appeared to be complete in 3–5 min. The inhibition of electron transport was much greater when untreated mitochondria were added to the respiratory control medium equilibrated with halothane than in the converse experiment. The degree of inhibition was, in all probability, a function of the final equilibrium concentration of halothane as determined by the relative solubility of halothane in mitochondria and in the medium in relation to the relative volume of mitochondria to suspending medium. The experimental results are consistent with a much greater solubility of halothane in the mitochondria than in the aqueous medium.

Energy-Dependent Swelling

It has been shown previously (13) that phosphate-induced swelling of mitochon-

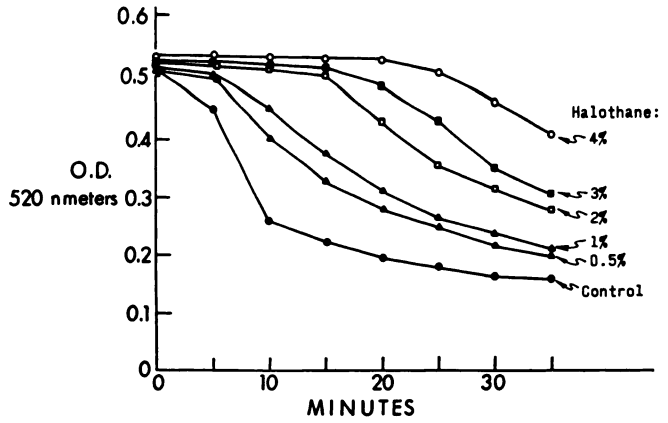

FIG. 7. *Effect of halothane on energy-dependent, phosphate-induced swelling with succinate as substrate*
Experimental conditions were the same as in Fig. 6, except that 14 μl of 0.5 M succinate were added instead of 3-hydroxybutyrate.

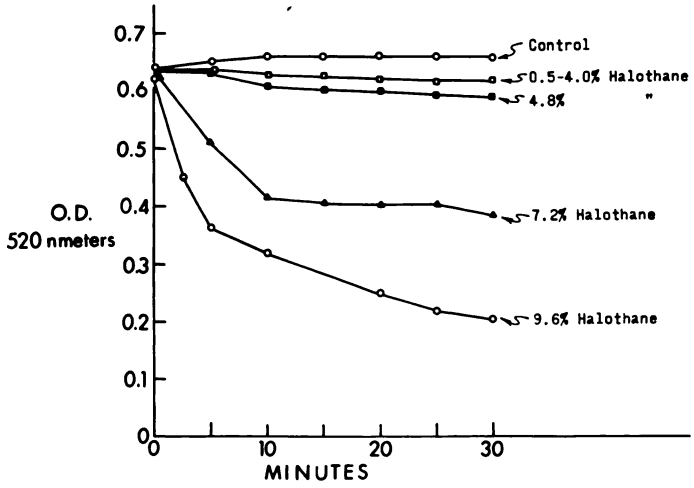

FIG. 8. *Halothane-induced, energy-independent swelling*
Optical density plotted against time. The test medium consisted of 3.5 ml of 0.33 M sucrose with 25 mM Tris-HCl (pH 7.35, 26°), *with no phosphate or substrate added.* The medium was equilibrated with the indicated halothane concentrations. Then 46 μl of mitochondrial suspension (protein, 10 mg/ml) were added to give a final protein concentration of 150 μg/ml.

dria requires high-energy intermediates. Figures 6 and 7 illustrate the concentration-dependent inhibition of phosphate-induced swelling by halothane. With 3-hydroxybutyrate (Fig. 6), 2% halothane reduced swelling to a very low rate, and 4% halothane stopped it completely. It is noteworthy that although there was no measurable effect of 0.5–2% halothane on the State 3 oxidation rate with succinate as substrate in polarographic studies (Fig. 2), there was a concentration-dependent retardation of the rate of phosphate-induced swelling of mitochondria in this concentration range (Fig. 7).

Energy-Independent Swelling

The possibility that halothane might induce swelling at higher concentrations or in the absence of phosphate and substrate was investigated. At concentrations up to 4% there was no swelling. High concentra-

tions of halothane (5–10%) caused concentration-dependent swelling of mitochondria suspended in 0.33 M sucrose–25 mM Tris without added phosphate or substrate (Fig. 8).

DISCUSSION

The data confirm the observation (2) that NAD^+-linked substrate oxidation is inhibited in a concentration-dependent relationship when isolated mitochondria are exposed to halothane in the clinically useful concentration range. Final quantitative evaluation of such an effect *in vivo* must await further experimentation. Both in the work of Cohen and Marshall (2) and in the present study, the mitochondria were maintained at 0° during exposure to halothane because of stability considerations. At 0° more halothane would be dissolved in the mitochondria than at 25°. The medium, on the other hand, was equilibrated at the temperature at which the test was to be conducted. Our experiments with either the medium alone or the mitochondria alone, equilibrated with halothane, indicate that the medium contributed the larger portion of the halothane.

We were very much interested in the question whether changes in respiration, respiratory control, or phosphorylation were secondary to deterioration of the mitochondria under conditions *in vitro*. This appeared not to be the case. In order to stabilize the mitochondria, bovine serum albumin and EDTA were used during isolation and in the test medium for oxygen consumption studies. The greatest stabilizing effect is probably due to bovine serum albumin in the medium (14). The most consistently rapid State 3 rates were obtained when Mg^{++} was included in the respiratory control medium. None of the components which stabilized the mitochondria altered the sensitivity of the NAD^+-dependent substrate oxidation to low concentrations of halothane. Thus, the use of stabilizing test media and very tightly coupled mitochondrial preparations increased the significance of the observed inhibition of electron transport by halothane.

Cohen *et al.* (4) suggested that halothane-induced loss of respiratory control was an oxygen-dependent process, which implied protection of mitochondria if previous treatment was performed with nitrogen as the carrier gas. Our data indicate that the mechanism by which halothane affects 3-hydroxybutyrate oxidation is not dependent on the presence of oxygen during prior treatment. It should be pointed out that the rapid onset of the effect when mitochondria were added to the halothane-containing medium in the cuvette is also consistent with this conclusion. Although an oxygen-dependent change seems unlikely, it should be recognized that the anaerobic mitochondria were exposed to halothane in the presence of oxygen in the cuvette for 3–5 min before the ADP tests. If an oxygen-dependent effect exists, it must occur very rapidly.

The studies with succinate oxidation, in which electron transport was not inhibited by 0.5–2% halothane, indicate a 20% increase in the State 4 rate, suggesting a very limited degree of uncoupling. At 3–3.5% halothane, the State 4 rate of respiration was nearly doubled, a result consistent with greater degrees of uncoupling.

Cohen and Marshall (2) reported increases in State 4 respiratory rate at halothane concentrations greater than 1.5%. Gatz and Jones (6), in a preliminary communication, reported that halothane had an effect resembling that of dinitrophenol in rat brain mitochondria with succinate as substrate. Comparison with the latter study must await details on experimental methods and the magnitude of the effects.

The concentrations at which the major release from respiratory control was seen in isolated rat liver mitochondria exceeded any tissue concentration likely to be reached within the clinical concentration range of halothane. Further evaluation of uncoupling effects at anesthetic concentrations is needed to determine whether this action contributes to the syndrome of malignant hyperpyrexia during halothane anesthesia *in vivo* (6, 15). Uncoupling is the most common explanation for increases in State 2 and 4 rates of respiration. However, loss

of respiratory control cannot be used as a definitive measure of the efficiency of energy conservation.

The question whether there is some uncoupling of phosphorylation is not easily studied with NAD^+-dependent substrates. Partial uncoupling should increase the State 4 rate, but this increase might be obliterated by partial inhibition of electron transport (2). The ADP responses and ADP:O ratio with partial inhibition of NAD^+-dependent respiration indicate that any uncoupling by 0–2% halothane must be of a very limited degree. There is respiratory control with a response to ADP when the State 3 rate of respiration is inhibited 80%.

In addition to the uncoupling-like effects, halothane at 3–4% also had significant effects on electron transport with succinate (Fig. 4). Three per cent halothane produced measurable inhibition of the State 3 rate, while 4% completely eliminated the response to ADP. The State 3 and State 4 rates became identical. The addition of dinitrophenol, pentachlorophenol, carbonyl cyanide *m*-chlorophenylhydrazone, or hexokinase + glucose did not increase the inhibited rate of oxygen consumption in the presence of 4% halothane. These data on inhibition of succinate oxidation suggest that halothane at higher concentrations (3–4%) acts at points in electron transport in addition to NADH dehydrogenase. Swelling studies indicate that these high concentrations of halothane do not cause general dissolution of structure. Cohen *et al.* (4) observed no inhibition of succinate oxidation, using 10 mM succinate and a different test medium. The basis for this difference in observations must be resolved by further experimentation.

The studies with mitochondrial swelling provide additional information. First, in the 0–4% range of halothane, no swelling occurred in sucrose-Tris medium. Thus, halothane did not increase membrane permeability and permit the entry of sucrose. Above 5% halothane, however, there was energy-independent swelling of mitochondria without added substrate and phosphate. This indicates an increased membrane permeability to and entry of sucrose, Tris, or chloride. The changes in optical density do not suggest disintegration of the mitochondria.

Halothane inhibits phosphate-induced swelling, a process known to require high-energy intermediates. With 3-hydroxybutyrate, the rate and degree of swelling were inhibited in a concentration-dependent manner closely correlated with the degree of inhibition of electron transport. When succinate was the substrate, low concentrations of halothane slowed the rate of phosphate-induced swelling in spite of the fact that electron transport was not altered. In addition to the possibility that uncoupling may occur, halothane may interfere with the linkage of high-energy intermediates to ion transport processes, or with the function of the phosphate carrier located within the mitochondrial membrane.

Snodgrass and Piras (16) published a study on the effects of halothane on rat liver mitochondria. The techniques of exposure and the concentrations used were very different from ours in most of their experiments. In our study and in that of Cohen and co-workers (2, 4, 5), the main emphasis has been on the direct action of low concentrations of halothane. For example, in our experiments, placing fresh mitochondria in a medium equilibrated with 2% halothane vapor inhibited 3-hydroxybutyrate oxidation about 70%. Equilibration with 2% halothane vapor produced 1 mM halothane in the aqueous medium at 25°. In the study of Snodgrass and Piras, virtually all the effects observed resulted from very high concentrations of halothane (4–17 mM), and nearly all experiments were performed with mitochondria that had been exposed to halothane at 4°, then centrifuged out of the halothane-containing solution and resuspended in fresh sucrose-Tris for testing. Thus, in most cases, the mitochondria were exposed to possible lipid-dissolving or -displacing actions, separated from those lipids by centrifugation, and then tested in the presence of an unknown but very low concentration of halothane. The loss of lipids essential for mitochondrial electron transport and/or phosphorylation could explain some of their results. Such proce-

dures probably produce effects that would not be observed in the presence of low concentrations of halothane, and effects dependent on the presence of halothane during the actual test could be missed. The loss of functional lipid could account for loss of dinitrophenol-stimulated ATPase and uncoupled phosphorylation without justifying the classification of halothane as "a true uncoupling agent." The conclusion of Snodgrass and Piras that NADH oxidation is not inhibited could be the result of testing mitochondria after most of the halothane had been removed. Thus, although interesting effects on electron transport and phosphorylation were observed, they cannot be compared directly with the present findings.

In considering the possible role of the effects reported here in various pharmacological actions on brain, liver, and other tissues *in vivo*, it is worth pointing out that clinical anesthesia results when whole tissue concentrations of halothane are 1.0–1.5 mM in brain and liver. Such concentrations would be in equilibrium with 0.7–1.0% halothane in alveolar gas, concentrations which would produce about 0.3 mM in water and 0.8 mM in blood (17, 18). Thus effects produced by equilibration of mitochondria *in vitro* with 1.0% halothane might occur to similar degrees *in vivo*. The rapid onset and reversibility of the inhibition of ADP-stimulated, NAD^+-linked electron transport by low concentrations of halothane should be emphasized in relating this study to its possible actions *in vivo*.

Lowe (18) has presented a detailed study on tissue distribution of anesthetic agents, including halothane. His human tissue/gas partition coefficients for halothane at 37° were as follows: H_2O, 0.88; blood, 2.24; liver, 4.48; kidney, 2.96; brain white matter, 6.8; brain gray matter, 3.96; muscle, 2.92; and fat, 171. Equilibration of mitochondria with halothane vapor, as was done in the present study, seems the best way of approximating concentrations which might be reached *in vivo*. The high partition coefficient with fat calls attention to a point which must be considered in later studies on mechanisms, for it is known that mitochondrial membranes have a much higher proportion of lipid than whole tissue.

Mitochondria in many tissues might be affected without detection during halothane anesthesia. Brain mitochondria would probably also be affected. This is not to say that effects on mitochondria are the cause of anesthesia, for plasma membrane effects are undoubtedly involved also. However, the mitochondrial membranes have proven to be a sensitive system with many measurable parameters for the study of the action of halothane on membrane systems. These experiments do not answer the question whether halothane produces its effects by interaction with lipids, hydrophobic portions of proteins, or some other mechanism such as stabilization of clathrates of water.

The effects of repeated or prolonged exposure to low concentrations of halothane on liver composition and enzyme activities have been reported by Platt and Cockrill (19) and by Schimassek, Kunz, and Gallwitz (20). The changes observed showed a complex pattern, but in the latter study increases in several citric acid cycle substrates suggested alterations in mitochondrial function. It remains to be determined exactly how the chronic effects relate to the immediate direct effects and to secondary effects on both mitochondria and other parts of the cell.

REFERENCES

1. G. P. Hoech, Jr., R. S. Matteo and B. R. Fink, *Anesthesiology* **27**, 770 (1966).
2. P. J. Cohen and B. E. Marshall, *in* "Toxicity of Anesthetics" (B. R. Fink, ed.), p. 24. Williams & Wilkins, Baltimore, 1968.
3. G. E. Kenny and B. R. Fink, *in* "Toxicity of Anesthetics" (B. R. Fink, ed.), p. 11. Williams & Wilkins, Baltimore, 1968.
4. P. J. Cohen, B. E. Marshall, J. E. Harris, J. H. Lecky and B. S. Rosner, *Fed. Proc.* **27**, 705 (1968).
5. P. J. Cohen, B. E. Marshall and J. H. Lecky, *Anesthesiology* **30**, 337 (1969).
6. E. E. Gatz and J. R. Jones, *Fed. Proc.* **28**, 356 (1969).
7. K. Ahmad, H. G. Schneider and F. M. Strong, *Arch. Biochem. Biophys.* **28**, 281 (1950).
8. B. Chance, L. Ernster, P. B. Garland, C.-P. Lee, P. A. Light, T. Ohnishi, C. I. Ragan

and D. Wong, *Proc. Nat. Acad. Sci. U. S. A.* **57**, 1498 (1967).
9. M. Klingenberg, *in* "Flavins and Flavoproteins" (E. C. Slater, ed.), p. 522. Elsevier, Amsterdam, 1966.
10. B. Chance and G. R. Williams, *Nature* **175**, 1120 (1955).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
12. C. R. Hackenbrock, *J. Cell Biol.* **37**, 345 (1968).
13. F. E. Hunter, *Proc. 5th Int. Congr. Biochem. (Moscow)* **5**, 287 (1963).
14. I. Boime, E. E. Smith and F. E. Hunter, *Arch. Biochem. Biophys.* **128**, 704 (1968).
15. R. Wilson, T. Dent, D. Traber, N. McKoy and C. Allen, *J. Amer. Med. Ass.* **202**, 111 (1967); C. R. Stephen, *J. Amer. Med. Ass.* **202**, 106 (1967).
16. P. J. Snodgrass and M. M. Piras, *Biochemistry* **5**, 1140 (1966).
17. R. A. Butler, *in* "Uptake and Distribution of Anesthetic Agents" (E. M. Papper and R. J. Kitz, eds.), p. 274. McGraw-Hill, New York, 1963.
18. H. S. Lowe, *in* "Theory of Applied Gas Chromatography" (H. F. Kroman, ed.), p. 194. Grune & Stratton, New York, 1968.
19. D. S. Platt and B. L. Cockrill, *Biochem. Pharmacol.* **18**, 445 (1969).
20. H. Schimassek, W. Kunz and D. Gallwitz, *Biochem. Pharmacol.* **15**, 1957 (1966).