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EFFECT OF HALOTHANE UPON RABBIT BRAIN MITOCHONDRIA

HYLTON SMITH

Department of Pharmacology, School of Pharmacy, Sunderland Polytechnic, Chester Road, Sunderland SR1 3SD, England

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Abstract—Mitochondrial function is altered in the presence of halothane and the results in this study suggest that a weak, oligomycin-like effect is produced. At higher concentrations of halothane, up to 4%, some uncoupling may occur. However, there is some doubt as to whether the halothane concentration in the mitochondria in intact brain ever reaches that observed in the isolated mitochondrial preparation exposed to halothane. If this were so, then the uncoupling effect may not be a physiological phenomenon at 4% halothane.

HALOTHANE is used widely as a general anaesthetic but its mechanism of action remains obscure. Amongst other physiological changes, decreased cerebral oxygen consumption has been observed in man anaesthetized with halothane.[1] A depression in oxygen consumption in rat brain slices in the presence of halothane has also been described[2] but the significance of these findings in relation to the phenomenon of anaesthesia in the intact animal is unknown. One hypothesis of anaesthesia is that a change in biochemical activity occurs in specific neurones due to partial and reversible blocking of oxidative phosphorylation in mitochondria. Such a concept implies that neurones in the brain associated with consciousness and pain are blocked by the anaesthetic. However, there is no evidence to suggest that halothane is taken up selectively in any part of the brain, thereby resulting in anaesthesia.

Measurement of oxygen consumption in whole brain or brain slices does not allow a precise definition of the role of mitochondria because of the presence of other cellular organelles. Because of this, the effect of halothane upon isolated mitochondria has been studied. Much of the work attempting to define the biochemical site of action of halothane has been performed on isolated rat liver mitochondria and the results extrapolated to explain the mechanism of action on brain mitochondria. The reason for this is that purified brain mitochondria free from synaptosomes are difficult to prepare. However, from these experiments, a depression in mitochondrial function has been reported but there is disagreement as to the site and type of inhibition within the electron transport chain. One proposal is that at concentrations of halothane below 2%, the rate of NADH linked substrate oxidation, but not that of succinate oxidation, is depressed.[3,4] At these concentrations of halothane there is no evidence of uncoupling of oxidation from phosphorylation. Harris *et al.*[4] using isolated rat liver mitochondria, supplemented by studies on beef heart mitochondria and electron transport particles, propose that halothane inhibits electron transport by blocking NADH–Co-Q reductase in the vicinity of the rotenone sensitive site of complex I.

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Above 2% halothane, there is a gradual and progressive uncoupling of phosphorylation.[3–6] Using a crude rat brain mitochondrial preparation, Gatz and Jones[6] describe the uncoupling effect as similar to that of DNP on the rate of succinate oxidation, implying that uncoupling is occurring at a phosphorylation site beyond site I. The published data on proposed sites of action of halothane on the respiratory chain and phosphorylation is summarized in Fig. 1.

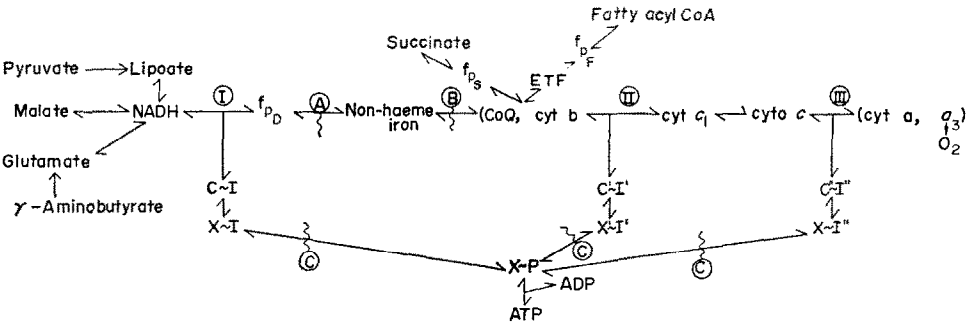

FIG. 1. Showing the proposed sites of halothane blocking relative to the point of entry of various substrates associated with electron transport and phosphorylation; I, II and III represent the sites of phosphorylation; f_pN, f_pS and f_pF are appropriate flavoprotein dehydrogenases; ETF is the electron transferring flavoprotein C ~ I, X ~ I, X ~ I', X ~ P, etc., are high energy intermediates of phosphorylation; A and B are proposed sites of halothane block (Harris *et al.* 1971, Miller *et al.* 1969); C is the site of action of oligomycin.

There are two criticisms of the published work attempting to define the effect of halothane upon mitochondrial function. The first is that purified brain mitochondria have not been used. This has been resolved in the present study by using a method recently described for isolating pure brain mitochondria.[7]

The other criticism is that all previous studies on the isolated mitochondrial preparation have involved exposing the prepared mitochondria to concentrations of halothane considered to be equivalent to those used in clinical anaesthesia. The mitochondria have therefore been equilibrated with gaseous halothane up to 4% in air, or an equivalent volume of liquid halothane has been added to the mitochondrial preparation. The fundamental question in such *in vitro* studies is whether the concentration of halothane, and by implication, the change in function of isolated mitochondria is similar to that occurring in the intact mitochondria in the brain of the anaesthetized animal.

An attempt has been made to justify the use of the isolated mitochondrial preparation by comparing the halothane concentration in this preparation to mitochondria which have been separated from the brain of anaesthetized animals.

The purpose of this paper is to present data on the effect of halothane on the purified isolated brain mitochondrial preparation.

METHODS

Preparation in vivo. Exposure of intact animals to halothane. Animals were exposed to a known concentration of halothane for 10 min using a calibrated Fluotec vaporisor. The range of concentrations of anaesthetic was varied between 0·5 and 4·0%.

The animals were then sacrificed and mitochondria prepared and analysed as described below. A 10-min period of exposure to halothane was used because, although equilibration with the anaesthetic was not achieved in this time,[8] it is the initial changes following induction that are of interest in this investigation.

Preparation of mitochondria. Male albino rabbits, weighing 1·5 kg, were sacrificed by dislocating the cervical vertebrae. The skull was opened with bone forceps and the whole brain scooped out into ice-cold 0·25 M buffered-sucrose solution. This procedure was completed in under 45 sec. The time factor is important because anoxia causes rapid deterioration of mitochondria and this is reflected in poor respiratory control ratios. The subsequent preparation of the mitochondria was identical to that described by Clark and Nicklas.[7] The technique utilizes a simple discontinuous Ficoll gradient which separates mitochondria from synaptosomes. The activity of the mitochondria is dependent upon the presence of K^+ and the concentration of K^+ in the medium corresponds closely to that found in the brain intracellular fluid. All procedures were carried out at a temperature below 5° and the centrifuge tubes were filled with fluid and stoppered to reduce any loss of halothane to a minimum. The final volume of mitochondrial suspension was adjusted so that the protein concentration was 15 mg/ml. An average rabbit brain weighed 5 g and yielded a mitochondrial pellet of about 20 mg.

Isolated mitochondrial preparation. Equilibration of isolated brain mitochondria and medium with halothane. The technique is essentially that used by Miller and Hunter.[3] Gaseous halothane delivered through a Fluotec vaporisor was passed through a 250 ml tonometer containing 1 ml of mitochondrial suspension and after complete displacement of residual air, the tonometer was equilibrated for 10 min at 30° with continual rotation. A similar flask containing the incubation medium was also equilibrated with the halothane gas. 0·2 ml of the suspension was then transferred to the electrode chamber together with halothane-equilibrated incubation medium. Mitochondria were exposed to halothane in air by this technique over the range 0·5–4·0 per cent.

Analysis of mitochondria for halothane

Mitochondria from the in vivo *preparation.* A 1 ml aliquot of the sucrose suspension of mitochondria was centrifuged in a refrigerated MSE high speed 18 at 5000 *g* for 5 min. One ml of carbon tetrachloride was added to the pellet and this was shaken mechanically for 20 min at room temperature. The extraction was repeated with another 1 ml of carbon tetrachloride. The supernates, together with 1 ml aliquots of each of the various media used in the separation of the mitochondria were also mixed with 1 ml aliquots of carbon tetrachloride and treated in the same way as the mitochondrial pellet. Five μl aliquots from each extraction procedure were injected onto the GLC column. The concentration of halothane in the *in vivo* preparation was calculated by summating the halothane content of the mitochondrial pellet and all of the fractions used to isolate the mitochondria.

Mitochondria from the isolated mitochondrial preparation after equilibration with halothane. A 1 ml aliquot of the sucrose suspension of mitochondria was centrifuged as before. One ml of carbon tetrachloride was added to the pellet and this was shaken mechanically for 20 min at room temperature. The extraction was repeated with another 1 ml of carbon tetrachloride. 5 μl aliquots of the organic solvent extract were injected onto the GLC column.

The analysis was by the method of Wolfson and Ciccarelli.[9] The column consisted of 5% SE 30 on 60/80 Chromosorb W and its dimensions were 210 × 0·4 cm. Recordings were made on a Pye series 104 chromatograph. The carrier gas was nitrogen, maintained at a flow rate of 25 ml/min. The oven temperature and detector oven temperature were controlled at 55 and 250°, respectively. Attenuation was adjusted to the 50 × 1 setting, but this was varied for greater sensitivity as appropriate in the aqueous sucrose or Ficoll solutions from above.

Measurement of oxygen consumption in brain mitochondria. Respiration was measured at 30° using a Rank-type oxygen electrode. The polished platinum electrode was polarized at −0·5 V with respect to the reference electrode in order to avoid the errors due to polarographic reduction of halothane.[10]

The incubation medium was that described by Nicklas *et al.*[11] with a potassium ion concentration of 100 mM. The medium contained the following substances at the quoted final concentrations: 225 mM mannitol; 75 mM sucrose; 100 mM potassium chloride; 10 mM Tris–chloride; 0·05 mM EDTA. The medium was adjusted to pH 7·4 with 2 m Tris–base.

0·2 ml of mitochondrial suspension containing 3 mg of protein was mixed with 2·8 ml of incubation medium in the electrode chamber. 0·05 ml of the appropriate substrate, adjusted to pH 7·4, was added to this, the substrate being either 100 mM sodium succinate or 100 mM sodium pyruvate together with 100 mM sodium malate.

The various states of respiration referred to in the text are those defined by Chance and Williams.[12] State 2 respiration was produced by adding 50 μl of 10 mM ADP. State 3 represents the rate of respiration following state 2 after the addition of substrate. State 4 represents the rate of respiration in the presence of substrate but after the depletion of added ADP. Results are expressed as microgram atoms of oxygen consumed per milligram of mitochondrial protein per minute. DNP-stimulated respiration (uncoupled respiration) was produced by adding 10 μl of 10 mM DNP. Protein was estimated by the method of Gornall *et al.*[13]

Measurement of DNP-stimulated ATPase activity in rabbit brain mitochondria. This was essentially the method of Beechey.[14] 1·1 mg of mitochondrial protein was suspended in 0·2 ml of 0·25 M sucrose–1 mM EDTA solution at pH 7·4. At zero time, this suspension of mitochondria was added to 0·8 ml of a reaction mixture containing 0·125 M sucrose, 63 mM Tris–chloride buffer (pH 7·4), 2·5 mM ATP and 3 mM ethanolic DNP. The reaction was maintained at 20° for 20 min and it was terminated by adding 0·1 ml of 40% w/v trichloracetic acid. Liberated phosphate was estimated by the method of Fiske and Subbarow[15] and the ATPase activity expressed as μM of inorganic phosphate liberated per hour per milligram of protein.

All reagents were of analytical quality. The halothane used throughout this investigation was Fluothane (I.C.I.).

RESULTS

In an initial experiment it was necessary to establish the validity of the isolated brain mitochondrial preparation as a model to study the effects of halothane. Thus the halothane concentration of mitochondria obtained from the brain of intact anaesthetized rabbits was compared to isolated rabbit brain mitochondria exposed after separation to halothane in a tonometer. The comparative data, covering the range 0·5–4·0% halothane, is shown in Fig. 2. The isolated mitochondrial preparation con-

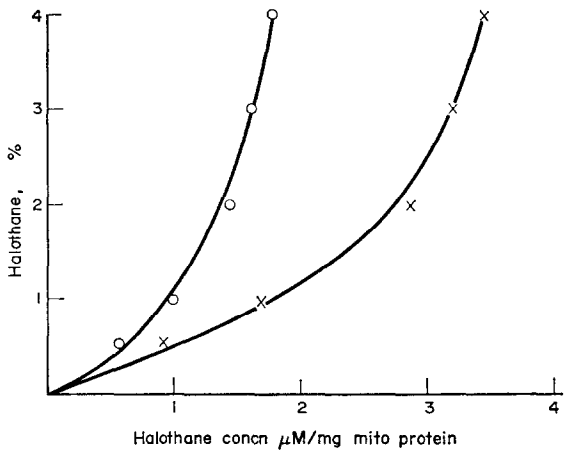

FIG. 2. Halothane concentrations in rabbit brain mitochondria. Comparison of *in vivo* and *in vitro* conditions. ○, Brain removed from anaesthetized rabbit; mitochondria subsequently isolated. ×, Isolated rabbit brain mitochondria preparation exposed to halothane in a tonometer.

tained more halothane than the *in vivo* preparation when both were exposed to identical concentrations of gaseous halothane for a 10-min period. There appears to be approximately a 2-fold difference between the two preparations. Thus the isolated mitochondrial preparation can be considered to be a valid model, provided it is accepted that it may contain more halothane than occurs in mitochondria in the intact anaesthetized animal when the concentration of halothane to which it is exposed is above 1 per cent. Conversely, the difference in halothane concentration between the two preparations may reflect a loss of halothane from the *in vivo* preparation during the separation procedure for the mitochondria.

Table 1 shows the effect of exposure of up to 4% halothane on the rate of state 3, state 4 and DNP-stimulated respiration on the isolated mitochondrial preparation. It would appear that in the presence of malate and pyruvate as substrate, state 3 respiration is significantly inhibited by halothane, even at 0·5% concentration. However, state 4 respiration remains unaltered when exposed to concentrations of up to 2% halothane but it is increased at concentrations of 3% halothane and above. This suggests some degree of uncoupling. DNP stimulated respiration remained responsive and unaltered when exposed to halothane concentrations of up to 4%.

On the contrary, halothane did not alter the rate of state 3 respiration during succinate oxidation in concentrations up to 3% although some inhibition was observed at 4% halothane. State 4 respiration was also not affected by 3% halothane but a slight increase in respiration rate occurred at 4% halothane. DNP-stimulated respiration remained unaltered up to a concentration of 4% halothane in the presence of succinate.

Thus the oxidation of NADH-linked substrates is inhibited in the presence of halothane at low concentrations when stimulated by ADP but not in the presence of DNP. Succinate oxidation is less affected by halothane except after exposure to 4% halothane. However, in view of the apparently higher concentration of halothane in the isolated mitochondrial preparation compared to the *in vivo* preparation, the physiological significance of these results is speculative. The facts suggest that halothane may act on oxidative phosphorylation in a similar way to oligomycin, that is by

TABLE 1. EFFECT OF HALOTHANE ON THE RATE OF OXIDATION IN RABBIT BRAIN MITOCHONDRIA

Halothane concn in equilibrating gas	Rate of respiration (ng atoms O/min/mg/protein)					
	Malate/pyruvate as substrate			Succinate as substrate		
	State 3	State 4	DNP stim.	State 3	State 4	DNP stim.
0	52·2 ± 1·1	16·3 ± 1·5	53·1 ± 1·8	34·2 ± 1·5	16·3 ± 0·9	38·1 ± 1·8
0·5	*42·7 ± 1·7	15·9 ± 1·3	52·7 ± 1·7	36·8 ± 1·7	15·9 ± 1·8	37·7 ± 1·7
1·0	*35·6 ± 1·8	16·4 ± 1·2	53·4 ± 1·7	35·6 ± 1·8	16·4 ± 0·9	35·9 ± 1·7
2·0	*32·7 ± 1·7	17·6 ± 1·4	53·0 ± 1·4	32·7 ± 1·7	17·6 ± 0·7	36·0 ± 1·4
3·0	*32·9 ± 1·6	*19·9 ± 1·6	55·4 ± 1·3	32·9 ± 1·6	†18·1 ± 1·9	38·5 ± 1·3
4·0	*19·4 ± 1·4	*19·7 ± 1·4	55·3 ± 1·5	23·4 ± 1·9	†19·9 ± 2·6	35·8 ± 1·5

Results represent the mean observations on six animals (± S.E.M.).
* $P = < 0.001$. Other results not significant.
† $P = < 0.01$.

FIG. 3. Effect of halothane on DNP-stimulated ATPase in isolated rabbit brain mitochondria.

inhibiting mitochondrial ATPase. The effect of halothane on DNP-stimulated ATPase was therefore investigated. Figure 3 reveals that a slight but progressive inhibition of this enzyme occurred. At 2% halothane the degree of inhibition was about 10 per cent, increasing to about 50 per cent when exposed to 4% halothane.

A slight increase in state 4 respiration was also observed at the higher concentrations of halothane in the presence of both malate and pyruvate and succinate as substrates. This could indicate some uncoupling of phosphorylation but, again, the change may be occurring at mitochondrial halothane concentrations which are not normally encountered in the anaesthetized, intact animal.

DISCUSSION

The concentration of halothane in the brain necessary to induce anaesthesia has been quoted as approx. 1·5 mM.[3] This occurs at equilibrium after exposure to 1% halothane in the alveolar gas. Such information gives no indication of the effective concentration of halothane in mitochondria. However, it is essential to have some knowledge of mitochondrial halothane concentration if this is to be related to a hypothesis of anaesthesia involving change in mitochondrial function.

In this preliminary study, no attempt has been made to selectively isolate those areas of brain alleged to be primarily involved in anaesthesia. Instead, mitochondria isolated from the whole brain of anaesthetized rabbits have been analysed for halothane. These results have been compared to the halothane concentration in the isolated mitochondrial preparation which was exposed to halothane after separation of the mitochondria. There is an obvious difference in halothane concentration between the two preparations. One explanation for this difference is that halothane may have been lost from mitochondria during the isolation procedure from whole anaesthetized brain although every attempt was made to minimize any loss. Another explanation is that both preparations were exposed to identical concentrations of halothane for 10 min yet the *in vivo* preparation is much more complex with several additional physiological barriers, each with a varying affinity for halothane, before the halothane reaches the

brain mitochondrial membrane. Thus, the halothane concentration in the cytosol of the intact brain cells could be lower than in the isolated mitochondrial preparations exposed to gaseous halothane. Nevertheless, there is not such a marked difference between the two preparations to invalidate the isolated mitochondrial preparation as a model for this study. As a preparation, it exhibited good respiratory control and P:O ratios suggested tightly coupled mitochondria with intact membranes.

It is stated that inhibition of electron transport occurs when isolated mitochondria, incubated with NADH-linked substrates, are exposed to gaseous halothane within a concentration range up to 2%.[3] This inhibition could not be relieved by the addition of DNP. At concentrations of halothane higher than 2%, partial inhibition of succinate oxidation occurred. Some degree of uncoupling of phosphorylation was also reported, which was reflected in an increase in respiration during state 3.[3] Finally at concentrations of halothane far in excess of the clinical range, definite uncoupling from all three sites was described.[3,5,6]

In this study, it appears that halothane depresses NADH-linked respiration when stimulated by ADP but this is relieved by the addition of DNP so that the respiratory chain is not inhibited. This is contrary to the concept that halothane selectively blocks the respiratory chain at a point before the entry of succinate into the chain. The finding that inhibition of DNP-stimulated ATPase occurs after exposure to halothane substantiates the hypothesis that an interference with high energy intermediates of phosphorylation is occurring. In fact, the results suggest an inhibitory effect on phosphorylating electron transport, that is, an oligomycin-like effect, rather than a blocking of electron flow along the respiratory chain. The less pronounced effect on respiration when succinate was used as a substrate could be due to the fact that tighter respiratory control exists at site I of phosphorylation than at sites II and III. In addition to this weak oligomycin-like effect, there may be an additional factor at the higher concentrations of halothane. The slight increase in state 4 respiration observed with both malate and pyruvate and succinate is consistent with some uncoupling of phosphorylation, possible in a way similar to the action of DNP.

However in view of the discrepancy in mitochondrial halothane concentration between the *in vivo* preparation and the isolated mitochondrial preparation, some caution should be used in the interpretation of the data. Assuming that Fig. 2 represents the true situation existing in the intact brain, then the concentration of halothane may never be high enough to produce the changes observed about 3% halothane in the intact mitochondrial preparation. Thus the uncoupling phenomenon may not occur in the intact animal. On the other hand, a weak oligomycin-like effect has been observed after exposure to 1% halothane in the isolated mitochondrial preparation, a situation which may be equated to the *in vivo* preparation exposed to 4% halothane.

The effect of halothane upon mitochondrial function in the intact animal remains obscure. Halothane has been shown to depress oxidative phosphorylation in isolated mitochondria but the critical question remains unanswered; does this preparation represent the actual conditions occurring in the intact brain of the anaesthetized animal?

Assuming that some depression in the rate of oxidation of NADH-linked substrates develops in the presence of anaesthetic concentrations of halothane, then this could lead to an overall depression of energy production because neither succinate nor fatty acyl Co-A can replace NADH-linked substrates. Succinate must be formed by the

tricarboxylic acid cycle via NADH-linked substrates and its steady state concentration in brain is low.

The effect of halothane upon the catabolism of γ-amino butyric acid is an interesting speculation. This amino acid is considered to be an inhibitory transmitter at certain syanpses in the mammalian nervous system[16] and it is rendered pharmacologically inactive by transamination to glutamate.[17] The latter substance is NADH-linked and therefore a depression of the oxidative phosphorylation process could lead to an accumulation of γ-amino butyric acid with possible inhibition of neuronal transmission.[4]

CONCLUSION

The amount of halothane present in mitochondria isolated from the intact, whole brain of anaesthetized rabbits has been compared to the amount present in isolated brain mitochondria subsequently exposed to gaseous halothane within the range 0·5–4·0 per cent. More halothane was found to be present in the latter preparation but it appears that the isolated mitochondrial preparation can be used as a valid model for studying the effects of halothane on mitochondrial function.

Mitochondrial function is altered in the presence of halothane and the results in this study suggest that a weak, oligomycin-like effect is produced. At higher concentrations of halothane, up to 4%, some uncoupling may occur. However, there is some doubt as to whether the halothane concentration in the mitochondria in intact brain ever reaches that observed in the isolated mitochondrial preparation exposed to halothane. If this were so, then the uncoupling effect may not be a physiological phenomenon at 4% halothane.

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