The Effect of Halothane on Rapid Axonal Transport in the Rabbit Vagus

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The effect of halothane on rapid axonal transport of labeled protein in the rabbit vagus nerve was measured using liquid scintillation counting. Observations were made in vivo and in vitro. No significant depression of the flow occurred with high "clinical" concentrations of halothane. In vitro, with 4 per cent halothane, the flow rate was slowed by 40 per cent, and with 10.4 per cent halothane the flow ceased. Although almost complete recovery of the flow could be obtained after exposure to 7.8 per cent halothane, no recovery of the flow was possible following 10.4 per cent halothane. Persistence of the control rate of rapid axonal transport with levels of halothane as high as 3 per cent argues against inhibition of rapid axonal transport per se as a factor in the mechanism of general anesthesia. It further argues against Allison and Nunn's depolymerization theory of anesthesia, since rapid axonal transport probably depends on intact microtubules. (Key words: Microtubules; Axonal transport; Rapid axonal transport; Halothane; Vagus nerve isolated; General anesthesia; Mechanism of anesthesia.)

Table 1. Comparison of Distributions of Radioactivity in the Left and Right Vagus Nerves (Mean ± SD)*

<table>
<thead>
<tr>
<th>Segment Distance from Ganglion (mm)</th>
<th>3.5 Hours after Injection</th>
<th>5.5 Hours after Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left (3)</td>
<td>Right (3)</td>
</tr>
<tr>
<td>5</td>
<td>74 ± 4</td>
<td>76 ± 5</td>
</tr>
<tr>
<td>10</td>
<td>73 ± 2</td>
<td>81 ± 2</td>
</tr>
<tr>
<td>15</td>
<td>92 ± 7</td>
<td>87 ± 5</td>
</tr>
<tr>
<td>20</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>25</td>
<td>91 ± 14</td>
<td>91 ± 1</td>
</tr>
<tr>
<td>30</td>
<td>63 ± 3</td>
<td>61 ± 2</td>
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<tr>
<td>35</td>
<td>12 ± 9</td>
<td>28 ± 23</td>
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<tr>
<td>40</td>
<td>11 ± 6</td>
<td>9 ± 6</td>
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<tr>
<td>45</td>
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<tr>
<td>50</td>
<td></td>
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<tr>
<td>55</td>
<td></td>
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<tr>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Figures in parentheses represent numbers of nerves examined.

It has been suggested that the state of general anesthesia is dependent on depolymerization of neuronal microtubules.† Accordingly, if intact microtubules are necessary for the rapid axonal transport of proteins, as many suppose,‡ then this transport should be impaired during anesthesia. Fink and Kennedy§ recently attempted to observe such impairment in optic nerve fibers during halothane anesthesia, but obtained generally negative results. The present study describes the effect of halothane on rapid axonal transport in the vagus nerve in vivo and in vitro. In vitro it was possible to use levels of halothane higher than those compatible with survival of the animal.

Methods

The general plan of procedure was to label the rapidly-transported protein in rabbit vagus

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Dr. Kennedy was awarded First Prize in the 1971 ASA Essay Contest for this paper.
nerves by injecting radioactive leucine into the calamus scriptorius area. The cells in the vicinity, including those of the dorsal nucleus of the vagus, take up the labeled amino acid and incorporate it into protein. Some of this protein is propelled somatofugally in axons of the vagus nerve. At various time periods following injection of label, the animals were killed and the cervical vagus nerves removed for incubation with or without halothane. Distribution of radioactivity in the vagus nerves was then determined. Electron microscopic observations were also made.

Male albino rabbits 11 weeks old, weighing 2.5 to 3.0 kg, were studied. Labeling of proteins transported in the vagus nerves was effected according to a modification of the method of Sjöstrand. Rabbits were anesthetized in a metal hutch, receiving a gas flow of 3 per cent halothane, 50 per cent O₂, and nitrogen. The anesthetized animal was placed prone on a plastic pan over a supporting thoracic pad and the head and neck fixed and flexed by traction on the ears. Anesthesia was maintained during the surgical procedure with 1.5 to 2.0 per cent halothane delivered through a mask. Following exposure of the brain stem through a midline suboccipital incision, the roof of the fourth ventricle was removed. 30 μl of neutralized L-leucine-4,5-²H (specific radioactivity 20 Ci/mM, concentration 1 mCi/ml, New England Nuclear) contained in a glass pipet with a tip diameter of 30 to 50 μ were injected within a few seconds 2 mm beneath the floor of the fourth ventricle in the median sulcus 4 mm from the obex. The volume delivered was controlled with a microsyringe connected to the pipet by polyethylene tubing.

**STUDIES IN VIVO**

Following injection of label, the 12 control animals were allowed to awaken and the others kept anesthetized with halothane. The control animals were killed 2.5, 3.5, 4.5, or 5.5 hours following injection of label. In figures 1, 2, 4, and 5, activity in the most radioactive 5-mm segment or "peak" is taken as 100. Vertical bars represent ±1 SEM. Figures in parentheses are the number of nerves studied at the various time intervals.

**Table 2. Effect of Halothane on Rapid Axoplasmic Flow in Vivo**

<table>
<thead>
<tr>
<th>Halothane Partial Pressure Inspired (atm %)</th>
<th>Time after Injection (Hours)</th>
<th>Peak Distance from Ganglion (mm ±SD)</th>
<th>Peak Activity (Counts/Min ± SD)</th>
<th>Number of Nerves</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.5</td>
<td>5 ± 0</td>
<td>377 ± 248</td>
<td>8</td>
</tr>
<tr>
<td>0</td>
<td>3.5</td>
<td>20 ± 3</td>
<td>348 ± 216</td>
<td>6</td>
</tr>
<tr>
<td>0</td>
<td>4.5</td>
<td>35 ± 4</td>
<td>82 ± 21</td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>5.5</td>
<td>55 ± 3</td>
<td>312 ± 122</td>
<td>9</td>
</tr>
<tr>
<td>1.0</td>
<td>5.5</td>
<td>55 ± 0</td>
<td>308 ± 48</td>
<td>2</td>
</tr>
<tr>
<td>1.5</td>
<td>5.5</td>
<td>50 ± 4</td>
<td>255 ± 153</td>
<td>8</td>
</tr>
</tbody>
</table>

**Fig. 1.** Radioactivity in control vagus nerves, in vitro, 2.5, 3.5, 4.5, and 5.5 hours following injection of label. In figures 1, 2, 4, and 5, activity in the most radioactive 5-mm segment or "peak" is taken as 100. Vertical bars represent ±1 SEM. Figures in parentheses are the number of nerves studied at the various time intervals.
hours after injection of label; the five anesthetized animals were all killed 5.5 hours after injection. Four of the second group inspired 1.5 per cent halothane, and one inspired 1 per cent halothane. Anesthesia was maintained with controlled ventilation, as previously described. Blood gases were determined in arterial samples obtained from the ear and ventilation and oxygenation were adjusted to maintain $P_{O_2}$ 100 to 130 torr and $P_{CO_2}$ 25 to 30 torr. Just prior to sacrifice, 5 ml of arterial blood were drawn from the ear and the content of halothane determined by the method of Fink and Morikawa. The temperatures of both experimental and control animals were measured rectally and kept at 39 ± 0.5°C with a heating pad.

**STUDIES IN VITRO**

For these studies, anesthetized animals received label as above and were allowed to re-

**TABLE 3. Effect of Halothane on Rapid Axoplasmic Flow in vitro**

<table>
<thead>
<tr>
<th>Halothane (atr %)</th>
<th>Period of Incubation (Min)</th>
<th>Peak Distance from Ganglion (mm ± SD)</th>
<th>Peak Activity (Counts/Min ± SD)</th>
<th>Number of Nerves</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>30 ± 1</td>
<td>300 ± 107</td>
<td>3</td>
</tr>
<tr>
<td>0</td>
<td>160</td>
<td>45 ± 3</td>
<td>236 ± 101</td>
<td>16</td>
</tr>
<tr>
<td>2.0</td>
<td>160</td>
<td>45 ± 5</td>
<td>188 ± 108</td>
<td>6</td>
</tr>
<tr>
<td>2.6</td>
<td>160</td>
<td>45 ± 4</td>
<td>193 ± 124</td>
<td>2</td>
</tr>
<tr>
<td>3.3</td>
<td>160</td>
<td>45 ± 8</td>
<td>194 ± 100</td>
<td>5</td>
</tr>
<tr>
<td>4.0</td>
<td>160</td>
<td>30 ± 3</td>
<td>213 ± 120</td>
<td>4</td>
</tr>
<tr>
<td>4.6</td>
<td>160</td>
<td>25 ± 4</td>
<td>240 ± 39</td>
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<tr>
<td>5.2</td>
<td>160</td>
<td>25 ± 3</td>
<td>122 ± 40</td>
<td>4</td>
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<td>7.8</td>
<td>160</td>
<td>20 ± 4</td>
<td>353 ± 35</td>
<td>2</td>
</tr>
<tr>
<td>10.4</td>
<td>160</td>
<td>5 ± 0</td>
<td>430 ± 151</td>
<td>2</td>
</tr>
</tbody>
</table>

**FIG. 3.** Slowing of rapid axoplasmic flow by halothane in vitro. The per cent of the control rate was obtained from figure 4 by dividing the peak distance in nerves exposed to halothane by the peak distance in control nerves. Vertical bars represent ±1 SEM.
awaken, and were killed 2.5 hours after the injection. Preliminary experiments showed that at this time radioactivity had entered the proximal cervical vagus. Vagus nerves were removed for incubation and placed on a double layer of Whatman #1 filter paper in 150-mm Petri dishes containing 10 ml of Medium 199 (Earle's #115 EECIX, Grand Island Biological Company, Oakland, California), in a 2.5-liter prewarmed humidified desiccator. The desiccator was evacuated, filled with a mixture of 95 per cent $\text{O}_2$ and 5 per cent $\text{CO}_2$; re-evacuated, and finally refilled to 720 torr; when halothane was added it was introduced before the second fill by evaporating a weighed amount from a vial connected to the desiccator. The amount required was calculated from the ideal-gas laws. The desiccators were incubated at 38.5°C. Tests showed that the partial pressure of oxygen in the medium exceeded 350 torr within 5 minutes after the final filling and that the temperature of the atmosphere in the desiccator reached 38.5°C.

<table>
<thead>
<tr>
<th>Table 4. Reversal of Inhibition of Rapid Axoplasmic Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Halothane (atm %)</strong></td>
</tr>
<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>5.2</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>7.3</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>10.4</td>
</tr>
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<td></td>
</tr>
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</table>
within 15 minutes after placement in the incubator.

Control incubations were carried out for periods of 100 and 160 minutes to determine a reference \textit{in-vitro} rate of rapid axoplasmic flow. The effect of halothane was studied in paired nerves by incubating one nerve in a halothane-free atmosphere for 160 minutes at 38.5°C and the other after addition of halothane 2.0, 2.6, 3.3, 4.0, 5.2, 7.8, or 10.4 atm per cent. Two to five nerves were studied at each partial pressure.

Reversibility of the halothane effect was studied in pairs of nerves incubated in halothane, 5.2, 7.8, or 10.4 atm per cent, for 160 minutes. At the end of this period the distribution of activity in one of the nerves was determined, and the other was incubated for an additional 180 minutes in an atmosphere of 95 per cent O$_2$–5 per cent CO$_2$.

**Distribution of Radioactivity in Vagus Nerves**

At the end of the period of treatment, the vagus nerves were cut sequentially in 5-mm segments, starting at the peripheral end of the nodose ganglion. Each segment was placed in a vial and digested overnight in 1 ml of Soluene TM 100 (Packard Instrument Company, Inc., Downers Grove, Illinois 60515) at 50 to 60°C. After digestion, the vials were cooled in darkness to minimize chemiluminescence; 14 ml of toluene-permafluor scintillation fluid were added to each vial and the radioactivity measured in a Packard 3340 liquid scintillation spectrometer. Ten-minute counts were repeated until there was less than 5 per cent difference between consecutive counts. Counting efficiency determined by the external automatic standard technique was 30–33 per cent for all samples. Since quenching was uniform, counts are reported uncorrected for quenching at 30 per cent efficiency but corrected for background activity (about 20 counts/min).

In order to normalize the results for graphing, the relative activity in a segment of nerve is reported as a percentage; the activity in the most radioactive segment or "peak" segment of the nerve is taken as 100 per cent. The actual mean activity in the "peak" segment of each nerve group is stated in the tables.

**Electron Microscopy**

Tissues for electron microscopy were obtained from vagus nerves incubated under control conditions or in the presence of halothane. They were fixed for three hours in a solution containing 3 per cent glutaraldehyde, 1 per cent sucrose, and Millonig's phosphate buffer.
at pH 7.4. Those exposed to halothane were fixed in presence of halothane. Subsequently the tissues were rinsed in buffer plus sucrose, postosmicated, dehydrated, and embedded in Epon. Sections were stained with uranyl acetate and lead citrate, and were examined in an AEI S01 electron microscope at 60 kv.

Results

Experiments in Vivo

Comparison of radioactivity distribution in left vagus nerves with those in right vagus nerves 3.5 hours and 5.5 hours after injection showed that distribution patterns on the two sides were similar (table 1). Student’s t test shows that differences between the relative activity in corresponding segments on the two sides are not statistically significant (P > 0.1). Therefore, one nerve could be used as a control for the other in studying rates of rapid axoplasmic flow. When plotted, the distribution patterns appeared as waves, each with an obvious crest or peak (fig. 1). From the locations of the peaks in animals sacrificed 2.5 hours and 5.5 hours after injection, a normal in-vitro rate of 17 mm/hour was calculated (table 2).

In animals receiving halothane for 5.5 hours the peak of radioactivity advanced a little less far down the vagus than in the controls (table 2), but the difference was not significant (P > 0.1), indicating that the axoplasmic flow rate was probably not slowed during halothane anesthesia. It may be inferred that uptake of leucine and incorporation into protein were not significantly retarded by anesthesia either. The virtual absence of effect occurred notwithstanding blood levels of halothane reaching 0.8 to 1.0 atm per cent, total absence of response to noxious stimuli, and moderate to marked depression of arterial blood pressure.

Experiments in Vitro

In the nerves examined after a period of incubation under control conditions in vitro, a wave of radioactivity located some distance from the nodose ganglion was again found (fig. 2). However, the wave was narrower than the corresponding in-vitro control wave, since continued movement of labeled protein into the cervical vagus from the brain stem was no longer possible. The wave retained the same narrow configuration during 100 and 160 minutes of incubation, indicating that dispersion by diffusion was not significant. Diffusion of label either intra- or extra-axonally would have tended to flatten and broaden the wave. From the location of the peak at 100 and 160 minutes, an in-vitro rate of travel of 15 mm/hour for the rapidly transported proteins was calculated (table 3). Energy for this movement must be supplied by the axon and/or its Schwann cells, since the cell body was disconnected from the axon.

By comparing the locations of peaks in nerves exposed to halothane with those in control nerves, the average effect of halothane on rate of transport may be calculated. There was no significant effect on axoplasmic flow rate when the partial pressure of halothane was below 4 per cent. With 4.0 per cent halothane vapor, the rate of flow decreased to 60 per cent of control, and with 10.4 per cent vapor the flow ceased (table 3, fig. 3). The distribution of activity in the wave did not change appreciably with the inhibiting doses of halothane, suggesting that all components of the flow were slowed to the same extent (fig. 4).

Although slow diffusion of anesthetic into the nerve could retard the onset of inhibition and lead to an underestimate of the potency of the drug, there are several reasons for thinking that this did not occur in the present experiments. First, tests showed that propagation of the compound action potential was blocked completely in one minute with 8 per cent halothane. Second, if halothane were diffusing slowly into the nerve, axoplasmic transport in the outer nerve fibers would be affected sooner than those near the center, resulting in a flattened distribution curve. However, no change in the shape of the distribution curve was observed. Third, the almost-complete recovery of axoplasmic flow after in-

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Fig. 6. Electron micrograph of vagus nerve incubated in Medium 199 and 95 per cent O2–5 per cent CO2 for an hour. In the normal myelinated and unmyelinated axons numerous microtubules (mt), neurofilaments (nf), profiles of smooth endoplasmic reticulum (SER), and mitochondria (M) are present. ×45,000.
libition with 7.8 per cent halothane argues in favor of ready diffusion out of the nerve. Diffusion may, however, become a limiting factor when using drugs of lower lipid solubility than halothane.

**Reversibility of Halothane Effect in Vitro**

Since inhibition of rapid axoplasmic flow could have been due to irreversible cellular damage or cell death, additional experiments were carried out to test the reversibility of the inhibition. Nerves were exposed to concentrations of halothane which slowed the flow and were then reincubated in a halothane-free atmosphere for a further 180 minutes. During reincubation following 5.2 and 7.8 per cent halothane, radioactivity continued to advance distally in the vagus (table 4, fig. 5). However, after 10.4 per cent halothane no further movement of activity occurred on reincubation. This suggests that damage to the transport system had occurred, since it is unlikely that sufficient halothane remained bound to nerve tissue to maintain the inhibition.

**Electron Microscopy**

Examination of the nerve ultrastructure after exposure to 7.8 per cent halothane (fig. 7) revealed no change in microtubular structure, number, or distribution from the control (fig. 6); axonal neurofilaments and smooth endoplasmic reticulum were also unaffected. Some Schwann cells and mitochondria were swollen, but most remained normal. After a three-hour exposure to 10.4 per cent halothane, some axons had reduced numbers of microtubules, but most axons were unaffected (fig. 8). Most Schwann cells and mitochondria, however, were swollen. The irreversibility of inhibition of rapid axonal transport after 10.4 per cent halothane could well be caused by this damage to Schwann cells and mitochondria.

**Discussion**

The cervical vagus of the rabbit offers a convenient mammalian nerve model for the study of rapid axonal transport (RAT). Transported protein in both vagus nerves can be labeled by means of a single midline injection of L-leucine-4,5-3H in the vicinity of the vagal motor nucleus, an area easily accessible. Both vagus nerves can be quickly excised in adequate length of uniform diameter, thin enough to allow rapid penetration of drugs, nutrients, and gases.

The results of the present experiments in vitro confirm our previous findings with the optic nerve, indicating that RAT is not appreciably slowed by halothane anesthesia. Since RAT is probably dependent on microtubules, these results suggest that the microtubular structure of the vagus nerve was intact. It could be argued that certain central nervous system microtubules behave differently during anesthesia and are more readily depolymerized by anesthetics than those in peripheral nerve. However, available evidence suggests that the CNS microtubules are remarkably stable. During hexobarbital narcosis the normal number of microtubules appears to be present in the neuronal processes of the rat brain. Microtubular structure in human brains is maintained for at least two hours after death.

In the rabbit vagus nerves studied in vitro, the effects on rapid axonal transport occurred with considerable consistency in both left and right nerves and in different animals. Rates of about 15 mm/hour were consistently found in both nerves. This rate is 10 to 15 per cent slower than the normal of 17 mm/hour found by Sjöstrand and by us, indicating that the ability to support axonal transport may be somewhat impaired in vitro. Nevertheless, the preparation appears quite suitable for the study of dose–response relationship of volatile anesthetics and rapid axonal transport.

In the experiments in vitro axonal transport was not affected in nerves exposed to 3.25 per cent halothane, a lethal dose more than double the highest measured in the blood of deeply anesthetized rabbits. As indicated above, the persistence of RAT with this level of halothane argues against any alteration in microtubular function. Furthermore, the electron micrographs show that the microtubules in the vagus nerves exposed to 7.8 per cent halothane for three hours were essentially normal.
in number. Thus, no alteration in function or structure of the microtubules was demonstrated even with supra-anesthetic doses of halothane.

Experimental production of a graded decrease in the rate of axonal transport has not been reported previously. Although other investigators have shown that with increasing doses of colchicine less material is transported, they have not quantified their results in terms of effect on rate. Kreutzberg, using the rat sciatic nerve, found that at the crushed end of the nerve 900 µg of colchicine produced less acetycholinesterase accumulation than 400 µg. Dahlström found that the accumulation of catecholamine granules in rat sympathetic ganglia was less with 20 per cent colchicine than with 2 per cent colchicine. More recently, James injected colchicine into the ventral horn of the spinal cord of the chicken and observed a greater inhibition of the total amount of protein flowing into the axons of the sciatic nerve with 3 µg than with 1 µg. In such experiments with colchicine, reversal of the drug effect has not been shown, leaving open the possibility that the effect was due to irreversible injury.

On the other hand, our results with halothane indicate that 70 per cent inhibition of rapid axoplasmic flow in vitro is compatible with almost complete reversibility. This appears to be one of the first demonstrations that inhibition of rapid transport can be reversed by simple removal of the inhibiting drug. It should be mentioned that Ochs (personal communication) has found that iodoacetate inhibition of the transport is reversed by addition of pyruvic acid.

The mechanism by which halothane affects the rapid axoplasmic flow is unknown. It is possible that halothane acts to prevent the formation of high-energy compounds needed for maintenance of rapid axoplasmic flow. Ochs has established that the flow is dependent on oxidative metabolism, and it may be pertinent that halothane is an inhibitor of oxidative metabolism in brain slices. However, the level of halothane necessary to inhibit the rapid axoplasmic flow in peripheral nerve is appreciably higher than the level that inhibits oxidative metabolism in brain slices.

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References