Anesthesiology
V 39, No 5, Nov 1973

Halothane Stereoisomers:

Lack of Stereospecificity in Two Model Systems

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To test the possibility that anesthetic potency might be dependent on stereochemical configuration, effects of the *d* and *l* optical isomers of halothane were compared. Two model systems, the isolated cervical sympathetic ganglion of the rat and synthetic phospholipid bilayer membranes spin-labeled to permit electron spin resonance (ESR) spectroscopy, were studied. The two halothane isomers did not differ in ability to depress synaptic transmission in the ganglion, or ability to increase the mobility of fatty-acid chains in the lipid bilayer. Since there is evidence that both models are relevant to anesthesia, these results support the theory that anesthesia by the inhalation agents is a physical phenomenon in which the stereochemical configuration of the anesthetic molecule plays at most a minor role. (Key words: Stereoisomers; Theories of anesthesia; Synaptic transmission; Lipid bilayers; Electron spin resonance.)

GENERAL ANESTHESIA produced by the inhalation agents is presently considered to involve physical phenomena rather than interaction at receptor sites necessitating specific molecular conformation. The more promising suggestions include a relationship of the anesthetic molecule with structured water, or more likely, a simple solution of the anesthetic in the lipid phase of nerve cell membranes. Support for the concept of a physical mode of action is provided by the good correlation observed between the partial pressure required for anesthesia and physical properties such as oil–water partition coefficients.[1-2] In addition, the wide variety of molecules with anesthetic properties, including some inert gases, makes the likelihood of a common physical property a more reasonable possibility. The opportunity to test the effect of molecular conformation on anesthetic potency in a volatile agent was provided by the synthesis of *d* and *l* optical isomers of halothane (2-bromo-2-chloro-1,1,1-trifluroethane).[3] § In theory, stereoisomers have identical physical properties, differing only in the direction in which they rotate polarized light. Therefore, any difference in activity between the two implies an action at a site with specific stereochemical requirements. Limited supplies of each isomer prevented direct measurement of MAC. We therefore compared the effects of the two optically active preparations on two different model systems: synaptic transmission in the isolated superior cervical sympathetic ganglion of the rat, and molecular order of lipid bilayers observed by electron spin resonance (ESR) techniques. The advantages of these as research preparations and their relevance to the phenomenon of anesthesia are discussed below.

Methods

SYNAPTIC TRANSMISSION

Male Sprague-Dawley rats weighing 300–400 g were anesthetized with pentobarbital (60 mg/kg), the superior cervical ganglion exposed under a binocular microscope, dissected free, and transferred to a perfusion chamber. The chamber was perfused with bathing fluid at room temperature equilibrated with 95 per cent O_2 and 5 per cent CO_2, and maintained at a *p*H between 7.37 and 7.41. The ganglion and its nerves were carefully cleaned of adherent blood vessels and connective tissue and mounted on electrodes (fig. 1).

The preganglionic nerve was stimulated with single shocks (0.5 msec duration) once every 10 seconds either through a glass suction electrode or by a pair of insulated platinum elec-

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Received from the Department of Anesthesiology, Stanford University School of Medicine, Stanford, California 94305. Accepted for publication May 15, 1973. Supported by NIH Program Project Grant GM-12527; presented in part at the Annual Meeting of the American Society of Anesthesiologists, Atlanta, Georgia, October 16–20, 1971.

§ Provided through the courtesy of Drs. F. Edamura and M. Chenoweth of the Dow Chemical Co.

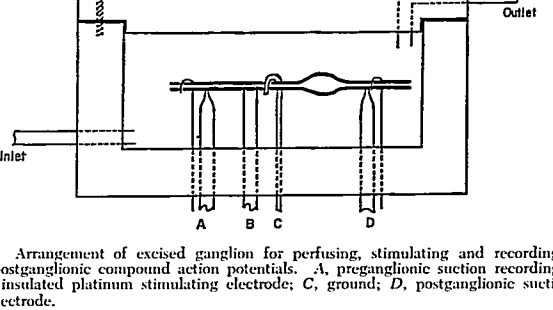

FIG. 1. Arrangement of excised ganglion for perfusing, stimulating and recording both pre-and postganglionic compound action potentials. *A*, preganglionic suction recording electrode; *B*, insulated platinum stimulating electrode; *C*, ground; *D*, postganglionic suction recording electrode.

trodes embedded in the nerve. The postsynaptic compound action potential (PSAP) was recorded extracellularly through a glass suction electrode placed on the postganglionic nerve about 1 mm from the ganglion. Potentials were amplified and displayed on an oscilloscope screen. In three of the experiments the response to each stimulus throughout the experiment was photographed by a Grass Kymograph Camera (Grass Instruments). Portions of the other experiments were recorded on tape for later photography and analysis by a Computer of Average Transients (CAT) (Technical Measurements Corporation).

Halothane has limited solubility in aqueous solutions (345 mg/100 ml at 23 C)[5] and is significantly soluble in the Teflon of the perfusion chamber. In spite of this, preliminary experiments showed that the predetermined concentration of 10 mg/100 ml halothane in the bath surrounding the ganglion could be reliably achieved by observing the following procedure. Freshly oxygenated bathing solution was placed in a 100-ml glass syringe together with a magnetic stir bar. To this a measured quantity of halothane was added from a microliter syringe. The large syringe was capped and placed on a magnetic stirring device for one hour, with care taken that the globule of halothane, usually visible on the bottom of the syringe, should be broken up early in the mixing procedure. A Harvard infusion pump was used to deliver the halothane-equilibrated solution to the recording chamber at a rate of 1 ml/min. The bathing solution was run in for 5 minutes and the pump was stopped. The chamber was then allowed to equilibrate with halothane for 5 minutes, and the halothane-containing solution was run in for an additional 10 minutes. In preliminary work with ^{14}C racemic halothane, samples of the chamber effluent showed that for the final 5 minutes of this 20-minute perfusion period the concentration of halothane in the recording chamber remained constant and was identical to that in the syringe. The oxygen tension in the syringes was in excess of 450 torr at the end of a seven-hour experiment. A solution prepared as above but without the addition of halothane and stored in the syringe for seven hours had no effect on the potentials recorded from the postganglionic nerve.

After satisfactory responses from the postganglionic nerve had been achieved, the teflon recording chamber was sealed by a tight-fitting teflon lid. The preparation was allowed to stabilize for an additional hour while being stimulated at the rate used for the experiment. Following stabilization, the solution containing one of the isomers was allowed to flow into the perfusion chamber according to the schedule described above. The preparation was then allowed to recover for 40 minutes, and was subsequently perfused with the other isomer. Exposure to the isomers was alternated, each preparation being exposed to each isomer three times. In three of the experiments the *d* iso-

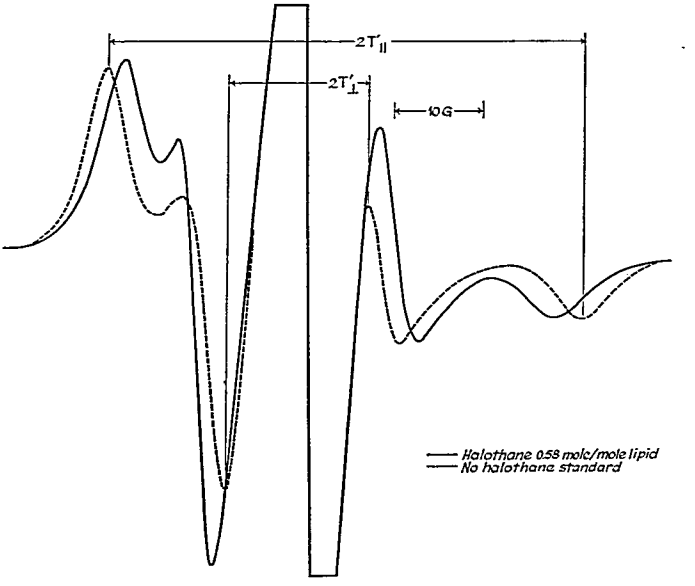

FIG. 2. An electron spin resonance spectrum of a spin-labeled phospholipid in a phospholipid vesicle, illustrating the effect of adding halothane to the vesicle suspension. The changes observed in the splittings ($2T'_{\perp}$ and $2T'_{\parallel}$) are a measure of the potency of the anesthetic. The calibration mark, 10G, indicates a span of ten Gauss in the magnetic field sweep. (Reproduced from Trudell et al.[6])

mer was given first, in the other three the *l*. The six complete experiments thus represent a total of 36 trials with halothane, 18 with each isomer.

For statistical analysis the amplitudes of the last ten postganglionic responses during exposure to halothane were averaged and compared with the last ten responses of the preceding recovery period. Per cent depression was calculated as the difference between the average heights of the compound action potentials at the end of the recovery period and at the end of exposure to halothane, divided by the former quantity. Consecutive trials of *d* and *l* isomers were paired in a standard version of the t test with paired variables.

ORDER IN PHOSPHOLIPID BILAYER MEMBRANE MODELS

A suspension of phospholipid vesicles was prepared as previously described[6] by sonicating a mixture of *l*-phosphatidylcholine, synthetic *l*-phosphatidylcholine containing a nitroxide spin-label on the β-fatty acid chain, and water in the ratio 10, 0.1, 90 by weight. Portions of the suspension (0.5 ml) were placed in Pasteur pipettes which had been sealed at the long end. To each experimental preparation, racemic halothane or one of the two halothane isomers was added, to produce a concentration of 0.16 moles of halothane per mole of lipid, based on a lipid/water partition coefficient of

13 ± 3.[6] Solutions of racemic halothane and of each isomer were made in triplicate.

Sonication of the phospholipid mixture produces double-walled spheres or vesicles. In these vesicle walls, polar phosphate headgroups face outward in contact with the aqueous solution, while the hydrocarbon chains of the fatty acids extend inward toward the bilayer center.

The spontaneous organization of the phospholipids induced by ultrasonic vibration produces a bilayer remarkably similar in structure and thickness to that seen in electron micrographs of cell membranes.[7] The mobility of the fatty-acid "tails" within the bilayer can be analyzed by ESR techniques. In our preparation one phospholipid in a hundred has a nitroxide spin label attached rigidly to the fatty-acid chain. Using an ESR spectrometer, one observes the resonant absorption of energy by the free electron located on the nitroxide spin label. The combination of magnetic field and microwave frequency which produces this resonant condition is sensitive to the motion of the free electron. Since the free electron is rigidly attached to the fatty-acid chain, observing the motion of the electron provides information about the motion of the fatty-acid chain. As the bilayer becomes more disordered or fluid, these chains increase the amplitude of their wagging and wobbling motions. Figure 2 illustrates ESR spectra of spin-labeled phospholipid vesicles, with and without the addition of racemic halothane. The changes in the hyperfine splittings labelled "$2T'_{II}$" and "$2T'_{\perp}$" reflect the increase in conformational mobility associated with the addition of halothane to a lipid bilayer. The spectra were measured on a Varian 4500 ESR spectrometer at 20 ± 0.1 degrees.

Results

Effects on Synaptic Transmission

Statistical analysis of the depression of PSAP produced by the isomers is presented in table 1. When successive trials are paired, there is an average difference in PSAP depression of 5 per cent, with the *l* isomer more potent than the *d* isomer. This is not significantly different from zero. Taking a space of two standard errors from the mean as a confidence interval, the largest potency difference consistent with these data is that the mixture in which the *l* isomer predominates is 13 per cent more potent than that enriched in the *d* isomer.

Table 1. Depression of Synaptic Transmission by Halothane Stereoisomers*

Per Cent Depression of Postsynaptic Compound Action Potential		Mean Difference $l - d$
l	*d*	
44.5 (3.5)	39.4 (3.6)	5.0 (4.2)

* Results are the means of 18 trials with each isomer on ganglia from six animals. Adjacent trials were paired to arrive at the figure for mean difference. Figures in parentheses are standard errors of the mean.

When each experiment is considered separately, in five of the six there was no consistent difference between per cent depression produced by the two isomers. An example of their similarity in effect is shown in figure 3. There was also no difference in latency of response, in dose–response curves as estimated by the time course of depression and of recovery, or in the waveform of the averaged compound action potential as displayed on the CAT (fig. 4).

In the one remaining experiment there was a large consistent difference between the two isomers: the *l* isomer produced an average depression of 61 per cent and the *d* isomer a depression of 22 per cent in this experiment. In view of the fact that most of the difference and the variance are contributed by one experiment (the only one in which differences were large and consistent), we believe that this experiment represents an error in preparation of one of the halothane solutions. This was the fourth experiment in the preplanned series of six. It would have been desirable to extend the series to insure that this was indeed an experimental error, but the limited supplies of the isomers did not permit this. In the complete series of six experiments, the difference between the isomers does not approach statistical significance.

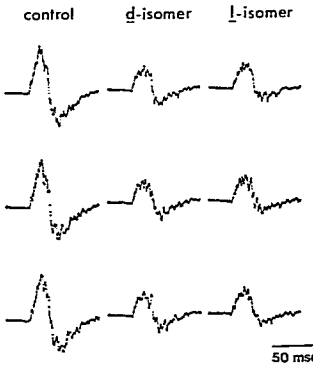

FIG. 3. Effects of the *d*- and *l*-optical isomers of halothane on amplitude of the postganglionic extracellularly recorded compound action potential. Each column represents the responses to three consecutive stimuli. Calibration marks are 50 msec and 5 mV.

EFFECTS ON LIPID MEMBRANE STRUCTURE

Electron spin resonance spectra similar to figure 2 were obtained for each of the suspensions treated with *d*-, *l*-, or racemic halothane. The $2T'_{II}$ and $2T'_{\perp}$ splittings were measured in each spectrum. The combined error in sample preparation and spectrum measurement techniques is approximately ± 10 per cent. Within this error limit, no difference between the spectral splittings caused by equal concentrations of *d*-, *l*-, and racemic halothane was seen.

Discussion

The results are consistent with the hypothesis that our samples of the *d* and *l* optical isomers of halothane do not differ in ability to depress transmission through the superior cervical ganglion of the rat, or in ability to increase disorder in a lipid bilayer membrane. The isomer preparations had calculated *d*/*l* ratios of 30/70 in the sample in which the *l* form predominated and 75/25 in the one in which the *d* enantiomer was the majority species (F. Edamura, personal communication). An actual potency ratio of 1.5/1 between the pure enantiomers would correspond to an observable ratio of 1.19/1 between the samples in our possession; the existence of a potency ratio of this magnitude could have been detected in both the isolated ganglion and the lipid membrane preparations. Stereospecificity in halothane effects on synaptic transmission and on lipid bilayers is therefore low or nonexistent.

SIGNIFICANCE OF THE RESULTS WITH RESPECT TO ANESTHESIA

The synthetic bilayered membranes produced by sonication of phosphatidylcholine in aqueous solution bear a close resemblance to electron micrographs of the central bilayer of cell membranes.[7] A number of models of cell membrane structure have been proposed, including the original Davson-Danielli protein-lipid–protein "sandwich"[8] and the fluid mosaic model of Singer and Nicholson.[9] In each, a bimolecular layer has been accepted as the arrangement of the structural lipids which make up a large part of the membrane.

The lipid region of the nerve cell membrane is also well accepted as the site of action of general anesthetics. Evidence in support of this premise is the close correlation between lipid solubility and anesthetic potency, a correlation better than that observed for any other physical property of the anesthetics.[2] In addition, recent work in this laboratory has shown that anesthetic effects on lipids are reversed by the application of high pressures.[10] Inasmuch as anesthesia itself is reversible by pressure, the criterion of pressure reversibility must be met by any model for anesthetic action. The anesthetic-produced alteration of lipid conformational mobility meets this criterion.

The sympathetic ganglion has often been used as a model for the study of anesthetic action on nerve cells.[4, 11] There are three types of synaptic transmission in this ganglion, a fast excitatory response mediated by acetylcholine acting on receptors of the nicotinic type; a slow excitatory response, also cholinergic but associated with muscarinic receptors; and a slow adrenergic inhibitory response.[12] There is evidence that nicotinic transmission is much more sensitive to halothane than the muscarinic pathway in sympathetic ganglia,[13] and this halothane-sensitive nicotinic pathway was presumably the one primarily activated by the low-frequency single stimuli employed in

the present experiments. However, any stereospecificity in halothane effects on postsynaptic elements of the other two pathways ought to have appeared in the results as a differential alteration in the excitability of the postganglionic neurons.

Although nicotinic receptors are rare or nonexistent in the mammalian central nervous system, the cation permeability increase associated with this type of transmission is typical of that implicated in synaptic transmission at several sites in the CNS.[14] The muscarinic and adrenergic forms of ganglionic transmission also have close counterparts in the CNS.[15, 16] The relevance of the present findings in the ganglion to anesthesia, therefore, depends on the certainty with which synaptic transmission of these or similar types can be identified as the site of anesthetic action at the cellular level. At present this identity is far from certain.

The concentrations of halothane used in the present work were chosen on grounds of experimental convenience, although in neither case are they far from concentrations used clinically to produce anesthesia. The halothane concentration of 10 mg/100 ml used in the ganglion experiments was chosen because exposure to this concentration of racemic halothane in preliminary experiments produced approximately 50 per cent depression of ganglionic response. At this level of depression differences between the isomers in both directions would have been clearly revealed. A concentration of 10 mg/100 ml is well within blood concentrations produced during clinical anesthesia[5] but probably higher than concentrations in cerebrospinal fluid water, the relevant phase for brain cells.

In the experiments on lipid bilayers, the concentration of 160 mmoles of halothane per liter of lipid is approximately three times that predicted by Meyer and Overton to obtain in membrane lipids during general anesthesia. Since previous studies have shown a linear change in spectral splittings as a function of

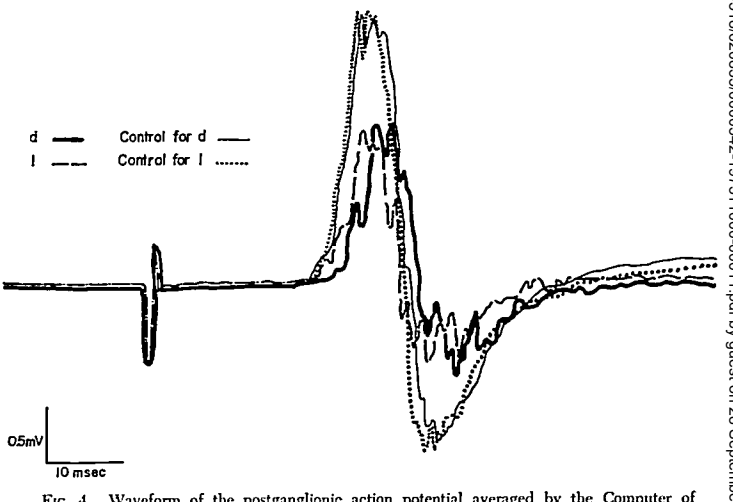

FIG. 4. Waveform of the postganglionic action potential averaged by the Computer of Average Transients (CAT). Ten responses of the same ganglion are averaged in each category: two control series after 40 minutes of perfusion with halothane-free medium; after 20 minutes in the *d*-isomer; after 20 minutes of exposure to the *l*-isomer.

halothane concentration,[6] the higher-than-physiologic concentration of halothane used in the present experiments was chosen to make the observed changes as large as possible.

Stereospecificity has not previously been investigated in volatile or gaseous general anesthetic agents. The results presented above demonstrate that in two different model systems there is no difference in potency between the two steric configurations of halothane. Evidence for the relevance of both models to anesthesia itself is strong. If the models are indeed relevant to anesthesia, the present results support the thesis that anesthesia by the gaseous and volatile agents consists of a physical alteration of the lipid component of cell membranes, independent of a requirement for stereospecific molecular configuration.

The authors thank Dr. Wayne Hubbell, Department of Chemistry, University of California, Berkeley, for measuring the spectra and assisting in the interpretation.

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Obstetrics

CORD BLOOD PROTEIN AND RDS The total protein concentration in venous blood from the umbilical cord was measured in 2,200 consecutive newborn infants. Thirty-four infants developed idiopathic respiratory distress syndrome (IRDS). Thirty-three of these infants had cord protein of less than 4.6 g/100 ml. Of 57 infants with low cord protein, low birth weight and immaturity, approximately half (33) developed IRDS. In infants with cord protein concentrations above 4.6 g/100 ml, the incidence of IRDS was less than 0.5 per cent regardless of gestational age or birth weight. (*Bland, R. D.: Cord-blood Total Protein Level as a Screening Aid for the Idiopathic Respiratory Distress Syndrome. New Eng. J. Med. 287: 9–13, 1972.*)