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Nonanesthetic alcohols dissolve in synaptic membranes without perturbing their lipids

(anesthesia cut-off/*Rana pipiens*/*Torpedo*/lipid order parameter)

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ABSTRACT **While many theories of general anesthesia postulate a lipid site of action, there has been no adequate explanation for the lack of anesthetic potency of the highly hydrophobic primary alkanols with more than 12 carbons (the cut-off). Some work suggests that these nonanesthetic alcohols do not dissolve in membranes. Other work contradicts this and suggests that an anesthetic site on a protein provides a better explanation. Here we show that both the anesthetic dodecanol and the nonanesthetic tetradecanol are taken up equally well into the tissues of animals and into isolated postsynaptic membranes. When a group of *Rana pipiens* tadpoles were treated with dodecanol, half were anesthetized by 4.7 μM (free aqueous concentration), and the corresponding concentration in the tissues was found to be 0.4 mmol per kg wet weight. Prolonged exposure (92 hr) to tetradecanol produced even higher tissue concentrations (0.7 mmol per kg wet weight), yet no anesthetic effects were observed. Furthermore, general anesthetics are thought to act on postsynaptic membranes but both alkanols partitioned into postsynaptic membranes from *Torpedo* electroplaques. The spin label, 12-doxyl stearate, was incorporated into these membranes. The lipid order parameter it reported was decreased by the anesthetic alcohols (octanol, decanol, and dodecanol), whereas the nonanesthetic alcohols either did not change it significantly (tetradecanol) or actually increased it (hexadecanol and octadecanol). Thus, although lipid solubility is unable to account for the pharmacology of the cut-off in potency of the long-chain alcohols, lipid perturbations provide an accurate description.**

The structural diversity of general anesthetics has led to the development of theories which assume that anesthetics must dissolve in and perturb the lipid bilayer of excitable membranes rather than bind to a site on a protein, and which predict that all lipophilic compounds will be anesthetics (for reviews see refs. 1–3). Thus, it has always been puzzling that highly lipophilic primary alcohols with more than 12 carbons are not general anesthetics (4) (an effect referred to as the cut-off in anesthetic potency). One possible explanation for the cut-off is that the nonanesthetic alcohols are not taken up into membranes in sufficient concentration. However, recent work shows that these 1-alkanols do partition into lipid bilayers (5), questioning the previous measurements in which biomembranes were used (reviewed in ref. 1).

Other explanations for the cut-off are possible. Nonanesthetic alcohols may dissolve in membrane lipids but not significantly perturb them (6), or the cut-off in anesthetic potency may be due to the specificity of anesthetic–protein interactions. Indeed, luciferase, a pure protein sensitive to anesthetics, was not inhibited by those alcohols with more than 16 carbons (7). In comparison, previous work places the cut-off for general anesthesia in tadpoles between dodecanol and tetradecanol (4, 8, 9). However, it is possible that the cut-off *in vivo* may have been underestimated because of experimental conditions. For most anesthetics, the free aqueous concentration does not differ significantly from the measured total concentration of anesthetic. However, long-chain alcohols are so insoluble in water that significant depletion of the alcohol may occur by adsorption to the apparatus (10) or uptake into animals, leading to uncertainty in their final free aqueous concentrations.

To reassess the issues raised by these reports, we have reexamined the cut-off both *in vivo* and in postsynaptic membranes. First, we have redetermined the position of the cut-off for normal alcohols in aquatic animals by using radiolabeled agents to monitor both the free aqueous concentration to which the animals were exposed and the uptake of the anesthetic and nonanesthetic alcohols into their tissues. Second, we reexamined the ability of long-chain alcohols to partition into and disorder postsynaptic membranes. For this work, we chose to use high specific activity acetylcholine receptor-rich membranes purified from *Torpedo* electroplaques, whose lipid composition (11) is broadly similar to that of the central nervous system (12), rather than postsynaptic membranes from brain, which are of heterogeneous origin.

EXPERIMENTAL METHODS

Anesthetic Concentration (EC_{50}) and Total Alcohol Concentration in Tadpoles. *Rana pipiens* tadpoles were exposed to anesthetics in oxygenated aqueous solutions of ^{14}C-labeled alkanols (ICN and New England Nuclear) and their ability to right themselves was tested as described (13). Animals were observed until a stable response level had been obtained and were then killed, weighed, dissolved in BTS-450 (Beckman), and stored so as to minimize chemiluminescence, and their alkanol content was assayed by liquid scintillation spectroscopy.

Alkanol Partitioning into Membranes. Postsynaptic membranes from *Torpedo* electroplaques were prepared by differential and sucrose density-gradient centrifugation as described (14). In some experiments, 1 ml of the membrane suspension was mixed with 4 ml of a saturated aqueous solution of ^{14}C-labeled alkanol, and in other experiments 10 μl of an ethanolic stock solution of radiolabeled alcohol was added to 1 ml of the membrane suspension (8.8 mg of protein). These mixtures were layered on top of continuous gradients, made from two cycles of freezing and thawing 1.2 M sucrose, and centrifuged to equilibrium (64,000 × *g*, 4 hr,

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4°C), and fractions were collected and assayed for protein and alcohol content. The purified electroplaque membranes contained 1.5 nmol of acetylcholine sites per mg of protein, and 400–425 phospholipid and 200–250 cholesterol molecules per acetylcholine binding site.

Order Parameter Measurements in Membranes. 12-Doxyl stearate [12-(2,2-dimethyl-*N*-oxyloxazolidine) stearate] was deposited from a methanolic stock solution by evaporation for 6 hr under vacuum. *Torpedo* electroplaque membranes were spin labeled by gently shaking them for 6 hr at 4°C with the spin label. Final probe concentration was <1% of membrane lipids (mol/mol). Membranes were then washed by three cycles of sedimentation (6000 × *g*, 10 min, 4°C) and resuspended in 3 vol of ice-cold *Torpedo* Ringer's solution (14) to minimize signal from unbound spin label. Small aliquots (<25 μl) of ethanolic stock solutions of each ^{14}C-labeled alcohol were added to 250 μl of the spin-labeled membranes (4 mg per ml of protein) and incubated at room temperature for 30 min. Membranes were then washed free of ethanol, as indicated by gas chromatography, using three cycles of sedimentation (6000 × *g*, 10 min, 4°C), resuspended in equal volumes of fresh buffer, and loaded into glass capillary tubes (o.d. 2 mm), which were then flame-sealed on ice. Electron spin resonance spectra were obtained at 20.0 ± 0.1°C with a Bruker ER200 operating at 9.5 GHz, 3250 G, modulation amplitude of 0.32 G, frequency of 100 kHz and 10 mW of microwave power. Order parameters (*S*) were calculated by the method of Hubbell and McConnell (15) from the hyperfine splittings, which were corrected for solvent polarity (16). Following spectroscopy, the samples were sedimented (6000 × *g*, 15 min) and the supernatants were discarded. The pellets were dispersed in scintillation cocktail and the ^{14}C-labeled alcohol content was determined.

RESULTS

Tetradecanol Is Not an Anesthetic, Although It Is Taken up by Tadpoles. When we exposed groups of tadpoles to [^{14}C]-dodecanol, the aqueous concentration fell by almost 20% over 2 hr, and the animals reached a steady level of anesthesia with an EC_{50} of 4.7 μM. Exposure to supersaturated solutions of [^{14}C]tetradecanol for up to 92 hr did not cause anesthesia. The concentration of dodecanol in tadpoles was found to be 0.4 mmol per kg wet weight at the anesthetic concentration. The concentration of [^{14}C]tetradecanol was 0.7 mmol per kg wet weight after 92 hr of exposure, demonstrating that the cut-off cannot be explained by lack of uptake of the alcohols into the tadpoles (Table 1). Indeed, Requena *et al.* (10) failed to block nerves with tridecanol even after perfusing them for a week.

Alkanols Partition into Membranes. When highly lipophilic ^{14}C-labeled alkanols dissolve in lipids, the depletion of the aqueous concentration is so severe that minor hydrophilic radiolabeled impurities may make a major contribution to aqueous-phase radioactivity (5). To avoid such problems, we did not aim to determine partition coefficients but only to determine whether nonanesthetic alkanols could exist in biomembranes in higher concentrations than those predicted by the lipid solubility hypothesis of general anesthesia. After a period of equilibration, mixtures containing the membranes and ^{14}C-labeled alkanols were centrifuged down sucrose density gradients (Fig. 1). A peak of radioactivity, coincident with the narrow membrane band, was found for both anesthetic and nonanesthetic alkanols.

Higher alkanol concentrations were obtained by adding alkanols mixed with ethanol to the same concentration of membranes as described above and then removing the ethanol. Similar results were obtained. In one experiment, [^{14}C]dodecanol was 0.5–0.75 μM in the aqueous phase and 70 mM in membrane lipid (phospholipid plus cholesterol). For [^{14}C]tetradecanol, the corresponding figures were 0.1–0.2

Table 1. Anesthetic uptake of long-chain 1-alkanols

Agent	Aqueous concentration	Exposure time, hr	Alkanol, mmol per kg wet weight of tadpole
Dodecanol	4.7 μM	2	0.4
Tetradecanol	Saturated solution	65	0.5
		92	0.7

R. pipiens tadpoles were exposed to ^{14}C-labeled alkanols in oxygenated aqueous solutions at the concentrations indicated, and their ability to right themselves was tested. Animals were observed until a stable response level had been obtained and were then killed and weighed, and their alkanol content was analyzed. Dodecanol anesthetized half the animals at this concentration, but no anesthetic effects were observed with tetradecanol.

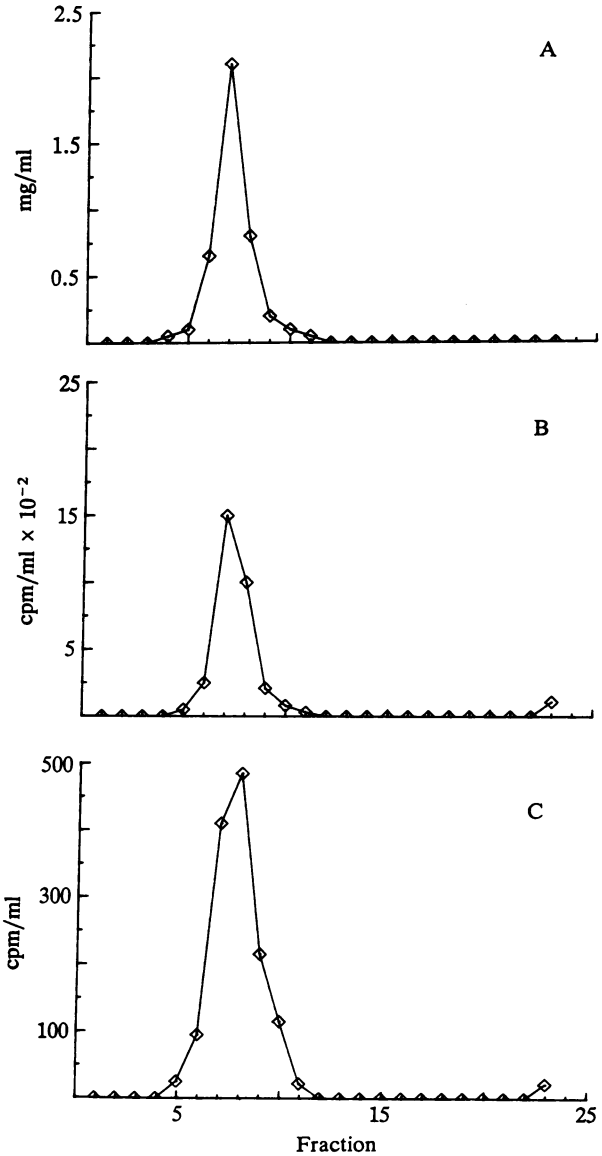

FIG. 1. Anesthetic and nonanesthetic alkanols both partition into membranes. Distribution of protein (*A*), [^{14}C]dodecanol (*B*), and [^{14}C]tetradecanol (*C*) in sucrose density gradients containing postsynaptic membranes from *Torpedo* electroplaques. In the experiment shown, [^{14}C]dodecanol and [^{14}C]tetradecanol were undetectable in the aqueous fractions, whilst their concentrations in membrane lipid were estimated to be about 12 and 0.6 mM, respectively, in the peak fractions.

μM and 15 mM. Because of the possibility of water-soluble radiolabeled impurities being concentrated in the aqueous phase (see above), these aqueous concentrations represent upper limits of alkanol concentration and any partition coefficients calculated would be lower limits. In fact, these limiting partition coefficients are higher than those previously reported in biomembranes (17). However, the important point is that with both alkanols, the apparent aqueous concentration is much less than that in a saturated solution (22 and 1.5 μM, respectively), and consequently the alkanol should be dissolved in the membrane. Considerably higher concentrations than this should be achievable before phase separation of the alkanol in the membrane.

From our data, we estimate that the membrane concentration of both these alkanols can readily exceed that occurring during general anesthesia ($\approx$50 mM). This is consistent with data of Bull *et al.* (18), which showed that erythrocytes perfused with supersaturated solutions of tetradecanol and hexadecanol expanded erythrocyte membrane area as efficaciously as dodecanol and shorter alcohols.

The Lipid Order Parameter Is Decreased by Dodecanol But Not by Tetradecanol. Although the exact nature of the lipid perturbation leading to general anesthesia is not established, we chose to use lipid order parameter because in many previous studies it has proved to be a good predictor of the pharmacology of general anesthesia, even though it does not suggest a very satisfactory mechanism (1–3). The membrane concentration of alkanol was established with [^{14}C]alkanols, thus resolving problems of depletion of the alkanol into membranes and adsorption to the apparatus, which was a problem we found to be quite serious. The anesthetic alkanols all caused a linear decrease in order parameter with increasing concentration in the membrane (Fig. 2). We were thus able to determine the disordering efficacy of each alcohol at equal membrane concentration (expressed as mol fraction in the membrane lipid; Fig. 2). The anesthetic alcohols all had positive disordering efficacies (3, 9) (i.e., they caused a negative change in order parameter with increasing alcohol concentration), whereas the nonanesthetic alcohols caused no significant disordering (tetradecanol) or ordered the membrane. In fact, disordering efficacy declined approximately linearly with carbon chain length (r = 0.988), being zero at a predicted chain length of 13.9 carbons.

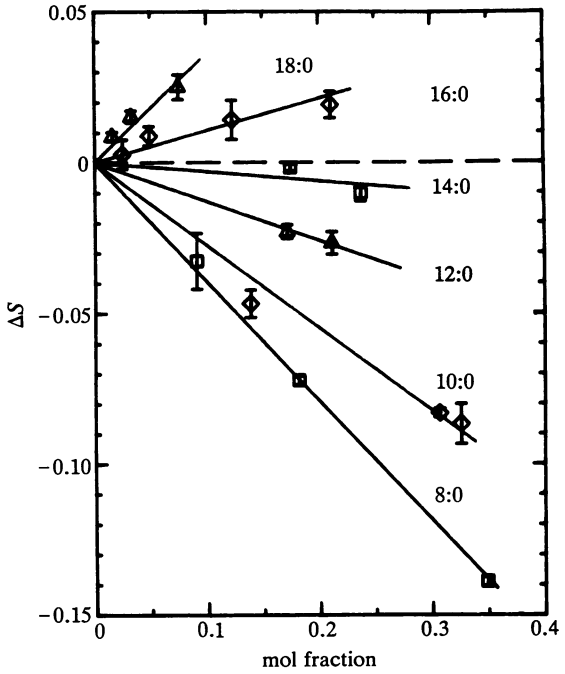

FIG. 2. Change in spectroscopic lipid order parameter (ΔS) as a function of mol fraction of alkanol in the membrane. The order parameter was measured from the electron spin resonance spectra of a fatty acid spin-labeled on the 12th carbon. The membrane alcohol mol fraction [alkanol/(alkanol + lipid)] was established with ^{14}C-labeled alkanols. mol of lipid was taken as measured phospholipid plus 0.5 mol of cholesterol per phospholipid (19). Lines were fit by the least-squares method and their origins do not differ significantly from zero. Each point represents the mean of at least three samples in a single experiment. For each agent, determinations were from at least two separate experiments, each performed with different batches of purified membranes. Error bars are SD. The slopes (± SE) for least-squares fits constrained to pass through zero were as follows: octanol, −0.39 ± 0.029; decanol, −0.27 ± 0.026; dodecanol, −0.13 ± 0.015; tetradecanol, −0.03 ± 0.018; hexadecanol, 0.11 ± 0.034; octadecanol, 0.36 ± 0.071. The slope for tetradecanol is not significantly different from zero ($P > 0.1$).

DISCUSSION

There appears to be no qualitative difference in the ability of the anesthetic dodecanol and the nonanesthetic tetradecanol to achieve effective levels in tadpoles (Table 1) in a postsynaptic membrane (see *Results*) or in lipid bilayers (5). The lipid solubility hypothesis of general anesthesia thus fails to account for the lack of potency of tetradecanol. However, although the lipid solubility hypothesis can be rejected, the lipid perturbation hypothesis remains plausible. Indeed, the data in Fig. 2 show that the ability of alkanols to disorder lipids in *Torpedo* postsynaptic membranes comes remarkably close to predicting the position for the cut-off in general anesthetic potency of the primary alkanols acting at some unknown membrane in the tadpole central nervous system.

By comparison, the cut-off predicted by luciferase occurs between hexadecanol and heptadecanol (7) and might possibly be even higher because it is unclear in these experiments how much of the added alkanol was available to the enzyme and how much was adsorbed to the cuvette used. Therefore, the suggestion (5, 7) that luciferase models the pharmacology of the site of general anesthesia better than do membrane lipids is premature. Indeed, if one takes a broader perspective, the lipid model not only successfully predicts cut-off, but also pressure reversal, the behavior of the inert gas series, and the relative potency of some structurally specific steroids (reviewed in ref. 3). On the other hand, firefly luciferase does not predict the behavior of the gases, although it may yet be shown to model them. It predicts incorrectly that hexanol and heptanol should have nearly equal potency (7), and the ability of steroids to inhibit luciferase correlates poorly with their anesthetic potency (20).

Why do the long-chain alcohols fail to disorder the membrane when they dissolve in it? One suggestion is that when the alkanols' chain length comes close to matching that of the lipids in the bilayer (for a review of early work, see ref. 1), its insertion does not perturb the acyl chains of the phospholipids. More recently, NMR studies of deuterated alcohols in synthetic saturated phospholipids lend some support to this notion and show that long-chain alcohols can indeed be oriented in the bilayer (21), but these studies need to be extended to biological membranes before detailed pharmacological conclusions can be drawn.

While pharmacological tests are incomplete, they tend to favor the membrane perturbation theory. On the other hand, the advantage of the luciferase model is that it has provided a mechanism of action—i.e., competition with aldehyde (22) or luciferin (7) cofactors. However, such a mechanism is unlikely to explain general anesthetic action on excitable membrane proteins, which are not thought to be modulated acutely by such cofactors. Bulk lipid properties, such as order parameters, while providing some idea of how anesthetics perturb lipids, seem unlikely to be directly responsible

for anesthetic action on excitable proteins. Thus, another as yet uncharacterized mechanism must be involved. One attractive possibility, which combines all the available evidence, is that lipids play a relatively specific role in maintaining the activity of some transmembrane excitable channels, and that anesthetics in some way disrupt the structural role of such lipids, perhaps displacing them competitively from the protein. For example, in the acetylcholine receptor, cholesterol, which preferentially distributes to the lipid–protein interface (23), is important for activity (24), and fatty acids are important for membrane insertion (25). Indeed, some studies suggest that alkanols act at the lipid–protein interface (26). Mechanistic studies on membrane proteins, although more difficult than on soluble proteins, may shed some light on these issues in the foreseeable future.

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1. Janoff, A. S. & Miller, K. W. (1982) in *Biological Membranes*, ed. Chapman, D. (Academic, New York), Vol. 4, pp. 417–476.
2. Franks, N. P. & Lieb, W. R. (1982) *Nature (London)* **300,** 487–493.
3. Miller, K. W. (1985) *Int. Rev. Neurobiol.* **27,** 1–61.
4. Ferguson, J. (1939) *Proc. R. Soc. London Ser. B* **127,** 387–404.
5. Franks, N. P. & Lieb, W. R. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5116–5120.
6. Zavoico, G. B., Chandler, L. & Kutchai, H. (1985) *Biochim. Biophys. Acta* **812,** 299–312.
7. Franks, N. P. & Lieb, W. R. (1985) *Nature (London)* **316,** 349–351.
8. Meyer, K. H. & Hemmi, H. (1935) *Biochem. Z.* **277,** 39–71.
9. Pringle, M. J., Brown, K. B. & Miller, K. W. (1981) *Mol. Pharmacol.* **19,** 49–55.
10. Requena, J., Velaz, M. E., Guerrero, J. R. & Medina, J. D. (1985) *J. Membr. Biol.* **84,** 220–238.
11. Popot, J.-L., Demel, R. A., Sobel, A., Van Deenan, L. L. M. & Changeux, J.-P. (1978) *Eur. J. Biochem.* **85,** 27–42.
12. Strichartz, G. R. (1977) in *Mammalian Cell Membranes*, eds. Jamieson, G. A. & Robinson, D. M. (Butterworths, London), Vol. 3, pp. 172–205.
13. Alifimoff, J. K., Firestone, L. L. & Miller, K. W. (1987) *Anesthesiology* **66,** 55–59.
14. Miller, K. W., Braswell, L. M., Firestone, L. L., Dodson, B. A. & Forman, S. A. (1986) in *Molecular and Cellular Mechanisms of Anesthetics*, eds. Roth, S. H. & Miller, K. W. (Plenum, New York), pp. 125–138.
15. Hubbell, W. & McConnell, H. M. (1971) *J. Am. Chem. Soc.* **93,** 314–326.
16. Gaffney, B. J. (1976) in *Spin Labeling Theory and Applications*, ed. Berliner, L. J. (Academic, New York), pp. 567–571.
17. Salle, V. L. (1978) *J. Membr. Biol.* **43,** 187–201.
18. Bull, M. H., Brailsford, J. D. & Bull, B. S. (1982) *Anesthesiology* **57,** 399–403.
19. Leibel, W. S., Firestone, L. L., Legler, D. W., Braswell, L. M. & Miller, K. W. (1987) *Biochim. Biophys. Acta* **897,** 249–260.
20. Banks, P. & Peace, C. B. (1985) *Br. J. Anaesth.* **57,** 512–514.
21. Westerman, P. W., Pope, J. M., Phonophok, N., Doane, J. W. & Dubro, D. W. (1988) *Biochim. Biophys. Acta* **939,** 64–78.
22. Middleton, A. J. & Smith, E. B. (1976) *Proc. R. Soc. London Ser. B* **193,** 173–190.
23. Ellena, J. F., Blazing, M. A. & McNamee, M. G. (1983) *Biochemistry* **22,** 5523–5535.
24. Fong, T. M. & McNamee, M. G. (1986) *Biochemistry* **25,** 830–840.
25. Olson, E. N., Glasser, L. & Merlie, J. P. (1984) *J. Biol. Chem.* **259,** 5364–5367.
26. Heidmann, T., Oswald, R. E. & Changeux, J.-P. (1983) *Biochemistry* **22,** 3112–3127.