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Anesthetic Potencies of Secondary Alcohol Enantiomers

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The Meyer-Overton rule has been interpreted to mean that general anesthetics act at a nonpolar site, either in a lipid bilayer or a protein. Optical isomers, also called enantiomers, are pairs of compounds with the same molecular formula and functional groups, but which differ in the arrangement of the groups around an "asymmetric" carbon atom and in the direction they rotate plane-polarized light. By definition, enantiomers that are anesthetics can distinguish between stereoselective and nonselective sites of anesthetic action. We used such enantiomers to determine whether anesthetics are stereoselective in their actions on animals by measuring the potencies of a homologous series of secondary aliphatic alcohols from 2-butanol through 2-octanol in tadpoles, using reversible loss of righting reflex as the endpoint. None of the isomeric pairs exhibited significant differences in potency. Anesthetic potency increased logarithmically with the number of carbon atoms in the hydrocarbon chain of the alcohol. The $ED_{50} \pm SE$ (mM) for the (+) and (−) forms of the alcohols, respectively, were as follows: 2-butanol 17 ± 1.2, 17 ± 1.1; 2-pentanol 4.7 ± 0.28, 4.8 ± 0.27; 2-hexanol 1.33 ± 0.068, 1.42 ± 0.079; 2-heptanol 0.32 ± 0.011, 0.33 ± 0.020; and 2-octanol 0.063 ± 0.0042, 0.061 ± 0.0032. These data demonstrate a lack of stereoselectivity in the interactions between the anesthetic secondary alcohols and their site of action in animals. (Key words: Anesthetics: alcohols. Enantiomers: alcohols. Potency, anesthetic: alcohols, ED_{50}. Theories of anesthesia: lipid solubility.)

A FUNDAMENTAL CONTROVERSY regarding the molecular mechanism of general anesthesia is whether anesthetics act directly on the proteins of excitable membranes or indirectly, through the lipids that surround them. Some investigators have used the Meyer-Overton rule[1,2] as evidence that the membrane's lipid bilayer is the primary anesthetic site,[3] whereas others have suggested that nonpolar sites on excitable proteins are the primary sites of action.[4] However, distinguishing between these two possibilities has been complicated.[5] For example, although anesthetics are capable of inhibiting the activity of a highly purified lipid-free protein system, firefly luciferase,[4] the inhibitory potencies of the anesthetics in this lipid-free system still correlate quite well with their lipid solubilities.[5]

Fortunately, several pharmacologic "tools" can be used to probe the nature of the anesthetic site. These include: 1) the "cut-off" in potency for the higher molecular weight members of homologous series of n-alkanes and alcohols[6,7]; 2) reversal of anesthesia by hydrostatic and helium pressure[8,9]; and 3) lack of selectivity between anesthetic stereoisomers.[10] Anesthetic cut-off is consistent with general anesthetic action at the lipid bilayer[11,12] but cut-off can also occur in the absence of lipid.[13] The predictions that follow from pressure reversal studies in animals are generally fulfilled by lipid theories[6]; however, a protein system, bacterial luciferase, also exhibits pressure reversal of inhibition by anesthetics.[14]

Thus, there is a need for further pharmacologic probes of the anesthetic target. To test whether the general anesthetic alcohols are stereoselective in their action on animals, we studied the anesthetic potencies of the enantiomers of a homologous series of alcohols from 2-butanol through 2-octanol in tadpoles.

Materials and Methods

Optical isomers (enantiomers) of secondary alcohols were purchased from Norse Laboratories (Newbury Park, CA) and had greater than 99% chemical purity that was confirmed by gas chromatography in our laboratory. Optical purity was greater than 98% for the isomers of 2-butanol through 2-hexanol, greater than 96% for 2-heptanol, and greater than 99% for 2-octanol. Aqueous solutions of the isomers of 2-butanol and 2-pentanol were prepared by stirring weighed aliquots of each compound in gas-tight vials for 6 h at room temperature. Because of their limited solubilities in water, solutions of the enantiomers of 2-hexanol, 2-heptanol, and 2-octanol were prepared by longer stirring (>12 h) in gas-tight vials at 4° C. Fresh solutions were prepared prior to each experiment.

Concentration–response experiments were performed at 20 ± 1° C on early prelimb bud tadpoles, *Rana pipiens*, approximately 1.0–1.5 cm in length (Carolina Biological Supply Co., Burlington, NC). Groups of five tadpoles were placed in covered 100 ml beakers in neutral solutions of the anesthetic alcohols in double-distilled water. No tadpole was used more than once in 48 h. Anesthesia was defined by loss of righting reflex (LRR), as described in detail previously for newts.[9] Briefly, after allowing 15 min for equilibration with an anesthetic solution, the animals were tipped manually with a flame-polished glass pipette.

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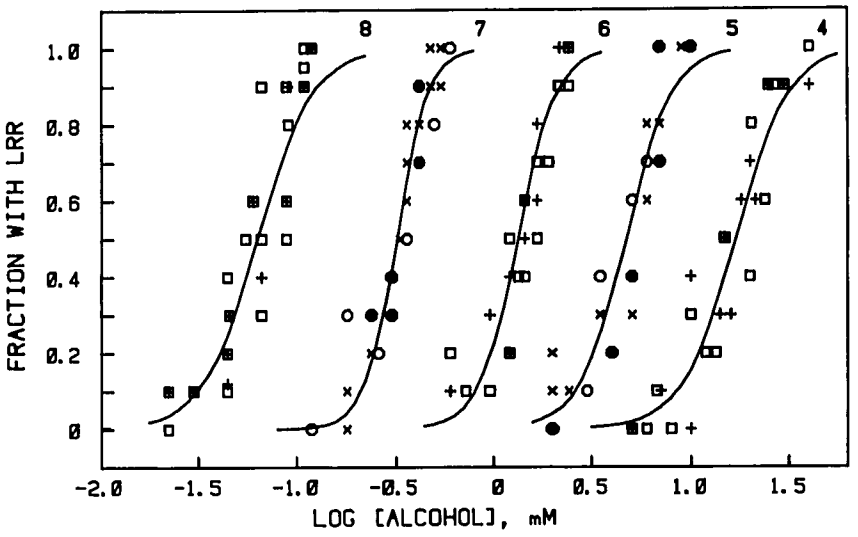

FIG. 1. Log concentration–response relationships for secondary alcohol enantiomers. (+) Enantiomers are represented by *pluses* or *crosses*. (−) Enantiomers are represented by *open squares* or *octagons*. Curves were fitted according to the method of Waud,[15] but only the curves for the (+) enantiomers are illustrated. Numerals above the curves represent number of carbon atoms in the alcohol. Each symbol represents the mean response of ten animals, scored quantally as described in "Materials and Methods." In some instances, symbols overlap. The total number of animals tested with each anesthetic appears in table 1.

Unresponsiveness for greater than 5 s was scored as LRR. Responses to all agents plateaued by 10 min and remained unchanged at 15 min and 30 min. After scoring, animals were placed in containers of neutral distilled water until recovery of righting reflex was confirmed.

Concentrations of 2-octanol and 2-butanol present during concentration–response assays were verified by gas chromatography (Beckman GC72, Poropak® "P" column packing (Waters Co., Milford, MA), column temperature 215° C). Anesthetics remained within 10% of their initial concentrations during the time course of assays.

Log concentration–response curves for each agent were based on a minimum of five different concentrations of anesthetic and ten animals per concentration. Entire studies were repeated, and the data pooled. Median effective doses (ED_{50}), slopes, and estimates of their respective standard errors were obtained according to the method of Waud[15] for quantal responses. The estimated variance in ED_{50} for each isomer in a pair was calculated from the standard error; the sum of these then yielded the estimated variance of the difference in ED_{50} between the (+) and (−) isomers. The ratio of this difference to its standard error was then referred to a standard normal distribution. The problem of multiplicity in comparing the slopes of all log concentration–response curves was addressed by using Bonferroni's inequality[16] to maintain the overall significance level at 0.05.

Results

Log concentration–response curves for the secondary alcohol enantiomers were sigmoidal in shape (fig. 1) and were steep, with slopes ranging from 3.1 to 5.4 (table 1). These slopes were not statistically different ($P > 0.1$), and thus the ED_{50} provides a satisfactory measure of relative potencies.

The anesthetic potencies (ED_{50}) and their standard errors are also shown in table 1. There were no significant differences in LRR potencies between pairs of optical isomers. The per cent error in ED_{50} values [$(SE/ED_{50}) \times 100\%$] ranged from 3.4 to 6.7; these values are comparable to those obtained previously in this laboratory, also using LRR in tadpoles as an endpoint.[11,17] Errors of this magnitude are also similar to those obtained by measuring either LRR or MAC in mice,[18] or MAC in humans.[19]

Stereoselectivity can also be expressed by a ratio of potencies for members of each pair.[20] Potency ratios for LRR in our study were calculated as $[ED_{50}\ (+)]/[ED_{50}\ (-)]$ and are presented in table 1. Values ranged between 0.94 for the enantiomers of 2-hexanol to 1.03 for the enatiomers of 2-octanol, but no systematic trends were observed. None of the potency ratios was significantly different from one ($P > 0.1$).

There was, however, a systematic increase in potency in the series from 2-butanol to 2-octanol, such that 2-octanol was some 280 times more potent than 2-butanol. The relationship between potency and number of carbon atoms was logarithmic, and a linear least-squares fit of log ED_{50} *versus* alcohol chain length produced a slope of -0.60 ± 0.013 (SE) with a correlation coefficient of -0.998 (fig. 2).

Discussion

Correlations between anesthetic potency and the physical properties of anesthetics indicate that the anesthetic site is hydrophobic in nature. However, these correlations do not make it possible to distinguish unequivocally among the various candidates for the anesthetic target, which include such hydrophobic domains as the bulk lipid bilayer,[21] patches on proteins,[22] lipid phase separations,[23]

TABLE 1. Loss of Righting Reflex Potency of Enantiomers of Secondary Alcohols in *Rana pipiens* Tadpoles at 20 ± 1° C

Alcohol	ED_{50} ± SE* (mM)	P†	Slope ± SE*	P†	n/T‡	Potency Ratio§
+2-butanol	17 ± 1.2	0.70	3.2 ± 0.59	0.73	3/130	1.00 ± 0.004
−2-butanol	17 ± 1.1		3.4 ± 0.52		3/160	
+2-pentanol	4.7 ± 0.28	0.92	3.7 ± 0.56	0.34	3/160	0.98 ± 0.081
−2-pentanol	4.8 ± 0.27		4.6 ± 0.87		2/110	
+2-hexanol	1.33 ± 0.068	0.40	4.4 ± 0.82	0.34	2/130	0.94 ± 0.074
−2-hexanol	1.42 ± 0.079		3.4 ± 0.64		3/150	
+2-heptanol	0.32 ± 0.011	0.73	5.4 ± 0.89	0.10	3/200	0.97 ± 0.069
−2-heptanol	0.33 ± 0.020		3.7 ± 0.75		2/120	
+2-octanol	0.063 ± 0.0042	0.69	3.1 ± 0.51	0.73	3/149	1.03 ± 0.086
−2-octanol	0.061 ± 0.0032		3.3 ± 0.45		4/220	
Range	0.061–17		3.1–5.4			0.94–1.03

* See "Materials and Methods" for curve-fitting procedure and estimation of standard errors.

† *P* = probability of a difference whose magnitude is at least as large as the actual difference, under the assumption that the true ED_{50} is the same for the two isomers.

‡ n = number of experiments; T = total number of animals in n experiments.

§ Potency ratio = ED_{50} (+)/ED_{50} (−)]. Errors propagated according to the method of Bevington.[38]

and the lipid–protein interface.[24] One pharmacologic strategy to elucidate more about the anesthetic site involves the use of pairs of optical isomers that are anesthetics.

Stereoisomers are compounds that have the same molecular formula and functional groups but differ in the arrangement of these groups around an asymmetric or "chiral" carbon atom. Stereoisomers whose structures are mirror images are called optical isomers or enantiomers.[25] Enantiomers have identical physical properties such as solubility and boiling point, whereas other stereoisomers (*e.g.*, geometric isomers and diastereomers) may differ in their physical properties.[25] Thus, with the latter isomer types, what appear to be stereoselective effects may actually be due to unequal concentrations in the appropriate biophase.

When stereoselective binding occurs, an enantiomer interacts at three points with a chiral center in its target molecule. Nonselectivity will be observed if the binding site does not have a chiral center, or if binding involves only two points of attachment. For example, the hydrocarbon core of a membrane's bilayer is achiral, and drug interactions with it are usually nonselective. Enantiomers of halothane are equipotent in their ability to disorder spin-labeled phosphatidylcholine bilayers[26]; similarly, enantiomers of a secondary alcohol disorder purified postsynaptic membranes equally (L. Firestone, K. Miller, unpublished data). The few reported instances of weakly stereoselective drug interactions with lipid bilayers have been associated with chiral centers in the lipid headgroups. Thus, adsorption of morphine to phosphatidylserine is stereoselective,[27] but ionic interactions between morphine and the charged phospholipid head groups make the major contribution to this interaction. Although enantiomers of delta[1]-tetrahydrocannabinol (THC) selectively disorder spin-labeled lecithin/cholesterol liposomes,[28] cholesterol must be present to express this effect. Cholesterol, which like THC has a rigid ring structure with asymmetric carbons, imparts sufficient chirality to the lipid bilayer to distinguish between THC isomers. If anesthetic enantiomers were to interact with the membrane bilayer, either at phospholipid head groups or "vicariously" through the chiral centers of cholesterol, then weak stereoselectivity would be anticipated.

Although the most likely candidate for the nonselective anesthetic site is the hydrocarbon core of the lipid bilayer, ligands can also interact with proteins nonselectively. One

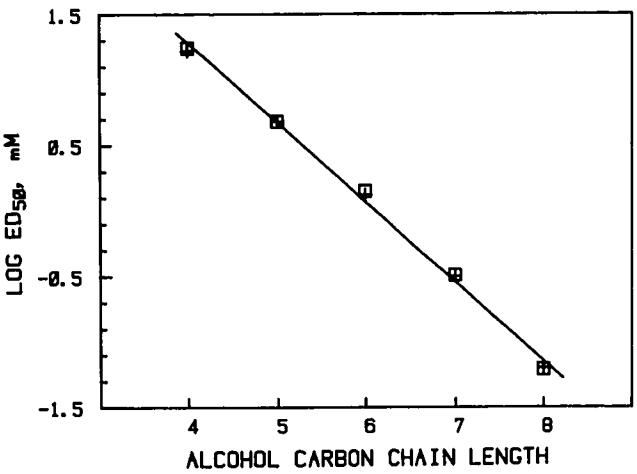

FIG. 2. Relationship between log ED_{50} and the number of carbons in the hydrocarbon chains of secondary alcohol enantiomers. (+) Enantiomers are represented by *crosses*. (−) Enantiomers are represented by *open squares*. The equation for the line is: y = [−0.60 ± 0.013 (SE)] x + [3.69 ± 0.078 (SE)]; r = −0.998.

such example is that the enantiomers of halothane induce a conformational change in hemoglobin equally.[29] But the relevance of anesthetic effects on soluble proteins has been criticized.[5] Perhaps a more pertinent example is a membrane-bound excitable protein like the nicotinic acetylcholine receptor from mammalian skeletal muscle. Here, the receptor-associated channel protein is nonselectively blocked by enantiomers of a toxin known to bind to it.[30] While our results do not preclude such nonselective anesthetic–protein interactions, they do suggest that the relevance of protein-based models of the anesthetic site, like firefly luciferase,[13,22] would be more convincing after a lack of stereoselectivity for alcohol enantiomers is demonstrated.

Potency ratios for enantiomeric pairs of anesthetic alcohols obtained in animals in our study were not significantly different from unit. This contrast with work in animals by other investigators. Potency ratios for the enantiomers of ketamine are approximately two in mice.[31] But stereoselectivity may have been only apparent because of stereoselective pharmacokinetic factors such as protein binding,[32] metabolism,[33] or even the production of active metabolites.[34] Differential metabolism occurs with the enantiomers of 2-butanol in the rat,[33] and such an effect could alter its apparent ED_{50}. This problem does not occur with tadpoles because of the large excess of drug in free solution. Because tadpoles are both small and aquatic, they rapidly equilibrate with drugs in aqueous solution. Thus, the concentration of drug in solution may be considered to be in equilibrium with that at the site of action *in vivo*.

It is particularly significant that enantiomers of 2-octanol do not exhibit stereoselectivity. This is because, when present, stereoselectivity is often a function of the potency of the ligand.[20,§] For example, horse liver alcohol dehydrogenase does not show stereoselectivity in the metabolism of enantiomers of 2-butanol, but the enzyme is stereoselective for 2-octanol, which has a higher apparent affinity.¶ Thus, if there were stereoselectivity in the anesthetic action of the homologous series of secondary alcohol enantiomers, it might not be detectable with compounds, such as 2-butanol, whose ED_{50} are in the millimolar range. However, in our study even the enantiomers of 2-octanol were equipotent. Because 2-octanol produced LRR at approximately 62 μM, which is on the same order as the affinities for many site-specific drugs, we can be more certain that weak stereoselective interactions were not overlooked. One example of a weak, yet stereoselective, interaction is binding of the enantiomers of N-methyl-5-phenyl-propyl-barbituric acid to the gamma-aminobutyric acid receptor-ionophore complex.[35] The potency ratio for these enantiomers is 6.6 even though their apparent dissociation constants (116 and 760 μM for the (−) and (+) enantiomers, respectively) exceed those of 2-octanol in our study (table 1). Although the longer chain secondary alcohols are even more potent (J. Alifimoff, L. Firestone, K. Miller, unpublished observations), these are not commercially available in resolved enantiomeric form.

Despite the theoretical opportunity that study of anesthetic enantiomers offers, the literature contains little data. Our results agree with those of Butler and Dickison,[36] who found that enantiomers of 2-butanol were equipotent in producing LRR in mice when administered intraperitoneally, but this is complicated by the possibility of differential metabolism as previously mentioned. Viditz** found (+) and (−) 2-butanol to be equipotent in tadpoles and fish, but concentrations were not monitored in that study. Both of these reports were limited to comparison of a single pair of enantiomers, rather than examination of a homologous series. Our work confirms these early experiments with 2-butanol and extends them with ultra-pure reagents and monitoring by gas chromatography to the enantiomers of 2-pentanol, 2-hexanol, 2-heptanol, and 2-octanol.

Our results for the chain length dependence of potency for the secondary alcohols in animals are close to those reported for the same compounds *in vitro*. In each of the few systems studied, including frog sciatic nerve,[37] cat stellate ganglion,†† and tortoise heart,†† only two alcohols were examined. Nonetheless, using the data available, we calculated that the slopes of log IC_{50} *versus* the number of carbon atoms in the alcohol ranged from −0.53 to −0.57. Our value of -0.60 ± 0.013 (fig. 2) is close to these, and thus for all these diverse assays, the potencies increase between three- and four-fold for each additional methylene group.

In summary, the potencies of a homologous series of anesthetic secondary alcohol enantiomers were investigated to determine the steric requirements of the anesthetic target. Our finding of lack of stereoselectivity indicates that interaction between anesthetics and their site(s) of action is nonselective and unconstrained by subtle spatial differences in anesthetic structure. Although our results with enantiomers cannot distinguish between a nonselective protein or lipid site, they do exclude the more specific types of anesthetic–protein interactions.

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