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Partitioning of long-chain alcohols into lipid bilayers: Implications for mechanisms of general anesthesia

(anesthetic target sites/cutoff effects/firefly luciferase)

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ABSTRACT **Alcohols act as anesthetics only up to a certain chain length, beyond which their biological activity disappears. Although the molecular nature of general anesthetic target sites remains unknown, presently available data support the hypothesis that this "cutoff" in anesthetic activity could be due to a corresponding cutoff in the absorption of long-chain alcohols into lipid-bilayer portions of nerve membranes. To test this hypothesis, we have developed an extremely sensitive biological assay, based on inhibition of the light-emitting firefly luciferase reaction, which is capable of measuring lipid-bilayer/buffer partition coefficients *K* for very lipid soluble compounds. Contrary to the hypothesis and reported data, we find a strictly linear increase in log(*K*) as the chain length increases [$\Delta(\Delta G^o)_{CH_2} = -3.63$ kJ/mol] for the primary alcohols from decanol to pentadecanol, with no hint of a cutoff. The fact that alcohols continue to partition into lipid bilayers long after their biological activity has ceased is consistent with the view that the primary target sites in general anesthesia are proteins rather than the lipid-bilayer portions of nerve membranes.**

For nearly a century it has been known that alcohols can act as general anesthetics (1, 2). As the chain length of an alcohol increases, so does its potency ($\equiv 1/ED_{50}$ concentration, where ED_{50} is the dose for 50% effect) as an anesthetic. Eventually, however, anesthetic potency begins to level off, and a point is soon reached beyond which anesthetic activity disappears (the so-called "cutoff effect"). For the primary alcohols, general anesthetic potency levels off after about undecanol (C_{11}) and completely disappears after tridecanol (C_{13}) (3, 4). Thus, while 1-dodecanol (C_{12}) is among the most potent of all the alcohols, 1-tetradecanol (C_{14}) is completely inactive. Although such behavior is consistent with protein mechanisms of general anesthesia (5), it presents a real problem for lipid theories. It has, however, been argued (4, 6, 7) that the observed cutoff in anesthetic potency is due to a corresponding cutoff in the ability of long-chain *n*-alcohols to partition into lipid bilayer portions of nerve membranes.

Presently available data on the partitioning of radiolabeled long-chain alcohols into biological membranes (8) and lipid bilayers (6) do in fact support the hypothesis (4, 6, 7) that the higher *n*-alcohols are inactive simply because they cannot attain high enough concentrations in lipid bilayers. However, labeling techniques are notoriously unreliable when measuring large membrane/buffer partition coefficients, since the presence of very small amounts of water-soluble impurities can produce severe underestimates. We have now reinvestigated this problem by developing a technique, based on the inhibition of firefly luciferase light output by anesthetics (5, 9) in the presence of membranes, which can reliably measure membrane/buffer partition coefficients having values up to at least 10 million (when expressed as ratios of molar concentrations).

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PRINCIPLE OF THE METHOD

The most important aspect of our method is the use of a light-emitting enzyme, sensitive to a wide range of simple organic compounds, to report on the very low aqueous concentrations of very lipophilic solutes, either in pure buffer or at equilibrium within a membrane suspension. By measuring inhibition of the light-emitting reaction by the solute, with and without membranes present, the membrane/buffer partition coefficient can be obtained knowing only (*i*) the ratio of membrane to buffer volumes and (*ii*) the ratio of the total amounts of solute added in the presence and in the absence of membranes.

We proceed to derive the relevant equations in a rather general form, with a view to the possible future use of the method with other enzymes. As long as the inhibition is linear (in the sense that linear Dixon plots are observed), the equations are independent of the nature of the inhibition (e.g., competitive, noncompetitive, etc.). For linear inhibition, the ratio *R* of enzyme activities in the absence (v_o) and in the presence (v) of inhibiting solute can be expressed as:

$$R \equiv \frac{v_0}{v} = (\text{constant})\, c_{buf} + 1, \qquad [1]$$

where c_{buf} is the concentration of inhibitory solute in the buffer, and the constant factor takes on a variety of forms, depending on the nature of the inhibition. (The constant factor may itself be a function of substrate levels, but in the measurements described here the substrate concentrations are fixed.)

Thus the ratio between the solute buffer concentrations in the presence (c_{buf}) and absence (c'_{buf}) of membranes is related to the corresponding enzyme activity ratios *R* and *R'*, respectively, by

$$\frac{c_{buf}}{c'_{buf}} = \frac{R-1}{R'-1}. \qquad [2]$$

(This assumes that the sensitivity of the enzyme to inhibition is not affected by the membranes.) But this ratio can also be obtained using

$$c_{buf} = \frac{n}{V_{buf} + K V_{mem}} \qquad [3]$$

and

$$c'_{buf} = \frac{n'}{V_{buf}}, \qquad [4]$$

where *n* and *n'* are the total amounts of solute added in the presence and absence of membranes, respectively, V_{buf} is the volume of buffer in both the presence and absence of membranes, V_{mem} is the volume of membranes, and *K* is the

membrane/buffer partition coefficient expressed as a ratio of molar concentrations (c_{mem}/c_{buf}).

Combining Eqs. 2–4 and rearranging gives

$$K = \frac{V_{buf}}{V_{mem}}\left[\frac{n(R' - 1)}{n'(R - 1)} - 1\right]. \quad [5]$$

Thus, knowing the ratio of buffer to membrane volumes, K can be determined by measuring the fractional inhibition (R^{-1}) in the presence of membranes, when n moles of solute inhibitor are added, and the fractional inhibition (R'^{-1}) in the absence of membranes, when n' moles of solute are added.

In practice, however, the most accurate procedure is to adjust the ratios n/n' and V_{buf}/V_{mem} until the observed inhibition is the same with and without membranes present. Under this "null" condition, $R = R'$ and Eq. 5 reduces to the very simple form

$$K = \frac{V_{buf}}{V_{mem}}\left[\frac{n}{n'} - 1\right]. \quad [6]$$

In addition to increased accuracy, the null procedure has the advantage that Eq. 6 holds for all types of inhibition, linear or otherwise.

MATERIALS AND METHODS

D-Luciferin, desiccated firefly lanterns, ATP (grade I), L-α-phosphatidylcholine (type V-E from egg yolks), dipalmitoyl-L-α-phosphatidic acid, cholesterol (chromatography grade), 1-undecanol, 1-tridecanol, 1-tetradecanol, and hexadecane were obtained from Sigma. Glycylglycine, 1-heptanol, 1-decanol, and 1-dodecanol were purchased from BDH. 1-Pentadecanol was obtained from Aldrich Chemical. All reagents were of the highest purity available and were used without further purification. Glass reaction vials (Spectravial 2) were obtained from BDH and acid-washed before use.

Pure crystals of the enzyme firefly luciferase were obtained from lanterns of the North American firefly *Photinus pyralis* using the affinity chromatography procedure of Branchini *et al.* (10). A stock solution of lipid bilayers (1.23 wt %) was prepared as follows. Egg lecithin, cholesterol, and phosphatidic acid were weighed out in mole ratios of 55:40:5 (assuming a molecular weight of 770 g/mol for egg lecithin) and dissolved in chloroform/methanol. This lipid solution was evaporated to dryness overnight under a vacuum. The lipid was then resuspended in buffer, with brief (about 2 min) sonication to aid dispersal. This stock solution was diluted as required. Lipid volumes were calculated assuming (11) a density of 1.014 g/cm^3.

Luciferase assays were performed by rapidly injecting (with an air-driven glass syringe) 2.5 ml of a buffered 10 mM ATP solution into a glass reaction vial holding 10–10.8 ml of a buffered solution containing firefly luciferase, luciferin, and 8.3 mM $MgSO_4$, with or without lipid membranes or the n-alcohol. The final concentration of luciferin was close to the K_m of the uninhibited enzyme (14 μM) and that of the luciferase enzyme was about 1 nM. The buffer was 25 mM glycylglycine, titrated to pH 7.8 with NaOH. The light output from the luciferase-catalyzed reaction was detected with a photomultiplier tube, and the signal was then amplified and stored on a digital oscilloscope. The enzyme activity was taken as the peak of the light output (9).

Typically, sufficient n-alcohol was added, in the absence of membranes, to reduce the control activity to about one-half ($R' \approx 2$). Next, using 5–10 times this amount of n-alcohol, increasing quantities of a lipid membrane suspension were then added until the activity was reduced to about one-half ($R \approx 2$) of the control value in the presence of the same amount of lipid. (In practice, the n-alcohol was always added after the lipid.) Having thus determined a pair of ratios V_{buf}/V_{mem} and n/n', which approximated the "null" condition, we then made multiple (five) determinations of R and R' and calculated the membrane/buffer partition coefficient K using Eq. 5.

The n-alcohols were added as ethanolic solutions. The final concentration of ethanol never exceeded 4% of its inhibition constant for luciferase; nonetheless, the same amount of ethanol was added to the controls. To check for possible adsorption of the n-alcohols onto the walls of the glass vials, we measured luciferase activities as a function of time; we could detect no loss of n-alcohol from solution, when left to stand in our acid-washed glass reaction vials for at least 30 min. We also considered the possibility that the free aqueous concentration of luciferin (a relatively hydrophobic molecule) might have been reduced in the presence of lipids, due to absorption into bilayers. However, spectrophotometric analysis of free luciferin concentrations showed that, even at the highest lipid concentrations used, any such depletion of luciferin was less than 2%.

We did find, however, that uptake of n-alcohol by the lipid membranes was not instantaneous; 15 min were sometimes required to attain complete equilibrium. The lipids were, therefore, left to equilibrate with the n-alcohol for at least this time before the luciferase enzyme was added and its activity assayed; controls were treated identically. In our calculations, we have assumed that, during the brief (<0.5 sec to peak light output) assay of luciferase activity, there was insufficient time for the n-alcohol in the lipid membranes to re-equilibrate, so we have taken V_{buf} in Eqs. 5 and 6 to be the value before injection of the ATP solution. We have also assumed that the sensitivity of the enzyme to inhibition was not directly affected by the membranes. Although this assumption could not be tested for the higher alcohols used, some experiments with more polar inhibitors suggested that the presence of the lipids might have decreased sensitivity slightly. If this were also true for the higher alcohols, then our calculated values of K would have been systematically overestimated. On the other hand, if the first assumption were incorrect, our calculated values of K would have been systematically underestimated (by up to 25%).

RESULTS

The basic idea behind the method can perhaps best be illustrated by the experiment shown in Fig. 1. Here, each trace gives the time course of light intensity observed after the rapid injection of ATP. The bottom trace shows that 5.8 μM 1-undecanol reduces the peak intensity to 14% of the control value. The middle trace shows that, in the presence of the same total amount of 1-undecanol, the addition of only 3.6 μl of lipid membranes restores activity to 52% of the control value. The free aqueous concentration of 1-undecanol, which is being monitored by the luciferase reaction, has obviously been reduced because the alcohol has partitioned into the lipid membranes. From these data and a knowledge of the buffer volume (10.3 ml), the partition coefficient of 1-undecanol between membranes and buffer could have been obtained directly from Eq. 5 as K = 16,200.

In practice, we chose to add less alcohol in the absence than in the presence of membranes and to so adjust the amount of lipid added that comparable luciferase activities (usually about 50% of controls) were observed in both cases. This "null" procedure made the measurement of K relatively independent of possible nonlinearities in both the enzyme inhibition pattern and the light detection system.

As a test of the method, we determined the partition coefficients of two n-alcohols (heptanol and undecanol) between n-hexadecane and buffer. We obtained values (± SE

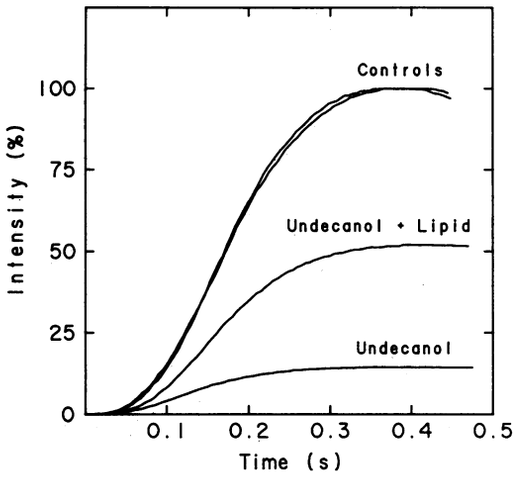

FIG. 1. Light output from the luciferase-catalyzed reaction after the injection of ATP. Lower trace, 74 nmol of 1-undecanol in the absence of membranes. Middle trace, 74 nmol of 1-undecanol in the presence of 3.6 μl of lipid membranes. Upper traces, controls with and without membranes, normalized to 100% peak intensity. The control signal in the presence of membranes was slightly higher than in the absence of membranes, due to light scattering increasing the total amount of light collected. ATP buffer (2.5 ml) was injected into 10.3 ml of a buffered solution containing luciferase, luciferin, and $MgSO_4$, with or without the membranes or 1-undecanol.

for five determinations) of $K = 7.6 \pm 0.5$ for 1-heptanol and $K = 2000 \pm 400$ for 1-undecanol. These results are plotted in Fig. 2, together with literature values obtained using a variety of other techniques. It is clear that our new method yields values consistent with these earlier measurements. In addition, although not exactly comparable, our values are also in excellent agreement with the measurements of Gilbert *et al.* (14) using closely related *n*-alkane/water systems. The slope

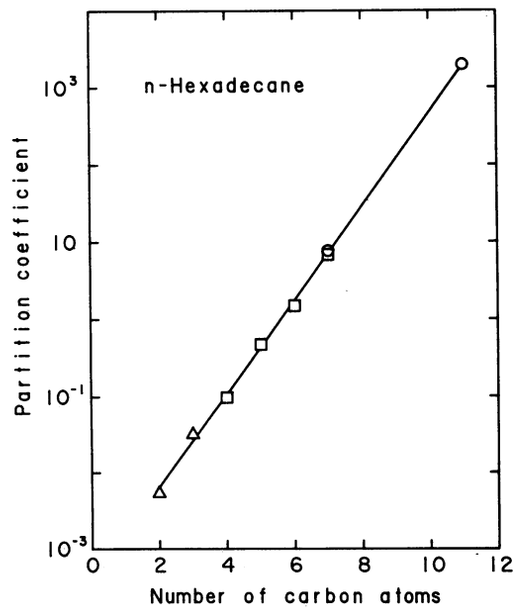

FIG. 2. Partition coefficients of *n*-alcohols between *n*-hexadecane and water. The values are expressed as ratios of molar concentrations. Data were derived from the following sources: ○, this paper; □, Aveyard and Mitchell (12); △, Franks and Lieb (13). The data of Aveyard and Mitchell (12) at 20°C have been corrected to the temperature of our determinations (23.5°C) using their average ΔH° of 30 kJ/mol. The slope of the regression line gives (mean ± SE) $\Delta(\Delta G^\circ)_{CH_2} = -3.46 \pm 0.05$ kJ/mol ($= -826 \pm 11$ cal/mol).

of the least squares line fitted to the data in Fig. 2 gives a value (± SE) for the incremental free energy of transfer (from buffer to *n*-hexadecane) for a methylene group of $\Delta(\Delta G^\circ)_{CH_2} = -3.46 \pm 0.05$ kJ/mol ($= -826 \pm 11$ cal/mol). This value is almost identical to that obtained by Smith and Tanford (15, 16) for the partitioning of *n*-carboxylic acids between heptane and buffer.

We went on to determine partition coefficients for the *n*-alcohols from decanol to pentadecanol between lipid bilayer membranes and buffer. The results are tabulated in Table 1 and plotted in Fig. 3. It is clear from Fig. 3 that, when the logarithm of the partition coefficient is plotted against the number of carbon atoms in the alcohol, the data points fall very accurately onto a straight line. From the slope of this line, the value (± SE) of the incremental free energy of transfer (from buffer to membranes) for a methylene group is $\Delta(\Delta G^\circ)_{CH_2} = -3.63 \pm 0.1$ kJ/mol ($= -867 \pm 24$ cal/mol).

DISCUSSION

Determination of Partition Coefficients. Our method makes it possible to obtain reliable values of membrane/water partition coefficients for compounds that are extremely lipid soluble. (In this paper, we have extended the range of *n*-alcohol values by over three orders of magnitude.) The general applicability of the method lies in the use of an enzyme (firefly luciferase) that is extremely sensitive to inhibition by a wide range of simple organic compounds (5, 9).

Because the enzyme reports on only the free aqueous concentration of the test compound (which is in equilibrium in a suspension of membranes), the method sidesteps one of the major problems with conventional methods, that of having to obtain a sample of aqueous buffer completely free of membranes. Failure to obtain a complete separation can cause considerable error. For example, if, after centrifuging a 1% membrane suspension, only 1% of the membranes remain as a contaminant in the supernatant, then the partition coefficient of a compound with a true $K = 10^6$ will be underestimated by two orders of magnitude.

Our method also avoids a particular problem that arises with radioactive tracer techniques. Here, the presence of minute levels of water-soluble impurities can again lead to severe underestimates of K. However, because inhibition of firefly luciferase increases in proportion to lipid solubility (9), the enzyme assay positively discriminates against the effects of water-soluble impurities. Consider, for example, a radio-

Table 1. Partition coefficients between lipid bilayers and buffer

n-Alcohol	Partition coefficient	V_{mem}	c_{buf}/c_{sat}
Decanol	$(3.9 \pm 0.6) \times 10^3$	9.8 μl	0.024
Undecanol	$(1.7 \pm 0.1) \times 10^4$	2.4 μl	0.017
Dodecanol	$(6.9 \pm 0.5) \times 10^4$	1.2 μl	0.032
Tridecanol	$(3.1 \pm 0.2) \times 10^5$	310 nl	0.055
Tetradecanol	$(1.4 \pm 0.2) \times 10^6$	66 nl	0.15
Pentadecanol	$(5.6 \pm 0.8) \times 10^6$	9.0 nl	0.25

Partition coefficients are expressed as ratios of molar concentrations. The errors are the random standard errors for five determinations. The lipid bilayers contained egg lecithin, cholesterol, and phosphatidic acid in molar ratios of 55:40:5, respectively. The membrane volumes have been corrected for the volume of *n*-alcohol in the membranes under the conditions of the determinations, using the correction factor $(1 + K_0 c_{buf} V_{alc})$, where K_0 is the partition coefficient calculated using Eq. 5 or 6 and the original lipid volume, c_{buf} is the free buffer concentration of the *n*-alcohol, and V_{alc} is the molar volume of the *n*-alcohol. This correction was ≤1% except for tetradecanol (9%) and pentadecanol (18%). The last column gives the fractional saturation of the buffer during the determination, using c_{sat} values calculated according to Bell (17) (see legend to Fig. 4). All measurements were made at 25°C.

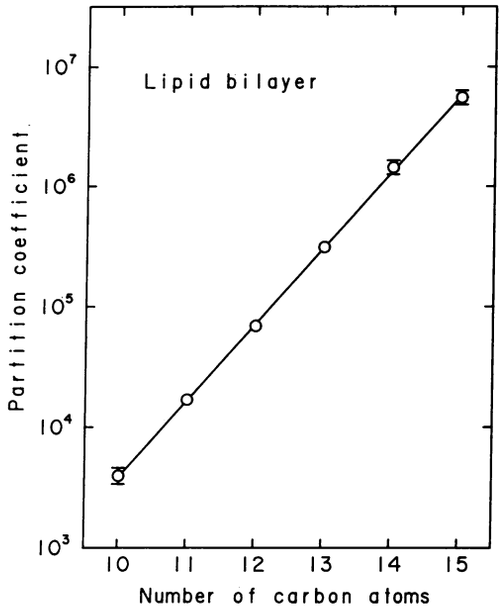

FIG. 3. Partition coefficients of higher *n*-alcohols between lipid bilayer membranes and buffer at 25°C. The data are listed in Table 1. The error bars give the random standard errors for five determinations; where no error bars are shown, the standard error was less than the size of the symbol. The slope of the regression line gives (mean ± random SE) $\Delta(\Delta G^o)_{CH_2} = -3.63 \pm 0.03$ kJ/mol (= − 867 ± 24 cal/mol). In view of the systematic uncertainties discussed, the error in $\Delta(\Delta G^o)_{CH_2}$ could be as large as ± 0.1 kJ/mol.

labeled compound with a true $K = 10^6$ that is contaminated with only 0.1% of a water-soluble impurity with $K = 1$. Centrifuging a 1% membrane suspension and counting the supernatant would result in K being underestimated by an order of magnitude (even if complete membrane separation could be achieved). With our luciferase method, on the other hand, the effect on K would be less than 0.1%.

Comparison with Previous Results on Lipid Bilayers. As a simple model for the lipid bilayer portions of nerve cell membranes, we chose to use negatively charged bilayers containing a high concentration of cholesterol. It has also been suggested (18) on other grounds that cholesterol and negatively charged lipids are necessary ingredients of "good" lipid models for the primary target sites in general anesthesia. Unfortunately, most previous determinations of *n*-alcohol partition coefficients have been for cholesterol-free lipid bilayers, and all of this work has been restricted to the lower *n*-alcohols. The most reliable and extensive data have been obtained by Hill (19, 20) and by Kamaya *et al.* (21) for fluid bilayers of dipalmitoyl lecithin. If we extrapolate their data from ethanol to the highest *n*-alcohols used (octanol and heptanol, respectively), we obtain an average partition coefficient for 1-decanol that is about twice the value for our cholesterol containing membranes.

Satisfactory agreement is also obtained when the slopes of the various data sets are compared. For dipalmitoyl lecithin, the slopes give $\Delta(\Delta G^o)_{CH_2} = -3.70$ kJ/mol and − 3.75 kJ/mol (using data from ethanol upwards in Table 1 of ref. 20 and Table 2 of ref. 21, respectively) with a mean value of − 3.73 kJ/mol. If corrected to the temperature (25°C) of our determinations, this becomes $\Delta(\Delta G^o)_{CH_2} = -3.62$ kJ/mol [using as a simple approximation (22) a constant $\Delta(\Delta H^o)_{CH_2} = -1.67$ kJ/mol]. This value is also in excellent agreement with our observed $\Delta(\Delta G^o)_{CH_2} = -3.63$ kJ/mol. Furthermore, these numbers are very similar to our value of $\Delta(\Delta G^o)_{CH_2} = -3.46$ kJ/mol for partitioning into *n*-hexadecane from water, showing that the binding environment for the hydrocarbon chains of *n*-alcohols in lipid bilayer membranes is very apolar even for alcohols containing as many as 15 carbon atoms.

Implications for General Anesthesia. The most important finding of this work is that, with increasing chain length, *n*-alcohols continue to partition into lipid bilayers long after they have ceased to be general anesthetics. Indeed, general anesthetic potencies (3, 4) level off after about C_{11} and disappear completely by C_{14}, whereas partitioning into lipid bilayers (see Fig. 3) is strictly linear from C_{10} to C_{15}, with no hint of a cutoff. Whether a cutoff will eventually appear is, of course, unknown, but the elegant experiments of Requena and Haydon (23) on the adsorption of *n*-alkyltrimethylammonium ions by lipid bilayers suggest that partitioning continues to increase up to at least C_{18}. Our results with the *n*-alcohols constitute a direct disproof of the suggestion (4, 6, 7) that the observed cutoff in anesthetic potency for the *n*-alcohols is due to a corresponding cutoff in partitioning into lipid bilayers.

Specifically, Miller and his colleagues (4, 6, 7) have argued that the higher *n*-alcohols are inactive simply because their maximum possible membrane concentrations fall well below the membrane concentrations (about 10–30 mM) achieved by the lower *n*-alcohols during general anesthesia. This suggestion can be tested using the membrane/water partition coefficients listed in Table 1. In Fig. 4, we have plotted the membrane concentrations attained by the higher *n*-alcohols at only 1/50th of their aqueous solubilities. [This fraction of a saturated solution of the lower *n*-alcohols is sufficient to cause general anesthesia (4, 24).] It is quite clear that the postulated threshold concentration (about 10–30 mM) is easily achieved by all of these higher *n*-alcohols, even when present at well below their saturated aqueous concentrations, yet even a saturated solution of 1-tetradecanol is completely inactive. In view of our results, it now seems likely that the published (6, 8) membrane/water partition coefficients for higher *n*-alcohols, used by Miller and his colleagues (4, 6, 7) in their calculations, are seriously in error.

We (13, 25, 26) have argued against lipid theories of general anesthesia mainly on quantitative grounds: the changes in the structure and properties of lipid bilayers caused by general anesthetic (ED_{50}) concentrations are usually barely detectable and can be mimicked by changes in temperature of only about 1°C. The data presented in this paper now provide an

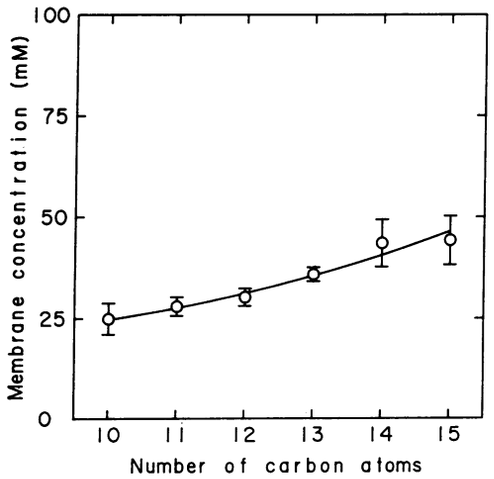

FIG. 4. Concentrations of the higher *n*-alcohols in lipid bilayer membranes when the free buffer concentration is only 2% of saturation. The values were calculated by multiplying the partition coefficients listed in Table 1 by 2% of the saturated aqueous concentrations c_{sat}, where $\log_{10} c_{sat} = -0.58\, N_C + 2.3$ when c_{sat} is expressed in mol/liter and N_C is the number of carbon atoms in the *n*-alcohol (17).

additional argument against these theories. Compare, for example, 1-dodecanol, which is a potent anesthetic, with 1-tetradecanol, which is completely inactive. The inactive tetradecanol can achieve, if anything, even higher membrane concentrations than dodecanol (see Fig. 4). Furthermore, when present at equal membrane concentrations, their effects on membrane properties are very similar (4). The general anesthetic properties of the *n*-alcohols can be accounted for in terms of binding to protein target sites of circumscribed dimensions: the leveling off in potency occurs when the site becomes full, and the cutoff point occurs when the concentration required for anesthetic activity falls below the aqueous solubility (5). Whether or not ingenious extensions of current lipid theories can be made that also account for the available potency data remains to be seen.

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