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Partial Molar Volumes of Some 1-Alkanols in Erythrocyte Ghosts and Lipid Bilayers†

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ABSTRACT: The partial molar volumes of 1-heptanol and 1-octanol in red cell ghosts, in egg phosphatidylcholine bilayers, and in water and phosphate buffer have been measured to a precision of better than 4% by using a density meter. In every case, the partial molar volume was independent of concentration in the range studied. In both membranes, the partial molar volume of each alcohol was close to its molar volume, whereas in aqueous solution it was considerably less. Comparison of the two membranes suggests that the major contribution to the partial molar volume arises from alcohol–lipid interactions in each case. Further comparison with partial molar volumes in bulk solvents suggests that on average the alcohols retain a hydrogen bond in the lipid bilayer. The magnitude of the volume change in ghosts is some 5 times less than the corresponding area changes previously reported by others [Roth, S. H., & Seeman, P. (1972) *Biochim. Biophys. Acta 255*, 190–198]. These two observations can only be resolved by assuming either that the bilayer expands anisotropically, experiencing a decrease in thickness with increasing volume, or that conformational changes in membrane-associated proteins can occur at constant volume to increase membrane area. Finally, these data are used to test the critical volume hypothesis of general anesthetic action. A volume change of 0.15% in red cell ghost membranes is found to be associated with anesthesia, which compares with a value of 0.2% predicted previously from pressure reversal of anesthesia studies. In egg phosphatidylcholine bilayers, a volume change of 0.36% is associated with anesthesia. The larger change in the lipid bilayer compared to the biomembrane originates solely in their different membrane/buffer partition coefficients.

Characterization of the thermodynamics of small hydrophobic molecules interacting with membranes may provide a basis for understanding the interactions between complex endogenous components of those membranes. Furthermore, small molecules may serve as structural perturbers, thus revealing information about membrane organization. In this paper, we have determined the volume changes which occur when alcohols interact with red cell ghost membranes.

Alcohols exert a wide variety of effects on membranes including effects on permeability (Kutchai et al., 1980a), transport (Sullivan et al., 1974; Kutchai et al., 1980b; Hara & Kasai, 1977), fluidity (Roth & Spero, 1976; Pringle & Miller, 1978; Zavoica & Kutchai, 1980), and excitability (Seeman, 1972; Swenson & Oxford, 1980). The anesthetic action of the alcohols has also been given considerable attention. Seeman and co-workers in particular have developed the idea that the action of alcohols may be mediated by membrane expansion, and this hypothesis is supported by the observation that anesthesia is reversed by pressure (Miller et al., 1973). Various attempts have been made to test this hypothesis by measuring the increase in surface area of erythrocytes exposed to alcohols (Roth & Seeman, 1972; Seeman, 1969; Seeman et al., 1969a,b) with the general conclusion that the area increase exceeds that calculated from the partition coefficient and molecular volume of the alcohols. One explanation for this would be that the alcohols were responsible, either directly or indirectly via lipid perturbations, for conformational changes in proteins which in turn result in a large area increase (Seeman, 1972, 1974). An alternative explanation suggested by several authors (Miller et al., 1973; Trudell, 1977) is that alcohols induce an anisotropic expansion of the membrane, such that its thickness decreases while its

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0006-2960/82/0421-2840$01.25/0

area increases. The protein conformational change explanation of Seeman (1972) was apparently confirmed by a density meter study which showed that ethanol expanded erythrocyte ghost and synaptic membranes more than lipid bilayers. However, this study was somewhat preliminary in nature, and it may be criticized on the grounds that the high aqueous concentrations of ethanol employed would have affected cytoplasmic proteins directly. A recent preliminary report challenges the earlier density measurements (Franks & Lieb, 1981). Furthermore, ethanol's partition coefficient in these membranes is not known, but studies in other membranes suggest it is less than one (Seeman, 1972; Katz & Diamond, 1974); consequently, the volume changes within the membrane would be difficult to deconvolute from the larger changes contributed by ethanol in the aqueous phase. Studies of more hydrophobic alcohols of known partition coefficient should allow the evaluation of the partial molar volume of the alcohol in the membrane and consequently resolve this uncertainty.

Excess volume dilatometry has recently been used by us to measure the partial molar volumes of small molecules in lipid bilayers (Kita et al., 1981). This method has the great advantage that the two components are contained separately in the dilatometer and are mixed within it, thus avoiding many sources of error, as previouly discussed (Kita et al., 1981). However, such dilatometers have one serious disadvantage in work with biomembranes, and that is the presence of mercury. This metal can be expected to react with disulfide bonds and sulfhydryl groups (Gurd & Wilcox, 1956). Indeed, preliminary experiments indicated that when acetylcholine receptor rich membranes from *Torpedo californica* (Sauter et al., 1980) were exposed to mercury, half the acetylcholine binding sites were lost within 24 h. To avoid this problem, we opted to use an all-glass density meter and to measure the density of a series of samples to which anesthetics were added. Because of the extra manipulations involved (for example, weighing and transfer to the density meter), this method is particularly inferior to the excess volume dilatometer when volatile agents are involved. We have thus ignored the typical volatile anesthetics in favor of the less volatile medium chain length alcohols. In the normal alkanol series, the membrane buffer partition coefficient increases (Seeman et al., 1971), but their aqueous solubility limit decreases (Bell, 1973) with chain length. Thus, the proportion of the measured volume change originating from the membrane increases, but the maximum volume change obtainable decreases, with increasing chain length. Hence, we have chosen heptanol and octanol for this investigation because they represent a good compromise between high partition coefficient and limited solubility. Our data show that the partial molar volume of each of these alcohols is close to its molar volume in both lipid bilayers and red cell ghosts. We thus find no evidence for an anomalous volume increase in biomembranes, and our data are consistent with, but do not prove, the anisotropic membrane expansion hypothesis.

Materials and Methods

Hemoglobin-free erythrocyte ghosts were prepared by the method of Dodge et al. (1963) from recently outdated human bank blood kindly supplied by J. W. Darnell. After the final wash, the ghosts were resuspended in 10 mM sodium phosphate buffer (pH 7.0). Lipid bilayer suspensions were prepared in the same buffer by sonication as described previously (Kita et al., 1981). Egg phosphatidylcholine (EPC)[1] was purchased from Lipid Products, Surrey, United Kingdom, and used without further purification. Cholesterol (Sigma) was twice recrystallized from methanol before use. The final concentrations of lipid and ghost suspensions were determined by dry weight after heating at 90 °C for 2 days and were corrected for buffer salts.

Density measurements were made by comparison with standards using a Metler-Paar DMA 601 (Graz, Austria) density measuring cell. This cell compares the natural frequency of a hollow glass U-shaped oscillator when filled with liquids of different density. The instrument has been described elsewhere (Kratky et al., 1969). Since density changes of the order of 10^{-6} g mL^{-1} should be detectable, and the thermal expansion coefficient of many liquids is in the range of 10^{-3}–10^{-4}/°C, good thermostating was mandatory. Water at 25.0 °C was circulated through the jacket of the density cell at 23 L min^{-1} from a water bath whose temperature was controlled to better than the sensitivity of our Beckman thermometer (about 2 mdeg) by a very high precision proportional temperature controller (PTC-40, Tronac Inc., Orem, UT). The latter bath was set within another thermostated water bath as previously described (Kita et al., 1981). The density meter was operated with the U vertical, rather than in the customary horizontal mode, so as to facilitate the egress of microbubbles when these occurred. Controls showed that density measurements were unaffected by the orientation of the cell.

Density standards were obtained by making up standard solutions of sodium chloride by weight. The densities of these solutions were obtained from the International Critical Tables (1926).

Weighed quantities of alcohols were added to samples containing aqueous solutions or membrane suspensions in glass bottles with ground glass or Teflon stoppers. Corrections were made for displaced air. Loss of alcohols was checked for by repetitive sampling. Mixing of the alcohols with lipid vesicles was achieved by rotating the bottles overnight. To avoid aggregation in samples containing red cell ghosts, we achieved mixing by rolling the tubes gently from time to time. Equilibration took 2–10 h depending on the alcohol and its concentration.

Method of Analysis

Partial Molar Volumes. If we consider that a small amount of anesthetic is added to the solvent water (buffer solution), the total volume between the unmixed (simple summation of the volumes of solute and solvent) and the mixed system has changed by

$$\Delta V = V_2 - V_1 \tag{1}$$

where V_1 and V_2 are the total volumes of the unmixed and mixed systems, respectively, and

$$V_1 = V_H + V_A = \frac{W_H}{d_H} + \frac{W_A}{d_A} \tag{2}$$

where V, W, and d are the volume, weight, and density of each sample, respectively. Subscripts H and A refer to water and alcohol, respectively. V_2 is obtained from the measured density, d_{AH}, of the sample after mixing completely since

$$V_2 = \frac{W_H + W_A}{d_{AH}} \tag{3}$$

The volume change, ΔV, is given from eq 1–3 as

$$\Delta V = \frac{W_H + W_A}{d_{AH}} - \left(\frac{W_H}{d_H} + \frac{W_A}{d_A}\right) \tag{4}$$

[1] Abbreviation: EPC, egg phosphatidylcholine.

The apparent excess molar volume, V_H^E, of anesthetic in water is defined as

$$V_H^E = \frac{\Delta V}{\Delta n_H} \quad (5)$$

where Δn_H is the moles of alcohol added to water. The volume change in the membrane suspension, ΔV_S, is obtained similarly as

$$\Delta V_S = \frac{W_S + W_A}{d_{AS}} - \left(\frac{W_S}{d_S} + \frac{W_A}{d_A}\right) \quad (6)$$

where d_{AS} is the density of the suspension after mixing with the alcohol and subscript S refers to the membrane suspension. ΔV_t is defined as the sum of the volume changes in the aqueous and membrane phases:

$$\Delta V_t = \Delta n_H V_H^E + \Delta n_M V_M^E \quad (7)$$

where Δn and V^E are the moles of alcohol and the apparent excess molar volume of alcohol in the respective phases defined by the subscripts. The total number of moles of alcohol in the membrane suspension, Δn_t, will be distributed between water and membrane with a membrane to water partition coefficient, $\lambda_{M/H}$, given by

$$\lambda_{M/H} = \frac{\Delta n_M}{W_M}\frac{V_H}{\Delta n_H} \quad (8)$$

and

$$\Delta n_t = \Delta n_H + \Delta n_M \quad (9)$$

where W_M is the weight of the membrane in the suspension. Rearranging eq 7 and combining with eq 8 and 9 yield

$$V_M^E = \frac{\Delta V_t}{\Delta n_t}(1 + A) - AV_M^E \quad (10)$$

where

$$A = \frac{V_H}{W_M}\frac{1}{\lambda_{M/H}} \quad (11)$$

Thus, V_M^E may be evaluated since V_H^E has been obtained from control experiments, $\lambda_{M/H}$ is known from the literature (Colley & Metcalfe, 1972; Jain et al., 1978; Seeman et al., 1971), and W_M/V_H is the measured membrane concentration in the suspension.

Density of Membranes. The control membrane volume is needed to determine the relative membrane expansion caused by the alcohols. The membrane density, d_M, is given by

$$d_M = \frac{W_m}{V_m} \quad (12)$$

and can be evaluated from measurements of the buffer's density, d_B, the membrane suspension's density, d_S, and the fractional dry weight of solids in the membrane suspension, w. These quantities are defined respectively as

$$d_B = \frac{W_B}{V_B} \quad (13)$$

$$d_S = \frac{W_M + W_B}{V_M + V_B} \quad (14)$$

and

$$w = \frac{W_p + W_M}{W_M + W_B} \quad (15)$$

where W and V are weight and volume, respectively, and the subscripts B, M, p, and S refer to buffer, membrane, sodium phosphate, and membrane suspension, respectively. The weight of sodium phosphate in buffer is a constant, a, where

$$a = W_p/W_B \quad (16)$$

Substituting eq 13–16 into eq 12 and rearranging yield

$$d_M = \frac{d_S d_B}{d_B - b(d_S - d_B)} \quad (17)$$

where

$$b = (w - 1)/(a - w) \quad (18)$$

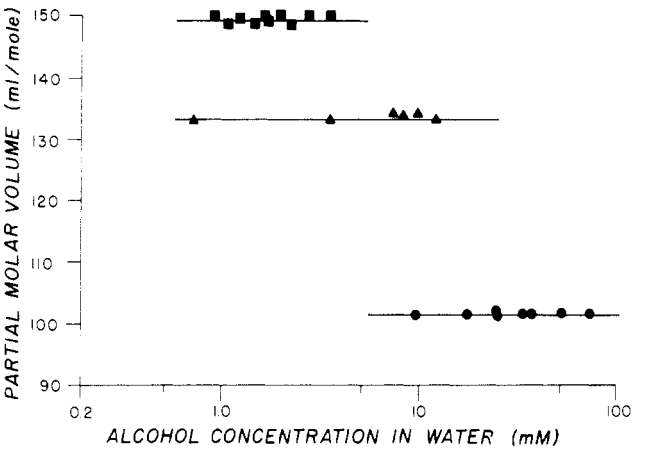

FIGURE 1: Partial molar volume of the alcohols in water at 25 °C vs. their concentration: (●) benzyl alcohol; (▲) 1-heptanol; (■) 1-octanol.

Results

Partial Molar Volumes in Water. We first determined the partial molar volume of benzyl alcohol in both water and lipid bilayers in order to compare the present method with our previous work which used excess volume dilatometry (Kita et al., 1981). The excess molar volume of benzyl alcohol in water V_H^E, was obtained from eq 5. The mean value of 28 runs (±standard deviation) was −2.2 ± 0.12 mL/mol. The molar volume, V^0, of benzyl alcohol at 25 °C is 103.83 mL/mol, and the partial molar volume of benzyl alcohol in water, $\bar{V}_H$, is thus 101.6 ± 0.12 mL/mol ($\bar{V}_H = V^0 + V_H^E$). This value may be compared to our previous value of 101.07 ± 0.067 mL/mol (Kita et al., 1981). Both our determinations compare well to the only other available literature values of 101.2 mL/mol interpolated at 25 °C from data obtained by using a pycnometer (Friedman & Sheraga, 1965).

Figure 1 shows a plot of partial molar volume vs. concentration of benzyl alcohol in water. The intercept obtained by least-squares fitting of $\bar{V}_H$ against concentration was 101.57 ± 0.044 mL/mol. This is the partial molar volume at infinite dilution, $\bar{V}^\infty$. The slope of the plot was not significantly different from zero in this range of concentration. Thus, the partial molar volume is independent of benzyl alcohol concentration within the precision of our data.

Figure 1 also shows our data for heptanol and octanol in water. The data are tabulated in Table I. The partial molar volumes at infinite dilution in water are in good agreement with the values of Vikingstad (1979) which were 134.6 and 150.5 mL/mol, respectively.

Partial Molar Volumes in Membranes. The excess molar volume of benzyl alcohol in EPC–cholesterol (2:1) bilayer membranes was obtained from the measured excess volume in suspensions of sonicated vesicles in water by using eq 10 with the value of V_H^E determined above. The mean partial

Table I: Mean Partial Molar Volumes, $\bar{V}$, and Partial Molar Volumes at Infinite Dilution, $\bar{V}^{\infty}$, of Solute Alcohols in Solvents[a]

solute	solvent	$\bar{V}$ (mL/mol)	$\bar{V}^{\infty}$ (mL/mol)
benzyl alcohol	water	101.6 ± 0.12	101.6 ± 0.04
(V° = 103.83)	EPC-chol[b]	105.5 ± 2.13	107.3 ± 0.98
heptanol	water	133.6 ± 0.42	133.3 ± 0.41
(V° = 141.70)	buffer soln	133.9 ± 0.03	134.1 ± 0.28
	EPC	139.4 ± 2.41	140.1 ± 2.41
	ghost	141.6 ± 5.06	
octanol	water	149.1 ± 0.71	149.1 ± 0.57
(V° = 158.01)	buffer soln	148.1 ± 0.43	147.1 ± 0.30
	EPC	158.6 ± 1.35	159.6 ± 0.66
	ghost	159.1 ± 4.02	157.7 ± 3.70

[a] Membrane/buffer partition coefficients used were as follows: for benzyl alcohol, 10.9 (Colley & Metcalfe, 1972); for heptanol and octanol between EPC and water, 170 and 378, respectively (Jain et al., 1978); and between red cell ghosts and buffer, 39.6 and 152, respectively (Seeman et al., 1971). [b] Cholesterol.

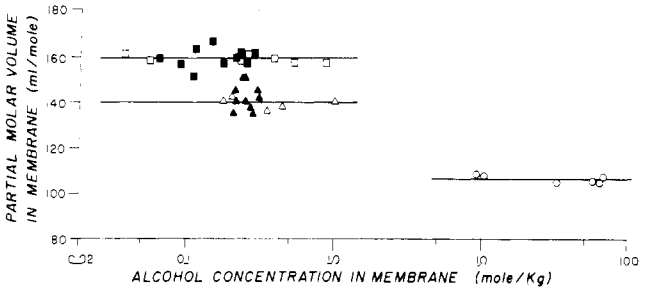

FIGURE 2: Partial molar volume of the alcohols in membranes at 25 °C vs. their concentration in the membrane: (○) benzyl alcohol in EPC–cholesterol (2:1); (△) 1-heptanol in EPC; (□) 1-octanol in EPC; (▲) 1-heptanol in ghost; (■) 1-octanol in ghost. The dry weight of membrane solids was about 3% for lipid bilayers and usually 0.6–0.9% for red cell ghosts.

molar volume, $\bar{V}_M$, of benzyl alcohol in these bilayers was found to be 105.5 ± 2.1 mL/mol, in good agreement with our previous value of 105.3 ± 0.25 mL/mol (Kita et al., 1981).

Figure 2 shows a plot of $\bar{V}$ vs. the concentration of benzyl alcohol in EPC–cholesterol (2:1) bilayers and of heptanol and octanol both in EPC bilayers and in red cell ghosts. The slopes are not significantly different from zero in any case shown in Figure 2, indicating that the partial molar volumes of these alcohols are independent of their concentration in this range. Note that the values of $\bar{V}^{\infty}$ in Table I are obtained by long extrapolation and that the narrow concentration range over which we were able to obtain accurate data with the biomembranes limits the precision of this extrapolation, especially in the case of heptanol in red cell ghosts.

The mean excess volumes of both heptanol and octanol were close to zero both in EPC bilayers and in red cell ghosts, indicating that within our errors the two membranes behave similarly.

Membrane Density. Evaluation of eq 17 for all the red cell ghost preparations yielded an average membrane density of 1.21 ± 0.049 (SD) g/mL. This compares to a value of about 1.2 g/mL which may be read from graphs in the work of Seeman (1974) and Franks & Lieb (1981). Similarly, we found a value of 1.01 ± 0.032 g/mL for egg lecithin which compares both to a value of 1.014 g/mL reported by Johnson & Buttress (1973) for lipid from the same source, but containing 4% phosphatidic acid, and to a value of 1.012 g/mL for egg lecithin bilayers (Huang, 1969).

Discussion

Methodology. As mentioned in the introduction, we chose to perform these measurements in an all-glass density measuring system rather than by excess volume dilatometry because of the presence of mercury in the latter. As expected, several limitations of the density determination method were encountered. Initial studies showed, for example, that it was very difficult to get reproducible results with extremely volatile agents such as halothane. This was presumably due to losses of this agent from the sample (its vapor pressure at 25 °C is 0.4 atm) and contrasts with the relative ease with which the data were obtained by us for halothane in the excess volume dilatometer. With the alcohols, the volatility problem was much less severe. The vapor pressure of octanol is about 10^{-4} atm at 25 °C, for example. Our errors in the partial molar volume measurements of alcohols in aqueous solution were generally comparable to those for corresponding measurements in the excess volume dilatometer (Kita et al., 1981). Scatter with the lipid suspensions, however, tended to be nearly an order of magnitude greater in the present method. This probably arose from small fluctuations in the lipid concentration between samples. Such fluctuations would not occur in the excess volume dilatometer where each complete experiment is carried out with a single loading of suspension. We cannot rule out that the use of microscopically heterogeneous suspensions in the oscillating tube dilatometer might also lead to variability. However, after a period of 15–20 min for temperature equilibration, the density of suspensions remained constant for up to 1 h.

Scatter with the red cell suspensions was greater than with lipid suspensions. This was expected because the obtainable concentration of membrane solids was lower (about 1% by weight compared to the 3% used with lipid). This difference is compounded because the membrane/buffer partition coefficients of the alcohols in red cell ghosts are 2–4 times less than those in lipid bilayers; thus, the fraction of the overall measured density change in the suspension contributed by the membrane is reduced. The somewhat smaller errors in both membranes for octanol compared to heptanol may be ascribed to octanol's larger partition coefficient. A further source of error with the biological membranes might arise from fluctuations in their composition from one preparation to another; however, errors between experiments were generally comparable to errors within an experiment.

In spite of the above problems, it proved possible to obtain values for the partial molar volumes of these alcohols in both lipid and biological membranes with a reproducibility that was better than 5%. We can only estimate our absolute errors by comparison with previous work. In aqueous solution, agreement was good with a considerable body of previous data (see Results and the section below). In lipid bilayers, our present value for benzyl alcohol in EPC–cholesterol agrees with that we previously obtained by using an excess volume dilatometer. The only other value for biomembranes is that determined by Franks & Lieb (1981) for halothane by using a similar method to ours. Their excess volume of mixing is +28 ± 14 mL/mol in red cell ghosts, +18 ± 24 mL/mol in bovine spinal cord lipids, and +10.5 ± 3.2 mL/mol in buffer alone. Their value in buffer contrasts with our value of −17 ± 0.6 mL/mol by excess volume dilatometry (Kita et al., 1981) and of −15 mL/mol deduced from the pressure dependence of halothane's aqueous partition coefficient (R. A. Smith and K. W. Miller, unpublished results). Thus, we believe that the large positive excess volume for halothane in red cell ghosts reported by Franks & Lieb (1981) is probably erroneous and may well result from the transfer of halothane vapor into microbubbles in their density meter during the course of the experiment.

Table II: Excess Volume of Mixing 1-Octanol with Various Solvents

solvent	excess volume, V_∞^E (mL/mol) 1-heptanol	1-octanol	ref
cyclohexane	+7.1[a]	+7.2	Stavely & Spice (1958)
n-heptane	+4.9[a]	+4.2	Stavely & Spice (1958)
1-decanol		+0.01	Pflug & Benson (1968)
water	−8.4	−8.9	this work
sodium dodecanoate micelle	−2.8	−2.3	Vikingstad (1979)
EPC	−1.6	+1.6	this work
red cell ghost	−0.1	−0.3	this work

[a] Data interpolated from those for a series of *n*-alcohols.

This problem caused difficulty in our own preliminary studies with the excess volume dilatometer (Kita et al., 1981). For these reasons, assessment of the absolute errors in our partial molar volume determinations in biomembranes must await further work.

Partial Molar Volumes. The partial molar volume of a given alcohol has the same value in both the bilayer and the biomembrane (Table I). We would not expect more accurate data to reveal a difference of more than 5%. This conclusion is in direct conflict with the finding of Seeman (1974) that ethanol expanded red cell ghosts and synaptosomes much more than lipid bilayers. The suggestion that the rather preliminary data on ethanol is in error is supported by another recent study (Franks & Lieb, 1981). This equality of the partial molar volumes in the bilayer and the biomembrane strongly suggests that the dominant contribution arises from alcohol–lipid interactions in each case. This does not rule out the possibility that a proportion of the alcohol molecules might be engaged in alcohol–protein interactions provided no conformational change giving rise to a detectable contribution to the *total* volume occurs.

Do our data tell us anything about the comparative states of the lipid bilayers in egg phosphatidylcholine and red cell ghosts? The high proportions of protein and cholesterol in the latter membrane certainly do influence barbiturate–membrane interactions (Korten et al., 1980). We did not examine phospholipid–cholesterol bilayers here because the required partition coefficients were not available, but in a previous, more accurate study, we showed that the partial molar volumes of halothane and methoxyflurane were both increased some 5% when 33 mol % cholesterol was added to egg phosphatidylcholine (Kita et al., 1981). These effects are small and in the present study might have gone undetected, but if the effects of cholesterol and protein had been additive (Korten et al., 1980), we would probably have detected this.

In Table II, the excess volumes of heptanol and octanol in a number of solvents are summarized. Transferring 1 mol of octanol from the pure liquid, or from solution in another alcohol, to a hydrocarbon results in a positive volume change of greater than 4 mL. This is probably due to the resultant loss of hydrogen bonding, because transfer of *n*-octane to another hydrocarbon results in a negligible volume change (Coburn & Grunwald, 1958). Less extensive data are available for 1-heptanol, but a similar conclusion probably holds. Now on transfer to egg lecithin neither heptanol nor octanol behaves as though they have been transferred to a hydrocarbon, suggesting that for a considerable fraction of time a hydrogen bond is retained by each alcohol, presumably at the membrane–aqueous interface because alcohols do not hydrogen bond in apolar solvents (Coburn & Grunwald, 1958). This interpretation is supported by our previous comparison of the solution properties of *n*-alkanes and 1-alkanols in bilayers (Miller et al., 1977). A similar conclusion may hold for red cell ghosts, but the accuracy of our data is hardly sufficient in this case to support a detailed analysis.

In micelles of sodium dodecanoate, both alcohols exhibit negative excess volumes (Table II), although smaller changes are seen in shorter chain detergents (Vikingstad, 1979). These data are again consistent with the notion that on average no change in hydrogen bonding occurs on transfer from alcohol to micelle, although some additional contraction of the structure may also occur. In this case, it is known that the transfer of *n*-octane to the micelle is accompanied by almost no change in partial molar volume (Vikingstad, 1978).

The thermodynamic changes on transferring a nonpolar molecule into water have traditionally been used to define the hydrophobic effect that makes a substantial contribution to membrane stability [for a recent review, see Tanford (1980)]. This is usually discussed in terms of an unfavorable entropy change but is also expressed in large negative excess volumes of mixing (Table II; Friedman & Sheraga, 1965). The linear relationship noted by the latter workers between the chain length, *n*, of the 1-alkanols from methanol to pentanol and their partial molar volumes in water may be extended by our work and that of Vikingstad (1979) up to decanol. The combined data give a good fit ($r = 0.9999$) to the equation

$$\bar{V}^\infty = 15.92(\pm 0.06)n + 22.6(\pm 0.38) \qquad (19)$$

where the figures in parentheses are standard deviations. Thus, the molar volume of a methylene group in water is 15.9 mL. Similarly, the molar volumes of methylene groups in 1-alkanols are 16.7 mL, giving an excess volume for transfer from the pure liquid to water (hydrophobic effect) of −0.8 mL/mol. It will be interesting to see how these values compare to values for transfer of methylene groups from membranes to water. Our present data do not attempt to answer this question, but a study of a wider range of alcohols in lipid bilayers using our more accurate excess volume dilatometer should be capable of yielding useful data.

Equation 19 also reveals that when the last methylene group is removed leaving water ($n = 0$), the predicted partial molar volume is 22.6 mL. This extrapolation from higher *n* does not contain the information that in the absence of carbon the last hydrogen becomes a site for hydrogen bonding. The difference between the extrapolated value and water's true molar volume (18.1 mL) is 4.5 mL and must largely reflect the addition of a hydrogen bond. This value is in good agreement with that deduced above from the data in Table II.

Do Alcohols Partition into Membranes? Recently, measurements of partitioning into biomembranes using a hygroscopic desorption technique have yielded partition coefficients several orders of magnitude lower than those obtained by centrifugation techniques. This discrepancy was assigned to the cosedimentation of hemimicelles of lipid and alcohol in the latter technique (Conrad & Singer, 1981). If this explanation is accepted, then we have merely demonstrated that partial molar volumes in EPC bilayers and ghost lipid hemimicelles are equal.

Such a conclusion runs counter to a vast array of data demonstrating the relationship between lipid solubility and cell membrane permeability [for a review, see Diamond & Wright (1969)]. So that a control could be provided in our own work, ghosts equilibrated with 0.5 mM [^{14}C]octanol were spun overnight into a sucrose gradient which contained the same octanol concentration. A protein band centering about 1.1 M sucrose was coincident with a peak in [^{14}C]octanol. No octanol

peak was detected above the gradient where lipid vesicles might be expected. Thus, either there are no hemimicelles or they are firmly attached to the ghost membrane. The latter possibility contrasts with the ease with which the hygroscopic desorption technique apparently detached these hemimicelles.

Furthermore, a recent reexamination of chlorpromazine binding to erythrocyte ghosts using the hygroscopic desorption technique has shown that substantial partitioning occurs at low concentrations (Bondy & Remien, 1981). Results comparable to those of Conrad & Singer (1981) were only obtained at concentrations high enough to cause membrane damage.

Comparison with Membrane Area Changes. Seeman and co-workers have determined the area increase induced by alcohols in erythrocyte ghosts (Seeman et al., 1969a,b; Roth & Seeman, 1972). Their conclusion that the area increase exceeds the expected volume increase has been confirmed by more recent studies using cells impaled on a micropipet (Bull et al., 1980). Seeman's original estimate of the degree to which the area increase exceeded the expected volume increase was inflated by the assumption that the partial molar volume of alcohols in membranes was less than their molar volumes. However, even after correction for this assumption, we find that area increases are 3–5 times larger than expected on the basis of our volume experiments. The origin of this anomaly is undetermined. It is possible that it might originate either from alcohol–cytoskeleton interactions or from perturbation of the lipid region inducing a constant volume conformational change in proteins leading to an increase in area (Seeman, 1972).

Another possibility is that the expansion of the lipids in the membrane may occur anisotropically with the membrane thickness actually decreasing as the area expands. We have argued for the possibility of anisotropic expansion previously (Miller et al., 1973; Kita et al., 1981). The concept is supported by analogy with the known anisotropic thermal expansion of bilayer structures (Rand & Pangborn, 1973). If we assume anisotropic expansion, we can calculate from our own volume change data and from the measured area change of 6% (Roth & Seeman, 1972) that the thickness should decrease 4% in the presence of 0.74 mM octanol. The more recent work of M. H. Bull and J. D. Brailsford (unpublished results) suggests a slightly smaller area expansion under these conditions and a consequent thickness decrease of only 2.7%. These calculations serve to define the magnitude of the expected thickness changes in red cell ghosts.

Such small changes in the lipid bilayer thickness of a complex biomembrane could be difficult to detect experimentally. In the squid axon, changes in capacitance in the presence of anesthetics have been used to infer a thickness increase (Haydon & Kimura, 1981), whereas in frog sciatic nerves, X-ray studies could detect no thickness changes under physiological conditions. Thickness increases, when observed, were associated with irreversible effects (Padron et al., 1980).

While work in biomembranes remains equivocal on the question of anisotropic expansion, in lipid bilayers both X-ray and neutron diffraction (Franks & Lieb, 1979; White et al., 1981) and capacitance measurements (Reyes & Latorre, 1979) all indicate little or no thickness change under conditions where excess volume dilatometry indicates bulk expansion. Thus, in the lipid bilayer anesthetic-induced expansion clearly occurs anisotropically.

Anesthetic Action. A decade ago, two lines of evidence implicated membrane expansion as a mechanism of general anesthetic action: first, the work of Seeman and collaborators on erythrocyte ghost–anesthetic interactions [for a review, see Seeman (1972) and Roth (1979)] and, second, the pressure reversal of anesthesia (Miller et al., 1973). The critical volume hypothesis provided a theoretical framework for linking these two lines of evidence, but at that time the necessary physicochemical parameters were not available for membranes. Instead, olive oil was used as a model; it suggested that a membrane expansion of 0.2% was required for anesthesia in poikilotherms at 20–25 °C. Now Table III shows that equianesthetic doses of three anesthetics cause a reasonably consistent expansion of the red cell ghost of 0.15%. Similarly, in EPC, our new alcohol data are consistent with our previous work with volatile agents and yield an expansion of 0.36%. The standard deviations of the mean expansions are both less than 20%, which is certainly not greater than the combined errors in the physiological and thermodynamic data. The difference between the two membranes results from the larger partition coefficients for EPC, as can be seen when membrane expansion is compared on a membrane concentration basis (Figure 3).

The data in Table III are quite consistent with the critical volume hypothesis. They also rule out the trivial explanation that pressure reversal is caused by the pressure dependence of the partition coefficient. For example, the ghost/buffer partition coefficient for octanol will only decrease 5% over a hundred atmospheres. This is important because the partial molar volumes of the four anesthetics in Table III do not vary sufficiently to unequivocally distinguish between membrane solubility and membrane expansion hypotheses.

The expansion associated with general anesthesia is rather small. Other physiological effects, such as nerve block, occur

Table III: Membrane Expansion at Equianesthetic Concentrations

agent	ED_{50} (μM)[c]	% expansion: red cell ghost	% expansion: EPC
heptanol	190[d]	0.13	0.45
octanol	61[e]	0.18	0.37
halothane[a]	230[f]	0.13[g]	0.30[f]
methoxyflurane[b]	210[f]		0.34[f]
		0.15 ± 0.029[h]	0.36 ± 0.064[h]

[a] $CF_3CHClBr$. [b] $CH_3OCF_2CCl_2H$. [c] Anesthetic concentration in aqueous phase bathing tadpoles. [d] M. H. Bull and J. D. Brailsford, unpublished results. [e] Pringle et al. (1981). [f] Kita et al. (1981). [g] Franks & Lieb (1981). [h] Mean ± SD.

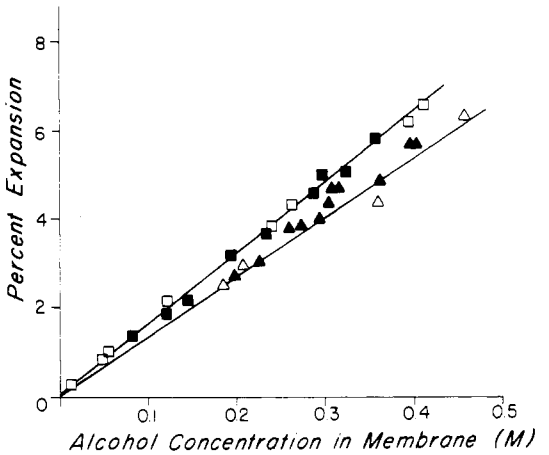

FIGURE 3: Fractional membrane expansions of the alcohols in the biomembrane and the lipid bilayer are the same when compared as a function of alcohol concentration in the membranes. The symbols have the same meaning as in the previous figures. Concentration in both membranes is in moles per liter of membrane. The densities of the bilayer and red cell ghost were 1.01 and 1.21 g/mL, respectively.

at concentrations up to an order of magnitude higher and will be associated with correspondingly greater expansions. To ascertain if such changes are of functional significance, one needs to consider the mechanism by which dimension changes in the surrounding bilayer might effect an excitable protein's function. This is complicated by the uncertainty over whether anisotropic expansion occurs in biomembranes (see above). However, it is clear that the hypothesis requires membrane proteins to vary in their sensitivity to such perturbations because the majority of such proteins continue to function during anesthesia. Reconstitution of $(Na^+ + K^+)$-ATPase into lipids of varying chain length throws some light on this question (Johannsson et al., 1981). Addition of methylene groups to short chain lipids initially caused an increase in the enzyme's activity, but between 16 and 20 carbons no increase was observed and above this point activity declined again. A $(Ca^{2+} + Mg^{2+})$-ATPase showed similar behavior, but maximum activity was achieved over a narrower range of chain lengths. Thus, the response of such systems to anesthetics will depend on the preexisting relationship between lipid and protein. That the $(Na^+ + K^+)$-ATPase exhibits maximum activity over such a wide range of thickness is consistent with its insensitivity to general anesthetics (Halsey et al., 1970).

The hypothesis also requires temperature-induced expansion to be qualitatively different from anesthetic-induced expansion; otherwise, increases of the order of a few degrees should cause anesthesia (Simon & Bennett, 1980). However, addition of thermal energy should affect the whole system, whereas anesthetics will cause a differential expansion of the lipid alone. Thus, expansion by heat and anesthetics cannot be considered equivalent.

Conclusion. Thus, we have demonstrated that the partial molar volumes of anesthetics in lipid bilayers and erythrocyte ghosts differ by less than 5%. This resolves the uncertainties created by an early density meter study (Seeman, 1974). Contrary to that study, we found that lipid bilayers expand more than ghosts because partitioning into the former is greater. Our results are consistent with the critical volume hypothesis of anesthetic action, but further studies are required to define the role of anisotropic expansion and the mechanisms by which dimension changes in the lipid couple to protein function.

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Stabilization of the Quaternary Structure of Transcarboxylase by Cobalt(II) Ions†

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ABSTRACT: When dilute solutions of transcarboxylase are incubated at 25 °C in an alkaline 50 mM buffer, the enzyme rapidly loses activity. This loss of activity is accompanied by the dissociation to enzymatically inactive subunits. The inclusion of 2 mM Co^{2+} in the buffer reduces both dissociation and the loss of enzymatic activity. This stabilization does not take place with 2 mM Mg^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, Ca^{2+}, or Cu^{2+}, but there is a slight protection by Zn^{2+}. At Co^{2+} concentrations of less than 2 mM, the stabilization decreases. The cobalt involved in the stabilization is not that required for catalysis as evidenced by the fact that the "catalytic" cobalt does not exchange with added free Co^{2+} under the conditions that prevent loss of enzymatic activity. The stabilizing effects of Co^{2+} were also observed toward inactivation with guanidinium chloride and by heat. It is proposed that Co^{2+} shifts the equilibrium of the dissociation of transcarboxylase toward the associated form and thus enzymatic activity is retained at alkaline pH.

Transcarboxylase (methylmalonyl-CoA:pyruvate carboxyltransferase, EC 2.1.3.1) is a biotin-containing enzyme found in the propionic acid bacteria [for reviews, see Wood & Zwolinski (1976) and Wood (1979)]. An illustration of the quaternary structure of the enzyme and its dissociation to enzymatically inactive subunits is shown in Figure 1. By use of transcarboxylase isolated from cells grown in ^{65}Zn or ^{60}Co, it has been demonstrated that the outer $5S_E$ subunit contains Co^{2+} and Zn^{2+} (Northrop & Wood, 1969; Ahmad et al., 1972; Fung et al., 1974). These two metals are tightly bound and dissociate only at a very low pH or in the presence of sodium dodecyl sulfate (Ahmad et al., 1972). Even at pH 9, EDTA[1] fails to remove these metals. These metals will be referred to as the "catalytic" metals.

In this report, we show that the presence of 2 mM exogenously added Co^{2+} ions protects transcarboxylase against dissociation and loss of activity at pH 8 and 9. These Co^{2+} ions are distinct from the catalytic metals and the protective effect is specific for Co^{2+}. Co^{2+} also protects against denaturation of the enzyme by guanidinium chloride and treatment at 50 °C.

Materials and Methods

The 26S transcarboxylase was isolated by the method of Wood et al. (1977) and assayed as described by Wood et al. (1969). For the tests of the effects of cobalt on enzymatic activity, the mixtures with and without Co^{2+} were incubated at 25 °C and exposed to air unless otherwise stated, under the conditions described in the text and figure legends. Aliquots were removed at the stated times and assayed. Unless otherwise stated, the Co^{2+} concentration was 2 mM and the buffer was 50 mM Hepes (Na^+), pH 8 or 9. Transcarboxylase containing ^{60}Co was isolated as described by Ahmad et al. (1972) and the $6S_E$ subunit by dissociation of 26S transcarboxylase at neutral pH, followed by glycerol gradient centrifugation (Wood et al., 1977). The $12S_H$ subunit was purified as recently described by Bahler et al. (1981). Reductive methylation was with [^{14}C]formaldehyde and $NaCNBH_4$ as described by Jentoft & Dearborn (1979). ^{60}Co was purchased from New England Nuclear and had a specific activity of 123 Ci/mg. Urea and guanidinium chloride were from K & K Laboratories, Rare and Fine Chemicals, Plainview, NY. All other chemicals were reagent grade or better.

Results

Effect of Co^{2+} on the Activity of Transcarboxylase Incubated at pH 8. The results shown in Figure 2 were obtained when transcarboxylase was diluted into 50 mM Hepes buffer, pH 8, at 25 °C, with and without the addition of 2 mM Co^{2+}, and was assayed with time. The inclusion of 2 mM Co^{2+} in the incubation buffer results in a pronounced protection of the activity of the enzyme. Similar results are obtained when the experiment is done at pH 9. The effect of Co^{2+} is most pronounced at low protein concentrations (≤1 mg/mL). No protective effect was observed with 2 mM Mg^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, Ni^{2+}, or Ca^{2+}, but a slight protection was noted in the presence of Zn^{2+} (Figure 2). If Co^{2+} is added to samples that have been incubated without Co^{2+} for 5, 10, or 20 min, there is no reactivation of enzyme. Inactivation of transcarboxylase

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GdmCl, guanidinium chloride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/82/0421-2847$01.25/0