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Thermodynamics of All-or-None Water Channel Closure in Red Cells

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Summary. The relation of osmotic to diffusional water permeability of human red blood cells was compared after treating the cells with different concentrations of PCMBS (*p*-chloromercuribenzene sulfonate). After subtracting the PCMBS-insensitive permeability (presumably the water permeability of the lipid bilayer) from each, the ratio of osmotic to diffusional permeability remains invariant (≈11) as more and more water channels are inhibited by increasing concentrations of PCMBS. This result implies that the channels close in an all-or-none way and suggests a two-state model. Analysis of the dependence of osmotic water permeability on PCMBS concentration in terms of the model reveals a 1 : 1 stoichiometry and a dissociation constant for the PCMBS/membrane receptor complex of about 0.019 mM at 37°C. Temperature dependence studies show that the reaction is entropically driven ($\Delta H^\circ \approx 25$ kcal/mol, $\Delta S^\circ \approx 100$ cal/mol-deg) and suggest the involvement of hydrophobic interactions.

Key Words PCMBS · red cell · water · channel · permeability · erythrocytes

Introduction

The principal evidence for the existence of water channels in the human red cell membrane rests on the findings that 1) water permeability is high; 2) the activation energy for water transport is low (Vieira et al., 1970); and 3) the ratio of osmotic to diffusional water permeabilities is considerably greater than one (Paganelli & Solomon, 1957). In contrast, the water permeability through lecithin cholesterol bilayers is about ten times lower, the activation energy is high (about 12 to 14 kcal/mol) (Haydon, 1969) and the ratio of osmotic to diffusional permeabilities equals one (Cass & Finkelstein, 1967; Everitt, Redwood & Haydon, 1969; Hanai, Haydon & Redwood, 1969). When mercurial reagents are applied to red cells, their water transport properties resemble lipid bilayers; i.e., red cell water permeability decreases by a factor of ten, activation energy increases to 11 to 12 kcal/mol and the ratio of osmotic to diffusional permeability approaches one (Macey & Farmer, 1970; Macey, Karan & Farmer, 1972). These results are the basis for the assertion that mercurials close water channels. Aside from the recent implication that the mercurials are reacting with a cryptic SH group of Band 3 (Solomon et al., 1981), virtually nothing is known about the mechanism.

This paper begins with the assumption that water permeates the membrane via two parallel mechanisms, channel transport, and lipid bilayer diffusion. Letting P denote the measured water permeability, (either osmotic or diffusional), p the channel permeability and q the lipid bilayer permeability, we write

$$P(C) = p(C) + q \tag{1}$$

where we assume that p depends on C, the mercurial concentration, but q does not. Supporting evidence for q insensitivity to these reagents follows from the fact that water permeability of chicken red cells, which presumably have no water channels is not influenced by PCMBS (*p*-chloromercuribenzene sulfonate) (Brahm & Wieth, 1977). By showing that the ratios g_o of osmotic to diffusional permeabilities for the channel remain invariant as the channel is progressively inhibited by more and more mercurial, we suggest that the channels are closed in an all-or-none mechanism. It follows that when all of the channels have interacted with PCMBS (e.g., at 2 mM), then $p = 0$ and $P = q$. We then go on to show that channel closure can be accurately described by a simple receptor binding reaction with a 1 : 1 stoichiometry. Finally by studying the temperature dependence of the parameters of the scheme: mercurial + receptor $\rightleftharpoons$ channel closure, we con-

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Table 1.

PCMBS (mM)	τ_f (msec)	τ_d (msec)	P_f [(cm/sec) × 10^3]	P_d [(cm/sec) × 10^3]	P_f/P_d	p_f/p_d
0.00	336	12.4	21.5	3.67	5.9	10.6
0.05	397	13.9	18.2	3.28	5.6	11.2
0.10	484	14.6	14.9	3.12	4.8	10.1
0.15	534	16.3	13.5	2.80	4.8	11.9
0.20	551	16.0	13.1	2.85	4.6	10.9
0.30	627	17.0	11.5	2.67	4.3	11.3
0.35	733	18.6	9.86	2.45	4.0	12.6
0.40	822	18.2	8.78	2.50	3.5	10.1
0.50	887	19.1	8.16	2.38	3.4	11.2
0.70	1566	20.8	4.61	2.20	2.1	7.3

Permeabilities P_f and P_d are calculated from Eqs. (1) and (2) assuming $V/A = 4.56 \times 10^{-5}$ cm (Brahm, 1982). The ratio p_f/p_d is calculated from Eq. (3) using a value of $q = 0.084\ P_f(0)$ as given by the intercept of the straight line plot of Fig. 1. Temperature = 25°C.

clude that the overall reaction is entropy driven. These results have been presented earlier in a preliminary report (Macey et al., 1979).

Materials and Methods

Sample Preparation

Human blood was collected (in acid-citrate-dextrose) and stored at 4°C until used (no longer than two days). Prior to use, the blood was washed three times in a high K^+ medium (mM: 140 KCl, 27 sucrose, 10 NaCl, 5 HEPES buffer; pH = 7.4) which will maintain a constant cell volume even after prolonged exposure to PCMBS. In several experiments it was necessary to incubate blood (at 3% hematocrit) 2 to 3 times for 45-min intervals in high K^+ media containing the desired concentration of PCMBS to ensure that the final PCMBS concentration was not compromised by adsorption onto or into the cells. In other experiments the PCMBS in the medium was directly assayed by methods of Benesch and Benesch (1962) and Patai (1974). For comparisons of diffusional and osmotic permeability, measurements were made simultaneously from samples of the same blood which had been pre-incubated with various levels of PCMBS. Both osmotic and diffusional permeability measurements were done at 15% hematocrit in order to minimize any variations in the degree of inhibition of sample pairs.

Permeability Measurements

Osmotic water permeability was measured by the small perturbation method (Farmer & Macey, 1970). Cells originally equilibrated in an isotonic solution were suddenly exposed to a hypertonic medium of 1.15× isotonic and the resulting cell shrinkage was followed in a photometric stopped-flow device. Osmotic permeability coefficients were calculated from the time constants τ_f of the tail ends of the cell shrinkage curves (Macey, 1979).

Diffusional permeability estimates were obtained with NMR techniques. Water proton spin-spin relaxation times, τ_2, of blood samples containing 20 mM extracellular Mn^{2+} were measured at 298 K in a Bruker CXP 300 spectrometer or a Bruker WP80 SY equipped with ASPECT 2000 computers. Mn^{2+} was added to the high K^+ extracellular medium just prior to separating the sample for comparative osmotic and diffusional permeability measurements. (Control experiments showed no effects of extracellular Mn^{2+} on osmotic permeability, while measurements of Brahm (1982) show no effects of extracellular Mn^{2+} on diffusional permeability.) Using a Carr-Purcell Meiboom-Gill sequence, sample points were taken on the top of each echo; the time interval was typically 0.1 msec. This provided a very fast and reproducible method for measuring spin-spin relaxation times in this system.

The curves obtained for the decay of the successive echo amplitude was resolvable into two exponential components: a fast component and a slow component. The time constant characteristic of the slower component, which is related to the water diffusion exchange time τ_d, was determined either from the linewidth of the "resonance" obtained by a Fourier transform of the spin echo amplitude decay or from the decomposition of this decay by a nonlinear least-squares computer program. Results obtained by both methods were in excellent agreement.

The measured relaxation time from the red cell results from the product of two decay processes: (1) the desired water exchange across the cell membrane, which is superimposed on (2) the ordinary spin-spin relaxation of water inside the red cell. Estimates of the latter are generally obtained by measuring the relaxation time τ_P of packed pellet (containing no Mn^{2+}); water exchange time τ_d across the membrane is then given by $\tau_d^{-1} = \tau_2^{-1} - \tau_p^{-1}$. Since τ_2 is generally much smaller than τ_p, the method is relatively insensitive to the accuracy of the estimate of τ_p. Most of the experiments were performed on the 300-mHz spectrometer, where τ_p was typically in the neighborhood of 50 msec. This is much shorter than the values of about 140 msec (e.g., Conlon & Outhred, 1972) usually reported from measurements with an 80 mHz spectrometer. Accordingly, we repeated some measurements in an 80-mHz machine and found values (i.e., τ_p = 125 mHz) that agreed with published data. Further, in the Mn^{2+}-doped samples, where τ_2 is small (<20 msec), the two spectrometers gave nearly identical results. We suggest that this discrepancy for long time constants is due to spin diffusion in a nonhomogeneous field (Brown, 1983). This artifact is more serious at longer time constants in the higher field because the field gradient is steeper. Accordingly, we consider values obtained at 80 mHz (τ_p = 125 msec) as the best approximation and have used it in our data.

Results

The Ratio of Osmotic to Diffusional Channel Permeability is Invariant

Table 1 shows our data for calculating the osmotic and diffusional permeabilities as a function of C, the concentration of PCMBS. Data is tabulated in terms of τ_f, the time constant for relaxation of cell volume following an osmotic perturbation, and τ_d, the water exchange time constant. (In general, subscript f refers to osmotic (filtration) measurements, while subscript d refers to diffusional experiments.) The relevant permeabilities are calculated by the following expressions (Macey, 1979): The diffusional permeability P_d is given by

$$P_d = \frac{V}{A\tau_d} \tag{2a}$$

and the osmotic permeability P_f is given by

$$P_f = \frac{b}{V_w \Pi_{iso}} \left(\frac{V}{A\tau_f}\right) \tag{2b}$$

where V/A is the ratio of cell water volume to membrane surface area, $b \approx 0.86$ represents a small correction factor relating cell water volume to osmotic dead space, V_w(= 18 ml) is the molar volume of water, and Π_{iso}(= 3×10^{-4} mol · cm^{-3}) represents isotonic osmolarity.

The control values (at $C = 0$) are consistent with results obtained in other laboratories. The control τ_d = 12.4 msec compares favorably with the value 11.0 msec obtained by Conlon and Outhred (1978) while the control $P_d(0) = 3.7 \times 10^{-3}$ cm/sec fits in the observed range of 2 to 4 $\times$ 10^{-3} cm/sec tabulated and discussed by Brahm (1982). Further, the osmotic permeability $P_f(0) = 2.2 \times 10^{-2}$ cm/sec agrees with the latest figures, 2.0×10^{-2} cm/sec obtained by Mlekoday, Moore and Levitt (1983).

The data of Table 1, as plotted in Fig. 1, support the conclusion that the ratio g_o of osmotic to diffusional permeability of the *channel* remains constant as the composite water permeability $P(C)$ is decreased by increasing concentrations of PCMBS; i.e., letting

$$g_o = \frac{P_f(C)}{P_d(C)} = \frac{P_f(C) - q}{P_d(C) - q} \tag{3}$$

we use Fig. 1 to show that g_o is independent of C. To this end, we solve Eq. (3) for $P_f(C)$, divide both sides of the result by $P_f(0)$ and use the identity $P_d(C) = P_d(0)P_d(C)/P_d(0)$ to arrive at

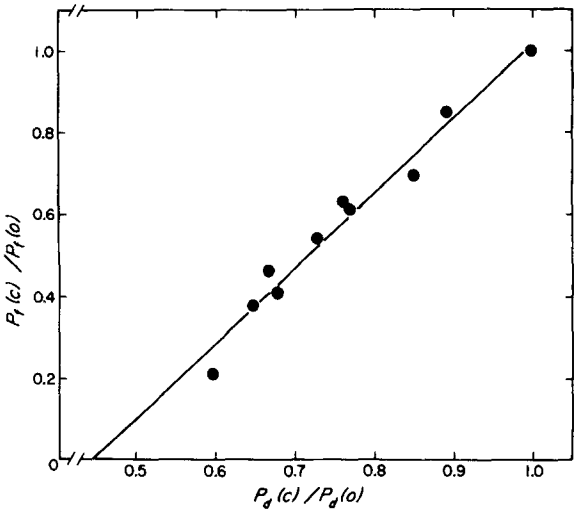

Fig. 1. Plot of $P_f(C)/P_f(0)$ *vs.* $P_d(C)/P_d(0)$ using data of Table 1 (T = 25°C)

$$\frac{P_f(C)}{P_f(0)} = \left(g_o \frac{P_d(0)}{P_f(0)}\right)\left(\frac{P_d(C)}{P_d(0)}\right) + (1 - g_o)\frac{q}{P_f(0)}. \tag{4}$$

The plot of $P_f(C)/P_f(0)$ *vs.* $P_d(C)/P_d(0)$ shown in Fig. 1 yields the straight line predicted by Eq. (4) and shows that g_o is indeed constant over a large range of $P(C)$. The value of g_o = 10.8 obtained from the slope represents the ratio of osmotic to diffusional permeability *for the channel*. The value $q/P_f(0)$ = 0.084 obtained from the intercept equals the fraction of osmotic permeability which is insensitive to PCMBS and presumably represents the fraction that passes through the lipid pathway. This estimate [$q/P_f(0)$ = 0.084] agrees reasonably with other estimates obtained by using saturating dosages of PCMBS (Macey et al., 1972; Naccache & Sha'afi, 1974). It is used with other data in Table 1 to construct the last column which shows values of g_o for each determination. It can be seen that as the concentration increases, $g_o = p_f/p_d$ hovers closely around 11. Deviations at high concentrations can be expected because the ratio defined by Eq. (3) becomes very sensitive to errors as $(P_d(c) - q) \to 0$. For example, in the case of 0.7 mM PCMBS, where less than 15% of the channels remain open, a 5% error in the estimate of P_d would be sufficient to raise the value of g_o from 7.3 to 10.

Contrast the invariance of $g_o = p_f/p_d$ with the ratio of composite permeabilities P_f/P_d which steadily decrease toward unity as expected when most channels are shut down, leaving simple diffusion through the bilayer as the primary mechanism for water transport. When saturating doses of PCMBS are used, P_f/P_d does in fact become unity (Macey et al., 1972). Values for osmotic and diffusional per-

Table 2. Control values for water permeability as derived from Table 1 and Fig. 1

	Permeability (cm/sec × 10^3)
Composite	
Filtration ($P_f = p_f + q$)	21.5
Diffusion ($P_d = p_d + q$)	3.67
Channel	
Filtration (p_f)	19.7
Diffusion (p_d)	1.86
Background "bilayer" (q)	1.81

meabilities in the channel and "bilayer" background are compared in Table 2.

Concentration Dependence of Channel Closure Shows a 1 : 1 Stoichiometry

Figure 2 shows the development of inhibition of osmotic water permeability as a function of time for different concentrations of PCMBS. Data are plotted in terms of τ_f which is inversely proportional to P_f. The effects of PCMBS saturate at concentrations above 1 mM after about 15-min incubation time while at lower subsaturation concentrations a longer time is required to reach a steady-state plateau. Notice that at maximal inhibition, $\tau_f(\max) = 10 \times \tau_f(\text{control})$ or correspondingly $q = P_f(\infty) = 0.1\ P_f(\text{control})$, which agrees with our estimate from the intercept in Fig. 1.

To analyze the equilibrium data obtained from the plateaus of the curves in Fig. 2, let us assume that whenever n molecules of PCMBS (C) react with a membrane receptor (M), the channel is closed, i.e.,

$$nC + M(\text{open}) \overset{K}{\rightleftharpoons} C_nM(\text{closed}) \tag{5}$$

where K is the association constant defined as

$$K = (C_nM)/(C)^n(M). \tag{6}$$

Note that the ratio $(C_nM)/(M)$ is simply equal to the ratio of the number of closed to open channels so that[1]

$$\frac{P(0) - P(C)}{P(C) - q} = \frac{(C_nM)}{(M)} = K(C)^n = KC^n. \tag{7}$$

[1] Although expressions (7), (8), and those that follow apply to either osmotic or diffusional permeabilities, the experimental results reported in the remaining parts of this paper are restricted to osmotic measurements.

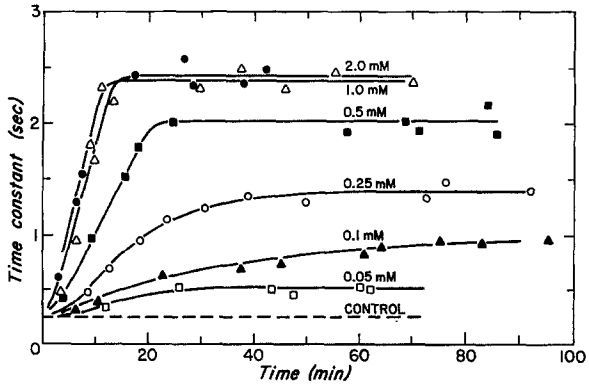

Fig. 2. Kinetics of inhibition of osmotic permeability by PCMBS. Plot of shrinkage time constant (proportional to $1/P_f(0)$) versus time of incubation with PCMBS. The indicated concentration of free PCMBS in the medium at the plateau was verified by titrating the supernatant fluid with GSH. Temperature = 37°C, pH = 7.0. High K suspension medium (mM: 140 KCl, 10 NaCl, 27 sucrose and 3 HEPES buffer) used to maintain constant cell volume after prolonged exposure to PCMBS

Solving Eqs. (6) and (7) for $P(C)/P(0)$ we have[1]

$$\frac{P(C)}{P(0)} = \frac{1 + (q/P(0))KC^n}{1 + KC^n}. \tag{8}$$

Data from Fig. 2 is replotted in Fig. 3 as a function of PCMBS concentration *measured in the medium* during the plateau period. Using a nonlinear least-square procedure, the illustrated data was fit to Eq. (8) yielding a value of 0.94 for n and $K = 39.4\ \text{mM}^{-1}$ ($K_{\text{dissoc}} = 0.025$ mM). Setting $n = 1$ (1 : 1 stoichiometry) and letting only K vary, resulted in the least-squares fit illustrated by the solid curve of Fig. 3. The agreement between empirical data (points) and the predictions of Eq. (8) with $K = 52.6\ \text{mM}^{-1}$ ($K_{\text{dissoc}} = 0.019$ mM) is excellent. Thus the equilibrium data is accurately described by Eq. (5) with a 1 : 1 stoichiometry. Note that the value of $K_{\text{dissoc}} = 0.019$ mM at 37°C is about one order of magnitude smaller than the corresponding constant at room temperature (Macey & Farmer, 1970).

Anomalous Temperature Dependence

The temperature dependence of osmotic water permeability in the PCMBS-inhibited cells is illustrated in Fig. 4. Note the peculiar minimum in the neighborhood of room temperature ($T = 298$K). This behavior can be accounted for by considering that the permeability $P(C)$ indicated in Eq. (8) depends on three factors $P(0)$, q and K, all of which have independent temperature characteristics. In particular,

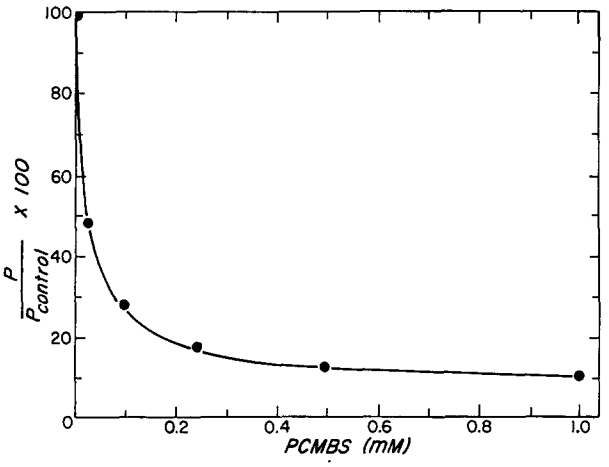

Fig. 3. Dependence of osmotic permeability on concentration of PCMBS. Data taken from plateaus of Fig. 2. Points represent experimental data. The solid continuous curve represents the "best fit" using Eq. (8) with $n = 1$ and $1/K\,K_{\text{dissoc}} = 0.019$ mM

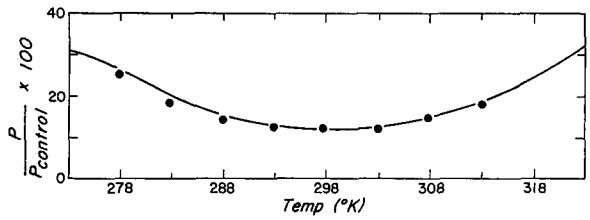

Fig. 4. Temperature dependence of osmotic water permeability in cells equilibrated in 1 mM PCMBS. Theoretical curve calculated from Eq. (12). Experimental conditions same as in Fig. 2

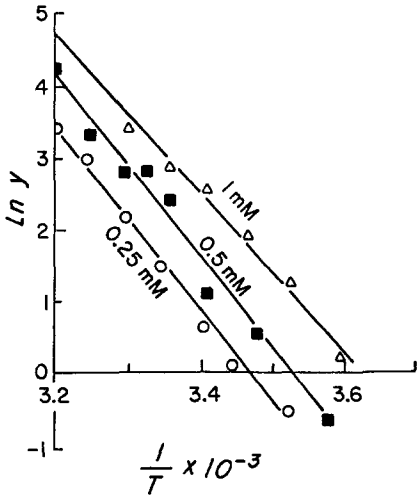

Fig. 5. Plot of ln y *vs.* $1/T$ as suggested by Eq. (12). Straight lines were fit by least squares. Concentrations of PCMBS are indicated on each line. Parameters for each are: △: (PCMBS) = 1 mM, $\Delta H^\circ = 22$ kcal/mol, $\Delta S^\circ = 93$ cal/mol-deg; ■: (PCMBS) = 0.5 mM, $\Delta H^\circ = 25$ kcal/mol, $\Delta S^\circ = 104$ cal/mol-deg; ○: (PCMBS) = 0.25 mM, $\Delta H^\circ = 25$ kcal/mol, $\Delta S^\circ = 102$ cal/mol-deg

if we denote dependence on the absolute temperature by the subscript T, we can write

$$K_T = \exp(-\Delta G^\circ/RT) = \exp[(-\Delta H^\circ/RT) + (\Delta S^\circ/R)] \tag{9}$$

$$\frac{P(0)_T}{P(0)_{298}} = \frac{\exp(-\Delta H^\dagger_{\text{app}}/RT)}{\exp(-\Delta H^\dagger_{\text{app}}/R \cdot 298)} \tag{10}$$

$$\frac{q_T}{q_{298}} = \frac{\exp(-\Delta H^\dagger_L/RT)}{\exp(-\Delta H^\dagger_L/R \cdot 298)} \tag{11}$$

where R is the universal gas constant, $\Delta H^\dagger_{\text{app}}$ is the activation enthalpy for water permeability in the absence of PCMBS, $\Delta H^\dagger_L$ is the activation enthalpy for the PCMBS-insensitive (lipid) path, and ΔG°, ΔH°, and ΔS° are changes in free energy, enthalpy and entropy associated with Eq. (5). Inserting Eqs. (9), (10) and (11) into (7), and taking the log of both sides of the results yields

$$\ln y = [(\Delta H^\circ/R)(1/T)] - (\Delta S^\circ/R) + \ln C \tag{12}$$

where

$$y = \frac{P(0)_{298}\exp\{-\Delta H^\dagger_{\text{app}}[(1/RT) - (1/298R)]\} - P(C)_T}{P(C)_T - q_{298}\exp\{-\Delta H^\dagger_L[(1/RT) - (1/298R)]\}}. \tag{13}$$

Thus, assuming ΔH° and ΔS° are relatively constant over the limited range of T used in these experiments, we may plot y *vs.* $1/T$ at constant C, and recover ΔH° and ΔS° from the slope and intercept. We have carried out this procedure using plateau data similar to that of Fig. 1 but at different values of T. $\Delta H^\dagger_{\text{app}}$ and $\Delta H^\dagger_L$ were assumed to be equal to 4.8 and 11.7 kcal/mol, respectively (Macey et al., 1972). Values for $P(0)_{298}$ and q_{298} were taken directly from the data. Results for these plots are shown in Fig. 5 at three different values of C (PCMBS). The experimental data fall reasonably well on straight lines as predicted by Eq. (12). The facts that the lines are straight and that consistent values for both ΔH° and ΔS° are retrieved from each line, support the assumption that these parameters are relatively constant within the experimental conditions employed.

Discussion

The original findings that $P_f/P_d > 1$ in normal red cells (Paganelli & Solomon, 1957) can be interpreted in terms of water-filled channels in two different ways. In both interpretations, the value de-

pends on the amount of water per channel, but not on N, the number of open channels. Thus the first interpretation assumes large channels of radius r where diffusional permeability is directly proportional to r^2 (cross-sectional area of channel), and to N. The osmotic permeability is also proportional to N, but its dependence on r is stronger than r^2 (e.g., in the case of ideal laminar flow it depends on r^4). Thus the ratio of these two permeabilities will depend on r (the geometry of the channel) but will be independent of N. The second interpretation assumes very narrow channels so that channel water can only move via single-file diffusion. In this case, the ratio of osmotic-to-diffusional permeabilities depends solely on the number of water molecules per channel (Lea, 1963; Dick, 1965; Levitt, 1974; Finkelstein & Rosenberg, 1979).

After subtracting the background bilayer permeability from our observed permeabilities we find that the ratio $g_o = p_f/p_d$ remains invariant as water permeability is progressively inhibited by more and more PCMBS. This implies that when PCMBS acts on a channel it closes completely; the amount of water (or geometry) of each open channel remains invariant, and it is only N, the number of open channels, that changes when PCMBS is added. Further, if there is some statistical distribution of channel sizes (or amount of water per channel), then our results imply that PCMBS attacks these channels at random, i.e., all channels are equally sensitive to PCMBS.

If we assume that the channels are so narrow that single-file diffusion is the prevailing mode of transport, then our measured value $g_o = 10.8$ indicates that there are approximately 11 water molecules per channel. All-or-none closure would be a reasonable expectation for single-file channels.

Calculation of the thermodynamic parameters of ΔH° and ΔS° requires knowledge of the temperature dependence of the water permeability ($P(0)_T$) in the uninhibited membrane. As seen in Eq. (10), this dependence is provided by $\Delta H^\dagger_{app}$ which is simply the empirical slope obtained by plotting ln $P(0)_T$ *vs.* $1/T$. It represents a composite of the activation enthalpies of the channel $\Delta H^\dagger_p$ and the lipid bilayer $\Delta H^\dagger_L$. Considering the expression $P = p + q$ as a function of $1/T$ and expanding both sides in a Taylor's series reveals that

$$\Delta H^\dagger_{\text{app}} = \frac{\Delta H^\dagger_p p + \Delta H^\dagger_L q}{p + q}.$$

The values of ΔH°, ΔS° and ΔG° calculated from measurements of temperature dependence of the PCMBS reaction with water channel control sites yield the following values:

$\Delta H^\circ \approx 25$ kcal/mole

$\Delta S^\circ \approx 100$ cal/mole/°K

$\Delta G^\circ = \Delta H^\circ - TS^\circ \approx -6$ kcal/mole at $T = 37$°C.

Thus the reaction indicated by Eq. (5) is energetically unfavorable (positive ΔH°), but is entropically driven.

If, instead of using the estimates for ΔH° and ΔS°, ΔG° is calculated directly from the equilibrium constant (from Fig. 3), a value $\Delta G^\circ = -6.74$ kcal/mole is obtained. Since ΔG° results from the difference of two large numbers, the discrepancy between the two estimates could easily result from small errors, e.g., a simple 3% increase in the estimate for ΔS° from 100 to 103 cal/mol/°K would be sufficient to change the calculated ΔG° from -6 to -6.93 kcal/mole.

The thermodynamic parameters listed above are not characteristic of simple mercaptide formation. Although the entropy changes are positive and usually rather large ($\Delta S^\circ = 27$ cal/mol-deg for the reaction of Hg^{++} with cysteine; $\Delta S^\circ = 54$ cal/mol-deg for Hg^{++} with glutahione; and $\Delta S^\circ = 68$ cal/mol-deg for Hg^{++} with thyoglycolate), the large negative ΔG° of simple mercaptide formation, ranging between -55 kcal/mol to -59 kcal/mol, necessitates a negative ΔH° (Webb, 1966, p. 749). However, our results refer not only to reaction of the mercurial with receptor site (presumably an SH group) but also to any subsequent changes that are involved in closing the channel. The fact that the values for ΔH° and ΔS° are noncharacteristic of common mercaptide formation should make these parameters particularly useful in attempts to identify the receptor site.

Positive enthalpies and large positive entropies are found in some macroassembly processes which are believed to be driven by hydrophobic interactions (Edelhoch & Osborne, 1976). For example, assembly of microtubules is accompanied by a $\Delta H^\circ = 29$ kcal/mol and $\Delta S^\circ = 100$ cal/mol-deg (Inoue & Sato, 1967). Formation of tobacco mosaic virus double discs (34 subunits) from the trimer species involved a $\Delta H^\circ = 30$ kcal/mol and $\Delta S^\circ = 124$ cal/mol-deg (Banerjee & Lauffer, 1966). Similar results on association of subunits to form flagellin and to form sickle cell hemoglobin fibrils are alluded to by Edelhoch and Osborne (1976, pp. 208–209).

Hydrophobic interactions are entropy driven presumably because water molecules surrounding exposed hydrophobic surface are more limited in their degrees of freedom. Removing a hydrophobic surface out of the water phase (e.g., by folding a side chain into the protein interior) increases the entropy of the surrounding water so that even though ΔH° is usually positive, the large positive ΔS° greatly favors removal of hydrophobic inter-

face. Kauzmann (1959) indicates that for each nonpolar aliphatic side chain that leaves an aqueous environment to enter a nonpolar region, a gain in entropy of the order of 20 cal/mol-deg may be expected.

If we assume the large entropy change is primarily due to hydrophobic interactions, then it follows that channel closure is accompanied by the disarray of ordered water. This is contrary to what would be expected if channel closure were due to the immobilization or "freezing" of water in the channel. Although it is tempting to associate the inferred changes in water structure with water lying within the channel, there is no evidence that this must be so.

Throughout this paper we have presumed that following maximal inhibition by PCMBS, water permeation takes place exclusively through the lipid bilayer portions of the membrane. Evidence in support of this has been alluded to in the Introduction. However, it should be noted that the validity of our results and conclusions are not dependent on this assumption. In this context, a more precise designation for the "lipid path" would be the PCMBS-insensitive pathway. The PCMBS-sensitive pathway has characteristics commonly attributed to channels; the PCMBS-insensitive path does not.

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