WATER PERMEABILITY OF THE CHROMAFFIN GRANULE MEMBRANE

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ABSTRACT NMR spin-lattice relaxation rates of solvent protons have been used to measure the water permeability coefficient of the chromaffin granule membrane. The technique involves labeling the chromaffin granule interior with Mn^{+2}, which provides an efficient relaxation pathway for intravesicular solvent protons. Added Mn^{+2} spontaneously accumulates in the chromaffin granule matrix in the presence of the divalent cation-specific ionophore A23187 and is maintained against a large concentration gradient. In this way, the internal proton relaxation rate is readily augmented to values some 10^2–10^3 times greater than that in the extravesicular water space. Transmembranal water transport permits solvent protons in the extravesicular water space, in which most of the observed NMR signal orginates, to sample the highly relaxive environment of the chromaffin granule matrix. By this process, water permeation shortens the observed relaxation rate. The diffusive water permeability coefficient of the chromaffin granule membrane has been measured over the temperature range 0–38°C. The permeability coefficient measured at 25°C is comparable to a previously reported value for planar lipid bilayers composed of ox brain lipids and cholesterol ($P_d \approx 0.37$–0.53 (10^{-3}) cm·s^{-1} at 25°C) but is substantially less than values for the plasma membranes of erythrocytes and *Chlorella*. Hypothesized hydrophilic "pores," thought to provide parallel permeation pathways in the latter membranes, appear to be absent in chromaffin granule membranes. The water permeation rate exhibits Arrhenius temperature behavior and does not reflect a phase transition at 32°–34°C observed previously in ESR spin-label studies of chromaffin granule ghosts.

INTRODUCTION

Chromaffin granules are the storage site of epinephrine in the adrenal medulla and are close structural and functional analogues of neurotransmitter storage vesicles. These organelles are limited by a bilayer membrane that is rich in phospholipids and cholesterol (Winkler et al., 1970; Winkler, 1976). This communication describes the application of proton NMR relaxation enhancements to the determination of the diffusive water permeability coefficient, P_d, of the chromaffin granule membrane.

Water permeation rates have not previously been measured for membranes of intact subcellular organelles, although a substantial literature exists concerning water permeance through simple lipid bilayers (Finkelstein and Cass, 1968; Finkelstein and Cass, 1967; Lipschitz-Farber and Degani, 1980; Andrasko and Forsén, 1974, Haran and Shporer, 1976), through the chloroplast thylakoid membrane (Sharp and Yocum, 1980*b*), and through plasma membranes of whole cells (Stout et al., 1978; Gutknecht, 1968; Dick, 1966; Conlon and Outhred, 1972; Shporer and Civan, 1975; Morariu and Benga, 1977; Conlon and Outhred, 1978; Andrasko, 1976; Viera et al., 1970; Fabry and Eisenstadt, 1975). An interesting result from these studies is that plasma membranes are often more highly permeant to water than would be expected from simple diffusion through the lipid phase, and the permeability is sensitive to protein-modifying reagents (Conlon and Outhred, 1978; Macey et al., 1973). These and other observations (Morariu and Benga, 1977; Conlon and Outhred, 1978; Macey et al., 1973) have supported the concept that plasma membranes contain hydrophilic proteinaceous channels which facilitate the transmembrane movement of water. The present experiments examine the permeation kinetics of water through the membrane of intact chromaffin granules. Comparison of these results with previous data on artificial lipid bilayers and on plasma membranes does not support the hypothesis that hydrophilic permeation pathways exist in the chromaffin granule membrane.

The experimental technique for measuring water permeation rates is based on a previous observation (Sharp and Sen, unpublished results) that chromaffin granules, in the presence of added Mn^{+2} and the divalent cation-specific ionophore A23187, strongly accumulate Mn^{+2} in the intravesicular water space. This accumulation is driven by the internal cation binding capacity of the chromaffin granule matrix and is facilitated by the resting pH gradient. Mn^{+2} provides an extremely efficient pathway for magnetic relaxation of the solvent protons. The accumulation of Mn^{+2} inside chromaffin granules provides a means of labeling the intravesicular water space as a region of greatly enhanced relaxation rate. The solvent proton

NMR signal in the present experiments derives most of its intensity from water molecules in the extravesicular space. The intrinsic relaxation rate of water in this space is relatively low (in the presence of Mn^{+2}/A23187) because of the nearly total intravesicular accumulation of Mn^{+2}. Water exchange across the membrane enhances the proton relaxation rate because external water protons experience a highly efficient paramagnetic relaxation pathway as they enter the internal environment. By varying the temperature and the internal Mn^{+2} concentration, an accurate determination of the water permeability coefficient is possible.

MATERIALS AND METHODS

Chromaffin granules were isolated from bovine adrenal glands by means of an isotonic sucrose-Ficoll-D_2O gradient (Trifaro and Dworkind, 1970). Before use in NMR experiments, the pure chromaffin granule pellets were washed once by resuspension in 0.31 M sucrose and 10 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES), pH 7.0, followed by centrifugation at 35,000 *g* for 20 min. Washed granules were kept at 4°C as a concentrated suspension (0.5 g wet pellet/ml) in 0.31 M sucrose - 10 mM BES, pH 7.0. Under these conditions, the granules were found to undergo negligible lysis over 36 h as monitored by ^1H NMR. Where indicated, chromaffin granules were preincubated in 20 μM A23187 (Calbiochem-Behring Corp. San Diego, CA) for 10 min at 37°C. Washed membranes were prepared by diluting the concentrated chromaffin granule suspension 1:3 in a buffer containing 0.145 M K_2SO_4, 10 mM BES, pH 7.0, and freezing and thawing five times in liquid nitrogen. This suspension was pelleted at 100,000 *g* for 1 h, and the pellet was resuspended at 2.7 mg protein/ml in the K_2SO_4/BES buffer.

NMR Measurements

Spin-lattice relaxation times $T_1 \equiv R_1^{-1}$ of the solvent water protons were measured at 20.7 MHz using the modified triplet sequence (Sharp, 1972; Sharp and Yocum, 1980*a*: $180_o-[\tau_1-(90_o-\tau_2-180_x-\tau_2-90_o)]_n$. Following the initial 180 pulse, each triplet samples, and restores, the z-component of the magnetization. This sequence determines T_1 in the course of a single decay and permits the time course of T_1 changes to be monitored as illustrated in Fig. 3. For all samples studied, $T_1 > T_2$ ($\leq$ 2 ms) $>> \tau_2$(0.1 ms); under these conditions the modified triplet sequence is highly accurate. The NMR spectrometer (Bruker Instruments, Inc., Billerica, MA, model B/KR 322s) and ^7Li field-lock have been described elsewhere (Sharp and Yocum, 1980*a*). Data were digitized, stored and analyzed in a Commodore Model 2001 microcomputer. Temperature control within ±0.5°C was achieved using a Bruker B-ST 100/700 temperature controller.

Data Analysis

For the purpose of describing chemical exchange effects on the proton resonance of water, the chromaffin granule suspension is separated conceptually into two aqueous domains, the intravesicular (i) and extravesicular (o) water spaces. Self-diffusion of water within either of these domains is assumed to be rapid on the NMR time scale (the validity of this point is discussed by Sharp and Yocum, 1980*b*. Each water space is characterized by a proton spinlattic relaxation rate, $R_{1,i}$ or $R_{1,o}$, and is occupied by a specified fraction, f_i or f_o, of solvent molecules. In the absence of transmembrane chemical exchange, the z-component of the proton magnetization M_z following a 180° pulse is the sum of two exponentially decaying components:z $M_z(t) = M^o_{z,i}(1-2e^{-R_{1i}t}) + M^o_{z,o}(1-2e^{-R_{1o}t})$. Chemical exchange of water protons across the membrane transfers magnetization between the two water spaces, and, when $R_{1,i} \neq R_{1,o}$, this process modifies the decay rate to

$$R_\pm = (1/2)\,[R_{1,o} + \tau_o^{-1} + R_{1,i} + \tau_i^{-1}] \pm (1/2)\,[(R_{1,i}-R_{1,o})^2 + 2(R_{1,i}-R_{1,o})(\tau_i^{-1}-\tau_o^{-1}) + (\tau_i+\tau_o)^2]^{1/2}. \quad (1)$$

In the present experiments, most of the observed magnetization arises from ^1H_2O in the external space ($f_o/f_i \cong 40$). The intravesicular water space, while relatively small, is characterized by a relatively high relaxation rate ($R_{1,i}/R_{1,o} \sim 10^3$) due to the presence of sequestered Mn^{+2}. With internal Mn^{+2} concentrations in the range 2–20 mM (corresponding to external Mn^{+2} concentrations in the 0.05–0.50 mM), the relaxation rate of water in the intravesicular space exceeds that in the buffer by factors in the range 10^2–10^3.

Thus the chromaffin granule suspension in the presence of A23187 plus added Mn^{+2} can be described, from the standpoint of proton relaxation, as a two-site system where the internal site has low capacity and high relaxivity. In this situation, Eq. 1 reduces to (Luz and Meiboom, 1964)

$$R_1 = R_{1,o} + f_i(R_{1,i}^{-1} + \tau_i)^{-1}. \quad (2)$$

In our experiments, $R_{1,i}$ is a linear function of the internal Mn^{+2} concentration and can be written

$$R_{1,i} = R_{1,B} + R_M[Mn^{+2}]_i = R_{1,B} + R_M[Mn^{+2}]_A f_i^{-1}$$

where $R_{1,B}$ is the internal relaxation rate when $[Mn^{+2}]_i = 0$, and $[Mn^{+2}]_A$ is the average concentration of added Mn^{+2}. R_M is calculable in principle from the Solomon-Bloembergen-Morgan equations (Dwek, 1975). Thus Eq. 2 becomes

$$R_1 - R_{1,o} = f_i\{(R_{1,B} + R_M[Mn^{+2}]_A f_i^{-1})^{-1} + \tau_i\}^{-1}$$

or

$$(R_1 - R_{1,o})^{-1} = (f_i R_{1B} + R_M[Mn^{+2}]_A)^{-1} + \tau_i/f_i. \quad (3)$$

A plot of $(R_1-R_{1,o})^{-1}$ vs. $[Mn^{+2}]_A$ approaches linearity in the limit $[Mn^{+2}]_A >> (R_{1,B}\, f_i/R_M)$. Extrapolation to $[Mn^{+2}]_A = 0$ gives an intercept equal to τ_i/f_i, from which the internal residence time, τ_i, can be determined. Alternatively, an iterative multiparameter fit to Eq. 3 is possible. Both methods are used in the present work. The permeability coefficient for diffusion of water through the chromaffin granule membrane is given by $P_d = (V - V_{ex})/3\tau_i A$, where V, V_m are the total and excluded volumes and A is the surface area. Neglecting excluded volumes, $P_d \cong \bar{r}/3\tau_i$, where $\bar{r} \equiv \langle r^3\rangle/\langle r^2\rangle$ is (approximately) the mean chromaffin granule radius (115 nm [Coupland, 1968]).

Theoretical plots illustrating the qualitative behavior of R_1 vs. τ_i for various ratios of $R_{1,i}/R_{1,o}$ are shown in Fig. 1. In the limit of very slow exchange, $R_1 \approx R_{1,o}$. At the opposite extreme ($\tau_i \to 0$), R_1 approaches a weighted average of internal and external relaxation rates, $R_1 \approx (f_i R_{1,i} + f_o R_{1,o})$. Data in these limiting regions do not contain information about the exchange kinetics, but can be used to determine the internal and external relaxation rates, $R_{1,i}$ and $R_{1,o}$, if f_i is known. In the region of intermediate exchange rates, R_1 increases with increasing temperature (i.e., with decreasing τ_i). This temperature dependence is normally opposite that of $R_{1,i}$ and $R_{1,o}$, and can lead to the appearance of two extrema, a local maximum and a local minimum, in a plot of R_1 vs. temperature. These extrema are quite useful in recognizing the existence of a dominant chemical exchange contribution to R_1. This behavior is not entirely unambiguous evidence of chemical exchange processes, however, since the paramagnetic contribution to R_1 does not vary montonically with temperature. In appropriate circumstances, the dipolar term in the paramagnetic Hamiltonian can produce extrema in R_1 that are superfi-

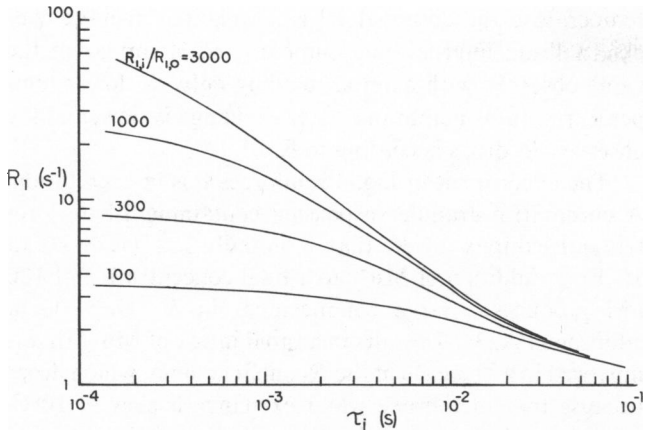

FIGURE 1 Theoretical temperature dependence of the spin-lattice relaxation rate, R_1, of a nuclear spin undergoing chemical exchange between a high capacity environment, o, and a low capacity environment, i. The low capacity environment is characterized by low fractional population ($f_i << f_o$) and efficient spin-lattice relaxation ($R_{1,i} >> R_{1,o}$). τ_i is the mean residence time in the low-capacity site. These plots assume $f_i = 0.025$ and $R_{1,o} = 1\ s^{-1}$.

cially similar to the effects produced by chemical exchange (see Dwek [1975] for a fuller discussion of this point). The paramagnetic part of R_1 is closely linear in the concentration of paramagnetic ions, whereas the chemical exchange contribution to R_1 is highly nonlinear in ion concentration in the intermediate or slow exchange regions (see Fig. 1). The qualitative temperature and concentration dependence of the chemical exchange contribution is quite distinctive and clearly identifies the region of influence of chemical exchange effects on the relaxation rate.

RESULTS

Mn^{+2}, when added either to the buffer or to a chromaffin granule suspension, produces a large paramagnetic increment in R_1 of the solvent protons. The former effect is shown in Fig. 2, where 100 μM Mn^{+2} is seen to raise R_1 of the buffer by a factor of ~3. In the presence of chromaffin granules, the increment is much larger (Fig. 3, ▼), about a factor of 10, although the precise increment is variable to a degree (see below). Mn^{+2}, like other di- and trivalent cations, adsorbs to the chromaffin granule membrane (Morris and Schober, 1977; Siegel et al. , 1978) and, in bound form, exhibits increased efficiency as a relaxation-producing agent of solvent protons. Surface binding greatly lengthens the reorientational correlation time of water molecules in the Mn^{+2} hydration sphere and results in an enhanced molar relaxivity as described by the Solomon-Bloembergen-Morgan equations (Dwek, 1975). Similar effects of surface binding on the molar relaxivity of Mn^{+2} in solutions of chloroplast thylakoid membranes have been described elsewhere (Sharp and Yocum, 1980*a*). Adsorbed Mn^{+2} equilibrates rapidly with the aqueous phase and can be displaced by added EDTA. The molar relaxivity of the Mn^{+2}·EDTA chelate is small compared with that of membrane-bound Mn^{+2} or of hexaquo-Mn^{+2} (Fig. 2, ●), and thus EDTA addition largely suppresses the Mn^{+2}-induced paramagnetic relaxation enhancement (Fig. 3, ▽ vs. ▼).

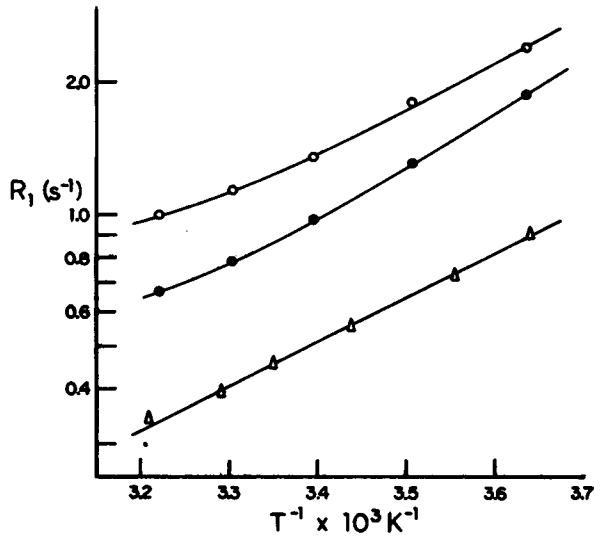

FIGURE 2 Temperature dependence of R_1 of the buffer (△), of the buffer plus 100 μ M $MnSO_4$ (○), of the buffer plus 100 μM $MnSO_4$ plus 1 mM EDTA (●).

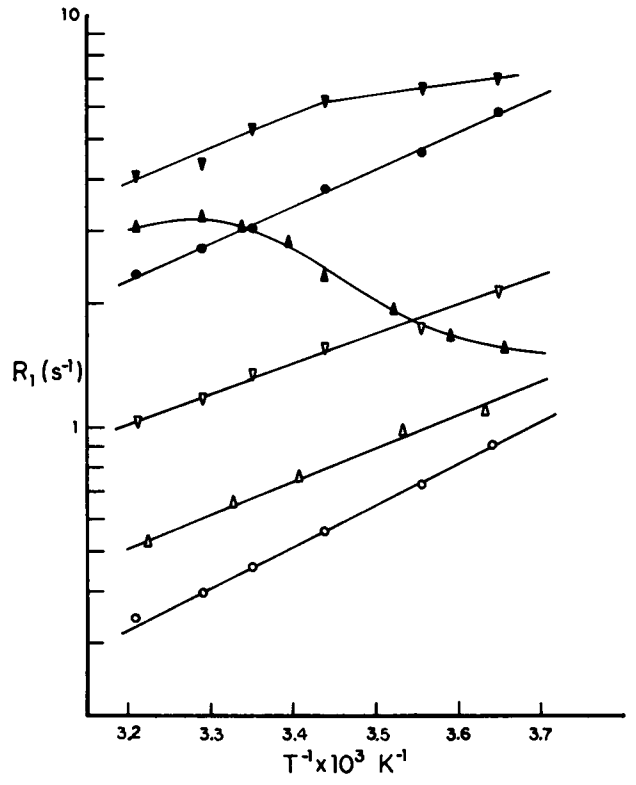

FIGURE 3 Temperature dependence of the proton spin-lattice relaxation rate of chromaffin granule suspensions and of the resuspending buffer. The curves, in ascending order, correspond to the following suspensions: (○), resuspension buffer (I); (△), washed membranes (2.7 mg protein/ml) suspended in buffer I; (▽), chromaffin granules (11.5 mg protein/ml in buffer I) plus 100 μM $MnSO_4$ and 1 mM EDTA; (▲), same as (▽) with 20 μM A23187 in place of EDTA; (▼), chromaffin granules (11.5 mg protein/ml in buffer I) plus 100 μM $MnSO_4$); (●) same as (▼) after five freeze-thaw cycles.

When chromaffin granules containing 100 μM Mn^{+2} (without EDTA) are intentionally lysed by several freeze/thaw cycles, the R_1 drops substantially (Fig. 3, ●). This drop probably reflects the ability of liberated ATP to bind Mn^{+2}, partially displacing it from the membrane into the aqueous medium in the form of a low molecular weight complex with relatively short reorientational correlation time. In this form, as is true for the Mn^{+2}·EDTA complex, the paramagnetic relaxivity is relatively low.

In the case of both lysed and intact granules, the R_1 enhancement increases strongly with decreasing temperature. In contrast, addition of the divalent cation-specific ionophore, A23187, to Mn^{+2}-containing chromaffin granule suspensions leads to the opposite temperature dependence: at low temperatures the R_1 enhancement is suppressed (Fig. 3, ▲). In a number of topological experiments to be reported elsewhere, we have shown that this behavior results from the fact that the added Mn^{+2} is strongly accumulated in the chromaffin granule matrix. Mn^{+2} sequestration inside chromaffin granules lowers the paramagnetic relaxation rate, $R_{1,o}$, in the extravesicular water space and greatly enhances the relaxation rate, $R_{1,i}$, of the matrix. At sufficiently high temperature ($T \gtrsim 30$°C in Fig. 3), water exchange across the membrane is rapid. At this temperature the observed R_1 is a weighted average over external and internal environments and is similar to the value observed with external binding only. At lower temperatures, transmembrane water exchange is slow, and the observed R_1 drops according to Eq. 3.

The experiment in Fig. 4 reinforces this interpretation. A chromaffin granule suspension containing 14 mg protein/ml exhibits an R_1 that is initially low (1.38 s^{-1} at 10.5°C). Addition of Mn^{+2} to a final concentration of 100 μM produces a large enhancement in R_1. Subsequent addition of A23187 results in a rapid influx of Mn^{+2} that is mirrored in a decrease in the R_1 enhancement, which drops because transmembrane water exchange is slow at 10°C. Intentional lysis, induced by several freeze-thaw cycles, releases the soluble contents including incorporated Mn^{+2} and results in an abrupt enhancement of R_1 to a value similar to that seen before A23187 addition (compare with Fig. 3). Subsequent addition of EDTA rapidly suppresses the R_1 enhancement. When the chromaffin granules are initially lysed by several freeze-thaw cycles before Mn^{+2}/A23187 addition, the uptake process seen in Fig. 4 does not occur (data not shown). If, in an experiment like that of Fig. 3, EDTA is added before freezing and thawing, EDTA has no effect on R_1 (data not shown). Thus the sequestration of Mn^{+2} in the presence of A23187 is essentially complete.

Fig . 5 shows the variation of R_1 of water protons with

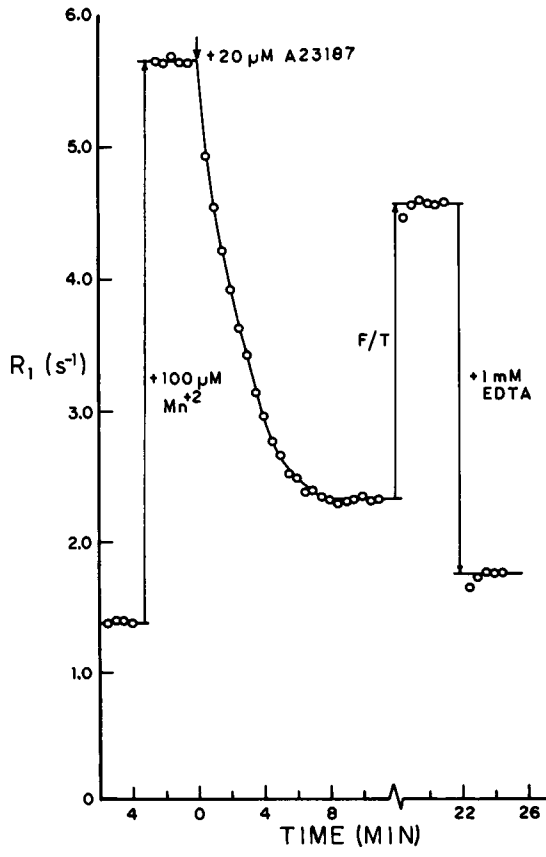

FIGURE 4 Changes in the proton spin-lattice relaxation rate of a chromaffin granule suspension (14 mg protein/ml) following the indicated additions or procedures. F/T indicates seven freeze-thaw cycles. Final concentrations are given. T = 10.5°C.

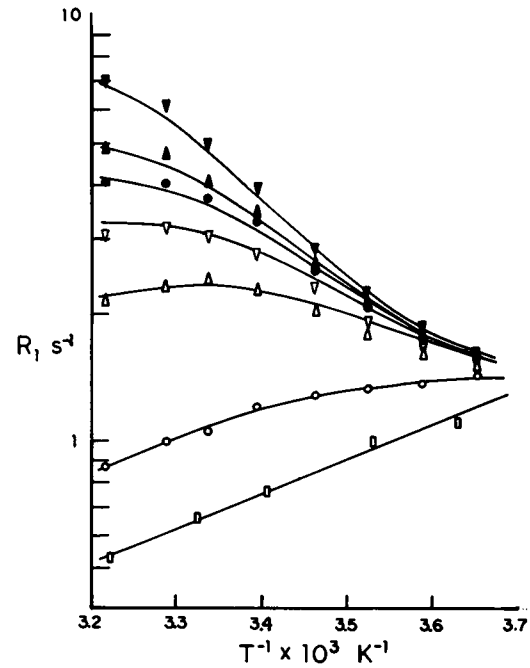

FIGURE 5 Temperature dependence of R_1 in the chromaffin granule suspension containing 11.5 mg protein ml^{-1} plus 20 μM A23187 and the indicated Mn^{+2} concentrations. Mn^{+2} concentrations, from top to bottom, are (▼), 400 μM; (▲), 200 μM; (●), 150 μM; (▽), 100 μM; (△), 50 μM; (○), no Mn^{+2}. (□) refers to washed chromaffin granule membranes (2.7 mg protein/ml). Solid lines are plots of Eq. 3 using parameters listed in Table I.

inverse temperature for a chromaffin granule suspension (11 mg protein/ml) containing A23187 and various concentrations of added Mn^{+2}. The background R_1 in a suspension of washed membranes is shown for comparison (□). In this latter suspension, the total protein concentration was equivalent to that of membrane protein in the suspensions of intact chromaffin granules, assuming that membrane-bound protein constitutes 23% of the total protein (Winkler, 1976).

The qualitative temperature and concentration dependence of R_1 in the Mn^{+2}-containing solutions is indicative of a dominant chemical exchange contribution to R_1 (see Fig. 1). Specifically, R_1 is an increasing, but very nonlinear, function of Mn^{+2} concentration at fixed temperature. Approximate values of τ_i were obtained from plots of Eq. 3. Typical plots are shown in Fig. 6; τ_i was calculated from the intercepts assuming an internal aqueous volume of 3.6 $\mu l \cdot (mg\ protein)^{-1}$ (Holz, 1979). A plot of τ_i vs. inverse temperature is shown in Fig. 7 (O---O). The kinetics of transmembrane water transport follows Arrhenius temperature dependence between 0° and 38°C with an activation energy of 14.7 kcal/mol.

A more accurate simultaneous fit of the data at all temperatures to the full nonlinear Eq. 3 was undertaken assuming Arrhenius temperature dependence of the intravesicular relaxation rates, $R_{1,i}$ and R_M, as well as of τ_i:

$$R_{1,i} = R^{\circ}_{1,i} \exp(E_i/RT) \tag{4a}$$

$$R_M = R^{\circ}_M \exp(E_M/RT) \tag{4b}$$

$$\tau_i = \tau_i^{\circ} \exp(E_d/RT). \tag{4c}$$

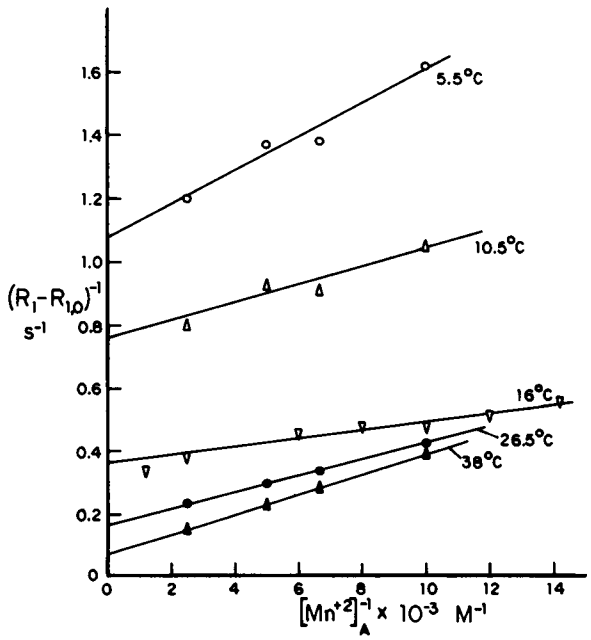

FIGURE 6 Plots of Eq. 3 in the region of high $[Mn^{+2}]_A (\geq 75\ \mu M)$. The chromaffin granule suspension contained 11.5 mg protein/ml plus 20 μM A23187 and the indicated average concentration of added Mn^{+2}.

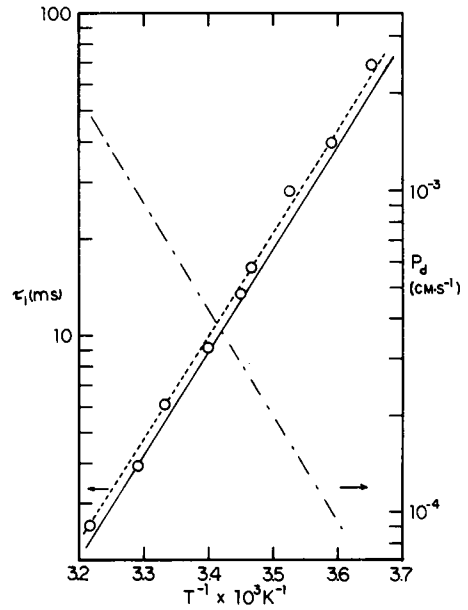

FIGURE 7 Plot of τ_i (the mean residence time of a water molecule inside a chromaffin granule) and P_d, the diffusive water permeability coefficient vs. inverse temperature. Circles and dashed line are values derived from intercepts of plots like those in Fig. 5. Solid line is the result of an iterative fit to the data in Fig. 4. Dash-dot (—·—) line is the diffusive water permeability coefficient.

The data were iterated on the six parameters, τ_i°, E_d, $R^{\circ}_{1,i}$, E_i, R°_M E_M. The resulting theoretical curves are given as solid lines in Fig. 5, and the iterated parameters are listed in Table I. The data conform well to theory, confirming not only that transmembrane water exchange exerts a dominant influence on R_1, but also that Mn^{+2} is strongly accumulated inside chromaffin granules against a concentration gradient in the presence of A23187. The assumption that R_M conforms to Arrhenius temperature dependence appears to be accurate, although such simple temperature dependence is not required by the Solomon-Bloembergen-Morgan theory (Dwek, 1975).

The iterated values of τ_i are shown as the solid line in Fig. 7. The fact that τ_i values obtained from the extrapolation procedure deviate from values obtained by the more accurate procedure of iterating Eq. 3 is expected because Eq. 3 is linear in $[Mn^{+2}]^{-1}$ only in the limit $[Mn^{+2}]^{-1} \rightarrow 0$. At higher $[Mn^{+2}]^{-1}$, the slope drops, thus tending to

TABLE I
PARAMETERS DERIVED FROM AN ITERATIVE FIT OF THE DATA IN FIG. 4 TO EQ. 3 OF THE TEXT. PARAMETERS ARE DEFINED BY EQUATIONS 4A–C.

Parameter	Value	Activation energy	Value (kcal/mol)
τ_i°	$1.08\ (10^{-13})s$	E_d	14.7
$R^{\circ}_{1,i}$	$2.76\ (10^{-3})\ s^{-1}$	E_i	2.96
R°_M	$0.121\ s^{-1} \cdot mM^{-1}$	E_M	3.44

overestimate τ_i. Nevertheless, the error is minor, and plots neglecting $R_{1,B}$ give τ_i to within ~10%.

In the course of these studies, variations in the magnitude of R_1 were observed in similarly prepared samples that were too large to attribute to differences in protein concentration or to lysis. In particular, slow increases in R_1 (over a period of minutes) frequently occurred during incubation of samples at 37°C. These variations reflect the fact that R_1 is a complex quantity and is influenced by all processes that alter the surface binding of Mn^{+2}. In the case of external Mn^{+2}, $R_{1,0}$ has been found to be a sensitive function of ionic strength and of pH, and it is also to be expected that it will depend on more subtle processes that influence surface charge density, such as, for example, the slow flip-flop reorientation of lipids. In the presence of internally sequestered Mn^{+2}, $R_{1,i}$ is strongly dominated by the two competing factors of transmembrane chemical exchange rate and the intravesicular paramagnetic relaxation rate. It was evident that R_1 variations observed after addition of A23187 were monitoring variations in either or both of these quantities. We particularly wished to examine whether the water exchange kinetics were undergoing variations due to some unknown process in the membrane. For this purpose, two different preparations which exhibited approximately maximal variations in R_1 were subjected to parallel sets of measurements as a function of temperature and Mn^{+2} concentration. These samples were preincubated at 37°C for 5–10 min in the presence of A23187 and Mn^{+2} to allow slow changes of the type described above to reach steady-state. Analysis of the two sets of data resulted in water permeability coefficients that differed by ~30% at 25°C. The lowest measured permeability corresponds to the data shown in Figs. 5 and 7 and gave values of $P_d = 0.37\ (10^{-3})$ cm·s^{-1} (25°C) and E_d = 14.7 kcal/mol. The preparation with the highest permeability gave values of $P_d = 0.53$ cm·s^{-1} and E_d = 12 kcal/mol. Thus the temporal variations observed in R_1 appear to reflect changes in both $R_{1,i}$ and in the water permeation rate. Changes in the latter do not appear to be major.

DISCUSSION

The diffusive water permeation coefficient, P_d, of the chromaffin granule membrane is given as a function of temperature in Table I and Fig. 7. These values are calculated from Eq. 3 using Coupland's (Coupland, 1968) estimate for the average chromaffin granule radius derived from electron microscopic studies: $\bar{r}$ = 115 nm. The uncertainty in the absolute value of P_d is believed to be ± 20%, due primarily to uncertainty in the average radius. Relative values of P_d are determined much more accurately (±5%) by the fitting procedure.

The preparative procedure we have used (Ficoll/D_2O density step) produces chromaffin granule pellets that are lightly contaminated with mitochondria (Trifaró and Dworkind, 1970). Mitochondrial contamination is probably not a significant source of error in the present experiments, however, because the Mn^{+2} relaxation label is selectively accumulated by ATP in the chromaffin granule matrix and is maintained against a large concentration gradient. Mitochondria accumulate Mn^{+2} in a process driven by ATP hydrolysis (Pressman, 1970), but it seems unlikely that significant uptake will occur in the absence of added ATP and in the presence of A23187. Mitochondria lack an obvious binding matrix that could compete with the high internal nucleotide levels of chromaffin granules. Furthermore, the basic internal pH of mitochondria would tend to expel Mn^{+2} in the presence of A23187, which exchanges 1 Mn^{+2} for 2H^+.

Comparative Water Permeance of Artifical and Natural Membranes

The diffusive water permeability coefficient has been determined for a variety of artificial lipid membranes, both planar (Finkelstein and Cass, 1968; Finkelstein and Cass, 1967) and vesicular (Lipschitz-Farber and Degani, 1980; Andrasko and Forsén, 1974; Haran and Shporer, 1976), as well as for plasma membranes of erythrocytes (Conlon and Outhred, 1972; Shporer and Civan, 1975; Morariu and Benga, 1977; Conlon and Outhred, 1978; Andrasko, 1976; Viera et al., 1970; Fabry and Eisenstadt, 1975; Macey et al., 1973) and nucleated cells (Stout et al., 1978; Gutknecht, 1968; Dick, 1966). Plasma membranes generally exhibit high water permeation rates with rather low activation energies relative to the background rate expected for water diffusion through a lipid bilayer. In addition, the permeability coefficient for diffusive water flux through plasma membranes reportedly differs significantly from the permeability coefficient measured under conditions of osmotic stress (Dick, 1966). These findings have supported the hypothesis that plasma membranes contain water-permeant proteinaceous pores. "Equivalent pore radii" of 4.–4.5 Å for human erythrocytes and 5.–6.2 Å for dog erythrocytes have been calculated from the hydraulic permeability (Viera et al., 1970).

This paper reports the first diffusive water permeability coefficients of an osmotically tight subcellular organelle. It is of interest to consider whether water permeation of the chromaffin granule membrane has similar properties to diffusive flow through simple lipid phases or whether the permeation properties support the existence of parallel permeation pathways.

A representative summary of water permeability coefficients for several artificial lipid membranes of erythrocytes and *Chlorella pyrenoidosis* is given in Table II. Older data for plasma membranes have been collected by Dick (1966) and show wide variations. Finkelstein and Cass (1968) have criticized these earlier experiments in which the importance of well-stirred surface layers was poorly understood. Table II summarizes only NMR measurements, which are not subject to this criticism, and some of the

TABLE II

DIFFUSION PERMEABILITY COEFFICIENTS (P_d) OF WATER ACROSS ARTIFICIAL AND NATURAL MEMBRANES.

Lipid	Temp °C	$P_d \times 10^3$ $cm\text{-}s^{-1}$	E_A kcal	Method
Planar bilayers				
egg lecithin	36	4.2*	—	Osmotic flow (1)**
egg lecithin cholesterol (50 mol%)	36	2.6*	—	Osmotic flow (1)
hydrogenated egg lecithin	36	1.7*	—	Osmotic flow (1)
ox brain lipids plus α-d,l-tocopherol plus cholesterol	36	1.0	—	Osmotic flow (2)
Vesicles				
dipalmitoyl phosphatidylcholine	25	0.34	19.3	NMR (^{17}O, ^1H) (3)
	25	0.26	15	NMR (^1H) (4)
egg lecithin	25	2.9	10.5	NMR (^{17}O, ^1H) (3)
egg lecithin— cholesterol (2:1 Wt%)	25	1.0	12 ± 2	NMR (^{17}O, ^1H) (5)
Chromaffin granule membrane	25	0.37–0.53	12.0–14.7	NMR (^1H) (this work)
	36	0.63‡		
Erythrocyte membrane	25	3.4‖	$6.7^{\S}_{(a)}$, $4.2^{\S}_{(b)}$	NMR (^1H) (6)
	25	3.9‖,¶	$7 \pm 1^{\S}_{(a)}$	NMR (^1H) (7)
	24	2.2‖	—	NMR (^1H) (8)
	25	2.2‖	$8.7^{\S}_{(a)}$	NMR (^{17}O) (9)
	25	2.8‖	$6^{\S}_{(c)}$	^3H-diffusion (10)
Chlorella plasma membrane	20	2.1	—	NMR (^1H) (11)

*Osmotic permeability coefficient.

‡Suspension with the lowest measured permeability.

§Biphasic: values refer to the high (*a*) ($T > 23$°C), low (*b*) ($T < 20$°C) or average (*c*) over the full temperature range.

‖Calculated from τ_i assuming $V/A = 0.37$ μm (Conlon and Outhred, 1978).

¶Calculated from the value cited at 37°C assuming $E_A = 7$ kcal/mol.

**The references for Table II are (1) Finkelstein and Cass, 1968; (2) Finkelstein and Cass, 1967; (3) Lipschitz-Farber and Degani, 1980; (4) Andrasko and Forsén, 1974; (5) Haran and Shporer, 1976; (6) Conlon and Outhred, 1978; (7) Morauiu and Benga, 1977; (8) Andrasko, 1976; (9) Shporer and Civan, 1975; (10) Viera et al., 1970; (11) Stout et al., 1978.

more recent ^3HHO diffusion measurements in which careful attention was given to the problem of surface stirring. Where comparisons can be made, there is approximate consistency among NMR measurements from different laboratories, even where quite different experimental approaches have been used. ^3HHO diffusion measurements for planar bilayers seem to be comparable to NMR measurements on vesicles of the corresponding lipids, and there is reasonable agreement between the two techniques when applied to erythrocytes. Significant variation is seen among the NMR results reported by the four laboratories that have studied erythrocytes. The two most recent studies (Morariu and Benga, 1977; Conlon and Outhred, 1978) have considered the reasons for these discrepancies in some detail, and have favored values in the range $P_d = 3-4 \times 10^{-3}$ cm·s^{-1} at 37°C.

Among simple lipid membranes, dipalmitoylphosphatidylcholine (DPPC) vesicles exhibit the lowest water permeability, $P_d \cong 0.3$ (10^{-3}) cm·s^{-1} at 25°C. Considerable variability among different lipids is evident, however. P_d for membranes composed of egg lecithin or egg lecithin-cholesterol mixtures are 5 to 10 times greater than values for DPPC membranes. The presence of cholesterol in the mixture markedly reduces the permeability coefficient (Finkelstein and Cass, 1968).

P_d for the chromaffin granule membrane is similar to the value for DPPC vesicles and is remarkably low compared with the value for lecithin-containing membranes or plasma membranes. Perhaps surprisingly, the values are also smaller than those for planar membranes composed of ox brain lipids plus d,lα-tocopherol and cholesterol. This lipid composition is probably not dissimilar to that of the chromaffin granule membrane, which is comprised primarily of phospholipids and cholesterol in a molar ratio of 1.0:0.6 (Dreyfus et al., 1977; Winkler and Westhead, 1980). The plasma membranes of *Chlorella* and erythrocytes have 2–3 times and 3–5 times, respectively, the water permeability of the chromaffin granule membrane.

Taken together, these data suggest that the background diffusive flux of water through the lipid phase can account

quite well for the water permeability properties of the chromaffin granule membrane. The presence of parallel proteinaceous channels for water flow is supported neither by the absolute magnitude of P_d, which is very close to the value expected for diffusive flux through the lipid layer, nor by the highly Arrhenius temperature dependence of P_d (see Fig. 6). It has been pointed out previously (Conlon and Outhred, 1978; Macey et al., 1973) that parallel diffusive paths are likely to lead to markedly non-Arrhenius temperature behavior of P_d, because the activation energy for the diffusion of water through lipids typically exceeds that of water self-diffusion in aqueous media by a factor of 2–4. E_A for water diffusion through simple lipid membranes is typically 10–20 kcal/mol (Table I), whereas E_A = 4–5 kcal/mol for the self diffusion of water in aqueous media (Simpson and Carr, 1958) or water adsorbed to hydrophilic surfaces (Morariu and Benga, 1977). Thus, parallel permeation pathways probably give rise to non-Arrhenius temperature dependence in P_d with apparent activation energies between those limits. Such behavior is in fact characteristic of erythrocyte membranes (Conlon and Outhred, 1978). In contrast, the water permeability of the chromaffin granule membrane is highly Arrhenius in character and has an activation energy indicative of water diffusion through lipid phases. Thus the data do not support the existence of multiple permeation pathways and are quite consistent with the expected properties of simple diffusion through the lipid bilayer.

Phase Transitions in the Chromaffin Granule Membrane

In artificial lipid membranes P_d is a very sensitive probe of phase changes in the lipid phase. A dramatic example of such effects is provided by DPPC membranes, which undergo a gel-to-liquid-crystal phase transition in the range 37–40°C (Lipschitz-Farber and Degani, 1980). The diffusion exchange rate increases fivefold across the transition, and the activation energy of the rate increases from 7.2 kcal/mol above 40°C to 19.3 kcal below the transition. In contrast, egg phosphatidylcholine does not undergo phase transitions, and the exchange rate has a constant activation energy of 10.5 kcal/mol from 0 to 65°C (Lipschitz-Farber and Degani, 1980). It is difficult to estimate the magnitude of changes in τ_i and E_d that accompany phase transitions in arbitrary lipid bilayers, but the highly Arrhenius behavior of τ_i in Fig. 6 would appear to argue strongly against the existence of phase transitions in the chromaffin granule membrane.

Previous measurements (Marsh et al., 1976) of the hyperfine order parameters of several lipid-soluble spin-labels in suspensions of isolated chromaffin granule membranes exhibit breaks in the temperature dependence at 32–34°C. Because similar transition temperatures were observed for several chemically disparate spin labels, an explanation based on local structural perturbations in the neighborhood of the spin label was ruled out, and the breaks were interpreted as the result of an intrinsic phase transition in the membrane. These data seem to contradict the results shown in Fig. 5, where an analogous transition is not evident in the water permeability coefficient.

Direct comparison of the two experiments is difficult, however, because the preparations are dissimilar. The spin-label experiments utilized membranes isolated by repeated hypotonic shock and resuspended in 10 mM BES, pH 7.4. The present experiments utilized intact chromaffin granules suspended in 300 mM sucrose, 10 mM BES, pH 7.0. Phase transitions are undoubtedly sensitive to the osmoticum and to factors that influence surface potential (e.g., pH, ionic strength). Lysis causes large perturbations of all these factors, especially for the inward-facing surface. In intact chromaffin granules, this surface faces a medium of very high ionic strength and low pH. Thus the apparent disagreement may result from a shift in transition temperature due to medium-related effects of this type.

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