Biochimica et Biophysica Acta, 690 (1982) 165–173
Elsevier Biomedical Press

BBA 71322

NMR STUDIES OF GLYCEROL PERMEABILITY IN LIPID VESICLES, ERYTHROCYTES AND THE ALGA *DUNALIELLA*

FRANCIS F. BROWN [a], ILENE SUSSMAN [b], MORDHAY AVRON [b] and HADASSA DEGANI [c,*]

[a] *Biochemistry Department, University of Oxford, Oxford (U.K.),* [b] *Biochemistry Department and* [c] *Isotope Department, The Weizmann Institute of Science, Rehovot (Israel)*

(Received March 23rd, 1982)

Key words: NMR; Lipid vesicle; Alga; Erythrocyte; Glycerol permeability; (Dunaliella salina)

Glycerol diffusional permeabilities through the cytoplasmic cell membrane of *Dunaliella salina*, the cell envelope of pig erythrocyte and egg phosphattidylcholine vesicles were measured by NMR spectroscopy employing the spin-echo method and nuclear T_1 relaxation. The following permeability coefficients (P) and corresponding enthalpies of activation ($\Delta H^{\ddagger}$) were determined for glycerol at 25°C: for phosphatidylcholine vesicles $5 \cdot 10^{-6}$ cm/s and 11 ± 2 kcal/mol; for pig erythrocytes $7 \cdot 10^{-8}$ cm/s and 18 ± 3 kcal/mol, respectively; for the cytoplasmic membrane of *D. salina* the permeability at 17°C was found to be exceptionally low and only a lower limit ($P < 5 \cdot 10^{-11}$ cm/s) could be calculated. At temperatures above 50°C a change in membrane permeability occurred leading to rapid leakage of glycerol accompanied by cell death. The data reinforce the notion that the cytoplasmic membrane of *Dunaliella* represents a genuine anomaly in its exceptional low permeability to glycerol.

Introduction

The halotolerant green alga *Dunaliella* can adjust its internal concentration of glycerol to accomodate large osmotic changes and can therefore grow in media containing a wide range of NaCl concentrations (0.1–5.5 M). *Dunaliella* cells lack a rigid cell wall and are enclosed only by a cytoplasmic membrane. Previous results have indicated [1,2] that glycerol serves as an intracellular osmoregulator in these cells and is synthesised or metabolized in response to changes in the external salt concentration. Little to no glycerol is found in the growth medium. The cytoplasmic membrane must therefore have two unique properties: (1) the ability to maintain a large concentration gradient of glycerol and (2) an exceptionally low permeability to glycerol. This study is concerned with the second of these properties.

Several previous studies on glycerol leakage from *Dunaliella* cells vary widely in their conclusions. Some data [3] indicate that *Dunaliella* are capable of retaining glycerol against a large concentration gradient with little to no leakage below 40°C. Above this temperature glycerol is leaked into the medium. Complete release occurs above 60°C and is paralleled by cell death. On the other hand, recent studies of the glycerol permeability and reflection coefficient of the plasmalemma of *Dunaliella parva* [4] have been interpreted to mean that this membrane does not exhibit an unusually low permeability towards glycerol and that significant amounts of glycerol diffuse continuously into the medium.

We approached this problem by comparing the glycerol permeability of the cytoplasmic membrane of *Dunaliella* with that of the pig erythrocyte and artificial phospholipid vesicular bilayers. All

* Correspondence should be addressed to: Hadassa Degani, Isotope Department, The Weizmann Institute of Science, Rehovot 76100, Israel.

three systems exhibit a pure diffusion mechanism (with no indication of active transport). Measurements in the three systems were performed under similar conditions by NMR spectroscopy. Recently developed techniques [5,6] allow a sensitive distinction to be made between environments inside and outside cells or vesicles and therefore provide a direct, non-invasive method to measure the rate of glycerol transport and/or its exchange diffusion. By using the appropriate procedures, NMR can be applied over a large range of rates with half-times between milliseconds and hours.

The data presented establish that the cell membrane of *Dunaliella* is indeed unique in exhibiting an exceptionally low permeability toward glycerol.

Materials and Methods

Algal samples

Dunaliella salina was grown in a medium which contained 1.5 M NaCl, 50 mM $NaHCO_3$, 5 mM KNO_3, 5 mM $MgSO_4$, 0.3 mM $CaCl_2$, 0.2 mM KH_2PO_4, 1.5 μM $FeCl_3$, 6 μM EDTA, 185 μM H_3BO_4, 7 μM $MnCl_2$, 0.8 μM $ZnCl_2$, 0.02 μM $CoCl_2$, 0.2 nM $CuCl_2$; pH 8.0. Algal cultures were grown under continuous illumination with fluorescent lamps (light intensity 350 ft-cd) at 26°C) with slow continuous shaking. One liter of algal suspension was harvested during the early steady-state phase by centrifugation at $4000 \times g$ for 7 min. All procedures were carried out at room temperature. The algae were washed once in a 1.5 M NaCl medium by centrifugation at $1500 \times g$ for 10 min. A second wash was performed as above using a minimum volume of medium containing 85% ^2H_2O, centrifuged in a 0.5 mm NMR tube at $1900 \times g$ for 10 min, and the supernatant discarded.

Viability tests

Due to the high cell concentration necessary for the NMR experiments, the cells were subjected to somewhat adverse conditions for extended periods of time. Therefore, experiments with cells prepared exactly as for the NMR measurements were performed in order to approximate the timing of cell death. This was done by incubating the cells in the NMR tube at 22°C and 40°C. Samples were removed at various times, diluted in growth medium and observed microscopically. At 40°C the cells began to show abnormal appearance after about 30 min of incubation and were seriously malformed after 90 min. Cells incubated at 22°C remained viable looking for at least 3.5 h.

Pig erythrocyte samples

Pig erythrocytes were obtained from fresh pig blood by washing five times with Krebs-Ringer buffer [7] at pH 7.4. The cell suspensions were then centrifuged at $2500 \times g$ for 5 min to give samples of 70–80% hematocrit as measured on a Hawkesly microhematocrit centrifuge.

Phospholipid vesicles

Vesicles were prepared from pure egg phosphatidylcholine. The lipid dispersions (50 mg/ml) were suspended in 50–100 mM $GdCl_3$ in aqueous solution and sonicated on ice under N_2 for 10–20 min with a Heat System W-375 Sonifier using a 0.375 inch tip and power level 5, pulsed mode (approx. 40% fractional power). The resulting suspension was centrifuged at $40000 \times g$ for 30 min at 4°C and the zone containing clear supernatant was removed for dialysis. 2 ml of vesicle suspension was dialysed three times for 24 h at 4°C against 3 liter of aqueous NaCl solution at a concentration equivalent to that of the $GdCl_3$. An additional short dialysis against equivalent ^2H_2O solutions (20 ml $\times$ 3) was carried out prior to the ^{1}H-NMR measurements. A control solution of $GdCl_3$ of the same concentration as that used in the preparation of the vesicles was dialysed simultaneously with the vesicles. Glycerol was added to the dialysed suspension and control solution to a final concentration of 0.1–0.3 M. The phospholipid contents of the vesicles was determined by ashing and analysing for inorganic phosphate.

NMR measurements

Rates of glycerol influx and efflux in pig erythrocytes and of glycerol efflux in *Dunaliella salina* were determined by proton spin-echo studies at 470 MHz on a FT spectrometer (built in the Biochemistry Laboratory, Oxford) and at 270 MHz on a Bruker WH-270 pulse FT spectrometer, respectively. Both instruments were equipped with variable temperature probes which maintained the temperature to within 1°C.

The ^1H and ^2H longitudinal relaxation rates in the vesicle suspension were determined by the inversion-recovery method at 270 MHz and 41.4 MHz, respectively, in the Bruker WH-270 spectrometer.

The paramagnetic reagent used for enhancement of field gradients in the *Dunaliella* and erythrocyte samples was Dy^{3+} diethylenetriaminepentaacetate which was added to give an overall concentration of up to 300 μM.

Data analysis

All permeability measurements were made by NMR, using procedures appropriate to the timescale of the processes involved.

The measurements on vesicles were made using a method developed by Lipschitz-Farber and Degani [6] which is appropriate to the fast exchange of glycerol between inside and outside of vesicles. In this method, the intravesicular solution is loaded with a non-perturbing paramagnetic material which greatly enhances the relaxation rate ($1/T_1^{io}$) of the glycerol nuclei within. The glycerol nuclei in the extravesicular region, which would in the absence of the vesicles have a relaxation rate $1/T_1^o$, would exhibit as a result of exchange with the paramagnetic enhanced intravesicular glycerol a rate given by:

$$\frac{1}{T_1} = \frac{1}{T_1^o} + \frac{G_i}{G_o}\left(\frac{1}{T_1^{io} + \tau^i}\right) \tag{1}$$

where G_i and G_0 are the fraction of glycerol molecules in the internal and external media respectively, and τ^i is the mean lifetime of the glycerol inside the vesicles. This lifetime is related to the permeability coefficient by:

$$P = \frac{r}{3} \cdot \frac{1}{\tau^i} \tag{2}$$

when r is the average radius of the spherical vesicles.

$1/T_1$ and $1/T_1^o$ were measured in the vesicle suspension and the control solution, respectively; the ratio G_i/G_o, which in these experiments is equal to the intra-to-extra-vesicular volume ratio, was calculated from the lipid concentration and the partial specific volume of the vesicle [8]. The exchange rate, $1/\tau^i$, over the temperature range studied, were determined by non-linear least squares fit of the data to Eqn. 1, assuming

$$\frac{1}{T_1^o}, \frac{1}{T_1^{io}} \propto \exp(\Delta H/RT)$$

and an Arrhenius temperature dependence for $1/\tau^i$ of the form:

$$\frac{1}{\tau^i} = \frac{kT}{h}\left(\exp - \left[(\Delta H^{\ddagger}/RT) - (\Delta S^{\ddagger}/R)\right]\right)$$

Permeability measurement of the erythrocytes and algae were made using a method developed by Brindle et al. [5] in which a small amount of a biologically inert non-penetrating material is added to the extracellular regions to induce extracellular field gradients and thereby reduce the signal from this region when using the Carr-Purcell spin-echo technique.

In the case of the erythrocytes, the method was applied for both influx and efflux studies while only efflux measurements were made for the algae. In both types of experiments the integrated intensity of the intracellular signal of the permeant molecule is monitored as a function of time. By using large enough field gradients and a long enough delay ($\tau > 70$ ms), it is possible to make the extracellular signal too small to observe. In the time-courses run with the Algae, data acquired under these conditions were related to data with $\tau = 10$ ms when the major part of both intracellular and extracellular glycerol was observed. This served as a check for any change in the total glycerol content, and instrumental stability.

In such flux experiments one usually measures the half life time, $t_{1/2}$, for the influx or efflux process. The relationship between $t_{1/2}$ and the permeability may be derived as follows:

The influx of material into a cell at any time, t, after addition to the extracellular region, may be given by the equation:

$$\frac{dC_i}{dt} = \frac{PA}{V_i}(C_o - C_i) \tag{3}$$

where P is the permeability in cm/s, V and A are the volume and the surface area of a cell, respectively, C is the concentration, and the subscripts

used are i (inside), o (outside) and T (total). substituting $C_i = n_i/V_i$, where n is the number of moles of substance then:

$$\frac{1}{V_i} \cdot \frac{dn_i}{dt} = \frac{PA}{V_i}\left(\frac{n_o}{V_o} - \frac{n_i}{V_i}\right)$$

cancelling and substituting for n_o

$$\frac{dn_i}{dt} = PA\left[\frac{n_T}{V_o} - n_i\left(\frac{1}{V_o} + \frac{1}{V_i}\right)\right] \quad (4)$$

Assuming V_o and V_i are constant, this integrates to

$$\frac{n_T}{V_o} - n_i(t)\left(\frac{1}{V_o} + \frac{1}{V_i}\right) = \exp\left[-PA\left(\frac{1}{V_o} + \frac{1}{V_i}\right)t\right]Q \quad (5)$$

when Q is the integration constant.

For an influx process, when $t = 0\, n_i = 0$, so that $Q = n_T/V_o$. Rearranging

$$n_i(t) = \frac{n_T V_i}{V_T}\left(1 - \exp\left[-PA\left(\frac{1}{V_o} + \frac{1}{V_i}\right)t\right]\right) \quad (6)$$

$n_T\, V_i/V_T$ is equal to the value of n at equilibrium ($n_{equilib.}$) obtained at $t = \infty$.

The NMR signal area, R, is proportional to the number of molecules producing it, namely to n. The area of an echo signal depends also on the apparent relaxation rate and therefore the extracellular and intracellular echo signals may differ in their integrated intensity per molecule. If the area per molecule in the extracellular region is S_o, and in the intracellular region is S_i, then the change in the signal area when one molecule passes from outside to inside is $S_i - S_o$. Thus

$$n_i(t)(S_i - S_o) = R(t) - R(0)$$

and

$$n_{equilib.}(S_i - S_o) = R(\infty) - R(0)$$

defining

$$\Delta(t) = \frac{n_i(t)}{n_{equilib.}} = \frac{R(t) - R(0)}{R(\infty) - R(0)}$$

substituting in Eqn. 6 and rearranging:

$$\Delta(t) = 1 - \exp\left[-PA\left(\frac{1}{V_o} + \frac{1}{V_i}\right)t\right] \quad (7)$$

At the half time, ($\Delta(t_{1/2}) = 1/2$), the permeability is given by:

$$P = \frac{\ln 2}{t_{1/2}}\left(\frac{A}{V_o} + \frac{A}{V_i}\right)^{-1} \quad (8)$$

V_o and V_i are related by the expression:

$$\frac{V_o}{V_i} = \left(\frac{1}{H} - 1\right)/b \quad (9)$$

where H is the fractional cell volume in the sample and b is the fractional accessible volume within the cell.

The same relationships hold for an efflux experiment except that $\Delta(t)$ is defined as:

$$\Delta(t) = \frac{R(t) - R(\infty)}{R(0) - R(\infty)}$$

The activation enthalpy for the process can be obtained as before from an Arrhenius plot.

Results

Glycerol permeability of egg phosphatidylcholine vesicles

The temperature variation of ^1H relaxation rates ($1/T_1$) of glycerol in a suspension of egg phosphytidylcholine vesicles containing excess Gd^{3+} is shown in Fig. 1. A similar experiment was performed with deuterated glycerol in H_2O. The temperature dependence of the rate constants ($1/\tau^i$) for diffusion exchange across the bilayer (from Eqn. 1) is illustrated in Fig. 2. Using an average r of $65 \cdot 10^{-8}$ cm, the permeability coefficient for both protonated and deuterated glycerol at 25°C was calculated from Eqn. 2, and was found to be $5.4 \cdot 10^{-6}$ cm/s. The activation enthalpies were 9.1 kcal/mol and 12.5 kcal/mol, respectively. Neither the effect of temperature on the energetics of transfer of glycerol from H_2O to ^2H_2O, nor the temperature dependence of the energetics of glycerol solvation in a membrane-like medium has been measured. Therefore, it is not known whether the origin of the discrepancy in the above enthalpies stems from the difference in the desolvation energies in the two solvents, or from the difference in the solution energies in the membrane or from both.

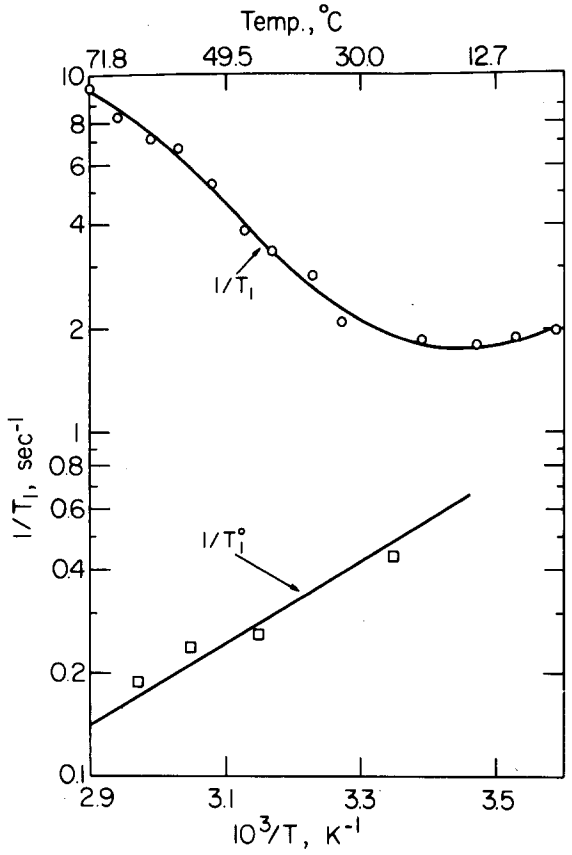

Fig. 1. Temperature dependence of ^1H longitudinal relaxation rate of a vesicular suspension of egg phosphatidylcholine. O——O, vesicles prepared with 50 mM $GdCl_3$ after dialysis; □——□, 50 mM $GdCl_3$ solution after dialysis (control). Measurements performed at 270 MHz as described in text.

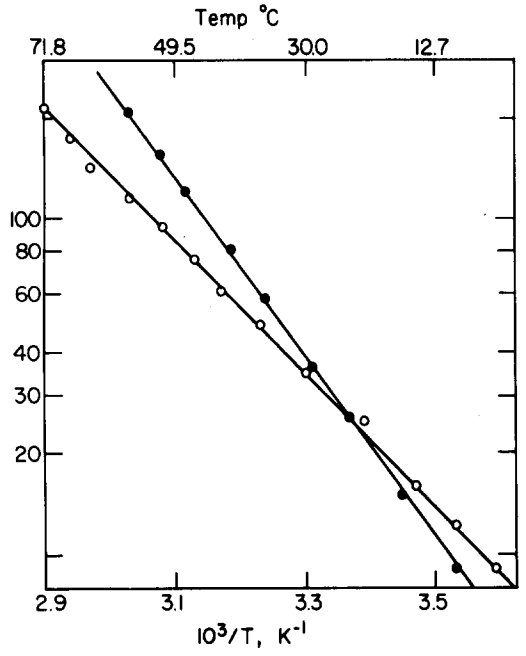

Fig. 2. Temperature dependence of glycerol diffusion exchange rate across phosphatidylcholine vesicles. O——O, from ^1H data in ^2H_2O (99.7%) solutions; ●——●, from ^2H data in H_2O solutions.

Glycerol permeability of pig erythrocytes

A characteristic set of raw data for a spin-echo influx experiment is shown in Fig. 3. A time-course extracted from such data is shown in Fig. 4 in comparison with that from an efflux experiment run under equivalent conditions. Although the $t_{1/2}$ differs slightly because of the difference in the hematocrit ($t_{1/2} = 7.5$ min in the influx and $t_{1/2} = 8.3$ min in the efflux), the permeability was found to be the same in each direction: $2.2 \cdot 10^{-8}$ cm/s at 10°C.

Table I shows data from an experiment illustrating the concentration dependence of the glycerol influx process. No significant variations in $t_{1/2}$ were observed as expected for a purely diffusive mechanism. It should, however, be pointed

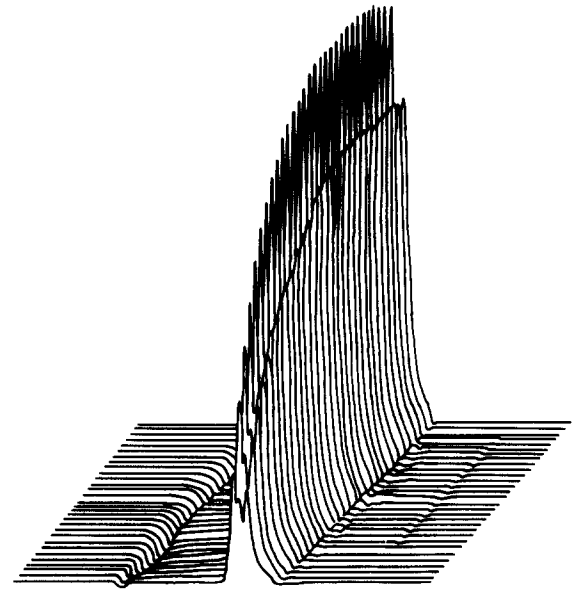

Fig. 3. Typical time-course of spin-echo spectra during a glycerol influx experiment in pig erythrocytes. Measurements at 470 MHz were obtained every 30 s. $\tau = 135$ ms.

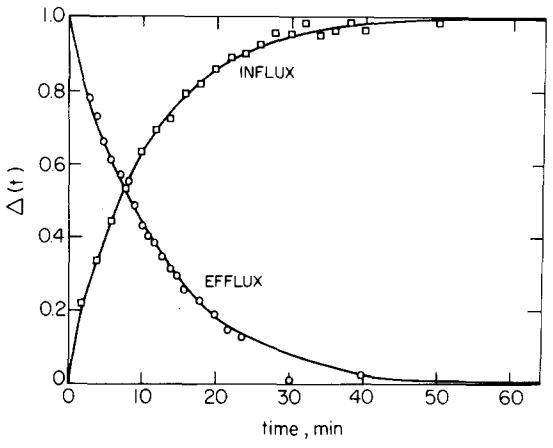

Fig. 4. Change with time of $\Delta(t)$ (Eqn 7), for glycerol influx and efflux in pig erythrocytes at 10°C. Pig erythrocytes were suspended in ^2H_2O Krebs-Ringer buffer at 10°C with hematocrit of 72% and 63% for influx and efflux experiments, respectively. In the efflux experiments 0.45 ml of cell suspension was preequilibrated with 67 mM glycerol for 10 min at room temperature and an additional 5 min at 10°C. Time-course efflux experiments were started 60 s after dilution with 0.15 ml buffer.

out that at relatively high concentrations of glycerol (> 150 mM), the NMR time-course for the influx exhibited an increasingly biphasic character. The first phase clearly belonged to the diffusion process, but the nature of the second, slower phase remains unclear. It is likely that the slower phase is due to some secondary changes in size or shape of the erythrocytes caused by the high concentration of glycerol, as the spin-echo method would be expected to be sensitive to such changes.

The temperature dependence of the influx rates is shown in Fig. 5, giving an enthalpy of activation of 18 kcal/mol. In calculating the permeability coefficient from Eqn. 8, the ratio A/V_i was obtained using the approximation:

$$\frac{A_{pig}}{A_{human}} = \frac{r^2_{pig}}{r^2_{human}}$$

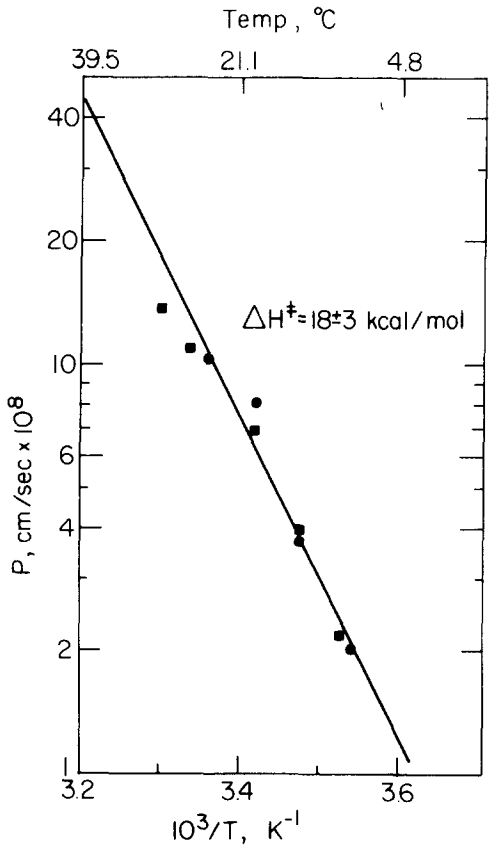

Fig. 5. Temperature dependence of glycerol permeability in pig erythrocytes. The data are representative of two separate experiments performed under similar condition.

so that $(A/V_i)_{pig} = (r^2/V_i)_{pig} \cdot (A/V_i)_{human}/(r^2/V_i)_{human}$. The following values were used: $(r^2/V_i)_{pig} = 2.85^2/93.5$ and $(r^2/V_i)_{human} = 3.2^2/137.6$ [9] and $(A/V_i)_{human} = 2.4 \cdot 10^4$ [10]. The permeability coefficient at 25°C was found to be $(1.0 \pm 0.2) \cdot 10^{-7}$ cm/s.

Glycerol permeability in the alga Dunaliella

The naturally high concentrations of glycerol within these cells dictated the choice of performing efflux experiments. Due to the very low glycerol

TABLE I

CONCENTRATION DEPENDENCE OF GLYCEROL INFLUX IN PIG ERYTHROCYTES AT 15°C

Experimental conditions as described in Fig. 4 for the influx study.

Glycerol (mM)	6	11.5	22	40	82	137
$t_{1/2}$ (min)	3.25	3.5	4.0	3.8	4.0	6.1

permeability of these cells the spin-echo method was applied in a slightly different manner than that for the erythrocyte experiments and will therefore be described in the following.

In a concentrated cell suspension (approx. 10^9 cells/ml) extracellular field gradients were produced by the introduction of Dy^{3+} diethylenetriaminepentaacetic. The intracellular glycerol was monitored at $\tau = 80$ ms. Dy^{3+} diethylenetriaminepentaacetic was added until the first indication of a decrease in the intracellular signal occurred. To check whether the extracellular signal had been completely eliminated, twice the intracellular concentration of glycerol was added to the extracellular medium, but no increase in signal was observed. This fixed the conditions for the maximum discrimination between intracellular and extracellular glycerol. The amount of Dy^{3+} diethylenetriaminepentaacetic was then fixed at this concentration for the rest of the experiments (300 μM).

The experiments were performed by comparing the integrated intensity for $\tau = 80$ ms, when no extracellular signal was seen, against that at $\tau = 10$ ms, when most of the glycerol from both compartments could be seen. Leakage into the extracellular

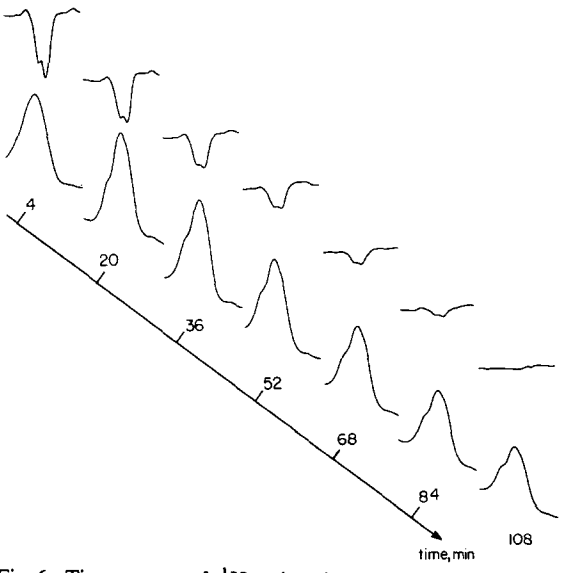

Fig. 6. Time-course of ^1H spin-echo spectra obtained in a *Dunaliella saline* suspension during glycerol efflux at 51°C (10^9 cells/ml). The upper spectra were obtained at $\tau = 80$ ms and lower spectra at $\tau = 10$ ms. Measurements were performed at 270 MHz.

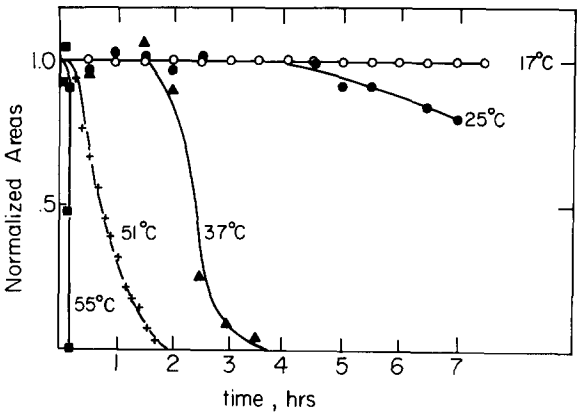

Fig. 7. Changes with time in the normalized area of the intracellular glycerol spin-echo signal. *D. salina* were suspended at 10^9 cells/ml and glycerol efflux was measured at the various temperatures indicated in the figure.

medium would be accompanied by a drop in the 80 ms signal but no change in the 10 ms signal. This would also occur with cell lysis up to the point where most of the cells were lysed in which case the signal would rise again due to the loss of field gradients since the distribution of Dy^{3+} diethylenetriaminepentaacetic within the system became homogeneous.

An example of signal elimination at 51°C in a time course experiment with *Dunaliella* is shown in Fig. 6. The results obtained at various temperatures are summarised in Fig. 7. In general, it would appear that each time-course may be described in terms of two components, a stable phase and an increased permeability phase.

During the stable phase, which was essentially non-detectable above 50°C, the intracellular signal remained constant within experimental error and represented a situation where no measurable leakage occurred. The leakage observed during the degradative phase was associated with cell death. It was therefore not possible to calculate either the permeability or the activation energy for the efflux process during the stable phase. However, it was possible to set an upper limit to the permeability, since each of the integrated intensity measurements were accurate to about 5%. At 17°C the glycerol leakage could not have been greater than about 1% over 7 h, while the measurements at 40°C (not shown) agreed within an accuracy of no more than 3% over the first 17 min. These would

correspond to efflux half times equal to or greater than 400 h and 7 h, respectively. Therefore, corresponding limits for glycerol permeability were, $5 \cdot 10^{-11}$ cm/s at 17°C and $3 \cdot 10^{-9}$ cm/s at 40°C. These permeabilities were calculated according to Eqn. 8. Cell volume was measured and found to be 10^{-10} cm^3 [11]; and the cell membrane area was calculated from Eqn. 9 assuming spherical shape for the cells with $r = 2.9 \cdot 10^{-4}$ cm for $H = 0.1$ and $b = 0.7$ [12].

Discussion

Variations of the same basic NMR technique were applied in order to study three membrane systems which proved to exhibit large differences in glycerol permeability. The technique employed in this study is both direct and non-invasive and as a result requires minimal assumptions or approximations. The method can be applied over the entire range of time scales observed for various transport processes (with $t_{1/2}$ greater than 0.1 ms). Other methods have been used previously to measure glycerol permeability including tracer techniques (also direct), the measurement of osmotically induced volume changes by photometry or light scattering, and the rate of lysis of cells.

The results obtained in this tudy, together with previous data published on similar systems, are summarised in Table II. In spite of the different methods employed, the trends are clearly visible. Pure lipid bilayers proved to be the most glycerol permeable membrane studied with intravesicular half times varying with temperature from 5–100 ms. The membrane of the pig erythrocyte is moderately permeable with a half time for influx or efflux of 1–10 min. The most impermeable membrane studied was that of *Dunaliella* with a half time for efflux of 10 h for *D. parva* and not less than 400 h for *D. salina* at 17°C.

The extreme impermeability of *Dunaliella* to glycerol was not, however, apparent from the measurements of the reflection coefficient published recently by Enhuber and Gimmler [4]. They obtained a value of 0.87 ± 0.06 for *Dunaliella parva* which is distinctly lower than the value of 0.99 ± 0.04 for bovine erythrocytes which have a similar permeability to the pig erythrocyte [15]. Reflection coefficients are a good measure of relative permeabilities and provide useful mechanistic information on the similarity of the diffusion pathways for solvent (water) and solute [16]. However, because the coefficient relates the solute permeability to that of the solvent, there are many molecules of biological interest which, though very permeable in a biological timescale, are much less permeable than water and thus have reflection coefficients close to 1. The value of 0.87 reported for *Duniella* suggests therefore a relatively high permeability to glycerol. The origin of this discrepancy is not

TABLE II

GLYCEROL PERMEABILITY COEFFICIENT AND ENTHALPY OF ACTIVATION IN SEVERAL SYSTEMS

Permeabilities at 20°C were calculated from values given in the literature at other temperatures using the corresponding activation energies.

System	P (cm/s)	$\Delta H^{\ddagger}$ (kcal/mol)	Method
Egg PC vesicles	$5 \cdot 10^{-6}$	11 ± 2	NMR
Egg PC liposomes	$2 \cdot 10^{-6}$ [a]	11	Osmotic [13]
Pig erythrocytes	$7 \cdot 10^{-8}$	18	NMR
Pig erythrocytes	$1 \cdot 10^{-7}$	–	Osmotic lysis [9]
Pig erythrocytes	$4 \cdot 10^{-7}$	20	Tracer [14]
Dunaliella salina	$< 5 \cdot 10^{-11}$ [b]	–	NMR
Dunaliella parva	$3 \cdot 10^{-10}$	–	Tracer [4]

[a] Calculated from the permeability of glycerol relative to urea determined by the osmotic method: 0.75 [19]; and from the permeability of urea determined in a membrane by a tracer technique: $2.3 \cdot 10^{-6}$ cm/s at 20°C [20].

[b] Upper limit obtained at 17°C.

clear. There is an explicit assumption that ion fluxes across the membrane under osmotic shock are zero. Though this is entirely reasonable it has not so far been demonstrated. Also the measurements for determining the reflection coefficient were made nearly 0.5 min after the osmotic shock was applied which according to the data of Rabinowitch et al. [17] is during the second and slower phase of water flow which follows an initial rapid flow phase, lasting a few seconds after an osmotic shock. For maximal accuracy reflection coefficient measurements should be performed over a 25–100 ms time range by stop-flow techniques [12]. Enhuber and Gimmler's measurements [4] may imply a relatively low water permeability compared with that of most membranes. However, we have recently measured using NMR: $P_w = 1.6 \cdot 10^{-3}$ cm/s at 20°C [18]; a value which is similar to that observed in many cell systems, such as erythrocytes.

The data presented here reinforces the notion that the membrane of *Dunaliella* represents a genuine anomaly in its exceptional low permeability to glycerol. The exact magnitude of this anomaly is hard to assess. The physical properties of the membrane which give rise to this uniqueness are at present unknown.

Acknowledgement

Helpful discussions with Drs. A. Ben-Amotz and I.D. Campbell in the course of this work and the assistance of Dr. T. Victor in data analysis are gratefully acknowledged. H. Degani is Incumbent of Wormser Career Development Chair.

References

1 Ben-Amotz, A. and Avron, M. (1973) Plant Physiol. 51, 875–878
2 Borowitzka, L.J. and Brown, A.D. (1974) Arch. Microbiol. 96, 37–52
3 Wegmann, K., Ben-Amotz, A. and Avron, M. (1980) Plant Physiol. 66, 1196–1197
4 Enhuber, G. and Gimmler, H. (1980) J. Phycol. 16, 524–532
5 Brindle, K.M., Brown, F.F., Campbell, I.D., Grathowhl, C. and Kuchel, P.W. (197() Biochem. J. 180, 37–44
6 Lipschitz-Farber, C. and Degani, H. (1980) Biochim. Biophys. Acta 600, 291–300
7 Krebs, H.A. and Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. 210, 344–351
8 Huang, C. (1969) Biochemistry 8, 344–351
9 Wesseles, J.M.C. and Veerkamp, J.H. (1973) Biochim. Biophys. Acta 291, 190–196
10 Carlsen, A. and Wieth, J.O. (1976) Acta Physiol. Scand. 97, 501–513
11 Ben-Amotz, A. and Avron, M. (1980) in Genetic Engineering of Osmoregulation (Rains, D.W., Valentine, R.C. and Hollaender, A., eds.), pp. 91–99, Plenum Press, New York
12 Gimmler, H. and Tobler, U. (1977) Z. Pflanzenphysiol. 83, 145–158
13 Cohen, B.E. (1975) J. Membrane Biol. 20, 205–235
14 Deuticke, B. (1977) Rev. Physiol. Biochem. Pharmacol. 78, 2–79
15 Owen, J.D., Steggal, M. and Eyring, E.M. (1976) J. Membrane Biol. 26, 287–299
16 Staverman, A.J. (1952) Trans. Faraday Soc. 48, 176–185
17 Rabinowitch, S., Grover, N.B. and Ginzburg, B.Z. (1975) J. Membrane Biol. 22, 221–230
18 Degani, H. and Avron, M. (1982) Biochim. Biophys. Acta 690, 174–177
19 Cohen, B.E. (1975) J. Membrane Biol. 20, 235–268
20 Poznansky, M., Tony, S., White, P.O., Milgram, J.M. and Solomon, A.K. (1976) J. Gen. Physiol. 67, 45–66