Changes in the ordering of lipids in the membrane of *Dunaliella* in response to osmotic-pressure changes

An e.s.r. study

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Changes in the ordering and motion of lipids in response to changes in the external solute concentration have been studied by using the 5-nitroxide stearate (5NS) and 16-nitroxide stearate (16NS) spin probes in the plasma membrane of the halotolerant unicellular alga *Dunaliella salina*. Increases in ordering of the 5NS probe and decreases in motion of the 16NS probe were observed in cells equilibrated over 18 h at increasing NaCl concentrations. These changes probably resulted from the influence of the high NaCl concentration on the charged phospholipid head groups of the membrane. A short-term (<100 min) decrease in the order parameter, S , of the 5NS probe was observed for cells swollen by exposure to a sudden decrease of NaCl concentration from 5.0 to 2.5 M. After 100 min the value of S for 5NS was close to the value obtained in cells that had been equilibrated in 2.5 M-NaCl for 18 h. Since the cells had regained their original size and shape by 100 min it was assumed that the short-term decrease in S was associated with the swelling. A similar result was obtained when the cells were suddenly changed from 3.0 M- to 1.5 M-sorbitol. Conversely, an increase in S was observed for cells shrunk when the external solute concentration was doubled from 1.5 M- to 3.0 M-NaCl. As the cells regained their original size and shape the value of S decreased to the value observed in cells that had been equilibrated in 3.0 M-NaCl for 18 h. It is suggested that the changes in S are related to the movement of lipid into or out of a reservoir of membrane material as the membrane shrinks or expands. This movement of lipid maintains the tension of the membrane below the value at which it is disrupted. Such changes in lipid ordering could provide a mechanism whereby information about external osmotic-pressure changes is transmitted across the cell wall.

The unicellular algae of the genus *Dunaliella* have three unusual properties. First, they can grow in NaCl solutions ranging in concentration from 0.5 M to 5.0 M. Secondly, they appear to achieve constant volume and to remain very nearly in osmotic equilibrium over this wide range of salinities by synthesizing glycerol, which is accumulated in the cell (Craigie & McLachlan, 1964; Ben-Amotz & Avron, 1973; Borowitzka & Brown, 1974; Ginzburg, 1978). The third property is that unlike other algae they have no rigid cell wall, only a naked cytoplasmic membrane. In the absence of sudden changes in external osmotic pressure they are oval,

motile cells, about 22 μ m on the long axis and 16 μ m on the short axis.

The accumulation of glycerol, which has been called a 'compatible solute' by Brown & Simpson (1972), appears to be closely regulated by the external water activity rather than by a specific solute effect, since increased concentrations of organic substances, such as sucrose, sorbitol and 2-deoxy-D-glucose, in the external medium also trigger glycerol accumulation (Borowitzka *et al.*, 1977). The alga initially responds to increasing water activity by swelling to about 30 μ m in diameter and then by dissimilating glycerol until the cell regains its usual size and shape. Since it has no rigid wall the cell must remain close to osmotic equilibrium, and some mechanism as yet unexplained must exist to prevent disruption of the cell membrane during swelling.

Abbreviations used: 5NS, 5-nitroxide stearate [2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyloxazolidine-3-oxyl]; 16NS, 16-nitroxide stearate [2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyloxazolidine-3-oxyl].

Nothing is known of the way in which information regarding the activity of water outside the cell is transmitted across the cell membrane to effect the regulation of the intracellular metabolic processes that are responsible for glycerol synthesis or dissimilation. It has been suggested (Kauss, 1978) that physical changes in the membrane caused by shrinking and swelling could provide this sensing mechanism. Recently, Fontana & Haug (1982) carried out an e.s.r. study on the plasma membrane of *Dunaliella primolecta*, in which they showed that the membrane fluidity decreased as the algae adapted to higher NaCl concentrations and suggested that this decrease may be the sensor of changing environmental salt concentrations. Their study, however, used cells that had been adapted for at least 30 generations to various salt concentrations, and it did not address the question of what happens during the transient swelling phases when the cells are either rapidly dissimilating or synthesizing glycerol. It, therefore, threw little light on how the intracellular glycerol content could be regulated in response to rapid changes in external salt concentration.

In the present paper we report e.s.r. data obtained during such changes, which suggest that significant exchange of lipid occurs between the plasma membrane and the interior of the cell, accompanied by membrane fluidity changes that could act as a transmembrane signal.

Materials and methods

Dunaliella

Dunaliella salina isolated from a solar salt pond in South Australia was cultivated in 200 ml batch cultures at 25°C and at a light intensity (photosynthetically active radiation) of approx. 200 $\mu\text{E}/\text{m}^2$ per s. The growth medium was seawater enriched with nutrients to half the concentration used by Guillard & Ryther (1962). NaCl was added to the medium to give concentrations that ranged from 1 to 5 M. The algae were harvested by centrifugation (500 g for 30 min). Although *Dunaliella* cells are osmotically robust, they are mechanically fragile. To eliminate possible damage by repeated centrifugation and washing, the cells were adjusted, after the initial harvesting, to the desired new solute concentration by dialysis against a large excess of solution under illumination for 18 h. The two suspending media used were seawater with 1.0–5.0 M NaCl added in 0.5 M steps and seawater with 1.0–4.0 M sorbitol added in 0.5 M steps. The final 2 h of dialysis was carried out under pressure with gentle stirring to bring the cells to a concentration of 10⁸/ml; the condition of the cells was checked microscopically during all manipulations. The cri-

terion for viability was that over 90% of the cells must be unbroken and motile.

E.s.r. spectroscopy

The nitroxide fatty acid spin labels 5-NS and 16-NS were purchased from Syva (Palo Alto, CA, U.S.A.).

The spin labels were added to the cells in two ways. In the first method a thin film of label was prepared by placing 0.25 ml of chloroform containing the desired concentration of spin label in a small vial and evaporating the chloroform with a stream of dry N₂. *Dunaliella* (10⁷ cells in 100 μl of the desired medium) were added and the vial was shaken at 20°C for 10 min. Alternatively, the desired concentration of label in 0.1 μl of ethanol was added to 30 μl of medium containing 3.3 $\times$ 10⁶ cells and the mixture was vortex-mixed for 30 s. Both methods gave identical spectra but the second was used throughout these experiments because it gave more reproducible concentrations of label. Label concentrations were expressed in terms of μmol of label/10⁸ cells.

E.s.r. spectra were recorded by means of a Varian V4502 spectrometer fitted with a Deltron (Sydney) model DCM 20 temperature control accessory, which maintained the temperature of the sample at $\pm 0.05^\circ\text{C}$ of the temperature selected. Spectra were recorded at 20°C. A 100 kHz modulation amplitude was used at 0.2 mT and power of 6 mW. Capillary samples were used, each containing 10⁶–10⁷ cells in 50 μl . The output of the instrument was connected to a Hewlett-Packard 9825A computer-controller through a 3437A systems voltmeter. This facility was used to perform spectral subtraction and double integration.

Where the high-field hyperfine extremum could be resolved, as in the case of 5NS, the polarity-corrected order parameter, S (Hubbell & McConnell, 1971), was calculated as described by Griffith & Jost (1976). Care was taken with the polarity correction because comparisons were made over a wide range of media with differing polarities.

The order parameter of spin-labels is frequently used as a measure of membrane lipid ordering (Kury *et al.*, 1974; Kury & McConnell, 1975). Where the spin-label motion was nearly isotropic, as in the case of 16NS, and where the high-field hyperfine extremum could not be resolved, an empirical motion parameter τ_0 was calculated. This gives an approximation of the rotational correlation time of a spin-label in a given solvent (Keith *et al.*, 1974) and is obtained from the relationship:

$$\tau_0 = 6.5 \times 10^{-11} W_0 - [(h_0/h_{-1})^2 - 1] \quad (1)$$

where h_0 and h_{-1} are the height of the mid- and high-field lines respectively and W_0 the width of the mid-field in mT. External medium polarity effects are

minimal in the mid-bilayer region probed by 16NS (Griffith *et al.*, 1974).

Because of the need to distinguish spectral changes due to alterations in motion from those produced by probe-probe interaction, spectra were obtained over a wide range of probe concentrations. It was established that probe-probe interaction did not occur at probe concentrations of $0.1 \mu\text{M}$ probe/ 10^8 cells, and unless otherwise stated this concentration was used in all experiments.

The measurement of probe-probe interaction has been discussed extensively by Sauerheber *et al.* (1977). Two criteria for determining the extent of probe-probe interaction were used; the first was the measurement of W_0 of the mid-field line:

$$W_0 = W_{\text{dip}} + W_{\text{ex}} \quad (2)$$

where W_0 is the linewidth in the absence of interaction, W_{dip} is the line-broadening caused by magnetic dipolar interactions and W_{ex} is contributed by spin exchange. The other measure (m) was based upon the observation that $T_{\perp}$, but not $T_{\parallel}$, broadens with increasing probe concentrations in liver and heart plasma membranes:

$$m = \frac{2}{3} \frac{T_{\parallel} - T_{\perp}}{T_{\parallel} - a_N}$$

where a_N is the isotropic coupling constant and $T_{\perp}$ and $T_{\parallel}$ are equal to half the distance between the inner and outer hyperfine extrema of the first differential spectral curve. A value of m of less than 0.95 was taken as indicative of probe-probe interaction.

Cell volume changes

Cell volume changes in *Dunaliella* that had been subjected to osmotically-induced swelling were measured directly by photomicrography, or indirectly by electrical means with a Coulter model TaII Counter, by the procedures described by Rabinowitch *et al.* (1975) and Grover *et al.* (1969a,b, 1972). A cylindrical orifice with a diameter of $140 \mu\text{m}$ and a length of $100 \mu\text{m}$ was used. Coincidence of cells in the orifice was minimized by diluting the suspension until no further change in volume distribution was found upon further dilution. At least 5×10^4 cells were used in each run. As observed by Rabinowitch *et al.* (1975) for the moderately halotolerant *D. parva*, our *D. salina* cells behaved as classical Boyle-Van't Hoff osmometers according to the equation:

$$\pi_0 = (V - b) = \phi R T n_1$$

where π_0 is the osmotic pressure of the outside solution, V is the cell volume, b the non-osmotic volume of the cell, ϕ the average osmotic coefficient of the intracellular solute, R the gas constant, T the absolute temperature and n_1 the number of moles of solute in the cell (Dick, 1972). The electrical method

was used because its rapidity and inherent averaging characteristics enabled simultaneous volume and e.s.r. measurements. The volumes obtained electrically were 0.48 of the volumes calculated from direct photographic measurement. Cell-surface areas were calculated from the average volumes obtained using the Coulter Counter, after multiplying the latter by 1/0.48.

Results

The effect of increasing NaCl concentration on the spectral parameters of 5NS and 16NS in the membrane of D. salina

5NS gave a typically anisotropic spectrum at all concentrations of NaCl, with a marked increase in the order parameter, S , at the higher salt concentrations. Increasing salt concentrations also decreased the probe-to-cells ratio at which spin-spin interaction occurred, as measured by an increase in ΔH and m . 16NS gave a much more isotropic spectrum than 5NS and it was not possible to calculate a value for S . τ_0 values were calculated instead. It was found that τ_0 for 16NS was much less affected by increasing NaCl concentration and the probe-to-cells ratio at which spin-spin interaction occurred was much lower at all NaCl concentrations than for 5NS. These data are summarized in Table 1.

The effect of increasing sorbitol concentration on the spectral parameters of 5NS and 16NS in the membranes of D. salina

Table 2 shows that there is a significant effect of increasing sorbitol concentration on the order parameter of 5NS, although the increase is considerably less than that observed for NaCl. τ_0 values for 16NS are barely affected by the increased sorbitol concentration and the latter had little effect on the concentration of either probe at which spin-spin interaction occurred.

The effect of rapid changes in solute concentration on membrane lipid ordering

A suspension of *D. salina* (2×10^8 cells/ml in 5.0M-NaCl) was rapidly mixed with an equal volume of distilled water so that the final NaCl concentration was 2.5M. Portions were taken initially at 3min, then at 10min intervals over a period of 150min and the order parameter for 5NS was determined for each portion. The experiment was repeated with sorbitol diluted from 3.0M to 1.5M. The results are plotted in Fig. 1(a). Order parameters were also obtained in the same way for 5NS in cells that had been suddenly transferred from 1.5M- to 3.0M-NaCl and 1.0M- to 2.0M-sorbitol by the addition of the appropriate volume of 5.0M-NaCl or 3.0M-sorbitol. The results are plotted in Fig. 1(b). It can be seen from Fig. 1(a) that a sudden increase

Table 1. *Changes in spectral parameters of 5NS and 16NS in Dunaliella exposed to increasing NaCl concentration*
Each value is the average of three spectra from separate samples. Maximum variation within each group of three was $\pm 6\%$.

Probe concn. ($\mu\text{mol}/10^8$ cells) ...	5NS									16NS					
	0.01			0.05			1.0			0.01		0.05		1.0	
	ΔH			ΔH			ΔH			$10^{10} \times \tau_0 \Delta H$		$10^{10} \times \tau_0 \Delta H$		$10^{10} \times \tau_0 \Delta H$	
	S	(mT)	m	S	(mT)	m	S	(mT)	m	(s)	(mT)	(s)	(mT)	(s)	(mT)
[NaCl] (M)															
0.5	0.612	0.36	0.97	0.612	0.36	0.97	0.616	0.36	0.97	13.51	0.26	13.51	0.26	13.86	0.26
1.0	0.630	0.36	0.97	0.632	0.36	0.97	0.641	0.36	0.96	14.2	0.26	14.32	0.26	14.61	0.26
2.0	0.670	0.37	0.97	0.679	0.38	0.95	0.696	0.39	0.94	15.31	0.27	15.56	0.27	17.85	0.28
3.0	0.730	0.38	0.97	0.738	0.39	0.93	0.749	0.40	0.93	16.61	0.27	16.56	0.27	17.85	0.28
4.0	0.770	0.39	0.97	0.781	0.40	0.91	—	0.41	0.90	17.41	0.20	17.67	0.28	20.37	0.30
5.0	0.805	0.40	0.97	0.821	0.40	0.90	—	0.41	0.90	17.83	0.29	18.92	0.29	23.65	0.31

Table 2. *Effect of increasing sorbitol concentration on spectral parameters of 5NS and 16NS in Dunaliella*
Each value is the average of three spectra from separate samples. Maximum variation within each group of three was $\pm 5\%$.

Probe concn. ($\mu\text{mol}/10^8$ cells) ...	5NS									16NS					
	0.01			0.05			1.0			0.01		0.05		1.0	
	ΔH			ΔH			ΔH			$10^{10} \times \tau_0 \Delta H$		$10^{10} \times \tau_0 \Delta H$		$10^{10} \times \tau_0 \Delta H$	
	S	(mT)	m	S	(mT)	m	S	(mT)	m	(s)	(mT)	(s)	(mT)	(s)	(mT)
[Sorbitol] (M)															
0.5	0.618	0.36	0.97	0.618	0.36	0.97	0.620	0.36	0.97	13.61	0.26	13.65	0.26	13.73	0.26
1.0	0.635	0.36	0.97	0.635	0.36	0.97	0.645	0.36	0.96	14.30	0.26	14.37	0.26	14.44	0.26
2.0	0.661	0.37	0.97	0.668	0.37	0.97	0.673	0.37	0.96	15.25	0.27	15.18	0.27	15.28	0.27
3.5	0.685	0.38	0.97	0.693	0.38	0.97	0.696	0.37	0.96	15.87	0.27	16.05	0.27	16.25	0.27

Fig. 1. *Changes in order parameter, S, for 5NS in the membranes of D. salina when the solute concentration is rapidly decreased (a) and increased (b)*
(a) $\circ$, 5.0M- to 2.5M-NaCl; $\bullet$, 3.0M- to 1.5M-sorbitol. (b) $\circ$, 1.5M- to 3.0M-NaCl; $\bullet$, 1.0M- to 2.0M-sorbitol.

in water activity is accompanied by a sharp decrease in ordering, which then slowly increases to the values established for 5NS at either 2.5M-NaCl or 1.5M-sorbitol. Conversely, when the water activity was suddenly decreased (Fig. 1b) there was a marked increase in ordering, which then slowly decreased to the values established for 5NS at either 3.0M-NaCl or 2.0M-sorbitol.

Relationship between membrane lipid ordering and cell-surface area

To determine whether the changes in ordering that occurred in response to sudden changes in water activity were related to changes in cell size,

Fig. 2. The relationship between changes in the order parameter S and cell-surface area when *D. salina* are rapidly transferred from media of low to high water activity

(a) 5.0M- to 2.5M-NaCl; (b) 3.0M- to 1.5M-sorbitol.

simultaneous e.s.r. and cell size measurements were carried out on cells that had been rapidly transferred from 5.0M- to 2.5M-NaCl. Cell-surface area is plotted against S in Fig. 2(a). The same experiment was carried out using sorbitol, the water activity being increased suddenly by decreasing the solute concentration from 3.0M to 1.5M. A very similar relationship was observed between cell-surface area and S for 5NS (Fig. 2b) as in the case of the NaCl experiment.

Internalization of label during shrinking of osmotically-swollen cells

A cell suspension (2×10^8 cells/ml) was rapidly transferred from 4.0M- to 2.0M-NaCl and then labelled with 5NS. Two portions of cells were taken at 15 min intervals over a 90 min period. One was made 0.05M with respect to ascorbic acid and the e.s.r. spectrum of both recorded. The cells were maintained in light throughout the experiment so as to maintain any internalized spin label in an oxidized state (Fontana & Haug, 1982). At the end of the 90 min the cells were rapidly transferred to 1.0M-NaCl and the proportion of ascorbate-accessible spin label measured. Order parameters were measured and the concentration of spin label in both the control and ascorbate-treated samples was determined by double integration. The results are summarized in Table 3. It can be seen that with increasing time an increasing proportion of the spin label became inaccessible to reduction by the ascorbic acid and that this inaccessible spin label had a significantly lower order parameter than that obtained for cells that had been equilibrated with 2.0M-NaCl and then labelled (Table 1). If the cells are reswollen the label becomes accessible to the ascorbate again.

Discussion

Our results show that changes in external solute concentration induce short- and long-term changes in ordering and motion in the lipids of the plasma membrane of *Dunaliella salina*, as reflected in the behaviour of the 5NS and 16NS probes. The short-term changes were observed over 150 min as the cells adjusted to rapid alterations in external solute concentration; the long-term changes were observed in cells that had been equilibrated for 18 h

Table 3. Effect of reduction with ascorbic acid on the concentration and order parameter of 5NS probe in osmotically-swollen *Dunaliella*

Time (min) ...	0	15	30	45	60	75	90	Re-swollen cells
S (control)	0.618	0.640	0.655	0.655	0.668	0.673	0.675	0.601
S (ascorbate treated)	*	0.589	0.583	0.581	0.580	0.579	0.575	*
% of ascorbate/control†	93	74	46	33	28	27	24	85

* Concentration too low to be measured accurately.

† $100 \times$ concn. of spin label after ascorbate treatment/concn. of spin label in control.

at a given solute concentration, and are similar to those observed by Fontana & Haug (1982). The observations that the changes are much less marked with increasing concentrations of sorbitol and that the outer region of the bilayer probed by 5NS is much more strongly affected by increasing NaCl concentration than the inner region probed by 16NS suggest that the long-term increase in ordering associated with increasing salt concentration is due mainly to electrostatic effects of the NaCl on the charged phospholipid head groups.

Dunaliella behaves as an ideal osmometer and the short-term changes in ordering reported by the 5NS probe possibly reflect changes in membrane properties brought about by changes in cell volume. As the external osmotic pressure decreases, the cells become distended. Initially, this would result in the membrane stretching elastically. If the number of molecules of lipid in the membrane is conserved the tension of the membrane increases with increasing area until the cell lyses, as happens with the osmotically-induced swelling of erythrocytes (Evans & Skalak, 1980). Since lysis does not happen over the 2:1 range of water activities to which *D. salina* can be suddenly subjected, even though the cell-surface area increases by 400%, the number of lipid molecules in the membrane cannot possibly remain constant. It has been suggested (Wolfe & Steponkus, 1981) that plant protoplasts have a reservoir of membrane material associated with the membrane that is incorporated in or removed from it as the cell shape responds to osmotic change. That a similar mechanism must exist in *D. salina* is suggested by the results of the experiments in which ascorbic acid was added to cells that had been spin-labelled and then were held for various times after the water activity had been doubled. These experiments suggest that, as the swollen cell shrinks, substantial amounts of the lipid probed by the 5NS probe are transferred to the interior of the cell.

It should be noted that the lipid that became progressively inaccessible to ascorbate reduction as the cells returned to their normal size had a lower order parameter than that obtained for the total lipid. Assuming that this non-reduced fraction represents interiorized lipid removed from the membrane, then the increase in ordering observed with time and decreasing cell volume (Figs. 1 and 2) must be brought about by removal from the membrane of this material with a lower order parameter. Lipid is added or removed as the cell swells or shrinks and the number of protein molecules is conserved. The other explanation could be that specialized domains of lipid with a lower order parameter are involved in the transfer of lipid from the interior to the plasma membrane. Both mechanisms could provide a means of signalling exterior osmotic changes as it is generally accepted

that changes in lipid motion can affect the activity of membrane-associated enzymes (Weiss, 1974; Sandermann, 1978; McMurchie & Raison, 1979), which could initiate glycerol synthesis or dissimilation.

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