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The structure of membrane lipids of the extreme halophile, *Halobacterium cutirubrum*, in aqueous systems studied by freeze-fracture

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The structures formed by the two major membrane lipids of the extreme halophile, *Halobacterium cutirubrum*, namely diphytanyl ether analogues of phosphatidylglycerol phosphate and glycolipid sulphate, dispersed in either water, 1 M NaCl or 5 M NaCl were examined by freeze-fracture electron microscopy. In water, both lipids formed lamellar phases which were highly hydrated. Dispersion in 1 M NaCl caused the bilayers to stack more tightly. The presence of 5 M NaCl, mixed phases were observed at 20°C consisting of both lamellar and non-lamellar structures. Studies of binary mixtures of the two lipids in 5 M NaCl in mole ratios of 1:2, 2:1 and 3.5:1 indicated that phase separation takes place and that glycolipid sulphate tended to form bilayers at the growth temperature whereas phosphatidylglycerol phosphate preferentially formed a non-bilayer arrangement in the presence of salt. Total polar lipid extracts *H. cutirubrum* formed mixed phase systems that reflected the proportions of the major lipid components. Thermotropic studies performed by thermally quenching dispersions at temperatures ranging from −30°C to 70°C indicated that bilayers were formed at lower temperatures in both pure lipids and mixtures of lipids whereas there was a preference for what gave the appearance of inverted cubic phases at high temperatures. These observations are consistent with the notion that non-bilayer lipids are required to package the intrinsic membrane proteins into a lipid bilayer matrix.

Introduction

The extreme halophiles, amongst which *Halobacterium cutirubrum* is a member, are characterized by growth conditions that demand saturated or nearly saturated salt solutions [1,2]. The habitat of these organisms include salt lakes, such as the Dead Sea, and salt flats formed by evaporation of sea water which expose the organisms to high levels of ultraviolet light. The constituents of the cell membrane of these organisms must be adapted to fulfil the usual membrane barrier functions as well as to provide a suitable matrix into which the membrane proteins are embedded. The lipid constituents are distinguished from those found in the membranes of most other organisms in that the hydrocarbon substituents are saturated phytanyl groups linked by ether bonds to glycerol carbons 2 and 3. It has been suggested [3] that the saturated phytanyl groups provide stability to the membranes against peroxidative damage, that the ether-linked alkyl groups are stable to chemical hydrolysis over a wide pH range and that the attachment to glycerol

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carbons 2 and 3, rather than carbons 1 and 2 in conventional membrane glycerolipids, affords protection against attack by phospholipases of other organisms. The other remarkable feature about these lipids is their highly charged and hydrated polar head groups. The major lipid of both the red and purple membrane of *H. cutirubrum*, the diphytanyl ether analogue of phosphatidylglycerol phosphate (65% by wt. of the total polar lipid), contains two charged phosphate groups while the next most abundant lipid, glycolipid sulphate (25% by wt. of the total polar lipid) contains a polar group consisting of an β-D-galactopyranosyl-3′-sulphate-α-D-mannopyranosyl-α-D-glucopyranosyl group linked to *sn*-2,3-diphytanylglycerol. Hydration of the head group of these lipids involves numerous polar hydroxyl groups in addition to the charged sulphate group. Reviews of the chemistry and biochemistry of these membrane lipids have been published elsewhere [4,6].

The osmotic behaviour of structures formed by these lipids in aqueous systems has been examined by Chen et al. [7] who reported that dispersions of total polar lipid extracts from *H. cutirubrum* in water formed large liposomes consisting of concentric lamellar arrangements as seen in negatively-stained preparations under the electron microscope and which acted as ideal osmometers in KCl and NaCl solutions in the concentration range 5 to 200 mM. At higher salt concentrations the arrangement of the lipids changed so that the structures were no longer osmotically active nor strongly birefringent and it was argued that charge shielding at high ionic concentrations would lead to a decrease in the surface area occupied by the polar group of the lipid relative to the phytanyl chains. It was further suggested that the constraints on packing of the phytanyl chains would be different in such conditions and this suggested that interactions of the lipids with the membrane protein constituents was necessary to preserve stability of the membrane at high salt concentrations. Some calorimetric studies of total polar lipids and fractions thereof have also been reported [7] which indicate the presence of endothermic transitions at subzero temperatures and on this basis it was concluded that the dispersed polar lipids are in a liquid-crystalline configuration at ambient temperature. Similar conclusions were derived from studies of aqueous dispersions of phosphatidylglycerol phosphate using ^{31}P-NMR and calorimetry in the temperature range 5–60°C in which there was no evidence of any thermotropic transition within the lamellar phase [8]. In the presence of high salt concentrations (4 M NaCl or 0.1 M $MgCl_2$), however, transitions localized in the interfacial region of the dispersed polar lipids have been reported on the basis of spin label and specific volume measurements in the temperature range −11°C to 45°C [9].

The phase behaviour of membrane lipids dispersed in water contrasts markedly with some studies of the thermal properties of intact membranes from *Halobacteria* which are reported to undergo transitions at high temperature [10] associated with changes in the crystal lattice of lipid and protein [11] while other studies using ^{31}P-NMR could detect no phase changes within the membrane [8]. Other calorimetric [12] and spin label [13] studies of these membranes found evidence for phase transitions in the temperature range 27–40°C with the hydrocarbon chains in a relatively rigid state. In a comparison of the flexibility of lipids in membrane preparations from *H. cutirubrum* with that of dispersions of extracted lipids using spin-labelled fatty acid probes in which a *N*-oxyldimethyloxazolidine substituent was located at different positions along the acyl chain, it was found that the lipids in the membrane were in a relatively immobilized state while the lipids in aqueous dispersion were comparitivly mobile even towards the ether linkage of the phytanyl chains to the glycerol [14]. All these studies pointed to a strong interaction between the lipid and the protein in the membranes of the halophilic bacteria which leads to a considerable modification of the phase behaviour of the isolated lipids.

In the absence of more detailed structural information about the diphytanyl lipids in aqueous systems it is difficult to assess the effects that interaction with membrane proteins will have on the phase behaviour of these lipids. The present freeze-fracture study is aimed to provide information on the physical structure of the major lipid constituents of *H. cutirubrum* in aqueous dispersions and mixtures of these lipids in proportions approximating those present in the membranes of the bacterium.

Materials and Methods

Preparation of lipids. Total polar lipids of *H. cutirubrum* were prepared by extraction of whole cells by the procedure of Bligh and Dyer [15] as described previously [16,17]. The polar lipids were separated from the neutral lipids by acetone precipitation [18] and fractionated by barium salt precipitation and preparative TLC [4,19,20].

A chloroform solution of the total polar lipids was diluted with a 10-fold excess of methanol and cooled to 4°C. The methanol-insoluble lipids were removed by centrifugation and the phosphatidylglycerol phosphate was precipitated by the addition of 20% (w/v) $BaCl_2$ in water (1–2 ml). The supernatant was found to contain primarily glycolipid sulphate. Phosphatidylglycerol phosphate and glycolipid sulphate were converted to the ammonium salt forms and purified by preparative TLC in an acid solvent system consisting of chloroform/90% acetic acid/methanol (30 : 20 : 4, v/v). The lipids were visualized with iodine and eluted from the silica with chloroform/methanol (1 : 1, v/v). The solutions were concentrated under reduced pressure and the residual lipids converted to the ammonium salt forms [19,20], precipitated from a minimum of chloroform with a 10-fold excess of acetone and dried in vacuo.

Hydration of lipids. Dried lipids were weighed into small sample tubes. Lipid mixtures were dissolved in a small volume of chloroform/benzene (2 : 1, v/v) and the solvent removed under a stream of dry nitrogen. Remaining traces of solvent were removed by vacuum desiccation. Aliquots of water or salt solution were added to the dried lipids to give a lipid concentration of 20% (w/v). Hydration was assisted by heating to 80°C in some cases and in others allowed to equilibrate over several days at 20°C in sealed tubes.

Freeze-fracture electron microscopy. Dispersions were sandwiched between two thin copper sample mounts (Balzers Union, 12 057/1205JT) and thermally equilibrated for at least 1 min at the required temperature using a regulated nitrogen gas flow monitored by a thermocouple placed adjacent to the specimens. Thermal quenching was achieved by liquid nitrogen jet freezing. Platinum-carbon replicas were prepared of the fracture surface exposed at a temperature of −115°C in a Polaron freeze-fracture device. The replicas were cleaned in chloroform/methanol, (2 : 1, v/v) before examination under a Philips EM 301G electron microscope.

Negative staining. Lipid dispersions were mixed with an equal volume of 2% (w/v) ammonium molybdate and applied to a carbon-coated sample grid.

Results

The structures of phosphatidylglycerol phosphate and glycolipid sulphate hydrated with water were examined using freeze-fracture electron microscopy and negative staining methods. Electron micrographs of replicas prepared from dispersions thermally quenched from 20°C showed that both lipids form lamellar structures but the phosphatidylglycerol phosphate forms small liposomal-like structures with relatively few layers (Fig. 1a) whereas the glycolipid sulphate forms large multilamellar liposomes (Fig. 1c). Dispersion of the lipids in 1 M NaCl caused the bilayers to stack more tightly with removal of aqueous medium from the interbilayer regions. Close apposition is observed in the bilayers of phosphatidylglycerol phosphate (Fig. 1b) and glycolipid sulphate (Fig. 1d) in which lenses of aqueous medium may be seen in cross fracture faces exposing the interior of large multilamellar structures.

The irregular spacing between the bilayers of lipid dispersed in water can be seen more clearly in negatively-stained preparations (Fig. 2). Penetration of stain into specimens shown in Figs. 2a and 2c confirms that the lipid is arranged in multilamellar structures in which the layers are not evenly separated.

The presence of relatively high concentrations of salt in the aqueous phase causes a dramatic change in the structure of the lipids. Electron micrographs of freeze-fracture replicas obtained from dispersions of phosphatidylglycerol phosphate and glycolipid sulphate in 5 M NaCl showed (Fig. 3) that both lipids exhibit heterogenous phase behaviour but the predominant form of phosphatidylglycerol phosphate is a lamellar structure with evidence of non-bilayer lipid arrangements closely associated with the lamellae (Fig. 3a). The most common form of the glycolipid sulphate

appeared to be large multilamellar aggregates (Fig. 3b).

To determine the effect on membrane structure and stability of these two lipids in the membrane of *H. cutirubrum* mixtures of phosphatidylglycerol phosphate and glycolipid sulphate corresponding

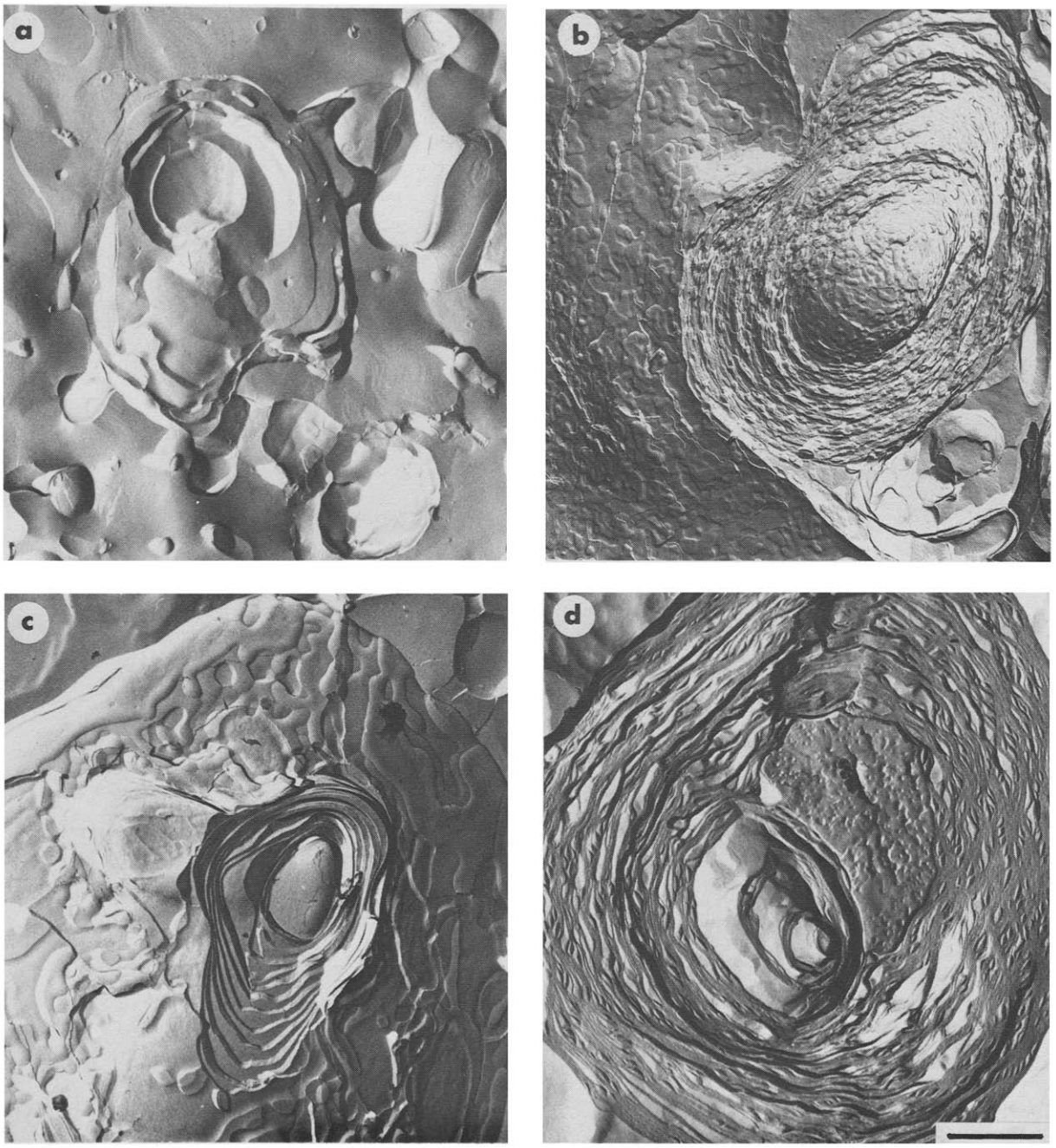

Fig. 1. Electron micrographs of freeze-fracture replicas of phosphatidylglycerol phosphate dispersed in (a) water and (b) 1 M NaCl; glycolipid sulphate dispersed in (c) water and (d) 1 M NaCl. Dispersions were all thermally quenched from 20 °C. Bar represents 500 nm.

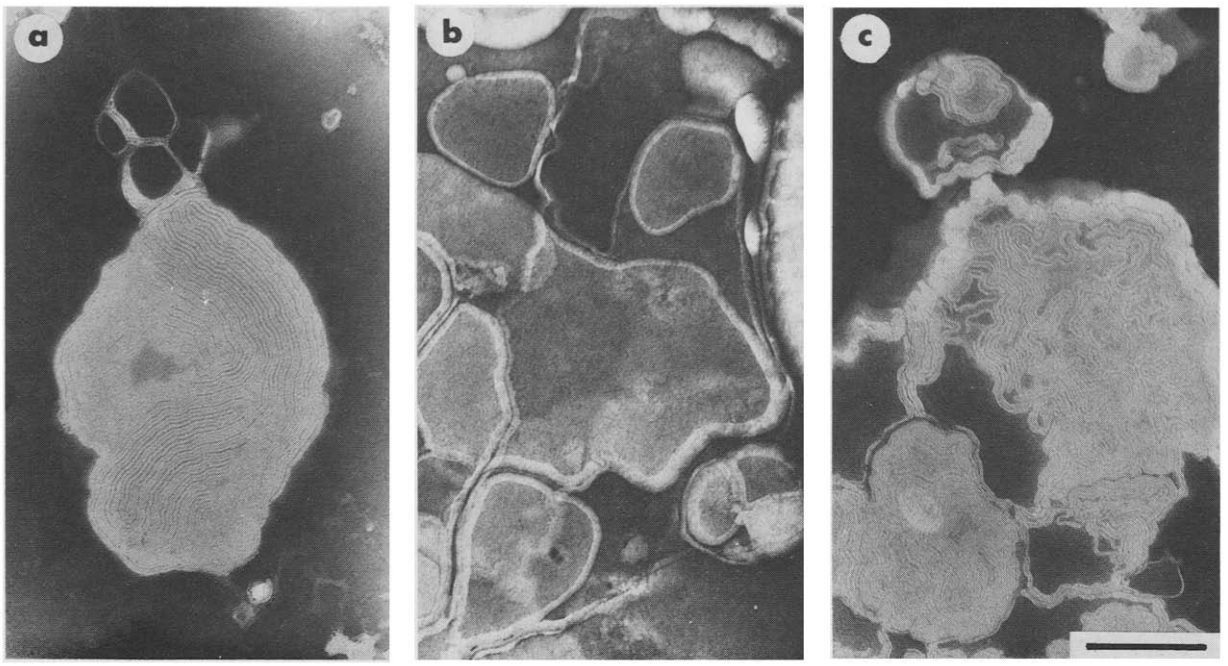

Fig. 2. Electron micrographs of negatively-stained aqueous dispersions of (a) phosphatidylglycerol phosphate, (b) glycolipid sulphate, (c) total polar lipids of *H. cutirubrum*. Bar represents 250 nm.

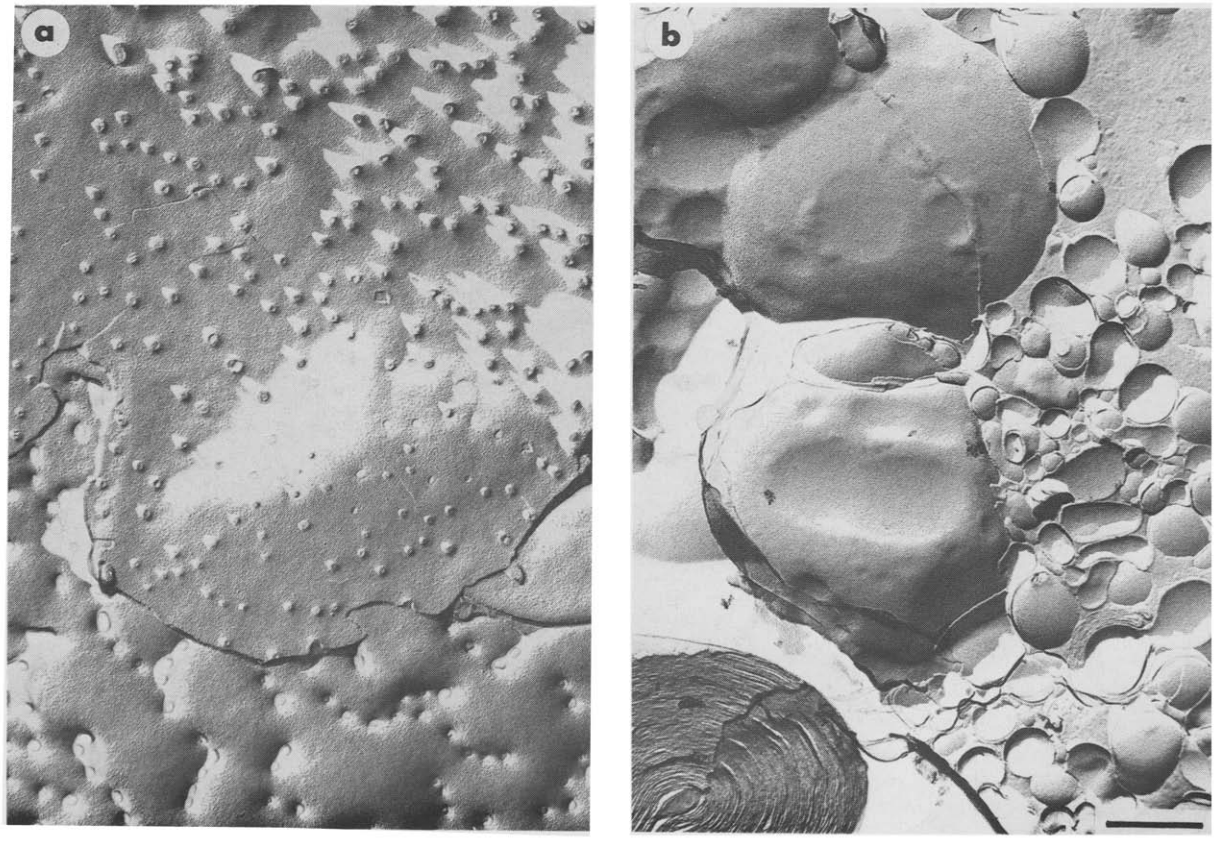

Fig. 3. Electron micrographs of freeze-fracture replicas prepared from (a) phosphatidylglycerol phosphate and (b) glycolipid sulphate dispersed in 5 M NaCl and thermally quenched from 20 °C. Bar represents 250 nm.

to ratios that occur in the outer leaflet of the purple membrane (assuming glycolipid sulphate to be entirely in the outer leaflet [21]), the purple membrane [17] and the cell membrane [17], in which both red and purple membranes are components, were examined. The mole ratios of phos-

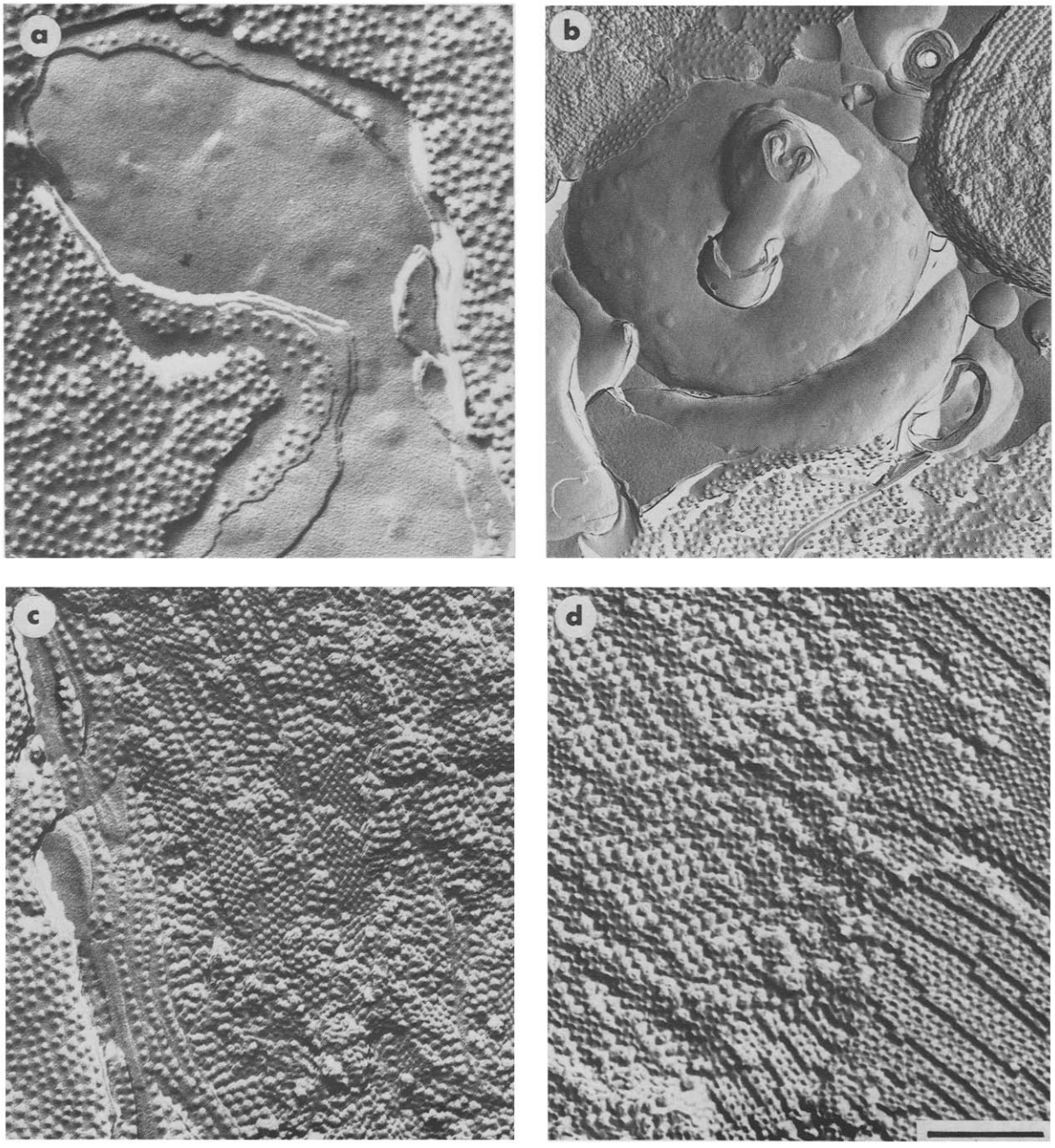

Fig. 4. Electron micrographs of freeze-fracture replicas prepared from mixed dispersions in 5 M NaCl of phosphatidylglycerol phosphate: glycolipid sulphate in mole ratios of (a) 1:2, (b) 2:1, (c) 3.5:1. The electron micrograph shown in (d) is that of a dispersion of total polar lipids of *H. cutirubrum* prepared in the same way as the lipid mixtures. Bar represents 250 nm in a, c and d; 500 nm in b.

phatidylglycerol phosphate to glycolipid sulphate were 1 : 2, 2 : 1 and 3.5 : 1, respectively. The total polar lipids of *H. cutirubrum* dispersed in 5 M NaCl were also examined; this mixture contained minor amounts of phosphatidylglycerol and phosphatidylglycerol sulphate [4], but was otherwise identical in composition to the sample with a mole ratio of phosphatidylglycerol phosphate: glycolipid sulphate of 3.5 : 1. The results of the freeze-fracture experiments with the various lipid mixtures are summarized in Fig. 4 in which electron micrographs of typical regions of the freeze-fracture replicas are shown. In general, all replicas show evidence of lamellar and what appear to be cubic phases as well as a variety of intermediate phases including tubular inverted micelles which often resemble hexagonal-II structure. The conclusions from careful examination of a number of replicas prepared from each mixture is that as the proportion of phosphatidylglycerol phosphate to glycolipid sulphate in the mixture is increased there is an increase in the proportion of the lipid structure in non-lamellar arrangement at the expense of structure that is purely lamellar (cf. Figs. 4a, b and c). The heterogeneity of the overall structure is exemplified by the 2 : 1 mole ratio mixture (Fig. 4b) which shows evidence of lamellar, cubic and a variety of intermediate phases all in close juxtaposition.

Studies of all the mixed lipid systems in 5 M NaCl, including the total polar lipids of *H. cutirubrum* (Fig. 4d), indicated that phase separations are likely to have resulted during preparation of the dispersions or during the freeze-fracture process. The latter effects could be largely excluded because there was no evidence of salt crystallization or distortions in the fracture faces to indicate that ice crystals had formed during the thermal quenching. All lipid dispersions were heated to 80°C prior to thermal equilibration and

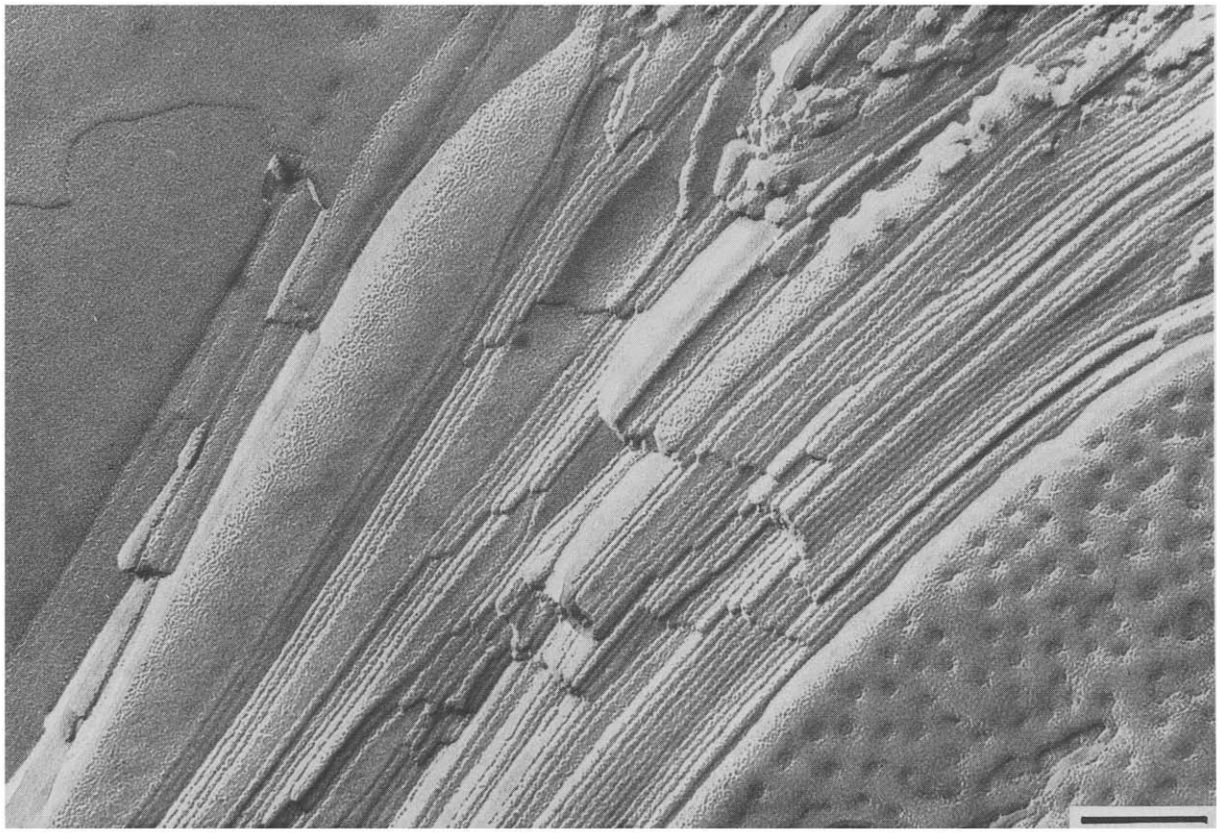

Fig. 5. Electron micrograph of a freeze-fracture replica prepared from a dispersion in 5 M NaCl of phosphatidylglycerol phosphate and glycolipid sulphate in a mole ratio of 2 : 1. The lipids were hydrated and thermally quenched from 20°C. Bar represents 100 nm.

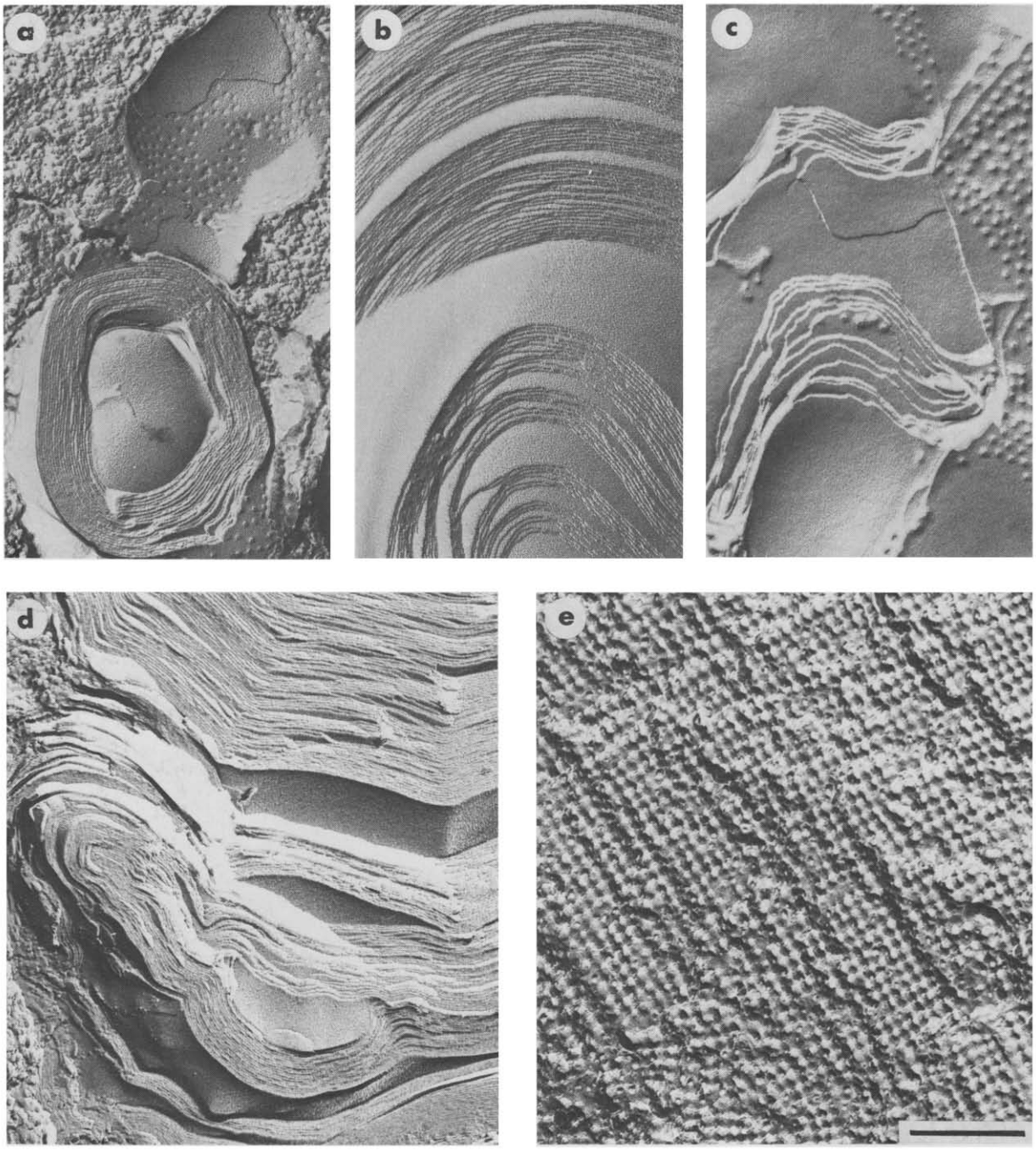

Fig. 6. Electron micrographs of freeze-fracture replicas of dispersions in 5 M NaCl of (a) phosphatidylglycerol phosphate, (b) glycolipid sulphate, (c) 2:1 molar ratio mixture of phosphatidylglycerol phosphate/glycolipid sulphate all thermally quenched from 0°C. Total polar lipid dispersions in 5 M NaCl thermally quenched from (d) −30°C, and from (e) 70°C are also shown. Bar represents 250 nm.

thermal quenching to ensure even hydration and to facilitate dispersion. To check the effects of exposure of the samples to high temperature, a mixed dispersion containing a mole ratio of 2 : 1 phosphatidylglycerol phosphate to glycolipid sulphate was hydrated at 20°C and thermally quenched from the same temperature. The predominant features of replicas prepared in this way were tubular micelles and what appeared to be hexagonal-II structure; the proportion of the lipid in pure lamellar or cubic structure was considerably less (Fig. 5). This suggested that heating dispersions to high temperature induces a phase separation within the lipid mixture. Moreover, the structure that forms in the mixture is not typical of either of the components of the mixture when dispersed alone in 5 M NaCl and thermally quenched from this temperature. To confirm that phase separations take place during heating, a sample prepared as indicated in Fig. 5 was heated to 80°C for 5 min, thermally equilibrated at 20°C and examined by freeze-fracture methods. The replica was identical to that shown in Fig. 4b.

To examine the effect of temperature on the phase behaviour of polar lipids from *H. cutirubrum*, dispersions of lipid in 5 M NaCl were thermally equilibrated at designated temperatures before thermal quenching. Pure lipid dispersions of phosphatidylglycerol phosphate (Fig. 6a) and glycolipid sulphate (Fig. 6b) both showed a predominance of lamellar structure when thermally equilibrated and quenched from 0°C. The phospholipid formed small liposomal structures with evidence of associated non-lamellar structures. The glycolipid sulphate, on the other hand, consisted of large multilamellar liposomes with only occasional regions of the fracture face exposing non-bilayer structures. Mixed lipid dispersions thermally quenched from 0°C (Fig. 6c) indicated close association between lamellar and non-lamellar structures throughout the replica surface suggesting that phase separations between the two components of the mixture had been created by lowering the temperature. Studies of dispersions of total polar lipid extracts in 5 M NaCl thermally quenched from −30°C and 70°C were undertaken and the results are shown in Fig. 6d and e, respectively. At the lower temperature an almost homogeneous lamellar phase was formed. Thermal equilibration at the higher temperature by contrast resulted in formation of a predominantly cubic phase with other non-lamellar structures located at intervals throughout the replica.

Discussion

The behaviour of total polar lipid extracts of *H. cutirubrum* in water and those of phosphatidylglycerol phosphate and glycolipid sulphate isolated therefrom are typical of that observed in acidic phospholipids [22] or mixtures of acidic and zwitterionic lipids [23,24]. Such lipids are found to form bilayer structures which take up water into the intercalated regions between the bilayers so that the lipid-water phase contains a relatively high water content. No precise values of limiting water contents in such lipid-water systems are available but X-ray diffraction measurements suggest that, at least up to concentrations of 70–80% (v/w) water, a single lamellar swelling phase exists [22]. At higher water contents the stacking order of the bilayers is decreased and, at a point that appears to be independent of the type of lipid, the multilamellar structures break up and each lamellae of the aggregate seals off in the form of single bilayer vesicles. The systems examined in the present study contain 80% (v/w) water which may represent an extensively hydrated but not necessarily a fully hydrated state of the *H. cutirubrum* lipids. Nevertheless, it can be noted from a comparison of structures formed by phosphatidylglycerol phosphate and glycolipid sulphate that the former reaches a more hydrated state at the same water content as that of the glycolipid sulphate (Figs. 1 and 2). Thus the predominant form of the phospholipid is small uni- or oligolamellar bilayer vesicles whereas the glycolipid sulphate exists as multilamellar liposomes albeit greatly swollen with intercalated water. This may indicate that charge repulsion between adjacent molecules of the phospholipid is greater than that experienced by neighbouring glycolipid sulphate molecules arranged in bilayer configuration. Recent monomolecular film studies confirm this interpretation (Kates, M., Stewart, L.C. and Tocanne, J.F., unpublished observations).

Shielding charges on these acidic lipids with 5 M NaCl causes a shift in dominance of the polar

group on the phase structure to that of the phytanyl chains. The phases formed by total polar lipids and the two major lipid components are much less hydrated and the most striking feature is the appearance of non-lamellar phases (Figs. 3 and 4). These take on a number of recognisable forms including inverted micelles sandwiched between bilayers, attachment sites between adjacent bilayers, tubular micelles all of which have been described in membrane lipids of various origin [25]. It is perhaps noteworthy that even with pure lipid fractions isolated from the total polar lipid extracts there is no clear evidence that a homogeneous lipid phase exists in 5 M NaCl even over a relatively wide range of temperature. What is clear, however, from the studies of the isolated lipids and mixtures of the two major lipids is that the glycolipid sulphate tends to form multibilayer structures in salt concentrations and temperatures akin to the growth conditions of the bacterium whereas the phosphatidylglycerol phosphate tends to form a mixed lamellar and non-lamellar phases (Fig. 6a). On the basis of the earlier studies of Chen et al. [7] phase separation of these non-bilayer structures must occur when the salt concentration exceeds about 200 mM when osmotic behaviour becomes non-ideal.

The thermotropic study confirms that non-lamellar arrangements of the individual lipids or mixed dispersions in 5 M NaCl tended to revert to a lamellar phase on reducing the temperature and that a bilayer arrangement is the preferred phase at low temperature. The reverse was true on heating the dispersions but the equilibrium structure of the pure lipids or their mixtures at high temperature is not clear. Unlike many other membrane lipids such as phosphatidylethanolamines [26] and monohexosyldiacylglycerols [27], which tend to form hexagonal-II phases at high temperature, the diphytanyl lipids of *H. cutirubrum* appear to prefer reversed cubic phases presumably with disordered arrangements of the phytanyl chains. It should be noted, however, that mixtures of mono- and digalactosyldiacylglycerols from wheat leaves have been shown to form reversed cubic phases at low water contents [28]. The presence of the side chain methyl groups of the phytanyl chains would seem to prevent close packing that would be required in formation of the hexagonal-II structure and the formation of an inverted cubic phase may not be unexpected.

Detailed structural analyses of the purple membrane of halophiles has indicated a crystalline packing of the lipid and the protein in the membrane [29] with the arrangement of the lipid exclusively in a bilayer configuration. Since the preferred arrangement of the dispersed lipid in salt solutions in which the membrane is stable is a non-bilayer configuration we may infer that the interaction of the lipid with the protein imposes a bilayer arrangement on the lipid in the membrane. Other membrane components such as squalene are also known to influence the phase behaviour of the membrane lipids [30].

It has been suggested that the function of non-bilayer forming lipids in membranes is to package the intrinsic proteins in such a way that irregular protein surfaces could be effectively intercalated within the hydrocarbon phase [31]. The bulky nature of the hydrocarbon substituents characteristic of the phytanyl ether lipids would be well suited to this role. Furthermore, such non-bilayer forming lipids appear also to be responsible for promoting oligomeric complexes between membrane protein components such as the protein-pigment complexes of the photosynthetic membrane [31,32] and the calcium pump protein of sarcoplasmic reticulum [33]. Whether the diphytanyl lipids are able to influence the activation energy of membrane processes of *H. cutirubrum* has yet to be determined but the high proportion of membrane protein in contact with the phytanyl groups [17] suggests that the packaging of these proteins into the membrane may be an important function of these lipids.

Acknowledgements

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