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The effect of salinity on the phase behaviour of total lipid extracts and binary mixtures of the major phospholipids isolated from a moderately halophilic eubacterium

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The effects of molar NaCl concentrations on the phase behaviour of the total lipid extracts and binary mixtures of the major phospholipids, namely phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), isolated from the moderately halophilic eubacterium, *Vibrio costicola*, grown in 1 M and 3 M NaCl containing media have been studied using X-ray diffraction and freeze-fracture electron microscopy. The effect of both the PE/PG ratio and alterations in fatty acid composition were examined by using binary mixtures which mimicked the PE/PG ratio found in the native bacterial membranes. We show that the samples exhibited complex phase behaviour, including the formation of non-bilayer phases, which depend upon the salinity of both the bacterial culture medium and the suspending solution. The total lipid from bacteria cultured in 1 M NaCl-containing medium and dispersed in 1 M NaCl exhibited a mixture of L_α and hexagonal-II phases at the optimum growth temperature of the organism (i.e., 30°C), whereas the same lipid dispersed in 3 M NaCl showed only a hexagonal-II phase down to a temperature of +3°C. The total lipid extracted from 3 M NaCl cultures showed only lamellar phases over the temperature range studied (+50°C to −50°C), but the phase transition temperatures of the various lamellar phases were generally higher when the lipid was dispersed in 3 M compared with 1 M NaCl. The phase behaviour of the binary mixtures was similar but not identical to that of the corresponding total lipid extracts and it is suggested that the minor lipid components (diphosphatidylglycerol, lysophosphatidylethanolamine and lysophosphatidylglycerol) play a part in determining the phase behaviour of the native membranes. These results show that the PE/PG ratio and fatty acid composition of the individual phospholipids, which are normally regulated by *Vibrio costicola* in vivo in response to culture medium salinity, are both important in maintaining a stable bilayer structure within the membrane.

Introduction

Biological membranes contain a mixture of lipids which, in isolation and under physiological conditions, can be grouped into those which form bilayers and those which aggregate into non-bilayer structures such as hexagonal-II and cubic phases. The group which typically forms bilayers includes molecular species of phosphatidylcholines, phosphatidylglycerols [1] and diglycosyldiacylglycerols [2]. Lipids forming non-bilayer structures include the phosphatidylethanolamines [3], monoglycosyldiacylglycerols [2] and plasmenylethanolamines [4]. The phase behaviour of all these lipids is affected not only by the polar head-group and fatty acid substituents, which define the lipid class and molecular species respectively, but also by the presence of solutes in the dispersing solution [5]. In order to form a functional biological membrane the mixture of lipids must be balanced precisely to include appropriate proportions of lipids from the two groups [5,6]; in addition, specific interactions between proteins and the different groups of lipids must be considered.

The stability of the bilayer is of crucial importance for cell viability, although the formation of transient non-bilayer mesophase structures or perturbed arrangements in the vacinity of integral membrane proteins cannot be excluded. It has been suggested that the reason why non-bilayer-forming glycolipids and phospholipids comprise a significant proportion of the total membrane lipid in many biological membranes is be-

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cause local regions forming non-bilayer structures are necessary for processes such as membrane fusion [7,8] and in the transmembrane movement of macromolecules [9]. However, the precise functional role of non-bilayer structures in biological membranes remains unknown.

Despite the importance of non-bilayer-forming lipids for membrane structure and stability the phase behaviour of lipids from relatively few organisms has been studied. The adaptation of cells to changes in the fatty acyl-chain composition is the best documented response which causes a modification to the ratio of non-bilayer- to bilayer-forming membrane lipids, and has been studied in *Acholeplasma laidlawii* (see Ref. 10 for a review) and *Clostridium butyricum* [4]. As the degree of unsaturation of the hydrocarbon chains is increased, *A. laidlawii* decreases the ratio of monoglucosyldiacylglycerol to diglucosyldiacylglycerol [2]. Unsaturated species of monoglucosyldiacylglycerol form non-bilayer structures at physiological temperatures but are stabilised in a bilayer phase by the addition of unsaturated species of diglucosyldiacylglycerol [11]. Similarly, in *C. butyricum* the ratio of non-bilayer- to bilayer-forming lipids was decreased when the membrane lipids were highly enriched with *cis*-unsaturated fatty acids and it was shown that this change in relative proportions was necessary to produce a lamellar arrangement of lipid mixtures [4].

Halotolerant and halophilic bacteria offer a good opportunity to study the function of non-bilayer-forming lipids in native membranes, since these organisms modify the ratio of non-bilayer- to bilayer-forming lipids in their membranes in response to alterations in NaCl concentration of the culture medium. For example, the moderately-halophilic eubacterium *Vibrio costicola*, in common with many other Gram-negative halophilic and halotolerant eubacteria [12], increases the ratio of phosphatidylglycerol to phosphatidylethanolamine from less than 0.5 : 1 in 1 M NaCl- to over 1 : 1 in 3 M NaCl-containing media [13] and there are also concomitant changes in fatty acid composition [14]. It has been suggested that such increases in the proportion of anionic lipids (usually phosphatidylglycerol) in moderate halophiles in response to raised salinity counter the high Na^+ concentration at the membrane surface [15]. However, the actual increase in amount of anionic lipids could only account for negative-charge shielding by cations of the order of millimolar, and not molar, salt concentrations [16] and this theory does not account for the equimolar concentration of Cl^-. Similar changes in anionic lipid composition have also been demonstrated in response to non-ionic solutes in *V. costicola* [17] and a range of halotolerant food-spoilage bacteria [18]. Therefore, the lipid changes are not related specifically to NaCl or ionic effects but are probably linked to water activity. An alternative hypothesis is that the modification to the membrane phospholipid ratio is necessary in order to preserve the integrity of the membrane bilayer in the face of an increased tendency of phosphatidylethanolamine to form non-bilayer phases as a consequence of raised external salinity [12].

Phosphatidylethanolamine and phosphatidylglycerol together comprise more than 80% of the total membrane lipid of *V. costicola*. We have demonstrated previously that the phase behaviour of the purified phosphatidylethanolamine and phosphatidylglycerol depends on not only the salinity of the solution used to resuspend the lipids [19] but also that of the bacterial culture medium as well which influences the fatty acyl composition of the phospholipids [14]. The major fatty acids of both phosphatidylethanolamine and phosphatidylglycerol from *V. costicola* grown in 1 M or 3 M NaCl-containing medium are 16 : 1*c*9, 16 : 0 and 18 : 1*c*11 [14]. The phospholipids have a predominantly *sn*-1 saturated, *sn*-2 unsaturated fatty acyl chain configuration which is affected little by the salinity of the growth medium. The fatty acyl residues of phosphatidylethanolamines and phosphatidylglycerols from *V. costicola* respond independently to changes in the salinity of the growth medium. In comparison with phosphatidylethanolamine, there are larger salinity-dependent changes in the fatty acid composition of phosphatidylglycerol. The phosphatidylglycerol isolated from 1 M NaCl cultures has a larger C18/C16 ratio and is more saturated than the phosphatidylglycerol from 3 M NaCl cultures; an increase in 16 : 1*c*9, from 30.7 mol% in 1 M NaCl cultures to 38.5 mol% in 3 M NaCl cultures, is associated with a corresponding decrease in 18 : 1*c*11 content. The minor (i.e., < 3.0 mol%) components 16 : 1*c*11 and 18 : 1*c*13 are present only in phospholipid extracted from 3 M NaCl cultures. The fatty acids of phosphatidylethanolamine from cultures grown in 1 M and 3 M NaCl have the same average chain length and unsaturation index. The main difference in the fatty acid composition of the phosphatidylethanolamines is a decrease in 18 : 1*c*11 from 19.2 mol% in 1 M NaCl cultures to 14.9 mol% in 3 M NaCl cultures [14].

The two phospholipids exhibited complex phase behaviour which was dependent on the salinity of both the bacterial culture medium and the phospholipid resuspending solution [19]. The phosphatidylethanolamine from cultures grown in 1 M or 3 M NaCl-containing media displayed a hexagonal-II phase which was present at the growth temperature of the organism (i.e., 30°C) and this phase persisted at temperatures up to 20 C° lower when the lipid was dispersed in 3 M compared with 1 M NaCl. The L_α phase of phosphatidylethanolamine isolated from 1 M NaCl-grown cultures was favoured less when the lipid was resuspended in 3 M compared with 1 M NaCl. The $L_\beta \rightarrow L_c$ phase transition temperature of the phosphatidylethanolamines

coincided with ice formation. In contrast, the phosphatidylglycerol samples exhibited only lamellar phases over the temperature range +50°C to −50°C. An increase in salinity of the resuspending solution increased the $L_\alpha \leftrightarrow L_\beta$ and $L_\beta \leftrightarrow L_c$ phase transition temperatures, indicating that the L_β and L_c phases were both stabilised by the higher NaCl concentration. The $L_\alpha \leftrightarrow L_\beta$ and $L_\beta \leftrightarrow L_c$ phase transition temperatures of phosphatidylglycerol isolated from 3 M NaCl cultures were higher than those from 1 M NaCl cultures. Formation of separate large domains of hexagonal-II phase lipids in the bacterial membrane would impair the cellular permeability barrier. This in turn would disrupt the ionic gradients which the cell requires for survival, as well as allowing the influx of the Na^+ and Cl^- present in the high salinity media, both of which would result in cell death. This hypothesis also explains why ionic and non-ionic solutes have similar effects, as long as they induce the formation of non-bilayer phases, possibly through dehydration of the phosphatidylethanolamine headgroups.

In the present study we have examined the effect of salinity upon the phase behaviour of total-lipid extracts and binary mixtures of phosphatidylethanolamine and phosphatidylglycerol, isolated from *V. costicola* grown in 1 M and 3 M NaCl-containing media, by dynamic X-ray diffraction and freeze-fracture electron microscopy. Binary mixtures were chosen to mimic the ratios of PE/PG found in the native bacterial membrane using phospholipids extracted from cultures grown at low or high salinity so that the effect of both the PE/PG ratio and alterations in fatty acid composition could be examined.

Materials and Methods

Vibrio costicola (NRC 37001) was grown aerobically at 30°C in a complex liquid medium containing 0.3% (w/v) Proteose Peptone (Difco), 0.3% (w/v) Bacto Tryptone (Difco) and the appropriate concentration of NaCl (AnalaR grade) as 10 litre cultures in a Microferm fermentor (New Brunswick). Bacteria were harvested in the late-exponential growth phase using a Sharples continuous-flow centrifuge operated at 25000 rpm.

Total lipid was extracted using the method of Bligh and Dyer as described by Kates [20]. Non-lipid contaminants were removed from the total-lipid extract by liquid/liquid partition chromatography using Sephadex G-25 (fine grade, Pharmacia) as described by Wells and Dittmer [21]. The purified total-lipid extract was fractionated into its component phospholipids by carboxymethyl cellulose chromatography (CM 52 sodium form, Whatman) [22]. Phospholipid samples were shown to be more than 98% pure on the basis of two-dimensional thin-layer chromatographic separations and quantitation by phosphorus and fatty acid analyses [23].

Lipid samples were prepared from stock solutions in chloroform; before use the solvent was removed using a stream of dry nitrogen gas, the sample placed under vacuum for 16 h and hydrated with 80 vol.% of the appropriate NaCl solution (pH 7.0).

Samples for freeze-fracture were thermally quenched from the desired temperature using a jet of liquid nitrogen. A Polaron freeze-fracture machine was used to fracture the specimens at −115°C, and platinum-carbon replicas of the fracture surface were prepared. The replicas were cleaned using a solvent consisting of $CHCl_3$/MeOH (1 : 1, v/v) before examination using a Philips EM301 transmission electron microscope operated at 100 kV.

X-ray diffraction studies were performed using a monochromatic focused X-ray beam at station 8.2 of the Daresbury Synchrotron Laboratories (Daresbury, U.K.). A cylindrically bent single crystal of germanium [24] and a long float-glass mirror were used to select monochromatic X-rays and to focus the beam horizontally to provide a beam size of 0.3 × 3.0 mm with about 10^{10} photons · s^{-1} at 2.0 GeV and 200 mA of electron beam current with the Wiggler operating at 5.0 tesla. A flat-plate camera was used with a linear-wire detector constructed at Daresbury. The detector contained 512 channels each of 0.193 mm. The detector response was determined by recording the signal from a fixed source accumulated over 1 h. X-ray scattering data were acquired in 255 consecutive time frames with an acquisition time for each frame of 4 s and a dead time between frames of 50 μs. Data were stored on a VAX 11/785 computer and the experimental data sets were corrected for detector response using the OTOKO program at the Daresbury Laboratories. Calibration of the spacings was obtained using Teflon [25] and dipalmitoylphosphatidylcholine in the L_β phase [26] as standards. The configuration of the camera and detector was such that diffraction spacings smaller than 0.35 nm were not detected. The resolution of the camera/detector configuration was 0.15 nm for a diffraction spacing of 5.0 nm and 0.01 nm for a diffraction spacing of 0.36 nm. Samples were mounted between thin mica sheets set 1 mm apart and placed in a vertically-mounted THM600 cryostage (Linkam Scientific Instruments, Tadworth, U.K.) abutting to an electrically-heated silver block. A flow of nitrogen gas at −150°C was passed internally through the sample holder. A TMS90 control system fitted with a remote control unit (Linkam) provided the appropriate amount of power to the heating block to maintain the sample at the desired temperature. Temperature scans were performed by programming the control system to maintain the desired rate of heating or cooling between preset temperature limits. A scan rate of 10 C° · min^{-1} was chosen to limit the

exposure of the sample to the X-ray beam so as to avoid radiation damage and to be consistent with previous studies [19]. Two thermocouples placed adjacent to the sample in the sample holder were used to monitor the temperature internally. The anaiogue signals from these were recorded digitally, together with the data file, on the VAX 11/785 computer. Samples recovered from the apparatus were subjected to thin-layer chromatography and showed no signs of degradation.

Results

Assignment of mesophases

The effect of salinity on the phase behaviour of total membrane lipids and binary mixtures of purified phosphatidylethanolamine and phosphatidylglycerol extracted from *Vibrio costicola* grown in 1 M and 3 M NaCl-containing media has been examined using freeze-fracture electron microscopy and dynamic X-ray diffraction. Mesophases were assigned on the basis of wide- and small-angle X-ray scattering profiles (Fig. 1) [27,28] and complementary freeze-fracture electron microscopy (Fig. 2). The lamellar and hexagonal-II phases were distinguished by their different small-angle diffraction profiles. Although small-angle Bragg diffraction peaks in the ratio $1:1/\sqrt{3}:1/\sqrt{4}:1/\sqrt{7}$ (Fig. 1d) can describe cubic and hexagonal mesophases, freeze-fracture electron micrographs of the phase showed structures consistent with a hexagonal rather than a cubic arrangement (Fig. 2a). Since these studies were with diacylphospholipids a hexagonal-II phase, rather a hexagonal-I phase, was assigned. The lamellar phases were subdivided according to their wide-angle scattering

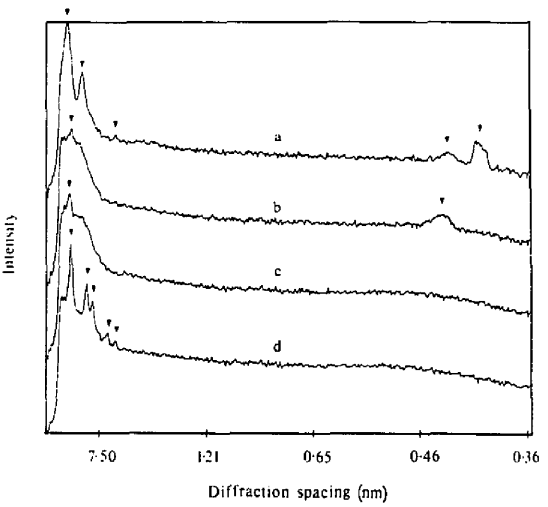
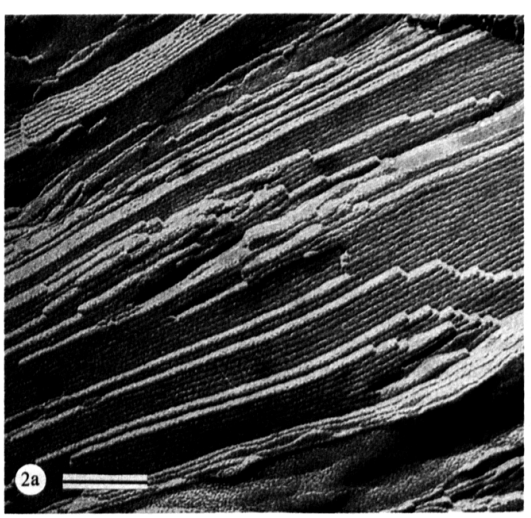


Fig. 1. The dependence of X-ray scattering intensity on the diffraction spacings of total-lipid extracted from *Vibrio costicola* grown in 1 M NaCl-containing medium and dispersed in 80% by vol. 1 M NaCl (pH 7.0). Phases were assigned as follows: (a) L_c (−25°C), (b) L_β (−10°C), (c) L_α (+10°C) and (d) hexagonal-II (+30°C). In the low angle region (diffraction spacing > 0.50 nm) the arrows (▼) indicate the Bragg diffraction maxima taken with increasing orders of magnitude (not all the maxima are distinct in every phase). The wide-angle diffaction maxima (▼) indicate the spacings of the acyl chains. Data acquisition time for each frame was 4 s.

patterns which reflect the packing of the acyl chains. An L_c phase was assigned when there were two or three diffraction peaks between 0.42 nm and 0.36 nm (Fig.



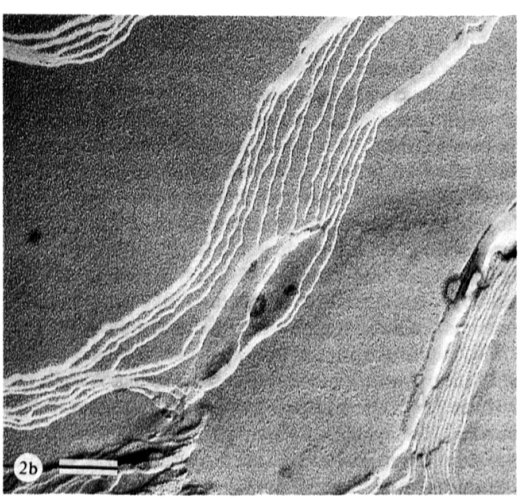


Fig. 2. Electron micrographs of freeze-fracture replicas prepared from total-lipid extracted from *V. costicola* grown in 1 M NaCl-containing medium and dispersed in 80% by vol. 1 M NaCl (pH 7.0). Samples were thermally quenched from (a) +40°C or (b) −20°C. Bars represent 100 nm.

1a), and this mesophase was confirmed by the close-packed lamellar sheets observed in electron micrographs of freeze-fracture replicas (Fig. 2b). In the L_β phase a single, sharp diffraction peak was produced by the hexagonally-packed acyl chains (Fig. 1b) and the disordered nature of the acyl chains in the L_α phase gave rise to a diffuse scattering profile (Fig. 1c).

Phase behaviour of purified total-lipid extracts

The phase assignments and diffraction spacings for the total membrane lipids extracted from *V. costicola* grown in 1 M NaCl-containing medium (i.e., '1 M total lipid') dispersed in 1 M NaCl are shown in Fig. 3a. When 1 M total lipid was cooled, a hexagonal-II phase was observed between +50°C and +18°C; the hexagonal-II and L_α phases coexisted between +30°C and +18°C. On further cooling, the L_α phase converted to the L_β phase at +3°C and a $L_\beta \rightarrow L_c$ transition occurred at −12°C. The lamellar repeat distances of the L_α and L_β phases were 5.6 nm and 6.4 nm respectively; the lamellar repeat distance of the L_c phase decreased from 4.7 nm at −12°C to 4.1 nm at −50°C. On reheating, no L_β phase was observed and instead the L_c phase converted directly to the L_α phase at −2°C. The phase transitions showed hysteresis, with the temperatures which marked the end of the L_c and L_α phases and the start of the hexagonal-II phase being higher on heating than cooling by 10, 4 and 10 C°, respectively.

When the 1 M total lipid was dispersed in 3 M NaCl and cooled, the hexagonal-II phase persisted down to −5°C (Fig. 3b). The L_β phase was observed between +3°C and −13°C and it coexisted with the hexagonal-II phase between +3°C and −5°C. The $L_\beta \rightarrow L_c$ transition occurred at −13°C. The lamellar repeat distance of the L_c phase decreased from 4.8 nm at −13°C to 4.1 nm at −50°C. When the total-lipid sample was subsequently reheated, the sequence of phases was reversed but the phase transitions occurred at different temperatures; the $L_c \rightarrow L_\beta$ transition occurred at −18°C, the L_β phase persisted to +5°C and the hexagonal-II phase formed at −4°C.

When the total lipid extracted from 3 M NaCl grown cultures (i.e., '3 M total lipid') was dispersed in 1 M NaCl and cooled, there was an $L_\alpha \rightarrow L_\beta$ transition at +3°C (Fig. 3c). The abrupt increase in the first-order diffraction spacing of the L_α phase at +26°C is a result of single-lamellar vesicles forming multi-layer liposomes. The L_β and L_c phases coexisted between −12°C, when the L_c phase formed, and −25°C, when the L_β phase ended. The lamellar repeat distances of the L_β and L_c phases were 5.6 nm and 6.4 nm, respectively. Reheating the sample resulted in a reversal of the phase sequence; the $L_c \rightarrow L_\beta$ transition occurred at −7°C and the $L_\beta \rightarrow L_\alpha$ transition was present at +2°C.

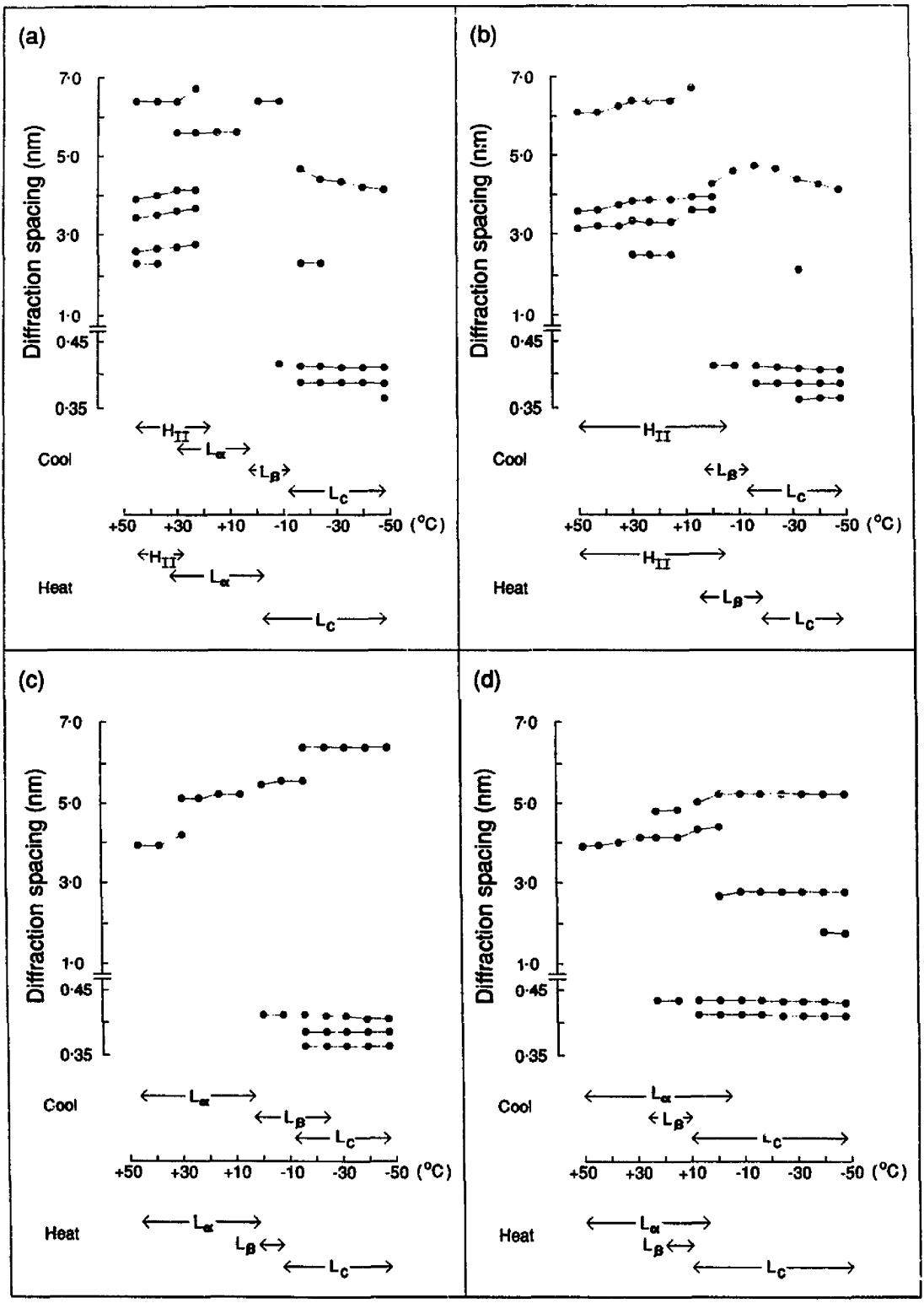
When the 3 M total lipid was dispersed in 3 M NaCl and cooled, it formed an L_α phase between +50°C and −5°C (Fig. 3d) with a lamellar repeat distance which increased from 3.9 nm at +50°C to 4.4 nm at −5°C. The L_β phase, which had a lamellar repeat distance of 4.8 nm, was present together with the L_α phase between +26°C and +10°C. An $L_\beta \rightarrow L_c$ transition occurred at +10°C and the L_c phase, which had a lamellar repeat distance of 5.2 nm, persisted to −50°C. The phase transition temperatures showed little hysteresis on reheating. The $L_c \rightarrow L_\beta$ transition occurred at +10°C, the L_β phase persisted to +20°C and the L_α phase was present between +4°C and +50°C.

Phase behaviour of phosphatidylethanolamine / phosphatidylglycerol binary mixtures

In order to examine the effects of the phosphatidylethanolamine/phosphatidylglycerol (i.e., PE/PG) ratio on the phase behaviour of *V. costicola* lipids, binary mixtures of purified phosphatidylethanolamine and phosphatidylglycerol isolated from cultues grown in 1 M or 3 M NaCl-containing media (i.e., 1 M PE/PG and 3 M PE/PG, respectively) were prepared with a PE/PG ratio of 2 : 1 (i.e., equivalent to the ratio present in the membranes of *V. costicola* grown in 1 M NaCl-containing media) or 1 : 1 (i.e., equivalent to the ratio in 3 M NaCl cultures).

The phase assignments and diffraction spacings for binary mixtures of purified phosphatidylethanolamine and phosphatidylglycerol isolated from *V. costicola* grown in 1 M NaCl-containing media (i.e., 1 M PE/PG) prepared with a PE/PG ratio of 2 : 1 (i.e., 1 M PE/PG (2 : 1)) dispersed in 1 M NaCl and 3 M NaCl, and 1 M PE/PG prepared in with PE/PG ratio of 1 : 1 (i.e., 1 M PE/PG (1 : 1)) dispersed in 3 M NaCl are shown in Fig. 4a, b and c, respectively. When 1 M PE/PG (2 : 1) dispersed in 1 M NaCl was cooled, the sequence of phases observed was hexagonal-II $\rightarrow L_\alpha \rightarrow L_\beta \rightarrow L_c$ (Fig. 4a). The hexagonal-II phase was present between +50°C and +5°C. The L_α phase was first observed at +25°C and converted to the L_β phase at −5°C. The $L_\beta \rightarrow L_c$ phase transition temperature was −36°C. On subsequent reheating, the temperatures of the transitions showed some hysteresis; the $L_c \rightarrow L_\beta$ transition occurred at −11°C and the $L_\beta \rightarrow L_\alpha$ transition at +5°C, whilst the hexagonal-II phase was first observed at +10°C.

When a dispersion of 1 M PE/PG (2 : 1) in 3 M NaCl was cooled, it formed a hexagonal-II phase between +50°C and −27°C (Fig. 4b). The L_β phase existed between −12°C and −29°C, coexisting for most of that thermal range with the hexagonal-II phase. The $L_\beta \rightarrow L_c$ transition occurred at −29°C. The sequence of transitions was reversible and showed hysteresis on reheating; the $L_c \rightarrow L_\beta$ transition occurred at −12°C, the L_β phase was present between −12°C and −2°C and the hexagonal-II phase between −15°C and +50°C (Fig. 4b). When 1 M PE/PG (1 : 1) dispersed in


(a)
Diffraction spacing (nm)
7·0
5·0
3·0
1·0
0·45
0·35
Cool
Heat
+50 +30 +10 -10 -30 -50 (°C)
(b)
(c)
(d)

3 M NaCl was cooled, the hexagonal-II phase was present between +50°C and −11°C (Fig. 4c). The L_α phase was observed between +5°C and −5°C, and always coexisted with the hexagonal-II phase. The $L_\alpha \rightarrow L_\beta$ transition occurred at −5°C and the $L_\beta \rightarrow L_c$ transition was observed at −21°C. Reheating the sample showed that the sequence of phases was reversible, with the $L_c \rightarrow L_\beta$ transition occurring at −20°C and the $L_\beta \rightarrow L_\alpha$ transition at −10°C; the L_α phase disappeared at +6°C and the hexagonal-II phase formed at +2°C (Fig. 4c).

The phase assignments and diffraction spacings for purified phosphatidylethanolamine and phosphatidylglycerol isolated from *V. costicola* grown in 3 M NaCl-containing media (i.e., 3 M PE/PG) prepared with a PE/PG ratio of 1 : 1 (i.e., 3 M PE/PG (1 : 1)) dispersed in 3 M NaCl and 1 M NaCl, and 3 M PE/PG prepared with a PE/PG ratio of 2 : 1 (i.e., 3 M PE/PG (2 : 1)) dispersed in 1 M NaCl are shown in Fig. 5a, b and c, respectively. When 3 M PE/PG (1 : 1) dispersed in 3 M NaCl was cooled the sequence of phases was $L_\alpha \rightarrow L_\beta \rightarrow L_c$ (Fig. 5a). The $L_\alpha \rightarrow L_\beta$ transition temperature was +3°C and the $L_\beta \rightarrow L_c$ transition occurred at −12°C. On reheating, the L_c phase persisted to +5°C. The L_β phase was present as a mixture with the L_c phase between −5°C and +5°C, at which temperature the L_α phase was formed at the expense of both the L_β and L_c phases.

When 3 M PE/PG (1 : 1) dispersed in 1 M NaCl was cooled, the hexagonal-II phase was observed between +50°C and +5°C (Fig. 5b). The L_α phase was present between +11°C and −12°C and coexisted with the hexagonal-II phase between +11°C and +5°C. The L_α phase converted directly to the L_c phase at −12°C. On subsequent reheating the sequence of transitions observed during cooling was reversed. Some hysteresis was observed in all the transition temperatures; the $L_c \rightarrow L_\alpha$ transition occurred at −5°C, the L_α phase finished at +25°C and the hexagonal-II phase was present from +10°C to +50°C (Fig. 5b).

When 3 M PE/PG (2 : 1) dispersed in 1 M NaCl was cooled, the hexagonal-II phase was observed between +50°C and +11°C (Fig. 5c). The L_α phase coexisted with the hexagonal-II phase between +18°C and +11°C. The L_β phase was observed between −5°C and −15°C, and coexisted with the L_α phase between −5°C and −10°C. The $L_\beta \rightarrow L_c$ transition occurred at −15°C. The sequence of transitions was reversed on subsequent reheating and hysteresis was observed in all the transition temperatures. The $L_c \rightarrow L_\beta$ transition occurred at −1°C, whilst the L_α phase was formed at −2°C. The L_β phase always coexisted with the L_α phase between −1°C and +6°C. The L_α phase ended at +20°C and the hexagonal-II phase was observed between +14°C and +50°C (Fig. 5c).

Discussion

As part of an investigation of membrane stability in the moderately-halophilic eubacterium *Vibrio costicola*, which can grow in up to 3.5 M NaCl [29], we report the effects of molar NaCl concentrations on the phase behaviour of total-lipid extracts and binary mixtures of the major phospholipids, phosphatidylethanolamine and phosphatidylglycerol, mixed in the proportions found in membranes of the bacterium cultured in 1 M and 3 M NaCl.

Increasing the NaCl concentration, from 1 M to 3 M, of the solution used to disperse total-lipid extracts isolated from cells grown in 1 M NaCl-containing medium (i.e., '1 M total lipid') results in stabilisation of the hexagonal-II phase over a wider thermal range by 23 C° (Figs. 3a and 3b). The high salinity, in stabilising the hexagonal-II phase, causes the hexagonal-II $\rightarrow L_\alpha$ and $L_\alpha \rightarrow L_\beta$ phase transitions to merge so that there is no separate L_α phase. When the 1 M total lipid of *V. costicola* is dispersed in 1 M NaCl (Fig. 3a) at the bacterial growth temperature (i.e., 30°C), it forms a mixture of hexagonal-II and L_α phases, whereas when it is dispersed in 3 M NaCl only the hexagonal-II phase is present between +50°C and −5°C (Fig. 3b). We have demonstrated previously that purified phosphatidylethanolamine isolated from *V. costicola* grown in 1 M NaCl-containing medium forms a hexagonal-II phase and that the temperature of the hexagonal-II → lamellar phase transition decreases with increasing salinity, whereas purified phosphatidylglycerol isolated under the same conditions forms only lamellar phases between +50°C and −50°C [19]. Since phosphatidylethanolamine comprises more than 50% of 1 M total-lipid extracts, it is to be expected that the salinity-induced phase changes of this phospholipid would dominate the phase behaviour of the total-lipid extract. When phosphatidylethanolamine and phosphatidylglycerol isolated from *V. costicola* grown in 1 M NaCl-containing medium are mixed in a ratio of 2 : 1, they show a similar response as purified phosphatidylethanolamine to an increase in salinity of the dispersing solution in that the

Fig. 3. Diffraction spacings (nm) and phase assignments calculated from X-ray diffraction data for total-lipid extracted from *V. costicola* grown in 1 M (a and b) or 3 M (c and d) NaCl-containing media and dispersed in 80% by vol. 1 M (a and c) or 3 M (b and d) NaCl (pH 7.0) during cooling and heating scans between +50°C and −50°C at 10 C°·min^{-1}. Diffraction spacings for a given phase were identical during heating and cooling. The spacings belonging to a particular phase have been connected by lines, and values observed during cooling are shown; the temperature spans of each phase during cooling and heating are summarised.

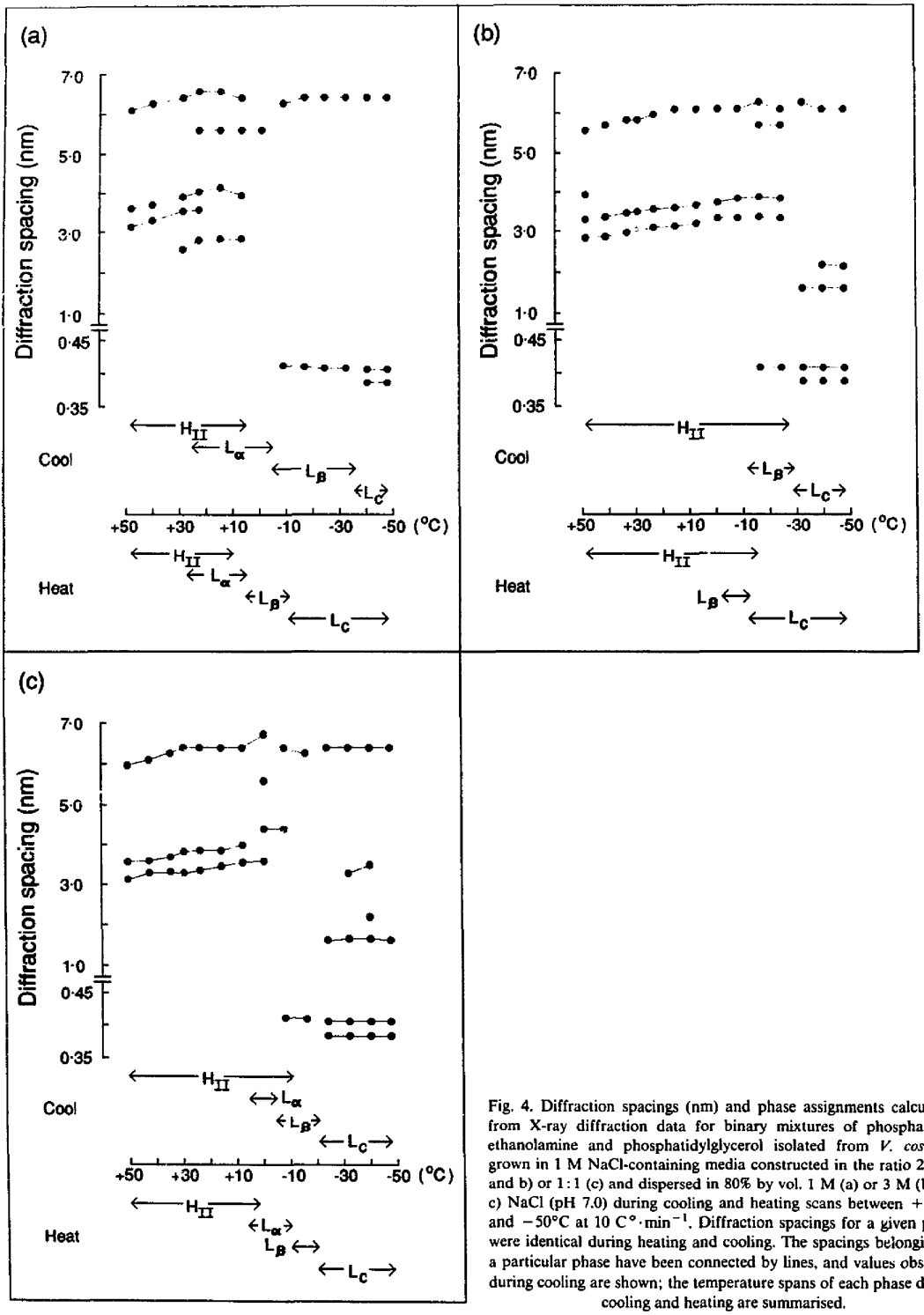


Fig. 4. Diffraction spacings (nm) and phase assignments calculated from X-ray diffraction data for binary mixtures of phosphatidylethanolamine and phosphatidylglycerol isolated from *V. costicola* grown in 1 M NaCl-containing media constructed in the ratio 2:1 (a and b) or 1:1 (c) and dispersed in 80% by vol. 1 M (a) or 3 M (b and c) NaCl (pH 7.0) during cooling and heating scans between +50°C and −50°C at 10 C°·min^{-1}. Diffraction spacings for a given phase were identical during heating and cooling. The spacings belonging to a particular phase have been connected by lines, and values observed during cooling are shown; the temperature spans of each phase during cooling and heating are summarised.

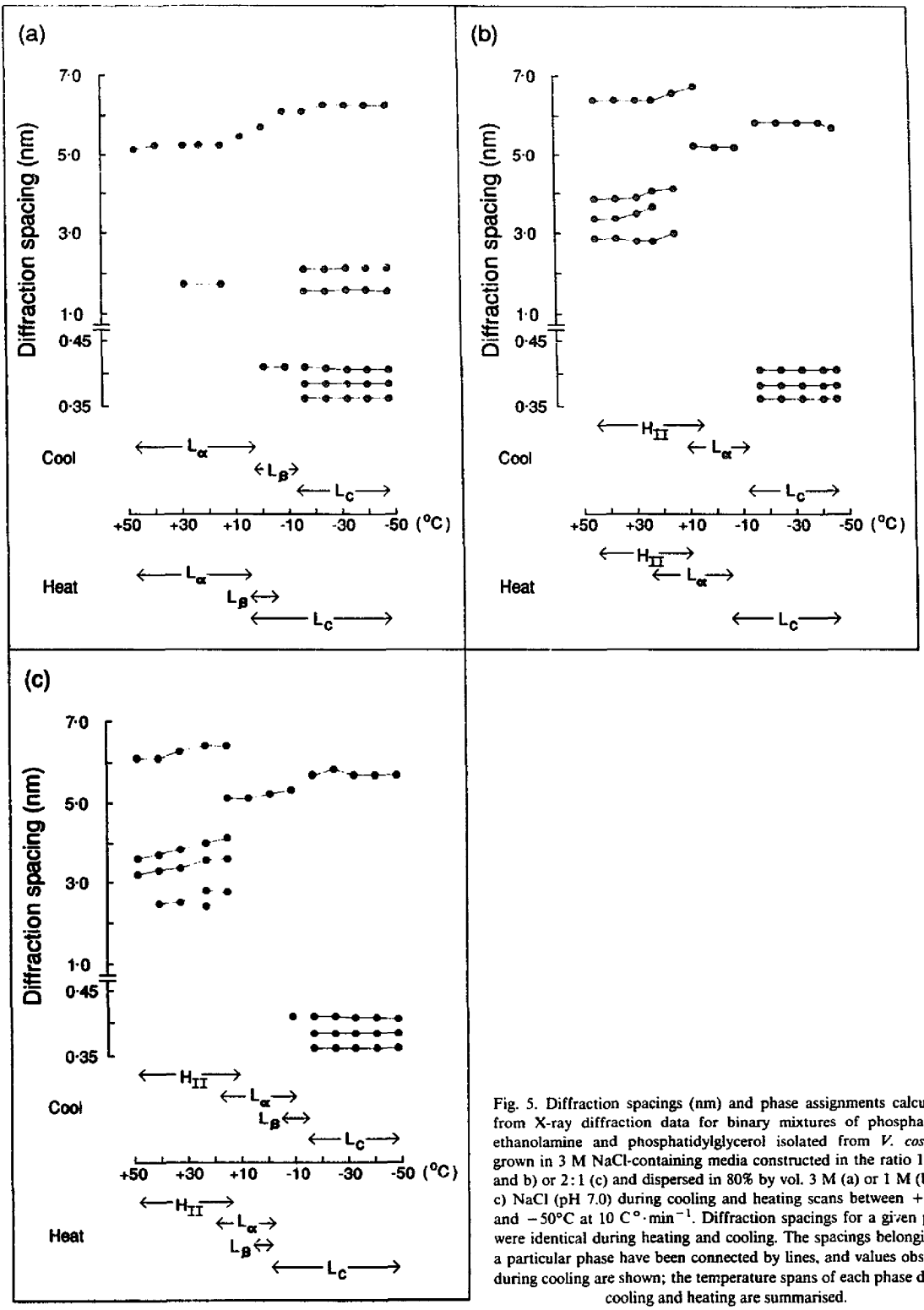


Fig. 5. Diffraction spacings (nm) and phase assignments calculated from X-ray diffraction data for binary mixtures of phosphatidylethanolamine and phosphatidylglycerol isolated from *V. costicola* grown in 3 M NaCl-containing media constructed in the ratio 1:1 (a and b) or 2:1 (c) and dispersed in 80% by vol. 3 M (a) or 1 M (b and c) NaCl (pH 7.0) during cooling and heating scans between +50°C and −50°C at 10 C°·min^{-1}. Diffraction spacings for a given phase were identical during heating and cooling. The spacings belonging to a particular phase have been connected by lines, and values observed during cooling are shown; the temperature spans of each phase during cooling and heating are summarised.

hexagonal-II phase persists at lower temperatures in 3 M compared with 1 M NaCl (cf. Fig. 4a and 4b).

When discussing the relevance of studies with total-lipid extracts to the phase behaviour of membranes, the role of membrane proteins and lipid–protein interactions must be considered. A number of examples are known in which the structures formed by the lipid extracted from biological membranes differ greatly from the bilayer arrangement that is believed to represent the lipid matrix in the native membrane. These membrane systems include the photoreceptor membrane for which it has been proposed that the protein rhodopsin plays a structural role in maintaining the membrane lipids in a bilayer configuration by suppressing the hexagonal-II and isotropic phases that the extracted lipids prefer to form [30]. A similar effect of protein in chloroplast thylakoid membranes has been proposed by Gounaris et al. [31]. It has also been demonstrated that the intrinsic membrane protein glycophorin can stabilise the bilayer configuration of dioleoylphosphatidylethanolamine by preventing the natural tendency of the phospholipid to form the hexagonal-II phase [32]. Hence, the phase behaviour of lipids in the biological membrane differs from that of the extracted lipids. Thus, it is assumed that the formation of the hexagonal-II phase observed in 1 M total lipid of *V. costicola* at the bacterial growth temperature is suppressed by membrane proteins such that a bilayer arrangement dominates in the native membrane, whilst the ability to form isolated non-bilayer domains could be retained by a lateral separation of lipids and proteins.

The total-lipid extract from *V. costicola* grown in 3 M NaCl-containing medium (i.e., '3 M total lipid') forms a lamellar configuration over the temperature range studied (+50°C to −50°C) when dispersed in either 1 M or 3 M NaCl (cf. Fig. 3c and 3d). Thus, it appears that the total lipid from *V. costicola* grown in 3 M NaCl could provide the bacterium with a membrane which would be suitable for growth in either NaCl concentration because a lamellar phase is the structure compatible with a non-leaky, functional membrane [33]. However, we know that NaCl-dependent changes in membrane lipid [13] and fatty acid [14] composition occur and may be a prerequisite for optimum growth of *V. costicola* in media containing different NaCl concentrations [34,35]. The question therefore remains as to why these changes are necessary when it appears that the lipid and fatty acid composition of 3 M total lipid are adequate to form a lipid bilayer at high or low salinity. The growth of *V. costicola* is 4-times faster in 1 M compared with 3 M NaCl-containing medium [17]. This indicates that, although the membrane composition of 3 M NaCl-grown bacteria is sufficient for growth, the composition of membranes from 1 M NaCl-grown bacteria is associated with faster growth of the organism. This may be related to the ability of 1 M total-lipid extracts to form non-bilayer structures at growth temperature.

The effect of salinity on the phase behaviour of binary mixtures of phospholipids isolated from *V. costicola* was also examined. Increasing the NaCl concentration, from 1 M to 3 M, of the solution dispersing the binary mixture of phosphatidylethanolamine and phosphatidylglycerol isolated from 1 M NaCl cultures and prepared in a 2:1 ratio (i.e., 1 M PE/PG (2:1)) decreases the temperature of the corresponding phase transitions and results in the absence of the L_α phase (Fig. 4a and b). The phase behaviour of this binary mixture appears to be dominated by the phase behaviour of the phosphatidylethanolamine component in that the hexagonal-II phase is formed, since purified phosphatidylglycerol has been shown to form only lamellar phases [19]. When binary mixtures of phospholipids isolated from 1 M NaCl-cultures are dispersed in 3 M NaCl, reducing the PE/PG ratio from 2:1 (i.e., equivalent to the phospholipid ratio present in 1 M NaCl-grown cultures) to 1:1 (i.e., the ratio in 3 M NaCl cultures) results in partial stabilisation of the lamellar phases (i.e., the hexagonal-II → lamellar transition temperature is raised by 16 C°); in addition, the L_α phase is observed (cf. Fig. 4b and 4c). These observations show that increasing the proportion of phosphatidylglycerol relative to that of phosphatidylethanolamine can stabilise a lamellar phase. The ability of phosphatidylglycerol to stabilise a bilayer configuration in unsaturated phosphatidylethanolamine has been reported previously [36]. The presence of more than 20 mol% of egg-phosphatidylglycerol caused egg-phosphatidylethanolamine, which in isolation will preferentially form a hexagonal-II phase, to form a lamellar phase.

Increasing the NaCl concentration, from 1 M to 3 M, of the solution dispersing the binary mixture of phosphatidylethanolamine and phosphatidylglycerol isolated from 3 M NaCl-cultures and prepared in a 1:1 ratio (i.e., 3 M PE/PG (1:1)) results in the formation of the hexagonal-II phase and the absence of the L_β phase at the lower salinity (cf. Fig. 5a and b). Thus, as was observed with the 1 M PE/PG (2:1) samples, resuspending the binary mixture at a NaCl concentration other than the salinity of the growth medium results in a destabilisation of the lamellar phase. However, formation of the hexagonal-II phase in 1 M NaCl but not 3 M NaCl cannot be explained on the basis of a direct consequence of NaCl concentration on the phase behaviour of phosphatidylethanolamine: based on evidence from model systems a reduction in NaCl concentration of the resuspending solution should result in an increase in the hexagonal-II → lamellar transition temperature, whereas in the present experiments using phospholipids of biological origin a decrease in the hexagonal-II → lamellar transition temperature was observed. The explanation for this difference is not known,

but it highlights the importance of performing relevant experiments with suitable lipids of biological origin.

Increasing the PE/PG ratio from 1 : 1 to 2 : 1 (i.e., from the ratio found in 3 M NaCl cultures to that found in 1 M NaCl cultures) of the binary mixtures of phospholipids isolated from 3 M NaCl cultures and dispersed in 1 M NaCl results in a small stabilisation of the L_α phase with respect to the hexagonal-II phase. Hence, as was observed for the binary mixtures of phospholipids isolated from 1 M NaCl-containing medium, a PE/PG ratio equivalent to that found in membranes of bacteria grown in a NaCl concentration equal to that used to resuspend the samples stabilises the lamellar phases, when compared to the phase behaviour of the binary mixtures which have a PE/PG ratio different to that found in membranes of cells grown in the same salinity as used to disperse the samples.

Binary mixtures were constructed to determine whether a stable bilayer arrangement could be produced by modifying only the PE/PG ratio to suit the external NaCl concentration. Phosphatidylethanolamine and phosphatidylglycerol isolated from 1 M (or 3 M)-grown cells were prepared in the ratio found in the membrane of 3 M (or 1 M) grown cells and dispersed in 3 M (or 1 M) NaCl (cf. Figs. 4c and 5c). Neither of these mixtures exhibits the same phase behaviour as the corresponding total lipid (i.e., 1 M (3 M) total lipid dispersed in 3 M (1 M) NaCl) or the equivalent binary mixture (i.e., 1 M PE/PG (2 : 1) dispersed in 3 M NaCl, or 3 M PE/PG (1 : 1) dispersed in 1 M NaCl, respectively). For example, when 1 M PE/PG (1 : 1) is dispersed in 3 M NaCl a hexagonal-II phase is observed between +50°C and −11°C, whereas 3 M total lipid, and the equivalent binary mixture of 3 M phosphatidylethanolamine and phosphatidylglycerol (i.e., 3 M PE/PG (1 : 1)), both form lamellar phases throughout the temperature range studied (+50°C to −50°C). These differences probably result from the altered fatty acid compositions of the purified phosphatidylethanolamine and phosphatidylglycerol, which depend on the salinity of the bacterial culture medium [14]. These results emphasise the need for the correct fatty acid composition of the constituent phospholipids in vivo, as well as the appropriate PE/PG ratio, for the proper functioning of the bacterial membrane in cultures growing in media having different salinities.

The effect of the ratio of phosphatidylethanolamine and phosphatidylglycerol on the phase behaviour of binary mixtures of the phospholipids isolated from *V. costicola* was also examined. The binary mixture corresponding to the major phospholipids in 1 M NaCl-grown cells (i.e., 1 M PE/PG (2 : 1)) exhibits a phase behaviour which is similar, but not identical, to 1 M total lipid dispersed in 1 M or 3 M NaCl (cf. Fig. 4a and b with Fig. 3a and 3b). The phase behaviour of a binary mixture of phosphatidylethanolamine and phosphatidylglycerol isolated from 3 M NaCl-grown bacteria (i.e., 3 M PE/PG (1 : 1)) is similar to the 3 M total lipid only when it is dispersed at the growth salinity of 3 M NaCl (cf. Fig. 5a and b with Fig. 4c and d). The 3 M PE/PG (1 : 1) dispersed in 1 M NaCl exhibits a hexagonal-II phase which is not observed with 3 M total lipid (cf. Figs. 5b and 3c). These differences are assumed to result from the presence of minor lipid components (i.e., diphosphatidylglycerol, lysophosphatidylethanolamine and lysophosphatidylglycerol) and suggest that they play a part in determining the phase behaviour of total-lipid extracts. At NaCl concentrations below 1.5 M diphosphatidylglycerol exists in the L_α phase, whereas raising the NaCl concentration above 2.5 M causes diphosphatidylglycerol to form exclusively the hexagonal-II phase [37]. These results are particularly relevant to the present study of *V. costicola* because in 1 M NaCl diphosphatidylglycerol would be expected to form a lamellar phase, whereas in 3 M NaCl it would form a hexagonal-II phase. This would increase the tendency for hexagonal-II phase formation in the membrane, as would the presence of lysophosphatidylethanolamine [38], whereas lysophosphatidylglycerol would be expected to form lamellar or hexagonal-I phases.

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References

1 Findlay, E.J. and Barton, P.J. (1978) Biochemistry 17, 2400–2405.
2 Wieslander, Å., Ulmius, J., Lindblom, G. and Fontell, K. (1978) Biochim. Biophys. Acta 512, 241–253.
3 Lewis, R.N.A.H., Mannock, D.A., McElhaney, R.N., Turner, D.C. and Gruner, S.M. (1989) Biochemistry 28, 541–548.
4 Goldfine, H., Johnston, N.C., Mattai, J. and Shipley, G.G. (1987) Biochemistry 26, 2814–2822.
5 De Kruijff, B., Cullis, P.R., Verkleij, A.J., Hope, M.J., Van Echteld, C.J.A. and T.F. Taraschi (1984) in Enzymes in Biological Membranes (Martinosi, A., ed.), pp. 131–203, Plenum Press, New York.
6 Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420.
7 Ellens, H., Siegel, D.P., Alford, D., Yeagle, P.L., Boni, L., Lis, L.J., Quinn, P.J. and Bentz, J. (1989) Biochemistry 28, 3692–3703.
8 Siegel, D.P., Banschbach, J., Alford, D., Ellens, H., Lis, L.J., Quinn, P.J., Yeagle, P.L. and Bentz, J. (1989) Biochemistry 28, 3703–3709.
9 Cullis, P.R., De Kruijff, B., Hope, M.J., Verkleij, A.J., Nayar, R., Farren, S.B., Tilcock, C., Madden, D.T. and Bally, M.B. (1983) in Membrane Fluidity in Biology, Vol. 1, Academic Press, New York.
10 McElhaney, R.N. (1984) Biochim. Biophys. Acta 779, 1–42.
11 Wieslander, Å., Christiansson, A., Rilfors, L. and Lindblom, G. (1980) Biochemistry 19, 3650–3655.
12 Russell, N.J. (1989) J. Bioenerg. Biomembr. 21, 93–113.
13 Hanna, K., Bengis-Garber, C., Kushner, D.J., Kogut, M. and Kates, M. (1984) Can. J. Microbiol. 30, 669–674.

14 Sutton, G.C., Quinn, P.J. and Russell, N.J. (1990) Curr. Microbiol. 20, 43–46.
15 Hiramatsu, T., Yano, I. and Masui, M. (1980) FEMS Microbiol. Lett. 7, 289–292.
16 Russell, N.J. and Kogut, M. (1985) Microbiol. Sci. 11, 345–350.
17 Adams, R., Bygraves, J., Kogut, M. and Russell, N.J. (1987) J. Gen. Microbiol. 133, 1861–1870.
18 Bygraves, J.A. and Russell, N.J. (1988) Food Microbiol. 5, 109–116.
19 Sutton, G.C., Quinn, P.J. and Russell, N.J. (1990) Chem. Phys. Lipids 56, in press.
20 Kates, M. (1986) Techniques of Lipidology: Isolation, Analysis and Identification of Lipids (2nd Edn.), Elsevier, Amsterdam.
21 Wells, M.A. and Dittmer, J.C. (1963) Biochemistry 2, 1259–1263.
22 Christie, W.W. (1982) Lipid Analysis (2nd Edn.), Pergamon Press, Oxford.
23 Hitchcock, C.A., Barrett-Bee, K.J. and Russell, N.J. (1986) J. Gen. Microbiol. 132, 2421–2431.
24 Helliwell, J.R., Greenough, T.M., Carr, P.D., Rule, S.A., Moore, P.R., Thomson, A.W. and Worgan, J.S. (1982) J. Phys. E15, 1363–1372.
25 Bunn, C.W. and Howell, F.R. (1954) Nature 174, 549–551.
26 Ruocco, M.J. and Shipley, G.G. (1982) Biochim. Biophys. Acta 691, 309–320.
27 Luzzati, V. (1968) in Biological Membranes, Vol. 1 (Chapman, D., ed.), pp. 71–123, Academic Press, New York.
28 Luzzati, V. and Tardieu, A. (1974) Annu. Rev. Phys. Chem. 25, 79–94.
29 Kushner, D.J. (1978) in Microbial Life in Extreme Environments (Kushner, D.J., ed.), pp. 318–368, Academic Press, London.
30 De Grip, W.J., Drenthe, E.H.S., Van Echteld, C.J.A., De Kruijff, B. and Verkleij, A.J. (1979) Biochim. Biophys. Acta 558, 330–337.
31 Gounaris, K., Brain, A.P.R., Quinn, P.J. and Williams, W.P. (1983) FEBS Lett. 153, 47–52.
32 Taraschi, T.F., De Kruijff, B., Verkleij, A. and Van Echteld, C.J.A. (1982) Biochim. Biophys. Acta 685, 153–161.
33 Singer, S.J. and Nicolson, G.L. (1972) Science 175, 720–731.
34 Kogut, M. and Russell, N.J. (1984) Curr. Microbiol. 10, 95–98.
35 Russell, N.J., Kogut, M. and Kates, M. (1985) J. Gen. Microbiol. 131, 781–789.
36 Farren, S.B. and Cullis, P.R. (1980) Biochem. Biophys. Res. Commun. 97, 182–191.
37 Seddon, J.M., Kaye, R.D. and Marsh, D. (1983) Biochim. Biophys. Acta 734, 347–352.
38 Slater, J.L., Huang, C.-H., Adams, R.G. and Levin, I.W. (1989) Biophys. J. 56, 243–252.