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^{31}P NUCLEAR MAGNETIC RESONANCE AND FREEZE-FRACTURE ELECTRON MICROSCOPY STUDIES ON *ESCHERICHIA COLI*

I. CYTOPLASMIC MEMBRANE AND TOTAL PHOSPHOLIPIDS

ELLIOTT BURNELL [a,*], LOEK VAN ALPHEN [b,c,**], ARIE VERKLEIJ [c] and BEN DE KRUIJFF [c]

[a] *Department of Biochemistry,* [b] *Department of Molecular Cell Biology, Microbiology Section, and* [c] *Institute for Molecular Biology, State University, Transitorium 3, Padualaan 8, 3584 CH Utrecht (The Netherlands)*

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Summary

1. At the growth temperature the total phospholipids isolated from *Escherichia coli* cells give rise to ^{31}P-NMR spectra which indicate the existence of lamellar, isotropic and hexagonal phases. These phases are also detected by freeze-fracture electron microscopy. In particular, the isotropic phase may contain lipidic particles (possibly inverted micelles) associated with the lamellar phase.

2. The cytoplasmic membrane isolated from *E. coli* cells grown at 37°C is mainly lamellar at 25°C, whereas at 37 and 45°C the presence of some almost isotropic phospholipid motion is indicated. The possible significance of the isotropic phase for the functioning of the cytoplasmic membrane is discussed.

Introduction

^{31}P-NMR spectroscopy is useful for detecting the structural symmetry of liquid crystalline phospholipid dispersions. In non-oriented samples the proton decoupled NMR spectrum is dominated by the powder pattern from the phosphorus chemical shift anisotropy which is axially symmetric due to the (at least

* On leave from: Chemistry Department, University of British Columbia, Vancouver, B.C., V6T 1W5, Canada.

** To whom reprint requests should be sent at (present address): Laboratorium voor de Gezondheidsleer, Mauritskade 57, 1092 AD Amsterdam, The Netherlands.

3-fold) symmetric motion of the phospholipids about the direction perpendicular to the lipid—water interface. For lipids with planar (e.g. lamellar) symmetry, a 90° powder pattern singularity is observed to high-field of the peak position for isotropic symmetry. For cylindrical symmetry (as found for the hexagonal-II phase) the singularity is to low field [1].

A study of the structural arrangement of the phospholipids in *Escherichia coli* membranes is of great interest because ^{31}P-NMR studies have shown that the major lipid component, phosphatidylethanolamine, undergoes a lamellar-to-hexagonal-II phase transition at 55°C [2]. Whether or not the total phospholipids extracted from *E. coli* cells exhibit hexagonal and other non-bilayer phases is an interesting question. Of even more importance from a biological point of view is whether or not non-bilayer phases exist in the intact membranes of such cells. Therefore, in this paper a combined ^{31}P-NMR and freeze-fracture electron microscopy study of the total phospholipids and the cytoplasmic membrane isolated from *E. coli* K12 cells is presented.

The cytoplasmic membrane in Gram-negative bacteria such as *E. coli* occurs next to an outer membrane in the cell envelope [3]. The phospholipid composition of both membranes is almost the same [4]. Freeze-fracture electron microscopy observations on the cytoplasmic membrane have revealed the existence of fracture faces with particles which are known to have lateral mobility because of the observed phase separation [5]. Deuterium NMR studies have also been used to characterize the cytoplasmic and outer membranes of *E. coli*, and the existence of non-bilayer phases was suggested [6]. In these studies it was shown that most of the phospholipids participate in the gel-to-liquid crystalline phase transition. This phase transition was found to be roughly 7°C higher for the cytoplasmic membrane compared to the outer membrane.

This paper, which focusses on the arrangement of the isolated phospholipids and on the phospholipids in the cytoplasmic membrane, is the first of a series of three papers on the membranes of *E. coli*. The study of the organization of the phospholipids in the outer membrane is much more difficult because of the presence of lipopolysaccharide, which contains six phosphates [7,8]. In the second paper of the series [8] a physical study of lipopolysaccharide will be presented, and in the third paper [9] a study of the arrangement of the phospholipids and lipopolysaccharides in the outer membrane will be described.

Materials and Methods

Bacterial strain and growth conditions

Cells of *E. coli* K12 strain CE1163, which lacks phospholipase A in the outer membrane [9], were grown exponentially (unless otherwise indicated) in Brain Heart Infusion medium [10] at 37°C under vigorous aeration in a fermentor to an absorbance of 2 at 660 nm.

Isolation and analysis of the cytoplasmic membrane

After conversion of the cells to spheroplasts with lysozyme and EDTA, membranes were isolated and separated according to a procedure [4] basically consisting of that of Osborn et al. [3], modified in that the spheroplasts were

lysed after centrifugation by resuspending in buffer without sucrose. The quality of the separation appeared to be good [9]. The isolated cytoplasmic membranes were resuspended in about 1 ml 10 mM Tris-HCl/150 mM NaCl, pH 7.4, in ^2H_2O (approx. 0.2 g/ml).

Isolation and characterization of total phospholipids, and preparation of samples

Phospholipids were isolated from intact cells according to the method of Bligh and Dyer [11], and the lipid composition was determined as described previously [12]. Fatty acids were analyzed in the form of their methyl esters by gas-liquid chromatography as described in Ref. 13. Hydration of the phospholipids was performed as follows: a chloroform solution of total phospholipids was dried under N_2 in an NMR tube; after storage under vacuum overnight the lipid film was dispersed in ^2H_2O containing 2 mM EDTA, 25 mM Tris-HCl, pH 7.4, with 100 mM NaCl (unless indicated otherwise), by shaking on a Vortex mixer and then frozen to −70 °C.

Freeze-fracture electron microscopy

Glycerol was added as a cryoprotectant to the membranes and the phospholipid samples. Samples were quenched from 22 or 37 °C, as indicated, and fractured in a Denton freeze-etch apparatus as described previously [14]. Electron micrographs were made with a Philips 301 electron microscope.

Nuclear magnetic resonance spectroscopy

^{31}P-NMR measurements were performed on a Bruker WH 90 NMR spectrometer operating at 36.4 MHz. Spectra were recorded under conditions of 18 W broad-band proton decoupling using a sweep width of 12 kHz. Zero ppm was taken as the position for the isotopic signal of external 85% orthophosphoric acid. A line broadening of 10 Hz was used (unless otherwise indicated).

Results

Characterization of isolated total phospholipids

The lipid and fatty acid analyses of the total phospholipids of *E. coli* K12 strain CE1163 are reported in Table I. The results are in good agreement with those previously published [4,12,15]. These extracted lipids were studied by NMR and freeze-fracture electron microscopy. Such studies can be used for the understanding of the organization of the phospholipids in both the outer and the cytoplasmic membranes because the composition of the phospholipids of both membranes is almost identical [4]. Representative NMR spectra are shown in Fig. 1. In the absence of salt, after freezing the samples to −70 °C, the signal from the total phospholipids is almost completely from lamellar structures (Fig. 1A). On the other hand, samples dispersed in buffer containing 100 mM NaCl (a situation that more closely mimics that found in the cell) and frozen to −70 °C were mainly lamellar at 25 °C (Fig. 1E), with a small isotropic signal. At 31, 37 and 45 °C, a hexagonal phase signal appears in addition to the isotropic signal which grows in intensity with increasing temperature (Figs. 1B, C, D). Such samples, upon cooling to 25 °C, appeared isotropic with a minor

TABLE I

PHOSPHOLIPID AND FATTY ACID COMPOSITION

Phospholipids were extracted from whole cells and analyzed with thin-layer chromatography. The relative amount of the individual phospholipids was determined by phosphate analysis of the chromatography scrapings. Fatty acids were liberated from the total phospholipids in methanolic alkali and analyzed gas-chromatographically in the form of their methyl esters.

Phospholipid	Amount (% of total)	Fatty acid		Amount (% of total)
Phosphatidylethanolamine	74	Myristic	C14:0	3.2
Phosphatidylglycerol	19	Palmitic	C16:0	40.1
Cardiolipin	3	Palmitoleic	C16:1	27.3
Lysophospholipids	3	9,10-Cyclopropane palmitic	C17:0Δ	5.0
		Stearic	C18:0	0.8
		Oleic	C18:1	18.9
		9,10-Cyclopropane stearic	C19:0Δ	2.5
		Unknown		2.2

hexagonal component, hence indicating that there is a large hysteresis in the phase transition.

Total phospholipids were also extracted from stationary phase cells, and the results obtained were qualitatively the same as those reported above for

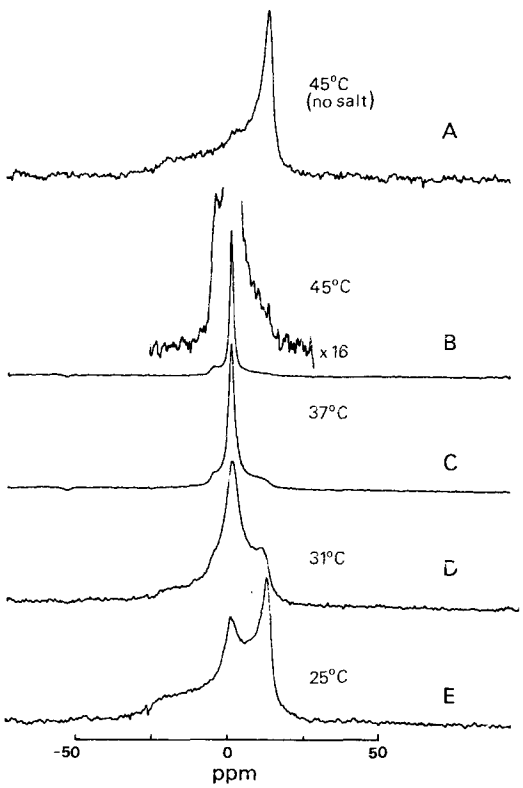

Fig. 1. 36.4 MHz ^{31}P-NMR spectra of an aqueous dispersion of the total phospholipids from cells of *E. coli* K12 strain CE1163. Phospholipids were hydrated in $^{2}H_2O$ containing 25 mM Tris-HCl/2 mM EDTA, pH 7.4, without addition of NaCl (A) or in the presence of 100 mM NaCl (B—E). Samples were frozen to —70°C before running the spectra.

exponential cells indicating that the presence of cyclopropane fatty acyl chains does not have a large influence on the phase behaviour.

In order to compare the results of the total phospholipids with those already published for pure phosphatidylethanolamine [2] which was isolated from stationary phase cells of *E. coli* B, the total phospholipids extracted from this same strain were also studied. The ^{31}P-NMR and freeze-fracture results are qualitatively similar to those found for *E. coli* K12 strain CE1163. The important result is that at the growth temperature (37°C) the total phospholipids exhibit far less of a tendency to form a bilayer phase than does the major lipid component, phosphatidylethanolamine.

Freeze-fracture electron microscopy experiments were also performed on the total phospholipids extracted from cells of strain CE1163 (Fig. 2). In the presence of salt, lipid dispersions that were heated to 25°C and then freeze-fractured show mainly smooth fracture faces with a few particles and pits (Fig. 2A). On the other hand, lipid dispersions that had been incubated at 37°C for 30 min showed mainly lamellar and hexagonal phases with particles (Figs. 2B, C). The hexagonal phase was difficult to detect by electron microscopy, probably because the quenching rate is not sufficiently fast to prevent the hexagonal-to-lamellar phase transition.

Characterization of the cytoplasmic membrane

Both freeze-fracture electron microscopy and NMR experiments were performed on the isolated cytoplasmic membrane. The freeze-fracture electron microscopy results showed that the cytoplasmic membrane formed large vesicles with the typical cytoplasmic membrane particle pattern (Fig. 3 and Ref. 5).

The ^{31}P-NMR results on this membrane are presented in Fig. 4. As shown in Fig. 4C, at 25°C the ^{31}P-NMR spectrum indicates mainly bilayer lipids with some minor narrow resonances possibly originating from non-phospholipid phosphorus. With increasing temperature a broad spectral component grows near the isotropic position in the spectrum such that at 45°C a considerable fraction of the total signal is present in this signal (Figs. 4A, B). Upon lowering the temperature to 25°C, the original 25°C spectrum is again obtained indicating that the appearance of the spectral feature is reversible. The singularity associated with the feature is 1 ppm upfield of the peak associated with isotropic phospholipid motion in Fig. 1. Such a shift indicates a decrease by a factor of 0.9 of the width of the bilayer signal. Such a situation would arise from inverted micelles (imbedded in a bilayer) if their phospholipids were in rapid exchange with those in the bilayer and if the phospholipids spent 90% of their time in the micelle. Alternatively, the signal could also arise from diffusive motion around oblate ellipsoidal shaped structures. Hence, at higher temperature some of the phospholipids in the inner cytoplasmic membrane undergo nearly isotropic motion.

With respect to the bilayer component of the cytoplasmic membrane spectra (Fig. 4), one can see from the reduction in the spectral width of this component that the observed chemical shift anisotropy is reduced at higher temperature. This reduction, which could be interpreted in terms of increased motion, agrees with the general reduction of the deuterium splittings for the chain

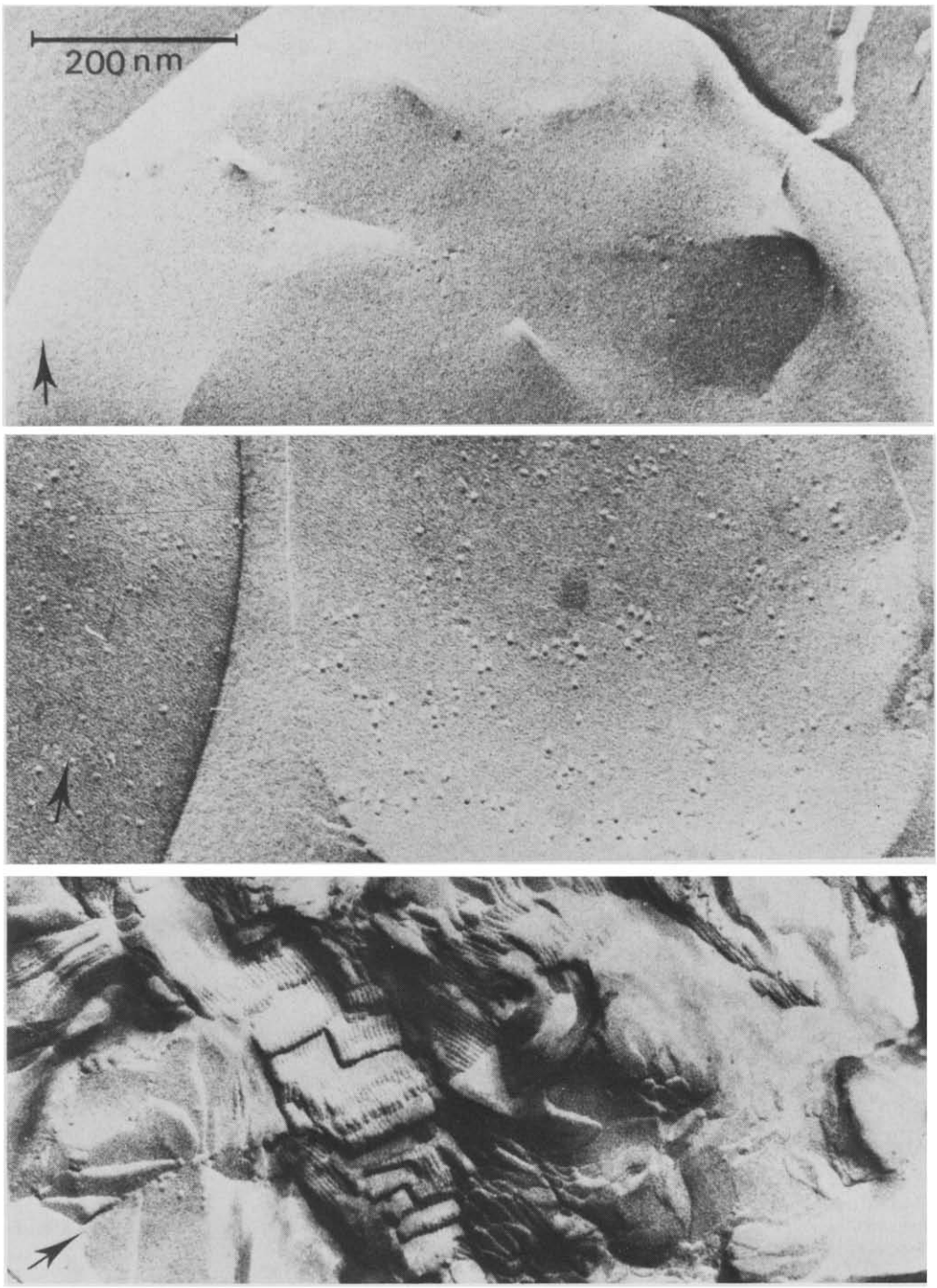

Fig. 2. Freeze-fracture electron micrographs of total lipids of *E. coli* K12 strain CE1163 dispersed in ^2H_2O containing 25 mM Tris-HCl/2 mM EDTA/100 mM NaCl, pH 7.4, at 25°C, top (A) and 37°C (B, middle; C, bottom). The arrow indicates the direction of shadowing.

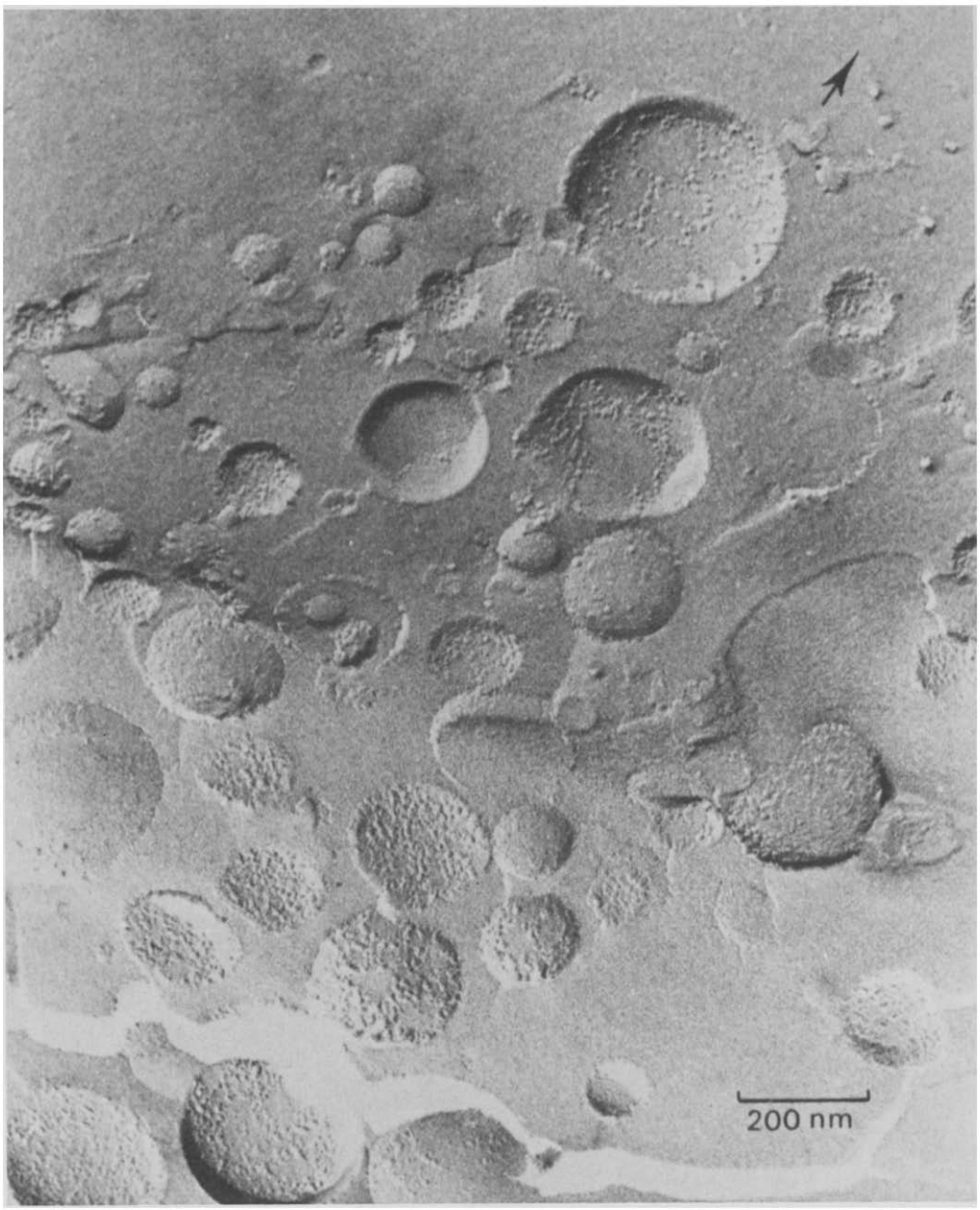

Fig. 3. Freeze-fracture electron micrograph from the cytoplasmic membrane of *E. coli* K12 strain CE 1163 dispersed in ^2H_2O containing 10 mM Tris-HCl/150 mM NaCl, pH 7.4, at 22°C. The arrow indicates the direction of shadowing.

methylene positions that has been observed by Davis et al. [6].

It should be noted that at 45°C the spectral feature just to be left of the isotropic lipid peak is a narrow isotropic peak, as deduced by subtracting spectra taken at different temperatures. This peak is probably not from phos-

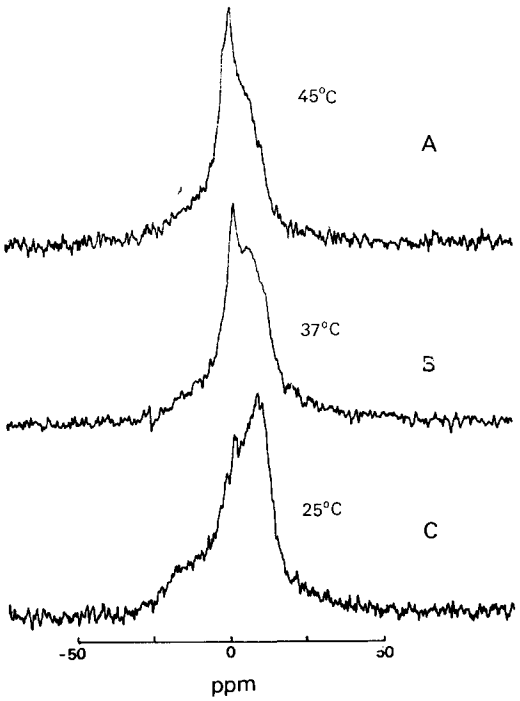

Fig. 4. 36.4 MHz ^{31}P-NMR spectra of an aqueous dispersion of the cytoplasmic membrane of *E. coli* K12 strain CE1163 dispersed in $^{2}H_2O$ and containing 10 mM Tris-HCl/150 mM NaCl, pH 7.4.

pholipid, and washing with buffer does decrease its intensity.

In summary, the phospholipids isolated in the cytoplasmic membrane of *E. coli* by the modified Osborn procedure are mainly bilayer at 25°C, and at the growth temperature (37°C) some of the phospholipids appear to have almost isotropic motion.

Discussion

Pure phosphatidylethanolamine, the major lipid component in the membranes of *E. coli*, is known to undergo a bilayer-to-hexagonal phase transition at 55°C [2]. The total phospholipids extracted from *E. coli*, however, have a strong preference for non-bilayer structures at much lower temperatures. The presence of salt, which is necessary for the formation of these non-bilayer phases in the total phospholipids, was found to be of no consequence for the phase behaviour of the phosphatidylethanolamine [2]. Hence, the tendency to form non-bilayer structures is probably due to the influence of the negatively charged lipids, phosphatidylglycerol and cardiolipin, present in the total lipids (Table I). A possible source of the isotropic NMR signals is indicated by the electron microscopy observations in which complementary particles and pits are observed (Fig. 2). These particles and pits, which might well be inverted micelles, were observed recently in different phospholipid systems [16,17]. The most striking result of the present study on the isolated phospholipids is that at physiological temperatures the total phospholipids extracted from *E. coli*

cells do show a definite preference for non-bilayer structures, including those in which isotropic motion occurs.

The phospholipids isolated in the cytoplasmic membrane of *E. coli* by the modified Osborn procedure are mainly in the bilayer state, indicating that the phase behaviour of the membrane differs from that of the isolated phospholipids; this difference is possibly due to the presence of proteins. However, with increasing temperature, a sizeable fraction of the lipids undergoes almost isotropic motion on the NMR time scale. Similar ^{31}P-NMR results have been observed for the endoplasmic reticulum of rat, beef and rabbit liver [18,19], and also for the sarcoplasmic reticulum of the rabbit muscle [20,21]. Although the exact source of the isotropic phospholipid motion is unknown, it is possibly due to transitory non-bilayer structures such as inverted micelles or short hexagonal-II segments. These structures could have important biological functions. Both the endoplasmic reticulum and sarcoplasmic reticulum are metabolically very active, and are the site of synthesis of many lipids. The rapid transbilayer movement of phospholipids which has been observed in these systems [22,23] could well involve non-bilayer structures such as inverted micelles. In many respects the cytoplasmic membrane of *E. coli* has functions similar to those systems mentioned above. This membrane is metabolically very active, and all the lipids for the cell are synthesized in it [15]. The phospholipids are synthesized in its inner leaflet and rapid transbilayer movement of these lipids is known to occur [24]. The lipopolysaccharide is also synthesized in the cytoplasmic membrane [25], and both this latter lipid and the phospholipids are translocated to the outer membrane. Non-bilayer phases may well be involved in some of these processes.

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References

1 Seelig, J. (1978) Biochim. Biophys. Acta 515, 105—140
2 Cullis, P.R. and De Kruijff, B. (1978) Biochim. Biophys. Acta 513, 31—42
3 Osborn, M.J., Gander, J.E., Parisi, E. and Carson, J. (1972) J. Biol. Chem. 247, 3962—3972
4 Lugtenberg, E.J.J. and Peters, R. (1976) Biochim. Biophys. Acta 441, 38—47
5 Haest, C.W.M., Verkleij, A.J., de Gier, J., Scheek, R., Ververgaert, P.H.J. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 356, 17—26
6 Davis, J.H., Nichol, C.P., Weeks, G. and Bloom, M. (1979) Biochemistry 18, 2103—2112
7 Prehm, P., Stirm, S., Jann, B., Jann, K. and Boman, H.G. (1976) Eur. J. Biochem. 66, 369—377
8 Van Alphen, L., Verkleij, A., Burnell, E. and Lugtenberg, B. (1980) Biochim. Biophys. Acta 597, 502—517
9 Burnell, E., van Alphen, L., Verkleij, A., de Kruijff, B. and Lugtenberg, B. (1980) Biochim. Biophys. Acta 597, 518—532
10 Lugtenberg, B., Peters, R., Bernheimer, H. and Berendsen, W. (1976) Mol. Gen. Genet. 147, 251—262
11 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—917

12 Van Alphen, L., Lugtenberg, B., van Boxtel, R. and Verhoef, K. (1977) Biochim. Biophys. Acta 466, 257—268
13 De Kruijff, B., Demel, R.A. and van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 255, 331—347
14 Ververgaert, P.H.J.Th., Elbers, P.F., Luitingh, A.J. and van den Berg, H.J. (1972) Cytobiologie 6, 86—96
15 Cronan, J.E. and Vagelos, P.R. (1972) Biochim. Biophys. Acta 265, 25—60
16 Verkleij, A.J., 'Mombers, C., Leunissen-Bijvelt, J. and Ververgaert, P.H.J.Th. (1979) Nature 279, 162—163
17 De Kruijff, B., Verkleij, A.J., van Echteld, C.J.A., Gerritsen, W.J., Mombers, C., Noordam, P.C. and de Gier, J. (1979) Biochim. Biophys. Acta 555, 200—209
18 De Kruijff, B., van den Besselaar, A.M.H., Cullis, P.R., van den Bosch, H. and van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 514, 1—8
19 Stier, A., Finch, S.A.E. and Bösterling, B. (1978) FEBS Lett. 91, 109—112
20 Davis, D.G. and Inesi, G. (1971) Biochim. Biophys. Acta 241, 1—8
21 Van den Besselaar, A.M.H.P., de Kruijff, B., van den Bosch, H. and van Deenen, L.L.M. (1979) Biochim. Biophys. Acta 555, 193—199
22 Zilversmit, D.B. and Hughes, M.E. (1977) Biochim. Biophys. Acta 469, 99—110
23 Van den Besselaar, A.M.H.P., de Kruijff, B., van den Bosch, H. and van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 510, 242—255
24 Rothman, J.E. and Lenard, J. (1977) Science 195, 743—753
25 Osborn, M.J., Gander, J.E. and Parisi, E. (1972) J. Biol. Chem. 247, 3973—3986