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

III. THE OUTER MEMBRANE

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Summary

1. The outer membrane of a phospholipase A-deficient mutant of *Escherichia coli* K12, isolated without the use of EDTA and lysozyme, showed the same freeze-fracture morphology as that seen in cells and remained stable for hours as observed by ^{31}P-NMR.

2. ^{31}P-NMR spectroscopy of the isolated outer membranes revealed that the lipopolysaccharide exists in the same physical state as in phospholipid-lipopolysaccharide liposomes and is most probably arranged in a bilayer at 37°C. The outer membrane contains most or all of the phospholipids at 37°C, and all the phospholipids at 20°C, as a bilayer.

3. The ^{31}P-NMR spectroscopy of the outer membranes from a mutant strain lacking the major outer membrane proteins b, c and d (60% of the total outer membrane protein) yields virtually the same spectrum as the wild-type outer membranes, although most of the particles and pits which were observed in wild-type outer membranes in freeze-fracture electron microscopy were absent.

4. Whereas treatment of wild-type outer membranes with calcium ions has no effect on the ^{31}P-NMR spectrum, treatment with EDTA results in more motion of the lipopolysaccharide.

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Introduction

The cell envelope of Gram-negative bacteria contains a cytoplasmic and an outer membrane separated by a peptidoglycan layer [1]. The outer membrane has a relatively simple protein composition [2] and has almost the same phospholipid composition as the cytoplasmic membrane [3]. In contrast to the cytoplasmic membrane, it contains lipopolysaccharide [1]. This membrane is of particular interest since it enables the bacterium to live in the intestine in the presence of detergents and bile salts without impeding the permeation of nutrients [4,5]. It is highly asymmetric in that lipopolysaccharide is located exclusively in the outer monolayer [6] and most of or all the phospholipids are in the inner monolayer [7—9].

Freeze-fracture electron microscopy studies of the outer membrane in wild-type cells have shown the presence of fracture planes which are occupied by particles predominantly located on the outer fracture face and by complementary pits on the inner fracture face [10—13]. In mutant cells which lack most of the major outer membrane proteins there is a large reduction in the number of particles and pits [11—13]. It has been suggested that these particles contain hemi-micellar lipopolysaccharide structures which form the basis of pores through the outer membrane [14].

The major objective of this paper is to use ^{31}P-NMR, complemented with freeze-fracture electron microscopy, to investigate the organization of both the phospholipids and lipopolysaccharide in the *Escherichia coli* outer membrane. The interesting question is whether or not the phospholipids and lipopolysaccharide exist in non-bilayer structures in the membrane. For the phospholipids this is especially interesting because the total phospholipids extracted from *E. coli* bacteria are known to give ^{31}P-NMR spectra which are a superposition of isotropic, hexagonal and lamellar phase-type signals at 37°C (paper I in this series [15]). The understanding of the physical state of the lipopolysaccharide is of interest in relation to the occurrence of particles and pits which have been observed in freeze-fracture experiments on the outer membrane. With a view to interpreting the ^{31}P-NMR spectral features of lipopolysaccharide in the membrane, model studies on isolated lipopolysaccharide were described in paper II of this series [16]. These studies indicated that native lipopolysaccharide is most probably lamellar at the growth temperature. Moreover, particles and pits were observed in freeze-fracture electron microscopy. It was suggested that these structures could give rise to isotropic-type signals of lipopolysaccharide.

In this paper the ^{31}P-NMR spectra of wild-type outer membranes are reported. These spectra are compared with those of an outer membrane protein b, c and d deficient mutant which lacks most of the particles and pits. Since EDTA extracts lipopolysaccharide from the outer membrane [17] and reduces the number of particles and pits [11,12] the effect of EDTA and Ca^{2+} on the physical state of lipopolysaccharide in the outer membrane of wild-type cells is described.

For the purpose of the ^{31}P-NMR studies a new procedure is described for the isolation of large amounts of stable outer membranes which occur in large structures with the same freeze-fracture morphology as in cells.

Materials and Methods

Strains and growth conditions

A derivative of *E. coli* K12 strain AB1133 was used throughout this study since the freeze-fracture morphology of cells of this strain has been investigated extensively [11]. An outer membrane detergent-resistant phospholipase A-deficient (*pldA*$^-$) strain was constructed. The *pldA* marker was introduced via transduction of strain AB1133*metE* with a bacteriophage P1 lysate of strain S17*pldA* [18,19] by selection for *met*$^+$ clones. Among these transfuctants *pldA* clones were elected as follows: cell envelopes were isolated [2] from 100 ml culture of cells late-exponentially grown in yeast broth supplemented with [2-^{14}C]acetate in order to label the lipids [7]. The cell envelopes were incubated for 2 h at 37°C in 0.5 ml 10 mM Tris-HCl, pH 7.8, in the presence of 5 mM $CaCl_2$, and the phospholipids were extracted and analyzed as described previously [7]. One of the *pldA*$^-$ transductants, strain CE1163 which showed less than 5% phospholipid degradation in contrast to *pldA*$^+$ strains in which about 60% degradation occurred, was chosen for further studies.

Mutant strain CE1166, which is deficient in the outer membrane proteins b, c and d, was isolated from strain CE1163 in three steps by selecting clones that were spontaneously resistant towards bacteriophages TuIa, Me-1 and K3 sequentially as described previously [20]. This mutant is sensitive towards bacteriophage U3, indicating that the core structure of the lipopolysaccharide is intact [21].

For the isolation of membranes, cells were grown exponentially in Brain Heart Infusion medium [22] at 37°C under vigorous aeration in a fermentor to an absorbance of 2 at 660 nm and then harvested.

Isolation and analysis of outer and cytoplasmic membranes

Two procedures were followed:

Method I is basically the modified [3] Osborn [1] procedure which was described in the first paper of this series [15] for the isolation of the cytoplasmic membranes.

Method II was used in order to avoid the use of EDTA and lysozyme and is based on the procedure of Schnaitman [23]. Cells were washed twice with 0.9% NaCl, frozen at −20°C overnight, thawed and resuspended in 50 mM Tris-HCl, pH 8.0/1 mM $MgCl_2$/0.2 mM dithiothreitol. After addition of 50 μg/ml ribonuclease and 50 μg/ml desoxyribonuclease I (Boehringer, Mannheim, F.R.G., Analytical grade) the cells were lysed in a French pressure cell at an operating pressure of 1200 atm. After removal of unlysed cells by low speed centrifugation (20 min at 3000 × *g*), the lysate was centrifuged for 1 h at 17 000 × *g*. The supernatant contained most of the separated cytoplasmic membranes which were mainly small inside-out vesicles, most of which had a diameter of less than 40 nm (Fig. 1A). Because of the small size of these vesicles the NMR was in the motionally narrowed regime and hence these cytoplasmic membranes are not useful for studying the structural arrangement of the membrane phospholipids by NMR. The pellet, which was mainly outer membrane and intact cell envelope present in large structures, was washed with the same buffer supplemented with 0.2 M KCl, centrifuged for 45 min at 200 000 × *g*, resuspended

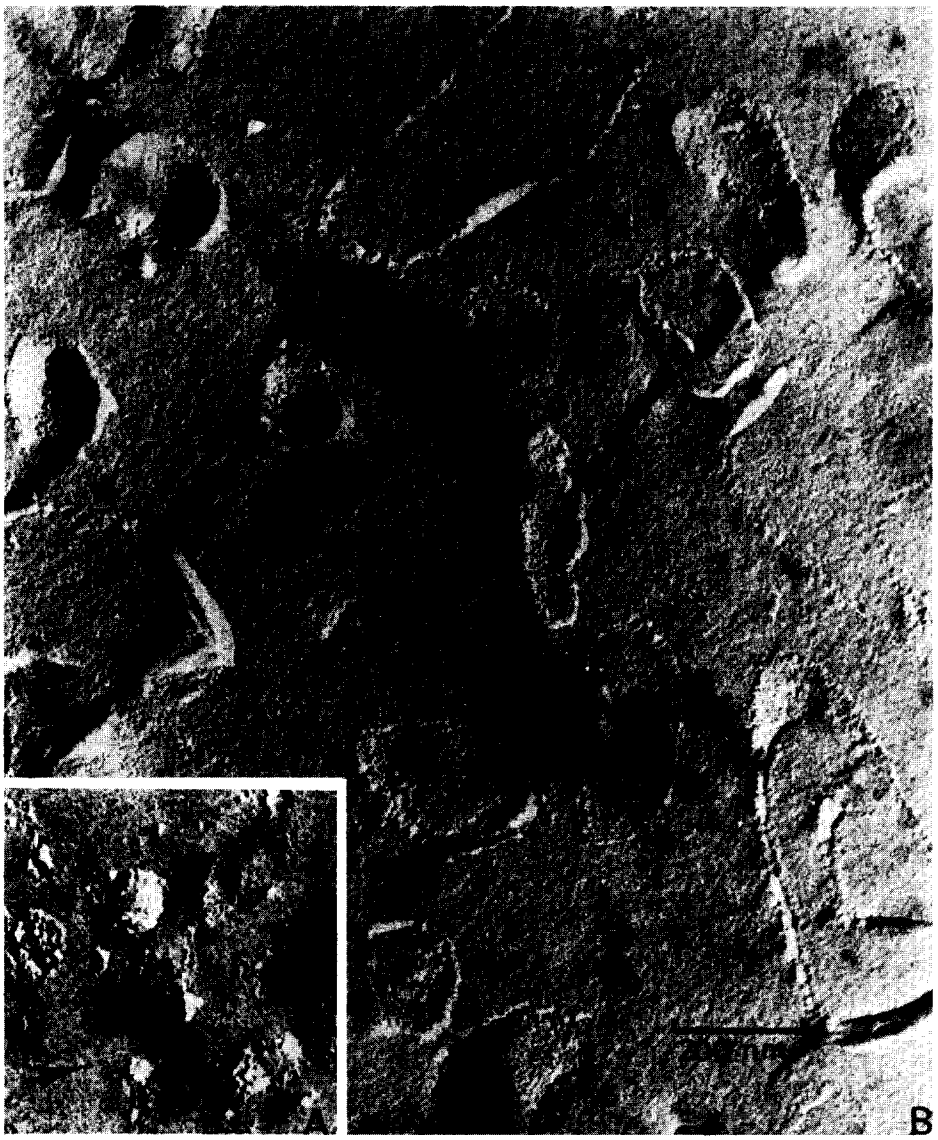

Fig. 1. Freeze-fracture electron micrographs of the membranes of *E. coli* K12 strain CE1163 (wild type) isolated according to method II. (A) cytoplasmic membrane, (B) outer membrane. Samples were quenched from 0°C. The arrow indicates the direction of shadowing.

in buffer without KCl, loaded on a stepwise sucrose gradient of 40—60% (w/w) with steps of 5% and a cushion of 65% sucrose, and centrifuged for 16 h at 21 000 rev./min in a Beckmann SW 27^{I} rotor. After isolation of the bands the buoyant density of the membrane fractions was determined and after dilution and addition of KCl (final concentration 0.2 M) the membranes were spun down by centrifugation for 1 h at 200 000 × *g*.

The quality of the separation of the membranes was determined by the NADH-oxidase activity [1], the ratio of the amounts of lipopolysaccharide

over protein, the density of the membranes, the sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns of the proteins [2] and by the freeze-fracture morphology. Protein was determined according to the method of Lowry et al. [24] and lipopolysaccharide by the determination of 3-deoxy-D-mannooctulosonic acid as described in Ref. 11. Total phosphate and lipid phosphate were determined as described previously [25].

Freeze-fracture electron microscopy

Freeze-fracture electron microscopy was carried out as described in paper I [15].

NMR

^{31}P-NMR experiments were performed as described in paper I [15]. Spin-lattice relaxation experiments were carried out using an inversion recovery ($180°$-τ-$90°$) pulse sequence where τ is the interpulse interval. The NMR signal following the $90°$ pulse was measured and Fourier transformed. The amplitude of the main spectral feature (the lamellar phase phospholipid singularity) was analysed as the sum of two exponentials. From the resulting relaxation times, assuming the relaxation to be independent of lamellar orientation, the value of τ which leads to a zero signal from either spectral component is readily calculated. Experiments were then carried out to measure the independent spectra using these values of τ.

Results

^{31}P-NMR spectra obtained from whole cells of *E. coli* changed rapidly with time, mainly with respect to the growth of an inorganic phosphate peak, but also with respect to other spectral features. Additionally, only about 1% of the signal is from ^{31}P in the phospholipids (unpublished result). Since it would be extremely difficult to draw definitive conclusions about the physical state of the membranes from such whole cell spectra, preparative procedures are necessary. These procedures may, to varying extents, damage the membrane. In our studies we have used different preparative procedures (methods I and II in Materials and Methods). As discussed later, method II is probably the less disruptive, and hence this method was used for most of the experiments.

Isolation and analysis of membranes

Our preliminary results with *E. coli* membranes yielded ^{31}P-NMR spectra which changed drastically with time. To obtain stable membranes a transductant lacking the outer membrane bound phospholipase A was obtained. Chemical analysis before and after NMR experiments where only minor spectral changes were observed (a sharp resonance grows at the position for isotropic phospholipid motion) showed that there was little chemical change in outer membrane samples that had spent several days at temperatures above 25°C. The only change detected was a slight increase (1%) in the amount of lysophospholipids, while both the amounts of protein and lipopolysaccharide and the protein pattern on gels were not changed (not shown). A new, rather mild, method for the purification of outer membranes was developed in order

to obtain large amounts of membranes, not modified by EDTA or lysozyme, in large structures. Such large structures are necessary to avoid motional narrowing of the NMR spectra due to isotropic motion of the membranes. The characteristics of the membranes isolated by this procedure (method II) and by the modified Osborn procedure (method I) are listed in Table I. The membrane separations appeared to be good with both methods, since only 2% of the total NADH oxidase activity, which is a marker enzyme of the cytoplasmic membrane, was found in the outer membrane fractions. Moreover, the isolated cytoplasmic membrane (method I) contained 10 times less lipopolysaccharide than the outer membrane. The amounts of lipopolysaccharide and phospholipid in the outer membranes agreed with previous results [7]. Gel electropherograms of the isolated membranes showed the characteristic patterns known for these membranes (not shown).

The gel-to-liquid crystalline phase transition of the outer membrane (method II) as measured by a differential scanning calorimetry heating scan ranged from 19 to 37°C.

Description of ^{31}P-NMR spectroscopy and freeze-fracture morphology of the outer membranes of wild-type cells

The freeze-fracture results showed that the outer membrane isolated by method II consisted of bilayer sheets larger than 100 nm in diameter and densely covered with particles on the outer fracture face (OM) and with pits on the inner fracture face (OM) (Fig. 1B) as was observed in whole cells [11]. The outer membranes isolated according to method I were visible as large spheres (also covered with particles and pits) with a diameter of more than 100 nm Fig. 2). ^{31}P-NMR of the outer membrane is complicated by the existence of ^{31}P in both phospholipid and lipopolysaccharide molecules. The signal from the lipopolysaccharide phosphorus represents about two-thirds of the total NMR signal as estimated from the molar ratio of lipopolysaccharide over phospholipid (Table I) assuming that each lipopolysaccharide molecule contains six

TABLE I

CHARACTERISTICS OF THE ISOLATED MEMBRANES

Membranes were isolated according to two methods (see Materials and Methods) and characterized by their density, their relative amounts of protein, phospholipid and lipopolysaccharide (the latter component being a marker for the outer membrane) and by the NADH-oxidase activity, as marker enzyme for the cytoplasmic membrane. CM, cytoplasmic membrane; OM, outer membrane.

Membrane	Isolation method	Ratio of lipopolysaccharide over protein (nmol/mg) *	Ratio of phospholipid over protein (μmol P/mg protein)	NADH-oxidase activity ** (%)	Density in g/cm^3
CM	I	17	0.61	98	1.16
OM	I	158	0.41	2	1.22 ***
OM	II	174	0.35	2	1.25 ***

* Lipopolysaccharide is considered as monomer [26].

** The values are compared with the total activity per mg protein.

*** A difference in the density of the outer membranes is due to the presence of the peptidoglycan layer which is attached to the outer membrane only in method II [23,27].

Fig. 2. Freeze-fracture electron micrograph of the outer membrane of *E. coli* K12 strain CE1163 isolated according to method I, quenched from 0°C. The arrow indicates the direction of shadowing.

phosphorus atoms [28]. The spectra obtained using membranes prepared by method II are shown in Fig. 3 (C—E). The NMR spectrum taken at 20°C indicates that at that temperature the phospholipids are organized as a bilayer (compare with Fig. 1A of paper I [15]). The broad resonance that dominates the spectrum is from bilayer lipopolysaccharide, as the overall width and shape of this broad spectrum is the same as obtained in paper II [16] for native lipopolysaccharide at 40°C (also see next section). At higher temperatures (37 and 45°C) the NMR spectrum indicates that the phospholipids and lipopolysaccharide are again mainly lamellar. However, at these temperatures an extra spectral feature is present in the spectra (Figs. 3C and D). Qualitatively, this feature looks like a rather broad, possibly isotropic, peak. The singularity associated with this feature lies close to (approx. 1 ppm downfield) the position expected for an isotropic phospholipid signal; however, it is certainly possible that the signal arises, at least in part, from the ^{31}P in the lipopolysaccharide. Because of the presence of this signal it is not possible to say that all the phospholipids and lipopolysaccharides are in a bilayer arrangement at 37°C. From the spectra of the outer membrane in Figs. 3C and D it can be seen that there is also a broad signal present in the higher temperature spectra, but

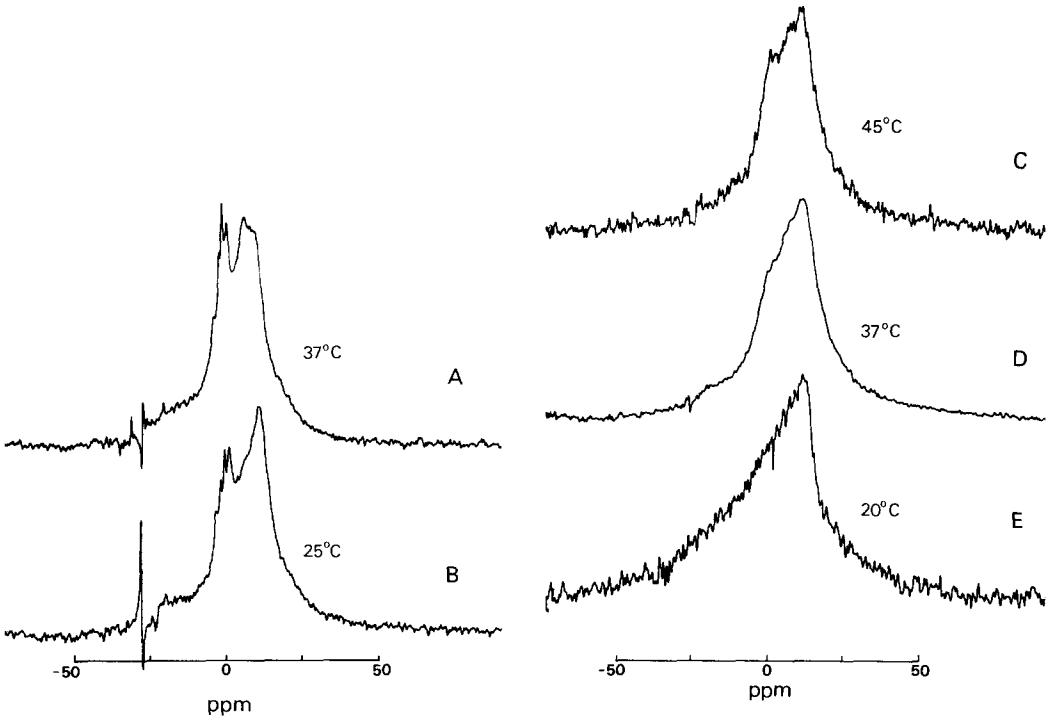

Fig. 3. 36.4 MHz ^{31}P-NMR spectra of aqueous dispersions of *E. coli* K12 strain CE1163 outer membrane: (A) and (B), method I; (C), (D) and (E), method II. All dispersions were in 10 mM Tris-HCl/150 mM NaCl, pH 7.4, and contained $^{2}H_2O$ to provide a lock signal. The zero corresponds with the peak position for 85% orthophosphoric acid.

the width is less than for the spectrum at 22°C. In fact the membrane spectrum at 37°C is identical to that for phospholipid-lipopolysaccharide liposomes at 37°C shown in Fig. 8 of paper II [16], with the exception that there is a small additional signal at the position for isotropic phospholipid in the spectrum from paper II. In paper II we argued that the spectrum of Fig. 8 is probably due to mainly lamellar phase phospholipid and lipopolysaccharide.

The NMR spectra obtained from membranes prepared using the isolation procedure of method I (Figs. 3A and B) are qualitatively similar to those discussed above with method II, but there are noticeable differences in that peaks appear in the spectrum (compare Figs. 3A and 3D). With the spectra from the method I membrane (Fig. 3A), the feature that appears in the 37°C spectrum just to the left of the lamellar phase singularity should be noted. This feature disappears upon lowering the temperature to 25°C. It lies close to one of the resonances found in the high resolution spectrum of native lipopolysaccharide reported in paper II [16], suggesting that at least some of the lipopolysaccharide in this sample is undergoing isotropic motion. Further evidence for this isotropic motion is given in Fig. 4A, where the result of subtracting the cytoplasmic membrane spectrum taken at 37°C (Fig. 4 of paper I [15]) from the outer membrane spectrum taken at 37°C (Fig. 3A) (both from method I) is

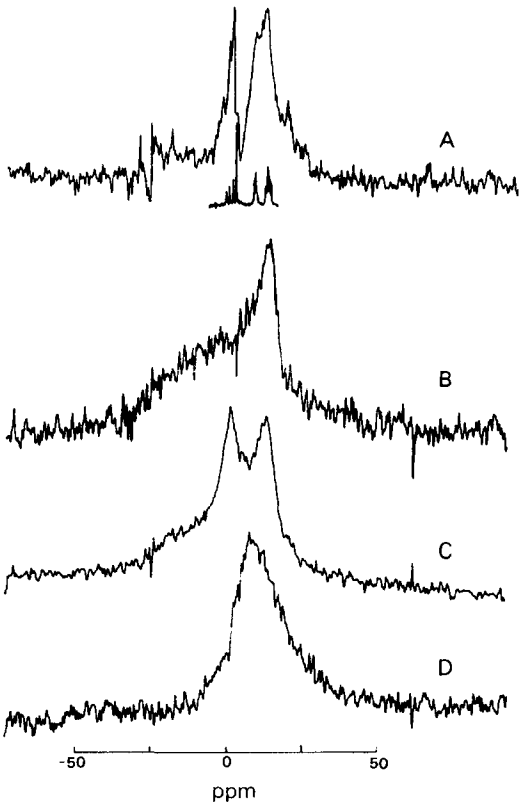

Fig. 4. 36.4 MHz ^{31}P-NMR difference spectra of dispersions of *E. coli* K12 strain CE1163 membranes in 10 mM Tris-HCl/150 mM NaCl, pH 7.4, containing $^{2}H_2O$. (A), Spectrum of outer membrane (at 37°C, method I, Fig. 3A) minus spectrum at 37°C of cytoplasmic membrane (Fig. 4B of paper I [15]). (B), Spectrum of outer membrane (at 20°C, method II, Fig. 3E) minus spectrum at lamellar native lipopolysaccharide (40°C. Fig. 6A of paper II [16]). (C) and (D), Spectra of outer membrane (37°C, method II) using 180°-τ-90° pulse sequence with τ = 16 ms for (C) and 48 ms for (D). Spectrum (C) is plotted upside down to that actually measured). Zero of the scale is as in Fig. 3. In A a high resolution spectrum of native lipopolysaccharide (Fig. 5B of paper II [16]) is included for comparison.

shown. The signal resulting from the subtraction is very close to the high resolution spectrum of native lipopolysaccharide (solubilized in sodium dodecyl sulfate) presented in Fig. 5B of paper II [16]. The increased motion of some of the lipopolysaccharide is accompanied by what appears to be a slight decrease in the intensity of the broad line part of the spectrum associated with the lipopolysaccharide. The above results strongly suggest that at least some of the lipopolysaccharide is mobilized by method I. Therefore, the remaining results in this paper were obtained with membranes prepared by method II.

Attempts to decompose the outer membrane ^{31}P-NMR spectrum into phospholipid and lipopolysaccharide parts

The outer membrane ^{31}P NMR-spectrum is a superposition of signals from lipopolysaccharides and phospholipids. In this section two different approaches

are used in an attempt to decompose the spectrum into contributions from the different phosphate groups, and hence enable a comparison with the spectra of dispersions of phospholipid and of lipopolysaccharide in papers I and II [15, 16]. Firstly, simple subtraction of spectra is performed to determine to what extent the outer membrane signals are superpositions of known signals. Secondly, the results of a two-pulse NMR experiment are used in an attempt to separate the different signals on the basis of their different relaxation times.

1. Subtraction of signals. In order to examine whether the outer membrane signal at 20°C is a superposition of signals from lamellar phase phospholipid and lamellar phase lipopolysaccharide, the spectrum of lamellar native lipopolysaccharide at 40°C (Fig. 6A of paper II [16]) was subtracted from the outer membrane spectrum at 20°C (in Fig. 3E). The resulting spectrum, shown in Fig. 4B, is characteristic of a lamellar phase phospholipid spectrum, such as that shown in Fig. 1A of paper I [15]. This indicates that at 20°C the outer membrane contains phospholipid and lipopolysaccharide in the same state as the samples that were used for comparison. Hence, the phospholipids are lamellar as far as the ^{31}P-NMR is concerned, and the lipopolysaccharide is in the same state as the native lipopolysaccharide at 40°C, which is probably also lamellar (paper II [16]). It is not worthwhile performing a similar subtraction using the outer membrane spectrum at 37°C because the broad line part of the lipopolysaccharide spectrum is narrower than that found for native lipopolysaccharide, but wider than that for electrodialyzed lipopolysaccharide and it is unlikely that anything would be learned definitively. In order to try to understand the spectrum at 37°C, the two-pulse experiment described below was performed.

2. Two-pulse experiment. An experiment carried out to measure the ^{31}P-NMR spin-lattice relaxation rate indicated that the signal as measured at the lamellar phase singularity position decays non-exponentially. The signal was analyzed as the sum of two exponentials. If we assume that there are only two broad line contributions to the spectrum, and that each of the contributions has a relaxation rate that is independent of the orientation of the local structure with respect to the magnetic field, then knowledge of the relaxation parameters enables us to choose pulse spacings such that one of the contributing signals is zero. Spectra obtained in this way are shown in Figs. 4C and D. At least some of the peak intensity at the position for isotropic phospholipid motion in Fig. 4C is due to a small amount of sample degradation during the time (20 h) needed to perform the experiment. The broader part of the spectrum in Fig. 4C does have the correct overall shape for a bilayer phospholipid spectrum, but there appears to be extra broad line intensity at the isotropic position. The right-hand side of Fig. 4D does correspond with the 40°C spectrum of lamellar native lipopolysaccharide (Fig. 6A of paper II [16]), while the left side seems to correspond more with the 40°C spectrum of electrodialyzed lipopolysaccharide dispersed in buffer (Fig. 6B of paper II [16]). Therefore it is likely that the ^{31}P in the phospholipid and some of those in the lipopolysaccharide (probably the low-field ones) have almost the same spin-lattice relaxation rates (12 s^{-1}) while the high field lipopolysaccharide ^{31}P probably have the larger spin-lattice relaxation rate (35 s^{-1}).

Hence, from the two types of manipulation experiment it can be concluded

that most of the phospholipid and lipopolysaccharide is lamellar. However, nothing has been learned about the extra minor resonance observed in both outer (37°C) and cytoplasmic (25°C) membranes which occurs near the isotropic phospholipid position. The understanding of this resonance from the two-pulse experiments is complicated by the slow growth of the decomposition peak at this position.

The effects of calcium and EDTA on wild-type outer membranes

Since calcium and EDTA are known to influence the physical and chemical properties of lipopolysaccharide in vitro and in cells [11,17,29], the effect of these components on the outer membrane (method II) of wild-type cells was studied. The addition of Ca^{2+} to a final concentration of 0.1 M did not lead to observeable changes in the NMR spectrum at 30°C. This result agrees with that for mixed phospholipid/lipopolysaccharide dispersions incubated with Ca^{2+} at 37°C (paper II [16]).

EDTA, however, does cause dramatic spectral changes, as shown in Fig. 5. The two isotropic-type peaks observed after the addition of 50 mM EDTA have a chemical shift which corresponds to lipopolysaccharide undergoing isotropic motion, and is similar to the spectrum of electrodialyzed lipopolysaccharide in Fig. 6B of paper II [16]. EDTA is known to extract lipopolysaccharide from cells [11,17]. Hence, EDTA probably extracts lipopolysaccharide from the membrane or, at least, causes changes that enable the phosphate groups to undergo more motion and this motion probably has isotropic symmetry. That extraction occurs is indicated by a density gradient sedimentation experiment

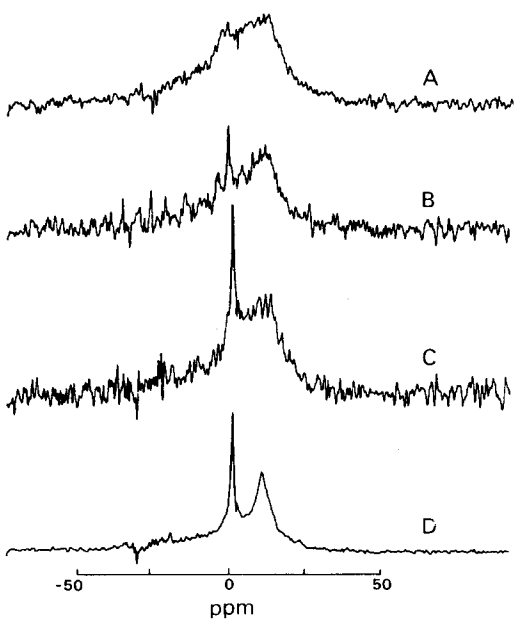

Fig. 5. 36.4 MHz ^{31}P-NMR spectra at 30°C of dispersions of *E. coli* K12 strain CE1163 outer membrane (method II) in 10 mM Tris-HCl/150 mM NaCl, pH 7.4, containing $^{2}H_2O$ and the following concentrations of EDTA: (A), 0; (B), 2 mM; (C), 10 mM and (D), 50 mM. The zero of the scale is as in Fig. 3.

in which the EDTA-treated membrane gave a band where NMR showed mainly lamellar phospholipids and no isotropic lipopolysaccharide peaks.

Mutant-lacking proteins b, c and d in the outer membrane

Understanding of the outer membrane can be gained using mutants which are deficient in the major outer membrane proteins b, c and d. This deficiency is compensated for by increased amounts of lipopolysaccharide and phospholipid, the latter being at least partly present in each mutant's outer monolayer [7]. The deficiency leads to an increase in the phospholipid/lipopolysaccharide ratio. Such mutant cells show far fewer particles and pits in the outer membrane than do wild-type cells [11,12]. The addition of Ca^{2+} to such mutant cells leads to the appearance of particles and pits in freeze-fracture electron micrographs [11]. Hence a study of the outer membrane of the protein b, c and d deficient mutant cells was undertaken both to gain some understanding of the particles and pits observed in freeze-fracture electron microscopy and to probe the various intramembraneous interactions.

Freeze-fracture electron microscopy of the outer membrane isolated according to method II showed almost completely smooth fracture faces (Fig. 6) as was observed in cells [11,12]. The spectrum of the mutant's outer membrane (Fig. 7) is rather broad, as was the case for the wild-type (method II) outer membrane (Fig. 3D). The broad part of the spectrum seems slightly less intense

Fig. 6. Freeze-fracture electron microscopy of the outer membrane of *E. coli* K12 mutant strain CE1166 ($b^- c^- d^-$) (method II). Samples were quenched from 0°C. The arrow indicates the direction of shadowing.

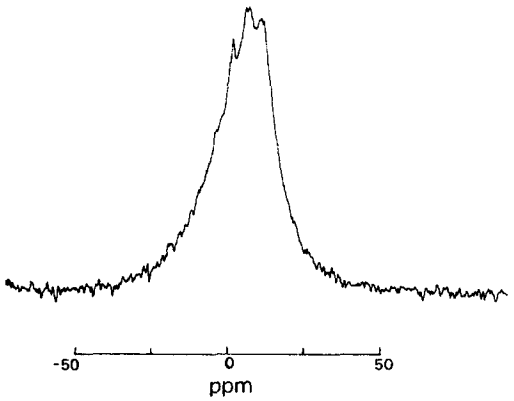

Fig. 7. 36.4 MHz ^{31}P-NMR spectrum at 37°C of a dispersion of the outer membrane of the *E. coli* outer membrane protein b, c and d deficient mutant strain CE1166 (method II) in 10 mM Tris-HCl/150 mM NaCl, pH 7.4, containing $^{2}H_2O$. The zero of the scale is as in Fig. 3.

than in the wild type, probably because of the lower lipopolysaccharide-to-phospholipid ratio. This difference in the intensity of the broad line was confirmed by a spectrum taken on a Bruker CXP 200 NMR spectrometer, which is designed to study broad lines. One feature is the relatively sharp peaks which are similar to those observed with the method I wild-type outer membrane, but shifted slightly in position. These peaks could represent a small fraction of lipopolysaccharide that has different, probably more, motion than in the outer membrane from wild-type cells. However, the integrated signal intensity present in these peaks is small.

The addition of calcium to this membrane completely disrupts the structure, as indicated by freeze-fracture electron microscopy. The NMR spectrum of this sample gave a sizeable isotropic-like peak about 1 ppm to low field of that expected for isotropic phospholipid. This effect of calcium on the mutant outer membrane contrasts to the results obtained for model phospholipid-lipopolysaccharide liposomes and wild-type outer membranes, where no effect was observed. This effect remains unexplained.

Discussion

The outer membrane of *E. coli* is a heterogeneous system containing proteins, phospholipids and lipopolysaccharides. Our ^{31}P-NMR studies have given information about the physical state of the latter two components. The several different approaches used to study the outer membrane of wild-type cells all indicate that with membranes freshly prepared by method II, there is no evidence for non-bilayer phases up to 20°C, where the NMR is indicative of total lamellar phase. At 37°C (even with a fresh sample) and at 45°C, there may be small amounts of non-bilayer phases for either or both of phospholipid and lipopolysaccharide. Since isotropic peaks sometimes appear during the long times needed to obtain spectra with a good signal-to-noise ratio, and since the spectrum of lamellar lipopolysaccharide is not sufficiently well understood, we cannot say definitely whether or not non-bilayer phases do exist in the outer

membrane. However, they definitely do not dominate the spectrum, and any isotropic motion is on such a time scale as to be associated with only relatively large structures. This preference for a bilayer arrangement is in contrast to the non-bilayer phases preferred by the total phospholipids at 37°C (paper I [15]). The ^{31}P-NMR does agree exactly with the bilayer organization observed (in paper II [16]) for phospholipid-lipopolysaccharide liposomes, although it should be noted that the distribution of lipopolysaccharide and phospholipids in the outer membrane, in contrast to these liposomes, is very asymmetrical [6—9].

The results obtained using two different preparation procedures suggest that method I disrupts the membrane in that it leads to more motion of at least some of the lipopolysaccharide although lipopolysaccharide was not extracted from the membrane (Table I and Refs. 17 and 11). A comparison of outer membranes prepared by methods I and II suggests some reasons for this. In method I the use of lysozyme results in degradation of the peptidoglycan, which has been shown to result in a transbilayer redistribution of lipopolysaccharide [6]. Moreover, the use of EDTA in this preparation procedure probably causes changes in the outer membrane, since cells become sensitive to inter alia lysozyme action by such treatment [7,8]. In fact the presence of EDTA has a marked effect on the NMR spectrum of the method II outer membrane. These results, together with the seemingly increased mobility of lipopolysaccharide with method I, indicate that the various NMR spectral features observed with method I are due to the preparation procedure. Hence, method II is a preferable preparation procedure.

The final question is whether or not anything can be learned from our ^{31}P-NMR studies about the particles and pits observed in the freeze-fracture electron microscopy studies of the outer membrane. The NMR signal from phospholipid and lipopolysaccharide is the same for outer membranes of wild-type and mutant cells and for phospholipid-lipopolysaccharide liposomes.

The very minor differences among these spectra could be interpreted as indicating more motion for a small fraction of the phosphate groups in samples which did not show particles and pits. Hence, the particles do not represent any different type of motion for a large fraction of either phospholipid or lipopolysaccharide. It has been suggested that these particles contain hemi-micelles of lipopolysaccharide [10—12,14]. If this is the case the NMR results indicate that there is probably little diffusional motion of the lipopolysaccharide around the curved surfaces of the particles and that it is likely that the major outer membrane proteins b, c and d, which are also thought to be present in the particles and which are probably complexed with lipopolysaccharide [14], can immobilize lipopolysaccharide in the particles.

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