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THE OUTER MEMBRANE OF *PROTEUS MIRABILIS*

II. THE EXTRACTABLE LIPID FRACTION AND ELECTRON-PARAMAGNETIC RESONANCE ANALYSIS OF THE OUTER AND CYTOPLASMIC MEMBRANES

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SUMMARY

1. The lipid fraction extracted from the outer and cytoplasmic membranes of *Proteus mirabilis* with chloroform/methanol consisted almost entirely of phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylglycerol.

2. The phospholipid content of the cytoplasmic membrane was more than twice that of the outer membrane (38 % as against 18 % of the total dry weight) and the proportions of the three phospholipids differed somewhat in the two membranes. Yet, the fatty acid composition of the extractable lipids was essentially the same in both membranes.

3. The freedom of motion of spin-labeled fatty acids in the outer membrane of *P. mirabilis* depended markedly on temperature and on the position of the nitroxide group on the hydrocarbon chain of the probe, suggesting that the local environment of the probe is an associate lipid structure with the properties of a bilayer. Nevertheless, the mobility of the probe was more restricted in the outer membrane than in the cytoplasmic membrane, indicating a higher viscosity of the outer membrane.

4. Chloroform/methanol completely removed the phospholipids from the outer membrane, leaving the lipopolysaccharide moiety intact. The motion of spin-labeled fatty acids in the extracted membranes was, however, highly restricted, suggesting that, in the native outer membrane, the local environment of the probe is composed of phospholipids rather than lipopolysaccharide. Aqueous acetone extraction removed only 75–80 % of the phospholipids of the outer membrane. Nevertheless, the mobility of the spin-labeled fatty acid remained highly restricted, suggesting the existence of two phospholipid environments in the outer membrane differing in the nature of their association with the lipopolysaccharide and protein moieties.

INTRODUCTION

The isolation of the outer and cytoplasmic membranes of *Proteus mirabilis*, described in the accompanying communication [1], enabled a comparison of their

lipid composition and an assessment of the physical state of their hydrophobic core. The present communication provides data on the composition of the lipid fraction which is readily extractable with chloroform/methanol. The composition of the non-extractable lipid A moiety of the lipopolysaccharide component of the outer membrane will be dealt with in a forthcoming communication.

The physical state of the hydrophobic core of the *P. mirabilis* membranes was investigated by electron-paramagnetic resonance spectroscopy (EPR) of spin-labeled fatty acids incorporated into the membranes [2]. The results reported in this paper suggest that the molecular organization and the freedom of motion of the hydrocarbon chains of the lipids differ in the two membranes enveloping *P. mirabilis* cells.

MATERIALS AND METHODS

Organism and growth conditions. Proteus mirabilis strain 19 was kindly provided by Professor H. H. Martin (Technische Hochschule, Darmstadt, Germany). The organisms were grown at 37 °C in 2-1 vols of Nutrient Broth (Difco) supplemented with 10 μCi of uniformly labeled [^{14}C]glycerol (19.5 mCi/mmol) or 15 μCi of either [9,10-^{3}H]oleic acid (2.1 Ci/mmol) or [9,10-^{3}H]palmitic acid (0.5 Ci/mmol). The organisms were harvested and cytoplasmic and outer membrane fractions were isolated as described in the accompanying paper [1]. Protein in the isolated membrane fractions was determined according to Lowry et al. [3] and the preparations were kept at −20 °C until used.

Lipid extraction. Lipids were extracted from ^{14}C-labeled cytoplasmic and outer membrane preparations by chloroform/methanol (2 : 1, ref. 4) or by aqueous acetone. For the acetone extraction one volume of membrane suspension (5 mg membrane protein per ml) was mixed with nine vols of acetone. To each 10 ml of the suspension 5 μl of a 28 % NH_4OH solution was added and extraction was performed for 30 min at −5 °C. The extracted material was sedimented by centrifugation and extracted five more times as described above. Radioactivity in the lipid extracts and in the extracted membrane residue was measured in a Packard Tri-Carb scintillation spectrometer using a dioxane/toluene scintillation liquor [5].

Separation of neutral from polar lipids. Neutral lipids were separated from polar lipids by silicic acid chromatography. The lipid extracts were applied to columns (6 × 100 mm) of activated silicic acid (100 mesh, Mallinckrodt Chemical Works, St. Louis, Mo.) prewashed with chloroform. Elution was carried out successively with 20 ml of chloroform and 20 ml of chloroform/methanol (1 : 1) to separate the neutral lipids and the polar lipids, respectively. The lipid fractions were dried under a stream of nitrogen, weighed and redissolved in 0.5 ml of chloroform/methanol (2 : 1).

Thin-layer chromatography. The polar lipid fraction was chromatographed on silica gel HR plates (0.5 mm thick), prepared from a slurry containing 40 % silica gel HR and 0.6 % of prewashed Florisil (Hopkin & Williams Ltd., Chadwell Heath, England) in 0.1 M Na_2CO_3. The plates were developed with chloroform/methanol/acetic acid /water (100 : 50 : 16 : 8, by vol.). Lipid spots were detected by iodine vapor, then scraped off the plate and their radioactivity measured as described above. In some experiments the plates were sprayed for the detection of phospholipid spots [6] and α-glycol-containing lipids [7]. Ninhydrin-positive lipids were detected with the Ninhydrin spray reagent (Merck, Darmstadt, Germany). For gravimetric deter-

mination about 10 mg of lipid was applied to the chromatoplate as a band and chromatographed. The resulting bands, detected by iodine vapor, were scraped off the plates and the lipids were eluted twice with chloroform/methanol (2 : 1). The extracts were filtered through an ultrafine sintered glass filter, evaporated to dryness under a stream of nitrogen and weighed.

Gas-liquid chromatography. Methyl esters of the fatty acids were prepared by heating the lipid samples for 18 h in anhydrous methanol containing 10 % (w/w) HCl at 72 °C in a sealed Pyrex ampoule. The resultant methyl esters were extracted with light petroleum ether (boiling point, 40–60 °C) and subjected to gas-liquid chromatography in a Packard model 840 instrument equipped with a polar column (200 × 0.3 cm, 15 % of diethylene glycol adipate on Chromosorb W). Fatty acids were identified by their retention time relative to that of standard methyl ester mixtures (Supelco, Inc., Bellefonte, Pa.).

Paramagnetic resonance spectroscopy. Membranes were spin labeled with *N*-oxyl-4′-4′-dimethyloxazolidine derivatives of 5-ketostearic acid and 12-ketostearic acid (Syva, Palo Alto, Calif.) by exchange from bovine serum albumin as previously described [8]. Electron paramagnetic resonance spectra of the spin-labeled membranes were obtained by use of a Varian E-4 spectrometer. The molecular motion is reported as $2T_{\parallel}$, the hyperfine splitting that has been previously shown to be related to the freedom of motion of the nitroxide radical [2] and as τ_0, an empirical parameter calculated from the expression used by Henry and Keith [9]:

$$\tau_0 = 6.5 \cdot 10^{-10} \cdot W_0 \left(\left(\frac{h_0}{h_{-1}} \right)^{\frac{1}{2}} - 1 \right)$$

where W_0 is the line width of the mid-field line and h_0 and h_{-1} are the heights of the mid- and high field lines on a first derivative absorption spectrum. Greater freedom of motion is associated with smaller values of either $2T_{\parallel}$ or τ_0.

Electron microscopy. For thin sectioning, pellets of native or extracted membrane preparations were fixed at room temperature by the Ryter-Kellenberger procedure [10] for 16 h. Post-fixation, embedding, sectioning and staining were performed as described in the accompanying paper [1]. The thin sections were examined in a Phillips EM-300 electron microscope.

RESULTS

Analysis of cell fractions obtained by sonic disruption of *Proteus mirabilis* showed the readily extractable lipids to be located almost exclusively in the cytoplasmic and outer membrane fractions, where it comprised about 38 % and 18 % of the dry weight, respectively [1]. When subjected to silicic acid chromatography only 5 % of the lipids could be eluted with chloroform, the rest being eluted with chloroform/methanol (1 : 1). Hence, the lipids of *P. mirabilis* membranes are almost entirely polar lipids.

Table I shows that *P. mirabilis* incorporated to about the same degree both radioactive oleic and palmitic acids from the growth medium into the extractable lipid fraction. However, while essentially all of the radioactive oleic acid could be recovered in the extractable lipid fraction, about 5 % of the radioactive palmitic acid

TABLE I

INCORPORATION OF LABELED FATTY ACIDS INTO THE EXTRACTABLE LIPID AND LIPOPOLYSACCHARIDE COMPONENTS OF THE OUTER MEMBRANE OF *P. MIRABILIS*

Cells grown with:	Radioactivity in outer membrane components			
	Extractable lipid		Lipopolysaccharide*	
	cpm	% of total	cpm	% of total
[^{3}H]Oleic acid	90 560	99.2	640	0.6
[^{3}H]Palmitic acid	85 700	95.3	4 150	4.6

* Extracted with phenol/water according to Westphal et al. [11].

resisted intensive extraction of the outer membrane fraction with chloroform/methanol. This residual radioactivity could be recovered in the water phase of the phenol/water extract [11] of the outer membrane, indicating that the palmitic acid was incorporated into the lipopolysaccharide, presumably into the lipid A moiety.

Thin-layer chromatography of the polar lipid fractions obtained from cytoplasmic and outer membranes revealed that the major component was a ninhydrin-positive phospholipid migrating with the same R_F as authentic phosphatidylethanolamine. On a dry weight basis this component accounted for 55–60 % of the polar lipid fraction of the cytoplasmic membrane and 75–80 % of that of the outer membrane. Diphosphatidylglycerol and phosphatidylglycerol were also identified in the polar lipid preparations of both membranes. The diphosphatidylglycerol accounted for about 30 % of the polar lipid of the cytoplasmic membrane and 15–20 % of that of the outer membrane. Because of its low content a precise gravimentric determination of the phosphatidylglycerol could not be made.

Table II shows the incorporation of radioactive lipid precursors into *P. mirabilis* phospholipids. The distribution of the label in the various phospholipids obtained from cells grown with oleate and palmitate was in good accord with the dry

TABLE II

INCORPORATION OF LABELED LIPID PRECURSORS INTO THE VARIOUS POLAR LIPID COMPONENTS OF THE CYTOPLASMIC AND OUTER MEMBRANES OF *P. MIRABILIS*

Component	% of total radioactivity in polar lipids					
	Cytoplasmic membrane from cells grown with:			Outer membrane from cells grown with:		
	[^{14}C]-Oleate	[^{14}C]-Palmitate	[^{14}C]-Glycerol	[^{14}C]-Oleate	[^{14}C]-Palmitate	[^{14}C]-Glycerol
Phosphatidyl-ethanolamine	61.9	55.5	54.1	80.6	70.5	73.3
Diphosphatidylglycerol	28.9	31.7	27.6	15.1	24.5	13.7
Phosphatidyl-glycerol	9.2	12.7	20.2	4.2	4.7	13.0

TABLE III

FATTY ACID COMPOSITION OF THE EXTRACTABLE LIPIDS OF THE CYTOPLASMIC AND OUTER MEMBRANES OF *P. MIRABILIS*

Preparation	Fatty acid (% of total)					
	14 : 0	16 : 0	16 : 1	18 : 0	18 : 1	19 : 0
Cytoplasmic membrane	3.1	42.3	28.7	1.6	21.9	2.1
Outer membrane	5.1	44.4	26.6	1.9	20.0	1.3

weight data presented above. [^{14}C]glycerol, on the other hand, was incorporated more intensively into the phosphatidylglycerol fraction than could be expected from the gravimetric data. Thus, labeling membrane lipids with either palmitate or oleate may be useful for the estimation of the relative amounts of the different phospholipids in the membranes. The labeling intensity of the phosphatidylglycerol band indicated that the polar lipid fraction of the cytoplasmic membrane contains about 11 % phosphatidylglycerol while that of the outer membrane contains less than 5 % phosphatidylglycerol.

The fatty acid composition of the extractable lipid of the cytoplasmic and outer membrane preparations is presented in Table III. It appears that the fatty acid composition of the two membranes is qualitatively and quantitatively almost identical. The major fatty acids were palmitic acid (~43 %), hexadecenoic acid ($C_{16:1}$; ~27 %) and octadecenoic acid ($C_{18:1}$; ~20 %). No C17 cyclopropane-containing fatty acids were found and only small amounts (~2 %) of the C19 cyclopropane-containing fatty acids could be detected. Also noteworthy was the absence of β-hydroxymyristate

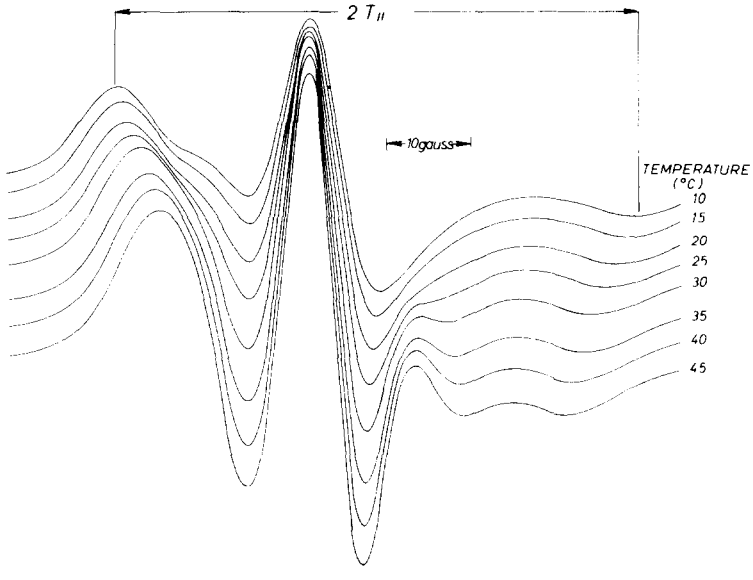

Fig. 1. The EPR spectra of the spin-labeled fatty acid 5-nitroxystearate in the outer membrane fraction of *P. mirabilis* at various temperatures.

found to be the major fatty acid (~60) of the lipid A moiety of the *P. mirabilis* lipopolysaccharide. The detailed fatty acid analyses of the *P. mirabilis* lipopolysaccharide and our notions on the organization of these fatty acids in the lipopolysaccharide molecule will be presented elsewhere.

Fig. 1 shows the pronounced temperature dependence of the hyperfine splitting ($2T_{||}$) of 5-nitroxystearate in the outer membrane of *P. mirabilis*. Although the spectra were similar to those obtained with spin-labeled cytoplasmic membranes, the hyperfine splitting was different (Fig. 2), indicating a greater freedom of motion of the spin-labeled fatty acid in the cytoplasmic membrane. With both outer and cytoplasmic membrane fractions the hyperfine splittings were markedly affected by changing the position of the nitroxide group on the hydrocarbon chain of the fatty acid derivative, decreasing as the nitroxide was moved away from the polar end of the molecule (Fig. 2). The greater molecular motion of the spin-labeled fatty acid in the cytoplasmic membrane was further demonstrated in a plot of τ_0 versus $°K^{-1}$ (Fig. 3), where τ_0 was the empirical motion parameter calculated according to Henry and Keith [9] from spectra of 12-nitroxystearate incorporated into the membrane preparations. Fig. 3 also shows a discontinuity at 36 °C in the Arrhenius plot of the data obtained with the outer membrane. The transition point was characterized by a fusion activation energy of $\Delta E = 8.5$ kcal/mol above and $\Delta E = 15.1$ kcal/mol below it. The plot of the data obtained for the cytoplasmic membrane was straight with an activation energy of $\Delta E = 7.7$ kcal/mol.

Chloroform/methanol (2 : 1) extracted over 99.9 % of the outer membrane phospholipids as estimated from the decrease in radioactivity upon extraction of

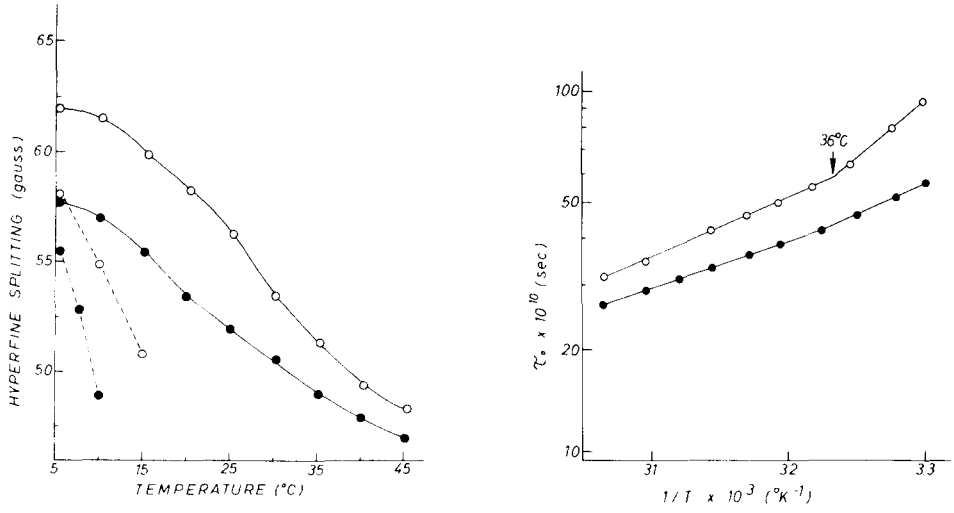

Fig. 2. Temperature dependence of the hyperfine splitting ($2T_{||}$) of the spin-labeled fatty acids 5-nitroxystearate (solid lines) and 12-nitroxystearate (broken lines) in the outer membrane fraction (○) and in the cytoplasmic membrane fraction (●).

Fig. 3. Arrhenius plots of τ_0 values calculated from EPR data obtained with the spin-labeled fatty acid 12-nitroxy stearate in the outer membrane fraction (○) and in the cytoplasmic membrane fraction (●).

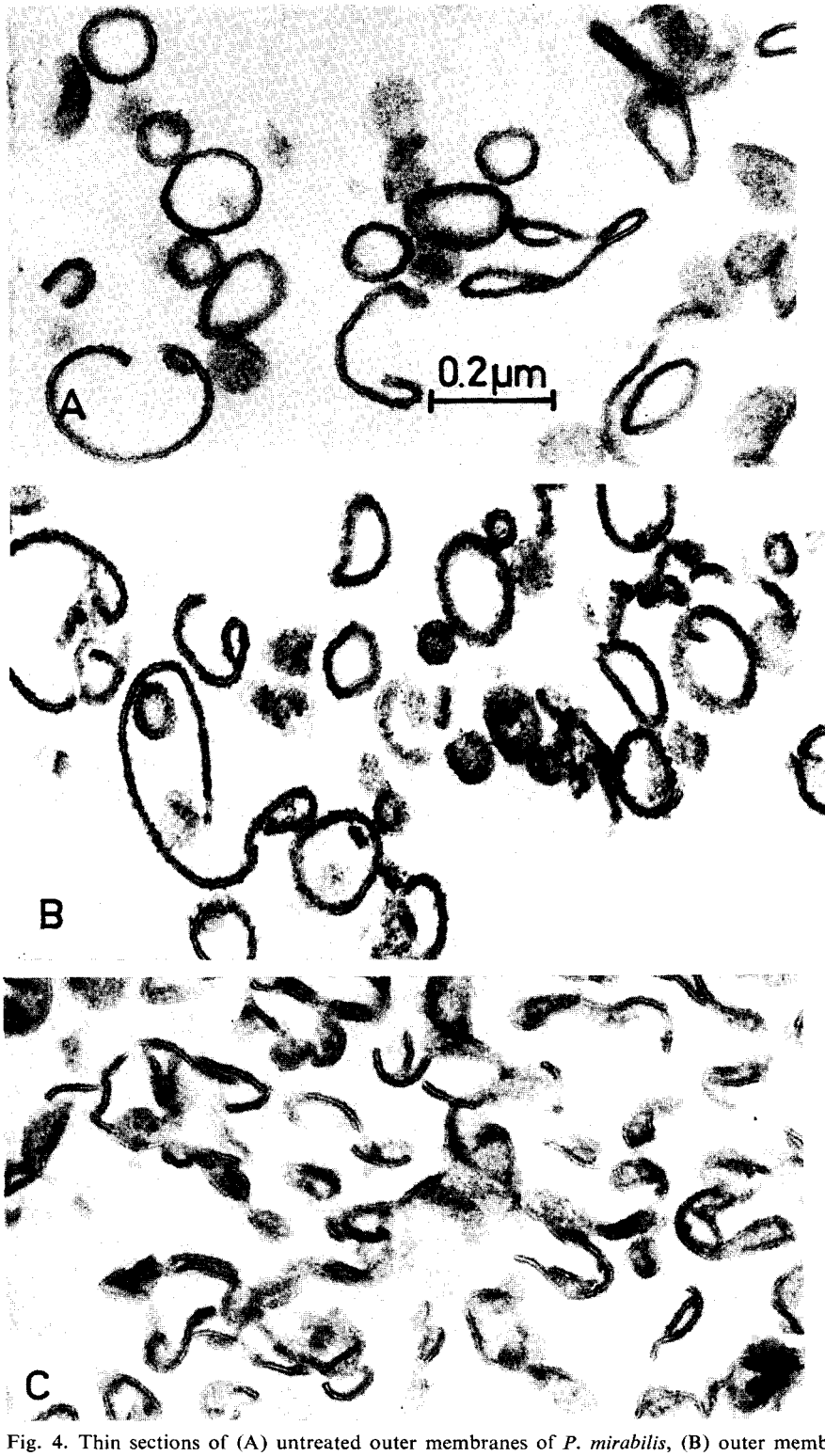

Fig. 4. Thin sections of (A) untreated outer membranes of *P. mirabilis*, (B) outer membranes extracted with aqueous acetone, (C) outer membranes extracted with chloroform/methanol.

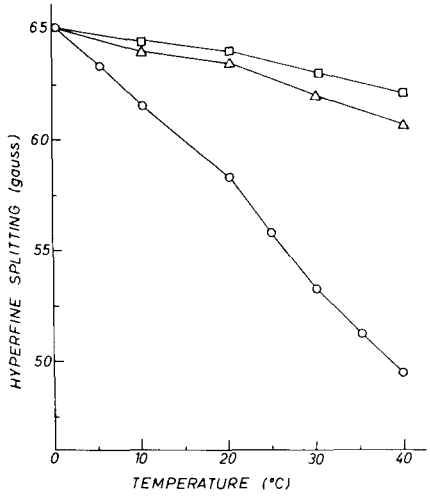

Fig. 5. Temperature dependence of the hyperfine splitting ($2T_{\parallel}$) of the spin-labeled fatty acid 5-nitroxystearate in untreated outer membranes (○) and in outer membranes extracted with aqueous acetone (△) or with chloroform/methanol (□).

outer membranes of cells grown with [^{14}C]oleic acid. However, the extracted outer membranes still retained their typical double-track shape in section (Fig. 4). When the spin label 5-nitroxystearate was added to the extracted outer membrane preparations the EPR spectra revealed a highly restricted freedom of motion of the probe as indicated by the very slight effect of increasing temperatures (Fig. 5). This suggests that in the chloroform/methanol extracted membranes the spin label is located in a highly rigid matrix. Very similar results were obtained with outer membrane preparations extracted with aqueous acetone (Fig. 5) though the most extensive acetone extraction still left over 20 % of the phospholipids in the outer membrane. It is of interest to note that almost all of the phospholipids of the cytoplasmic membrane can be extracted with aqueous acetone (Table IV).

DISCUSSION

The extractable lipid fraction of both the outer and cytoplasmic membranes of *P. mirabilis* appears to consist almost entirely of phospholipids, as was found for the related *Proteus* P18 strain by Nesbitt and Lennarz [12]. We could not find any significant quantities of free fatty acids or neutral lipids, which were found to comprise a large proportion of the extractable lipids of the cell envelope of *Pseudomonas aeruginosa* and *Brucella abortus* [13].

Although the phospholipid content of the cytoplasmic membrane was more than twice that of the outer membrane, the phospholipid composition of both membranes was qualitatively the same. This can be expected in view of the finding that the enzymes

TABLE IV

AQUEOUS ACETONE EXTRACTION OF [^{3}H]OLEIC ACID-LABELED LIPIDS OF THE OUTER AND CYTOPLASMIC MEMBRANES OF *P. MIRABILIS*

Extractions were carried out for 30 min at 0-5 °C with acetone / water / ammonia (90 : 10 : 0.05, by vol.).

Preparation	Outer membrane		Cytoplasmic membrane	
	Radioactivity* (cpm/mg protein)	% of total	Radioactivity* (cpm/mg protein)	% of total
Unextracted membranes	151 000	100.0	143 000	100.0
Membranes extracted twice	48 600	32.0	13 500	9.4
Membranes extracted 5 times	37 500	24.8	7 000	4.9
Membranes extracted 10 times	33 500	22.0	4 300	3.1

* The data represent the radioactivity values remaining in the membranes after extraction.

involved in the biosynthesis of the phospholipids are localized in the cytoplasmic membrane only [14, 15]. Hence, the phospholipids of the outer membrane originate in the cytoplasmic membrane. Yet, the proportions of the three phospholipids detected in *P. mirabilis*, phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylglycerol, were not the same in the outer and cytoplasmic membranes, suggesting that the phospholipids have different rates of turnover or transfer from the cytoplasmic to the outer membrane.

In composition, the phospholipids of the extractable lipid fraction of *P. mirabilis* resemble those of membranes of other enteric bacteria. However, the relative amounts of the major phospholipids differ [13, 16, 17]. Thus, the percentage of diphosphatidylglycerol in the extractable lipid fraction of *P. mirabilis* exceeded that of phosphatidylglycerol, whereas in *Salmonella typhimurium* [16] and *Escherichia coli* [17] phosphatidylglycerol exceeded diphosphatidylglycerol. These quantitative differences may well be due to differences in the age of the culture used for membrane preparation since phosphatidylglycerol is known to be converted to diphosphatidylglycerol at the later growth phases [18, 19]. Nevertheless, phosphatidylethanolamine appears to be the dominating phospholipid in all cases. The percentage of phosphatidylethanolamine in the phospholipid fraction of the outer membrane of *P. mirabilis* is higher than in the corresponding fraction of the cytoplasmic membrane, as was also found for *Salmonella typhimurium* [16] and *Escherichia coli* [20].

The fatty acid composition of the extractable lipids of the outer and cytoplasmic membrane of *P. mirabilis* was found to be qualitatively and quantitatively almost identical, supporting the common origin of this lipid fraction. It differed significantly from the fatty acid composition of the extractable lipids of the related *Proteus* P18 strain [12], mainly in having much smaller amounts of cyclopropane-containing fatty acids and much larger amounts of the $C_{16:1}$ and $C_{18:1}$ fatty acids. Since $C_{16:1}$ and $C_{18:1}$ are known to be the precursors of the cyclopropane fatty acids [21] the difference in fatty acid composition between our strain and *Proteus* P18 may be the result of differences in the age of the cultures.

The marked temperature dependence of the mobility of spin-labeled fatty acids in the outer membrane of *P. mirabilis*, as well as the dramatic increase in mobility when the nitroxide radical was moved away from the polar head group of the probe, suggests that the local environment of the spin-labeled fatty acid in the outer membrane is an associate lipid structure with the properties of a bilayer [2]. Nevertheless, the mobility of the probe was more restricted in the outer membrane than in the cytoplasmic membrane indicating the higher viscosity of the lipid matrix of the outer membrane.

Why does the fluidity of the two membranes differ though their phospholipid composition is very much the same? The difference is apparently not due to the incorporation of the probe solely into the lipopolysaccharide moiety since the complete removal of the extractable lipids from the outer membrane, which leaves the lipopolysaccharide moiety intact, resulted in an almost complete immobilization of the spin-labeled fatty acid, indicating the highly viscous nature of lipopolysaccharide. The fact that a single homogeneous low-field peak was consistently observed in the outer membrane suggests that the probe is localized in an homogeneous matrix, apparently in the more fluid phospholipid domain, thus excluding the possibility that there is a partition of the probe between the highly viscous lipopolysaccharide and the phospholipid domains. Hence, the spin-labeled fatty acid seems to be localized mainly in a phospholipid bilayer structure. If so, why is the mobility of the spin label more restricted in the outer than in the cytoplasmic membrane? Three possible explanations may be offered. (1) The outer membrane differs from the cytoplasmic membrane in its protein composition and disposition and, as was previously demonstrated [22], membrane proteins may markedly affect the probe's freedom of motion in the membrane. (2) The lipid bilayer of the outer membrane is made up of phospholipid molecules interdigitated by the highly saturated acyl chains of the lipopolysaccharide molecules. The saturated acyl chains will act to reduce the fluidity of the bilayer. (3) The phospholipid moiety of the outer membrane is organized in small pockets surrounded by lipopolysaccharide and/or protein, whereas in the cytoplasmic membrane the phospholipid forms a more continuous bilayer. The adjacent lipopolysaccharide and protein zones may act to restrict the mobility of the probe in the phospholipid pockets.

The finding that about 20 % of the phospholipids of the outer membrane resisted extraction with aqueous acetone, conditions which suffice to extract essentially all the phospholipids from the cytoplasmic membrane (Table IV), indicates the existence of two lipid environments. It may be suggested that the part of the phospholipid fraction which resists extraction with acetone is more tightly bound to the outer membrane, probably due to its close association with the hydrophobic portions of the lipopolysaccharide or protein molecules. It could be expected that the mobility of a spin-labeled fatty acid incorporated into membrane regions containing the tightly-bound phospholipids will be more restricted.

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