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INTERACTION OF LIPOPOLYSACCHARIDE WITH DETERGENTS AND ITS POSSIBLE ROLE IN THE DETERGENT RESISTANCE OF THE OUTER MEMBRANE OF GRAM-NEGATIVE BACTERIA

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Summary

In the presence of $MgCl_2$, amounts of detergents which disrupted phospholipid vesicles caused lipopolysaccharide I from *Proteus mirabilis* to aggregate and form vesicular, membrane-like structures. Vesicle formation with *P. mirabilis* lipopolysaccharide II containing longer O-polysaccharide chains was extremely poor. Lipopolysaccharides of *Salmonella minnesota* R mutants (chemotypes Ra, Rc and Re) displayed a growing tendency for vesicle formation with increasing deficiency of the R core polysaccharide. Lipopolysaccharides of chemotypes Rc and Re produced vesicles even in the absence of $MgCl_2$ and detergent. Spherical aggregates consisting of *P. mirabilis* lipopolysaccharide I, $MgCl_2$ and detergent were unable to either entrap or retain [^{14}C]-sucrose, [^{3}H]inulin or [^{3}H]dextran. On the other hand, *S. minnesota* R mutant lipopolysaccharides of chemotypes Rc and Re could entrap all three saccharides and retain them for at least short periods of time. Leakage of [^{3}H]-inulin out of Re-lipopolysaccharide vesicles was greatly retarded by addition of $MgCl_2$ to the vesicle system. Incorporation of *P. mirabilis* lipopolysaccharide I or *S. minnesota* Rc lipopolysaccharide into phospholipid vesicles protected these model membranes from disruption by detergent. This suggested a similar protective function of lipopolysaccharide in the outer membrane of enteric bacteria against the action of surfactants occurring in their normal intestinal habitat.

Introduction

The relative resistance of Gram-negative bacteria to surface active agents is a property reflecting the unique character of the outer membrane complex of the cell wall of these organisms. This resistance has been most thoroughly documented through the extensive use of selective culture media containing surface-active agents for the specific isolation of enteric bacteria.

In previous work [1] we used a phospholipid model membrane system to show that some specific protein components contribute to the detergent resistance of the outer membrane in the Gram-negative bacterium *Proteus mirabilis*.

In the present investigation we have studied the possible function of lipopolysaccharide in the interaction of the outer membrane with detergents. The ability of S- and R-forms of lipopolysaccharide to aggregate into membrane-like vesicles in the presence of divalent cations is described, and the permeability properties of these artificial membranes were examined. Also, the ability of lipopolysaccharides to protect phospholipid vesicles from disruption by detergent was investigated.

Methods

Bacterial strains. P. mirabilis strain 19 of this laboratory was cultivated as previously described [2] on *Proteus* L medium. *S. minnesota* R mutants R60 (chemotype Ra), R5 (chemotype Rc) and R595 (chemotype Re) [3] were cultivated in a fermenter using the batch method as previously described [4].

Lipopolysaccharides and phospholipids. Lipopolysaccharides types I and II were extracted from *P. mirabilis* 19 by the phenol/water method [5] and further purified according to Gmeiner [6]. The procedure described by Galanos et al. [7] was used to extract lipopolysaccharides from R mutants of *S. minnesota*. All lipopolysaccharide preparations were electrodialyzed [8]. The absence of phospholipids in lipopolysaccharide preparations was determined by fatty acid analysis [9]. Phospholipids were extracted from *P. mirabilis* as previously described [9]. Phospholipids contained approx. 80% phosphatidylethanolamine, 12% phosphatidylglycerol, 4% disphosphatidylglycerol, 1% lysophospholipid and 2% "neutral lipids" [9].

Production of vesicular structures from lipopolysaccharides. 10 mg of *P. mirabilis* lipopolysaccharide I were suspended in 1.0 ml of a buffer containing 0.15 M NaCl and 0.01 M Tris · HCl, pH 7.3, plus an optimal concentration of $MgCl_2$ (see text), and sonicated for 1 min in an ice bath using a Branson Model S-125 apparatus (Branson, Danbury, Conn., U.S.A.) at power level 4 with the microtip to thoroughly suspend the lipopolysaccharide. To the sonicated mixture, an optimal amount of detergent (see text), e.g. sodium dodecyl glycol ether sulfate, known as Texapon N-25 (Henkel and Cie, Düsseldorf, G.F.R.), sodium dodecyl sulfate, Triton X-100 (Serva, Heidelberg, G.F.R.) or sodium deoxycholate (Merck, Darmstadt, G.F.R.) was added and the mixture immediately filtered on a 1.5 × 30 cm column of Sepharose 4B equilibrated with the above buffer. The vesicle fraction eluted with the void volume of the column.

For production of vesicles from *S. minnesota* R lipopolysaccharides 10 mg of lipopolysaccharide were suspended in 1.0 ml of a buffer containing 0.15 M NaCl and 0.01 M Tris · HCl, pH 7.3. In some cases (see text) 0.01 M $MgCl_2$ was added. The mixture was sonicated as above for *P. mirabilis* lipopolysaccharide vesicles and immediately filtered on Sepharose 4B. Vesicles eluted with the void volume of the column.

Permeability measurements on lipopolysaccharide vesicles. Either 1.9 ·

10^6 cpm [^{14}C]sucrose (Amersham-Buchler GmbH, Braunschweig, G.F.R.), $1.2 \cdot 10^7$ cpm [^{3}H]inulin (M_r = 5000) or $3.2 \cdot 10^6$ cpm [^{3}H]dextran (M_r = 20 000; New England Nuclear Corp., Dreieich, G.F.R.) were added to the above lipopolysaccharide mixtures before sonication. In some cases non-radioactive carrier saccharides, e.g. 3.4 mg sucrose, 50 mg inulin (Merck, Darmstadt, G.F.R.) or 150 mg dextran (Serva, Heidelberg, G.F.R.) were included. The vesicle fraction was isolated after gel filtration on Sepharose 4B to remove non-entrapped radioactive saccharides. Radioactivity associated with the resulting vesicle fraction (time 0 vesicles) was measured in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instruments GmbH, Frankfurt, G.F.R.) and 2-keto-3-deoxyoctonate content was determined according to Waravdekar and Saslaw [10]. After various periods of incubation at room temperature, the vesicle fraction was re-chromatographed on Sepharose 4B and the resulting vesicle fraction (time X vesicles) was measured for radioactivity and 2-keto-3-deoxyoctonate content as before. The percentage radioactivity remaining in the vesicle fraction after time period X was calculated.

Production of phospholipid-lipopolysaccharide vesicles. Phospholipid vesicles were prepared by mixing 12.5 μmol phospholipid with 1.0 ml of a buffer containing 0.1 M NaCl, 2.5 mM $MgCl_2$ and 0.02 M Tris · HCl, pH 7.3. Mixed vesicles containing phospholipid and lipopolysaccharide were obtained by adding 2.5—20.0 mg lipopolysaccharide to the above mixture plus $6.0 \cdot 10^5$ cpm [^{3}H]dextran. The resulting solution was then sonicated for 4 min in an ice bath under a stream of nitrogen using a Branson Model S-125 apparatus at power level 1 with the microtip.

Protection of phospholipid vesicles from dissociation by detergent. Phospholipid vesicles prepared as described above containing entrapped [^{3}H]dextran were recovered in the fraction eluting with the void volume of the column after filtration on Sepharose 4B (0.9 × 20 cm column). The amount of phospholipid in the vesicle fraction was determined by total phosphorus analysis [11]. Varying amounts of sodium deoxycholate were added and the vesicle fraction was again filtered on the column. The minimum amount of detergent (0.2% (w/v)) which caused the release of all radioactivity from the vesicles completely without changing the elution pattern of the phospholipids (all phospholipid still recovered in the void volume) was chosen for subsequent protection tests. For this purpose, varying amounts of either *P. mirabilis* S-form lipopolysaccharide I or *S. minnesota* R-form lipopolysaccharide of chemotype Rc were added to phospholipids for production of phospholipid-lipopolysaccharide vesicles containing [^{3}H]dextran as described above. Vesicle fractions were isolated after filtration on Sepharose 4B, treated with 0.2% (w/v) sodium deoxycholate and again filtered on Sepharose 4B. From the amount of [^{3}H]-dextran per μmol phospholipid remaining in the resulting vesicle fraction, the percentage protection was calculated.

It should be noted that vesicles which were not treated with detergent retained [^{3}H]dextran fully for at least 48 h, even if subjected to repeated gel filtration on Sepharose 4B.

Purification of radioactive saccharides. [^{3}H]Inulin and [^{3}H]dextran both contained material which tended to bind to lipopolysaccharide. The contaminating material was removed by adsorbing it to lipopolysaccharide I of *P.*

mirabilis. The lipopolysaccharide with the adsorbed contaminant was then sedimented by centrifugation for 2 h at 145 000 × *g*. The procedure was repeated with fresh portions of lipopolysaccharide until no more radioactive material was sedimented. After the final absorption, the supernatant following centrifugation was filtered on Sepharose G-50 to remove any traces of lipopolysaccharide.

Electron microscopy. Samples for electron microscopy were either negatively stained with 2% (w/v) potassium phosphotungstate, pH 7.2 (Merck, Darmstadt, G.F.R.) on carbon films rendered hydrophilic by glow discharge in air [12] or prepared by the agar-filtration technique [13] and shadowed with platinum/paladium. For preparation of thin sections [14], lipopolysaccharide-detergent-$MgCl_2$ vesicles were prefixed in 2.5% (v/v) glutaraldehyde for 2 h and postfixed for 90 min in 1% (v/v) OsO_4. The fixed samples were embedded in Epon 812 (Roth, Heidelberg, G.F.R.), sectioned with an LKB ultramicrotome (LKB Instrument GmbH, Gräfelfing, G.F.R.) and subsequently stained with uranyl acetate and lead citrate [15]. Preparations were observed in a Siemens Elmiskop IA electron microscope.

Other materials. Sodium dodecyl [^{35}S]sulfate was purchased from New England Nuclear Corp., Dreieich, G.F.R. and sodium deoxy[*carboxy*-^{14}C]-cholate from Amersham-Buchler GmbH, Braunschweig, G.F.R.

Results

Production of vesicle-like aggregates from P. mirabilis lipopolysaccharide with defined concentrations of detergent and $MgCl_2$

Previous reports [16—19] have indicated that lipopolysaccharide has the potential ability to form membranous, vesicle-like structures.

In the present investigation, highly purified lipopolysaccharide from *P. mirabilis* as seen in the electron microscope had a polymorphous ribbon-like appearance and was by itself incapable of aggregating to form coherent structures of higher order. However, mixtures of this lipopolysaccharide with anionic or neutral detergents and $MgCl_2$ in strictly defined concentrations led to the formation of complexes which, in negatively-stained preparations or those made by the agar-filtration technique, had the appearance of spherical structures of uniform size (Fig. 1D and Fig. 2C). In thin sections, the same aggregates are seen as vesicle-like structures or double track membranous sheets (Fig. 1E). Appearance and dimensions of such membranes are reminiscent of a lipid bilayer.

The effect of the anionic detergent Texapon N-25 on lipopolysaccharide aggregate formation is presented in Fig. 1. In all cases the procedure for production of vesicular structures with *P. mirabilis* lipopolysaccharide I as described in Methods was followed. The buffer contained 0.1 M $MgCl_2$ and the concentration of detergent was varied as indicated. $MgCl_2$ alone was not sufficient for vesicle formation with *P. mirabilis* lipopolysaccharide I (Fig. 1A), even in a concentration of 0.1 M. The lipopolysaccharide fraction was in the form of ribbons which tended to aggregate. After addition of increasing amounts of Texapon N-25, the ribbons coalesced (Fig. 1B) and formed round, flat bodies (Fig. 1C). At the optimal detergent concentration of 0.31% (v/v,

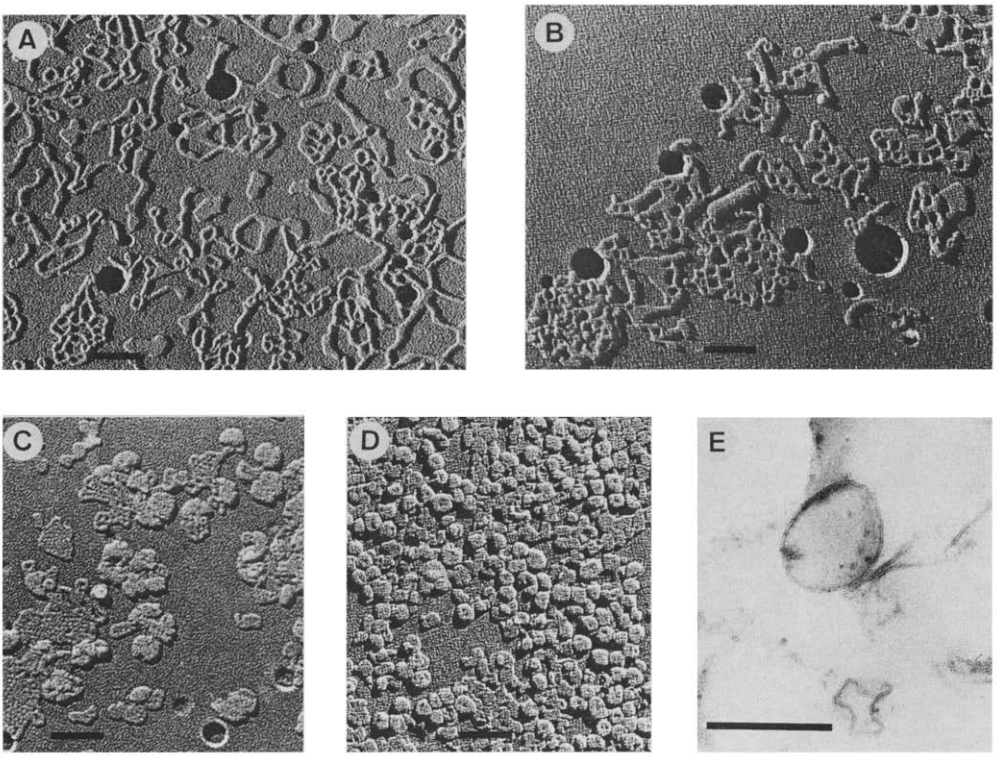

Fig. 1. Effect of detergent on *P. mirabilis* lipopolysaccharide I. Electron micrographs of 1% lipopolysaccharide plus 0.1 M $MgCl_2$ and (A) 0%, (B) 0.10%, (C) 0.21% and (D) and (E) 0.31% Texapon N-25 after filtration on Sepharose 4B. Preparations A—D were made using the agar-filtration technique. Preparation E is a thin section stained with uranyl acetate and lead citrate. Bars represent 200 nm.

Fig. 1D), spherical bodies of fairly uniform size (approx. 80—100 nm in diameter) were produced. Thin sections of these aggregates (Fig. 1E) showed closed structures bounded by a membrane-like bilayer. Greater amounts of detergent added to the system led to a gradual disruption of the aggregates into smaller particles (not shown).

The requirement for $MgCl_2$ could be illustrated in the same manner (not shown). No vesicle formation occurred with lipopolysaccharide I and detergent in the absence of $MgCl_2$. Increasing concentrations of $MgCl_2$ in the presence of 0.31% Texapon N-25 caused lipopolysaccharide I to aggregate into round forms until at the optimal concentration of 0.1 M $MgCl_2$, spherical structures of uniform size were produced.

Other detergents tested could also effectively form spherical aggregates with *P. mirabilis* lipopolysaccharide I. For a 1% (w/v) solution of lipopolysaccharide, 0.75% (w/v) sodium dodecyl sulfate plus 0.1 $MgCl_2$ were required, while 0.075% Triton X-100 (v/v) plus 0.04 M $MgCl_2$ were optimal. In both cases, the structures formed were similar in appearance to those produced with Texapon N-25. In contrast, aggregates produced by 0.1% (w/v) sodium deoxycholate plus 0.05 M $MgCl_2$ were extremely large and varied in size (Fig. 2A). The marked difference in appearance of these aggregates may well be a reflection of

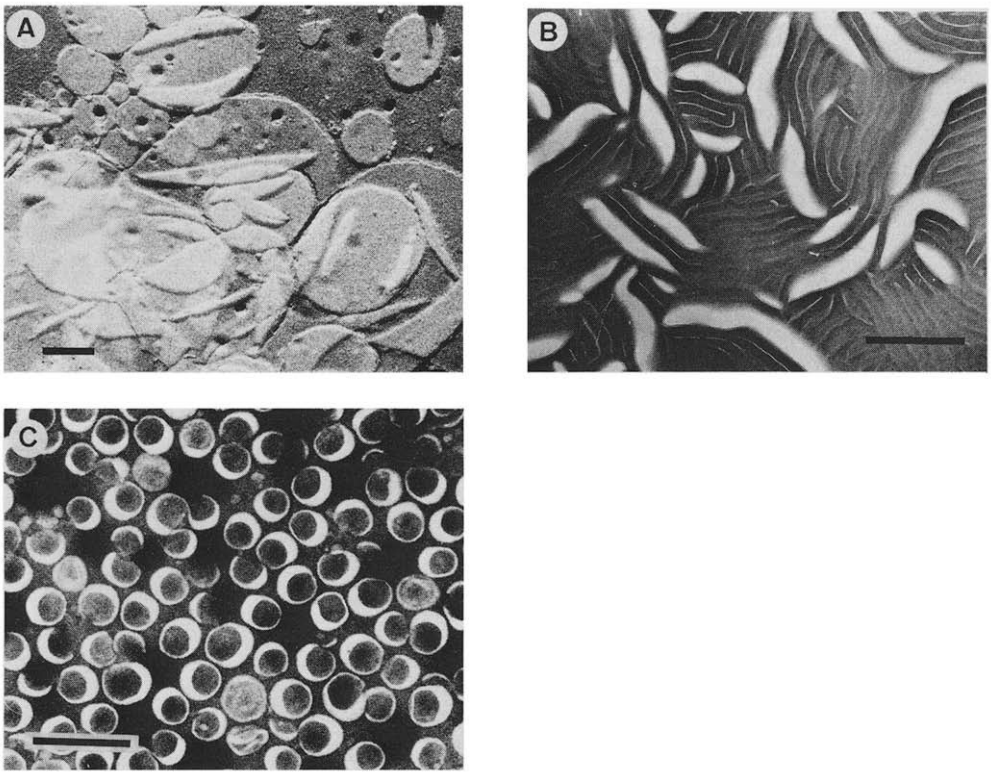

Fig. 2. (A) *P. mirabilis* lipopolysaccharide I after treatment with 0.10% sodium deoxycholate plus 0.05 M $MgCl_2$. Preparation was made using the agar-filtration technique. (B) *P. mirabilis* lipopolysaccharide II and (C) *P. mirabilis* lipopolysaccharide I after treatment with 0.31% Texapon N-25 plus 0.1 M $MgCl_2$. Preparations were negatively stained with potassium phosphotungstate. Bars represent 200 nm.

the molecular structure of the bile salt which is quite different from the structures of the other detergents used. Because of the known curvature-enhancing capacity of the latter group of detergents such as Triton X-100 and sodium dodecyl sulfate [20], they might facilitate the formation of uniformly curved structures from lipopolysaccharide.

The amount of detergent actually bound to the lipopolysaccharide aggregate fraction was estimated using sodium dodecyl [^{35}S]sulfate and sodium deoxy-[*carboxy*-^{14}C]cholate. After Sepharose 4B filtration of the mixture, approx. 0.23 μmol sodium dodecyl sulfate/μmol 2-keto-3-deoxyoctonate and 0.09 μmol sodium deoxycholate/μmol 2-keto-3-deoxyoctonate were found associated with the lipopolysaccharide fraction.

A second type of lipopolysaccharide, termed lipopolysaccharide II, can be isolated from *P. mirabilis*; it is highly water soluble and contains longer polysaccharide chains than the lipopolysaccharide I described above [6]. In the electron microscope, preparations of this lipopolysaccharide show lamellar or myelin-like structures with regular spacings of about 20 nm (Fig. 2B). This behaviour of lipopolysaccharide II points up the typical ampholytic character of the lipopolysaccharide as well as the greater length of the hydrophilic O-polysaccharide chains. Only a minute fraction of this material formed spherical

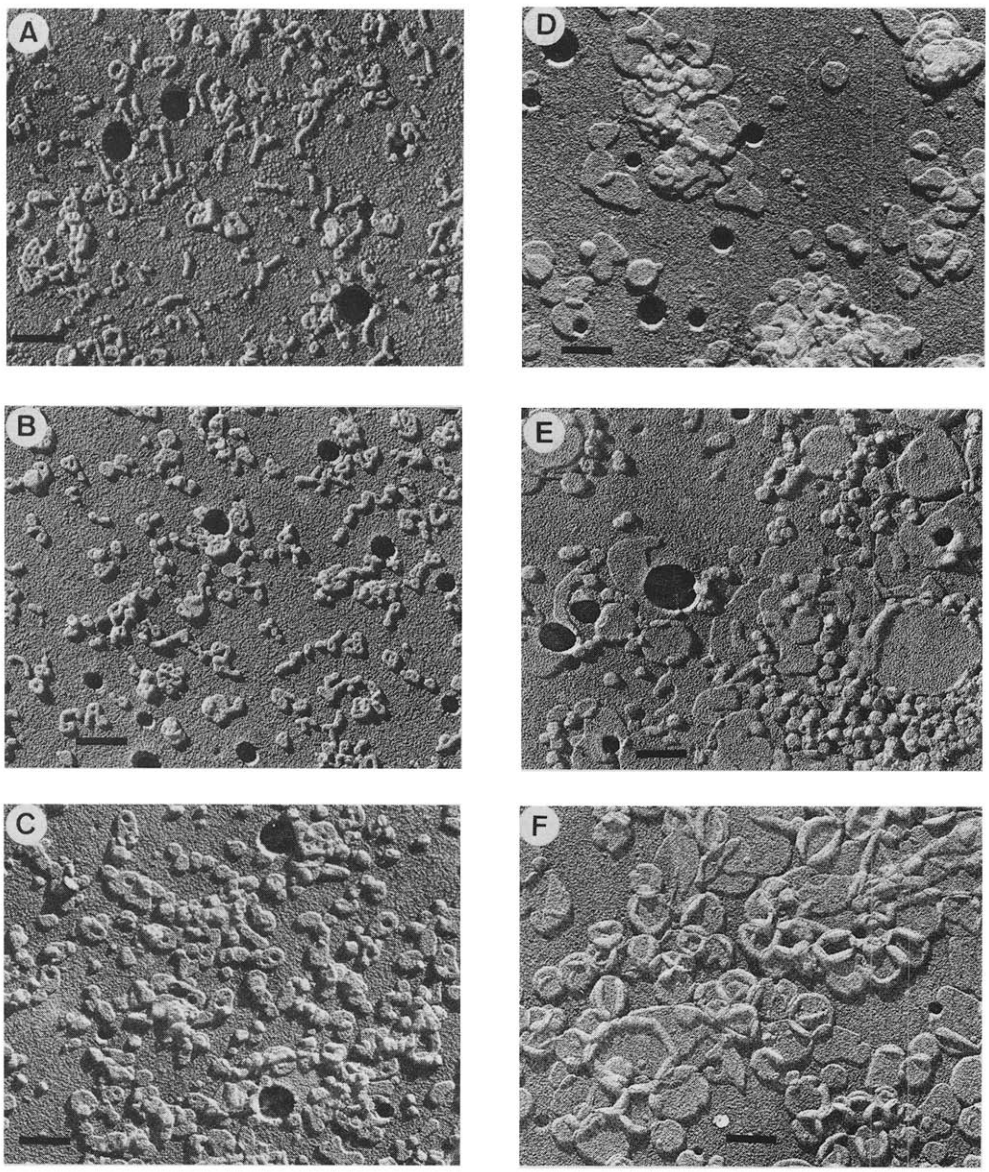

Fig. 3. Effect of the length of R core polysaccharide and $MgCl_2$ on vesicle formation with *S. minnesota* R mutant lipopolysaccharides. Lipopolysaccharide from Ra mutant (complete core) without $MgCl_2$ (A) and with 0.01 M $MgCl_2$ (B); from Rc mutant (2-keto-3-deoxyoctonate (KDO), heptose, glucose) without $MgCl_2$ (C) and with 0.01 M $MgCl_2$ (D); from Re mutant (KDO) without $MgCl_2$ (E) and with 0.01 M $MgCl_2$ (F). Preparations were made using the agar-filtration technique. Bar represents 200 nm.

bodies after addition of as much as 0.62% (v/v) Texapon N-25, which actually disaggregated most of the lipopolysaccharide II. As a comparison, a negatively-stained preparation of lipopolysaccharide I after treatment with Texapon N-25 and $MgCl_2$ is presented (Fig. 2C).

Production of vesicles from S. minnesota R lipopolysaccharides

Results of experiments with lipopolysaccharides I and II from *P. mirabilis*

suggested that good 'vesicle' formation was dependent upon the length of the O-polysaccharide chain. We therefore initiated studies with lipopolysaccharides from mutants displaying increasing deficiencies in the R core portion of the lipopolysaccharide molecule. We chose *S. minnesota* R mutants of chemotypes Ra (containing the complete core), Re (containing 2-keto-3-deoxyoctonate, heptose and glucose) and Re (containing only 2-keto-3-deoxyoctonate) for this work because these lipopolysaccharides have been well characterized [3].

Lipopolysaccharides from the mutants showed a growing tendency for vesicle formation with increasing deficiency of the R core polysaccharide even in the absence of $MgCl_2$ and detergent (Figs. 3A, 3C and 3E). $MgCl_2$ enhanced the vesicle-forming capacity of lipopolysaccharides from the Rc and the Re mutants (Figs. 3D and 3F), but had no apparent effect on the Ra lipopolysaccharide (Fig. 3B).

Permeability properties of vesicle-like structures of lipopolysaccharide

The ability of lipopolysaccharide aggregates to entrap and retain [^{14}C]-sucrose, [^{3}H]inulin or [^{3}H]dextran was determined in order to establish some measure of the quality of membrane vesicle formation. *P. mirabilis* lipopolysaccharide I spherical aggregates containing detergent and $MgCl_2$ were unable to either entrap or retain any of the saccharides tested. Therefore, these lipopolysaccharide aggregates were not closed vesicular structures as they appeared in thin sections in the electron microscope, or they were highly permeable even to large molecular weight hydrophilic molecules.

In contrast, entrapment of all three saccharides by *S. minnesota* Rc or Re lipopolysaccharide aggregates and retention of these molecules for at least short periods of time indicated that these structures were indeed closed vesicles.

Both vesicle systems of mutant lipopolysaccharides entrapped comparable amounts of [^{14}C]sucrose and [^{3}H]inulin in the absence of $MgCl_2$ (Table I). Addition of 0.01 M $MgCl_2$ to the vesicle buffer enhanced the amount of [^{3}H]-inulin entrapped in Re-lipopolysaccharide vesicles approx. 4-fold (from 40 to 167 μl/mmol 2-keto-3-deoxyoctonate). Amounts of $MgCl_2$ greater than 0.01 M caused the mutant lipopolysaccharides to precipitate so heavily that permeability measurements could not be made. In general, the results indicate that both vesicle systems in the absence of $MgCl_2$, as well as the Rc-lipopolysaccharide vesicle system in the presence of $MgCl_2$ were leaky for sucrose and inulin.

Studies measuring the rate of release of saccharides from Rc- and Re-lipopolysaccharide vesicles confirm this result (Table II). Even in the presence of 0.01 M $MgCl_2$, most of the [^{14}C]sucrose and [^{3}H]inulin was released from Rc-lipopolysaccharide vesicles after the first 2 h incubation period. A residual amount of radioactivity remained associated with the vesicle fraction thereafter. In contrast, the release of [^{3}H]inulin from Re-lipopolysaccharide vesicles was greatly retarded when compared to the diffusion of the smaller [^{14}C]-sucrose molecule out of the same vesicles.

It should be stressed that the designation of these measurements as 'rate' of release of saccharides is only relative. We are not just measuring the passive diffusion of saccharides out of the vesicles with time, but because of the

TABLE I

EFFECT OF $MgCl_2$ ON THE AMOUNT OF RADIOACTIVE SACCHARIDES ENTRAPPED IN *SALMONELLA MINNESOTA* Rc- AND Re-LIPOPOLYSACCHARIDE VESICLES

Values listed in the table represent the mean of 2—4 determinations.

	μl saccharide per mmol 2-keto-3-deoxyoctonate			
	Rc-Lipopolysaccharide vesicles		Re-Lipopolysaccharide vesicles	
	No $MgCl_2$	0.01 M $MgCl_2$	No $MgCl_2$	0.01 M $MgCl_2$
[^{14}C]Sucrose	48	56	63	57
[^{3}H]Inulin	40	38	40	167

mechanics of the gel filtration step to remove non-entrapped radioactive material from the vesicle fraction, we are probably forcing some diffusion of saccharides during this step. However, on a relative basis, our method demonstrated that [^{3}H]inulin diffused more slowly out of Re-lipopolysaccharide vesicles than out of Rc-lipopolysaccharide vesicles when $MgCl_2$ was present, and that inulin diffused more slowly out of these Re-lipopolysaccharide vesicles than did the smaller [^{14}C]sucrose molecule. The total time required for the gel filtration step was approx. 15 min.

The stabilizing effect of $MgCl_2$ on membranes is well documented and optimal concentrations of divalent cations can evidently promote stronger association of lipopolysaccharide molecules in the outer membrane of Gram-negative bacteria [21]. In this regard, the apparent stabilizing effect of $MgCl_2$ on Re-lipopolysaccharide vesicles can be understood. Since $MgCl_2$ did not enhance vesicle formation with the Ra mutant lipopolysaccharide (Figs. 3A and 3B) and was ineffective in retarding the release of inulin from Rc-lipopolysaccharide vesicles (Table I), the length of the R core polysaccharide is apparently critical for the production of truly stable vesicles with lipopolysaccharide and divalent cations.

Evidence that the saccharides were actually entrapped in the vesicles and not just adsorbed to lipopolysaccharide is provided by the fact that the saccharides

TABLE II

RATE OF RELEASE OF RADIOACTIVE SACCHARIDES FROM *SALMONELLA MINNESOTA* Rc- AND Re-LIPOPOLYSACCHARIDE VESICLES

Vesicles were prepared with buffer containing 0.01 M $MgCl_2$. Percentages given in the table represent mean values of 2—3 determinations.

	Percentage radioactivity remaining in vesicle fraction after various periods of incubation at room temperature					
	Rc-Lipopolysaccharide vesicles			Re-Lipopolysaccharide vesicles		
	2 h	6 h	24 h	2 h	6 h	24 h
[^{14}C]Sucrose	31	22	n.d.	27	21	16
[^{3}H]Inulin	18	20	17	87	63	15

n.d., not determined.

were extensively absorbed with lipopolysaccharide to remove material binding to ampholytes before use (see Methods). Also, detergent added in high enough concentrations to disrupt lipopolysaccharide aggregates (for example, 0.6% sodium deoxycholate) caused the complete release of entrapped radioactive saccharides from the vesicles.

Protection of phospholipid vesicles from dissociation by detergent

We showed previously that two outer membrane proteins of *P. mirabilis* can protect phospholipid vesicles from dissociation by detergent when incorporated into the model system [1].

In view of the formation of highly ordered aggregates of S-type lipopolysaccharide with detergents as demonstrated in the foregoing sections, we also tested the ability of lipopolysaccharide incorporated into vesicular phospholipid membranes to protect such mixed membranes from disaggregation by detergent action.

Incorporation of either *P. mirabilis* S-form lipopolysaccharide I or *S. minnesota* Rc mutant lipopolysaccharide into phospholipid vesicles as described in Methods gave good protection against dissociation by 0.2% (w/v) sodium deoxycholate, an amount which caused the complete release of [^{3}H]-dextran from phospholipid vesicles. Evidence for the association of lipopolysaccharide with phospholipid in the vesicle system was provided by buoyant density analysis of the mixture using sucrose gradient ultracentrifugation [1]. The vesicle mixtures employed above did not contain free lipopolysaccharide.

On a molar basis, the *P. mirabilis* S-type lipopolysaccharide was more effective than the much smaller *S. minnesota* Rc mutant molecule. Specifically, 10 mg of the S-form lipopolysaccharide added to 12.5 μmol phospholipid gave 100% protection, while 14 mg of the R mutant lipopolysaccharide was required for the full effect. Thereafter, the degree of protection diminished with increasing amounts of lipopolysaccharide added in both cases. For example, 12.0 mg of *P. mirabilis* lipopolysaccharide provided only 80% protection and 16.0 mg of *S. minnesota* Rc-lipopolysaccharide gave only 55% protection. Evidently, at higher ratios of lipopolysaccharide to phospholipid, association of the two molecules is no longer constructive and protection of the phospholipid bilayer by lipopolysaccharide becomes less effective.

Divalent cations are not necessary for phospholipid vesicle formation, but were apparently essential for full protection of model membranes from detergent action. Omission of $MgCl_2$ altogether, or even a reduction in concentration below 2.5 mM caused a decrease in the protective effect of lipopolysaccharide (data not shown).

Discussion

Although the relative resistance of the cell wall of Gram-negative bacteria to dissociation by detergents is well documented, the actual molecular basis of this resistance has never been clearly defined.

Our studies indicate that treatment of isolated lipopolysaccharide with detergent in concentrations that normally disrupt plasma membranes [22] actually led to aggregation of lipopolysaccharide into membrane-like vesicles

when $MgCl_2$ was present. Thus, in this system membrane structure was augmented rather than destroyed by detergents as in the case of membranes based on a phospholipid bilayer.

It is attractive to speculate that the detergent molecules in ordered structure at or above critical micell concentrations, interact through their hydrophobic moieties with the lipid A portion of lipopolysaccharide, thereby substituting for the phospholipid molecules which are the normal partners of lipopolysaccharide, divalent cations and proteins in the construction of the native enterobacterial outer membrane.

In the lipopolysaccharide-detergent system, Mg^{2+} at optimal concentration was most probably required for the neutralization of the numerous negative charges and proper spacing of the lipopolysaccharide molecules before hydrophobic interaction could occur.

The balance of hydrophilic and hydrophobic properties of the lipopolysaccharide molecule appears to be the decisive factor in the formation of membrane-like vesicles. The complete, amphipathic lipopolysaccharide of *P. mirabilis* formed vesicular structures only in combination with Mg^{2+} and detergent, which presumably substitutes for native polar membrane lipid. On the other hand, the lipopolysaccharides of the *S. minnesota* R mutants, which lack the O-polysaccharide chain, displayed a growing tendency for independent vesicle formation with increasing deficiency of the R core polysaccharide. Indeed, the most R core-deficient lipopolysaccharide of *S. minnesota* R595 (chemotype Re) is extremely hydrophobic and forms vesicles spontaneously, even in the absence of detergent and $MgCl_2$.

We further demonstrated that lipopolysaccharide incorporated into phospholipid bilayers was able to protect these model membranes from disruption by detergent.

In the native outer membrane of Gram-negative bacteria, the stability to the disaggregating effect of detergents evidently depends upon the interplay of the complete system of components in their normal spatial arrangement. Isolated, ion-free lipopolysaccharide [17,23,24] or lipopolysaccharide plus phospholipid and protein in extensively washed, ion-depeleted, isolated outer membranes [25] are solubilized even by low concentrations of anionic detergents. In the case of isolated outer membranes [25], the solubility of components may also be due to the greater accessibility of detergent to the inside of membranes, whereas in the living cell, the detergent may not readily penetrate from the outside. An asymmetric distribution of constituents in the outer membrane has been proposed where phospholipid occupies essentially the inside of a lipid bilayer structure and lipopolysaccharide the outside, with the O-polysaccharide chains extending to the cell surface [26,27]. In combination with our data, this model suggests that in vivo, the ability of the outer membrane to withstand detergent action depends to a large degree on the property of its ordered structure with lipopolysaccharide mainly on the outside and in first contact with detergent. The inside phospholipid leaflet would be the primary target for detergent-induced disaggregation of the outer membrane, if and when the surfactant reaches this area.

The structure of our artificial membrane consisting of random mixtures of phospholipid and lipopolysaccharide cannot be expected to correspond

precisely to that proposed in the model of an asymmetric outer membrane of Gram-negative bacteria. Nevertheless, we observed protection of the phospholipid model membranes from disruption by detergents by incorporated lipopolysaccharide and also by some outer membrane proteins [1]. This lends strong support to the conclusion that the preferential presence of large amounts of lipopolysaccharide and proteins together with very little phospholipid in the outer leaflet of the native outer membrane is the decisive factor in the effective detergent resistance of this membrane.

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