Effect of Variations in Lipopolysaccharide on the Fluidity of the Outer Membrane of Escherichia coli

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The lipid hydrocarbon chains in the outer membrane of gram-negative bacteria appear from previous experiments to be less mobile than in the cytoplasmic membrane. To determine whether lipopolysaccharide, a unique outer membrane component, is a cause of this restricted mobility, outer membranes differing in the amount of lipopolysaccharide, and the length of the polysaccharide side chain, were prepared from Escherichia coli 55. Cytoplasmic membranes were prepared for comparison. The probes, 5- and 12-doxylstearate, were introduced into these membranes, electron spin resonance spectra were analyzed, and the order parameter (S) and empirical motion parameter (τ∞) were calculated. Outer membrane preparations containing long chain lipopolysaccharide were much less fluid by these criteria than were preparations containing short chain lipopolysaccharide. Removing about 40% of the lipopolysaccharide from the former preparations greatly increased their fluidity. The lipid in the cytoplasmic membrane preparations was more fluid than in the outer membrane and cytoplasmic membranes were similar to each other regardless of the composition of the outer membrane. These results indicate that lipopolysaccharide, and especially the polysaccharide portion, directly or indirectly causes the restricted mobility of the lipid hydrocarbon chains observed in the outer membrane.

The outer membrane of gram-negative cells differs in many physiological and biochemical properties from the cytoplasmic membrane. Although their lipid content is similar, their proteins are totally different, and only the outer membrane contains lipopolysaccharide (1, 2). While the cytoplasmic membrane has permeability properties common to many biological membranes, being impermeable to most ionized and organic molecules, the outer membrane is a molecular sieve, rather freely permeable to molecules of less than Mw = 700, although larger ones are excluded (3, 4).

Several recent studies have suggested that some or all of the lipid of the outer membrane is less mobile than is the lipid of the inner membrane. Cheng et al. (5) found, by using fluorescence polarization, that the outer membrane of Escherichia coli showed more microviscosity than the inner membrane.

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Rottem et al. (6), studying Proteus mirabilis, found that nitroxide-labeled fatty acids moved less freely in the outer than in the inner membrane. Finally, in the only reported experiments using membranes of defined lipid composition, Overath et al. (7) showed that inner and outer membranes of E. coli had the same transition temperature as indicated by x-ray and fluorescence measurements. But much less of the lipid of the outer membrane participated in this transition than was the case for lipid of the cytoplasmic membrane.

To determine the reason for the restricted mobility of the outer membrane, we have studied the effect of variations in lipopolysaccharide composition and content on the mobility of spin-labeled fatty acids in the outer membrane. The results indicate that reduction in either the polysaccharide side chain length or reduction in lipopolysaccharide concentration increases the fluidity of the outer membrane.

MATERIALS AND METHODS

Bacterial Strains and Media—A mutant of Escherichia coli 0111:B4, designated J9, that lacks uridine diphosphate galactose-4-epimerase, was used. This mutant uses exogenous galactose solely for the synthesis of lipopolysaccharide. It grows normally without galactose but makes an incomplete lipopolysaccharide lacking most of its carbohydrate (8).

The growth medium was made by mixing equal volumes of proteose peptone-beef extract medium (1) and basal salts medium with the glucose omitted (9). The proteose peptone No. 5 was routinely tested as previously described to insure that it lacked galactose (10).

Growth and Labeling of Bacteria—For cells grown in the absence of galactose, 20 ml of an overnight culture grown in the above medium containing 1 mM glycerol was used to inoculate 1 liter of the same medium containing 1 mM [14C]galactose, 40 μCi/mmole. The cells were grown at 37° with vigorous aeration and harvested at a density of 4 × 10^6/ml.

EDTA Treatment—The cells were sedimented by centrifuging at 12,000 × g for 5 min at 4°. Half of the cells were used for membrane preparation without further manipulation (see below). The other half was resuspended in 50 ml of 0.12 M Tris-HCl, pH 8.0, at room temperature and warmed to 37° with gentle shaking. EDTA (1 mM final concentration) was added and shaking was continued for 2 min. The culture was sedimented at 12,000 × g for 3 min at room temperature. The cell pellet was used for membrane preparation as described below.

Membrane Preparation—All steps were at 0-4° unless otherwise indicated. All solutions were prepared in distilled water that was...
also deionized by passage over Dowex 50 H+ (B). Both the EDTA-treated
and the control cell pellets were resuspended in sucrose-Tris, ex-
posed to lysozyme, and thereafter slowly diluted with EDTA, exactly
as described by Osborn et al. (1). The resultant spheroplasts were
lysed osmotically as described (1) and the membranes were sedi-
mANTED for 1.5 hr at 142.000 × g. The pelleted contained 80 to 95% of the
membranes as measured by content of [3H]glycerol precipitable by
trichloacetic acid. The membranes were resuspended in 5 ml of a
solution containing 5 mM EDTA (25% sucrose for membranes
from cells grown without galactose and 30% sucro.se for membranes
from cells grown with galactose). The suspension was layered on
gradient containing 6 ml each of 30, 35, 40, 45, and 50% sucrose (for
membranes from cells grown without galactose) or 35, 40, 45, and
55% sucrose (for membranes from cells grown with galactose). G.:al
solutions were made in 5 mM EDTA. The gradients were centrifuged
41 to 50 h at 51,000 g. The centrifugation was stopped by placing
1 ml of a 5% w/v sucrose solution containing 0.1 M KCl, pH 7.5, in
each tube. The gradients were centrifuged for an additional 30 min
at 51,000 g. The centrifugation was terminated by diluting the
membranes to 1/5 of their original volume with a fresh solution of
EDTA (12). The relative content of triglyceride in membranes from
cells grown without galactose was measured in the following manner:
Cells, mem-
nembranes, or the material released from EDTA-treated cells, or all
three, were extracted with phenol to isolate the lipopolysaccha-
dride (13), the dialyzed aqueous extract was treated with RNase,
dialyzed, and concentrated (14). The content of 2-keto-3-deoxyoctulonate,
a component present only in lipopolysaccharide, was estimated by
the trioxobismuthin acid assay (35). A typical preparation yielded the
following results: lipopolysaccharide from 500 ml of control cells, 23.0
μg of 2-keto-3-deoxyoctulonate; lipopolysaccharide from 500 ml of
EDTA-treated cells, 12.6 μg; lipopolysaccharide extracted from ma-
terial released by EDTA treatment, 11.0 μg. Since the stoichiometry of
the trioxobismuthin acid precipitable to lipopolysaccharide, or all
remaining in the cells, in many experiments the per cent loss of
lipopolysaccharide due to EDTA treatment was estimated by meas-
uring only the lipopolysaccharide recovered from the supernatant
rather than assaying the lipopolysaccharide that remained in the
cells or membranes.

The relative content of lipid in membranes was assayed by meas-
uring the trioxobismuthin acid precipitable [3H]glycerol. This com-
ound was proven to be only in lipid by the following criteria. (a) 85
to 99% of the [3H]glycerol co-purified with the membrane fraction;
(b) 86 to 100% of the [3H]glycerol was extractable in the lipid frac-
tion by the method of Bligh and Dyer (16).

Protein was assayed by the method of Lowry et al. (17). Sodium
dodecyl sulfate gel chromatography was performed as described by
Aimes (5).

RESULTS
Preparation and Composition of Membranes—Cells were
injected in the presence and absence of galactose as described
under "Materials and Methods." Half of each culture was then
exposed to EDTA as described and membranes were prepared
from all four samples. These membranes were centrifuged to
equilibrium and membrane fractions isolated as previously
reported (1, 10).

The properties of the membranes thus isolated are shown in
Table I. The identification of the peaks was based on their
content of lipopolysaccharide, an outer membrane constituent,
and their content of succinic and lactic dehydrogenases, which
are found in the inner membrane. Since cells treated with
EDTA yielded more unseparated membrane material than
control cells, in some experiments the outer membrane frac-
tions were pooled, diluted to reduce the concentration of su-
crose, and recentrifuged to equilibrium to purify them further.
The contamination of outer membranes by inner membranes,
when judged by the specific activity of lactic and succinic
dehydrogenases, was the same whether or not the cells had been
treated with EDTA.

The density of the outer membrane from cells grown with
galactose was shown previously to be higher than the density of
membranes grown without galactose, presumably because of the
higher polysaccharide content of the former (10). In accord with those results, loss of lipopolysaccharide by EDTA
treatment lowers the density of the outer membrane slightly,
without affecting the density of the inner membrane (Table I).
Outer membranes from EDTA-treated cells contained 50 to
70% as much lipopolysaccharide, and 75 to 100% as much lipid
relative to protein as did control outer membranes consistent
with previous results on the effect of EDTA treatment on
whole cells (14). It was of interest that, while lipopolysaccha-
rde was always released by EDTA, in some experiments lipid
was released as well, while in some it was not (compare Table

T', r = 2.74 + 1.96 x 1.723

where T', r = 2.74 + 1.96 x 1.723

0.053

0.091

The motion parameter r(r) was calculated according to Henry and
Keath (20) from the expression:

r = 6.5 x 10^15 W_s \left( \frac{h_1}{h_2} \right)^{1/2} - 1 S

where W_s is the line width of the midline and h_1 and h_2 are the
heights of the mid- and high field lines on a first derivative absorp-
tion spectrum. Since the departure from isotropic tumbling is related
to the proximity of the nitrogen atom to the carbonyl group of
the fatty acid, it was shown that 12-doxylstearate, though far from
being spherical, moves in a nearly isotropic fashion, warranting the use
of Kivelson's formula (21) for determining rotational correlation time
(r(r)). Nevertheless, the spectra reported here are too slow for the
one-line shape theory to apply (r(s) > 10^9 s); thus, for comparative
purposes, the empirical parameter r_0 is used (22). Only r_0 values
faster than 10^9 s are presented, as the high field lines at values
closer than 10^9 s showed nonexistent variability.

ESR Methods—Outer and cytoplasmic membrane preparations
were labeled with N-O-Acetyl-4',4'-dimethyloxazolidine derivatives of 5-
ketocaric acid and 12-ketoerolic acid (hereinafter called 5-dox-
ylsteraric acid and 12-doxylsteraric acid) (Syva, Palo Alto, Calif.) by ex-
clution as follows. Membranes suspended in 0.12 M Tris-HCl, pH 7.5 (0.35 ml), were incubated for 30
min at 4 °C with 1 ml of 2.5 mM of the above probes dissolved in 5% aqueous solution bovine serum albumin (F. V. M. W.).
Four milliliters of 0.25 mM Tris-HCl, pH 7.5, solution was then added, and the mem-
branes were sedimented by centrifugation at 140,000 × g for 5 h. The pellet was washed once and resuspended in 0.05 to 0.12 ml of 0.123
M Tris-HCl, pH 7.5, solution. The samples were then transferred to a
disposable pipette, sealed at one end, and electron paramagnetic resonance spectra were obtained using a Varian E-5 spectrometer
equipped with a temperature control accessory (Varian Associates, Palo Alto, Calif.).

The freedom of motion of the spin-labeled fatty acids in the mem-
brane preparation was assessed from the order parameter (19) and from T_1, an empirical motion parameter (20). The order parameters
(S) are related to the mean angular deviation of the labeled fatty
acid chain from (1) an average orientation in the membrane. Low values of order parameter are associated with higher freedom of motion
of membrane lipids. Since the observed values of T_1 differ from the
real value by a factor related to (T_1 - T_1^0), order parameters were
calculated according to the following equation (19):

S = T_1 - T_1^0 - C / T_1 + 27^0 K + 2C

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Fluidity Changes in Outer Membrane of E. coli

I. Experiments 2 versus 3; however, the ESR results obtained, and described below, were the same for both sorts of experiments.

The protein composition of the outer membrane of Escherichia coli J5 has been reported not to vary when the cells are grown with or without galactose (23). In these experiments, the composition of outer membranes from cells grown with and without galactose, with or without EDTA treatment, was the same as indicated by identical protein bands after sodium dodecyl sulfate gel electrophoresis.

The gels provided another important control: since proteins affect the fluidity of phospholipid membranes (24), it was important to determine whether there might be sufficient differential binding of lysozyme by EDTA-treated versus control cells to affect the results. Examination of the gels revealed that little or none of the protein (less than 5%) in any of the outer membrane preparations banded in the region of lysozyme.

EDTA Used during Preparation of Membranes Does Not Release Lipopolysaccharide—EDTA is present during the membrane preparation used on both EDTA-treated and control cells. Osborn et al. (1) have commented that no lipopolysaccharide loss occurs during such membrane preparation; however, due to the importance of this point for the current investigation, it was necessary to determine whether lipopolysaccharide loss occurs during manipulation of these particular cells grown under these conditions. Cells were grown in the presence of galactose and the culture divided. Spheroplasts were prepared from one portion of the culture exactly as described under "Materials and Methods," except that the spheroplasts were not lysed but sedimented by centrifugation for 10 min at 13,000 x g. Less than 10% of the lipopolysaccharide, as measured by trichloroacetic acid-precipitable [14C]galactose, was in the supernatant after such a procedure. The remaining cells, treated with EDTA as described under "Materials and Methods," released 50% of their lipopolysaccharide to the supernatant.

ESR Results—Table II shows that at 37° the order parameter of 5-doxylstearate and 12-doxylstearate in outer and cytoplasmic membranes of Escherichia coli grown with or without galactose differed.

| Lipid | Lipopolysaccharide | Density | Sucinic dehydrogenase | Lactic dehydrogenase |
|-------|--------------------|---------|-----------------------|----------------------|
| cpm [14C]glycerol/mg protein | g/ml | units/mg | units/mg |
| 1, grown without galactose | Outer membrane, no EDTA | 46,600 | 23 | 1.21 | 0.025 |
| Outer membrane, EDTA-treated | 45,500 | 14 | 1.19 | 0.024 |
| Inner membrane, no EDTA | 33,000 | 1 | 1.16 | 0.14 |
| 2, grown with galactose | Outer membrane, no EDTA | 96,600 | 10,600 | 1.24 | 0.0087 |
| Outer membrane, EDTA-treated | 100,300 | 9,700 | 1.22 | 0.011 |
| Inner membrane, no EDTA | 103,300 | 1,200 | 1.16 | 0.093 |
| 3, grown with galactose | Outer membrane, no EDTA | 130,000 | 9,100 | 1.24 | 0.083 |
| Outer membrane, EDTA-treated | 101,000 | 8,700 | 1.19 | 0.080 |
| Inner membrane, no EDTA | 112,000 | 1,600 | 1.16 | 0.080 |
| Inner membrane, EDTA-treated | 111,000 | 860 | 1.16 | 0.080 |

a The specific activity of the added glycerol was 0.05 mCi/mmol for Experiment 1 and 0.1 mCi/mmol for Experiments 2 and 3. Comparable amounts of protein (±15%) were isolated in outer membrane preparations under all conditions.

b The units are: Experiment 1, 2-keto-3-deoxyoctulonate, μg/total preparation; Experiments 2 and 3, [14C]galactose, cpm/mg of protein.

c Differences in the third place are highly significant and reproducible.

d Not determined.

Table II

Order parameters for 5-doxylstearate and 12-doxylstearate in outer and cytoplasmic membranes of Escherichia coli grown with or without galactose

Spectra were recorded at 35-37°. The calculation of order parameters is described under "Materials and Methods."

| 5-Doxylstearate | 12-Doxylstearate |
|-----------------|-----------------|
| Outer mem- | Cytoplasmic mem- | Outer mem- | Cytoplasmic mem- |
|brane |brane |brane |brane |
| Cells grown with galactose (long polysaccharide chain) | 0.75 | 0.48 | 0.69 | 0.32 |
| Cells grown without galactose (short polysaccharide chain) | 0.57 | 0.47 | 0.52 | 0.32 |
The results indicate that outer membranes containing long chain polysaccharide restrict the motion of the probe more than membranes containing short chain polysaccharide.

The restricted molecular motion of the spin-labeled fatty acid in the outer membrane of glucose-grown cells was further demonstrated in an Arrhenius plot of the motion parameter ($r_a$) versus $\theta$ (Table II). At each temperature tested, the motion parameter was higher for outer membranes containing long chain lipopolysaccharide than for those containing short chain lipopolysaccharide. The Arrhenius plot for both membrane preparations revealed straight lines with activation energies of 15 and 6 kcal/mol for outer membranes with long and short chain lipopolysaccharide, respectively (Fig. 2). These results substantiate and expand those reported above, indicating that the fluidity of the outer membrane is reduced when the carbohydrate moiety of the lipopolysaccharide is long.

Outer membranes from galactose-grown cells that had been treated with EDTA and thus had lost 40% of their lipopolysaccharide, showed a marked increase in the freedom of motion of the spin label relative to untreated cells. This increase was manifested as a decrease in order parameter determined at 37°C (Table III) and also as a decrease in the empirical motion parameter at various temperatures (Fig. 2). In contrast, the effect of EDTA treatment was negligible for outer membrane preparations from cells grown without galactose. These results will be discussed below.

**DISCUSSION**

The addition of galactose to a culture of a mutant lacking galactose epimerase, such as *E. coli* J5, permits the synthesis of longer polysaccharide side chains on the lipopolysaccharide (8). The outer membranes contain the same quantity and types of phosphotidyl, the same quantity and types of proteins (22), and the same number of lipopolysaccharide monomer units (8), regardless of polysaccharide content. Thus, the only variable introduced is the length of the polysaccharide side chain. Exposure of the cell to EDTA removes 35 to 50% of the lipopolysaccharide, with a small amount of outer membrane protein and variable amounts (0 to 30%) of the phospholipid (14). Thus, the effect is primarily on the lipopolysaccharide content.

The current results show that varying only the length of the carbohydrate chain of lipopolysaccharide greatly affects the mobility of a lipid probe added to outer membrane preparations. Reducing the lipopolysaccharide content by EDTA treatment increases the mobility of the probes in a membrane containing a long chain lipopolysaccharide, and the results were independent of whether or not phospholipid was also lost. The important moiety of lipopolysaccharide is not the lipid A, but the polysaccharide, since outer membranes containing the same amount of lipid A but less polysaccharide (strain J5 grown without galactose) show mobility more similar to cytoplasmic membranes; furthermore, reducing the lipopolysaccharide content of these membranes has a negligible effect. Presumably, differences still observed between outer membranes containing short polysaccharides and the cytoplasmic membrane may be due to factors other than the lipopolysaccharide moiety.

An intrinsic limitation of electron spin resonance measurements is the ability of the probes to sample conditions only in the portion of the membrane in which they are contained. If the probes were located in vastly different domains in each experimental situation, interpretation would be difficult. Such a possibility appears unlikely in these experiments because approximately equal amounts of probe were absorbed by outer membranes regardless of their lipopolysaccharide chain length, or whether or not they had been exposed to EDTA. Furthermore, both removal of long chain lipopolysaccharide by EDTA and abbreviation of chain length, which should perturb the membrane in different ways, resulted in internally consistent results.

Parenthetically, in the current experiments, no melting point of lipids (or break in the Arrhenius curve, Fig. 2) can be obtained since the membranes were not derived from a fatty acid auxotroph grown on a specific unsaturated fatty acid, and
lipids of wild type E. coli exhibit only a very gradual melting curve over the temperatures assayed (25).

Although at first glance it may appear strange that the presence of polysaccharide side chains should affect the mobility of a probe in the lipid bilayer, there is at least one possible interpretation. It is known that polysaccharide chains can interact with each other, as measured by x-ray analyses (26). If lipopolysaccharide molecules interact with a certain percentage of the phospholipid of the outer membrane, then interaction of polysaccharide side chains might restrict mobility both of lipopolysaccharide and of the associated lipid.

This hypothesis is consistent with data from two sources. First, as mentioned above, x-ray analysis indicates that polysaccharides can interact with each other to form 2-, 3-, and 4-membranes of other cells. They suggested that the remainder of the outer membrane lipids was immobilized by interaction with protein. Both their data and the data of the current paper can interact with each other, as measured by x-ray analyses (26). If lipopolysaccharide molecules interact with a certain percentage of the phospholipid of the outer membrane, then interaction of polysaccharide side chains might restrict mobility both of lipopolysaccharide and of the associated lipid.

This possibility that polysaccharides can modulate properties within the membrane bilayer offers an attractive additional role for the varied glycoproteins and glycolipids found in the membranes of eukaryotic and prokaryotic cells. Perhaps interaction among such polysaccharides can help localize the molecules on which they occur into domains of functional importance to the cell.

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Table III

| Membrane preparation | With galactose (long polysaccharide) | Without galactose (short polysaccharide) |
|----------------------|-------------------------------------|----------------------------------------|
| From control cells   | 0.75                                | 0.57                                   |
| From EDTA-treated cells | 0.67                  | 0.56                                   |

Fig. 2. Arrhenius plots of $\tau_0$ values calculated from ESR spectra of 12-doxylstearate incorporated into Escherichia coli outer membrane preparations from cells grown with or without galactose. The possibility that polysaccharides can modulate properties within the membrane bilayer offers an attractive additional role for the varied glycoproteins and glycolipids found in the membranes of eukaryotic and prokaryotic cells.
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