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Release of Lipopolysaccharide in *Escherichia coli* Resistant to the Permeability Increase Induced by Ethylenediaminetetraacetate

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SUMMARY

Mutants of *Escherichia coli* that fail to show the characteristic increase in permeability induced by brief treatment with ethylenediaminetetraacetate have been isolated. They were examined to see whether they release lipopolysaccharide as expected during such a treatment. Mutants derived from two strains released only 20 to 40% less lipopolysaccharide than their parents, but further analysis of one mutant and its parent showed that this represented reduction of a specific lipopolysaccharide fraction. Both organisms released two lipopolysaccharide components differing in sedimentation rate in sucrose density gradients, but while both released similar amounts of the faster moving component, the mutant released only about one-third as much of the slower moving component. These results suggest that release of the slower component may be required for the permeability increase induced by EDTA.

Brief treatment of *Escherichia coli* with ethylenediaminetetraacetate increases cell permeability and releases part of the lipopolysaccharide of the cell wall into the medium (1, 2). The relationship of these two phenomena has not been defined. If they are related, then mutants selected for absence of the permeability effect might not be expected to release LPS.[1] This paper describes the isolation and characterization of two mutants resistant to the permeability-promoting effect of EDTA. These mutants release LPS, but in reduced amounts. Further study of one mutant revealed that its LPS, like that of its parent, yielded two fractions on sucrose gradient ultracentrifugation, and that one of these was released in normal, and the other in reduced amounts.

EXPERIMENTAL PROCEDURE

Materials and Methods—Actinomycin D was the gift of Dr. A. Patchett of Merck Sharp and Dohme, and colitose and 3-deoxy-octulosonate were gifts from Dr. E. Heath. Actinomycin D-methyl-^{14}C (9.09 μCi per mg) and D-galactose-1-^{14}C (48.1 mCi per mmole) were obtained from New England Nuclear. Yeast transfer ribonucleic acid was obtained from Miles Laboratory.

Additional colitose was prepared from LPS released by EDTA treatment of *E. coli* 0111:B4 (3). LPS was hydrolyzed in 0.2 N H_2SO_4 at 95° for 15 min and neutralized with saturated $Ba(OH)_2$. The sugars thus released were subjected to descending chromatography with the solvent of Colombo *et al.* (4) and detected with alkaline $AgNO_3$. The area corresponding to authentic colitose was eluted. After periodate oxidation both at 55 and 23°, the isolated material and authentic colitose gave identical absorption spectra in the thiobarbituric acid assay. The isolated material showed no detectable contamination by other sugars (less than 5%) on rechromatography.

Triton-toluene scintillation fluid (5) was composed of 2 parts toluene containing 0.0225% bis(phenyloxazolyl)benzene and 0.825% diphenyloxazole and of 1 part Triton X-100 (Packard Instrument Company). Assuming 10^9 cells are equivalent to 0.25 mg, dry weight (6), dry weight of cells was calculated from colony counts. Protein was determined by the method of Lowry *et al.* (7).

Strains—The parental strains are: (*a*) *E. coli* AB1105 (*his*, *pro*, *thi*, *lac* Y, *gal* K (8)) obtained from Dr. E. Maxwell; (*b*) *E. coli* J-5, a mutant lacking uridine diphosphate galactose 4-epimerase (9) and derived from *E. coli* 0111:B4. This strain was the gift of Dr. E. Heath. Mutant strains isolated in this study are NL101 (AB1105, *per*-1) and NL102 (J-5, *per*-2).

Growth of Cells—The following growth media were used: (*a*) minimal: a Tris-based minimal medium (2) with 0.5% glucose as carbon source; (*b*) enriched minimal medium: minimal medium supplemented with 0.2% yeast extract (Difco) and 0.2% casein hydrolysate (N-Z-Case, Sheffield); (*c*) minimal galactose medium: minimal medium containing 0.25% glucose and 0.25% galactose; (*d*) Trypticase soy minimal galactose medium: minimal galactose medium containing 10% by volume of Trypticase soy broth (Baltimore Biological Laboratories); and (*e*) Trypticase soy galactose medium: Trypticase soy broth containing 0.25% galactose. Minimal medium was supplemented with histidine, proline, and thiamine for growth of AB1105 and NL101. Cultures were grown at 37° with shaking to provide aeration. Turbidity was measured at 530 mμ.

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[1] The abbreviation used is: LPS, lipopolysaccharide.

EDTA Treatment—Cells were harvested at room temperature in the exponential phase of growth, washed and resuspended in 0.12 M Tris-HCl, pH 8.0, and treated with EDTA for 2 min as previously described (10). EDTA action was stopped by adding $MgCl_2$ at 2 to 5 times the EDTA concentration or by dilution into growth medium.

Determination of Permeability—The degree of permeability induced by EDTA was generally measured by assaying for uptake of actinomycin. This was done in three ways. (*a*) Cells were measured for loss in viability after exposure to the antibiotic. EDTA-treated cell suspensions were diluted at least 10-fold into growth medium containing 10 μg of actinomycin per ml and incubated in this medium at 37° for half a doubling time. Viable centers were assayed before and after incubation with actinomycin. (*b*) Uptake of actinomycin was assayed directly by exposing cells to ^{14}C-actinomycin in growth medium at 37° for times noted in the text. Uptake was terminated by chilling the cells to 0° (1). The cells were centrifuged at 4°, washed twice with 0.15 M NaCl at 0–4°, suspended in 0.15 M NaCl, and counted. (*c*) Uptake was also assayed by determining the sensitivity of RNA synthesis to the antibiotic. Incorporation of uracil-1-^{14}C into acid-precipitable material in the presence and absence of actinomycin was determined, and actinomycin sensitivity was calculated as previously described (10).

Permeability to *o*-nitrophenylgalactoside was assayed by comparing β-galactosidase activity of intact cells with that of toluenized cells as previously described (10).

Release of LPS—EDTA-treated and control cell suspensions were separated into cell pellet and supernatant fluid by centrifugation. The amount of LPS in each was determined by assaying for 3-deoxyoctulosonate (AB1105 and NL101) or for colitose (J-5 and NL102) after hydrolyzing samples for 15 min at 95°. Colitose was determined by the method of Cynkin and Ashwell (11) directly on resuspended pellets and supernatant fluids as previously described (3). 3-Deoxyoctulosonate was determined by the method of Weissbach and Hurwitz (12) on the supernatant fluid and on LPS extracted from the pellet. These fractions were prepared as follows. The supernatant fluid was dialyzed at 4° against 0.10 M sodium phosphate buffer, pH 7.0 (100 volumes), and then against three portions of distilled water. The pellet (containing cells from 100 ml of culture) was suspended in 1.0 ml of water and extracted twice with hot phenol (13). The combined aqueous phases were dialyzed against distilled water (250 volumes) yielding a preparation containing approximately equal amounts of LPS and RNA (13). When 3-deoxyoctulosonate was added to the crude LPS, and assayed, the values obtained were additive, indicating that the extract did not interfere with the assay.

Sucrose Density Gradient Centrifugation—Cells were grown in medium containing ^{14}C-galactose, treated with EDTA, and centrifuged. The supernatant fluids were dialyzed for 24 hours against 0.05 M sodium phosphate buffer, pH 7.0 (two 250-volume portions). Approximately 0.20 ml was applied to 4.6 ml of a 5 to 20% sucrose gradient in 0.05 M sodium phosphate buffer, pH 7.0. Gradients were centrifuged in the SW39 head of a Beckman model L-2 ultracentrifuge. Fractions of 0.2 ml of volume were collected through the bottom of the centrifuge tubes. Water (1.8 ml) was added to each fraction and 1.0 ml of the diluted fractions was counted. No appreciable quenching by sucrose was observed. An average of 85% of the counts applied to the gradients were recovered.

Lipid Extraction and Analysis—Samples were extracted at room temperature two to three times with an equal volume of 1-butanol. The combined butanol extracts were washed two to three times with water, concentrated by evaporation, applied to Silica Gel G thin layer plates (Mann Research Laboratories), and the chromatographs developed in a chloroform-methanol-water (65:25:4, v/v) solvent as described by Ames (14). After development, the plates were exposed to iodine vapors to stain lipid components, sprayed with ninhydrin to detect amino groups, and sprayed with the reagent of Dittmer and Lester (15) to detect organic phosphate.

RESULTS

Selection of Mutants

Although normal *E. coli* cells are impermeable to actinomycin, EDTA-treated cells admit enough actinomycin to cause a 95% or greater loss in viability. Mutants which do not show such increased permeability (*per* mutants) were selected by their ability to survive exposure to actinomycin after EDTA treatment. Since a small percentage of cells survive but are not mutants (10), the survivors were repeatedly exposed to the selection procedure until mutants became numerous. Two classes of mutants were obtained: permeability mutants of the type being sought, and mutants permeable to actinomycin after EDTA treatment but resistant to its action (actinomycin-resistant mutants). These latter will be described in a separate communication. *Per* mutants were distinguished from resistant mutants by screening for uptake of actinomycin after EDTA treatment.

Mutants of *E. coli* AB1105 were selected by the following procedure. A fully grown culture was treated with nitrous acid by the method of Kaudewitz (16). Approximately 2% of the cells survived. The cells were suspended in enriched minimal medium, to a density of about 3.0×10^8 cells per ml, incubated for 2 hours, and treated with EDTA. The treated cells were diluted with an equal volume of enriched minimal medium containing actinomycin (final concentration, 10 μg per ml), incubated for 45 min, further diluted with 4 volumes of enriched minimal medium and incubated overnight. The selection procedure was repeated four times; the survivors were diluted into enriched minimal medium, grown to the midexponential phase, treated with EDTA, diluted 10-fold into enriched minimal medium, and incubated overnight in the presence of 10 μg of actinomycin per ml. About 3% of the cells survived the first, second, and third treatments. Both the fourth and fifth treatments gave about 40% survival.

Survivors from the final selection were screened for uptake of actinomycin, taking advantage of the observation that actinomycin colors cells that take it up. Cultures grown from single colonies and from the parent strain were treated with EDTA, incubated with actinomycin (5 μg per ml) for 15 to 20 min, and centrifuged. A mutant (NL101), carrying a mutation designated *per*-1 on the basis of data presented below, gave a paler pellet than AB1105 cells. A single colony isolate was used to prepare the stock strain.

Mutants of *E. coli* J-5 were selected as described for AB1105 except cells were not exposed to mutagen prior to the selection procedure, and cells were grown in minimal, as opposed to enriched minimal, medium prior to EDTA treatment (following EDTA treatment, cells were incubated in enriched minimal medium). It was found with J-5 that the number of survivors

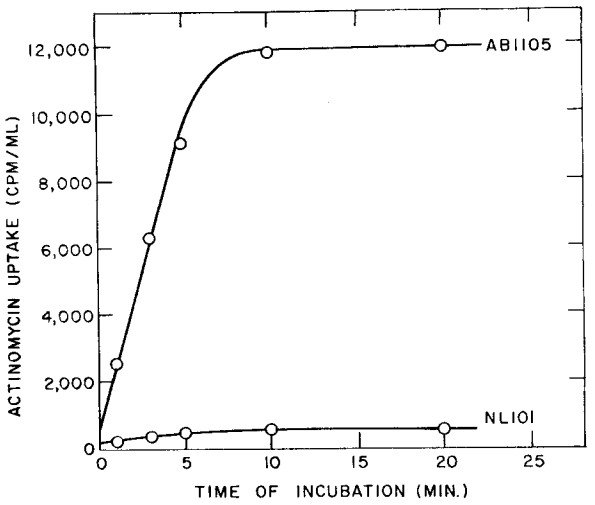

FIG. 1. Uptake of ^{14}C-actinomycin by EDTA-treated cells of NL101 and AB1105. NL101 and AB1105 were grown to approximately equal turbidity in minimal medium and treated with EDTA as described under "Experimental Procedure." Exposure to EDTA was terminated by dilution into 4½ volumes of previously warmed minimal medium. The diluted cells were incubated at 37°, 2 to 3 min later 4 μg of ^{14}C-actinomycin per ml were added (0 time), and samples were taken at various times thereafter.

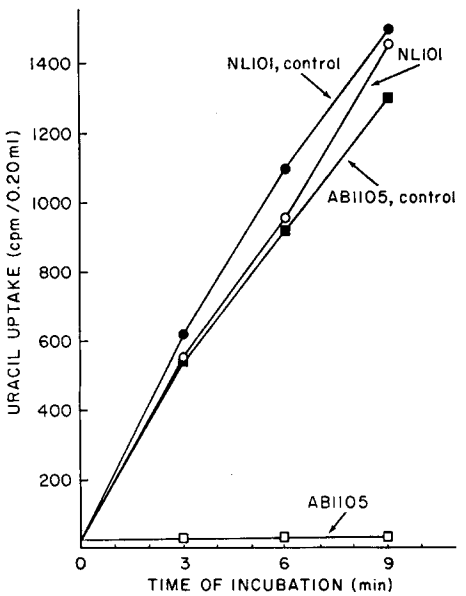

FIG. 2. Inhibition of RNA synthesis by actinomycin in EDTA-treated cells of NL101 and AB1105. Samples of EDTA-treated and control suspensions of NL101 and AB1105 (see legend to Table III) were incubated in minimal medium containing actinomycin. ^{14}C-Uracil was added at zero time, and at various times thereafter, 0.20-ml aliquots were precipitated with 5% trichloracetic acid at 4° and counted.

increased successively with each treatment: 0.0001, 0.0005, 0.75, and 36%. Survivors from the final passage were further screened. In this case, to distinguish *per* mutants from actinomycin-resistant mutants, survivors were treated with EDTA and tested for the absence of a noticeable lag in growth in medium containing actinomycin, since actinomycin-resistant mutants show such a lag.[2] One survivor (NL102) which did not show a lag was designated as carrying a *per*-2 mutation on the basis of data presented below. A single colony isolate was used to prepare the stock strain.

The organisms thus isolated are derivatives of the starting strains, and not contaminants, since NL101 has the same auxotrophic requirements for proline, histidine, and thiamine as AB1105; and NL102, like J-5, contains colitose as a constituent of its LPS.

In the growth media employed in this study, the *per* mutants grow at 37° at virtually the same rate as the parental strains. In minimal medium NL101 and AB1105 have a doubling time of 60 min and, in enriched minimal medium, both have a doubling time of 35 min. In trypticase soy minimal galactose medium, NL102 and J-5 have a doubling time of 53 min. In minimal medium, J-5 has a doubling time of about 75 min and NL102 has a generation time about 10% greater than this.

Response of NL101 to EDTA Treatment

Permeability—In contrast to the parent strain AB1105, NL101 (*per*-1) grown in minimal medium is virtually impermeable to actinomycin after EDTA treatment. Following EDTA treatment, uptake of ^{14}C-actinomycin by NL101 was less than 5% that of the parent strain (Fig. 1). In the parent strain uptake of actinomycin by EDTA-treated cells halts RNA synthesis (1). In contrast, actinomycin does not inhibit RNA synthesis in EDTA treated mutant cells (Fig. 2), a result consistent with little uptake of the antibiotic.

EDTA treatment of *E. coli* increases permeability to a variety of compounds (1). To see if impermeability of NL101 to actinomycin truly reflects resistance of a general permeability barrier to EDTA, entry of another normally excluded compound was studied. Since AB1105 is lactose permease negative, *o*-nitrophenylgalactoside normally cannot be hydrolyzed by internal β-galactosidase, but can be hydrolyzed after the cells are treated with EDTA (1). In contrast, NL101 remained impermeable to this compound after EDTA treatment (Table I).

The response of NL101 to EDTA was influenced by the composition of the growth medium. When grown in minimal medium, EDTA-treated cells of NL101 were practically impermeable to actinomycin (Figs. 1 and 2), but when grown in enriched medium they became partially permeable, incorporating about 30% as much actinomycin as the parent strain (Table II). Under these conditions, the mutant showed 50% survival after exposure to actinomycin; the parent strain, as expected, showed much less (2%) survival (Table II). In subsequent studies of LPS release, the more definitive minimal medium was used.

Release of LPS—NL101 and AB1105 cells were washed and resuspended in Tris, one portion of each treated with EDTA, and the suspensions separated into pellet and supernatant fluid by centrifugation. To determine the release of LPS, the pellets and the supernatant fluids were assayed for 3-deoxyoctulosonate, a compound which has been found only in LPS (17). As measured by percentage of release of 3-deoxyoctulosonate, AB1105 released about 50% of its LPS upon EDTA treatment, while NL101 released about 30% (Table III). The total amount of 3-deoxyoctulosonate in both strains was the same, about 0.0095 μmole per mg of cells, dry weight. Less than 2% of the protein of the

[2] M. J. Voll and L. Leive, manuscript in preparation.

TABLE I

Permeability of NL101 and AB1105 to o-nitrophenylgalactoside

Cells were grown in minimal medium and isopropylthiogalactoside (1.0 mM) was added to the cultures 30 min before harvesting and washing. Aliquots containing 3.4×10^7 NL101 cells or 2.9×10^7 AB1105 cells were assayed for β-galactosidase activity both before and after treatment with EDTA. To determine the total amount of β-galactosidase activity in the cells, samples were treated with toluene and then assayed (last column).

Strain and time of measurement	β-Galactosidase activity	
	Units measured	Total units assayable after toluene
NL101		
Before EDTA	0.04	3.3
After EDTA	0.18	4.2
AB1105		
Before EDTA	0.08	4.6
After EDTA	4.5	4.4

TABLE II

Uptake of actinomycin by NL101 and AB1105 grown in enriched medium

NL101 and AB1105 were grown in enriched minimal medium. The cells were washed, resuspended in Tris buffer, and divided into equal samples. One sample was incubated with EDTA and the other sample without. Two minutes after the addition of EDTA both samples were diluted into 4.0 volumes of previously warmed enriched minimal medium (approximately 8×10^8 viable centers per ml) and incubated at 37°. Two to three minutes later, 9 μg of ^{14}C-actinomycin per ml (1.2×10^5 cpm per ml) were added. Ten minutes after the addition of the labeled antibiotic, the amount of label taken up by the cells and the number of viable centers were determined.

Strain	EDTA treatment	^{14}C-Actinomycin uptake	Uptake caused by EDTA treatment	Viable centers per ml after actinomycin
		cpm/ml	*cpm/ml*	
NL101	+	2178	1518	3.8×10^8
	−	660		7.4×10^8
AB1105	+	6115	5685	1.5×10^7
	−	430		9.2×10^8

cells was found in the supernatant fluid of control or EDTA-treated cells (Table III), indicating that no appreciable lysis had occurred. Thus EDTA-treated NL101 cells release LPS but to a lesser extent than the parent strain.

A small amount of noncovalently bound phospholipid is released by EDTA treatment of another strain of *E. coli* (0111:B4) (3). Similar results have now been obtained with NL101 and AB1105. Cell pellets and supernatant fluids from the experiment of Table III were analyzed for phospholipids as described under "Experimental Procedure." Control cell supernatants yielded only unidentified traces of material while those from EDTA-treated cells of both parent and mutant yielded a component with the same R_F value and staining properties as phosphatidylethanolamine. The cell pellet fractions of both mutant and parent stains contained phosphatidylethanolamine, phosphatidylglycerol, and a component with the same staining

TABLE III

Release of LPS by NL101 and AB1105

NL101 and AB1105 were inoculated into 400 ml of minimal medium and grown to the midexponential phase. The cells were washed twice with 25-ml portions of Tris buffer and resuspended in 4.0 ml of Tris buffer. One-half of the resuspended cells were treated with EDTA. Treatment was stopped by adding Mg^{++} to 5 times the EDTA concentration. The other half (control cells) were treated in the same manner except EDTA was added after the Mg^{++}. Aliquots (.010 ml) were removed for determination of actinomycin sensitivity (Fig. 2), the cell suspensions were centrifuged, and the supernatant fluid was recentrifuged to remove residual cells. 3-Deoxyoctulosonate and protein were determined as described under "Experimental Procedure." The supernatant fluids of all samples contained between 0.10 and 0.22 mg of protein.

Strain	Dry weight	EDTA treatment	3-Deoxyoctulosonate		Total 3-Deoxyoctulosonate in supernatant
			Pellet	Supernatant	
	mg		*μmole*		%
NL101	22	+	0.144	0.064	31
	22	−	0.206	<0.007	<4
AB1105	24	+	0.118	0.112	49
	24	−	0.181	<0.005	<3

TABLE IV

Permeability and LPS release in EDTA-treated NL102 and J-5 grown in different media

Growth medium and strain	Sensitivity to 5 μg of actinomycin/ml	Colitose release	Total μmole of colitose per 1.0 optical density unit of cells
	%	%	
Minimal galactose[a]			
NL102	11	24	0.038
J-5	89	35	0.043
Trypticase soy minimal galactose[b]			
NL102	12	25	0.041
J-5	86	38	0.037
Trypticase soy galactose[b]			
NL102	40	31	0.041
J-5	95	52	0.041

[a] Cells washed one time in Tris buffer prior to EDTA treatment.
[b] Cells washed one time in 0.15 M NaCl prior to EDTA treatment.

properties and a R_F close to but not identical with beef heart cardiolipin. Both strains had roughly the same proportions of these lipids, with phosphatidylethanolamine by far the predominant component.

Response of NL102 to EDTA

To facilitate further studies of release of LPS by *per* organisms, NL102 (*per*-2) and its parent, J-5, were used. Their LPS can readily be assayed by measuring colitose, a sugar found only in the antigenic side chains of the LPS of these organisms; or by incorporation of radioactively labeled galactose, since lack of UDP-galactose 4-epimerase (9) insures that these strains incorporate exogenously supplied galactose only into LPS and its precursors (18).

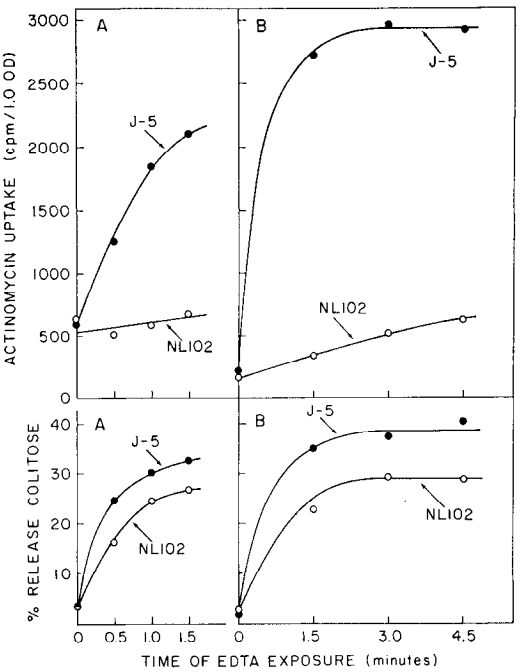

FIG. 3. Release of LPS and increased permeability as a function of time of exposure to EDTA. J-5 and NL102 were grown in tryptocase soy galactose minimal medium to approximately equal turbidities washed, and resuspended. In *A*, cells (4.5 ml; 1.2 × 10^{10} per ml) were equilibrated to 37°. A 1.0-ml sample (zero time sample) was removed and immediately mixed with EDTA (0.8 μmole) and Mg^{++}. To the remaining suspension, EDTA (2.4 μmoles) was added and at 30, 60, and 90 sec thereafter 1.0-ml samples were removed and immediately mixed with Mg^{++}. In *B*, cells (4.4 ml; 6 × 10^9 per ml) were treated in the same manner except that the amounts of EDTA were reduced by one-half. Samples were removed at 1½, 3.0, and 4½ min. To measure permeability, 0.20-ml (*A*) or 0.10-ml (*B*) aliquots of the samples were diluted to 2.0 ml with previously warmed minimal medium (minus PO_4) containing 5.5 μg per ml (*A*) or 5.9 μg per ml (*B*) ^{14}C-actinomycin and the amount of label taken up by the cells in 4 min at 37° was determined. To measure LPS release, the remainder of the samples was separated into pellet and supernatant fluid and the colitose content of each was determined.

Permeability and Release of LPS—Increased permeability, as measured by actinomycin sensitivity, and percentage of LPS release, as measured by colitose content, were determined for EDTA-treated NL102 and J-5 after growth in three different media (Table IV). In all three media the mutant released 30 to 40% less LPS than the parent strain. As measured by colitose, no difference in the LPS content of mutant and parent strains was found. NL102 is much less permeable to actinomycin after EDTA treatment than is its parent. It is least permeable after growth in minimal medium. As with NL101 (*per*-1), growth on rich medium (Trypticase soy galactose) increases the permeability induced by EDTA. J-5 and NL102 grow poorly on minimal medium, but it was found that supplementing minimal medium with 10% trypticase soy broth (trypticase soy minimal galactose) both improved growth and preserved the relative impermeability of NL102 after EDTA treatment, so trypticase soy minimal galactose medium was used in all further studies.

Kinetics of Increase in Permeability and Release of LPS—The release of LPS and the initial rate of actinomycin uptake as a function of time of exposure to EDTA were measured (Fig. 3). As in *E. coli* 0111:B4 (2), release of LPS and increase in permeability in J-5 occur rapidly, both processes approaching completion within 2 min of addition of EDTA. In J-5, the increase in permeability, as measured by initial rate of uptake of actinomycin, was found to parallel rather closely release of LPS. In the mutant strain, there is only a slight increase in permeability with time although there is a rapid and substantial release of LPS. At all times tested, NL102 released 20 to 30% less LPS than the parent strain. In other experiments, treating NL102 with 3 times the usual amount of EDTA did not appreciably increase LPS release or permeability to actinomycin.

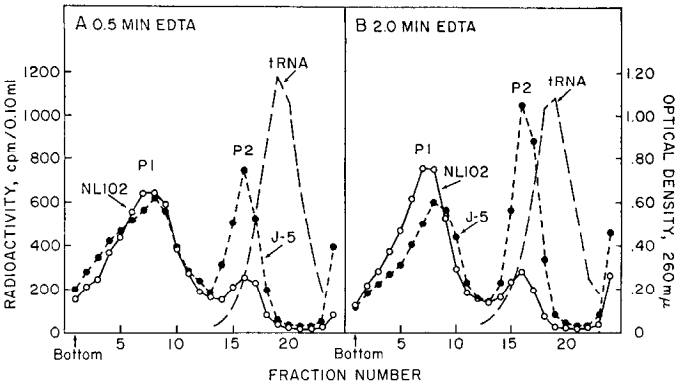

FIG. 4. Sucrose density centrifugation of LPS released from NL102 and J-5 by EDTA. NL102 and J-5 were grown for at least four generations in tryptocase soy galactose minimal medium containing 7.0 μCi per ml (*A*) or 5.5 μCi per ml (*B*) ^{14}C-galactose. Washed cells were resuspended at 20-fold concentration and treated with EDTA for 30 sec (*A*) or 120 sec (*B*). The dialyzed supernatant fluids from an equivalent amount of NL102 and J-5 cells were layered on a 5 to 20% sucrose gradient and centrifuged for 15 hours, at 73,000 × *g* and 4°. Less than 5% of counts applied to the gradient pelleted to the bottom of the centrifuge tube. In these experiments, increase in permeability following EDTA treatment was determined by assaying for viability after exposure to actinomycin as described under "Experimental Procedure." J-5 underwent a 97% reduction in viable centers when treated with EDTA for 30 sec, and a 99.6% reduction in viable centers when treated for 2 min. NL102 showed no reduction in viable centers in either case.

TABLE V

Release of lipopolysaccharide fractions

Strain	Time of EDTA treatment	Release of LPS[a]		
		Total[b]	P1[c]	P2[c]
	min	%	%	%
NL102	0.5	27	21.2	4.4
J-5	0.5	37	22.2	11.4
NL102	2.0	37	27.3	6.6
J-5	2.0	45	22.2	17.8

[a] LPS was measured as high molecular weight ^{14}C-galactose, determined as nondialyzable counts in the material released by EDTA and in the EDTA-treated cells as counts precipitable by cold 5% trichloracetic acid.

[b] Similar values were obtained on the basis of colitose content.

[c] Obtained by multiplying total % release of LPS (third column) by the fraction of counts in the gradient that fell under P1 or P2.

Sucrose Density Gradient Studies on Released LPS—The LPS of J-5 and NL102 was uniformly labeled by growing cells at least

four generations in Trypticase soy minimal galactose medium containing ^{14}C-galactose. The cells were then treated with EDTA for 30 sec or 2 min and the supernatant fluid was dialyzed and layered on a 5 to 20% sucrose gradient. The LPS released both by J-5 and NL102 sedimented as two components (Fig. 4): a narrow peak (P2) sedimenting slightly faster than a tRNA marker (S $\sim$ 4) and a faster sedimenting peak of broader distribution (P1). Both J-5 and NL102 released roughly equal amounts of the P1 component. In contrast, NL102 released significantly reduced amounts of P2 when compared to the parent strain. In the experiments presented in Fig. 4, the mutant strain released only about one-third as much P2 as the parent (Table V). Similar results have been obtained in other experiments.

The sedimentation constants of P1 and P2 were calculated with use of transfer RNA as a reference and assuming simple proportionality between s and distance traveled from the meniscus (19). Sedimentation values of approximately 6 S and 12 S for P2 and P1, respectively, were obtained.

Like AB1105 and 0111:B4, the supernatant of EDTA-treated J-5 cells contains noncovalently bound phospholipid.[3] When supernatants from J-5 and NL102 were extracted with butanol prior to centrifugation in a sucrose density gradient, a single peak of radioactivity was found at approximately the same position as P2, indicating that removal of this lipid reduces the sedimentation rate of P1 to a value approximating that of P2.

DISCUSSION

Mutants resistant to the permeability promoting effect of EDTA (*per* mutants) were isolated to help determine whether release of LPS is both necessary and sufficient for the EDTA-induced permeability effect. Some earlier observations suggested a functional relationship. Both the increased permeability and LPS release occur equally well at 0 and 37° (3), and both occur rapidly, being complete within a few minutes of EDTA exposure (2). Both reactions are prevented or terminated by divalent cations but divalent cations do not reverse either effect. Restoration to normal permeability does not require RNA, protein, or peptidoglycan synthesis but does require energy metabolism, an observation which would be compatible with a requirement for resynthesis of cell wall LPS (10). On the other hand, it was found with *E. coli* 0111:B4, that the amount of LPS released by EDTA over a pH range of 7.0 to 8.0 did not vary, whereas over this same range the induced permeability increased with increasing pH, indicating that these two effects could be partly dissociated (3).

The *per* strains isolated in this study showed normal growth rates and no significant differences in LPS content, but EDTA-treated cells of the mutants released less LPS than the parent strains. Further study of one strain revealed that this reflected reduced release of one specific LPS fraction. The possibility that the phenotype of the *per* mutants may result from multiple mutations cannot be excluded. The same phenotype was obtained in two independent selections and in two different strains, making this possibility less likely. However, since the cells of one strain were treated with mutagen, and the mutants selected after several cycles of an enrichment procedure, multiple mutations could accumulate. Preliminary studies suggest that some revertants of *per*-2 that become permeable after treatment with EDTA have not regained the original pattern of LPS release but instead may have further changes in their LPS. Genetic analysis of *per* mutants and their revertants, currently under way, will obviously be of great interest and help to clarify these phenomena.

Although the *per* mutation leads to lesser release of LPS, mere reduction in amount of LPS released in the mutant is insufficient to explain its failure to be permeable. As shown in Fig. 3, after 1½ min of EDTA treatment, NL102 released as much LPS as J-5 did at 30 sec but this release was associated with much less permeability in the mutant than in the parent. This result suggested that perhaps the release of a particular fraction of LPS is required for the increased permeability effect.

The LPS released by EDTA treatment of *E. coli* 0111:B4, the parent strain of *E. coli* J-5, was previously found to contain two major components (3). In the analytical ultracentrifuge, one component, F1, sedimented as a broad heterodisperse peak of 10 to 80 S and the second component, F2, as an ultrasharp peak of 5.4 S at infinite dilution. If, prior to ultracentrifugation, the released LPS was treated with chloroform-methanol to remove noncovalently bound lipid, F1 sedimented as a single symmetrical peak of 11 S, and F2 was unchanged. The two components were qualitatively identical in their chemical composition, but differed in the proportion of their carbohydrate components.

When the LPS released by EDTA from J-5 and its *per*-2 mutant was studied by sucrose density centrifugation, the released LPS showed two major components (P1 and P2) suggestive of the F1 and F2 components released by 0111:B4. Like F1, the sedimentation rate of P1 was reduced by organic solvent extraction; however, both before and after such extraction, the sedimentation rate of P1 was lower than found for F1. This could be the result of an effect of the sucrose gradient on the molecular structure of this component or might reflect disaggregation since the sucrose gradients utilized less than 1% the concentration of LPS as did the analytical ultracentrifuge studies. It is of course possible that P1 and P2 do not represent the two chemically different LPS species, F1 and F2, but only reflect differing degrees of association of LPS with phospholipid.

Although the LPS released by both parent and mutant showed the same two components, one of these components (P2) was appreciably reduced in the mutant. Thus the rather small reduction in total LPS release shown by the mutant represents a large decrease in a specific fraction. Also, in J-5, the increase in release of P2 between 30 sec and 2 min of EDTA treatment roughly paralleled the percentage increase in permeability during this time (Fig. 3 and Table V). Both these results suggest a relationship between release of P2 and increase in permeability. It cannot yet be concluded that this LPS release is necessary for the permeability effect to occur. The two effects could be only operationally associated; for instance, they might be independent secondary effects of some primary action of EDTA. The present results do, however, support a working hypothesis that release of this specific fraction of LPS is in some manner required for the EDTA-caused permeability change.

[3] S. B. Levy, personal communication.

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