

Outer Membrane of *Salmonella typhimurium*: Chemical Analysis and Freeze-Fracture Studies with Lipopolysaccharide Mutants

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The outer membrane layer of the cell wall was isolated from wild-type *Salmonella typhimurium* LT2 as well as from its mutants producing lipopolysaccharides with shorter saccharide chains. Chemical analysis of these preparations indicated the following. (i) The number of lipopolysaccharide molecules per unit area was constant, regardless of the length of the saccharide side chain in lipopolysaccharide. (ii) In contrast, in "deep rough" (Rd or Re) mutants producing the lipopolysaccharides with very short saccharide chains, the amount of outer membrane protein per unit surface area decreased to about 60% of the value in the wild type. (iii) In the wild type, the amount of phospholipids is slightly less than what is needed to cover one side of the membrane as a monolayer. In comparison with the wild type, the outer membrane of Rd and Re mutants contains about 70% more phospholipids, which therefore must be distributed in both the outer and inner leaflets of the membrane. Freeze-fracture studies showed that the outer membrane of Re mutants were easily fractured, but fracture became increasingly difficult in strains producing lipopolysaccharides with longer side chains. The convex fracture face was always nearly smooth, but the concave fracture face or the outer half of the membrane was densely covered with particles 8 to 10 nm in diameter. The density of particles was decreased in Re mutants to the same extent as the reduction in proteins, suggesting the largely proteinaceous nature of particles. A model for the supra-molecular structure of the outer membrane is presented on the basis of these and other results.

The cell walls of gram-negative bacteria consist of two layers, the outer membrane layer and the peptidoglycan layer. The outer membrane in turn contains lipopolysaccharide (LPS), proteins, and phospholipids. We have previously reported (2) that some mutations in LPS biosynthesis apparently inhibit the incorporation of proteins into the outer membrane; especially the outer membrane of "heptoseless" or Re-type mutants, which contain only 3-deoxy-octulosonic acid residues in the saccharide portion of LPS (14), contains drastically reduced amounts of the major outer membrane proteins. A similar situation has independently been discovered in a heptoseless mutant of *Escherichia coli* K-12 (12).

This paper presents the results of quantitative analysis and freeze-fracture studies on the outer membrane of LPS mutants of *Salmonella typhimurium* and proposes a model for the structure of *Salmonella* outer membrane.

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MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* LT2 and its mutants producing incomplete LPS were used (Table 1; Fig. 1). The Re mutant HN502 was newly isolated from TA2167 by the procedure of Ames and co-workers (1). SL1004 and SL1181 were kindly given to us by B. A. D. Stocker.

Chemicals. The chemicals used were of the best grade commercially available. 3-Hydroxytridecanoic acid was synthesized chemically (11). 2- ^3H glycerol and ^{32}P phosphate were obtained from New England Nuclear Corp.

Growth conditions. Cells were grown in L broth (5) (glucose omitted) at 37 C with vigorous aeration by shaking at 200 rpm on a New Brunswick rotatory shaker, model G52. The volume of the medium was always kept to 25% of the volume of the Erlenmeyer flask used. Under these conditions, the cells grew exponentially up to a cell density of about 1 mg (dry weight)/ml, and we took care to always use cells in mid-exponential phase, i.e., at the cell density of 0.25 to 0.5 mg (dry weight)/ml.

TABLE 1. *Properties of the strains used*

Strain	LPS produced	Molar ratio				Crystal violet sensitivity ^b	Phage C21 sensitivity ^c	Genotype
		Glucose ^a	Heptose	KDO	3-OH-14:0			
LT2	S	ND ^d	ND	ND	ND	0	R	Wild type
<i>his-642</i>	Ra	ND	ND	ND	ND	0	R	<i>his-rfb-642</i>
HN202	Rc	0.8	2.2	2.3	3.0	0.5	S	<i>galE503</i>
TA2167	Rc	0.8	2.1	ND	3.0	0	S	<i>galE506 hisC3076</i>
SL1004	Rd ₁	<0.1	2.0	ND	ND	2.8	S	<i>rfaG571 rfb met trp fla str</i>
SL1181	Rd ₂	<0.1	0.9	2.0	ND	4.2	R	<i>rfaF511 xyl met trp fla str</i>
TA2168	Re	<0.1	<0.1	1.8	3.0	5.0	R	<i>galE506 rfa-1009 hisC3076</i>
HN502	Re	<0.1	<0.1	1.3	3.0	5.5	R	<i>galE506 rfa-4309 hisC3076</i>

^a LPS composition. Sugars were assayed as described previously (2). 3-Hydroxytetradecanoic acid (3-OH-14:0, β -hydroxymyristic acid) was determined as described in Materials and Methods. KDO, 3-Deoxyoctulosonate (2-keto-3-deoxyoctonic acid).

^b Tested by placing a filter paper disk (8 mm in diameter), containing 10 μ g of crystal violet, on a lawn of cells on L agar plates. The values correspond to the radius of the inhibition zone minus the radius of the disk.

^c R, Resistant; S, sensitive.

^d ND, Not determined.

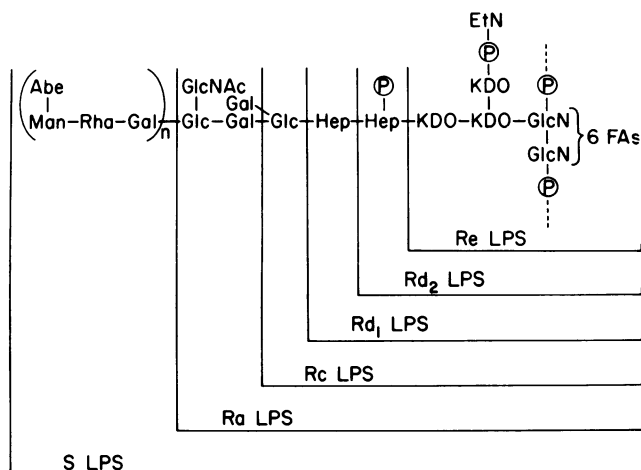


FIG. 1. LPS produced by the strains used (14). Abbreviations: Abe, abequeose; Man, D-mannose; Rha, L-rhamnose; Gal, D-galactose; GlcN, D-glucosamine; Hep, L-glycero-D-manno-heptose; KDO, 3-deoxy-D-mannooctulosonic acid; EtN, ethanolamine; P, phosphate; FA, fatty acids; and Ac, acetyl. This scheme shows a "monomer" unit; a molecule of LPS is usually thought to contain three such monomer units linked through pyrophosphate linkages (dotted line). Among the six fatty acid residues linked to the glucosamine disaccharide are three residues of 3-hydroxytetradecanoic acid (22). S, Ra, etc. refer to the structural type of LPS produced.

For studies in which the results were related to dry weight of the cells, the mid-exponential-phase cells grown as described above were washed and suspended in distilled water to an optical density at 600 nm (Perkin-Elmer 124 spectrophotometer) of 1.22, and portions of the suspensions were used for quantitative assay. The dry weights of cells in 300

ml of such suspensions were determined after lyophilization followed by drying in vacuo over silica gel. They were 131.0, 129.9, and 96.1 mg for LT2, HN202, and TA2168, respectively.

Preparation of radioactively labeled cells. L broth (5 ml) containing 20 μ Ci of 2-[³H]glycerol (3 nmol) was inoculated with 0.1 ml of an overnight

culture of an appropriate strain and was incubated at 37 C with shaking for 4 h. The cells were collected by centrifugation, washed twice with L broth, and finally suspended in 100 ml of L broth containing 0.05% nonradioactive glycerol. After 1 h of incubation at 37 C with shaking, the cells were collected and used for the isolation of outer and inner membranes. Under these conditions, the ^3H label is expected to be located almost exclusively in the glycerol residues of phospholipids.

^{32}P -labeled cells were prepared by growing cells to mid-exponential phase in 5 ml of L broth containing 0.1 mCi of [^{32}P]phosphate.

Isolation of outer and inner membranes. This was done essentially according to a modification (12) of the Schnaitman procedure (27), except for the following. (i) Cells were grown as described above. (ii) Cells were centrifuged rapidly (5 min) and were washed rapidly with ice-cold 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid (HEPES) buffer, pH 7.4. Cell pellets were stored frozen at -70 C. (iii) Cells (0.5 g, dry weight) were suspended in 100 ml of 10 mM HEPES buffer, pH 7.4, containing deoxyribonuclease and ribonuclease, and the suspension was passed through a French pressure cell. Neither MgCl_2 nor ethylenediaminetetraacetic acid was added at any stage during the isolation procedure. (iv) The isolated membranes were washed twice in 10 mM HEPES buffer and were purified by re-centrifugation through a similar sucrose gradient. The purified membranes were washed four times with distilled water and lyophilized. All operations after the harvesting of cells were carried out at 0 to 4 C, except as noted.

For preparation of membranes from [^3H]glycerol- or [^{32}P]phosphate-labeled cells, similar but scaled-down procedures were used. Sucrose gradient centrifugation was done in a Beckman SW41 rotor at 20,500 rpm for 15 h. These membrane fractions were stored frozen until analysis.

The possible contamination of the outer membrane fraction by the inner membrane was checked by assaying for cytochrome *b*, from the difference in optical density at 429 nm between unreduced and dithionite-reduced preparations (19). Reduced nicotinamide adenine dinucleotide oxidase was also assayed (19) in some preparations.

Determination of outer membrane components. Phospholipids were extracted by the Bligh-Dyer method (6) and quantitated by assaying for phosphorus after digestion in sulfuric acid (3). An average molecular weight of 700 was assumed for the calculation of phospholipid content.

With Re mutants, a portion of the incomplete LPS is extracted by the Bligh-Dyer method together with phospholipids (12). To correct for the extracted LPS, outer membrane fractions were prepared from ^{32}P -labeled cells of TA2168 and HN502. When the Bligh-Dyer extracts of these fractions were separated by chromatography on an ion exchange paper (Amberlite WB-2) with diisobutylketone-acetic acid-water (8:5:1, vol/vol/vol) as solvent, it was found that Re LPS ($R_f = 0.25$) contained 31% of the radioactivity of the extract. The amount of "lipid phosphorus" obtained from the outer membrane of Re mutants was

therefore multiplied by 0.69 to correct for this contamination by LPS.

For the determination of 3-hydroxytetradecanoic acid, 50 mg (dry weight) of whole cells or 10 mg (dry weight) of membrane fractions was hydrolyzed in 10 or 2 ml of 4 N HCl at 100 C for 15 h, together with a known amount of the internal standard, 3-hydroxytridecanoic acid. The hydrolysate was extracted three times with ether, and the fatty acids were converted into methyl esters by treatment with diazomethane. The esters were analyzed by gas chromatography on a 6-foot (ca. 183-cm) column of 10% polyethyleneglycolsuccinate on GasChrom P at 150 C. The amount of methyl 3-hydroxytetradecanoate was calculated by comparing its peak area with the peak area corresponding to methyl 3-hydroxytridecanoate.

Protein was determined with the Lowry procedure for "insoluble proteins" (13), with bovine serum albumin as standard.

Measurement of cell size. Cells of HN202 and TA2168 were grown as described above. Then 25% glutaraldehyde (filtered four times through activated charcoal and stored under N_2) was added to a final concentration of 3%, and the cells were fixed for 30 min on ice. They were then washed once with 0.1 M phosphate buffer, pH 7.2, and resuspended in distilled water. For light microscopic measurements this preparation was photographed directly with a Zeiss GFL microscope equipped with phase fluorite objectives. Prints were made at final magnification of 5,000 \times .

For electron microscopic measurements, drops of the same preparations were placed on 400-mesh copper grids that were coated with Formvar and carbon stabilized. The bacteria were viewed unstained in a Siemens-Elmiskop 1A electron microscope equipped with a decontamination device. Photographs were made with electron image plates (Eastman Kodak). Prints were then made at a final magnification of 21,000 \times . In both cases measurements were made from the prints.

Freeze-fracture and freeze-etching studies. Cells were grown as described above. The cells were then harvested by centrifugation and washed once with 0.1 M phosphate buffer, pH 7.2. They were then resuspended in 1.5% glutaraldehyde in phosphate buffer and fixed for 1 h at room temperature. The cells were centrifuged again and resuspended in glycerol-0.1 M phosphate buffer (1:4, by volume). After a minimum of 2 h the cells were pelleted, and the pellet was prepared for freeze fracture. For experiments involving etching, the glycerol was omitted. Freezing of samples was accomplished by immersing in the liquid phase of partially solidified Freon 22 cooled by liquid nitrogen.

Freeze fracture and freeze etching was carried out in a Balzers device. The stage temperature was -115 C for freeze fracture runs and -100 C when etching was desired. In etching experiments the etch time was 2 to 3 min. After fracturing, the samples were shadowed with platinum and coated with carbon. The replicas thus obtained were cleaned with 6% sodium hypochlorite, washed with distilled water, and placed on uncoated 300-mesh

copper grids. They were observed with a Siemens-Elmiskop 1A equipped with a decontamination device.

Quantitation of particles seen in freeze fracture. To count the number of particles observed in fractures of the outer membranes of the strains used, typical photographs were selected. Rectangular areas of calculated dimensions were drawn. Care was taken to select areas that were perpendicular to the electron beam, since an oblique angle would result in an apparent compaction of the number of particles seen per unit area. The typical particles seen in all strains were 8 to 10 nm in diameter. In optimal photographs of the Re mutants, small irregular "particles" (2 to 4 nm in diameter) could be seen in the smooth areas between the 8- to 10-nm particles. These smaller "particles" were not scored, since they could not be seen in the Rc mutant where the normal particles are very tightly packed and since their size was too small to permit us to distinguish between a real particle and an imperfection in the replica.

Other methods. Radioactivity was quantitated with an Amersham-Searle liquid scintillation spectrometer, with Bray's solution (8) as the scintillator fluid.

RESULTS

Isolation of outer and inner membranes.

For this study we needed a method which could reproducibly and easily separate outer membrane from inner membrane. The method described by Schnaitman (27) and by Koplow and Goldfine (12) was better suited for our purpose than that of Osborn and co-workers (19), since it is much simpler and permits the separation of membranes of Re mutants, a separation difficult to achieve with the Osborn procedure. The membranes were clearly separated into two bands (Fig. 2); the heavier band contained much LPS and thus corresponded to the outer membrane, and the lighter band contained cytochromes and reduced nicotinamide adenine nucleotide oxidase, thus corresponding to the inner or cytoplasmic membrane. The two bands were widely separated even in preparations from the Re mutants, and little or no intermediate band ("M band") is seen. These results could presumably be due to the fact that the outer membrane band contains attached peptidoglycan, which increases the density of the complex so that clean separation from the inner membrane band is always obtained. When the outer membrane fractions were examined for possible contamination by the inner membrane by assaying for cytochrome b_1 , the degree of contamination was found to be <2, <2, and 4% for strains LT2, HN202, and TA2168, respectively. On the other hand, significant amounts of LPS-specific component, 3-hydroxytetradecanoic acid, were always found in the inner membrane fractions

(Table 2). This may mean that our "inner membrane" fractions are contaminated by the outer membranes. Such contamination seems espe-

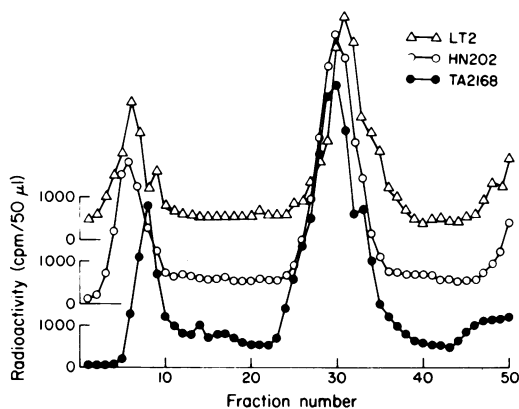


FIG. 2. Separation of the outer and inner membranes by sucrose gradient centrifugation. Cells labeled by growing in 2-[^3H]glycerol (see Materials and Methods) were used for the separation of membranes as described in the text.

TABLE 2. Quantitative analysis of membranes from various strains

Membrane preparation	Component ($\mu\text{g}/\text{mg}$ of membrane)		
	Protein	Phospholipid	LPS ^a (3-hydroxytetradecanoic acid)
LT2 (S)			
Outer	430	60	— (24.9)
Cytoplasmic	600	198	— (8.9)
HN202 (Rc)			
Outer	500	84	144 (31.0)
Cytoplasmic	600	194	31 (6.6)
TA2167 (Rc)			
Outer	510	101	155 (33.2)
Cytoplasmic	640	197	53 (11.5)
SL1004 (Rd ₁)			
Outer	408	172	165 (37.2)
Cytoplasmic	616	193	43 (9.7)
SL1181 (Rd ₂)			
Outer	437	161	173 (41.6)
Cytoplasmic	616	199	45 (10.8)
TA2168 (Re)			
Outer	412	174	161 (42.3)
Cytoplasmic	610	209	48 (12.7)
HN502 (Re)			
Outer	390	176	167 (43.9)
Cytoplasmic	650	198	17 (4.4)

^a The mass of LPS was calculated from the molecular weights of Rc, Rd₁, Rd₂, and Re LPS (10,230, 9,744, 9,168, and 8,355) calculated from the structural formulas (14) on the basis of assumption that each molecule of LPS contains nine 3-hydroxytetradecanoic acid residues (23, 25).

cially likely for the preparation from TA2168, an Re mutant, since its outer membrane fragments without the attached peptidoglycan would have a buoyant density approaching that of the inner membrane.

Quantitative analysis of isolated membrane preparations. The membrane fractions were washed in water, lyophilized, and then analyzed for various components (Table 2). Several observations can be made. (i) For those strains producing LPS of precisely known structure (i.e., Rc through Re), the sums of the weights of proteins, phospholipids, and LPS were about 75% of the dry weight of the outer membranes analyzed. This is a very good recovery, as the "membranes" contained peptidoglycan layer. (ii) Although the outer membrane is often thought to be unusually rich in proteins, the protein content per unit weight of the membrane is usually lower in the outer membrane than in the inner membrane. (iii) In an Re mutant of *E. coli*, the inner membrane is reported to contain three times as much LPS as the outer membrane in terms of total amounts present and probably about the same amount of LPS if the results are expressed as weight per unit weight of the membrane (12). Although our "inner membrane" was somewhat contaminated by the outer membrane, we could not find such an abundance of LPS in the inner membrane fractions of our Re mutants. Since in the work of Koplów and Goldfine most of the Re mutant membrane material applied to the sucrose gradient ended up in the "inner membrane" fraction (see Table 3 of reference 12), we suspect that the large amount of LPS found in that fraction was actually due to the extensive contamination of that fraction by outer membrane fragments. (iv) It is difficult, however, to compare figures on different strains, because the figures are based on the dry weight of the membrane, which is greatly affected by the varying length of the saccharide side chains of LPS.

Estimation of the surface area and number of LPS molecules per unit area. To make meaningful comparisons on the outer mem-

brane composition data of various strains, we had to estimate the surface area of cells and then correlate the surface area with the amounts of some outer membrane components. We chose an Rc (HN202) mutant and an Re (TA2168) mutant for this purpose. We determined the average cell diameter by measurement on the electron microscopic pictures (Table 3). Here great care was taken not to measure cells that appeared flattened. Since the cells were not stained, this is easily seen by the relative translucence of the cells. A constant diameter of 0.75 μm (standard deviation, 0.03 μm) was seen for both HN202 and TA2168. This observation was confirmed by light microscope measurements, although lower resolution and magnification limited the accuracy of the measurements.

We initially tried to measure the average cell length and calculate the cell surface on the basis of length and diameter of the cells. The average cell length, however, was difficult to determine, especially with HN202, because this strain exhibited some delay in cell division and consequently produced a number of very long cells. We therefore used an approach similar to the one adopted by Mühlradt and co-workers (17). That is, we determined the cell number per milligram (dry weight) and then calculated the average cell volume from this value and the specific volume of the cells. The partial specific volume is expected to be slightly larger than 2 $\mu\text{l}/\text{mg}$ (dry weight), as it is known that 1 g (dry weight) of *E. coli* cells contains about 2 ml of intracellular water (24). Mühlradt and co-workers (17) have actually measured the partial specific volume of *S. typhimurium* LT2 derivative and found a value of $7.2 \times 10^{11} \mu\text{m}^3/300 \text{ mg}$ (dry weight) = 2.4 $\mu\text{l}/\text{mg}$ (dry weight). We therefore used this value for the calculation of average cell volume. We also checked the validity of our values for diameter, partial specific volume, and cell number by calculating the average length of TA2168 cells from this cell volume and the diameter (0.75 μm), assuming that the cell can be approximated by a cylinder with two hemispherical ends. Such a calculation pro-

TABLE 3. Calculation of cell surface area

Strain	No. of cells/mg (dry wt)	Mean cell diam (μm)	Mean cell length (μm)		Cell surface ^a ($\mu\text{m}^2/\text{mg}$ [dry wt] of cells)
			Observed	Calculated ^a	
HN202	1.01×10^9	0.75		5.48	13.1×10^9
TA2168	2.19×10^9	0.75	2.47	2.56	13.3×10^9

^a Cells were assumed to be cylinders with hemispherical ends. Thus, the volume of a cell equals $(\pi/6)d^3 + (\pi/4)d^2(l - d)$, where d and l are diameter and length of the cell, respectively. The surface area of this cell is then $\pi d^2 + \pi d(l - d) = \pi dl$ (see reference 17).

duced the cell length of 2.56 μm , which was very close to the value actually measured on electron microscopic prints (2.47 μm). This agreement suggests that our values for diameter, etc. are not grossly incorrect.

The cell surface area was then calculated on the basis of cell diameter and average cell volume, as described by Muhlradt and co-workers (17). The results (Table 3) show that there is very little difference between the two strains in cell surface-dry weight ratios, even though the strains differed very much in cell length.

As a substance that is uniquely present in the outer membrane, we determined the amount of 3-hydroxytetradecanoic acid in the hydrolyzate of whole cells (Table 4). By using the surface area values in Table 3, we could then calculate the surface density of LPS (Table 4). The results clearly showed that the number of LPS molecules per unit surface area was constant, in spite of differences in the structure of the saccharide portion of LPS.

Surface density of outer membrane components. The results in the preceding section showed that a unit surface area of exponential-

phase *S. typhimurium* cells contained the same number of LPS molecules, even in a mutant producing an extremely defective (Re-type) LPS. Furthermore, Muhlradt and co-workers (17) found that the same is true even among unrelated strains of *S. typhimurium*. We therefore feel that we are justified in extrapolating this conclusion to all the strains we have studied in this work. The results of Table 2 have therefore been recalculated on the assumption that 1 μm^2 of the outer membrane contains 1.92×10^5 molecules of LPS (Table 5). The values listed in Table 5 are dependent on the values of surface area, which cannot be determined very accurately. The values therefore should be considered only very rough approximations. We should like to emphasize, however, the following points. (i) In the S and Rc strains, phospholipids present are barely enough to cover one side of the membrane. (ii) In "deep rough" mutants (Rd and Re), the amount of proteins per unit area is reduced to 52 to 57% of the "normal" value. This confirms the semiquantitative results presented earlier (2). (iii) In "deep rough" mutants there is a large increase in phospholip-

TABLE 4. Surface density of 3-hydroxytetradecanoate and LPS

Strain	3-Hydroxytetradecanoate			LPS (molecules/ μm^2 of surface area)
	$\mu\text{g}/\text{mg}$ (dry wt) of cells	$\mu\text{g}/\mu\text{m}^2$ of surface area	molecules/ μm^2 of surface area	
HN202	9.24	7.05×10^{-10}	1.74×10^6	1.93×10^5
TA2168	9.26	6.96×10^{-10}	1.71×10^6	1.95×10^5

TABLE 5. Amounts of outer membrane components found in unit surface area

Strain	LPS produced	Components present in 1- μm^2 section of outer membrane			Surface area covered by components ^a (%)	
		LPS ^b (molecules)	Phospholipids (molecules)	Protein (fg)	LPS ^c	Phospholipids ^c
LT2	S	1.92×10^5	1.45×10^6	12.0	44	78
HN202	Rc	1.92×10^5	1.63×10^6	11.3	44	88
TA2167	Rc	1.92×10^5	1.83×10^6	10.8	44	98
SL1004	Rd ₁	1.92×10^5	2.78×10^6	6.9	44	148
SL1181	Rd ₂	1.92×10^5	2.32×10^6	6.6	44	124
TA2168	Re	1.92×10^5	2.48×10^6	6.8	44	134
HN502	Re	1.92×10^5	2.42×10^6	6.3	44	131

^a Expressed as percentage of the membrane area covered. Thus complete coverage of both surfaces of the membrane will give a value of 200%.

^b One LPS molecule was assumed to contain three monomeric units (25), each of which contains three 3-hydroxytetradecanoate residues (23).

^c Each molecule of LPS and phospholipid is assumed to occupy 2.32 and 0.54 nm^2 of surface area, respectively (25).

ids, a change that presumably compensates for the decrease in proteins. Most importantly, in "deep rough" mutants many more phospholipids are present than could be accommodated on one side of the membrane.

Freeze fracture and freeze etching. Freeze fracture and freeze etching revealed a pattern similar to that seen in *E. coli* by van Gool and Nanninga (30). Etching studies provided a view of the outer surface of *S. typhimurium* cells. As can be seen (Fig. 3), the wild-type strain LT2 (so-called "smooth") does indeed have a smooth outer surface, presumably due to the long saccharide chains of LPS. A good representation of those strains missing most of the saccharide chain is seen in the etched appearance of *his-642*, a mutant that produces an Ra-type LPS

(Fig. 4). One can now see perturbations which seem to correspond to closely underlying particles, seen in the concave fracture face of the outer membrane (see below).

The results of freeze-fracture studies are shown in Fig. 5 through 10. Only two fracture planes were found. The inner plane is that of the plasma membrane. This fracture has a very characteristic appearance: the convex face contains many particles (Fig. 5, face b, and Fig. 6) and the concave face is nearly devoid of particles (Fig. 9, face b). In the various strains utilized in this study, as well as other derivatives of LT2, this appearance was invariant (see Fig. 6). It apparently is also similar to that seen in most other bacteria, gram-positive as well as gram-negative (4, 9, 18).

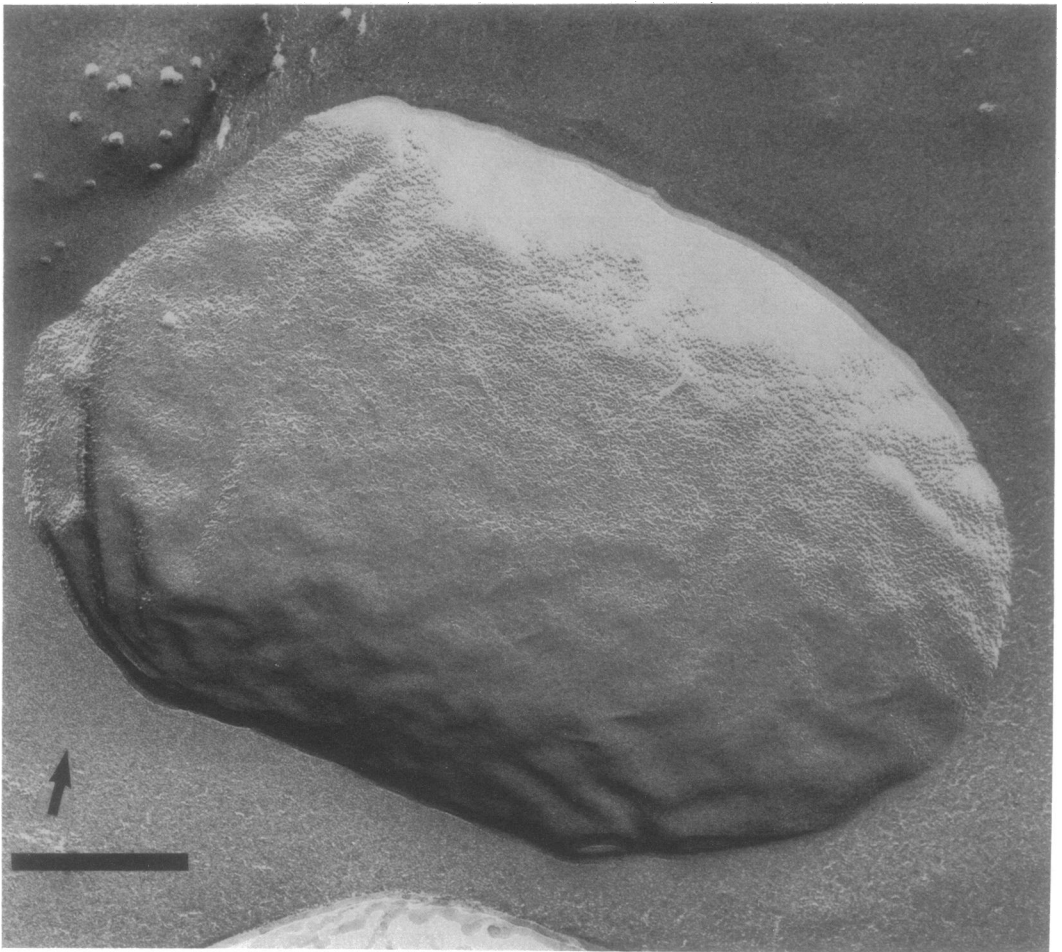


FIG. 3. Freeze etching of LT2, a wild-type strain. What is seen is the outer surface of the bacterium. Note the smooth appearance and lack of information on the underlying layers. The length of the bar corresponds to 0.2 μm in this as well as in subsequent figures. Arrows in this and subsequent figures show the direction of shadowing.



FIG. 4. Freeze etching of *his-642*, a strain with an *Ra*-type LPS and hence missing most of the saccharide chain (Fig. 1). Compare with Fig. 3, in which the wild-type phenotype is shown. Note what appears to be the outline of underlying particles. The "blebs" on the surface are likely vesicles of outer membrane material being excreted, a phenomenon often seen in these mutant strains.

The second, outer fracture plane is presumably located within the outer membrane. In this case, the particles seen almost exclusively remain with the outer leaflet, or the concave face, upon fracture (see Fig. 7; cf. Fig. 8 with Fig. 9). The inner leaflet of the outer membrane also has a very characteristic appearance: it is almost devoid of particles except for an occasional quite large and characteristic particle, the nature of which is not known (see Fig. 5, face a; Fig. 7, face b; and Fig. 8). Regardless of

differences in the appearance of the outer leaflet in various strains (see below), the appearance of the inner leaflet remained identical.

Etching was used to determine if the fracture corresponding to the outer membrane was indeed within the membrane and the particles thus seen were indeed intercalated with the membrane. This point is important because one may argue that the outer fracture plane is located between the outer membrane and the peptidoglycan layer, and that the particles seen



FIG. 5. Freeze fracture of TA2168, a "heptoseless" mutant. (a) The convex fracture face (inner leaflet) of the outer membrane. Note the few but prominent particles, and also the otherwise relatively smooth appearance. We speculate that this is because the majority of the outer membrane particles do not penetrate deeply into this leaflet. (b) Convex fracture face (inner leaflet) of the underlying cytoplasmic membrane. (c) Concave fracture face (outer leaflet) of the outer membrane. (d) Cross fracture of a cell.

in the concave face are actually lining the inner surface of the outer membrane (see reference 22). If so, the convex face of this fracture would correspond to the peptidoglycan layer with water-filled periplasmic space underneath, and etching after fracture would significantly alter

the appearance of this convex face. No such alteration was detected, however (Fig. 8). We therefore conclude, as did Branton (7) working with several eukaryotic cell types, that this finding unambiguously demonstrates that the fracture plane runs through the hydrophobic

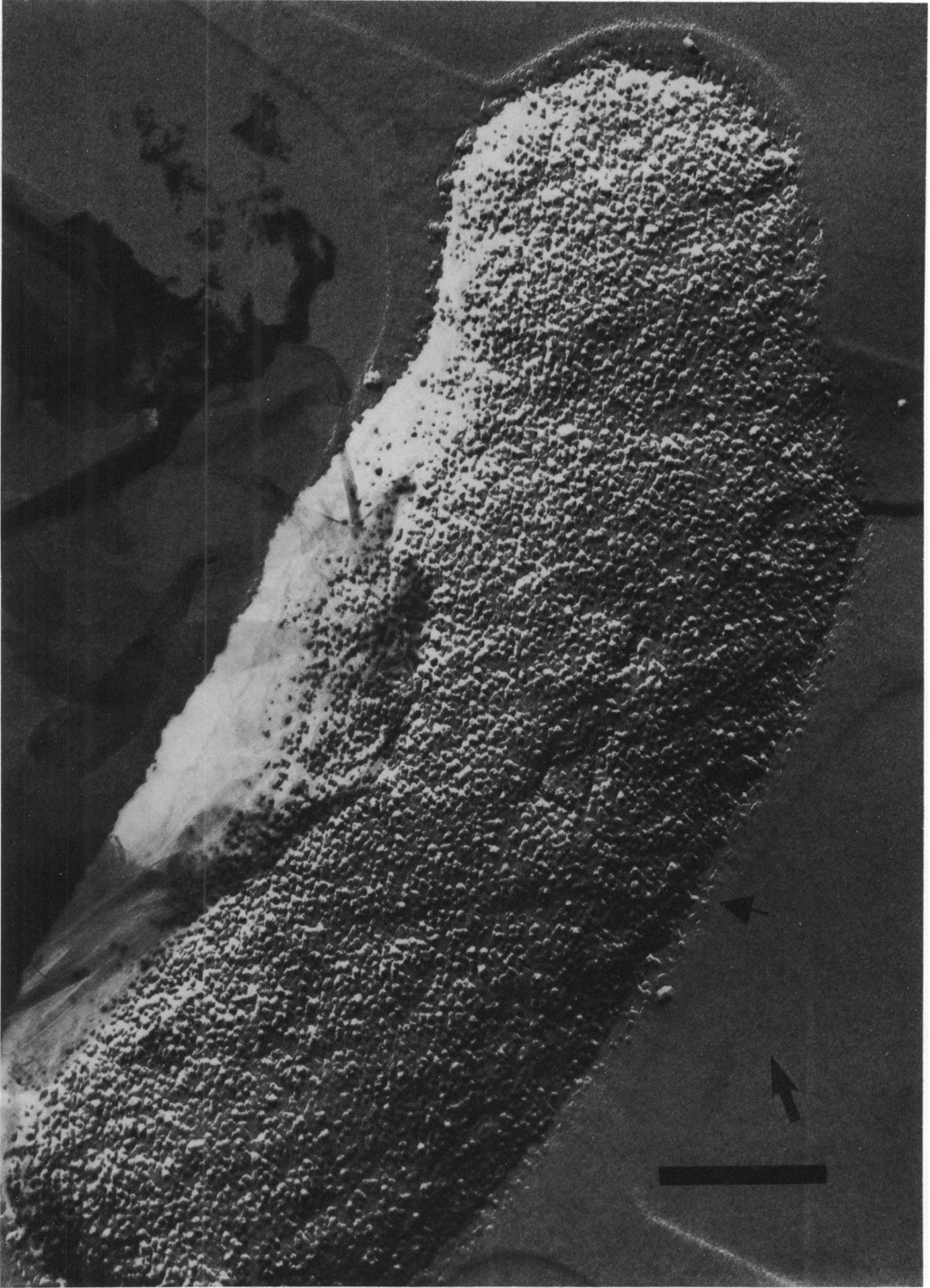


FIG. 6. Freeze fracture of LT2 (wild type) showing the convex fracture face (inner leaflet) of the cytoplasmic membrane. This appearance is typical of all strains investigated. At the border (arrow) one can see the cross-fractured outer membrane.



FIG. 7. Freeze fracture of TA2168, a "heptoseless" strain, showing both the convex and concave fracture faces of the outer membrane. (a) The concave fracture face (outer leaflet) of the outer membrane showing a sparser distribution of particles than the wild type (Fig. 9). See also Fig. 10. (b) The convex fracture face (inner leaflet) of the outer membrane, showing far fewer particles than the outer leaflet.

interior region of the membrane. van Gool and Nanninga (30) also reached the same conclusion in their freeze-fracture studies of *E. coli*.

The convex fracture face of the outer membrane sometimes takes on an appearance

rather similar to the outer surface of the cell (for example, see Fig. 8). However, the convex faces seen in nonetched, fracture pictures (Fig. 5 and 7) are most probably true fracture faces, as it is well known (7) that the fracture tech-

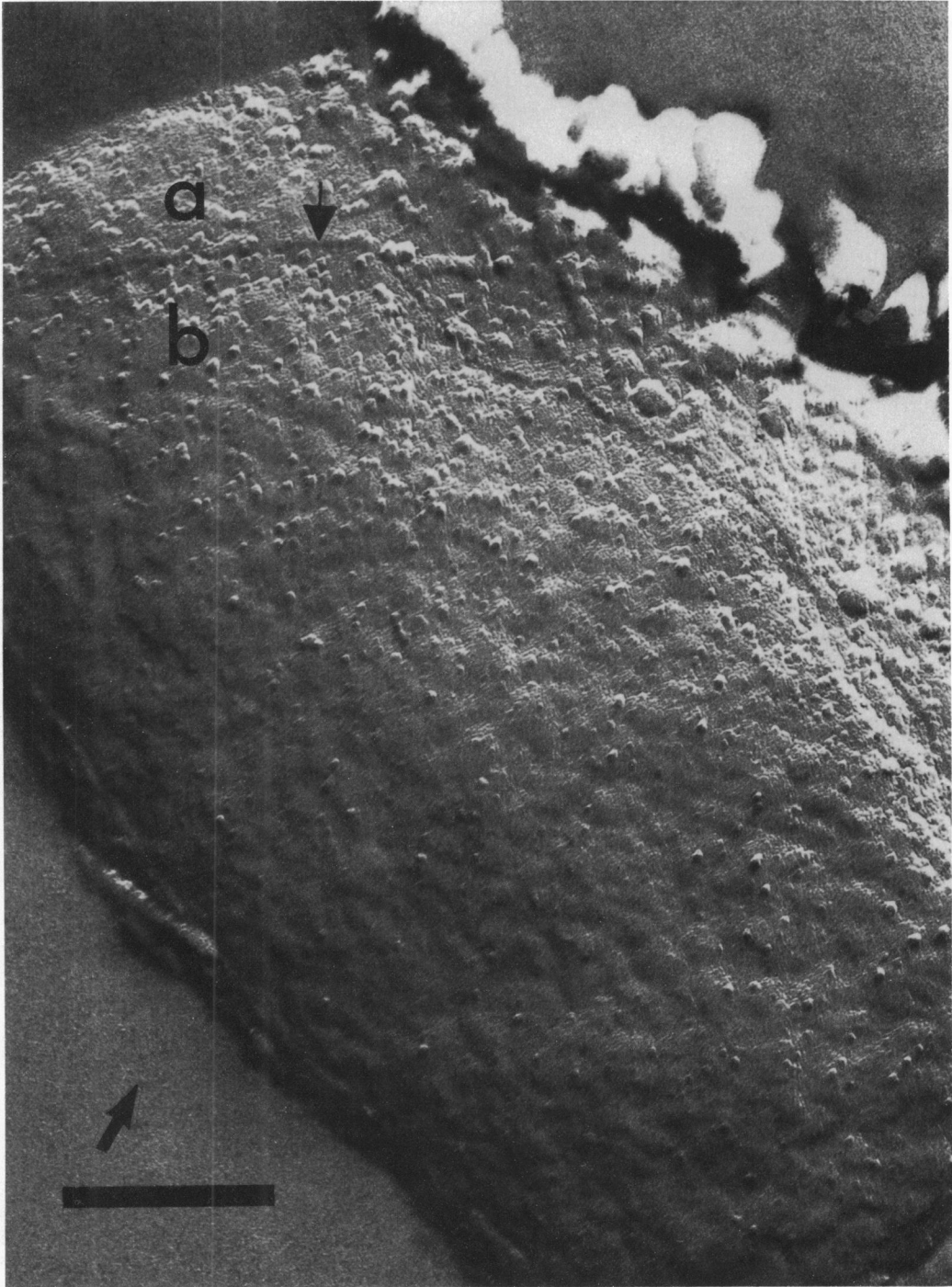


FIG. 8. Freeze fracture and freeze etching of TA2168, a "heptoseless" strain. (a) The outer surface of bacterium revealed after 2 min of etching at -100°C . (b) Convex fracture face (inner leaflet) of the outer membrane. Note that the appearance has not changed after etching for 2 min (compare with Fig. 5 and 7). Arrow shows the point of fracture.

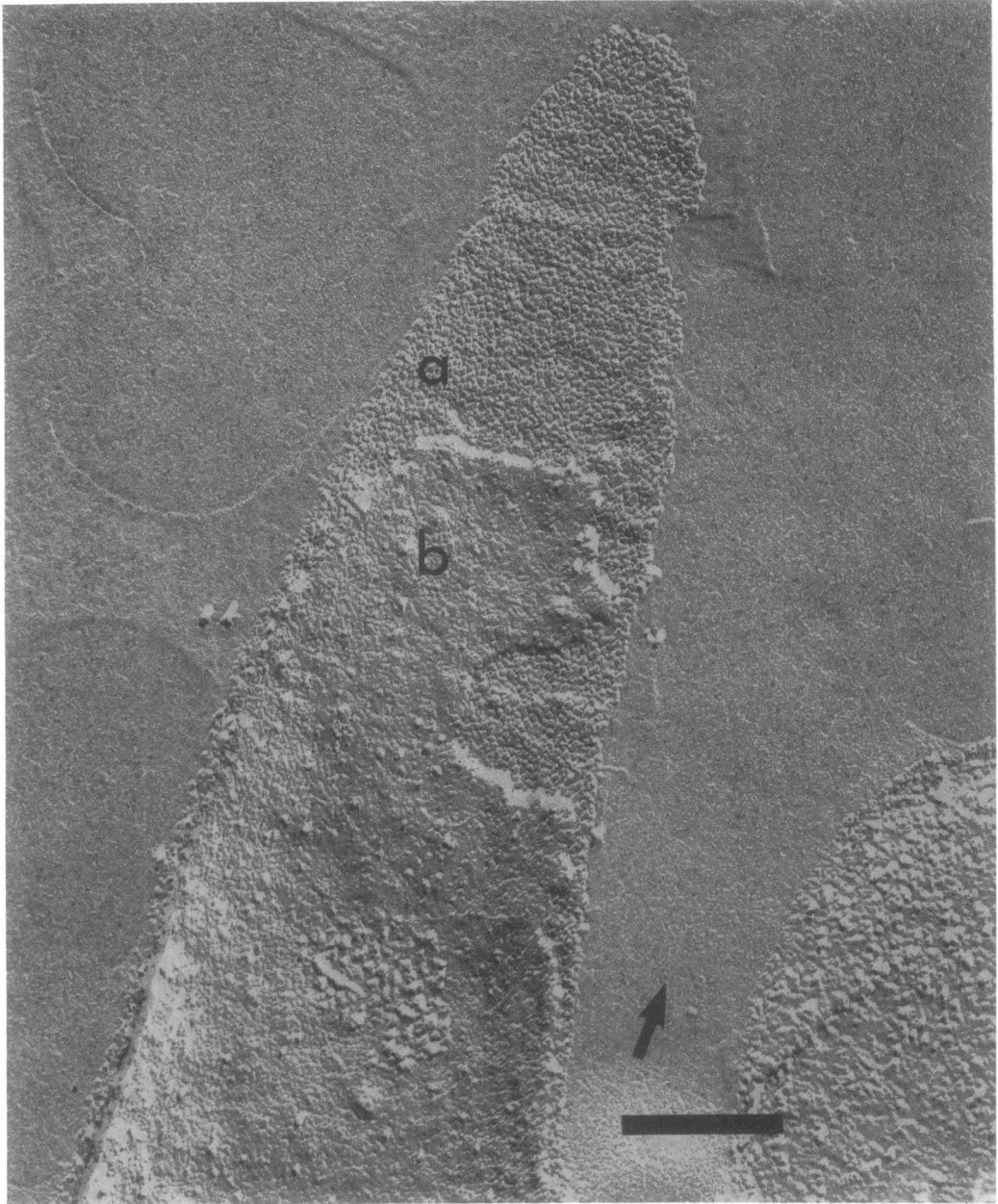


FIG. 9. Freeze fracture of HN202 showing the concave fracture faces (outer leaflets) of both membranes. (a) Concave fracture face of the outer membrane showing the dense packing of particles with almost no space between particles. Compare with Fig. 7 and 10. (b) Concave fracture face (outer leaflet) of the cytoplasmic membrane showing a sparse distribution of particles. Note that the appearance of this leaflet is rougher than the convex face of the outer membrane. There is much more evidence of the pits corresponding to the particles seen on the inner leaflet.

nique almost never results in cleavage along the surface of a membrane.

Another possible suggestion, however, is that under "nonetching" conditions of fracture

(-115 C) one may inadvertently etch away the contents of this watery space, and thus further etching under normal etch conditions (-100 C) would not cause any further change. But since

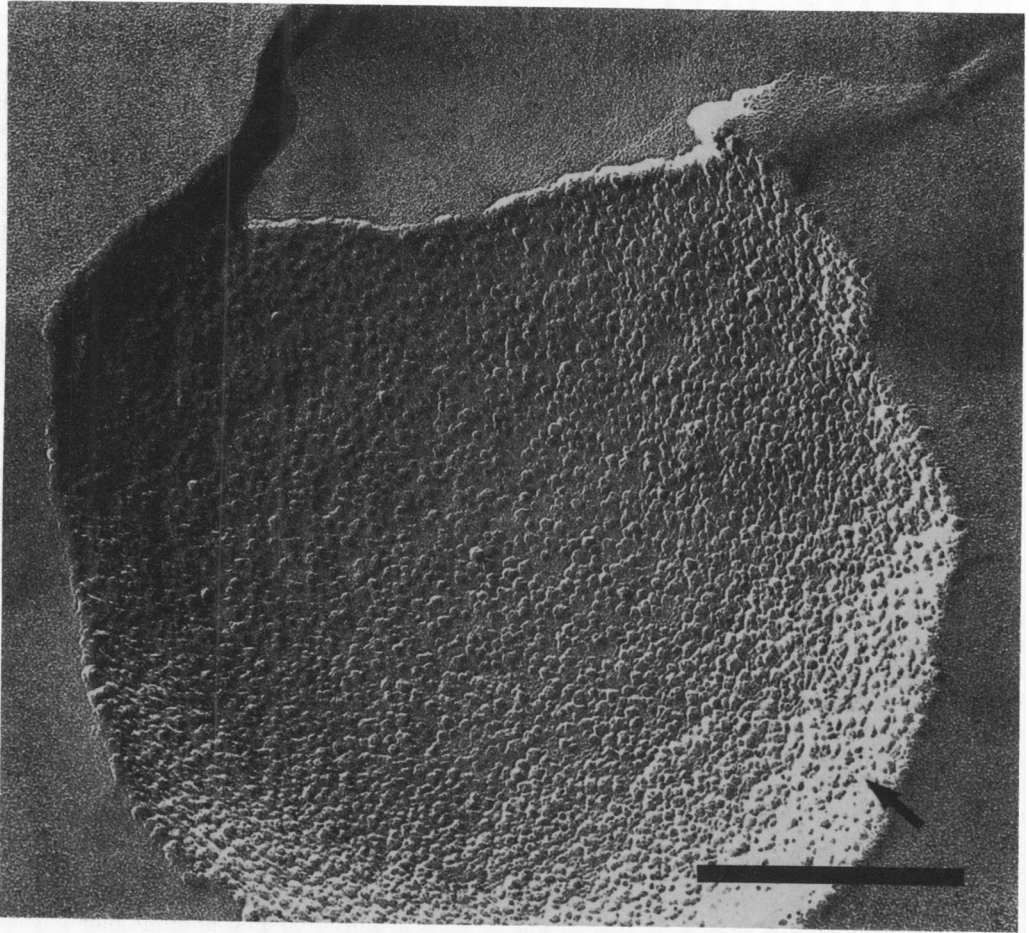


FIG. 10. Freeze fracture of TA2168, a "heptoseless" mutant. The fracture plane is through the outer membrane, showing the concave fracture face (outer leaflet). Note the smooth spaces between particles and the density of the particles as compared to Fig. 9.

replication occurs within seconds of the last pass of the knife, calculations show that under the conditions of fracture, the amount of etching would be much less than 3 nm, which is considered to be the resolution limit of the technique (15).

Differences between "deep rough" (Re) mutants and parent strains. The appearance of the outer leaflet, or concave fracture face, of the outer membrane was markedly different between S and Rc strains, on the one hand, and Re mutants on the other. In the former strains, there was a very dense arrangement of particles, which appeared to be in contact with each other (Fig. 9, face a); in the latter, the density of particles seemed much less, and "empty" areas were visible between particles (Fig. 7, face a, and Fig. 10).

When the particle densities were quantitated as described in Materials and Methods (Table 6), this visual impression was confirmed: in both of the Re mutants the density was only 62 to 65% of that found in the parent Rc strain. This finding corresponds well to the observation that the protein content per unit area in the outer membrane of Re mutants is only 59% of that in Rc strains (Table 5) and suggests that at least a large portion of these particles is composed of proteins.

Another difference between strains was in the ease of fracture of the outer membrane. (i) Fracturing of the S strain LT2 yielded almost all membrane fractures through the inner membrane (see Fig. 6), with only an occasional edge of the outer membrane being exposed. (ii) With the Rc strain HN202 the outer membrane frac-

TABLE 6. Density of particles seen on the concave fracture face of outer membrane

Strain	Photograph no.	No. of particles	Area (μm^2)	Particles/ μm^2
HN202	96764	301	0.035	8,600
	96766	560	0.0675	8,300
	96762	227	0.030	7,570
	97743	740	0.0875	8,460
	97740	315	0.0375	8,400
Average				8,320
TA2168	96828	218	0.040	5,450
	96827	436	0.090	4,840
	96831	197	0.040	4,930
	96670	831	0.016	5,190
	96833	193	0.040	4,830
Average				5,070
HN502	97476	419	0.060	6,700
	97820	877	0.018	4,870
	97822	760	0.014	5,430
	97826	958	0.0175	5,470
Average				5,410

tures occurred more often (see Fig. 9), although they were still less frequent than fractures through the inner membrane. (iii) Re mutants were fractured very easily through the outer membrane. In fact, it was more difficult to find faces corresponding to the inner membrane. Very often outer membrane fracture faces corresponding to entire halves of bacteria were found (Fig. 7, 8, and 10). This difference in fracturing ability was quite reproducible. We believe that this is most probably due to different amounts of phospholipids present in the outer membrane (see Discussion). After this work was completed, we noticed that similar differences in ease of fracture were found in a "deep rough" mutant of *E. coli* (M. E. Bayer, J. Koplów, and H. Goldfine, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, p. 167, K120).

DISCUSSION

We have previously reported (2) that mutants lacking most of the saccharide portion of LPS, i.e., "deep rough" mutants exemplified by the Rd or Re mutants, did not incorporate normal amounts of proteins into the outer membrane. Although this conclusion suffered from uncertainties related to possible differences in surface area between the mutants and parent strains, we have shown in this paper that Rd and Re mutants contained much less proteins per unit surface area of the outer membrane. Furthermore, we found that there was a compensatory increase in outer membrane phospholipids in Rd and Re mutants, whereas the number of LPS per unit area of the outer membrane remained constant in different strains.

Our analytical data put some constraints on the possible structure of the outer membrane. (i) The reduction in proteins and an apparently compensatory increase of phospholipids in Rd and Re mutants strongly suggest that at least some of the proteins in the parent strains are integral proteins and occupy significant portions of the hydrophobic region of the outer membrane. (ii) The outer membrane of the Rd and Re mutants contains more phospholipids than would be necessary for the formation of phospholipid monolayers covering the area of cell surface. This means that the outer membrane of Rd and Re mutants is most likely to contain phospholipid bilayer regions; no such constraint applies for the outer membrane of parent strains producing Rc and S-type LPS.

In addition, some other pieces of information are available on the structure of the outer membrane. (i) All LPS molecules are located in the outer half of the membrane (16; Y. Funahara and H. Nikaído, submitted for publication). (ii) One cannot detect any phospholipid head groups that are exposed on the surface of S or Rc strains, but such exposed head groups are readily detectable on the surface of Rd₁, Rd₂, and Re mutants (Y. Kamio and H. Nikaído, submitted for publication).

One can propose a tentative model of the outer membrane structure on the basis of results described above (Fig. 11). Thus, in the wild-type or Rc mutants, the outer half of the outer membrane is composed exclusively of LPS and protein molecules, whereas the inner half is essentially a phospholipid monolayer. In Rd and Re mutants, however, less proteins and more phospholipids are found. Consequently, phospholipids are now found both in the outer and the inner halves of the membrane.

This model is consistent with the results of freeze-fracture studies. (i) The concave fracture face of the outer membrane was filled with particles, which presumably are proteins (see Results; see also below); in contrast, few particles were found on the convex fracture face. These results suggest that the outer leaflet of the outer membrane contains most of the proteins, as predicted by the model of Fig. 11. An alternative explanation would be that most proteins span the entire thickness of the membrane but remain associated only with the outer leaflet upon fracture. If this were the case, we might expect to find pits on the convex face. One has to be careful because such pits might get obscured by contamination or suboptimal shadowing (29). However, we could not find any pits throughout numerous runs with varying intensities of shadow: the convex fracture

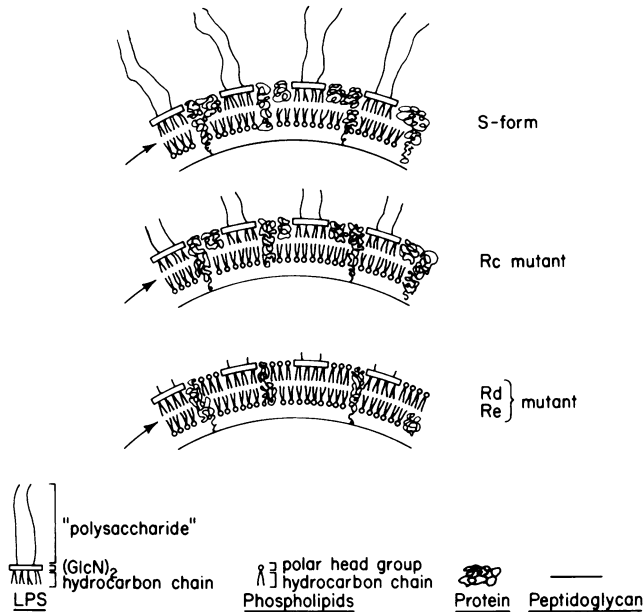


FIG. 11. Proposed model for the structure of the outer membrane in *S. typhimurium*. The arrow shows the presumed plane of fracture.

face always appeared quite smooth and structureless. (ii) That the particles are located mainly in the outer leaflet is also suggested by the observation that the outer surface of bacterium often showed patterns seemingly following the contours of the densely packed particles or protein aggregates underneath (cf. Fig. 4 with face a of Fig. 9). (iii) The outer leaflet of the outer membrane of Re mutants, according to the model (Fig. 11), should contain regions composed of phospholipids in between the protein aggregates. Freeze fracture of Re mutants indeed showed regions of smooth appearance, characteristic of phospholipid regions (7), between protein particles (Fig. 10 and face a of Fig. 7). (iv) The outer membrane of Rc mutants was slightly easier to fracture than that of the S parent, and that of Re mutants was fractured most easily. This observation is consistent with the model. In the Re mutant, fracture occurs easily because the structure of the outer membrane is closest to the phospholipid bilayer. In contrast, the outer membrane of the S strain has no phospholipid bilayer regions and thus is fractured only with extreme difficulty. The Rc mutants are intermediate in the ease of fracture, as expected from their slightly increased phospholipid content per unit area of the outer membrane (see Table 5).

Although the structure described in Fig. 11 is consistent with most of our observations, it is by no means the only hypothesis possible.

There are many possible alternatives to the main or minor features of the hypothesis. For example, the outer half of S and Rc outer membrane could contain phospholipid molecules, whose head groups are shielded underneath surface proteins. Similarly, many of the hydrocarbon chains of LPS and phospholipids may penetrate into the interior of protein molecules. Further study is needed to exclude these alternative interpretations.

Our most significant observation in comparing freeze-fracture studies with biochemical analysis of the outer membrane is the close correlation, in Re mutants, between the extent of protein reduction and that of reduction of the number of particles observed. This is more than a coincidence, since this correlation was demonstrated in two strains independently isolated. Our conclusion is that these particles are at least protein-containing entities. This, we felt, was interesting, since, in spite of the widely held belief that particles seen in freeze fracture do represent proteins, often the evidence is not so strong, except for Pinto da Silva's work with erythrocyte ghosts (20, 21). Thus, some experiments suffer from the fact that the systems are artificial (10, 28), and in other nonartificial systems only qualitative results have been obtained (26).

In the system we are studying, however, we have built-in differences in protein content in otherwise isogenic organisms and have ob-

served the differences in their natural state. We therefore feel that the reproducible correlation observed between protein content and particle number is very strong quantitative evidence showing that the particles are indeed largely proteinaceous in their composition.

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