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Supramolecular Structure of the Rigid Layer of the Cell Wall of *Salmonella, Serratia, Proteus, and Pseudomonas fluorescens.* Number of Lipoprotein Molecules in a Membrane Layer*

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ABSTRACT: The rigid layer of *Salmonella typhimurium* LT2 and *Serratia marcescens* consists of murein to which depending upon the size of the cell between 75,000 and 240,000 lipoprotein molecules are covalently bound. After digestion of the rigid layer of *Salmonella typhimurium* with trypsin, thermolysin, papain, and pronase, only lysine and arginine remained covalently bound on the murein. By analogy to *Escherichia coli* it is concluded that lysine at the N-terminal end of the lipoprotein constitutes the link between the lipoprotein molecules and the murein. On average, 1 lipoprotein molecule is bound to every tenth repeating disaccharide unit of the murein from which an average distance of about 100 Å between individual lipoprotein molecules along the polysaccharide chains is deduced. Some of the lipid is very tightly or even covalently bound to the protein so that the rigid layer is composed of murein–protein–lipid. Lysozyme digestion of the murein–lipoprotein complex of *S. typhimurium* yielded the lipoprotein with 2 repeating units of the murein still attached. From the amino acid composition and gel chromatography in 2% sodium dodecyl sulfate a molecular weight of 8000 is deduced for the lipoprotein. The lipoprotein is composed of about 60 amino acids of which about 65% are polar. It does not contain glycine, proline, histidine, cysteine, and phenylalanine. In contrast to *Salmonella*, *Serratia*, and *E. coli* no covalently linked lipoprotein could be found on the murein of *Proteus vulgaris*, *P. mirabilis*, and *Pseudomonas fluorescens*. Upon treatment of the cell envelope of *S. typhimurium* with trypsin the turbidity decreased rapidly, and in ultrathin sections it was observed that the cytoplasmic membrane and the cell wall, otherwise attached, are separated from one another.

Little is known about the structure of the rigid layer of the cell wall of Gram negative bacteria compared with the wealth of knowledge in Gram positive bacteria (see for the most recent review Ghuysen, 1968). This is partially due to the difficulty in isolating a pure rigid layer from the much more complex cell wall of Gram negative bacteria. It may also be due to the presence in the isolated rigid layer of additional material, roughly characterized as polysaccharide, protein, and lipid, beside the murein. Recently we were able to show for *Escherichia coli* (Braun and Rehn, 1969) that the protein associated with the rigid layer (Martin and Frank, 1962; Weidel and Pelzer, 1964) is in fact a lipoprotein which is covalently linked to the murein. It was found that on average 1 lipoprotein molecule is bound to every tenth repeating unit of the murein. The linkage consists of a peptide bond between the amino group of the N-terminal lysine of the lipoprotein and the carboxyl group of the optical L center of the diaminopimelic acid of the murein (Braun and Sieglin, 1970; Braun and Wolff, 1970). With trypsin the linkage between the lipoprotein molecules and the murein could be split specifically. This caused a rapid decrease in the absorbance of the cell envelope suspension, and in ultrathin sections the cytoplasmic membrane and the cell wall, normally closely associated, could be seen to be separated into two distinct layers.

We studied *Salmonella*, *Serratia*, *Proteus*, and *Pseudomonas fluorescens* to see whether the rigid layer of these Gram negative bacteria is composed of a similar structure to that of *E. coli*. *Salmonella* is known to have a continuous murein layer (Weidel *et al.*, 1963), the composition of which closely resembles that of *E. coli*. We should here like to present data which show that the total rigid layer of *Salmonella* and *Serratia* is composed of murein with covalently linked

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lipoprotein molecules. In contrast the rigid layers of *Proteus* and *Pseudomonas fluorescens* do not contain a covalently bound protein on the murein. In the case of *S. typhimurium* we will also demonstrate the specific effect of trypsin on the cell envelope.

Materials and Methods

Strains and Culture Conditions. The following strains were used: *Salmonella typhimurium* LT2, *Salmonella minnesota* mR 608 and *Salmonella usumbura* (both obtained from O. Lüderitz, Freiburg), *Serratia marcescens*, *Proteus mirabilis* ATCC 2505 which required thymine and nicotinic acid and which was streptomycin resistent (D. Helinsky, La Jolla), *Proteus mirabilis* VI and 1 Ca/5 (H. Martin, Darmstadt), *Proteus vulgaris* ATCC 13315, and *Pseudomonas fluorescens*. Unless otherwise indicated (see Table IV for *Proteus*), the cells were grown in Difco antibiotic medium 3 supplemented with additional growth factors according to the requirements of the strains; 2.5-l. bottles were inoculated with 10-ml overnight cultures and vigorously aerated at 37°, and the cells grown to a density of about 5×10^8 cells/ml. The cells were chilled with ice, spun down, and washed once with water; 3–5 g cells of was obtained. Larger quantities were grown in 16-l. bottles.

Isolation of the Rigid Layer. Cells were shaken with glass beads followed by differential centrifugation as described (Braun and Sieglin, 1970). The pellet consisting of the cell envelope was washed once with 0.01 M EDTA, pH 8, then suspended in water, and added dropwise into a boiling solution of 4% sodium dodecyl sulfate (W. Leutgeb, personal communication). The insoluble rigid layer was spun down and washed with water as described (Braun and Rehn, 1969). The sodium dodecyl sulfate treatment was repeated with the rigid layer of *S. minnesota*, *S. usumbura*, and *Proteus* and *Pseudomonas fluorescens*.

Enzymatic Digestion. The decrease of absorbance of the cell envelope suspension of *S. typhimurium* upon incubation with hydrolytic enzymes was measured at 578 nm as previously described for *E. coli* (Braun and Rehn, 1969). The thermolysin digestion followed the procedure outlined below for the large scale digestion.

Proteolytic Enzymes. Murein–lipoprotein (50 mg) was incubated with 1 mg of pronase P (Serva, Heidelberg) for 2 hr in 0.01 M Tris-HCl, pH 7.4 (Braun and Sieglin, 1970). The preparation of murein–lysine after the incubation of *Salmonella* cell envelopes with trypsin [twice crystallized (Serva) or TPCK-treated] followed the procedure described (Braun and Rehn, 1969). The digestion of the murein–lipoprotein complex of *S. typhimurium* with papain (Serva, Heidelberg) was carried out with 50 mg of the substrate and 1 mg of cysteine-activated enzyme in 25 ml of 0.01 M Tris-HCl, pH 6.4, for 4 hr at 37°. In the thermolysin digestion 1 mg of enzyme (Merck, Darmstadt) was incubated with 50 mg of murein–lipoprotein complex in 12 ml of 0.1 M Tris-HCl, pH 8.0–0.003 M $CaCl_2$ for 2 hr at 60°.

Lysozyme. The degradation of 40 mg of murein–lipoprotein complex of *S. typhimurium* with 1 mg of lysozyme (Boehringer, Mannheim) was performed in 20 ml of 0.01 M Tris-HCl, pH 7.4, for 15 hr at 34°. The reaction mixture was centrifuged for 30 min at 37,000*g* and the supernatant concentrated to 2 ml on a rotary evaporator at 40° and then chromatographed on a 30 × 2.5 cm Sephadex G75 (Pharmacia, Sweden) column with 0.01 M $NaHCO_3$. Fractions 21–27, 34–65, and 66–80 were lyophilized and the amino acid composition was determined.

Molecular Weight Determination. Lipoprotein (20 mg) obtained by lysozyme degradation of the murein–lipoprotein complex was dissolved in 2 ml of 4% sodium dodecyl sulfate–0.01 M $NaHCO_3$, together with 5 mg of horse heart cytochrome *c* (Boehringer, Mannheim) and dextran blue (Pharmacia). This mixture and the single components separately were chromatographed on a 90 × 2.5 cm Sephadex G-50 column (Pharmacia), which was equilibrated and run with 2% sodium dodecyl sulfate–0.01 M $NaHCO_3$. Three fractions of 3 ml each were collected per hr. Each fraction (1 ml) was, after hydrolysis with 2.5 N NaOH, tested with ninhydrin. Cytochrome *c* was determined at 415 nm, dextran blue at 578 nm.

Performic Acid Oxidation. The salt-free lyophilized murein–lipoprotein complex (2 mg) or the lipoprotein (2 mg) was oxidized according to Hirs (1956), slightly modified, in 2 ml of performic acid (9.5 ml of 98% formic acid, 0.5 ml of 30% perhydrol (Merck, Darmstadt)) for 6 hr at 0°. The reaction mixture was diluted to 30 ml with water and evaporated to dryness; this procedure was repeated twice and then the mixture was hydrolyzed with 2 ml of 6 N HCl for 23 hr at 100°.

Amino Acid Analyses. The amino acid analyses were performed with an Unichrom amino acid analyzer (Beckman, Munich) (Braun and Sieglin, 1970). The hydrolysis conditions are indicated in the tables. No corrections for the loss of muramic acid, serine, and glucosamine during hydrolysis were made.

Preparation of Ultrathin Sections of Cell Envelopes and Trypsin-Treated Cell Envelopes of S. typhimurium. Cells were grown in the minimal medium of Vogel and Bonner (1956) with 0.2% glucose and harvested during exponential growth phase. Cells (2 g) were suspended in 4 ml of buffer containing 0.01 M Tris chloride, pH 7.4–0.01 M EDTA and were disrupted for 7 min with 5 g of glass beads, 0.17–0.18 mm, in a Mickle type homogenizer. The mixture was diluted to 20 ml with the same buffer and centrifuged for 3 min at 4200 rpm. The sediment, consisting mainly of unbroken cells, was treated again by the same procedure. The supernatants were combined and centrifuged at 4200 rpm for 2 min and the sediment was discarded. Subsequent centrifugation for 5 min at the same speed sedimented the material for electron microscopy. This preparation contained about 5% of unbroken cells but further fractionation was avoided since extensive disintegration of the fragile inner membrane occurred during more centrifugations and suspendings. The cell walls were suspended to a protein concentration of about 5 mg/ml in 5 ml of 0.01 M Tris, pH 8.2, with 0.01 M $MgCl_2$ and 5 μg/ml of DNase and the suspension was divided into two parts. One half was left without further additions, the other one was incubated at room temperature for 30 min with 100 μg/ml of trypsin. Both samples were then fixed and embedded by the method of Kellenberger (Ryter *et al.*, 1958) and sectioned, stained, and viewed as described (Braun and Rehn, 1969).

Results

Isolation of the Rigid Layer. A one-step procedure for isolating the rigid layer of Gram negative bacteria in rather

TABLE I: Composition of the Murein–Lipoprotein Complex (Rigid Layer) of *Salmonella*.

	Murein–Lipoprotein Complex[a]					
	Untreated A	Performic Acid Oxidized B	Trypsin Digested C	Papain Digested D	Thermolysin Digested E	Pronase Digested F
Lysine	4.98	5.01	1.04	0.98	0.99	0.60
Arginine	3.95	4.03	0.42	0	0.54	0.50
Aspartic acid	15.02	14.78	0	0	0	0
Threonine	1.87	2.10	0	0	0	0
Serine	6.35	5.84	0	0	0	0
Glutamic acid	19.14	20.50	12.70	12.40	12.78	11.90
Alanine	27.55	31.24	19.40	18.97	18.82	19.10
Valine	3.74	3.97	0	0	0	0
Methionine	*b*	0.91[c]	0	0	0	0
Isoleucine	0.93	1.20	0	0	0	0
Leucine	3.84	4.04	0	0	0	0
Tyrosine	1.05	0.66	0	0	0	0
Muramic acid	8.95	9.47	8.72	8.42	9.10	8.93
Diaminopimelic acid	10.00	10.00	10.00	10.00	10.00	10.00
Glucosamine	9.72	9.57	8.92	8.67	9.42	8.86

[a] Samples A and C–F were hydrolyzed with 4 N HCl, 15 hr at 100°, sample B with 6 N HCl, 23 hr at 100°. The amino acid residues are referred to 10 residues of diaminopimelic acid which equals the number of murein repeating units per 1 lipoprotein molecule. The values for histidine, proline, glycine, cysteine, and phenylalanine were below 0.3 residue. Tryptophan was not determined. [b] Methionine was not completely separated from diaminopimelic acid. [c] Methionine determined as methionine sulfone.

pure form involves treatment of the cell envelope with hot 4% sodium dodecyl sulfate. Cells were first opened mechanically by shaking with glass beads thus, under our conditions, maintaining the cell envelope, as examined by electron microscopy, intact, and the cytoplasm can be removed by differential centrifugation. The cell envelope consisting of the cytoplasmic membrane and the cell wall is then dissolved in hot 4% sodium dodecyl sulfate except for the covalently linked supramolecular structure of the rigid layer which can then be easily obtained by centrifugation. It is important to use freshly grown or frozen cells, preferentially harvested in the logarithmic growth phase. With lyophilized cells or cells pretreated with phenol–water (Westphal *et al.*, 1952) we could not prepare a pure rigid layer. The purity of the rigid layer, as will be shown below, can best be checked by the absence of amino acids which are not constituents of the rigid layer or by the absence of β-hydroxymyristic acid and 2-keto-3-deoxyoctonic acid which both are typical constituents of the lipopolysaccharide.

Structure of the Rigid Layer (Murein–Lipoprotein Complex) of Salmonella and Serratia. Salmonella. The amino acid analysis of the rigid layer yielded the murein-specific constituents muramic acid and diaminopimelic acid and all common amino acids except histidine, glycine, proline, cysteine, and phenylalanine (Table I, A, B).

Alanine and glutamic acid are known to be constituents of the murein of *Salmonella* (Weidel *et al.*, 1963) but the other amino acids present in considerable amounts (aspartic acid, for example, exceeds that of diaminopimelic acid) should be constituents of a protein either covalently bound to the murein or intimately associated with it. The latter possibility is unlikely since the preparation with hot 4% sodium dodecyl sulfate should dissolve most of the protein material not chemically bound. On the other hand in *E. coli* B a glycogen apparently not bound to the murein is held back in the murein sacculus (Leutgeb and Weidel, 1963). In the following we present evidence that a defined protein is covalently bound to the murein, this protein being a lipoprotein where the lipid most probably is also covalently bound to the protein (murein–protein–lipid).

Degradation of the rigid layer with proteolytic enzymes resulted in a murein free of protein. The only amino acids which remained on the murein and which are not constituents of the murein itself were lysine and arginine. It appeared that lysine and arginine constituted the covalent link between the murein and the lipoprotein. After incubation with trypsin or papain or thermolysin the same amount of lysine stayed on the murein, this being 1 residue per 10 disaccharide units of the murein (Table I, C, D, E). When this lysine was taken as unity for calculating the amino acid composition of the lipoprotein integral values were obtained for most of the amino acids (Table I, A, B). The smallest values, methionine, isoleucine, and tyrosine, thus amounted to 1 residue which was a good indication that our basis for calculating the number of amino acid residues, and thus the molecular weight of the protein, was either correct or that the true molecular weight is a multiple of this figure. Since the lipoprotein molecules were apparently covalently linked

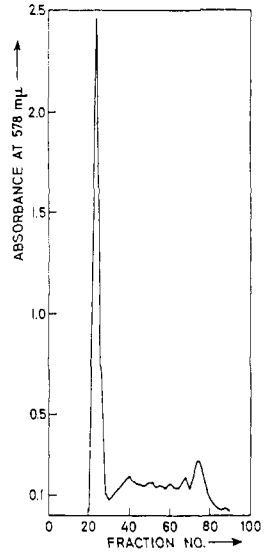

FIGURE 1: Gel chromatography on Sephadex G-75 of the degradation products of murein–lipoprotein complex obtained with lysozyme. The degradation products from 40 mg of murein–lipoprotein complex were run on a 30 × 2.5 cm column with 0.01 M $NaHCO_3$. Four fractions of 2.4 ml were collected per hr. Every fraction (0.2 ml) was tested with ninhydrin after alkaline hydrolysis and the absorbance was measured at 578 nm.

TABLE II: Composition of the Lipoprotein with the Murein Attachment Site after Lysozyme Degradation of the Murein–Lipoprotein Complex.[a]

	Lipoprotein with Murein Attachment Site			
	Untreated A	Performic Acid Oxidized B	Lipoprotein C	Murein Attachment Site D
Lysine	4.85	5.02	5	
Arginine	4.21	4.24	4	
Aspartic acid	15.21	15.01	15	
Threonine	1.82	2.19	2	
Serine	6.72	6.25	6	
Glutamic acid	8.92	9.51	7	2
Alanine	13.65	13.18	9–11	2–3
Valine	2.59	3.83	3–4	
Methionine	*b*	0.98[c]	1	
Isoleucine	0.85	0.96	1	
Leucine	3.60	4.28	4	
Tyrosine	0.98	0.52	1	
Muramic acid	1.83	1.80		2
Diaminopimelic acid	1.76	1.94		2
Glucosamine	2.05	1.87		2

[a] The lipoprotein (A) was hydrolyzed with 4 N HCl, 15 hr at 100°; after performic acid oxidation (B) it was hydrolyzed with 6 N HCl, 23 hr at 100°; (C) tentative amino acid composition of the lipoprotein after subtraction of the constituents of the murein attachment site (D). [b] Methionine was not completely separated from diaminopimelic acid. [c] Methionine determined as methionine sulfone.

to the murein we degraded the murein with lysozyme to determine the molecular weight of the lipoprotein. From earlier experience with *E. coli* we could assume that lysozyme should leave 1 or 2 repeating units of the murein on the lipoprotein (Braun and Wolff, 1970). On gel filtration with Sephadex G-75 (Figure 1) or Bio-Gel P 300 the lipoprotein was eluted with the void volume (fractions 21–27) indicating an apparent molecular weight of more than 300,000. In the fractions following the lipoprotein peak only constituents of the murein were found. The amino acid composition of the lipoprotein in the first peak is given in Table II, A and B, based on the same calculation as in Table I. The values for the amino acids occurring only in the lipoprotein agree well with those found in the intact murein–lipoprotein complex (Table I, A, B). Instead of 10 only 2 repeating units of the murein were still bound on the lipoprotein (see values for muramic acid, diaminopimelic acid, glucosamine). The composition of the murein repeating unit can also be obtained from the pronase-digested murein–lipoprotein complex (Table I, F) from which the values for alanine and glutamic acid, present in the murein repeating unit, were obtained. Since only two repeating units of the murein remained bound on the lipoprotein a quite accurate amino acid composition of the lipoprotein could be deduced, including the values of alanine and glutamic acid. In *E. coli* we found that the lipoprotein molecules replace the D-alanine at the attachment site on the murein. Therefore for 2 repeating units of the murein, only 2–3 alanine residues were listed in Table II, D. By subtraction of the murein attachment site from the lipoprotein an amino acid composition as given in Table II, C follows for the lipoprotein. The protein consists of about 60 amino acids from which a molecular weight of 7000 follows. In *E. coli* we have evidence that the size of the lipid is not more than 1500 (V. Braun and K. Hantke, unpublished). So far as can be judged at present the lipid of *Salmonella* is similar to that of *E. coli* (see below). The protein, the lipid, and the 2 repeating units of the murein together add up to a molecular weight of 10,000 in contrast to that found by gel chromatography of more than 300,000. This discrepancy could be due to aggregation. We found indeed that only in strong detergent solutions could complete disaggregation of the lipoprotein be achieved. We dissolved the lipoprotein obtained from the lysozyme degradation in 4% hot sodium dodecyl sulfate and chromatographed it on Sephadex G-50 with 2% sodium dodecyl sulfate. Under these conditions it cochromatographed with horse heart cytochrome *c* which has a molecular weight of 12,000 (Margoliash and Schejter, 1966). The correspondence of the molecular weights derived from the amino acid composition and that determined by gel chromatography is satisfactory and supports the calculation of the amino acid composition. The lysine remaining on the murein after trypsin, papain, or thermolysin digestion therefore corresponds to 1 amino acid of 1 lipoprotein molecule and we think that, as in the case of *E. coli* (Braun and Sieglin, 1969; Braun and Wolff, 1970), this lysine is the

N-terminus of the lipoprotein and forms the linkage to the murein.

S. usumbura cell envelopes were used for preparing the murein–lipoprotein complex after the lipopolysaccharide was extracted by the phenol–water procedure (Westphal *et al.*, 1952). In the case of *S. minnesota* we started from lyophilized cells. From neither preparation, even after repetitive treatment with 4% hot sodium dodecyl sulfate, did we obtain a pure rigid layer. Instead contaminating amino acids such as glycine, phenylalanine, and histidine in nonstoichiometric amounts were still present. But despite these by-products the analyses we obtained were clear enough to state that between *S. typhimurium*, *S. usumbura*, and *S. minnesota* no significant differences in the composition of the murein–lipoprotein complex exist.

Evidence for a Lipoprotein. After hydrolysis of the murein–lipoprotein complex with 4 N HCl or 6 N HCl at 100° (between 2 and 24 hr) material remained undissolved. A part of the residue was soluble in chloroform–methanol (2:1) or petroleum ether. Two moving spots were identified by thin-layer chromatography on silica gel (Figure 2, II). The spot with the lower R_F value cochromatographed with palmitic acid and in *E. coli* palmitic acid was identified by gas chromatography and mass spectrometry as the main component in this spot (Braun and Rehn, 1969). Nothing could be observed in the region where β-hydroxymyristic acid would chromatograph (Figure 2, I) which shows that no contaminating lipopolysaccharide was present. After degradation of the murein–lipoprotein complex with lysozyme the lipid stayed with the protein (Figure 2, III) which was purified by gel filtration (Figure 1) or by precipitation with acid. When the murein–lipoprotein complex was degraded with pronase, most of the lipid went into solution together with the solubilized peptides. A smaller part of the lipid and some peptides remained insoluble, as does the murein, but could be removed with the peptides from the murein by detergent treatment. The lipid stays firmly with the protein and is solubilized with the protein when the murein is degraded with lysozyme or when the protein is cleaved from the murein by degradation into peptides. Therefore, the lipid is bound to the protein, thus forming a lipoprotein. By no physical method including treatment with detergent solutions or organic solvents with different polarities could that portion of the lipid, containing after hydrolysis the palmitic acid spot, be removed from the protein. Either this portion represents a tightly bound contamination or this lipid is covalently linked to the protein as perhaps is the case in *E. coli*, where after extensive degradation of the protein with various proteases a peptide containing mainly serine, aspartic acid, and alanine could be isolated which remains bound to the lipid (V. Braun and K. Hantke, unpublished).

Number of Lipoprotein Molecules per Cell. The number of lipoprotein molecules per cell is a function of the size of the cell which largely depends on the growth conditions used and the growth state at the time of harvesting. Although the cell content and the cell envelope can vary qualitatively and quantitatively (see, *e.g.*, Maaloe and Kjeldgaard, 1966) a measure of the size of the cell surface is obtained by determination of the cell mass. From 200 g of *S. typhimurium* cells (wet weight) which corresponds to 44.8 g in the lyophilized state, 810 mg of murein–lipoprotein complex was obtained. After lysozyme digestion (6 mg of lysozyme in 240 ml of 0.001 M ammonium acetate, pH 6.9, 3 hr, 32°) 322 mg of lipoprotein was precipitated by lowering the pH to 3.2 with acetic acid. This lipoprotein contained only 1.5 disaccharide units of the murein. By repetitive extraction with warm chloroform–methanol 2:1, acetone, petroleum ether, and ether the lipoprotein lost 32% of its weight. To be sure that by this treatment no lipoprotein went into solution, a sample of the undegraded murein–lipoprotein complex was extracted in the same way. A weight reduction of 18% was obtained, again demonstrating that the murein part of the total complex contains no lipid. To account for possible loss in moisture during the treatment with organic solvents the same procedure was applied to several proteins including a crude enzyme extract from the pancreas. A weight loss of 3% was found. It therefore appears that 29% of material, tentatively assigned as hydrophobically bound lipid, could be removed by the treatment with organic solvents. No loss of protein occurred and no change in the amino acid composition was detected. The lipid component with the highest R_F value in Figure 2 was however absent. Other differences in the extracted lipoprotein were apparent in that, for example, the lipoprotein could no longer be precipitated with acid after lysozyme degradation of the murein–lipoprotein complex. We assume then that after the treatment with strong solutions of detergents and organic solvents the product consists mainly of a covalent structure. This means that 688 mg of murein–lipoprotein complex yields 229 mg of lipoprotein plus the murein attachment site.

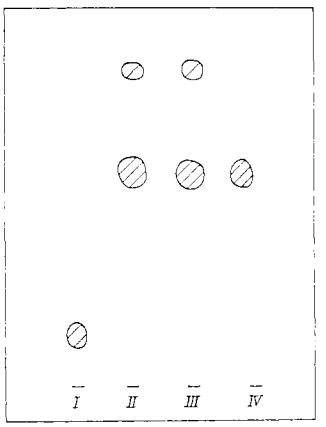

FIGURE 2: Thin-layer chromatography on silica gel. (I) β-Hydroxymyristic acid, (II) hydrolysate of the murein–lipoprotein complex, (III) hydrolysate of the lipoprotein obtained by lysozyme degradation of the murein, (IV) palmitic acid. Thin-layer plates of silica gel F_{254} on aluminium sheets (Merck, Darmstadt) were developed after heating for 10 min at 100° with a mixture of petroleum ether (60–80°)–ether–acetic acid (70:30:2). The spots were identified at 366 nm after the plate was exposed to iodine vapor and then dipped into a solution of 0.25% rhodamine B in ethanol.

From the same batch of cells the mass per cell was determined by counting the number of cells with the coulter counter. Grown in rich medium (Difco, M3) and harvested in the logarithmic growth phase one cell had a mass of 0.66×10^{-12} g. A weight of 44.8 g of cells is then equivalent to 6.78×10^{13} cells. With a molecular weight of 8500 for the lipoprotein with 1.5 repeating units of the murein still attached 229 g corresponds to 162×10^{17} lipoprotein molecules. Therefore one cell contains on the murein 240,000 lipoprotein mole-

TABLE III: Composition of the Murein–Lipoprotein Complex (Rigid Layer) of *Serratia marcescens*.

	Murein–Lipoprotein Complex[a]				Murein Present in C	Lipoprotein Calculated	
	Untreated A	Performic Acid Oxidized B	Untreated C	A Pronase Digested D	E	F	G
Lysine	5.00	5.00	5.00	1.1		5	5
Histidine	1.04	0.85	0.67			1	1
Arginine	4.37	3.97	3.74			4	4
Aspartic acid	15.00	15.00	15.00			15	15
Threonine	1.23	1.02	1.12			1	1
Serine	6.58	6.31	7.21			6	7
Glutamic acid	27.34	27.91	21.30	20.9	14	7	7
Alanine	46.79	46.90	41.13	33.8	28	13	13
Valine	3.50	3.72	3.78			4	4
Methionine		1.07[b]				1	
Isoleucine	1.28	1.23	1.13			1	1
Leucine	3.79	4.04	4.24			4	4
Tyrosine	0.96	0.72	0.92			1	1
Muramic acid	14.71	13.12	11.47	14.5	12		
Diaminopimelic acid	17.17	17.21	14.08	17	14		
Glucosamine	16.39	13.58	12.94	15.05	13		

[a] Two preparations of the murein–lipoprotein complex are given (A and C). The samples A and C were hydrolyzed with 4 N HCl, 15 hr at 100°; the sample B was hydrolyzed with 6 N HCl for 23 hr at 100°. [b] Methionine was determined as methionine sulfone. Amino acids not listed were below 0.3 residue. Tryptophan was not determined. The amino acid composition of the lipoprotein in preparation A was calculated (F) by subtracting the murein constituents determined after pronase digestion of the total complex listed in column D and for the values listed in column G by taking the amount of diaminopimelic acid (E) as being representative for the murein content in preparation C.

cules. For cells growing in a glucose minimal salt medium (Vogel and Bonner, 1956) and harvested in the logarithmic growth phase a cell mass of 0.346×10^{-12} g was determined and correspondingly the number of molecules per cell was calculated to be 125,000. For cells grown in the same medium to the stationary phase 75,000 molecules were determined and in an enriched glucose medium 168,000 molecules per cell were found.

Serratia marcescens. The rigid layer of *Serratia marcescens* was found to be similar to that of *E. coli* and *Salmonella* in that it consists of murein with covalently linked lipoprotein (Table III, A,B,C). The composition of the murein resembles that (Table III, D) found before (Mandelstam, 1962). But in the total rigid layer there was slightly less lipoprotein on the murein compared with *E. coli* or *Salmonella*. In *E. coli* and *Salmonella* we found a range of 10–12 murein repeating units per 1 lipoprotein molecule. In *Serratia* this range was between 14 and 17 (Table III, D,E). Again after pronase digestion lysine remained on the murein of *Serratia* (Table III, D), suggesting the same type of linkage between the lipoprotein molecules and the murein as in *E. coli* and *Salmonella*.

The amino acid composition and the size of the lipoprotein very nearly equals that of *E. coli* and *Salmonella*. Clear differences were recognized in the methionine content in that *Serratia* contains 1 residue, *Salmonella* 1, and *E. coli* 2 and in the threonine content where *Serratia* contains 1 residue, *Salmonella* 2, and *E. coli* 2. In addition we found in the lipoprotein of *Serratia* 1 histidine residue which may be a contaminant since the amount varies to some extent in different preparations.

Concerning the lipid, we found after acid hydrolysis the same main spot cochromatographing with palmitic acid as with *E. coli* and *Salmonella*.

Structure of the Rigid Layer of Proteus and Pseudomonas fluorescens. When the rigid layer of *Proteus vulgaris* and *Pseudomonas fluorescens* was prepared with hot 4% sodium dodecyl sulfate to our surprise a murein nearly free of protein was obtained (Table IV, A). Treatment twice with hot 4% sodium dodecyl sulfate was necessary to obtain preparations as pure as shown. Otherwise there remained with the murein residual protein (10–20% w/w) which had an amino acid composition totally different from the lipoprotein on the murein of *E. coli*, *Salmonella*, and *Serratia* in that it contained, for example, glycine as one of the major amino acids. Aspartic acid as the main amino acid is listed in Table IV to indicate the contaminating protein. Beside *Proteus vulgaris* we isolated the rigid layer from 3 strains of *Proteus mirabilis* including those for which a covalently linked protein was claimed (*Proteus mirabilis* VI and 1 Ca/5). To exclude dependency on culture conditions we grew these strains in the same medium as was used earlier (Martin, 1964). In none of these strains could we find a significant amount of a covalently linked protein on the murein after preparation of the rigid layer

TABLE IV: Composition of the Murein (Rigid Layer) of *Proteus*.[a]

	P. vulgaris A	*P. vulgaris* B	*P. mirabilis* C	*P. mirabilis* VI D	*P. mirabilis* 1 Ca/5 E
Glutamic acid	1.09	1.42	1.15	1.01	1.18
Alanine	1.85	1.84	1.93	1.95	1.89
Muramic acid	0.82	0.92	0.89	0.84	0.87
Diaminopimelic acid	1.00	1.00	1.00	1.00	1.00
Glucosamine	0.87	0.93	0.91	0.86	0.90
Aspartic acid	0.04	0.07	0.05	0.15	0.07

[a] All samples were hydrolyzed with 4 N HCl for 15 hr at 100°. The values are referred to diaminopimelic acid as 1 residue. A, B, D, and E were grown in the medium of Martin (1964); C was grown in Difco antibiotic medium 3. The rigid layers of A, C, and E were prepared with the standard procedure with one additional sodium dodecyl sulfate treatment. In B the cells were first shaken in 0.8% sodium dodecyl sulfate and then dropped into hot 4% sodium dodecyl sulfate. In D the cells were directly put into hot 4% sodium dodecyl sulfate, shaken again with glass beads, and put into hot 4% sodium dodecyl sulfate.

TABLE V: Composition of the Murein (Rigid Layer) of *Pseudomonas fluorescens*.[a]

	Murein	Murein after Pronase Digestion
Lysine	1.14	0
Arginine	1.46	0
Aspartic acid	1.49	0
Threonine	0.95	0
Serine	1.30	0
Glutamic acid	15.51	10.08
Alanine	24.75	17.86
Leucine	1.05	0
Tyrosine	0.44	0
Muramic acid	8.14	8.23
Diaminopimelic acid	10.00	10.00
Glucosamine	8.83	8.92

[a] Hydrolysis for 15 hr with 4 N HCl at 100°. Amino acids common in protein which are not listed were below 0.1 residue referred to 10 residues of diaminopimelic acid.

with the sodium dodecyl sulfate procedure. Since solubilization of the cell envelope with hot 4% sodium dodecyl sulfate proceeds at neutral pH, a chemical cleavage even of an especially sensitive bond between the murein and the protein is considered to be unlikely. To exclude enzymatic cleavage of the protein from the murein during isolation, cells after growth were directly added into boiling 4% sodium dodecyl sulfate and the insoluble part washed twice with the detergent solution. The composition of this material is listed in Table IV, D. Even under these unfavorable conditions the yield of protein material as indicated by the value of aspartic acid is only 15% of diaminopimelic acid. We therefore conclude that *Proteus* does not have an *E. coli*- or *Salmonella*-like covalently linked protein on the murein. We also did not find any indications of lipid in our murein preparations.

In the murein of *Pseudomonas fluorescens* (Table V), prepared with the standard procedure, a low amount of protein was present which we consider as contamination. Murein from cells directly treated with hot 4% sodium dodecyl sulfate without prior removal of the cytoplasm yielded a similar composition. Pronase treatment of such a murein preparation and subsequent washing with sodium dodecyl sulfate resulted in a murein totally free of protein (Table V). The composition of this murein is the same as in *E. coli*, *Salmonella*, *Serratia*, and *Proteus*.

Effect of Hydrolytic Enzymes on the Ultrastructure of the Cell Envelope of Salmonella typhimurium. When a cell envelope suspension of *S. typhimurium* was treated with hydrolytic enzymes, proteases, glycosidases including lysozyme, and phospholipases as described for *E. coli* (Braun and Rehn, 1969) the only strong effect, as measured by the decrease of the absorbance, was observed with proteases (Figure 3). This points to the essential role of proteins as structural elements in the cell envelope of *S. typhimurium*. This was also found for the inner mitochondrial (Fleischer *et al.*, 1967) and the mycoplasma membrane (Morowitz and Terry, 1969) where over 90% of the lipid could be extracted without major change in the appearance of the membrane. Compared

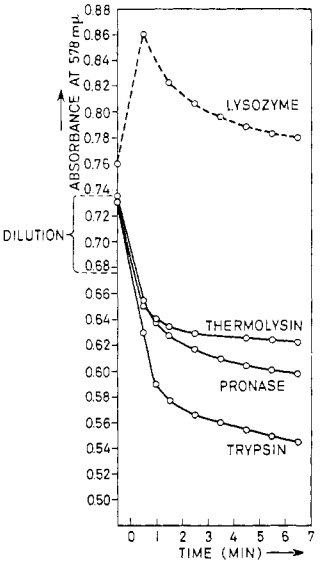

FIGURE 3: Decrease of absorbance of a cell envelope suspension of *S. typhimurium* incubated with various hydrolytic enzymes. The incubation was done with a ratio of enzyme/total cell envelope protein of 1:50 in 0.01 M Tris-Cl buffer at pH 8.2 (trypsin and thermolysin) and pH 7.4 (pronase and lysozyme). The decrease of the absorbance was measured at room temperature with a photometer "Eppendorf" 1101 M at 578 nm.

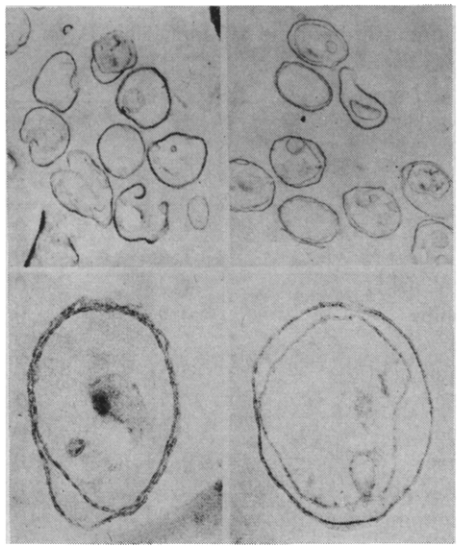

FIGURE 4: Survey pictures of ultrathin sectioned cell envelopes of *S. typhimurium* before (left) and after (right) trypsin treatment, magnification ×13,333; lower part: single cell envelope of *S. typhimurium* before (left) and after (right) trypsin treatment, magnification ×53,333.

with the decrease caused by pronase and thermolysin the decrease with trypsin is faster especially in the first 2 min.

When viewed in the electron microscope, the cell wall preparation under some precautions consists mainly of unbroken envelopes that appear as a combination of two membranes: an outer one that is presumably the true cell wall (plastic and rigid layer) and an inner one that is probably the cytoplasmic membrane (Figure 4 left part). After trypsin treatment for 20 min at room temperature an almost complete separation of the two membranes (Figure 4 right part) took place which were more or less closely associated before the enzyme treatment.

The decrease of the absorbance upon trypsin treatment of *Salmonella* cell envelopes was not as pronounced as with *E. coli*. This may be explained by the observation (see survey pictures, Figure 4, upper half left) that the cell envelopes show already parts of the inner layer detached from the outer layer which was not the case in *E. coli*. But it is important to note that after trypsin treatment in all cell envelopes the contact between the inner layer and the outer layer is abolished.

Discussion

According to the wide range of the cell size 75,000–240,000 lipoprotein molecules were found to be present in the rigid layer. Assuming negligible turnover in rapidly growing cells the number of polypeptide chains synthesized per growth cycle exceeds that of an induced enzyme like β-galactosidase or a derepressed enzyme like alkaline phosphatase. For example, in rich medium 50 polypeptide chains of alkaline phosphatase are made per second (Maaloe and Kjeldgaard, 1966) which within a doubling time of 25 min corresponds to 75,000 chains per cell. Under these growth conditions about 240,000 lipoprotein molecules are bound to the murein of 1 cell. Compared with the most abundant proteins under normal conditions, those of the ribosomes (Schleif, 1967), the lipoprotein molecules are present in about 10 times the amount of individual ribosomal proteins.

The lysine remaining on the murein after proteolytic digestion points to the same covalent linkage between the murein and the lipoprotein molecules for all 3 strains. In *E. coli* it was found that the lipoprotein molecules are linked by their N-terminus lysylarginine to the carboxyl group of the optical L center of diaminopimelic acid (Braun and Sieglin, 1970; Braun and Wolff, 1970). In *E. coli* it was mostly the case that digestion with trypsin or pronase resulted in only 1 lysine, or 1 lysine and 1 arginine, respectively, being left per 10 repeating units of the murein. In *Salmonella* and *Serratia* this was not as clear. In *Salmonella* less arginine and in *Serratia* no arginine remained on the murein after pronase treatment. These small differences in the digestibility of the murein–lipoprotein complex point to minor differences in the structure. The variability found in the amino acid composition is so far limited to methionine, threonine, and perhaps histidine where differences of 1 residue between the 3 strains were identified. Whether these are amino acid replacements or whether the lipoprotein molecules differ slightly in their length cannot be decided yet because the values for alanine and glutamic acid, present both in the murein and the lipoprotein, cannot be determined accurately enough.

In *Proteus* our findings do not support the existence of a covalently linked protein on the murein. However, it is difficult to obtain protein-free murein and it is likely that protein is intimately attached to the murein, but not chemically bound. This may explain earlier observations of protein present in murein preparations of *Proteus* (Martin, 1964) for which a covalent linkage was claimed. But already in those analyses the protein content of the murein preparations differed considerably for two closely related *Proteus* strains. In the light of our finding that the lipoprotein content and the lipoprotein composition of *E. coli*, *Salmonella*, and *Serratia* are nearly identical such a variability within *Proteus* (*Proteus mirabilis* VI and *Proteus mirabilis* 1 Ca/5) is very unlikely. The absence of the covalently linked lipoprotein on the murein of *Proteus* may also be responsible for the fact that from *Proteus* stable L forms can be obtained but with *E. coli* many attempts to isolate these have failed.

In *Salmonella*, on the average, for every tenth repeating unit of the murein there is 1 lipoprotein molecule covalently bound. This allows a tentative deduction concerning the molecular architecture of the rigid layer. The length of one β-1,4-linked disaccharide unit is 10.3 Å (Dweltz, 1961; Carlström, 1962). The polysaccharide chains of the murein are rigid molecules which follows also from model building with Dreiding models. One can, therefore, deduce that the average distance between lipoprotein molecules along the polysaccharide chains is about 100 Å. The same conclusion was drawn for *E. coli* (Braun and Rehn, 1969; Braun and Sieglin, 1970). Very recently in freeze-etched cells of *E. coli* closely packed elements of about 100 Å diameter were observed on the innermost layer of the cell wall (Nanninga, 1970), which elements are very likely the lipoprotein molecules on the murein.

When we isolated the lipoprotein after lysozyme degradation of the murein we found 1.5–2 disaccharide units of the murein still attached to 1 lipoprotein molecule. Lysozyme

cleaves the polysaccharide chains of the murein at the reducing end of *N*-acetylmuramic acid and disaccharide units are the smallest cleavage products (Salton and Ghuysen, 1960). It would be interesting to see how the sections of the polysaccharide chains between the lipoprotein molecules fit into the active center of the enzyme (Phillips, 1967; Rupley and Gates, 1967; Chipman and Sharon, 1969). From the dense packing of the lipoprotein molecules on the murein it is likely that the enzyme can only cleave the polysaccharide chains from that side of the murein network opposite the lipoprotein layer.

The specific effect of trypsin on the ultrastructure of the cell envelope of *E. coli* (Braun and Rehn, 1969) could also be observed in *S. typhimurium*. Upon incubation with trypsin the absorbance of *S. typhimurium* cell envelopes decreased rapidly while concomitantly the association of the cytoplasmic membrane and the cell wall was abolished (Figure 4). Quantitative differences were noted, however. The trypsin effect was not as pronounced as in *E. coli*. This may be due to several reasons. The sensitivity of the lysyl–lipoprotein peptide bond to trypsin on the murein attachment site in *Salmonella* is not as distinct. Arginine which is adjacent to lysine remains also on the murein to a considerable extent (Table I, C). Small differences in the structure of the cell wall surrounding the lipoprotein molecules may be the cause of a lower rate and a smaller specificity of trypsin upon this cleavage site. The different response upon trypsin treatment may also derive from the different lability of the inner membrane. In *E. coli* the cytoplasmic membrane stays quite firmly attached to the outer wall unless treated with trypsin and after trypsin treatment it remains mainly intact. In *Salmonella* the membrane is so labile that differential effects in electron microscopy appear only when special precautions are used while isolating the cell envelope before and after trypsin treatment. In breaking down, the inner membrane of *Salmonella* not only forms smaller vesicles retaining the usually observable membrane thickness but disintegrates into pieces—probably small sheets—with boundaries grossly widened.

We have the impression that the cause leading to rapid decrease of the turbidity and the separation of the two membrane systems upon trypsin incubation may result from some kind of tension arising from the association of the inner membrane with the outer wall which hinders the inner membrane from forming smaller vescicles. This is observed when the association of the two membranes is loosened by trypsin treatment and can also be seen by curled edges in sections of disintegrated parts of the cell envelope without trypsin treatment. Nevertheless the same conclusions for association of the two membranes and the role of the lipoprotein may be drawn for *Salmonella* as for *E. coli*. The detachment of the cytoplasmic membrane from the cell wall does not necessarily imply that the lipoprotein is directly involved in the fixation of the cytoplasmic membrane. Any structural transition in the cell wall caused by the cleavage of the lipoprotein from the murein could change the interaction of the two membrane layers. From the apparent distortion of the ultrastructure of the cell envelope by cleaving the lipoprotein molecules from the murein with trypsin we conclude that the lipoprotein layer plays an important structural role within the cell envelope in that it may integrate the hydrophilic murein network into the mostly hydrophobic cell wall. The murein network with the covalently linked lipoprotein molecules could serve as a firm basis for physical attachment of the other constituents of the cell wall—*i.e.*, polysaccharides, lipids, phospholipids, lipopolysaccharide, and protein which make up about 80% of the cell wall.

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