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Ultrastructural Study of *Salmonella typhimurium* Treated with Membrane-Active Agents: Specific Reaction of Dansylchloride with Cell Envelope Components

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Amino groups of cell envelope proteins, lipids, and lipopolysaccharides cannot be labeled in intact cells of *Salmonella typhimurium* G 30 by using 5-dimethylaminonaphthalene-1-sulfonylchloride incorporated in lecithin-cholesterol vesicles. However, application of membrane-interacting agents like tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, ethylenediaminetetraacetate (Na salt) (EDTA), divalent cations, and sublethal doses of the cationic antibacterial agents polymyxin B and chlorhexidine induced specific fluorescent labeling of envelope proteins and lipids but not of cytoplasmic compounds, with the exception of a soluble protein with a molecular weight of 46,000 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Treatment with Tris-hydrochloride buffer produced labeling of the heat-modifiable protein B/B^+ and of proteins with molecular weights of 26,000, 22,000, and below 17,000. A combination of Tris-hydrochloride and EDTA induced additional dansylation of the major protein A and of proteins of molecular weights 80,000, 60,000, and 44,000. Polymyxin B and chlorhexidine caused similar labeling patterns. In every case, except with divalent cation treatment, protein B/B^+ was the most prominently labeled species. Phosphatidylethanolamine was dansylated up to 30%. Lipopolysaccharide was not reactive under any condition or treatment. In addition, the peptidoglycan-bound lipoprotein did not react with dansylchloride in either intact or Tris-hydrochloride-treated cells. The results are discussed with regard to a possible localization of labeled and unlabeled compounds of the cell envelope on the basis of a model placing cell envelope amino groups into ion-ion interactions with anionic components of other envelope compounds like phosphate and carboxyl groups.

Gram-negative bacteria possess a complex envelope composed of the outer membrane, peptidoglycan, and the inner or cytoplasmic membrane (6, 20, 27, 35). It has been suggested that the outer membrane of *Salmonella typhimurium* (27) is about 60% covered by proteins (25), 20 to 25% by lipopolysaccharide (LPS) (22), and the remainder by phospholipids. Despite the predominance of proteins, we previously reported that no proteins were labeled in intact cells of *S. typhimurium* G 30 grown in glucose-salts medium (31) by using the surface-specific labeling method of Schmidt-Ullrich et al. (34) with dansylchloride-lecithin-cholesterol vesicles as applied to erythrocytes. In isolated envelopes, however, dansylation by this method, followed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), revealed that all Coomassie brilliant blue-stainable protein bands showed more or less fluorescence. This all-or-nothing effect was interpreted to be due to a lack of deprotonized amino groups for the reaction with dansylchloride (31). If present at the cell surface, all amino groups should therefore be involved in ion-ion interactions with electronegatively charged envelope compounds, thus preventing their discharge even at pH 9. To test this hypothesis, we investigated compounds which might neutralize the excess negative charges of the envelope (24), e.g., tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer or cations like Ca^{2+} or Mg^{2+}. Tris - hydrochloride - ethylenediaminetetraacetate (Na salt) (EDTA), which is known to release the acidic compound LPS from the envelope (17), and the cationic antibiotics polymyxin B and chlorhexidine, which complex with LPS (2) and acidic phospholipids (18, 36), were also investigated.

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The influence of these compounds on the dansylation of proteins and lipids is reported in this publication. From the observed specificity of dansylation, we propose a scheme for the localization of envelope components in the outer membrane of *S. typhimurium* G 30.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *S. typhimurium* G 30 lacking UDP galactose-4-epimerase (EC 5.1.3.2; from M. J. Osborn, Farmington, Conn.) was grown in a glucose-salts medium (31). In radioactive phospholipid labeling, glucose was replaced by glycerol (weight/weight), which did not lead to a different protein content or labeling. Growth was maintained with constant shaking (80 rpm) at 37°C. The cells were harvested in the logarithmic phase (7.5×10^8 cells per ml) by centrifugation for 20 min at 9,000 $\times g$ at room temperature.

Pretreatment of cells for dansylation. Before dansylation the bacteria were suspended in 0.4 volumes of the following solutions for 5 min at 23°C: 0.1 M Tris-hydrochloride; 0.1 M Tris-hydrochloride–2×10^{-4} M EDTA; 0.1 M Tris-hydrochloride–10^{-3} M EDTA; 0.1 M Tris-hydrochloride–10^{-2} M EDTA; 0.1 M Tris-hydrochloride–2×10^{-4} M EDTA–25 mM Ca^{2+}; 0.1 M phosphate (Na)–2×10^{-4} M EDTA; 25 mM $CaCl_2$; and 25 mM $MgCl_2$. All solutions were pH 7.8.

After centrifugation for 10 min at 9,000 $\times g$, the sediment was suspended in 1/10 the original volume in PSB (52 mM Na_2HPO_4, 77 mM NaH_2PO_4, and 155 mM sucrose), adjusted to pH 9 with NaOH.

For treatment with polymyxin B and chlorhexidine, the desired amounts of the antibiotics were added to the growth medium (7.5×10^8 bacteria per ml). After 10 min of incubation at 23°C, the cells were centrifuged and suspended in PSB, pH 9.

Cell envelopes were prepared by the suspension of the bacterial sediment in 0.05 volumes of PB (PSB without sucrose, pH 6.6), by addition of 10 μg of ribonuclease and deoxyribonuclease (Boehringer) each per ml and by sonification (Branson Sonifier, step 8, six times for 20 s each at 0°C). No intact cells were detectable by phase-contrast microscopy. After tripling the suspension volume with PB, intact cells were sedimented for 5 min at 3,000 $\times g$. Cell envelopes were centrifuged from the supernatant fluid for 1 h at 48,000 $\times g$ and resuspended in PSB by homogenization or by sonification (step 3, 20 s).

Dansylation procedure. Dansylation was performed by following the method of Schmidt-Ullrich et al. (34) with slight modifications as previously described (31). Labeling time was 1 h in the dark at room temperature.

Further treatment of dansylated samples. After dansylation, cell samples were centrifuged for 15 min at 3,000 $\times g$, washed twice with PSB (pH 8), and suspended in PB. Cell envelopes of those samples were prepared as described above but were suspended for the last step in deionized water to a protein content between 2 and 6 mg per ml. Cell envelope samples were centrifuged after dansylation for 1 h at 48,000 $\times g$, washed twice in PSB (pH 8), once in PB, and suspended in deionized water.

Storage of these samples was in the dark at −18°C. Usually, SDS-PAGE was performed the next day. However, no differences in the fluorescence pattern were detectable apart from bleaching in 2 or 3 months.

SDS-PAGE. SDS-PAGE was performed by using the discontinuous system of Laemmli (16) and the continuous system of Weber and Osborn (38) at polyacrylamide concentrations of 3.5 and 10% with a Desaga vertical gel plate apparatus as previously described (31). For longer gels (separation distance of 11 cm), a Hölzel separation chamber was employed. The samples were heated in the incubation buffer for 30 min at 37°C, 20 min at 70°C, or 2 min at 100°C; centrifuged for 2 min at 16,000 $\times g$; and applied to the gel.

Dansylated lysozyme and albumin (Boehringer; 15,000 molecular weight [15K], 30K, 45K, and 68K; 136K) and sometimes chymotrypsinogen (25K) and ovalbumin (45K) served as molecular-weight standards.

The gel was fixed in 7.5% acetic acid, viewed under an intense UV light source, and photographed with a Polaroid MP4 Land camera with film type 107 (black and white, 3,000 ASA) and a yellow filter. Exposure time was 15 to 45 s according to distance at f-stop 4.5. Pictures of Coomassie brilliant blue-stained gels were made with an orange filter at f-stop 32, 1/30 s.

Protein determination. Protein was determined by the method of Lowry et al. (19) with prior incubation of the samples in 0.66 N NaOH for 90 min at 60°C. Lysozyme (Boehringer) served as the standard.

Phospholipid assays. *S. typhimurium* G 30 was grown in glycerol-salts medium (to 6×10^8 cells per ml), sedimented, washed in 0.1 volume of salts medium without glycerol, and suspended in 0.02 volume of salts medium supplemented with 10^6 cpm of [^{14}C]glycerol per ml (1-[^{14}C]glycerol; 31 mCi/mmol; Amersham-Buchler). After incubation for 1 h at 37°C with intensive stirring, the bacteria were washed twice in salts medium without glycerol and suspended in PSB, pH 9. Further pretreatment and dansylation were done exactly as described. Subsequently, the samples were washed twice in PSB (pH 8), once in PB, and suspended in distilled water. For phospholipid isolation, we used the method of Folch et al. (8). Chromatography was done by the method of Wagner et al. (37) with chloroform-methanol-water (65:25:4). Radioactive spots were scraped off and counted in a scintillation cocktail of 4 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis-(5-phenyloxazolyl)benzene in 1 liter of toluene.

LPS isolation. LPS was isolated by the phenol-water method (39).

Chromatographic methods. Samples were hydrolyzed by heating for 1 h in 1 N HCl at 118°C or for 18 h in 6 N HCl at 118°C in closed Fiolax vials. After the samples were dried in vacuo, the residue was dissolved in methanol-NH_3 (95:5) for chromatography. Dansylated *O*-phosphorylethanolamine (O-P-Eth) was determined in the chromatographic system of Wagner et al. (37). Dansylamide and dansylethanolamine were separated in a two-dimensional system (40).

RESULTS

Dansylation of intact cells and isolated envelopes. As reported previously (31), no proteins and only a small amount of phosphatidylethanolamine (PE) (Table 2) could be dansylated in intact cells of *S. typhimurium* G 30 grown in glucose-salts medium. In accordance with these results, cross-linking of amino groups in intact bacteria was reduced in phosphate buffer (33). In contrast, all proteins stainable with Coomassie brilliant blue could be labeled in isolated envelopes (Fig. 1).

Dansylation of cells after pretreatment with Tris-hydrochloride and Tris-hydrochloride EDTA buffer. The application of the amino buffer Tris-hydrochloride, which is thought to activate only surface-localized groups, led to the labeling of proteins with apparent molecular weights of 26K, 22K, and below 17K and of the heat-modifiable major outer-membrane protein B/B$^+$ (29). The Tris-hydrochloride-EDTA combination, which had been reported to liberate about 50% of LPS without

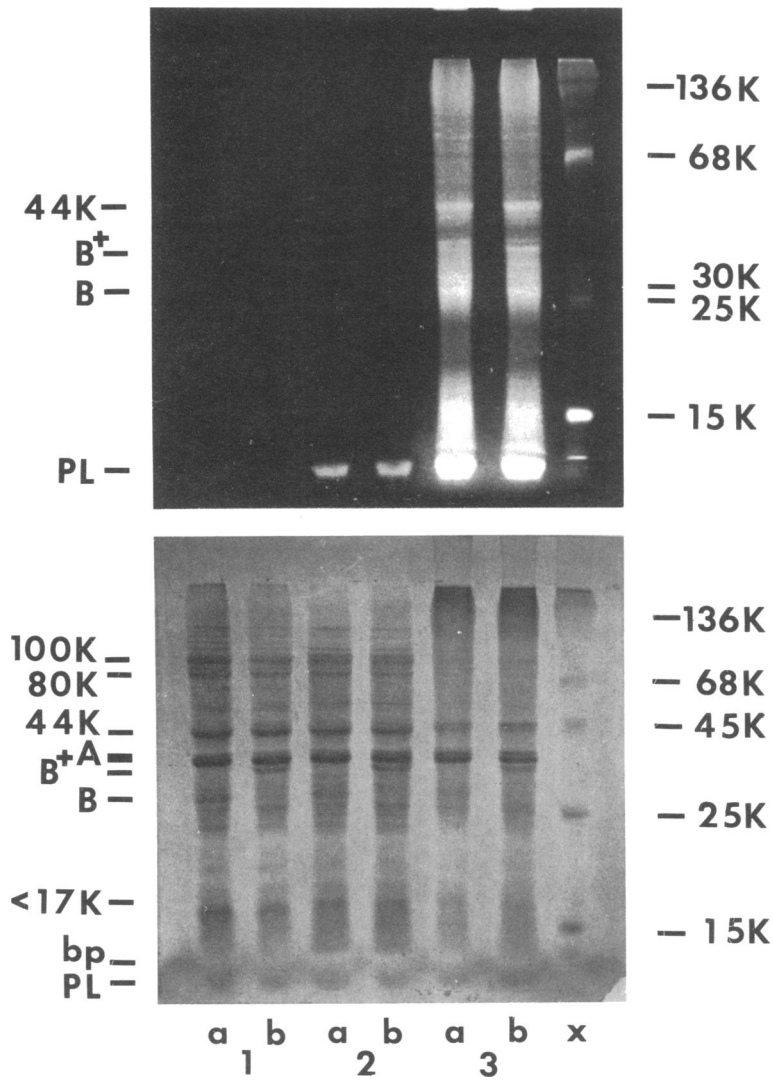

FIG. 1. *SDS-PAGE electrophoretogram, produced by the method of Laemmli, of cell envelopes from non-dansylated (1) and dansylated untreated intact cells (2) and dansylated isolated envelopes of S. typhimurium G 30 (3) after incubation for 20 min at 70°C (a) and for 2 min at 100°C (b). The Coomassie brilliant blue staining pattern is recorded below the fluorescence pattern. Abbreviations: x, molecular weight standard proteins; bp, bromophenol blue position; PL, phospholipids.*

affecting survival (17), enabled additional dansylation of proteins at 80K, 60K, 44K, and below 17K and of another major outer-membrane protein A similar to the matrix protein A of Rosenbusch (30). This protein A could be resolved in at least three distinct bands (below). Higher EDTA concentrations (10^{-2} M) intensified the fluorescence pattern without the appearance of newly labeled protein bands. However, 2×10^{-4} M EDTA in 0.1 M phosphate buffer did not produce fluorescence labeling.

Influence of polymyxin B and chlorhexidine. Polymyxin B and chlorhexidine are cationic membrane-active antibacterial agents. The cationic amino groups are able to neutralize the excess negative charges of the cell envelope and can replace cellular amino groups in ion-ion interactions. Their lipophilic regions might potentiate this disturbance by integrating into the lipid layer.

LPS and acidic phospholipids (2, 36) are receptors for polymyxin B. In freeze-etch electron microscopic pictures, extended aggregations were observed arising from the outer monolayer of the outer membrane which consist mainly of LPS-polymyxin complexes (32). By this mechanism, amino groups of proteins and PE could be exposed to dansylation. Under the conditions used (32), the minimal inhibitory concentrations were 4.2 μg of polymyxin B-sulfate per ml and 5.5 μg of chlorhexidine-diacetate per ml. The bactericidal concentrations were 5 μg of polymyxin per ml and 14 μg of chlorhexidine per ml. Since polymyxin penetrates into the cell interior and aggregates with nucleic acids and ribosomes once the minimal inhibitory concentration is reached (32), lower doses were used for the dansylation experiment.

Pretreatment with 0.5 μg of polymyxin B per ml enabled labeling of the protein B/B^+ and a protein below 17K. At 1 μg of polymyxin B per ml, a dansylation pattern similar to that obtained from Tris-hydrochloride treatment was obtained. At higher concentrations, more and more proteins were labeled, thus demonstrating a progressive destruction of the outer-membrane structure. The subsequent production of pores should also expose the cytoplasmic membrane for labeling.

A similar labeling pattern could be observed after chlorhexidine treatment. As in Tris-hydrochloride-, Tris-hydrochloride-EDTA-, and polymyxin B-treated cells, the protein B/B^+ was also preferentially labeled in chlorhexidine-treated bacteria.

Influence of Ca^{2+} and Mg^{2+}. About 40% of the LPS is bound to the envelope by divalent metal ions such as Ca^{2+} and Mg^{2+} (17). The addition of those ions should therefore strengthen the metal-dependent LPS-LPS binding by condensing such areas. In this fashion, the inner monolayer can be exposed by weakening the normal outer layer. Indeed, in Ca^{2+}-treated *Escherichia coli*, altered surface structures could be demonstrated by increased susceptibility to viral nucleic acids (21) and higher transformation rates (5).

Ca^{2+} treatment resulted in some faintly labeled protein bands. But here, the protein B/B^+ was only sparsely dansylated while the protein A showed dansylation comparable to that obtained with Tris-hydrochloride-EDTA treatment. A similar pattern was obtained by Mg^{2+} treatment.

Removal of dansylated proteins by washing procedures. The bacterial envelopes were washed twice more and suspended by thorough sonification. The major outer-membrane proteins B/B^+ and A were especially enriched by this treatment. In the Tris-hydrochloride-treated sample, weak fluorescence appeared at the position of protein A. The proteins of 26K, 22K, and below 17K, however, were no longer visible, indicating their localization at a peripheral site from which they could be rinsed away.

In addition to B/B^+ and A, two proteins below 17K seemed to be enriched in the Tris-hydrochloride-EDTA sample, thus pointing to positions not exposed by Tris-hydrochloride alone.

In dansylated isolated envelopes, many protein bands were fluorescent. Here, protein B/B^+ was not the predominantly labeled one, although, together with A, it represents the most abundant protein species as indicated by Coomassie brilliant blue staining, thus suggesting that its labeling was a specific one and not due to its preponderence.

Dansylation of soluble proteins. In the supernatant of dansylated samples from treated cells, one strongly fluorescent protein species at 46K could be found after Tris-hydrochloride-EDTA, polymyxin B, and chlorhexidine treatment, but not in the soluble fraction of Tris-treated and untreated dansylated intact cells (manuscript in preparation).

Characterization of the heat-modifiable membrane proteins in *S. typhimurium* G 30. By the application of the Laemmli electrophoretic system (16), the behavior of the major outer-membrane proteins B/B^+ and A was investigated. Protein B was fully soluble in SDS at 37°C and ran as 28K in SDS-PAGE. The position was not changed by incubation at 70°C; however, at 100°C, protein B was completely transformed into protein species B^+ having an apparent molecular weight of 34K.

Protein A was insoluble at 37°C, but completely soluble at 70°C, where it runs with 37 to 40K. That position was not changed at 100°C. In longer gels, the protein A could be separated into three distinct bands without detectable differences, depending on solubilization at 70 or 100°C.

Proteins similar to B and A have been detected in several enterobacterial species.

A protein at 46K behaved similar to protein A, although it was less soluble at 70°C in normally washed envelopes. Any relation to the single dansylated protein species in the soluble cell fraction is not clear.

If a continuous electrophoretic separation system (38) was applied, remarkable differences could be observed. Protein B, soluble at 37°C, was partially changed to protein B^+ at 70°C. This transfer was intensified at 100°C, but there was still protein present in the position of B. Protein A was insoluble at 37°C, but soluble at 70 and 100°C. Its position was identical with that of B^+, as found by Osborn et al. (27), and could not be separated in longer gels. At 37°C two further bands were located at 70 and 60K. At 70°C the lower band seemed to be reduced, whereas at 100°C both were missing. However, new bands appeared at 50 and 26K.

The heat-modifiable character of proteins has been interpreted as a different SDS loading due to the denaturation rate at different incubation temperatures (9) and/or as a temperature-dependent destruction of the β-structure of outer-membrane proteins (23). Both may be affected by distinct dissociation processes due to phosphate and Tris-hydrochloride ions.

Asymmetric localization of protein B/B^+. Extensive washing of cell envelopes resulted in different enrichment of fluorescence in B/B^+ and A, according to the pretreatment presented in Table 1. In Ca^{2+}-treated cells, protein B/B^+ was not enriched, thus indicating a further site from which it can be rinsed.

Dansylation of PE. The results of dansylation of PE are summarized in Fig. 2 and Table 2. Dansyl-PE was characterized by fluorescence, iodine reaction, phosphate staining, negative ninhydrin reaction, and co-chromatography with dansylated isolated PE. Dansylation in acetone of phosphatidylglycerol and cardiolipin did not result in fluorescent derivatives. In 6 N HCl dansyl-PE was completely hydrolyzed to dansylethanolamine, as shown by the chromatographic system of Zanetta et al. (40). In the samples pretreated with Ca^{2+}, the fluorescent spots seemed to be more intense than expected from the radioactivity. An association of the reaction vesicles with the bacterial cell or even a fusion with phospholipid exchange must have occurred. The reaction vesicles contained minor impurities of dansyl-PE.

Dansylation of LPS. LPS contains free amino groups from O-P-Eth and pyrophosphorylethanolamine (10, 28) bound to the core sugars heptose and ketodeoxyoctulonic acid (KDO). The hydrolysate (1 N HCl) of dansylated isolated LPS from *S. typhimurium* G 30 contained dansylated O-P-Eth as shown by co-chromatography with dansylated commercially available O-P-Eth. Its R_f value was 0.06 by the system of Wagner et al. (37). Moreover, two other fluorescent spots appeared, representing dansyl-glycine, whose origin is unknown but which may derive from tRNA, and a spot which ran at the position of no usual amino acid derivative. Whether or not it represented a stable dansylated dipeptide, an N-disaccharide, or a labeled O-P-Eth containing a sugar of LPS was not investigated. Hydrolysis of dansylated isolated LPS in 6 N HCl resulted mainly in dansylamide. A spot with dansyl-Eth derived from O-P-Eth showed minor fluorescence. Dansylglycine was present in 6 N HCl hydrolysates, whereas the unknown fluorescent spot fully disappeared. Since dansylated LPS remained in the phenol phase (39), envelopes of dansylated samples were hydrolyzed and applied to chromatography for dansylated O-P-Eth (1 N HCl; 37). Only a very faint, greenish fluorescence could be detected in samples from Tris-hydrochloride-EDTA-, polymyxin B-, and chlorhexidine-treated cells as well as in dansylated isolated envelopes at the position of the normally fluorescent-yellow dansylated O-P-Eth. Comparison of envelope samples hydrolyzed in 6 N HCl (40) for the products of dansyl-Eth and dansylamide all showed negligible fluorescence in the dansylamide position. Both can be explained by the assumption that LPS could not be labeled in *S. typhimurium* G 30 in situ. In Tris-hydrochloride-EDTA-treated and dansylated *E. coli* B and K-12, however, there was significant fluorescence from dansylated O-P-Eth and dansylamide in the respective hydrolysates.

TABLE 1. *Fluorescence intensity of proteins A and B as judged by SDS-PAGE (16) of cell envelopes (a) and twice-more-washed cell envelopes (b) from dansylated pretreated intact bacteria*

Protein	Fluorescence[a] with pretreatment:					
	1 μg of polymyxin per ml		25 mM calcium chloride		0.1 M Tris-hydrochloride-2 × 10^{-4} M EDTA	
	a	b	a	b	a	b
A	+/−	+/−	+/−	+	+/−	+
B	++	+++	+	+	+	+++

[a] +/−, Little or no fluorescence; +, ++, +++, moderate to strong fluorescence.

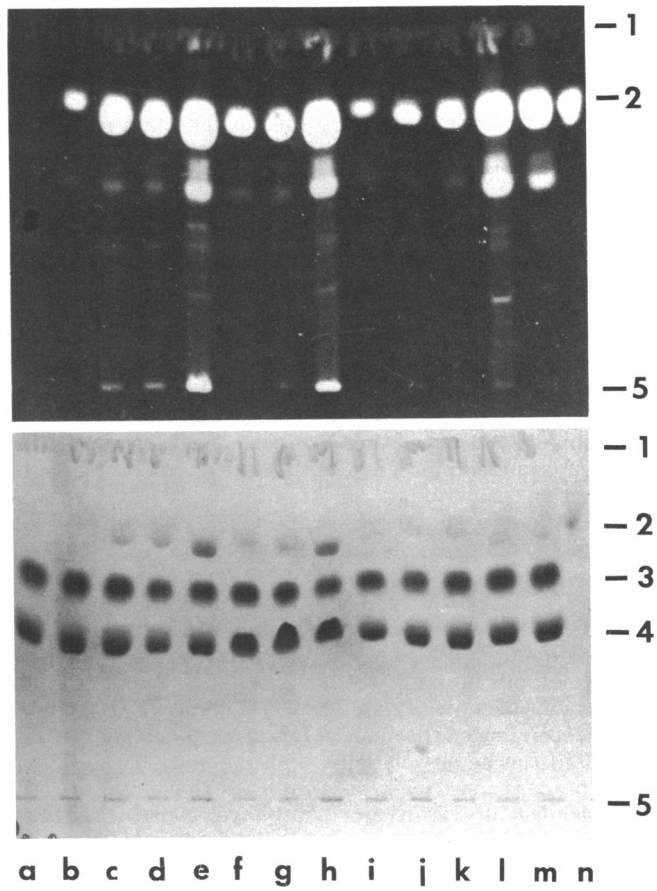

FIG. 2. *Separation of phospholipids in chloroform-methanol-water (65:25:4) from non-dansylated (a) and dansylated (b) untreated cells and dansylated pretreated intact cells of S. typhimurium G30. Pretreatment: 1, 2, or 3 μg of polymyxin B per ml (c, d, e); 1, 3 or 5 μg of chlorhexidine per ml (f, g, h); 0.1 M Tris-hydrochloride (i); 0.1 M Tris-hydrochloride–0.2 mM EDTA (j); 0.1 M Tris-hydrochloride–1 mM EDTA (k); Tris-hydrochloride-EDTA-Ca^{2+} (l); 25 mM Ca^{2+} (m); and dansyl-PE (n). The bacteria were grown in [^{14}C]glycerol. The fluorescence pattern (above) and the autoradiographic pattern (below) are shown. (1) Neutral lipids at the front position; (2) dansyl-PE; (3) PE; (4) phosphatidylglycerol and cardiolipin; (5) start. Some minor fluorescent spots were not identified, but may correspond to lyso-PE derivatives.*

TABLE 2. *Distribution of dansyl-PE and PE from nondansylated (a) and dansylated (b) untreated cells and dansylated pretreated intact cells (c to m) of S. typhimurium G 30 as well as from dansylated envelopes (CE_1, CE_2)*

Determination	Distribution (%) with pretreatment[a]:														
	a	b	c	d	e	f	g	h	i	j	k	l	m	CE_1	CE_2
PE	100	98.9	91.7	84.2	73.2	95.1	92.8	68.5	98.5	95.7	95.1	97.0	97.8	74.7	79.3
Dansyl-PE	—	1.1	8.3	15.8	26.8	4.9	7.2	31.5	1.5	4.3	4.9	3.0	2.2	25.3	20.7

[a] Symbols for pretreatment are the same as those described in the legends to Fig. 2. CE_1, Isolated cell envelopes suspended by sonification before dansylation; CE_2, isolated cell envelopes suspended by homogenization.

DISCUSSION

Experimental evidence presented in this paper suggests that the failure of amino group labeling in glucose-grown intact cells of *S. typhimurium* G 30 was due to the total protonization of these groups by ion-ion interactions with electronegative cell envelope groups. This conclusion is drawn from the fact that treatment with Tris-hydrochloride buffer, Tris-hydrochloride-EDTA, polymyxin B, chlorhexidine, and metal ions activated cellular amino groups either by displacing them and/or by neutralizing the ex-

cess negative charges of the envelope. Both will result in the liberation, deprotonization, and consequent dansylation of such amino functions. Therefore, the labeling spectrum of proteins and lipids obtained should indicate those locations where the destructive action of the membrane-disturbing agents has been implicated, directly or by a long-range order reaction.

Possible localization of labeled proteins. The suitability for surface labeling studies by the method used for erythrocytes has been reported (34). The failure to label intact cells of the bacterial strain used further showed that dansylchloride is tightly trapped in the vesicles. In this way, it seems to react only by direct contact with a corresponding amino group. With [^{3}H]dansylchloride we could show an 18-fold specific enrichment in the sedimentable envelope fraction over that of the supernatant of polymyxin-treated bacteria. Moreover, dansylation did not influence colony number and the phosphoenolpyruvate-dependent sugar transport system in polymyxin-treated cells (P. R. G. Schindler, Ph.D. thesis, Technical University of Munich, Munich, 1976). Therefore, by keeping the problems of labeling methods in mind (13), a hypothetical location scheme of activated sites in the bacterial envelope depending on the mode of action of the agents used can be postulated.

It seems justifiable to propose a surface location for those proteins labeled in Tris-hydrochloride, Tris-hydrochloride-EDTA, low polymyxin B, and low chlorhexidine concentrations. That was always the case for proteins 26K, 22K, and below 17K and for the major heat-modifiable outer-membrane protein B/B$^+$. Since the proteins 26K, 22K, and below 17K could be removed from the membrane by rinsing, they most likely are located on the periphery or loosely penetrate only one monolayer. The protein B/B$^+$, however, may span the outer membrane. As indicated by its excellent labeling after polymyxin B and chlorhexidine treatment, B/B$^+$ may be particularly involved in interactions with LPS, which is a primary receptor for polymyxin B (2). The release of metal-bound LPS by Tris-hydrochloride-EDTA followed by a weakening of the LPS-protein association also resulted in a favorable labeling of B/B$^+$. Because of the preponderance of B/B$^+$ in the outer membrane, as suggested from Coomassie brilliant blue staining, we propose that the main surface protein in *S. typhimurium* is B/B$^+$.

Proteins additionally labeled by Tris-hydrochloride-EDTA treatment and higher antibiotic concentrations may be located at the surface but involved in tighter interactions, although, alternatively, these treatments could lead to an exposure of inner sites.

The metal ion treatment enabled a preferential dansylation of the protein A as compared with that of B/B$^+$, thus indicating a failure of metal ions to replace amino groups. In this way, the condensation of LPS led to an exposure of inner monolayer components which may account for the dansylation obtained.

From this point of view, the matrix protein A in *Salmonella* should be located in the inner monolayer and associated with the peptidoglycan as shown for *E. coli* (30). Its exposure by Tris-hydrochloride-EDTA and divalent metal ions indicated that protein A is in contact with metal-bound LPS. According to the membrane model of Leive (17), metal-bound LPS is mainly localized at the adhesion zones. We therefore predict the location of protein A to be mainly at those sites where it may have stabilizing functions or be directly involved in the construction of pores. That proposed location would explain the phage receptor properties of protein A (26). A primary electrostatic disturbance by virus contact may uncover that protein and permits binding directly at or above those pores, followed by infection. The diminished content or lack of major outer-membrane proteins like A and B/B$^+$ (1, 12), which did not result in loss of vital functions, is compensated by a higher content of phospholipids (20). This may account for the proposal that protein A plays a functional role in bilayer construction similar to that of phospholipids. An obvious lack of amino groups at its surface, as shown by its weak labeling even in isolated envelopes, could be explained by the great hydrophobicity of protein A. Therefore, protein A may construct hydrophobic areas in the inner monolayer of the outer membrane upon which newly produced LPS and phospholipids will be held in place and can extend above the surface.

The dansylation of the lipoprotein was not investigated. One of the labeled proteins below 17K may be identical to the free form of the lipoprotein (11). Since we did not use lysozyme, the peptidoglycan-bound form (3) was in the insoluble sediment of the SDS incubation mixture, which was fluorescent in cells pretreated with Tris-hydrochloride-EDTA and higher antibiotic concentrations but not in dansylated intact or Tris-treated bacteria. Therefore, the bound lipoprotein in *Salmonella* is not located at the surface. A comparable result has been reported for wild-type *E. coli* (4). Our interpretation of surface-localized proteins is in contrast to the findings of Kamio and Nikaido (14) who proposed a surface arrangement for protein A but not for protein B/B$^+$. This may be explained by the assumption that there was no LPS protein B/B$^+$ substitution in intact bacteria with

the used activated dextran assay (14), although this protein is located at the surface. On the other hand, the protein A in our strain may lack surface amino groups and would not be labeled in this way. So we could label protein A even in untreated *E. coli* species, thus suggesting a surface location (manuscript in preparation). Nevertheless, in our *Salmonella* mutant, we propose a protein A localization extending from the peptidoglycan only to the inner monolayer, thus enabling a preferred fracturing between the outer and inner monolayers of the outer membrane as previously shown (32).

Dansylation of PE and LPS. The described pretreatments induced the dansylation of PE. The relative high dansylation rate at high antibiotic concentrations as compared with Tris-hydrochloride treatment may be due to either a retarded activation of the amino groups at the surface or their favored location in the inner monolayer. The fact that intact cells of *E. coli* will not be attacked by externally added phospholipases (7) may strengthen the latter explanation. More elucidation should result from the use of phospholipid-deficient or enriched mutants.

The failure to label LPS in *S. typhimurium* G 30 shows that available amino groups are sterically hindered in being placed into the interior of the three pyrophosphate-linked LPS subunits. That we could label LPS in *E. coli* B and K-12 (unpublished data) may be explained by the lack of KDO-linked O-P-Eth in *S. typhimurium*. KDO-linked O-P-Eth has been demonstrated in *E. coli* (28) but not in *S. typhimurium* (20); also we could not demonstrate LPS dansylation in the heptose-less mutant *S. typhimurium* SL 1102, which has only KDO as core sugar.

Concluding remarks. The reported dansylation, the proposed localization, and the behavior of the major heat-modifiable outer-membrane proteins A and B/B$^+$ are characteristic of the strain *S. typhimurium* G 30. In another report (in preparation), we will show the existence of profound differences in these respects when strains of *Salmonella*, *Escherichia*, *Enterobacter*, *Serratia*, and *Proteus* are compared, thus indicating numerous differences in membrane architecture within the group of enterobacteria.

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