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# Isolation and Partial Characterization of Membrane Vesicles Carrying Markers of the Membrane Adhesion Sites

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At areas of adhesion between outer membrane (OM) and inner membrane (IM) in gram-negative bacteria, newly synthesized membrane constituents are inserted, and bacteriophage infection occurs. We describe here the isolation of these sites from cell membrane fractions of *Salmonella anatum*. Sucrose density gradients yielded membrane vesicles of the OM and IM; their mutual cross-contamination was low, as measured by 2-keto-3-deoxyoctonate and β-NADH-oxidase activities. To mark the areas of lipopolysaccharide synthesis in the envelope (the adhesion sites), we infected *S. anatum* with phage ε15, which causes a rapid change (conversion) in the cell's O-antigenic composition from serogroup $E_1$ to $E_2$; lipopolysaccharide of type $E_2$ also serves as receptor for phage ε34. We found that the fractions of intermediate density (Int. M) from briefly converted cells bound both phage ε34 and $E_2$-specific antibody. In the electron microscope, ε34 was seen to have absorbed with a high degree of significance to the Int. M fraction of briefly converted cells, but not to the Int. M fraction of unconverted cells. Furthermore, the Int. M fractions of briefly converted cells coagglutinated anti-$E_2$-coated *Staphylococcus aureus*, whereas the OM and IM fractions showed comparatively little agglutination. In addition, Int. M material exhibited elevated phospholipase $A_1$ and $A_2$ activities comparable to those of the OM fraction; the IM was essentially phospholipase free. Our data indicate that this membrane fractionation allows one to isolate from Int. M regions a variety of activities associated with adhesion sites.

Cell envelopes of gram-negative bacteria consist of two layers, the outer membrane (OM), which also harbors the peptidoglycan layer, and the inner or cytoplasmic membrane (IM) (21). Both membranes face the periplasmic space, which seems to separate them from each other (28). A preparative separation of the cell envelope fraction into the two distinct membrane systems was achieved in sucrose gradients after mechanical disruption of the envelopes (2, 25, 31, 35, 37). However, the quantitative separation of the two membranes became questionable after ultrastructural data showed that, at several hundred discrete areas, both membranes adhere to each other (3, 5, 6). After their structural identification, a multitude of functions has been associated with these membrane adhesion sites: export for lipopolysaccharides (LPS) (5, 6, 26, 27), capsule polysaccharide (10), and major OM proteins (7). In addition, a wide variety of bacteriophages infect their hosts at these sites. Structurally similar membrane features exist at the areas of flagellar anchoring and F-pilus insertion (4, 7). This clustering of functions for macromolecular export and import focused our attention on the biological activities at the adhesion area. The functional and biochemical organization of these sites has been a topic of much speculation, and several models of membrane growth have been proposed (16, 30). This paper presents results on the isolation of envelope fractions which exhibit activities associated with membrane adhesion sites. Membrane vesicles exhibiting functional activities of the adhesion sites were recovered from regions of intermediate membrane density (Int. M) after sucrose density centrifugation of envelope vesicles. Int. M fractions were capable of binding ε-phages specific for newly formed LPS and of agglutinating antibody specific for newly formed LPS. They also contained at least half of the phospholipase activity of the cell envelope.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *Salmonella anatum* and *Salmonella ado* were from the collection of H. Uetake, Kyoto University. *Escherichia coli* B, originally from W. Weidel, had been maintained for many years in our laboratories. O-antigens of *S. anatum* are 3,10 ($E_1$). Phage ε15 and ε34 were originally from the collection of H. Uetake and were purified as described before (9). Phage ε34 was propagated in *S. ado*. After phage ε15 conversion, the O-antigen changes to 3,15 ($E_2$) (32, 41). *S. ado* is a defective lysogen of ε15 (41) and exhibits antigens 3,15. The

cultures were grown at 37°C with aeration to cell densities of $3 \times 10^8$ to $3.5 \times 10^8$/ml in 2 liters of 0.8% nutrient broth (Difco Laboratories) plus 0.2% yeast extract. (Difco) with 0.1% glucose as the carbon source. Turbidity was measured with a Klett-Summerson photometer after establishing the relation of turbidity, colony counts, and cell counts, using a Neubauer chamber and phase-contrast microscopy. To initiate production of new LPS by phage conversion, growing cultures of *S. anatum* were infected with phage ε15 (multiplicity of infection 10), and kept at 37°C for 6 to 8 min; they were then rapidly chilled by dilution into ice-cold medium and used for subsequent preparations, such as vesicle formation in the French Press.

**Antisera.** New Zealand white rabbits were immunized by subcutaneous and intramuscular injections of $10^8$ cells in weekly periods for 6 to 7 weeks. Heat-killed *S. anatum* (antigen $E_1$) or *S. ado* (antigens $E_1$ and $E_2$) cells were washed twice and suspended in 0.2 ml of 0.9% saline solution. Antibody titers determined by tube and slide agglutination were 1:160 to 1:320. Almost all of the rabbits used for antiserum production were observed to have a low primary agglutination titer (1:12) against the antigen (3, 10) $E_1$. This titer was reduced to 1:2 after adsorption to a *Salmonella anatum* cell suspension. To obtain anti-$E_2$, the coproduced anti-$E_1$ antibody was two times absorbed with $8 \times 10^9$ heat-killed *S. anatum* cells per ml of serum. For coagglutination of vesicle preparations, formaldehyde-killed *Staphylococcus aureus* ATCC 12598 (containing the Fc-binding protein A) was prepared as described (38); we used the antiserum as well as the immunoglobulin G for binding to the staphylococci. Vesicle fractions of ε15-converted *Salmonella anatum* were added to antibody-coated staphylococci, and the agglutination of the staphylococci was measured and timed by light microscopy at ×10 to 80 magnification. The vesicle agglutination served as a sensitive indicator for the presence of antigen $E_2$, which is produced (over adhesion sites) within a few minutes after addition of phage ε15 (5, 6). The onset of the conversion was tested by (i) agglutination of the infected cells with anti-$E_2$ serum and (ii) by following the absorption kinetics of phage ε34; this phage absorbs to LPS of antigenic type 3,15 ($E_2$) but not to (unconverted) 3,10 ($E_1$) (41). The phage adsorption was monitored as described before (8).

**Electron microscopy.** For electron microscopy, special care was taken to maintain vesicle preparations at 0 to 4°C, including the glassware, microscope grids, and tweezers. Fifty microliters of purified phage ε34 (equivalent to $10^{10}$ phages) was mixed with 0.5 ml of the vesicle preparation for 5 min; the mixture was subsequently added to carbon-coated copper grids. After 30 s, the grid was washed 2× in cold nutrient medium and 2× in distilled water, 3 to 5 s each and negatively stained in half-saturated uranylacetate, and the excess liquid was blotted off. To prevent condensation of water, the grid was dried in a vacuum desiccator which was gradually warmed to room temperature (9). The specimens were coded and then inspected in the electron microscope by two microscopists who were not provided with either the code or each other's data. Absorption of virus particles to membrane pieces and vesicles was recorded.

**Chemicals.** [1-$^{14}$C]oleic acid and [1-$^{14}$C]palmitic acid were purchased from New England Nuclear Corp. RNase A, phospholipase $A_2$, phosphatidylethanolamine, and fatty acids (unlabeled) were from Sigma Chemical Co. It should be noted that we observed in the commercially available DNase preparations a considerable phospholipase contamination similar to that described previously (39). We measured high phospholipase A activities in all Sigma DNase I samples (10 to 35% free fatty acids [FFA] were hydrolized from substrate after 2 h at 37°C). For vesicle isolation, we used purified DNase I which was essentially phospholipase A free; only 1% FFA were released after 2 h of substrate hydrolysis at 37°C. This enzyme has been purified by DEAE-Sephadex and phosphocellulose chromatography and was a gift from Kenneth Lipson, Case Western Reserve University, Cleveland. All other chemicals were from standard commercial sources.

**Breakage of cells and isolation of membrane fractions.** We modified the procedure given by Smit et al. (37). *Salmonella anatum* cells grown in 2.5 liters of nutrient broth under vigorous shaking to a final density of $3 \times 10^8$ cells per ml were quickly cooled and spun at 4°C for 10 min at 1,000 × *g*. All subsequent operations were carried out at 0 to 4°C. The resuspended pellet was taken up in M-9 medium containing 1 mM $MgSO_4$ (1) and washed twice. For the hydrolysis of cellular DNA and RNA, cells were suspended in 120 ml of the M-9 medium supplemented with 1 mM $CaCl_2$ and incubated for 15 min with: (i) 2-mercaptoethanol (2 mM), (ii) RNase A (5 mg, boiled for 10 min in a water bath), and (iii) purified DNase I (5 mg). The presence of $Mg^{2+}$ and $Ca^{2+}$ in the incubation medium is required for the complete hydrolysis of double-stranded DNA (12). The cell suspension was passed twice through a French pressure cell at 1,400 kg/cm$^2$. Cell debris was removed by low-speed centrifugation. The envelope fraction sedimented after 1 h in a Spinco Ti 60 rotor at 300,000 × *g*. The pellet was suspended in 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer, pH 7.4, and 1.5-ml portions of the suspension were layered on top of discontinuous sucrose gradients in the HEPES buffer. Gradients were prepared by layering the following amounts and concentrations, respectively, onto a 0.5-ml, 2.63 M sucrose cushion: 1.5 ml of 2.02 M, 5.25 ml of 1.44 M, and 4.0 ml of 0.77 M sucrose. After centrifugation in a Spinco SW40 rotor for 15 h at 55,000 × *g*, 16 to 18 fractions per tube were collected from the tube bottom, each fraction containing 0.5 to 0.6 ml. Protein was determined by the method of Lowry et al. (22) and compared with measurements at an optical density at 280 nm, using bovine serum albumin (BSA) as standard for both methods. The contribution of light scattering was negligible at the vesicle concentration used. All gradient fractions were dialyzed two times against a 100-fold volume of HEPES buffer. Protein concentrations of individual fractions were measured again after dialysis. The fractions were stored in cryotubes at −70°C.

Contamination of the vesicle fractions with OM and IM material was checked by assaying for 2-keto-3-deoxyoctonate (KDO) and for β-NADH-oxidase activity by the method of Osborn et al. (31).

**Electrophoresis of membrane fractions.** The protein composition of membrane vesicle fractions was analyzed by sodium dodecyl sulfate-acrylamide gel electrophoresis by the procedure described by Lugtenberg

et al. (23). Proteins 33K to 36K, components of the OM of *Salmonella typhimurium* (2), were the major proteins of *Salmonella anatum* outer cell membranes. The presence of some of these OM proteins in the Int. M and IM bands was in agreement with the data of Osborn et al. (31) on *Salmonella typhimurium* cell envelopes. For the quantitation of OM protein in Int. M and IM fractions of *Salmonella anatum*, we scanned stained slab gels in a densitometer (3380S Integrator; Hewlett-Packard Co.)

**Preparation of substrate for phospholipase assay.** Two microcuries each of [$^{14}$C]palmitic acid (16:0) and [$^{14}$C]oleic acid (18:1) were dried under $N_2$, and fatty acid-free BSA (2 mg/ml of double-distilled water) was added by mixing on a Vortex shaker. This dispersion, together with unlabeled oleic acid (18:1; [$^{14}$C]palmitic acid/oleic acid molar ratio, 1:3), was mixed with 1 ml of *E. coli* B ($4 \times 10^9$ cells) and 9 ml of nutrient broth. After incubation for 1 to 2 h on a shaking water bath (37°C), the culture was harvested by centrifugation at $5,000 \times g$ (10 min at 4°C), suspended in 5 ml of nutrient broth containing 2% BSA, and centrifuged again (as above). The pellet was taken up in 0.5 ml of 0.15 M NaCl and autoclaved for 30 min at 120°C. After two further washes ($2,000 \times g$ for 10 min) in sterile saline solution containing 2% BSA, the pellet was resuspended in saline solution without BSA, and samples of 0.1 ml were frozen at −70°C.

**Enzyme assay.** To estimate the activity of the enzyme, the incubation procedure of Scott et al. (36) was employed with some modifications. To 200 μl of 100 mM Tris-chloride buffer (pH 7.5) and 25 μl of 200 mM $CaCl_2$ were added 25 μl of labeled substrate (boiled *E. coli* containing approximately $4 \times 10^5$ cpm/ml). The reaction was initiated by rapidly adding 250 μl of the membrane vesicle fractions (1.5 mg of protein each) and incubating for 2 h at 37°C on a shaking water bath. The reaction was stopped by adding 3 ml of a mixture of chloroform-methanol (2:1 [vol/vol]). The solution was extracted overnight at room temperature. For the assay of the mixture of phospholipase $A_1$ and $A_2$ (17), we followed the procedure of Folch et al. (18) by extracting the FFA in a biphasic mixture of chloroform and methanol. The lipid-containing chloroform phase was dried under a stream of $N_2$, and the phospholipids and FFA were separated by thin-layer chromatography (Silica gel H plates; Analabs, Inc.) in a solvent system consisting of petroleum ether-diethylether-glacial acetic acid (80:20:1) (33). On the same thin-layer chromatogram plates, oleic acid, palmitic acid (unlabeled), and phosphatidylethanolamine were run as controls. In control experiments, the labeled substrate or phosphatidylethanolamine or both were incubated with commercial phospholipase $A_2$; in these controls, the recovery of $^{14}$C-FFA from substrate was 87%. Plates were stained in iodine vapor, the locations of stained FFA and other phospholipid derivatives were recorded, and the individual sections were scraped off and their radioactivity measured in an LS-233 liquid scintillation counter (Beckman Instruments, Inc.).

## RESULTS

Fractionated envelope preparations of *Salmonella anatum* separated into the two major protein peaks characteristic of the OM and IM fractions of *Enterobacteriaceae* (2, 25, 31, 37). We used gradient profiles with sharp and distinct separations between the OM and IM (Fig. 1). The distribution of markers for OM and IM vesicles, KDO and β-NADH-oxidase, respectively, is shown in Fig. 1A. The level of cross-contamination of OM and IM material appears to be negligible. These profiles were well reproducible; the position of protein peaks as well as the maxima in marker positions did not vary by more than one gradient fraction in over 30 gradients. The amount of the major OM proteins (33K, 36K) in vesicle preparations was measured by acrylamide gel electrophoresis; densitometer scans of OM protein revealed a ratio of 100:45:6.4 in OM, Int. M (fractions 7 or 8), and IM (fraction 13). The level of contamination with the OM protein in the IM vesicle fractions, therefore, seems to be extremely low. In a third of the experiments, a very low level of KDO was found in IM fraction 13 (3 μg/mg of protein).

**Phospholipase $A_1$ and $A_2$ activity of membrane vesicle fractions.** As further marker for envelope fractions, especially the OM, we measured the hydrolytic activity of phospholipase $A_1$ and $A_2$ by incubating membrane vesicles with fatty acid-labeled *E. coli* B as substrate. The rate of phosphoglyceride hydrolysis, determined by the amount of $^{14}$C at the positions of oleic and palmitic acid on thin-layer chromatograms, is shown in Fig. 1B. The rate of phospholipid degradation for each vesicle fraction is expressed in percentage of FFA liberated from the labeled substrate. Consistently high enzyme activities were observed in vesicles of the OM and Int. M regions, whereas only very little activity was detected in the IM. Control experiments showed that the enzyme contamination in the added (boiled) RNase A and purified DNase I preparations cleaved only 3.1% of the substrate. This free, non-vesicle-bound phospholipase banded in the lightest, upper fractions of sucrose gradients. Furthermore, we tested for nonspecific binding of any phospholipase that might have been freed during the membrane fractionation process; for this determination, vesicles of the Int. M were centrifuged ($250,000 \times g$, 5 h), and the phospholipase activity was determined in the supernatant and the pellet. Free enzyme (in the supernatant fraction) hydrolyzed only 3.2% of the labeled substrate, whereas 18.8% was present in the vesicle pellet. The remaining 78% of labeled substrate phospholipids were not hydrolyzed within 2 h. Vesicles of the Int. M region exhibited a higher total enzyme activity than did OM vesicles in several of the experiments (8 of a total of 20). The data suggest to us that a substantial phospholipase $A_1$ and $A_2$ activity is associated with the gradient fractions containing membrane adhesion sites, further supporting previous reports on the possible involvement of

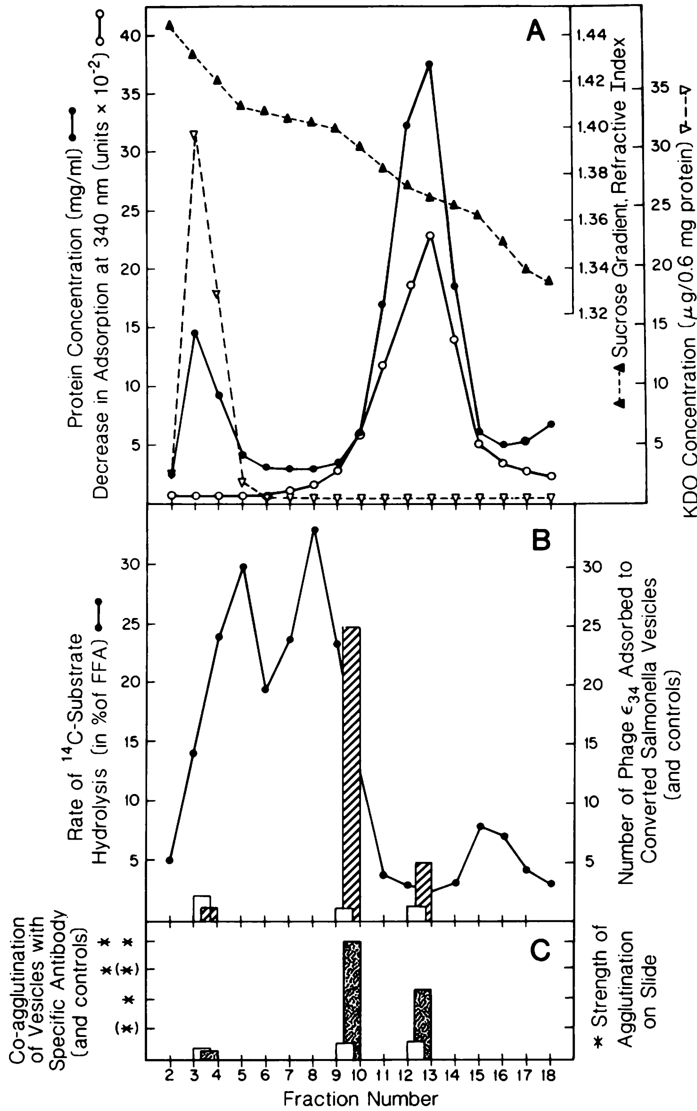


FIG. 1. Sucrose gradient centrifugation of total membrane fraction from *Salmonella anatum*. (A) Assays for protein content (●), β-NADH-oxidase (○), and concentration of KDO (▽) as described in the text. (B) Hydrolysis of labeled substrate ([$^{14}$C]oleic acid- and [$^{14}$C]palmitic acid-labeled *E. coli* phospholipids) by membrane fractions of *Salmonella anatum*. The rate of phospholipid hydrolysis is expressed as percent of FFA released after a 2-h incubation at 37°C with 1 mg of membrane protein of each fraction (●). Adsorption of phage ε34 to membrane vesicle fractions of converted *Salmonella anatum* exhibiting the $E_2$-antigen is represented by shaded columns. Adsorption of phage ε34 to unconverted *Salmonella anatum* exhibiting the $E_1$-antigen serves as control (plain column). (C) Coagglutination of membrane fractions with *Staphylococcus aureus* coated with specific antibody against the $E_1$-antigen. In controls, membrane vesicles of converted *Salmonella anatum* (exhibiting the new $E_2$-antigen) are agglutinated with specific antibody against $E_1$ (plain column). The strength of the slide agglutination (*) was determined by phase-contrast microscopy.

these areas in the regulation and turnover of OM phospholipids (13, 20). No attempt was made to study the hydrolysis of phosphoglycerides by phospholipases C and D; we were concerned here solely with the hydrolytic cleavage of fatty acids from the labeled substrates.

**Agglutination.** Coagglutination of anti-$E_2$-coated *Staphylococcus aureus* showed clearly the highest degree of agglutination with Int. M vesicles of the converted cells (Fig. 1C and 2), with the IM fraction exhibiting the antigen $E_2$ to a lesser degree.

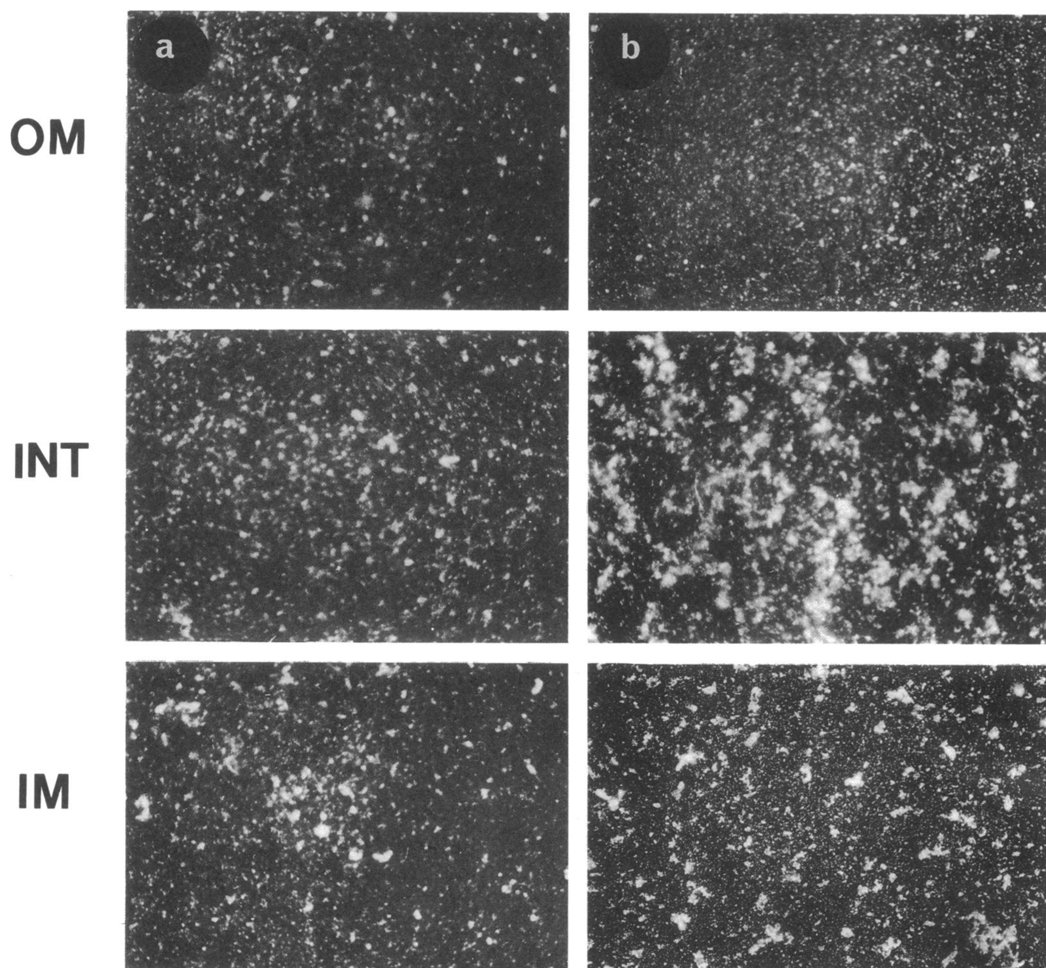


FIG. 2. Dark-field micrographs of coagglutination of *Salmonella anatum* membrane vesicles prepared 8 min after phage ε15 conversions. Three fractions of sucrose gradient (OM, Int. M, IM) were exposed for 12 to 15 min to a suspension of *Staphylococcus aureus* to which anti-$E_2$ immunoglobulin G had been adsorbed. The bacteria served as antibody-carrying beads of visible size. Note strong agglutination in Int. M fraction of converted cells (b) in comparison with nonconverted vesicle fraction (a).

**Electron microscopy.** Electron microscopy of the OM gradient fractions (3 and 4) shows the typical OM vesicle with its high-contrast (stain-excluding) profile (Fig. 3A), whereas the IM vesicles (fractions 13 through 15) were of lesser contrast and varied greatly in size and shape. These data are in agreement with earlier reports (31). Int. M fractions (9 through 11) show predominantly a vesicle arrangement composed of both OM and IM profiles, mostly forming a complex with each other (Fig. 3B). The arrangement becomes quite obvious when phage ε34 is present as a structural reference; the virus capsid, with its clearly defined polygonal contour and its adsorption organelles, can be seen as it attaches to vesicle complexes of converted cells (Fig. 4 and 5). In all preparations of Int. M regions, complexes between a high-contrast part (OM) and a low-contrast vesicle (IM) were the prevalent structures; only a few individual OM and IM vesicles were visible. To a limited degree, ε34 absorbed to IM vesicle fractions of converted cells; however, no OM vesicle features were seen in these complexes.

The quantitative evaluation of these microscopic preparations is shown in Fig. 1B and Table 1. We included in Table 1 also a column for virus particles observed in the vicinity of membrane vesicles, the vicinity meaning the distance of one virion diameter. The accumulation of virus particles in association with Int. M fragments from converted cells is striking. Sta-

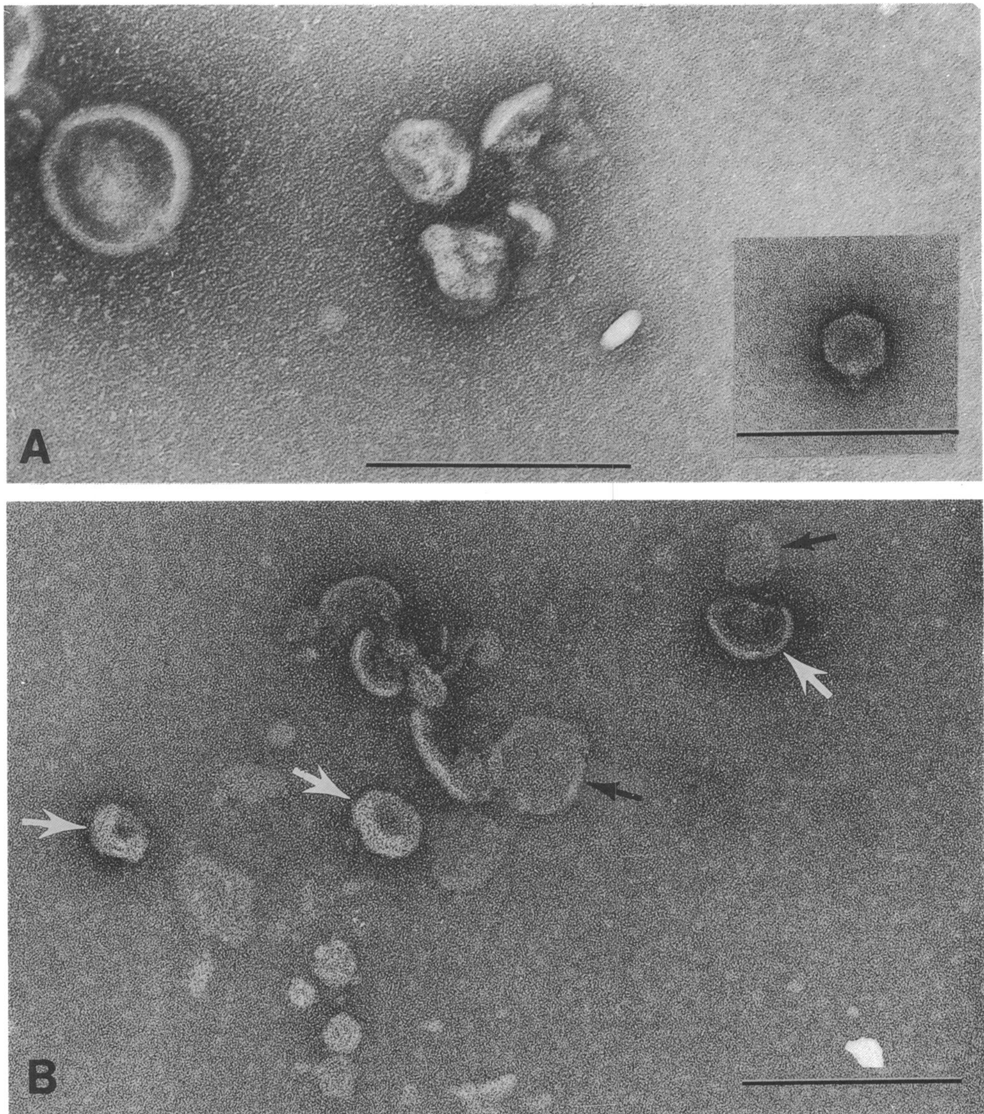


FIG. 3. (A) OM vesicles of *Salmonella anatum*, high-density region of sucrose gradient, negatively stained in uranyl acetate. (B) Int. M of sucrose gradient (unconverted cells) containing fractions of the OM (small vesicles of high contrast, white arrows) and IM (lower-contrast material, dark arrows). Phage ε34, added to the vesicles, is not attaching; the insert shows one of the few phages found in the preparation. Bar, 0.2 μm.

tistical evaluations show a highly significant rate of ε34 absorption to Int. M vesicles of converted cells and not to those of unconverted cells (Table 2). It should be emphasized here that these data are derived from double-blind observations at the electron microscope, with the microscopist and the individual preparing the coded specimen unaware of the specimen's fraction numbers.

## DISCUSSION

Transfer of the newly synthesized macromolecules from the IM to the OM is a prerequisite for cell surface growth. In rapidly growing *Salmonella* and *E. coli* strains, sites of transfer of LPS, capsule polysaccharides, and major OM proteins are distributed over the entire cell (for review, see reference 7) and were shown to be located at areas where the IM and OM are closely attached (fused) to each other (3). To label the area of adhesion sites, we used the conversion of the LPS by phage. After infection with phage ε15, the new LPS O-antigen, which also serves as receptor for phage ε34, was rapidly exported to the cell surface. We found that, after exposure of host cells to phage ε15,

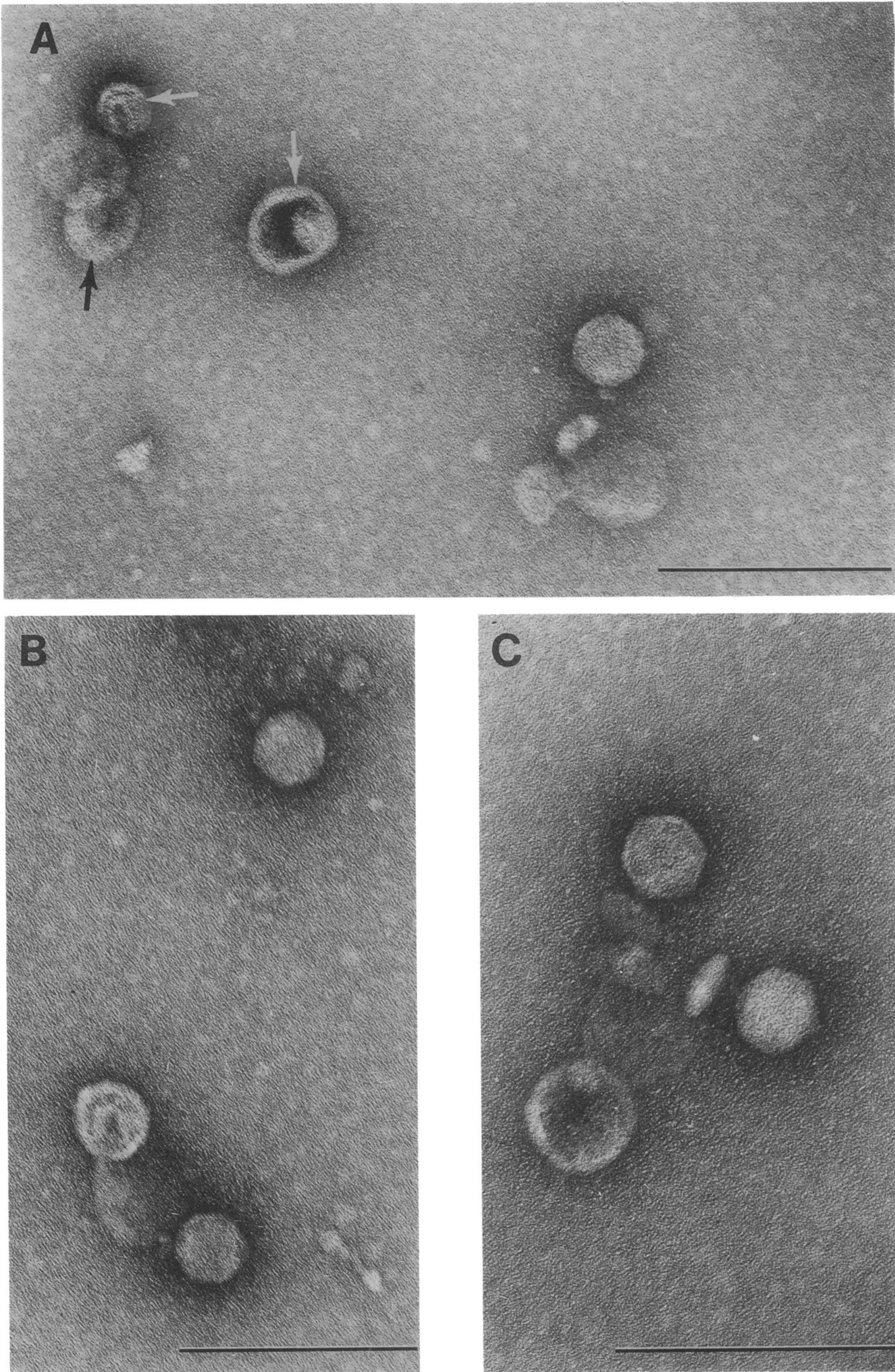


FIG. 4. Int. M region, converted cells; phage ε34 added. (A) OM vesicle (white arrows) and IM material (black arrow); right side shows OM-IM complex, with phage ε34 attached. (B) Free phage and adsorbed phage. (C) Two ε34 particles attached to vesicle complex. Bar, 0.2 μm.

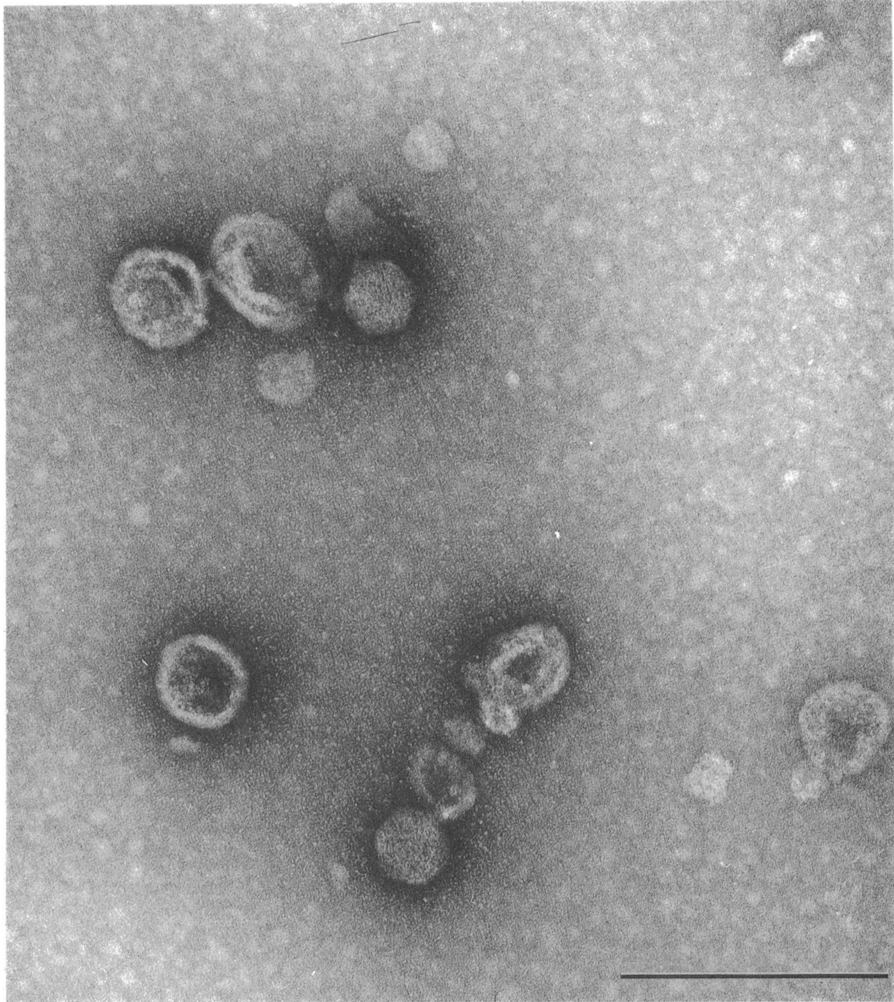

FIG. 5. Vesicle complex with OM-IM aspect and OM vesicle from the Int. M vesicle fraction; virions are attached to complexes. Bar, 0.2 μm.

the new antigen ($E_2$) is measurable as phage ε34 receptor within 1.5 to 2 min (M. E. Bayer, manuscript in preparation). However, the LPS export occurs at 20 to 40 sites per cell, or at about 10% of the total (average) number of adhesion sites per cell. When envelope fractions of such cells are prepared, the marker depicted by either antibody against antigen $E_2$ or by phage ε34 will therefore react only with the fraction showing the converted LPS. Tomita et al. (40) reported the migration of viral capsid markers from OM however, a redistribution of phage label fractions to vesicle fractions of intermediate density; may occur under similar conditions was reported by Crowlesmith et al. (15) in phage P22-labeled *Salmonella typhimurium* cells. Therefore, we employed phage or antibody adsorption after membrane fractionation, thus circumventing the danger of redistribution of markers during cell disruption and gradient purification. Those membrane fractions to which phage ε34 had adsorbed were visible as vesicle complexes composed of OM fragments and vesicular structures of lesser electron contrast (Fig. 3 and 4). The latter material is structurally very similar to that of the IM fraction. The composite fragments constitute the most abundant feature in Int. M fractions; they were rarely found in protein peak regions of IM fractions, and were not observed at all in those of the OM. In agreement with the clear separation of membrane fragments were data of sodium dodecyl sulfate gel electrophoresis; IM proteins were not found in the OM (after staining of the gels in Coomassie blue), whereas traces of OM proteins were seen in the IM fractions only

TABLE 1. Number of ϵ34 phages adsorbed to OM and Int. M vesicle fractions of *Salmonella anatum*[a]

| Fraction | No. of unattached phages | No. of phages attached to: | | No. of phages near[b]: | |
|---|---|---|---|---|---|
| | | OM | Int. M | OM | Int. M |
| OM[c] | 157 | 0 | 6 | 0 | 0 |
| OM converted | 109 | 3 | 2 | 5 | 0 |
| Int. M[c] | 134 | 2 | 3 | 1 | 8 |
| Int. M converted | 116 | 7 | 61 | 3 | 19 |
| IM[c] | 121 | 1 | 3 | 0 | 2 |
| IM converted | 109 | 2 | 12 | 0 | 5 |
| ϵ34, Control | 155 | 7 | 5 | 4 | 0 |

[a] Adsorption was measured by electron microscopy.
[b] "Near" designates an unattached virus particle positioned within one phage diameter from the vesicle surface.
[c] Membrane fractions derived from unconverted cells.

after overloading the gel. Very similar data were obtained with *E. coli* B: separation of the two major OM protein peaks paralleled that of *Salmonella anatum*, and the morphological appearance of the material in the various gradient fractions was also similar to that of the *Salmonella* strain.

Membrane-bound phospholipids are the major (90%) fatty acid-containing components in the enterobacteria (14); among them, phosphatidylethanolamine is the major phospholipid (24), and phospholipase A is a major hydrolyzing enzyme in the OM (34). Since phospholipase A (a mixture of $A_1$ and $A_2$) is now widely used as marker for the OM of gram-negative bacteria (11, 17, 19, 29), we studied the distribution of this enzyme with the intention to determine especially its activity in membrane fractions derived from the Int. M regions. Also, it had been previously suggested (42) that phospholipases may be useful tools in localizing phospholipid turnover and renewal processes in biological membranes. Such processes might occur specifically at membrane adhesion areas. We found that the phospholipase content predominates in the heavier and intermediate zones of the sucrose gradients. The graphic representation of the phospholipase activity consistently showed a valley between two peaks, as if the enzyme activity were associated specifically with two different structures, such as a lighter OM fraction and the still lighter fractions present at the Int. M region. The high phospholipase $A_1$ and $A_2$ activity in membrane fractions rich in label for adhesion sites supports the suggestion of a possible involvement of these sites in the turnover and regulation of envelope phospholipids (20). In addition, the material of Int. M exhibits the export sites for LPS which function as phage receptor and $E_2$-specific antigen; the Int. M region reveals in the electron microscope a complex between OM and IM vesicles.

TABLE 2. Chi-square evaluation of phage ϵ34 adsorption to vesicles of Int. M fractions[a]

| Phages | No. of phages adsorbed to vesicles | |
|---|---|---|
| | Unconverted cells | Converted cells |
| Attached to Int. M vesicles | 3 | 61 |
| Not attached to Int. M vesicles | 145 | 145 |

[a] Data are from Table 1. Result: $\chi^2_{(1)} = 42.4$ ($P < 0.001$).

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