JOURNAL OF BACTERIOLOGY, July 1976, p. 440-450
Vol. 127, No. 1

Printed in U.S.A.

Synthesis and Turnover of the Regularly Arranged Surface Protein of *Acinetobacter* sp. Relative to the Other Components of the Cell Envelope

KAREEN J. I. THORNE,* RHONDA C. OLIVER, AND AUDREY M. GLAUERT
Strangeways Research Laboratory, Wort's Causeway, Cambridge CB1 4RN, United Kingdom

Received for publication 7 April 1976

The formation of the components of the cell envelope of *Acinetobacter* sp. 199A was investigated by measuring the incorporation of [^{3}H]leucine into protein, [^{14}C]galactose into lipopolysaccharide, ^{32}P into phospholipid, and [^{3}H]diaminopimelic acid into peptidoglycan. Whereas the lipopolysaccharide and intrinsic protein of the outer membrane were stable, some of the regularly arranged surface protein, the *a*-protein, was lost into the growth medium. Only newly synthesized *a*-protein was lost. The peptidoglycan of the murein layer was also labile. Selective inhibition of the formation of individual components of the cell envelope with penicillin, chloramphenicol, and bacitracin showed that incorporation of protein into the outer membrane required the simultaneous formation of complete lipopolysaccharide. The converse was not true: protein synthesis was not required for lipopolysaccharide incorporation. Formation of the outer membrane and the murein layer proceeded independently.

The cell envelope of bacteria is a complex but well-defined structure. Gram-negative bacteria have an envelope that is multilayered, with plasma membrane, murein, outer membrane, sometimes a surface protein, and possibly also intermediate layers (7). The formation of the cell envelope therefore requires precise chemical and topological control and thereby provides a valuable experimental system for investigating regulation of synthesis in a multicomponent structure.

Although a considerable amount of work has been done on the regulation of plasma membrane formation (15), less is known about the other layers. Components of the outer membrane are synthesized at the plasma membrane (3, 19), but the mechanism whereby they are incorporated in controlled fashion is not understood. One of the components of the outer membrane is a regularly arranged surface protein. The synthesis and turnover of this protein is considered in particular detail in this paper. The characteristics of its formation provide a mechanism whereby the cell is always completely covered with surface protein. This indicates that the surface protein has an important biological function.

Since the outer membrane of *Acinetobacter* sp. strain 199A can be prepared (31) and individual components can be isolated, it is possible to use it to follow the synthesis and turnover of each component and to examine their dependence on one another and on the murein layer of the cell envelope. The outer membrane contains lipopolysaccharide, lipid, and at least three intrinsic proteins (28, 33), and carries a fourth protein in a regularly patterned layer, the *a*-layer or additional layer, on its surface (31, 32). Synthesis and turnover were measured by the incorporation and loss of radioactive precursors. Interdependence was investigated by selectively inhibiting the formation of each component with an appropriate antibiotic or inhibitor.

MATERIALS AND METHODS

Growth of bacteria. *Acinetobacter* sp. strain MJT/F5/199A (NCIB 10885) was grown in heart infusion broth (Difco, Detroit, Mich.) to which 0.01% $CaCl_2$ was added, at 25 to 30 C, with maximum aeration, either in 700-ml batches in an FE 007 fermentor (Biotec Ltd., Selsdon, South Croydon, U.K.) or in 100- to 120-ml batches in 500-ml Erlenmeyer flasks. The bacteria were grown either from a large inoculum of 10^8 cells/ml or from a small inoculum of 10^3 cells/ml. The mean generation time was 2 h.

Radioactive labeling. Radioactive precursors were added to the growth medium together with the bacterial inoculum. Protein was labeled from L-[4,5-^{3}H]leucine (58 Ci/mmol); lipopolysaccharide was labeled from D-[1-^{14}C]galactose (60 mCi/mmol); peptidoglycan was labeled from [G-^{3}H]2,6 diaminopimelic acid (DAP) (300 mCi/mmol); phospholipid was labeled from [^{32}P]phosphate. All radioactive chemicals were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. The radioactivity of the

cell fraction was measured in 1-ml aqueous samples in 10 ml of a standard Triton X-100-based scintillation mixture, by liquid scintillation. Lipids were counted in a toluene-based scintillation mixture.

Cell fractionation. (i) Cell walls. Cell walls were prepared as previously described (31) from cells broken in a French press. These walls contain less than 2% of the original plasma membrane, but have intact outer membranes.

(ii) Cytoplasmic protein. After breakage in the French press and treatment with deoxyribonuclease and ribonuclease (31), the cell walls were collected by centrifuging at 12,000 × *g* for 15 min. Cytoplasmic protein was recovered from the supernatant and from the first wash of the cell walls by precipitation with an equal volume of 2% phosphotungstic acid in N HCl. The precipitated protein was washed twice with 5% trichloroacetic acid.

(iii) Peptidoglycan. Peptidoglycan was solubilized by treatment of the isolated cell walls with egg white lysozyme (Armour Pharmaceutical Co., Eastbourne, U.K.) (100 μg/ml) in 50 mM sodium phosphate, pH 7.4, and 10 mM $MgCl_2$ for 1 h at 20 C.

(iv) *a*-Protein. The regularly arranged *a*-protein of the outer membrane was extracted from isolated cell walls by incubation with 2 M urea for 1 h at 37 C (33). The cell walls were removed by centrifuging at 12,000 × *g* for 15 min. The supernatant fluid, containing the *a*-protein, was dialyzed against distilled water to remove the urea and then centrifuged again at 12,000 × *g* for 15 min to precipitate contaminating bits of membrane (29). All samples were checked for purity of the *a*-protein and for completeness of extraction by electrophoresis on polyacrylamide gels in sodium dodecyl sulfate (SDS), where the *a*-protein gives a single band (molecular weight, 65,000) (33). Protein concentrations were determined by the method of Lowry et al. (13).

(v) *a*-Protein-depleted outer membrane. Outer membrane was prepared by incubating cell walls, from which *a*-protein had been removed, with lysozyme and $MgCl_2$ to remove peptidoglycan (31).

(vi) Lipopolysaccharide. Lipopolysaccharide was extracted from isolated cell walls, either by incubation with 10 mM ethylenediaminetetraacetate (EDTA) in 0.1 M ammonium bicarbonate (pH 8) at 20 C for 10 min (29) or with 45% phenol at 68 C for 10 min (10). Lipopolysaccharide was assayed chemically by the carbocyanine method (9).

(vii) Phospholipid. Lipid was extracted from the wet cell wall pellet with chloroform-methanol (1:1 [vol/vol]) at 7 C for 24 h. Insoluble material was removed by filtration.

Electron microscopy. Preparations for the electron microscopy of thin sections were fixed as pellets in 2.5% glutaraldehyde in 0.09 M cacodylate buffer (pH 7.2), containing 3 mM $CaCl_2$, for 30 min at room temperature (20 to 25 C), and then washed overnight or longer in cacodylate buffer at 4 C. The pellets were then postfixed in veronal-acetate-buffered OsO_4, pH 7.2, for 1 h at room temperature, stained with 0.5% uranyl acetate in veronal-acetate buffer for 1 h at room temperature, dehydrated in ethanol, and embedded in Araldite. Thin sections were cut with glass knives on an LKB Ultrotome III or a Cambridge Huxley ultramicrotome and stained with lead citrate. Specimens were examined in an AEI EM6 B electron microscope operating at 60 kV with a 50-μm objective aperture.

Inhibition by antibiotics. Batches of 100 ml of growth medium were inoculated with large inocula and then incubated for 45 min or 2 h with [^{3}H]leucine (5 to 100 μCi), [^{14}C]galactose (2 to 10 μCi), [^{3}H]DAP (10 to 20 μCi), or [^{32}P]phosphate (100 μCi) and various concentrations of penicillin G, chloramphenicol, or bacitracin (Sigma Chemical Co., St Louis, Mo.). Growth was stopped by the addition of sodium azide to 1 mM and rapid cooling to 0 C with crushed ice. Cells were then harvested by centrifuging at 5,000 × *g* for 15 min, washed with 50 mM sodium phosphate, pH 7.4, and fractionated.

The time course of bacitracin action was investigated by growing bacteria from a large inoculum in 700 ml of growth medium with the appropriate radioactive precursor for 2 h. Bacitracin (100 μg/ml) was then added and samples of 100 ml were removed at intervals, cooled to 0 C, inhibited with 1 mM sodium azide, harvested, washed, and fractionated.

Measurement of loss of radioactivity. (i) One-generation label. Log-phase bacteria were grown in 700 ml of growth medium for 2 h from a large inoculum in the presence of [^{3}H]leucine (100 μCi), or [^{14}C]galactose (8 μCi) or [^{3}H]DAP (20 μCi). The bacteria were harvested, washed twice with phosphate buffer, and suspended in 700 ml of unlabeled growth medium. Samples of 100 ml were removed at intervals, treated with 1 mM sodium azide, cooled to 0 C within 30 s, washed, and fractionated.

(ii) Many-generation label. Bacteria were grown in 120 ml of growth medium for 18 h from a small inoculum in the presence of [^{3}H]leucine (20 to 100 μCi), or [^{14}C]galactose (10 μCi) or [^{3}H]DAP (40 μCi). The bacteria that were still in log phase were harvested by centrifuging, washed twice with phosphate buffer, and suspended in 120 ml of unlabeled heart infusion broth. Samples of 25 ml were removed at intervals, treated with 1 mM sodium azide, cooled to 0 C, harvested, and washed. Before fractionation unlabeled bacteria were added to each sample to increase the bulk of material.

Immunology. Antiserum was prepared by injecting purified *a*-protein in Freund complete adjuvant (Difco, Detroit, Mich.) intramuscularly into rabbits (300 μg of protein/rabbit). Four weeks later a second intramuscular injection of 150 μg of *a*-protein was given. Blood (10 to 20 ml/rabbit) was taken 5 and 7 days after the second injection, and serum was prepared. Further samples of antiserum could be obtained from the same rabbits 5 and 7 days after a repeat injection of 150 μg of *a*-protein.

The specificity of the antiserum was tested on Ouchterlony plates. The antisera gave a single precipitin line against purified *a*-protein and the same line together with only traces of a second line against whole cell walls. No precipitin line was obtained with lipopolysaccharide isolated from *Acinetobacter* sp. 199A.

Immunoglobulin G was prepared from the antisera by chromatography on diethylaminoethyl-cellulose (22). This was coupled to Sepharose 4B using

cyanogen bromide-activated Sepharose 4B (Pharmacia Ltd., London, U.K.) (2), to produce an affinity column for the detection of radioactive a-protein in the bacterial growth medium. Purified a-protein bound to the column and could be eluted with 1% (wt/vol) SDS. Samples of 25 ml of growth medium were dialyzed extensively against water to remove [^{3}H]leucine, concentrated to 5 ml, and incubated at 4 C overnight in 10 mM $MgCl_2$ to precipitate a-protein. The precipitate was dissolved in 2 ml of water and bound to 1 ml of the affinity gel, in a small column, in 0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl. The gel was washed thoroughly with 0.1 M $NaHCO_3$ and 0.5 M NaCl to remove unbound protein. The a-protein was then eluted with 1% (wt/vol) SDS.

For immunoprecipitation the $MgCl_2$ precipitates were dissolved in water and 100 μg of unlabeled a-protein was added to increase the bulk of protein. Antiserum (0.1 ml) was added and the samples were left at 4 C overnight. Precipitated material was collected by centrifugation.

RESULTS

Incorporation of radioactive precursors. The synthesis of various cell envelope components was measured by the uptake of the appropriate radioactive precursor. The time course of [^{3}H]leucine incorporation into cell proteins is illustrated in Fig. 1. Incorporation into a-protein and outer membrane protein leveled off after 1 h (see also Fig. 3), which suggests that labeling of these fractions has reached a steady state. The distribution of radioactive isotopes between the cell fractions after 2 h of incorporation is shown in Table 1. About 7% of the incorporated leucine was found in the cell wall. Although the a-protein only represents 20% of the total wall protein (33), it comprised one-quarter of the radioactive protein. Further experiments, which will be described below, indicate that this may be due to turnover of part of the a-protein. The lysozyme-soluble "peptidoglycan" fraction also contained radioactive protein. This was to be expected since protein, possibly of periplasmic origin or perhaps a lipoprotein similar to that isolated from *Escherichia coli* (5), is released when *Acinetobacter* 199A cell walls are treated with lysozyme (28).

Most of the radioactive material in the cell wall from [^{3}H]DAP was recovered in the lysozyme-soluble peptidoglycan fraction. This has been shown by thin-layer chromatography to be at least 85% DAP (28). The presence of radioactivity in the a-protein and membrane protein fractions presumably reflects conversion of DAP to lysine.

After growth with [^{14}C]galactose, radioactive lipopolysaccharide could be extracted either with EDTA or with phenol. Only two-thirds as much radioactive lipopolysaccharide was extractable with EDTA as with phenol.

Inhibition by bacitracin. After 45 min of incubation with bacitracin, the incorporation of radioactive leucine into a-protein and the intrinsic outer membrane proteins were inhibited (Fig. 2). This was not due to growth inhibition since incorporation into cytoplasmic protein was unaffected. Even after the longer incubation of 2 h, when bacitracin does begin to inhibit cytoplasmic protein synthesis, owing to its inhibition of cell growth, the effect on the cell wall proteins is greater than that on cytoplasmic protein. After 45 min some inhibition of outer membrane phospholipid synthesis could also be detected.

The expected inhibition of DAP incorporation into peptidoglycan was observed (Fig. 2). Galactose incorporation into lipopolysaccharide was less affected. Since a mutant of *Acinetobacter* 199A lacking uridine 5'-diphosphophosphate galactose epimerase was not available, some of the galactose may have been converted to other

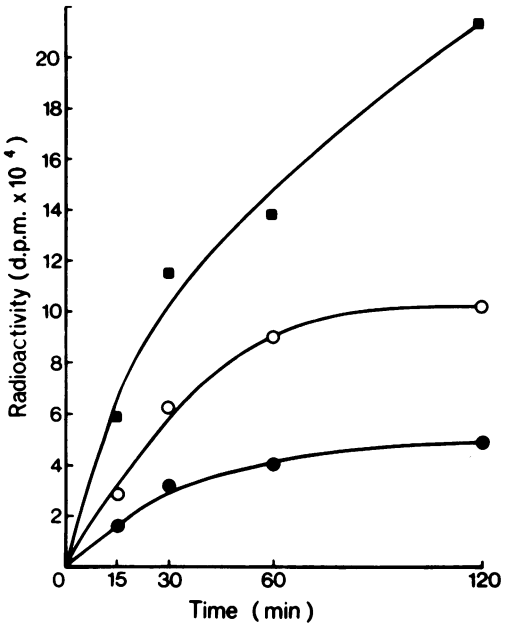

FIG. 1. *Incorporation of [^{3}H]leucine into cell proteins. Cells were grown from a large inoculum in 120 ml of growth medium containing 100 μCi of [^{3}H]leucine. Samples of 25 ml were removed after 15, 30, 60, and 120 min, treated with 1 mM sodium azide, and cooled rapidly in ice. The cells were then fractionated and analyzed as described in Materials and Methods. The radioactivity of the cell fractions was determined. Total disintegrations per minute: ●, a-protein; ○, intrinsic outer membrane protein; ■, one-tenth cytoplasmic protein.*

TABLE 1. *Incorporation of radioactive precursors into cell fractions after 2 h of growth from a large inoculum (100 ml of growth medium)*[a]

Radioactive precursor (μCi/100 ml of growth medium)	Disintegrations/min per 100 ml of growth medium					
	Cytoplasmic protein	Cell wall				
		Peptidoglycan	Lipopolysaccharide		α-Protein	Intrinsic membrane protein
			EDTA	Phenol		
[^{3}H]leucine (10 μCi)	227,000	1,420	ND[b]	ND	4,070	11,100
[^{3}H]DAP (14 μCi)	2,910	560	ND	ND	53	166
[^{14}C]galactose (2 μCi)	ND	ND	358	580	ND	ND

[a] Cells were grown in 100 ml of medium for 2 h from a large inoculum in the presence of 10 μCi of [^{3}H]leucine, 14 μCi of [^{3}H]DAP, or 2 μCi of [^{14}C]galactose. The cells were collected and the cell walls prepared and fractionated as described in Materials and Methods.

[b] ND, Not determined.

sugars. In addition, some of the incorporated galactose is probably in the core oligosaccharide, whose synthesis is not dependent on prenol phosphate and might not therefore be inhibited by bacitracin (18, 34). When the lipopolysaccharide was assayed chemically it was apparent that its formation was indeed inhibited by bacitracin (Tables 2 and 3). Some loss of lipopolysaccharide seemed also to have been induced, since the chemical assay showed lower levels than could be accounted for by inhibition of galactose incorporation (Fig. 2). Loss of radioactive lipopolysaccharide after addition of bacitracin can be seen in Fig. 7. The parallel inhibition of α-protein formation could also be detected chemically (Table 2).

The inhibition of incorporation of [^{3}H]leucine into α-protein and outer membrane protein was detectable 30 min after the addition of bacitracin (Fig. 3).

Inhibition by penicillin. The synthesis of peptidoglycan, measured as lysozyme-soluble DAP, was inhibited 85% by 50 μg of penicillin per ml (Fig. 4). This concentration of penicillin had little effect on the incorporation of [^{3}H]leucine into either the α-protein or the intrinsic outer membrane proteins, even after 2 h of incubation. Some inhibition is inevitable owing to the growth inhibitory effect of penicillin. This is reflected in a small inhibition of cytoplasmic protein synthesis.

Electron microscopy of thin sections of penicillin-treated cells (Fig. 5b) showed that the peptidoglycan layer d (Fig. 5) was thinner and somewhat less defined than in normal cells (Fig. 5a).

Inhibition by chloramphenicol. Synthesis of the protein components of the cell envelope was inhibited by chloramphenicol (Fig. 6). No difference was seen between the response of cytoplasmic and envelope proteins to chloramphenicol. Lipopolysaccharide synthesis, measured as radioactivity of phenol-extractable galactose, and peptidoglycan synthesis were unimpaired. The continued synthesis of peptidoglycan in the absence of protein synthesis resulted in cells with a thickened peptidoglycan layer (Fig. 5c). The outer membrane also became unbalanced since growth for 2 h with 20 μg of chloramphenicol per ml increased the concentration of lipopolysaccharide from 105 μg/mg of cell wall protein to 220 μg/mg of protein.

Loss of cell wall components. When bacitracin was added 2 h after the radioactive precursors (Fig. 7), and incubation continued in the presence of the radioactive precursors, it was discovered that some components of the cell envelope lost radioactivity. When the incorporation of [^{3}H]leucine into α-protein and of [^{3}H]DAP into peptidoglycan was inhibited by bacitracin, it was possible to see that these two components were losing radioactivity. In an attempt to discover whether α-protein and peptidoglycan were indeed lost from the cell envelope, cells were grown for 2 h (one generation) or for 18 h with radioactive precursors, then harvested, washed, and transferred to unlabeled growth medium. The radioactivity of the different cell wall components was then measured at intervals up to 3 h (Fig. 8). Specific activities could not be measured as unlabeled bacteria were added to increase the cell bulk before fractionation.

Both lipopolysaccharide and outer membrane protein were predominantly stable, whether they were labeled for one generation or many; the radioactivity of the intrinsic outer membrane protein was, however, slowly reduced after long labeling. Peptidoglycan radioactivity was lost under both conditions. The regularly arranged α-protein exhibited some unusual properties. Under both conditions there was an

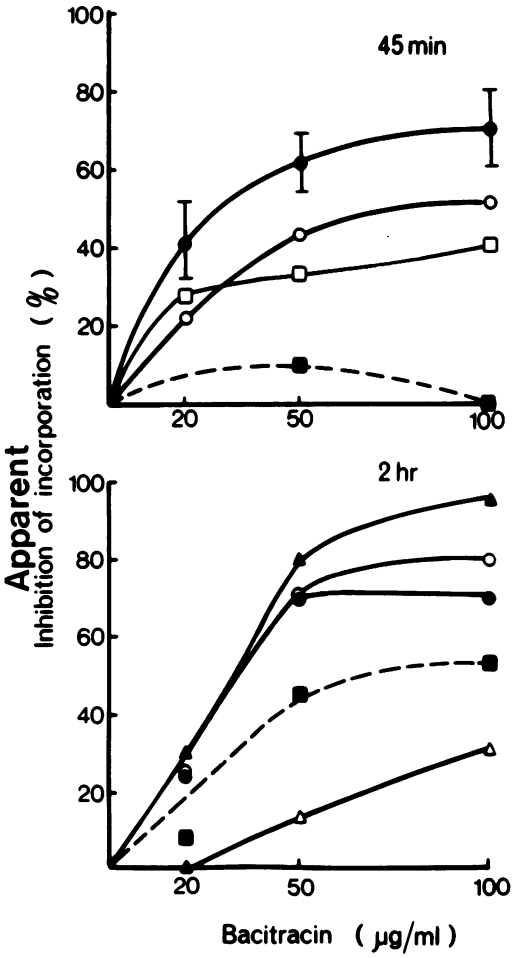

FIG. 2. *Inhibition of synthesis of cell envelope components by bacitracin. Cells were incubated for 45 min or 2 h in 100 ml of growth medium with 0 to 100 μg of bacitracin per ml and the appropriate radioactive isotope. Cell walls were then prepared, fractionated, and analyzed (Materials and Methods). Symbols: [³H]DAP incorporation into lysozyme-soluble peptidoglycan (▲); [³H]leucine incorporation into intrinsic outer membrane protein (○); [³H]leucine incorporation into a-protein (●), bar lines represent standard errors of the mean (three experiments); [³H]leucine incorporation into cytoplasmic protein (■); [¹⁴C]galactose incorporation into phenol-extractable lipopolysaccharide (△); ³²P incorporation into phospholipid (□).*

initial increase in the radioactivity of the *a*-protein when the cells were transferred to unlabeled growth medium. A somewhat similar, although less marked, residual incorporation into intrinsic outer membrane protein could also be seen, after short labeling. In cells that had been labeled for 2 h, the initial increase in activity of the *a*-protein was followed by a rapid loss. This loss was not detectable in cells labeled for 18 h, where most of the *a*-protein is probably many generations old. This suggests that only new *a*-protein is lost.

The tendency of new *a*-protein to be lost from the cell wall was confirmed in the following way. Cells were grown with [³H]leucine for periods from 1 to 18 h. They were then suspended in unlabeled medium, and the amount of radioactivity lost from the *a*-protein fraction after 2 h was measured (Table 4). The longer the bacteria were grown in labeled medium the smaller was the percentage of radioactive *a*-protein lost.

Detection of secreted *a*-protein. One possibility is that the *a*-protein that is lost from the cell wall is shed intact into the growth medium. If this is so, it should be possible to detect radioactive *a*-protein in the medium. Isolated *a*-protein has the ability to self-assemble in the presence of $MgCl_2$ into crystalline arrays which can be collected by centrifugation (29). Growth medium from bacteria growing with [³H]-leucine was dialyzed extensively against water to remove free leucine. Precipitation overnight at 4 C with 10 mM $MgCl_2$ gave a precipitate which contained most of the radioactivity of the growth medium (Table 5). The identity of the $MgCl_2$ precipitated with *a*-protein was tested immunologically and by gel electrophoresis. After electrophoresis on poly-

TABLE 2. *Inhibition of lipopolysaccharide (LPS) and a-protein synthesis by bacitracin*

Bacitracin (μg/ml)	Total LPS in cells from 100 ml of medium (μg)	Total *a*-protein in cells from 100 ml of medium (μg)
Before incubation		
0	180	310
After 2 h of incubation		
0	400	730
20	350	630
50	205	380
100	150	330

TABLE 3. *Effect of bacitracin on lipopolysaccharide (LPS) synthesis*

Incubation time (min)	Total LPS in cells from 100 ml of medium	
	Control (μg)	+ 10 mg of bacitracin (μg)
0	180	137
30	280	137
90	480	120

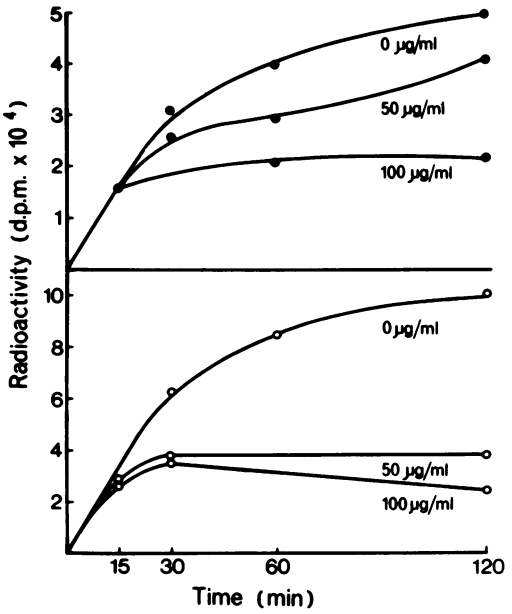

FIG. 3. *Effect of bacitracin on the time course of incorporation of [³H]leucine into a-protein and intrinsic outer membrane protein. Cells were grown from large inocula in batches of 120 ml of growth medium containing 100 µCi of [³H]leucine and bacitracin at concentrations of 0, 50, and 100 µg/ml. Samples of 25 ml were removed after 15, 30, 60, and 120 min, treated with 1 mM sodium azide, and cooled rapidly in ice. Cell walls were then prepared and fractionated as described in Materials and Methods. The radioactivity of the cell fractions was determined. Symbols: ●, a-protein; ○, intrinsic outer membrane protein.*

acrylamide gels in SDS (Fig. 9), all of the radioactive material was found in a single band. This had the same mobility as purified *a*-protein.

Addition of the specific anti-*a*-protein antiserum to the dissolved $MgCl_2$ precipitate, in the presence of 100 µg of added unlabeled *a*-protein to increase the bulk, resulted in precipitation of 323 counts/min out of the original 362 counts/min in the sample (or 91%).

Binding to immunoglobulin G coupled to Sepharose 4B was less effective (Table 6). Of the original radioactivity 53% did not bind. No activity could be eluted with 0.2 M glycine at either pH 2.8 or pH 10. For this reason 1% (wt/vol) SDS was used to break the antigen-antibody complex. A total of 42% of the original radioactive material was eluted with SDS.

The amount of *a*-protein secreted into the growth medium is apparently similar to the amount incorporated into the cell wall (Table 5).

DISCUSSION

There are interesting differences between the turnover of the different components of the envelope of *Acinetobacter* sp. 199A. The peptidoglycan of the murein layer is apparently labile; the outer membrane, as represented by lipopolysaccharide and intrinsic protein, is largely stable; newly synthesized *a*-protein is labile, but the older *a*-protein is stable. Although peptidoglycan turnover has been reported in several gram-positive bacteria (4, 6, 20, 21, 23) it has not previously been described in a gram-negative bacterium. *Acinetobacter* sp. 199A is, however, somewhat unusual since it has a fairly thick peptidoglycan layer (31) and sometimes stains positively with the Gram stain (30). About half of the peptidoglycan synthesized in a generation is resistant to hydrolysis. Turnover starts immediately at a rapid rate and then gradually slows down. This is in direct contrast to the turnover observed in gram-positive bacteria, where an initial lag is followed by an increasing rate of loss of peptidoglycan radioactivity (4, 6, 20, 21, 23). It has been postulated that the peptidoglycan has to reach the outside of the cell before it becomes accessible to autolytic enzymes (20, 21, 23). Conversely in *Acinetobacter* the peptidoglycan becomes less accessible to autolysis as the cell grows, possibly because the autolysin attacks

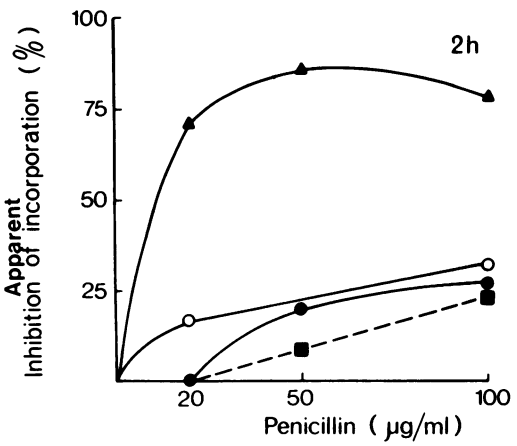

FIG. 4. *Inhibition of synthesis of cell envelope components by penicillin. Cells were incubated for 2 h in 100 ml growth medium with 0 to 100 µg of penicillin per ml and the appropriate radioactive isotope. Cell walls were then prepared, fractionated, and analyzed (Materials and Methods). Symbols: [³H]DAP incorporation into lysozyme-soluble peptidoglycan (▲); [³H]leucine incorporation into intrinsic outer membrane protein (○); [³H]leucine incorporation into a-protein (●); [³H]leucine incorporation into cytoplasmic protein (■).*

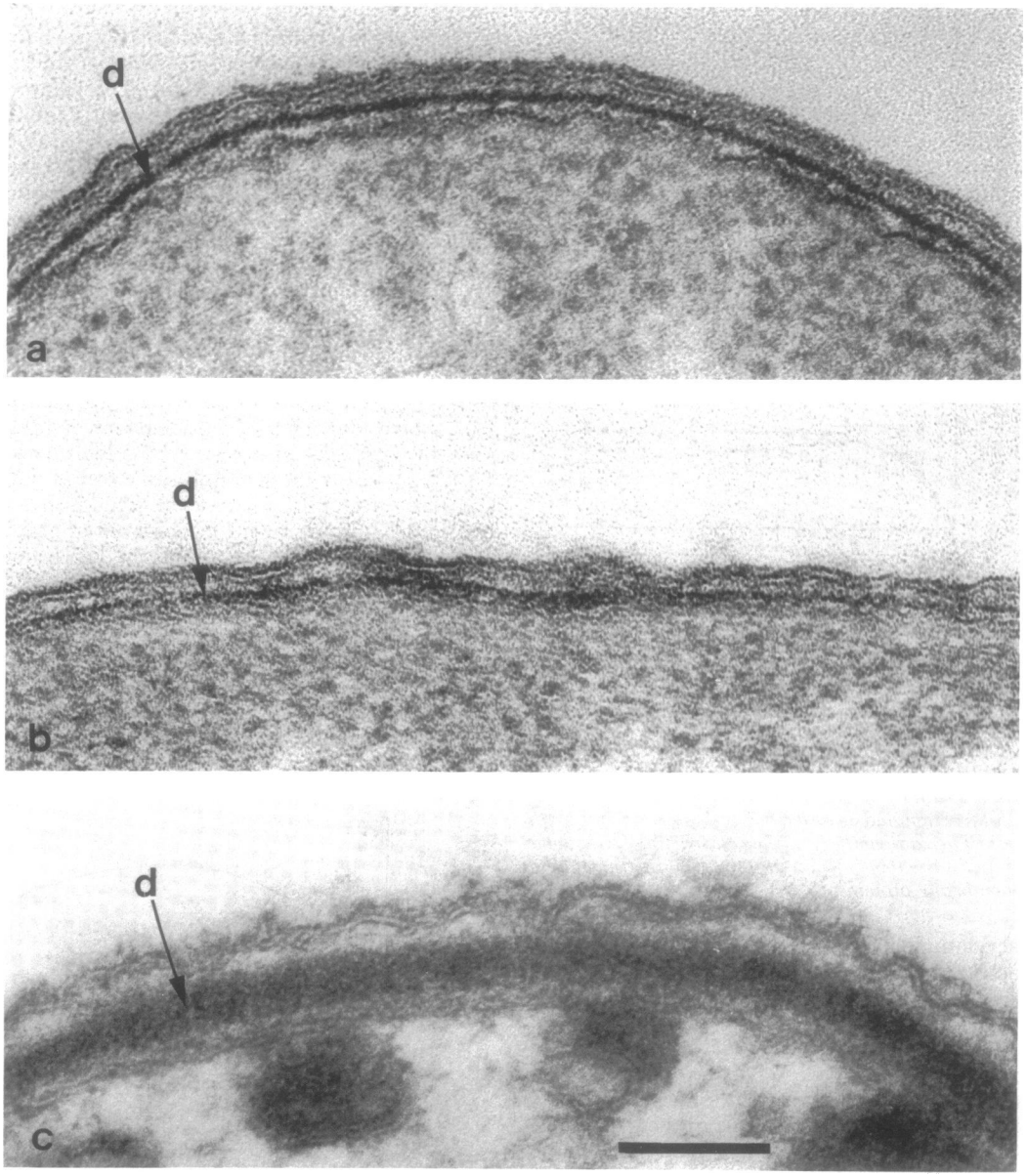

FIG. 5. *Effect of penicillin and chloramphenicol on the ultrastructure of the cell envelope. Thin sections of whole bacterial cells grown (a) without addition or (b) with penicillin (100 μg/ml) or (c) with chloramphenicol (50 μg/ml). The peptidoglycan-containing layer is labeled (d). The bar line represents 0.1 μm.*

the inner, plasma membrane side of the murein layer.

The loss of the *a*-protein provides information on the mechanism of its incorporation into the outer membrane. Bacteria that were labeled for a short time lost a considerably larger proportion of their radioactive *a*-protein than those that were labeled for a long time. Apparently, it is the newest *a*-protein that is lost, perhaps because excess is synthesized. Older protein has become an integral part of the outer membrane and has thereby acquired stability. The free excess *a*-protein can be detected in the growth medium where it retains its chemical and immunological properties. A somewhat similar process has been proposed (24) to explain the arrangement of surface subunits on the gram-positive *Clostridium thermosacchar-*

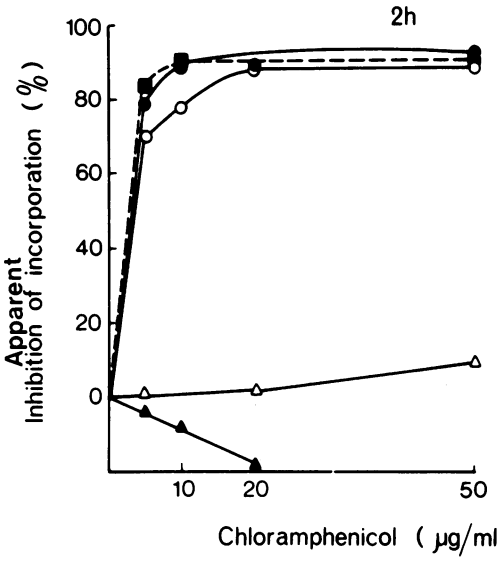

FIG. 6. *Inhibition of synthesis of cell envelope components by chloramphenicol. Cells were incubated for 2 h in 100 ml of growth medium with 0 to 50 μg of chloramphenicol per ml and the appropriate radioactive isotope. Cell walls were then prepared, fractionated, and analyzed (Materials and Methods). Symbols: [^{3}H]DAP incorporation into lysozyme-soluble peptidoglycan (▲); [^{3}H]leucine incorporation into intrinsic outer membrane protein (○); [^{3}H]leucine incorporation into a-protein (●); [^{3}H]leucine incorporation into cytoplasmic protein (■); [^{14}C]galactose incorporation into phenol-extractable lipopolysaccharide (△).*

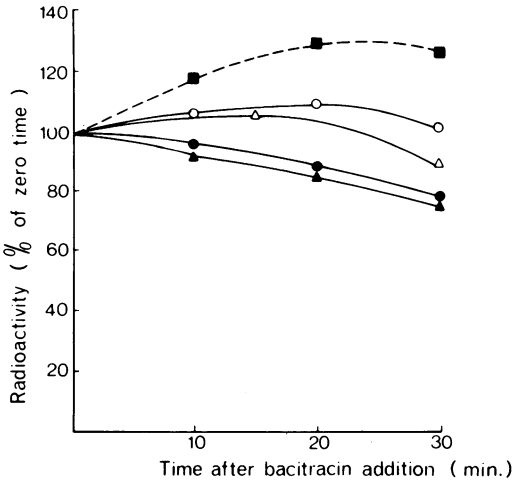

FIG. 7. *Time course of inhibition of synthesis of cell envelope components by bacitracin. Cells were grown for 2 h with the appropriate radioactive precursor before addition of bacitracin (100 μg/ml). Samples (100 ml) were removed at intervals after bacitracin addition for preparation of cell walls and fractionation (Materials and Methods). Symbols: [^{3}H]DAP incorporation into lysozyme-soluble peptidoglycan (▲); [^{3}H]leucine incorporation into intrinsic outer membrane protein (○); [^{3}H]leucine incorporation into a-protein (●); [^{3}H]leucine incorporation into cytoplasmic protein (■); [^{14}C]galactose incorporation into lipopolysaccharide (△).*

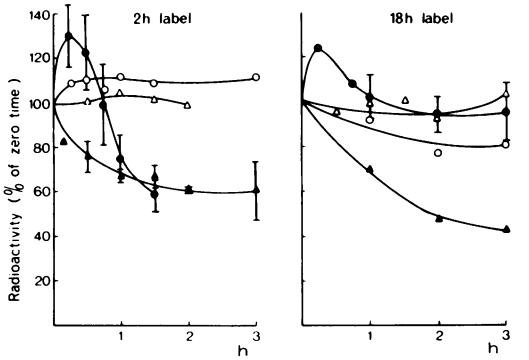

FIG. 8. *Loss of radioactivity from cell envelope components. Cells were labeled for 2 or 18 h with the appropriate radioactive precursor as described in Materials and Methods, then harvested, washed, and transferred to unlabeled medium. Samples were removed at intervals (Materials and Methods). Symbols: radioactivity of lysozyme-soluble peptidoglycan from [^{3}H]DAP (▲); radioactivity of intrinsic outer membrane protein from [^{3}H]leucine (○); radioactivity of a-protein from [^{3}H]leucine (●); radioactivity of lipopolysaccharide from [^{14}C]galactose (△). Standard errors of the mean are given for radioactivity of a-protein and of peptidoglycan labeled for 2 h (three experiments).*

olyticum and *C. thermohydrosulphuricum*. Here the subunits are believed to form initially small areas of pattern in the septal regions, where they are synthesized. The subunits then rearrange themselves to give large patterned areas which cover the whole surface of the cell. A necessary part of this theory is that the cell synthesizes excess protein, and that only enough is used to keep the surface covered. Although there is no direct evidence that biosynthesis of the outer membrane in gram-negative cocci is localized in the septal region, an apparent excess of material in the form of blebs can be seen in this area in freeze-etched preparations of *Acinetobacter* 199A (25).

Formation of the murein layer and of the outer membrane of strain 199A proceed independently. When peptidoglycan synthesis is inhibited by penicillin, bacteria have a thinner murein layer, but a normal outer membrane. Conversely, peptidoglycan synthesis continues leading to the formation of cells with thickened murein layers when synthesis of the outer membrane protein is inhibited with chloramphenicol (25).

TABLE 4. *Loss of a-protein after different labeling times*[a]

Labeling time (h)	Disintegrations/min		Percentage lost
	Before chase	After chase	
1	593	264	55.5
2	3,560	2,250	37
4	11,090	8,160	26
6	37,300	30,230	19
18	314,000	312,000	1

[a] Bacteria were grown in 700 ml of growth medium from a large inoculum in the presence of 40 μCi of [^{3}H]leucine. Samples of 100 ml were removed at intervals from 1 to 18 h, cooled to 0 C with crushed ice, harvested, and washed twice. Half of the washed cells was kept for measurement of radioactivity at zero time; the other half was resuspended in 100 ml of unlabeled growth medium and incubated for a further 2 h. The washed cells from 0 h and 2 h for each labeling time were then fractionated as described in Materials and Methods, and the a-protein was isolated.

TABLE 5. *Secretion of radioactive a-protein into the growth medium*[a]

Incubation time (min)	Radioactivity (dpm)		
	Growth medium		Cell wall a-protein
	Dialyzed medium	$MgCl_2$ precipitate	
15	19,650	14,400	16,000
30	38,400	23,200	32,000
120	42,200	37,000	50,000

[a] Cells were grown in 120 ml of growth medium containing 100 μCi of [^{3}H]leucine. Samples of 25 ml were taken after 15, 30 and 120 min, treated with 1 mM sodium azide, and cooled rapidly in ice. Cells were removed from the medium by centrifugation and used for the preparation of a-protein. The medium was dialyzed extensively against water, and a-protein was then precipitated with 10 mM $MgCl_2$ at 4 C overnight.

Interdependence is found between individual components of the outer membrane. Neither intrinsic protein, nor a-protein are incorporated into the outer membrane when lipopolysaccharide synthesis is modified by bacitracin. Bacitracin inhibits the regeneration of prenol phosphate from the prenol pyrophosphate formed during peptidoglycan biosynthesis (26). In the presence of bacitracin prenol phosphate is depleted (27), and the biosynthesis of those cell wall polymers which require it, peptidoglycan (26) and the O-antigen side chains of lipopolysaccharide (34), is inhibited. The O-antigen-deficient lipopolysaccharide formed in the presence of bacitracin behaves similarly to that seen in lipopolysaccharide mutants of *Salmonella typhimurium* (1) and *E. coli* (11). In both species the formation of modified lipopolysaccharide inhibited the incorporation of protein into the outer membrane, and in *S. typhimurium* lipopolysaccharide was lost into the growth medium. The modified lipopolysaccharide induced by bacitracin may be similarly lost into the medium. Certainly the reduction in level of total lipopolysaccharide is greater than can be accounted for by the inhibition of radioactive galactose incorporation. The small modifications introduced into the lipopolysaccharide by bacitracin have a large inhibitory effect on the total amount of lipopolysaccharide found in the outer membrane. This then interferes with the incorporation of proteins into the outer membrane.

This indicates that new protein is only taken into the outer membrane in combination with

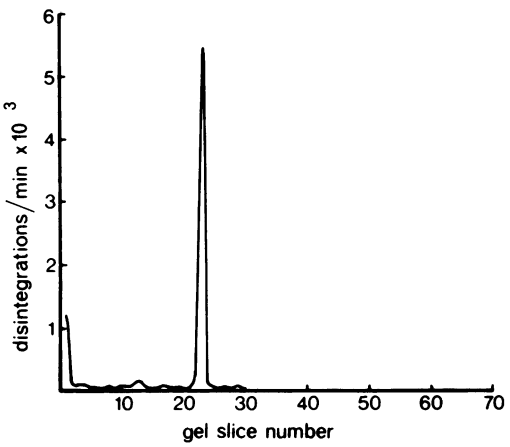

FIG. 9. *Identity of radioactive protein in growth medium with a-protein. The $MgCl_2$ precipitates described in Table 5 were dissolved in water, combined, and concentrated. The concentrate was separated by electrophoresis on polyacrylamide gels in SDS (33). Gels were cut into 1-mm slices, dissolved in 0.2 ml of 3 M hydrogen peroxide, and counted in a Triton X-100-based scintillant.*

TABLE 6. *Binding of a-protein to Sepharose 4B-immunoglobulin G (1-ml column)*

Fraction	Radioactive material	
	Disintegrations/min	Percentage of original
Original sample	16,300	100
Not bound to column	8,600	53
Eluted with 1% (wt/vol) SDS		
1st ml	3,400	21
2nd ml	2,140	13
3rd ml	1,270	8

complete lipopolysaccharide. The converse is not true. When protein synthesis is inhibited by chloramphenicol, lipopolysaccharide synthesis still continues, leading to an unbalanced outer membrane containing twice the normal concentration of lipopolysaccharide. Possibly, protein does not become incorporated in the membrane unless lipopolysaccharide is simultaneously available to form a complex with it. This is an extension of the mechanism proposed by Nikaido for outer membrane formation (17). Newly synthesized lipopolysaccharide first forms an EDTA-resistant complex with phospholipid (12). Protein is then incorporated, which puts the lipopolysaccharide into an environment from which it is extractable with EDTA.

In gram-positive bacteria the separate layers of protein A and peptidoglycan of *Staphylococcus aureus* are also formed independently (16), whereas two components of the same layer, teichoic acid and peptidoglycan, exhibit interdependence (14).

The cell wall fraction from *Acinetobacter* sp. 199A used in this work provides a particularly useful experimental system. It consists of the murein layer, the periplasmic space, the outer membrane, and the surface *a*-protein. It therefore contains not only the final products of formed outer membrane and *a*-protein, but also regions of the envelope which may include precursors of these layers, thus allowing the detection of the turnover of newly synthesized, incompletely incorporated *a*-protein. The residual incorporation of radioactive protein for up to 15 min after transference to unlabeled medium was also seen by Inouye et al. (8) in *E. coli* outer membrane. It should be possible to follow the transference of outer membrane components from their site of synthesis in the plasma membrane (3, 19) to their final destination.

ACKNOWLEDGMENTS

We acknowledge the support of the Medical Research Council (to K.J.I.T and R.C.O), the Sir Halley Stewart Trust (to A.M.G.), and the Wellcome Trust for the loan of the AEI-EM6B electron microscope.

We are indebted to R. A. Parker for skilled technical assistance.

LITERATURE CITED

1. **Ames, G. F., E. N. Spudich, and H. Nikaido.** 1974. Protein composition of the outer membrane of *Salmonella typhimurium:* effect of lipopolysaccharide mutations. J. Bacteriol. 117:406-416.
2. **Axén, R., J. Porath, and S. Ernback.** 1967. Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. Nature (London) 214:1302-1304.
3. **Bell, R. M., R. D. Mavis, M. J. Osborn, and P. R. Vagelos.** 1971. Enzymes of phospholipid metabolism: localisation in the cytoplasmic and outer membrane of the cell envelope of *Escherichia coli* and *Salmonella typhimurium*. Biochim. Biophys. Acta 249:628-635.
4. **Boothby, D., L. Daneo-Moore, M. L. Higgins, J. Coyette, and G. D. Shockman.** 1973. Turnover of bacterial cell wall peptidoglycans. J. Biol. Chem. 248:2161-2169.
5. **Braun, V., and K. Rehn.** 1969. Chemical characterization, special distribution and function of a lipoprotein of the *E. coli* cell wall. Eur. J. Biochem. 10:426-438.
6. **Daneo-Moore, L., J. Coyette, M. Sayare, D. Boothby, and G. D. Shockman.** 1975. Turnover of cell wall peptidoglycan of *Lactobacillus acidophilus*. The presence of a fraction immune to turnover. J. Biol. Chem. 250:1348-1353.
7. **Glauert, A. M., and M. J. Thornley.** 1969. The topography of the bacterial cell wall. Annu. Rev. Microbiol. 23:159-198.
8. **Inouye, M., J. Shaw, and C. Shen.** 1972. The assembly of a structural lipoprotein in the envelope of *Escherichia coli*. J. Biol. Chem. 247:8154-8159.
9. **Janda, J., and E. Work.** 1971. A colorimetric estimation of lipopolysaccharide. FEBS Lett. 16:343-345.
10. **Knox, K. W., J. Cullen, and E. Work.** 1967. An extracellular lipopolysaccharide-phospholipid-protein complex produced by *Escherichia coli* grown under lysine limiting conditions. Biochem. J. 103:192-201.
11. **Koplow, J., and H. Goldfine.** 1974. Alterations in the outer membrane of the cell envelope of heptose-deficient mutants of *Escherichia coli*. J. Bacteriol. 117:527-543.
12. **Levy, S. B., and L. Leive.** 1968. An equilibrium between two fractions of lipopolysaccharide in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 61:1435-1439.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
14. **Mauck, J., and L. Glaser.** 1972. On the mode of in vivo assembly of the cell wall of *Bacillus subtilis*. J. Biol. Chem. 247:1180-1187.
15. **Mindich, L.** 1973. Synthesis and assembly of bacterial membranes, p. 1-36. *In* L. Leive (ed.), Bacterial membranes and walls. Marcel Dekker Inc., New York.
16. **Movitz, J.** 1974. A study on the biosynthesis of protein A in *Staphylococcus aureus*. Eur. J. Biochem. 48:131-136.
17. **Nikaido, H.** 1973. Biosynthesis and assembly of lipopolysaccharide and the outer membrane of the gram negative cell wall, p. 131-208. *In* L. Leive (ed.), Bacterial membranes and walls. Marcel Dekker Inc., New York.
18. **Osborn, M. J.** 1969. Structure and biosynthesis of the bacterial cell wall. Annu. Rev. Biochem. 38:501-538.
19. **Osborn, M. J., J. E. Gander, and E. Parisi.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Site of synthesis of lipopolysaccharide. J. Biol. Chem. 247:3973-3986.
20. **Pooley, H. M.** 1976. Turnover and spreading of old wall during surface growth of *Bacillus subtilis*. J. Bacteriol. 125:1127-1138.
21. **Pooley, H. M.** 1976. Layered distribution, according to age, within the cell wall of *Bacillus subtilis*. J. Bacteriol. 125:1139-1147.
22. **Porter, R. R.** 1959. The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. Biochem. J. 73:119-126.
23. **Rogers, H. J., H. M. Pooley, P. F. Thurman, and C. Taylor.** 1974. Wall and membrane growth in bacilli and their mutants. Ann. Inst. Pasteur Paris 125:135-147.
24. **Sleytr, U. B., and A. M. Glauert.** 1975. Analysis of regular arrays of subunits on bacterial surfaces: evi-

dence for a dynamic process of assembly. J. Ultrastr. Res. 50:103–116.
25. **Sleytr, U. B., and M. J. Thornley.** 1973. Freeze-etching of the cell envelope of an *Acinetobacter* species which carries a regular array of surface subunits. J. Bacteriol. 116:1383–1397.
26. **Stone, K. J., and J. L. Strominger.** 1971. Mechanism of action of bacitracin: complexation with metal ion and C_{55}-isoprenyl pyrophosphate. Proc. Natl. Acad. Sci. U.S.A. 68:3223–3227.
27. **Thorne, K. J. I.** 1973. Identification of prenol intermediates of wall biosynthesis in growing cells of *Lactobacillus plantarum*. J. Bacteriol. 116:235–244.
28. **Thorne, K. J. I., M. J. Thornley, and A. M. Glauert.** 1973. Chemical analysis of the outer membrane and other layers of the cell envelope of *Acinetobacter* sp. J. Bacteriol. 116:410–417.
29. **Thorne, K. J. I., M. J. Thornley, P. Naisbitt, and A. M. Glauert.** 1975. The nature of the attachment of a regularly arranged surface protein to the outer membrane of an *Acinetobacter* sp. Biochim. Biophys. Acta 389:97–116.
30. **Thornley, M. J.** 1967. A taxonomic study of *Acinetobacter* and related genera. J. Gen. Microbiol. 49:211–257.
31. **Thornley, M. J. Glauert, A. M., and U. B. Sleytr.** 1973. Isolation of outer membranes with an ordered array of surface subunits from *Acinetobacter*. J. Bacteriol. 114:1294–1308.
32. **Thornley, M. J., A. M. Glauert, and U. B. Sleytr.** 1974. Structure and assembly of bacterial surface layers composed of regular arrays of subunits. Philos. Trans. R. Soc. London Ser. B 268:147–153.
33. **Thornley, M. J., K. J. I. Thorne, and A. M. Glauert.** 1974. Detachment and chemical characterization of the regularly arranged subunits from the surface of an *Acinetobacter*. J. Bacteriol. 118:654–662.
34. **Wright, A., M. Dankert, P. Fennessey, and P. W. Robbins.** 1967. Characterisation of a polyisoprenoid compound functional in O-antigen biosynthesis. Proc. Natl. Acad. Sci. U.S.A. 57:1798–1803.