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THE NATURE OF THE ATTACHMENT OF A REGULARLY ARRANGED SURFACE PROTEIN TO THE OUTER MEMBRANE OF AN *ACINETOBACTER* SP.

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SUMMARY

Acinetobacter 199A carries on the outer surface of its outer membrane a layer of regularly arranged protein subunits. The isolated surface protein assembles into the same regular array even in the absence of the underlying outer membrane. Cl^- is required for this self-assembly. Evidence is presented that the interaction of the surface protein with the outer membrane involves the linking of a carboxyl group in the surface protein to a negatively charged group in the outer membrane protein, via a divalent cation. The surface protein could be detached from the outer membrane by the protein perturbant urea, by the chelating agent EDTA and by replacing Mg^{2+} with Na^+. It could not be detached by treatment with phospholipases A and C or the detergents Tween 80 and sodium deoxycholate. The conditions favourable for re-attachment of surface protein to the cell wall were the presence of divalent cations and a pH of 3–5. Conversion of carboxyl groups in the surface protein to amine with carbodiimide and ethylene diamine interfered with reattachment. The surface protein did not attach to isolated cell wall lipid or lipopolysaccharide.

INTRODUCTION

Several species of Gram-negative bacteria carry a layer of regularly arranged subunits on the outer surfaces of their outer membranes, in either tetragonal or hexagonal array [1, 2]. These regularly arranged subunits are composed of acidic protein, of molecular weight 67 000 in *Acinetobacter* strain MJT/F5/199A [3] and of molecular weight 125 000–150 000 in *Spirillum serpens* [4].

In the present paper we describe investigations of the nature of the attachment of one of these regularly arranged proteins to the bacterial outer membrane. The chemical components of the outer membrane are protein, lipid, lipopolysaccharide and carbohydrate [5]. We have investigated which component of the outer membrane

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is responsible for the attachment of the surface protein and what type of bond is involved. A preliminary report of this work has already appeared [6].

MATERIALS AND METHODS

Preparation of cell walls and outer membranes

Acinetobacter sp. strain MJT/F5/199A (NCIB 10885) was grown in 700 ml batches of heart infusion broth (Difco, Detroit, Michigan, U.S.A.), to which 0.01 % $CaCl_2$ was added, in an FE 007 fermentor (Biotec Ltd, Selsdon, South Croydon, CR2 8YD, England), at a temperature of 25–30 °C. and harvested in mid-logarithmic phase. Cell walls were prepared as described previously [7] from cells broken in a French press. These walls contain less than 2 % of the original plasma membrane [5] but have intact outer membranes. Outer membranes were prepared from the cell wall fraction by treatment with lysozyme [7].

Preparation of purified surface protein

The surface protein was detached from isolated cell walls by treatment with 1 M urea [3]. A preparation of cell walls containing about 10 mg protein was incubated with 5 ml 1 M urea for 2 h at 37 °C. It was then centrifuged at 12 000 × *g* for 10 min. Urea was removed from the supernatant by dialysis against glass-distilled water, containing Amberlite resin IR-120 (H) (5 g/l) to remove traces of cations. After dialysis the extract was centrifuged again at 12 000 × *g* for 15 min to reduce contamination of the preparation by small pieces of membrane carrying attached surface protein. The soluble surface protein was stored frozen in plastic containers. Glass vessels were unsuitable as the protein tended to adhere to the surface.

Preparation of cell walls free of surface protein

The cell walls were extracted with 1 M urea to remove the surface protein, as described above, and then washed once with water. Electron microscopy showed the walls to be substantially free of regular pattern and electrophoresis on polyacrylamide gels revealed the presence of between 10 and 12 % of the original surface protein (see Results).

Isolation of bacterial lipopolysaccharide and lipid

Lipopolysaccharide was isolated from cell walls by extraction with phenol as described previously [5]. Lipid was extracted from the wet cell wall pellet, containing about 10 mg protein, with 10 ml chloroform/methanol (1 : 1, v/v) (Pronalys grade, May and Baker, Ltd, Dagenham, England) at 7 °C for 24 h. Insoluble material was removed by filtration. The lipid extract was evaporated to dryness and stored in chloroform/methanol (1 : 1, v/v) at 7 °C. Cardiolipin was purchased from Koch Light (Haverhill, Essex, England) and egg-yolk lecithin, Type III E, from Sigma (Kingston-upon-Thames, Surrey, KT2 7BH, England).

Measurement of reattachment of surface protein to cell walls

The reattachment of isolated surface protein to cell walls from which surface protein had been removed, was investigated by electron microscopy of negatively stained preparations and by chemical and electrophoretic measurement of protein

binding. Urea-treated cell walls were incubated with surface protein at a relative concentration similar to that seen in the original wall [3], for 2 h at room temperature, in polycarbonate centrifuge tubes. The cell walls were then collected by centrifuging at $12\,000 \times g$ for 10 min, and washed once with water to remove self-assembled surface protein. The walls were examined by electron microscopy to detect reformation of the surface pattern. A quantitative measure of the amount of binding of surface protein to the cell wall was obtained by two methods:

(a) Since the surface protein has a characteristic electrophoretic mobility in 10 % polyacrylamide gels containing 0.1 % sodium dodecylsulphate [3], the reappearance of this electrophoretic band in the cell wall pellet can be used to measure the extent of binding of the protein to the cell wall. The electrophoretic procedure of Weber and Osborn was used [8]. Before electrophoresis samples (10–50 μg protein) were incubated with 0.1 % sodium dodecylsulphate in 0.1 M sodium phosphate, pH 7.2, and 5 % mercaptoethanol in a total volume of 50–100 μl for 20 min at 70 °C. After electrophoresis proteins were detected by staining with 0.01 % Coomassie brilliant blue. Gels were scanned with a Joyce-Loebl densitometer (Gateshead-on-Tyne 11, England). The extent of binding was calculated from the ratio of the surface protein P to two other cell wall proteins I and II.

(b) The concentration of protein remaining in the supernatant after binding of surface protein to cell wall was also used to calculate the extent of binding. The amount of added surface protein, and the amount remaining unattached in the supernatant and the water wash after removal of the cell walls were determined by the Folin-Lowry method [9]. Electrophoresis of the supernatants on polyacrylamide gels in sodium dodecylsulphate was used to check that the protein was only surface protein. If other proteins were solubilised from the cell walls appropriate corrections to the protein determinations were made.

Modification of cell walls

After removal of the surface protein a number of different procedures were used to investigate the effect of modifying the structure of the cell wall on reattachment of surface protein.

(a) *Sodium periodate.* A sample of cell walls containing 2 mg protein was incubated at 0 °C for 1 h with 1 ml 18 mM sodium periodate in 40 mM sodium acetate, pH 4.4.

(b) *EDTA.* A sample of cell walls containing 2 mg protein was incubated with 1 ml 10 mM EDTA, pH 8, at 25 °C for 10 min.

(c) *Phospholipase C* (*EC* 3.1.4.3). Phospholipase C from *Bacillus cereus* was obtained from Sigma. A sample of cell walls containing 2 mg protein was incubated with 5 units phospholipase C in 1 ml 7 mM Tris · HCl, pH 7.3, 0.7 mM $CaCl_2$ and 35 % glycerol at 37 °C for 1 h.

(d) *Trypsin* (*EC* 3.4.4.4). A sample of cell walls containing 2 mg protein was incubated with 100 μg trypsin (British Drug Houses Ltd, Poole, Dorset, England) in 1 ml 20 mM Tris · HCl, pH 7.2, at 37 °C for 1 h.

(e) *Papain* (*EC* 3.4.4.10). A sample of cell walls containing 2 mg of protein was incubated with 2.5 units papain (Sigma) in 1 ml 0.1 M sodium phosphate, pH 6.0, and 0.6 mg/ml cysteine at 37 °C for 1 h.

(f) *Carboxyl modification with carbodiimide* [10]. Samples of about 2 mg of cell

walls in 0.5 ml water were mixed with an equal volume of 2 M glycinamide or ethylene diamine. After addition of 20 μl of 2.5 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma) the pH of the mixture was kept at 4.5 with 0.1 M HCl for 1 h. The reaction was then stopped with 0.2 ml 2 M sodium acetate, pH 3.5. The reaction mixture was dialysed overnight against 0.7 mM HCl at 7 °C.

After treatments (a) to (e) cell walls were collected by centrifuging at $12\,000 \times g$ for 10 min and washed once with 50 mM sodium phosphate, pH 7.4, before determination of their ability to bind surface protein.

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 1 h at room temperature, and then washed overnight or longer in cacodylate buffer at 4 °C. The pellets were then post-fixed 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.

For negative staining, grids coated with collodion-carbon films were floated face-downwards on the surface of the preparation to be examined, for about 1 min, and then on a solution of 1 % ammonium molybdate, pH 7.0. The grids were dried by touching their edges to filter paper.

Specimens were examined in an AEI EM6 B electron microscope operating at 60 kV with a 50 μm objective aperture.

RESULTS

Three methods were used to investigate the nature of the attachment of surface protein to underlying membrane. These were: attachment of surface protein to purified components of the outer membrane, reattachment of surface protein to cell walls under a variety of conditions, and elucidation of the treatments necessary to remove surface protein from the cell wall.

The tendency of the surface protein to self-assemble was examined in some detail first, since this process competes with the reattachment of the surface protein to the cell walls and leads to experimental difficulties.

Self-assembly of the surface protein

When surface protein is removed from cell walls it exhibits a strong tendency to self-assemble into two dimensional arrays (Fig. 1a). These arrays have the same dimensions as the original pattern on the bacterial surface [11, 12]. To investigate this process the surface protein was most frequently isolated by extraction from cell walls with 1 M urea, followed by removal of the urea by dialysis. Protein prepared by extraction with 10 mM EDTA (Fig. 1b) or with 0.05 % (w/v) Triton X-100 (Fig. 1c) also self-assembled after removal of the EDTA or Triton by dialysis. When the protein was extracted with 0.85 % (w/v) NaCl solution self-assemblies were seen even in the presence of NaCl (Fig. 1d). Fig. 1c illustrates how preparations of isolated surface protein are contaminated with small pieces of membrane carrying attached surface

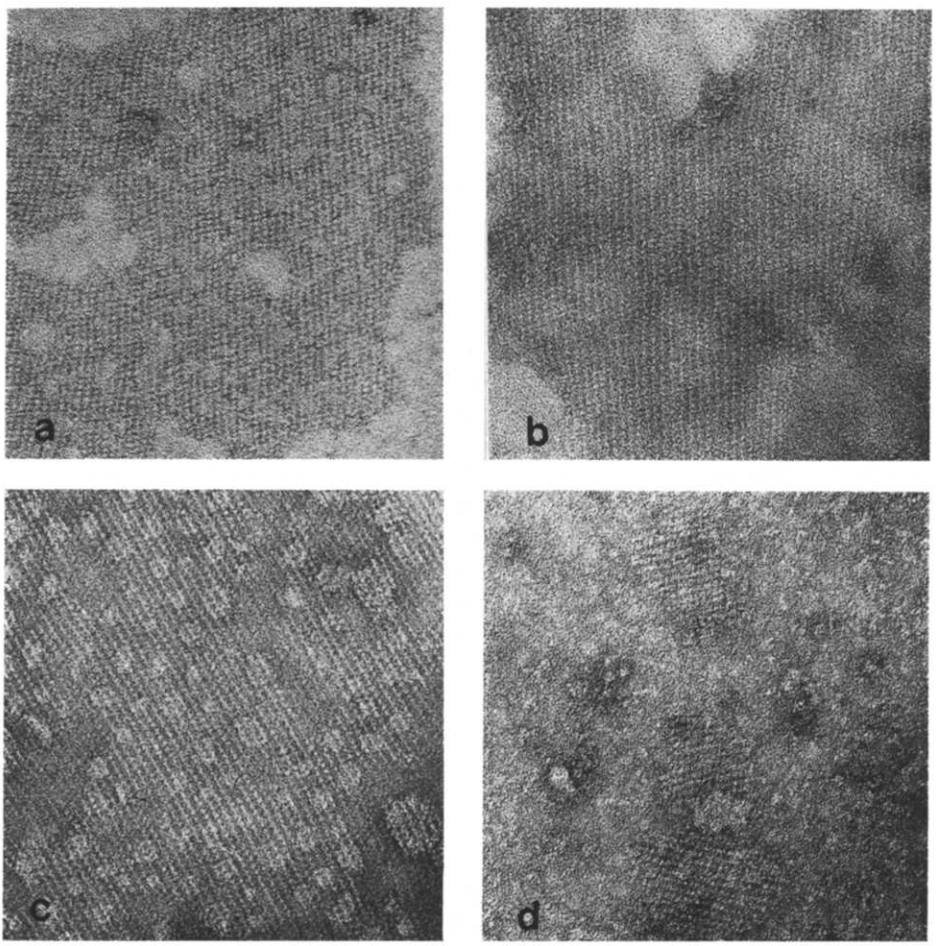


Fig. 1. Self-assembly of surface protein. All preparations were negatively stained with 1 % ammonium molybdate. (a) Urea extract, dialysed against water, then incubated with 10 mM $MgCl_2$. (b) EDTA extract, dialysed against water, then incubated with 10 mM $MgCl_2$. (c) Triton X-100 (0.05%) extract, dialysed against water, then incubated with 10 mM $MgCl_2$. (d) NaCl (0.85 %, w/v) extract. ($\times$ 120 000).

protein. Most of these were removed by centrifuging the extracts, after they had been dialysed against water to remove the extracting agents (see Materials and Methods).

The degree of self-assembly was determined by differential centrifugation. After centrifuging at 12 000$\times g$ for 10 min monomeric surface protein remained in solution while polymeric assemblies aggregated either as a pellet or at the surface of the solution. The aqueous phase was removed carefully with a Pasteur pipette, leaving the aggregates adhering to the centrifuge tube. The amount of protein remaining in solution was determined by the Folin-Lowry method [9].

The effect of different salts on the extent of self-assembly is shown in Table I. The salts have been arranged in order of effectiveness. It is evident that the nature of the anion is more important than the nature of the cation. Chloride is most effective,

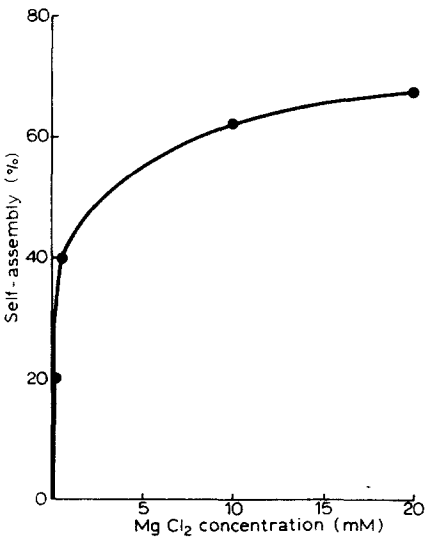


Fig. 2. Effect of $MgCl_2$ concentration on self-assembly. Experimental details are given in Table I.

TABLE I

EFFECT OF SALTS ON SELF-ASSEMBLY OF SURFACE PROTEIN

Isolated surface protein (70–120 μg/ml) was incubated at room temperature in polycarbonate centrifuge tubes for 2 h in distilled water with the addition of the appropriate salt. Self-assembled protein was removed by centrifuging at 12 000 $\times g$ for 10 min and the amount of free protein remaining in the supernatant was measured [9].

Salt added	Proportion self-assembled (% ±S.E.)
$CaCl_2$ (10 mM)	75±4 (2)
$MgCl_2$ (20 mM)	72±6 (3)
NaCl (20 mM)	63±6 (2)
$Mg(NO_3)_2$ (20 mM)	57
$NaNO_3$ (20 mM)	44
$MgSO_4$ (20 mM)	39
Na_2SO_4 (20 mM)	30
$ZnSO_4$ (20 mM)	28±10 (2)
Magnesium phosphate (5 mM)	24±3 (2)
Sodium phosphate (20 mM)	9±7 (2)

followed by nitrate, sulphate and finally phosphate. It appears that increasing the valency of the anion reduces its ability to promote self-assembly. The concentration of $MgCl_2$ required for maximum self-assembly is between 10 and 20 mM (Fig. 2).

Attachment of surface protein to purified components of the cell wall

Attempts were made to detect interaction of surface protein with purified components of the cell wall by electron microscopy. In aqueous solution the isolated

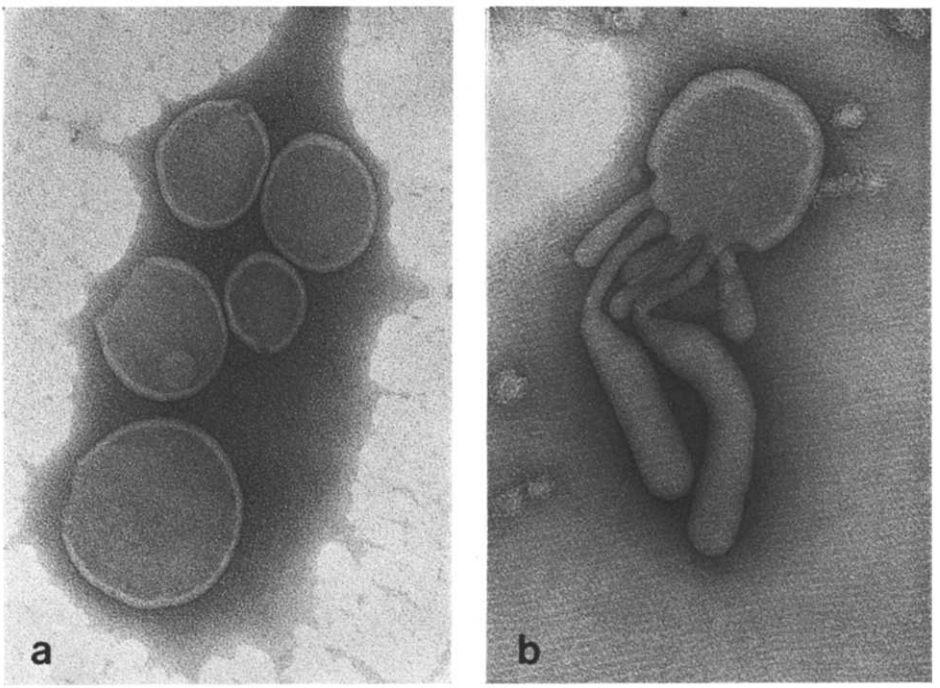


Fig. 3. Test for interaction of surface protein with lipopolysaccharide. (a) Lipopolysaccharide. (b) Lipopolysaccharide (100 μg) mixed with surface protein (150 μg) in 0.2 ml 10 mM $CaCl_2$ for 24 h at room temperature. Preparations were negatively stained with 1 % ammonium molybdate. (X 120 000).

lipopolysaccharide was in the form of vesicles (Fig. 3a). When surface protein was mixed with an equal amount of lipopolysaccharide in the presence of 10 mM $CaCl_2$ and left for 24 h at room temperature the protein formed self-assemblies which showed no interaction with the lipopolysaccharide (Fig. 3b).

The interaction of surface protein with lipid was tested using isolated cell wall lipid, a mixture of cell wall lipid and lecithin, and a commercial preparation of cardiolipin, the principal lipid of *Acinetobacter* outer membrane [5]. Electron microscopy of ultrasonically dispersed lipid revealed a mass of long, closely-packed tubes which broke up into smaller aggregates in the presence of lecithin. No interaction of protein with lipid was detected. This is illustrated in Fig. 4b where the arrays of surface protein are seen to be quite separate from the cardiolipin.

The lack of interaction of surface protein with lipid was confirmed by differential centrifugation [13]. A sample of bacterial lipid (0.5 mg) was ultrasonically dispersed with a Di Sontegrator (Ultrasonic Industries Inc., Albertson, Long Island, N.Y., U.S.A.) for 10 min in 0.7 ml 50 mM sodium phosphate, pH 4.2. It was incubated with surface protein (70 μg) in 10 mM $MgCl_2$ in a total volume of 1 ml for 2 h at room temperature. The lipid was then collected by centrifuging at 12 000 $\times$ g for 10 min and the concentration of protein in the supernatant assayed. No protein was removed from solution by the lipid.

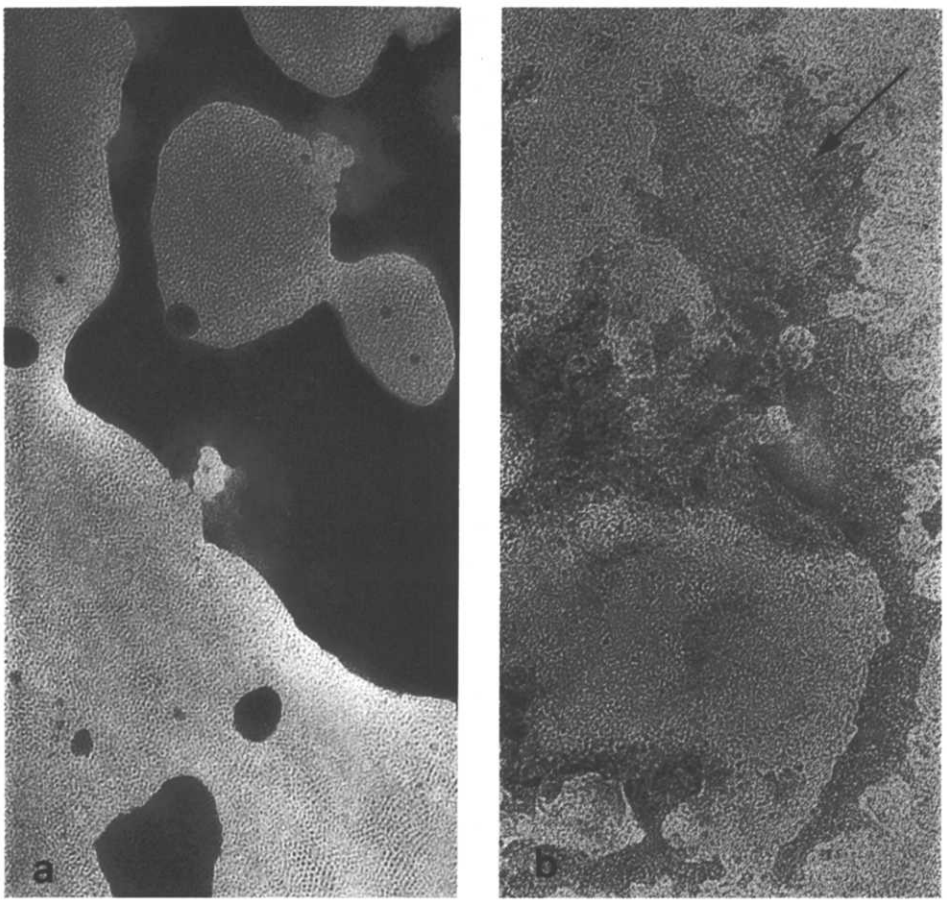


Fig. 4. Test for interaction of surface protein with cardiolipin. (a) Cardiolipin (0.8 mg) in 1 ml water, dispersed on a Whirlimixer (Fison's Scientific Apparatus, Loughborough, England) for 1 min. (b) Sample (a) mixed with 0.4 mg surface protein in 10 mM $CaCl_2$ and incubated at room temperature for 30 min. Negatively stained with 1 % ammonium molybdate. (X 120 000). Arrow indicates surface protein.

Reattachment of surface protein to urea-extracted cell walls

Under certain conditions isolated surface protein could be reattached to cell walls from which it had been removed. The extent of reattachment was determined by measuring the amount of protein bound to the wall and by the reappearance of the characteristic surface protein band after electrophoresis (see Materials and Methods). The reformation of the characteristic patterned layer on the bacterial surface was checked by electron microscopy. The array of subunits is clearly seen on the untreated cell walls (Fig. 5a). After removal of the surface protein no patterned layer is seen (Fig. 5b). When surface protein is incubated with cell walls some surface pattern begins to reappear but self-assemblies are also formed (Fig. 5c). Since these self-assemblies are precipitated together with the cell walls during differential centrifugation their presence interferes with the quantitative determination of reattachment. The fact that Cl^- is necessary for the formation of the self-assemblies, and that these

break up in water, means that they can be removed by simply washing the cell wall pellet with water (Fig. 5d). Although considerable reattachment of surface protein could be detected after 30 min incubation with cell walls, somewhat more was obtained after 2 h. The longer incubation time was therefore used. An increase in the fraction of surface protein bound to cell walls could be obtained by increasing the relative amount of cell wall present. It was, however, felt preferable to work with the relative concentration of protein and wall found in vivo.

The relative importance of different salts in the reattachment of surface protein is shown in Table II. The extents of reattachment as determined from the amount of protein bound and by electrophoresis are given separately for comparison. The agreement between the two methods is reasonably good with the exception of the results obtained in the presence of NaCl. The anomalously high figure for binding obtained electrophoretically, where the intensity of the surface protein band is compared with that of two other proteins in the membrane, is artificially elevated owing to extraction of some of the other membrane proteins during incubation in NaCl. In contrast to the monovalent anion requirement for self-assembly a divalent cation was required for reattachment to the bacterial surface. Sodium salts were considerably less effective than magnesium salts. Salts of calcium and zinc also promoted reattachment. The optimal concentration of $MgCl_2$ for reattachment is shown in Fig. 6. Only 2 mM $MgCl_2$ is required for reattachment, while 10–20 mM $MgCl_2$ was needed for self-assembly.

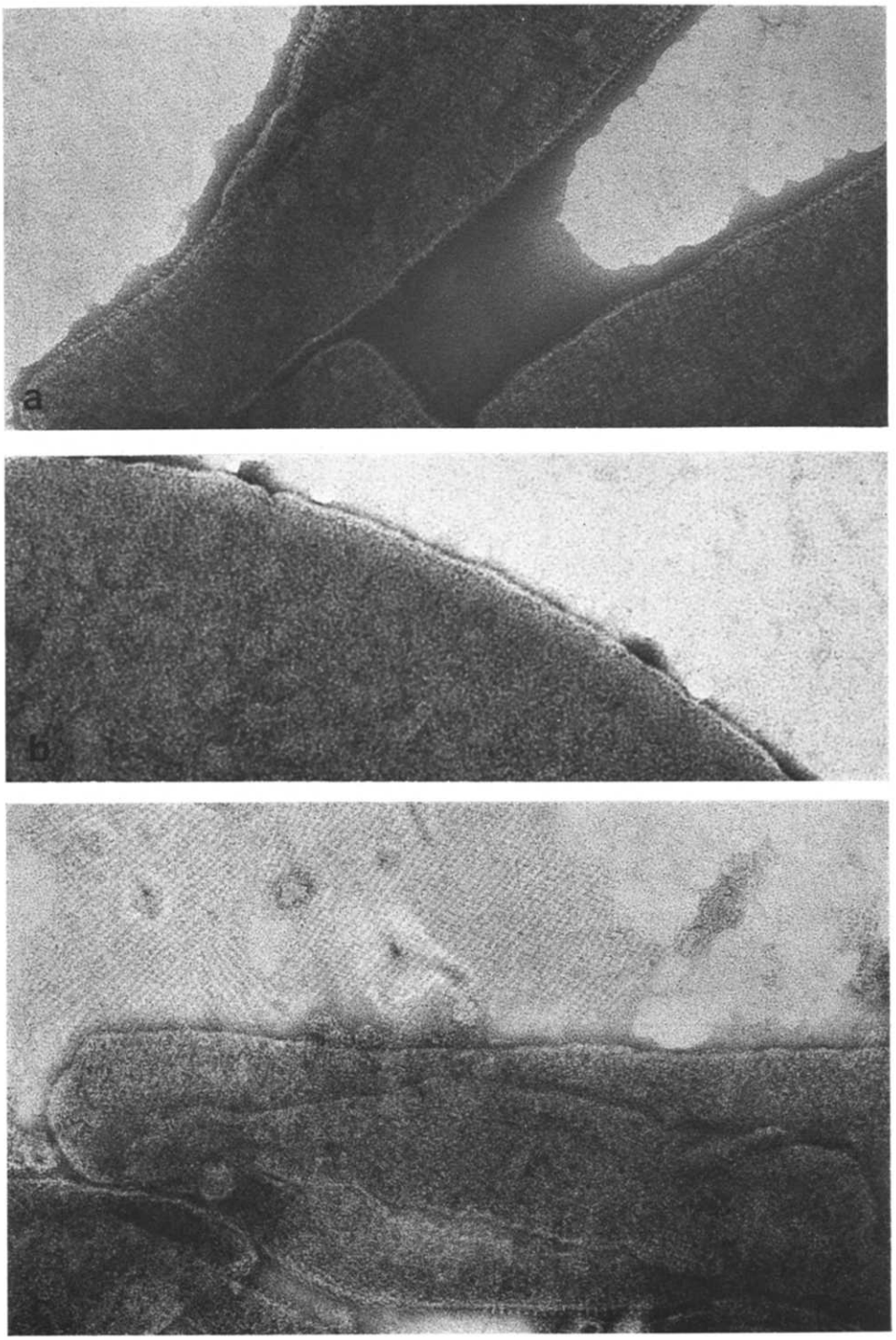
The influence of pH on reattachment of surface protein was also investigated. Some difficulty was experienced in selecting a suitable buffer for controlling the pH. High concentrations of Cl^- favoured self-assembly of the surface protein. Ionised carboxyl groups interfered with reattachment. Although phosphate was not a very effective buffer at the low pH values required it proved to be the most satisfactory salt for the present work as it counteracted the tendency of the protein to self-assemble. Buffers of the appropriate pH were obtained by titration of sodium phosphate with phosphoric acid. After reattachment of surface protein at different pH values the cell

TABLE II

EFFECT OF SALTS ON REATTACHMENT OF SURFACE PROTEIN TO CELL WALLS

Experimental details are given in Materials and Methods

Salt added	Proportion reattached (% ±S.E.)	
	Determined by protein assay	Determined electrophoretically
Magnesium phosphate (5 mM)	68±10 (2)	
$Mg(NO_3)_2$ (20 mM)	53±11 (2)	58
$MgSO_4$ (20 mM)	50±11 (2)	
$MgCl_2$ (20 mM)	42± 6 (5)	39±4 (6)
Sodium phosphate (20 mM)	19± 8 (5)	10.5±3 (4)
NaCl (20 mM)	18± 7 (6)	29.5±6 (4)
Na_2SO_4 (20 mM)	10.5± 2 (2)	
$NaNO_3$ (20 mM)	6	
No added salt	5± 4 (7)	6± 2 (5)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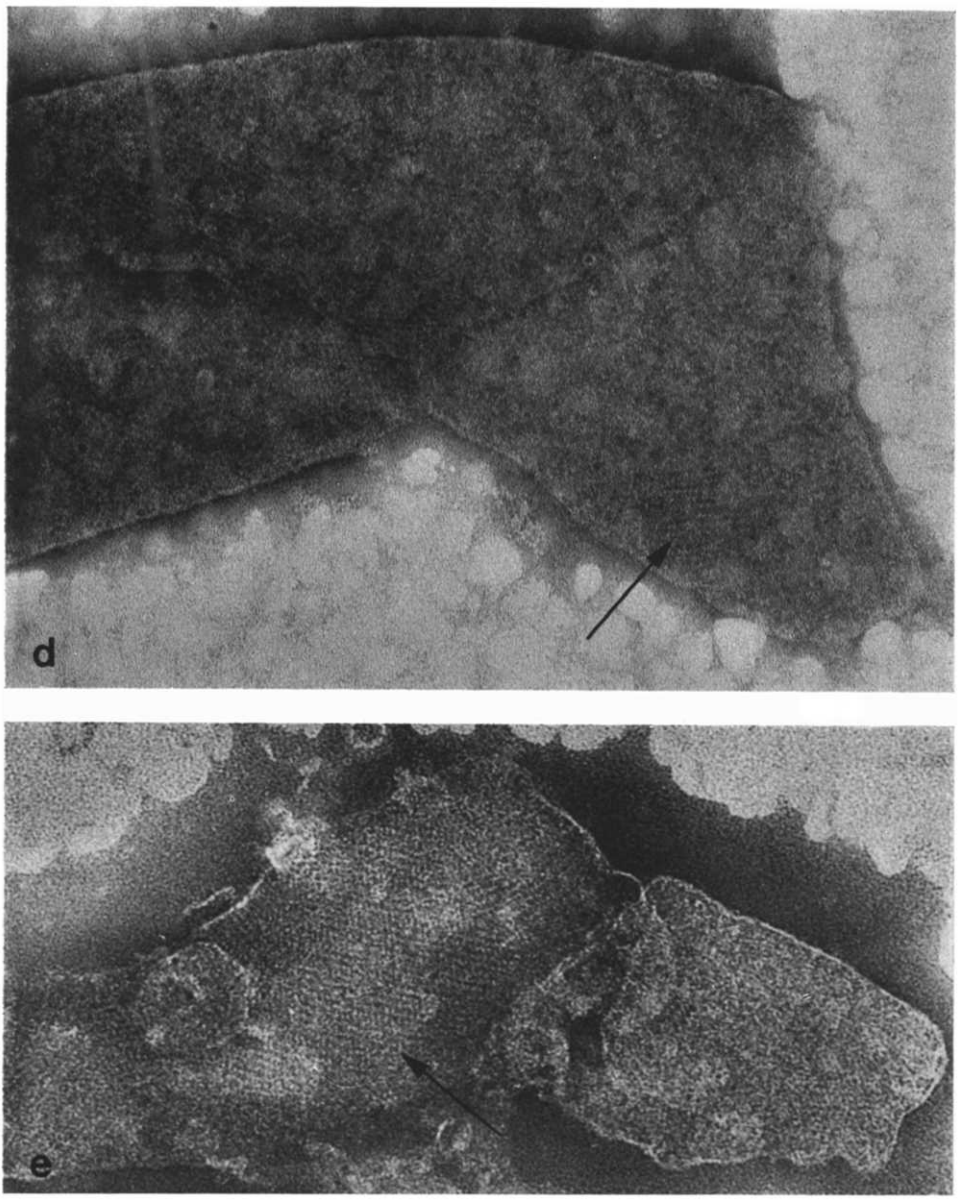


Fig. 5. Reattachment of surface protein to cell walls. (a) Untreated cell walls. (b) Cell walls extracted with urea to remove surface protein. (c) Reattachment of surface protein to walls extracted with EDTA. This illustrates the tendency of surface protein to both reattach to the cell walls and to self-assemble independently. (d) Reattachment of surface protein to cell walls at pH 4.4 followed by washing with water to remove self-assemblies. (e) Reattachment of surface protein to walls treated with carbodiimide and glycinamide. All preparations negatively stained with 1 % ammonium molybdate. (X 120 000). Arrow indicates surface protein.

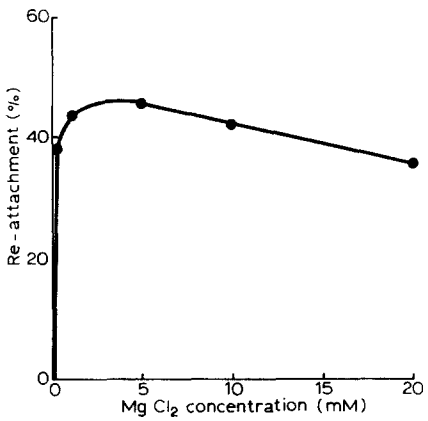


Fig. 6. Effect of $MgCl_2$ concentration on reattachment of surface protein. Experimental details are given in Materials and Methods.

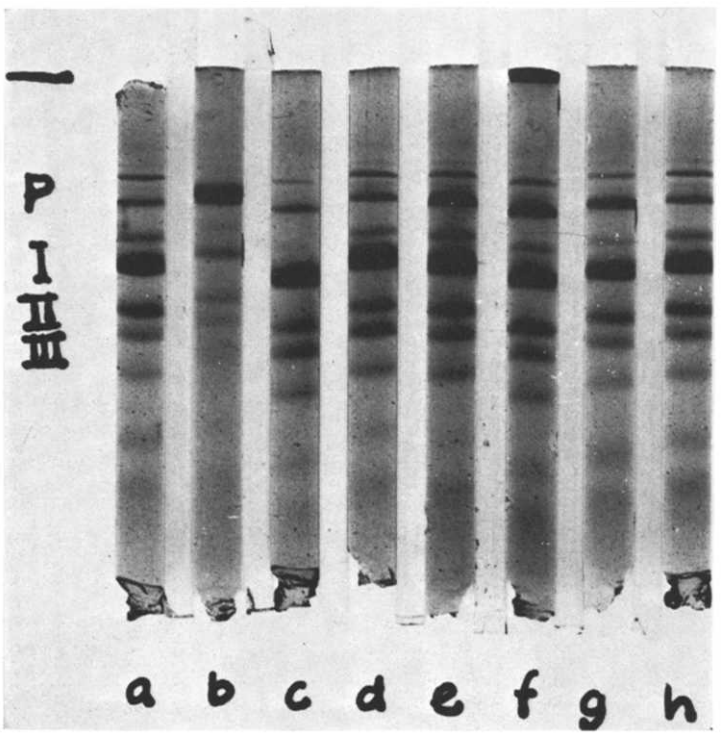


Fig. 7. Electrophoresis of cell walls after reattachment of surface protein at different pHs. (a) Cell walls extracted with urea. (b) Surface protein. (c) Reattachment at pH 2.3. (d) Reattachment at pH 2.4. (e) Reattachment at pH 2.85. (f) Reattachment at pH 3.6. (g) Reattachment at pH 4.4. (h) Reattachment at pH 5.7. P denotes surface protein.

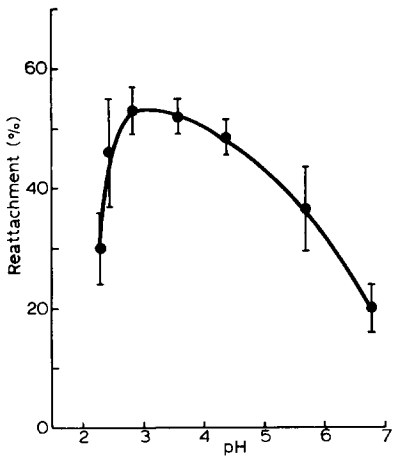


Fig. 8. Effect of pH on reattachment of surface protein to cell walls. Surface protein and cell walls were incubated together in 50 mM sodium phosphate and 10 mM $MgCl_2$ and the extent of reattachment determined as described in Materials and Methods. The bars represent ±S.E.

walls were analysed by electrophoresis on polyacrylamide gels (Fig. 7). Surface protein is labelled P; other cell wall proteins are labelled I, II and III. Maximum binding is seen between pH 2.85 and 3.6, as shown graphically in Fig. 8. There was a rapid fall in the amount of reattachment below pH 3. As the optimal pH 3 was rather acid, all further measurements of reattachment were done at pH 4.4.

Attempts were made to modify the structure of individual components of the urea-extracted cell wall specifically in order to prevent the binding of surface protein. Sodium periodate treatment of the cell walls, which oxidises carbohydrate [14], did not interfere with binding. EDTA extracted all the lipopolysaccharide, as assayed by the method of Aminoff [15], but the walls still bound surface protein (Fig. 5c). The lipid of the cell wall was modified by incubation with phospholipase C from *B. cereus*. After phospholipase treatment the remaining lipids were extracted and separated by thin-layer chromatography on silica gel G in chloroform/methanol/water (65 : 25 : 4, v/v). This showed that the enzyme had the same effect on *Acinetobacter* walls as on *Bacillus subtilis* protoplasts [16] and hydrolysed all of the phosphatidylethanolamine and phosphatidylglycerol, but only half of the cardiolipin. The binding of surface protein was unaffected.

The protein of the cell wall was attacked both enzymically and chemically. Electrophoresis showed that trypsin (100 μg/ml at 37 °C for 1 h) had no effect on the proteins of the cell wall. Papain hydrolysed all the proteins to small molecular weight peptides. When attempts were made to reattach the surface protein to the papain-treated cell walls, sufficient papain remained attached to the walls to hydrolyse the surface protein as well. Free carboxyl groups in the cell wall protein were converted to amide or amine by reaction with glycinamide or ethylene diamine in the presence of carbodiimide. After conversion to amide, the walls still bound surface protein in an ordered array (Fig. 5e); after conversion to amine, surface protein bound to the walls in a non-specific manner without forming a patterned layer. This binding could represent electrostatic interaction between a negatively charged carboxyl group on the

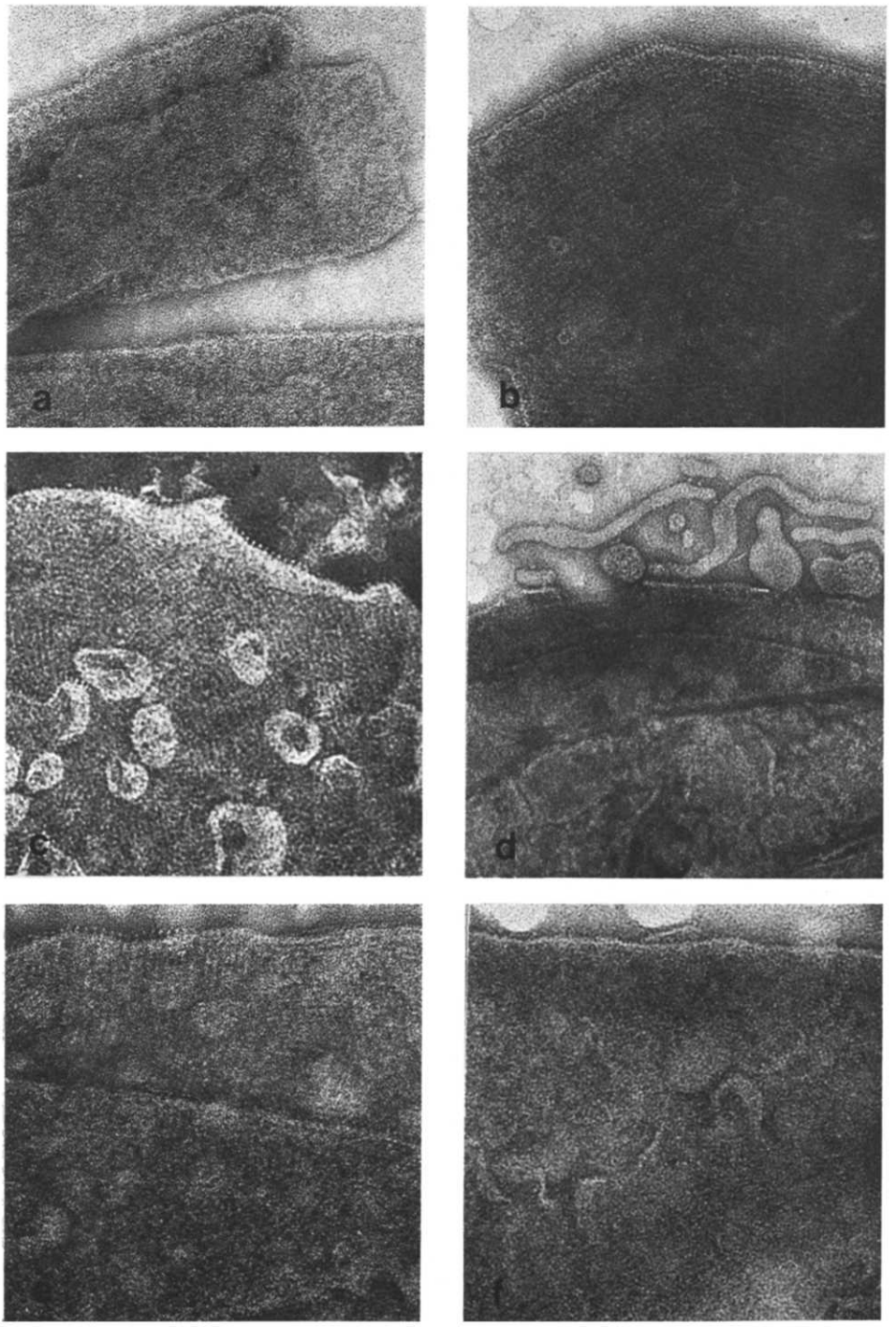
surface protein and the positively charged amine on the cell wall. When the carboxyl groups of the surface protein were converted to amino groups the protein no longer bound to the urea-extracted cell walls.

Detachment of surface protein from cell walls

The two most effective methods for removing surface protein from cell walls are extraction with 1 M urea (Fig. 5b) or extraction with 10 mM EDTA [3]. After treatment with EDTA all of the lipopolysaccharide was found in the EDTA extract, while the surface protein was not liberated until the cells were washed with water [3]. A number of other treatments have yielded useful information on the nature of the attachment of surface protein. The effect of EDTA can presumably be explained by the chelation of the divalent cations necessary for attachment to the surface. A similar effect was seen with NaCl. Treatment of intact cell walls with 0.85 % NaCl removed a large part of the surface protein (Fig. 9a), possibly by replacing Mg^{2+} with Na^{+}. The liberated protein was found in small self-assemblies and not as free monomers, owing to the presence of Cl^{-} (Fig. 1d).

The lipid of the walls was modified by treatment with phospholipase C without detaching the protein (Fig. 9b). Phospholipase A from *Crotalus terrificus terrificus*, hydrolysed the phosphatidylethanolamine of the bacterial cell wall, but not the cardiolipin or phosphatidylglycerol, without affecting the binding of the surface protein. Extraction with 1 % (w/v) Tween 80 (Fig. 10a) and with sodium deoxycholate (1 %, w/v) had no effect. Some effect was, however, observed with Triton X-100. When cell walls (1 mg protein/ml) were incubated with Triton X-100 (0.2 %, w/v) for 10 min at room temperature and then collected by centrifuging at 10 000 × *g* for 10 min and washed once with water, some of the walls were completely intact, with attached surface proteins; others had disintegrated (Fig. 10b). When the lower concentration of 0.05 % (w/v) Triton X-100 was used some unmodified walls were again found while others had only lost their surface protein (Fig. 10c). The amount of lipid solubilised by 0.05 % (w/v) Triton X-100 was measured using cells labelled with ^{32}P. The proportion of lipid-soluble ^{32}P which was extracted by 0.05 % Triton X-100 was found to be 80 %. Gel electrophoresis showed that surface protein was not the only protein solubilised by 0.05 % Triton X-100. All of the proteins were found in the supernatant after centrifuging at 12 000 × *g* to remove insoluble material. The solubilisation of the membrane proteins by Triton X-100 was prevented by the addition of 10 mM $MgCl_2$ (Fig. 10d). This resistance of the outer membrane of Gram-negative bacteria to Triton X-100 in the presence of $MgCl_2$ was also observed by de Pamphilis and Adler [17]. The effect of 0.05 % (w/v) Triton X-100 on purified outer membrane, from which the peptidoglycan has been removed with lysozyme, confirmed that all the proteins were solubilised in a non-specific fashion (Fig. 10e).

Fig. 9. Detachment of surface protein from cell walls. (a) Walls treated with 0.85 % (w/v) NaCl. (b) Walls treated with phospholipase C. (c) Walls incubated at pH 2.8 in 0.1 M glycine/HCl for 1 h at room temperature. (d) Walls incubated in 0.01 M NaOH for 1 h at room temperature. (e) Walls reacted with glycinamide and carbodiimide to convert carboxyl to amide. (f) Walls reacted with ethylene diamine and carbodiimide to convert carboxyl to amine. All preparations negatively stained with 1 % ammonium molybdate. (X 120 000).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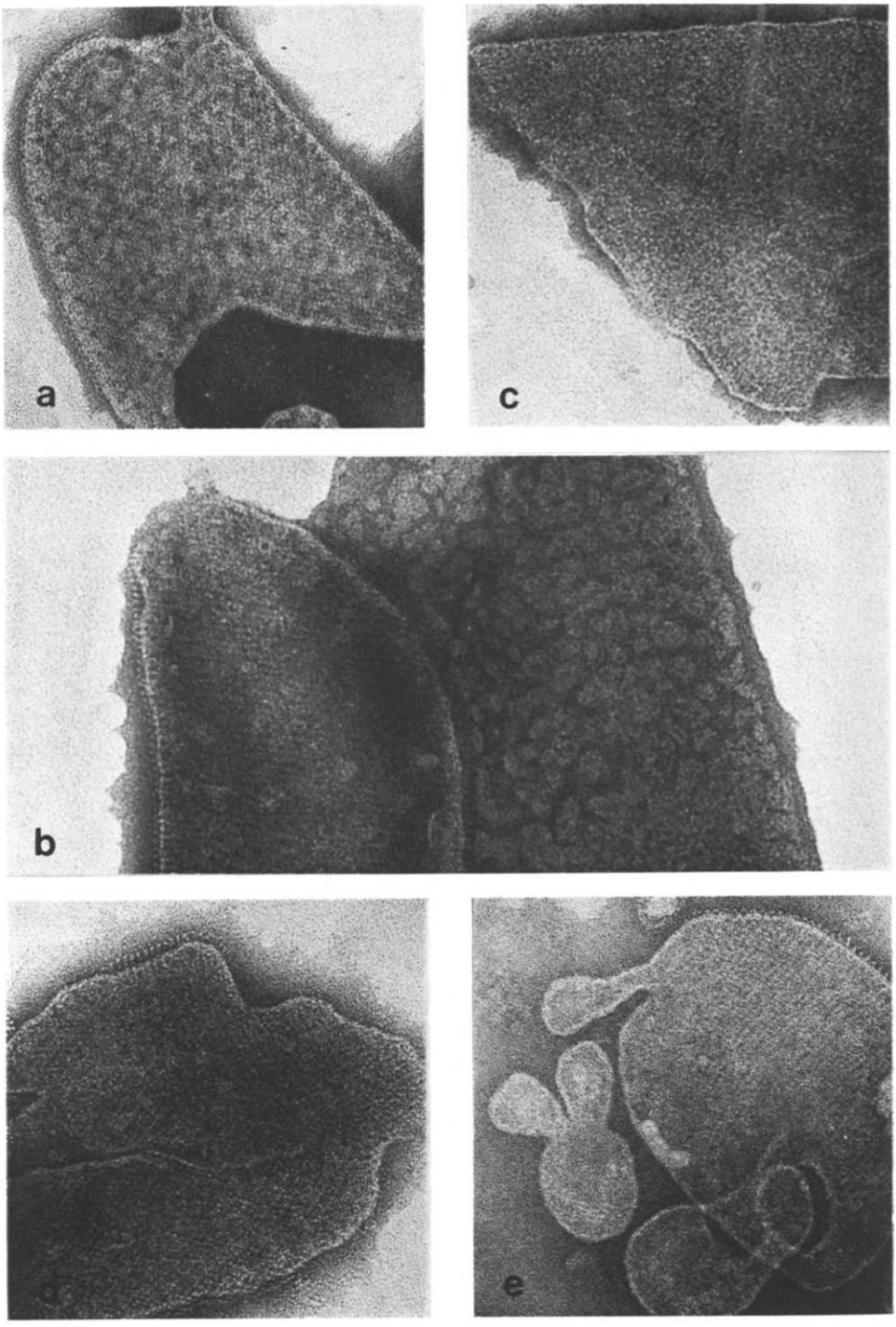
a
c
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d
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Fig. 10. Effect of detergents on cell walls and isolated outer membranes. (a) Walls treated with 1 % (w/v) Tween 80 for 10 min at room temperature. (b) Walls treated with 0.2 % (w/v) Triton X-100 for 10 min at room temperature. (c) Walls treated with 0.05 % (w/v) Triton X-100 for 10 min at room temperature. (d) Walls treated with 0.05 % (w/v) Triton X-100 for 10 min at room temperature in the presence of 10 mM $MgCl_2$. (e) Outer membranes treated with 0.05 % (w/v) Triton X-100 for 10 min at room temperature. All preparations negatively stained with 1 % ammonium molybdate. (f) Thin section of (d). (g) Thin section of (e). (X 120 000).

Although lipid and protein solubilisation was extensive the outer membrane still retained its unit membrane structure in thin sections of cell wall and outer membrane (Figs 10f and 10g).

The importance of the structure of the cell wall protein in the detachment of surface protein was also investigated. The attachment of the protein to the surface was remarkably stable to acid. The pattern is still present at pH 2.8 (Fig. 9c) although the structure of the wall is deteriorating. The pattern was less stable to alkaline conditions and was lost at pH 12 (Fig. 9d). At this high pH many of the proteins of the cell wall are solubilised. Conversion of the carboxyl groups of the cell wall protein to amide by reaction with glycinamide in the presence of carbodiimide did not cause loss of the surface protein (Fig. 9e). When the carboxyl was converted to amine, with ethylene diamine the pattern was lost (Fig. 9f). Electrophoresis of the cell wall pellet obtained after centrifuging at $12\,000 \times g$ for 10 min showed that the surface protein was still attached, presumably in a non-specific manner. This was in agreement with the observation that, after conversion of carboxyl groups to amino groups, surface protein could still be bound to the cell wall, but no pattern was formed.

DISCUSSION

Two types of bond are involved in the formation of the regularly arranged layer of surface protein; those within the layer and those to the underlying outer membrane. The stronger bonds seem to be between adjacent protein molecules in the plane of the surface array, since in isolation the surface protein has a strong tendency to self-assemble. The formation of self-assemblies with a pattern identical to that found on the bacterial surface shows that the arrangement of the protein is determined within the surface protein layer and not by underlying components in the outer membrane. This tendency to self-assemble has also been described for another surface protein, the "T-layer", from the Gram-positive *Bacillus brevis* [18].

The requirement for Cl^- in the formation of self-assemblies is somewhat unusual. Possibly it is needed to neutralise positive charges in the protein which might otherwise prevent aggregation. High concentrations of 0.5–5 M NaCl are known to be required to stabilise the cell walls of certain marine microorganisms such as *Pseudomonas* B-16 [19] and *Halobacterium cutirubrum* [20]. The only previously described involvement of a low concentration of chloride is the requirement of the *rod* B mutant of *B. subtilis* for 10–20 mM Cl^- for growth (Rogers, H. J. and Thurman, P., personal communication).

The four main components of the outer membrane to which the surface protein might be attached are lipopolysaccharide, lipid, carbohydrate and protein. Carbohydrate seems unlikely to be implicated as periodate-treated walls were as effective as normal in reattaching surface protein. Both lipopolysaccharide and lipid were excluded from the role of receptor by several different types of experiment. Lipopolysaccharide was found not to be involved since surface protein did not interact with isolated lipopolysaccharide, but did reattach to cell walls from which the lipopolysaccharide had been extracted with EDTA. In addition after EDTA treatment lipopolysaccharide was found in the first extract, while the surface protein was only obtained after subsequent treatment with water [3].

Surface protein did not attach to isolated lipid, whether assessed by electron microscopy or by differential centrifugation. Phospholipases A and C hydrolysed a considerable proportion of the lipids of the outer membrane without causing release of the surface protein. Pretreatment with phospholipase C also did not interfere with the reattachment of surface protein which had been previously removed with urea. The detergents Tween 80 and sodium deoxycholate did not release surface protein which again indicates that the receptor is not lipid. Triton X-100, however, at a concentration of 0.05 %, or a molar ratio of detergent to lipid of about 5 to 1, did release surface protein when 80 % of the lipid was solubilised. Since the other proteins of the cell wall were also released this result does not show that surface protein is directly attached to lipid. The liberation of protein together with lipid from erythrocytes after Triton X-100 treatment has been described in detail by Yu et al. [21]. The bacterial proteins were not selectively solubilised in the manner of the erythrocyte proteins. Ultimately the whole cell wall or outer membrane disintegrated.

The remaining component, and indeed quantitatively the main component [5], since it constitutes 80 % of the dry weight of the outer membrane, is protein. Considerable circumstantial evidence suggests that the receptor for surface protein is protein. Surface protein can be removed from the cell wall by treatment with the

protein perturbants urea, alkali and guanidine [3]. It is of interest that no other proteins were released from the bacterial membrane by these reagents. Steck and Yu [22] have shown that half of the erythrocyte membrane protein is selectively solubilised by protein perturbants. They postulate that these proteins are held in the membrane by bonds which depend on the configuration of the protein. A similar protein-protein interaction may be broken down when *Acinetobacter* walls are treated with protein perturbants to liberate surface protein from its receptor. The changes in protein conformation are evidently reversible since after removal of urea from both surface protein and residual cell wall the protein can both self-assemble and reattach to the bacterial surface.

The removal of surface protein by the proteolytic enzyme papain resulted in its digestion to small molecular weight peptides. Evidence for a protein receptor in the cell wall could not be obtained by using papain-treated walls since the enzyme remained tightly bound to the walls and hydrolysed added surface protein.

The conditions necessary for reattachment of surface protein suggest involvement of a receptor protein. A divalent cation was found to be necessary. This could be required to form a bridge between anionic groups in the surface and receptor proteins. The liberation of surface protein by treatment of cell walls with EDTA which chelates divalent cations, supports this theory. Extraction of surface protein with NaCl could be due to replacement of divalent cations with Na^+. The sharp drop in binding of surface protein below pH 3 suggests the involvement of an anionic group with a pK of about 2.5, possibly an α-COOH or phosphate residue. Buffers containing ionised carboxyl groups certainly interfered with binding.

More direct evidence for the involvement of COO^- was obtained by carbodiimide-mediated addition of various compounds to the carboxyl groups. A free carboxyl on the surface protein was found to be necessary for reattachment. When it was blocked by addition of ethylene diamine binding no longer occurred. When carboxyl groups in the cell wall proteins were converted to amides surface protein was still bound in an ordered array. When the carboxyl groups were converted to amines the protein bound non-specifically without forming a patterned layer. Possibly this resulted from an electrostatic attraction between negative groups in the surface protein and the newly introduced positive groups in the outer membrane. Similarly the conversion of carboxyl groups to amines in the intact cell wall resulted in loss of surface pattern without loss of surface protein. It is possible that instead of carboxyl groups other negative groups in the receptor protein may be responsible for binding surface protein. We have obtained preliminary evidence that some of the outer membrane proteins may be phosphorylated.

We conclude that two types of bond are involved in the maintenance of the patterned layer of protein on the surface of the outer membrane of *Acinetobacter*. Firstly there are bonds between the surface protein molecules which require Cl^-. These are responsible for the formation of the tetragonal pattern. Secondly, there are bonds between surface protein and underlying membrane which require divalent cations. The divalent cation forms a bridge between a carboxyl group in the surface protein and some other negatively charged residue, possibly phosphate, probably in protein in the membrane.

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