

CHEMICAL CHARACTERIZATION OF THE ISOLATED CELL SURFACE OF *AMOEB*A

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ABSTRACT

The cell surface has been isolated from uninucleate, freshwater, phagocytic amoebae by a new procedure. Several criteria were employed to demonstrate purity of the cell surface fraction. All morphological components of the tripartite surface were present in the isolated surface and the weight of the isolated surface was quantitatively accounted for by the components analyzed. Chemical analyses showed the presence of lipid, protein, and carbohydrate. Mannose was the predominant neutral sugar. Analyses for three different strains of *Amoeba* were similar. Phosphate was found to be the major anionic group in the cell surface material. Sulfate, uronic acid, sialic acid, muramic acid, and nonamidated glutamic acid and aspartic acid were absent. Evidence is presented suggesting that the phosphate is associated with an unidentified nonreducing polyol.

INTRODUCTION

In 1934, Mast and Doyle (1) described the process of pinocytosis by *Amoeba proteus*. Since then, freshwater carnivorous amoebae have served as experimental material for many studies on cell surface dynamics and ultrastructure (2-6). Most of the several different strains of carnivorous amoebae that have been studied are similar with respect to pinocytosis, phagocytosis, and cell surface ultrastructure.

The structural complexity of the cell surface of both multinucleate and uninucleate amoebae has been demonstrated by electron microscopy (2-4, 6). The characteristic structural features of the cell surface are (a) a unit membrane adjacent to the cytoplasm, (b) an amorphous layer external to the unit membrane, and (c) filaments projecting into the extracellular medium from the amorphous layer.

One of the striking attributes of the uninucleate and multinucleate, freshwater, carnivorous amoebae is the ability of the cell surface to adsorb a variety of cations (5). The binding of cations and subsequent pinocytotic events have been extensively studied by light and electron microscopy (3-8). It was demonstrated by electron microscopy that the surface filaments are the sites of cation adsorption, and hence contain the chemical groups responsible for the anionic nature of the amoeba cell surface (3, 9). There has been much speculation concerning the chemical nature of these anionic groups. However, no rigorous identification of the groups responsible for cation adsorption has been published.

As part of a study to elucidate cellular mechanisms controlling cell surface structure and function, we have selected uninucleate, freshwater,

phagocytic amoebae as a model system. The functional simplicity of the amoeba cell surface is evidenced by the apparent lack of catalyzed transmembrane transport processes (10, 11) and the presence of one or, at most, very few antigens (12).

An impetus to the study of the chemistry of the amoeba surface was provided when O'Neill (12, 13) isolated the cell surface of *Amoeba proteus* by a procedure involving shrinkage of cell components over a period of 7 days with a high concentration of sucrose. O'Neill presented many qualitative observations on the isolated cell surface and, although he reported the absence of sialic and muramic acids, he did not further characterize the anionic groups.

This report describes an improved method for isolating the tripartite amoeba cell surface, provides quantitative information on its chemical constituents, and identifies the anionic groups.

MATERIALS AND METHODS

Amoeba Cultures

The predominant strain of *Amoeba* used in this work is denoted T₁D₁₃, a freshwater, uninucleate, phagocytic strain. Strain T₁D₁₃ had previously been cultured at King's College, London (14), and has been cultured in the laboratory at the State University of New York at Buffalo since 1963. Strains Q and S were derived from micromanipulation experiments at King's College and have been cultured in Buffalo since 1963. Strain Q differs slightly from T₁D₁₃ in morphology and physiology, while strain S is markedly different.

Amoebae were cultured in 22 × 35 × 4.5-cm Pyrex dishes at 19°C. The culture medium was a modified Chalkley's solution (14) containing a trace of CaHPO₄. Amoebae were fed *Tetrahymena* according to Prescott and James (15).

Analytical Methods

Protein was determined by the method of Lowry et al. (16) with crystalline bovine serum albumin as a standard. Neutral sugar was determined by the phenol-sulfuric acid method (17) using a mixture of mannose, glucose, and galactose (9.2:1.7:1.0) as a standard. Individual neutral sugars were determined as their alditol acetates by gas liquid chromatography (18). Uronic acids were assayed by a carbazole method (19) and values corrected for the neutral sugar contribution using appropriate standards. Sialic acid was determined by the thiobarbituric acid method of Warren (20). Sulfate determination was

as described by Antonopoulos (21). Phosphate was determined by the method of Ames (22) and calcium by the method of Alonso et al. (23).

Hexosamines were separated on Dowex 50 H⁺ columns (Dow Chemical Co., Midland, Mich.) (24) after hydrolysis of samples in 4 N HCl at 105°C for 8 h. Colorimetric assay of hexosamines was as described by Boas (25) and values were corrected for 20% loss of standards.

Muramic acid was determined on the long column of a Beckman amino acid analyzer (Beckman Instruments, Inc., Palo Alto, Calif.) after hydrolysis of samples at 105°C in 4 N HCl for 8 h or in 6 N HCl for 4 h. The column was operated with the eluting buffer at pH 3.08 instead of the normal pH 3.28. Amino acids were determined on the amino acid analyzer after hydrolysis of samples in 6 N HCl for 22 h at 110°C. Destruction of serine and threonine was not corrected for.

Glutamine and asparagine were determined after extensive Pronase digestion of samples in 0.10 M ammonium formate, 1 mM CaCl₂, pH 7.3 at room temperature for 94 h. 0.38 mg Pronase was added to 11.35 mg strain T₁D₁₃ cell surface at 0, 36, and 60 h and digestion carried out under toluene. The digest was centrifuged at 27,000 g for 15 min, the supernate was applied to a Sephadex G-10 column (Pharmacia Fine Chemicals, Piscataway, N. J.) and eluted with water, and the free amino acid peak was pooled. This pooled material was then analyzed for glutamic and aspartic acids on the amino acid analyzer before and after hydrolysis in 2 N HCl for 30 min at 100°C.

Lipids were extracted with chloroform-methanol (26) and the residues weighed after evaporation of the solvents. Lipid content was determined for three different lots of amoeba cell surface.

Water content of lyophilized samples was determined by following weight gain of samples exposed to the air after drying at 65°C *in vacuo*. All values were corrected to a water-free basis.

Enzyme Assays and Digestions

Acid phosphatase activity was assayed at pH 3.6 in 0.05 M citrate buffer at 37°C with para-nitrophenyl phosphate as substrate. α -mannosidase, β -galactosidase, and β -N-acetylglucosaminidase activities were assayed at pH 4.5 in 0.05 M citrate buffer at 37°C with the corresponding *p*-nitrophenyl glycosides as substrates. Succinic dehydrogenase was assayed according to Pennington (27).

α -amylase, type II-A, was obtained from Sigma Chemical Co., St. Louis, Mo. Samples of cell surface were incubated in 0.05 M phosphate buffer, pH 6.98, with NaCl at a concentration of 5 mM. Digestion was carried out under toluene at room temperature for 24 h. Ribonuclease A, type XII-A, was obtained from Sigma Chemical Co. Samples were

water and centrifuged at 110 *g* for 5 min to obtain the final surface fraction. The procedure is summarized in Fig. 1.

Electron Microscopy

Cell surface was prepared for electron microscopy by fixation in 1% unbuffered osmium tetroxide, dehydrated in an ethanol series, and imbedded in Epon and thin sections were cut. Sections were stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope. Samples were also prepared for negative contrast observation by staining with 1% phosphotungstic acid, pH 7.0, without prior fixation.

RESULTS

Surface Isolation

Several methods of homogenization in combination with a variety of solvent systems were employed during the development of the procedure reported here. The method described was the only one found to give a cell homogenate with the cell surface present as large sacs and sheets. The homogenate gave suitable starting material from which the surface could be isolated and followed through the various steps by phase-contrast microscopy.

The isolated surface undergoes disruption in distilled water over a period of several minutes when observed by phase-contrast microscopy, but it retains its characteristic morphology in 50 mM CaCl₂. Fig. 2 is an electron micrograph of a negatively stained preparation showing clearly the preservation of surface filaments. The presence of the unit membrane in the isolated cell surface is shown in Fig. 3.

Purity of the Isolated Surface

Purity of the cell surface fractions is based on phase-contrast and electron microscope observations; acid phosphatase, succinic dehydrogenase, and glycosidase assays; and RNA and glycogen analyses.

Fig. 4 is a low power electron micrograph showing the absence of particulate contamination in the isolated surface fraction.

Much of the cellular acid phosphatase is contained within osmotically sensitive particles of low density and appears in the CaCl₂ washes. Much less than 1% of the total acid phosphatase activity was recovered in the surface fraction (Table

I). Alkaline phosphatase was absent from these cells. Succinic dehydrogenase was present in the cell homogenate but was absent from the isolated surface. Very low levels of α -mannosidase, β -*N*-acetylglucosaminidase, and β -galactosidase were present in the cell surface fraction, representing less than 1% of the initial cellular activity of these enzymes (Table I). The results of the above assays indicate the absence of lysosomal and mitochondrial contamination in the surface fraction. Although the intracellular localization of glycosidases was not determined, their absence from the cell surface fraction may be taken as a criterion of purity.

Absence of glycogen as a contaminant of the cell surface fraction was indicated by the fact that no glucose could be rendered dialyzable by amylase digestion of surface fractions. Neutral sugar analyses by gas-liquid chromatography showed that ribose was the most variable constituent, ranging from 2 to 3.9 mole percent of the total neutral sugars present. Over 80% of the ribose present could be rendered dialyzable by ribonuclease digestion as described. The levels of ribose found in the cell surface fraction correspond to less than 1% by weight RNA.

About 1 mg of lyophilized surface was obtained per milliliter of packed cells accounting for 4.5, 2.7, 10.6, and 19.3% of the cellular neutral sugar, protein, glucosamine, and galactosamine, respectively.

Composition of Cell Surface

The composition of the lyophilized cell surface fraction from strain T₁D₁₃ is shown in Table II. The most striking characteristic of the fraction is its very high content of neutral sugar.

Uronic acid, sialic acid, muramic acid, and sulfate were absent, and the only anionic group detectable was phosphate which accounted for over 10% of the weight of the cell surface material. Of this phosphate, 2% was due to phosphate in RNA, 3–5% was present as phosphate in phospholipids, 0–3% was present as nonesterified orthophosphate, and 90–95% was present as a polyol-associated phosphopolymer.

The lipid content is relatively low. From the phosphate content of the chloroform-methanol extracts, it is estimated that phospholipid accounted for 25% of the total lipid fraction. Neutral sugars were absent from the lipid extracts, indicating the lack of glycolipids.

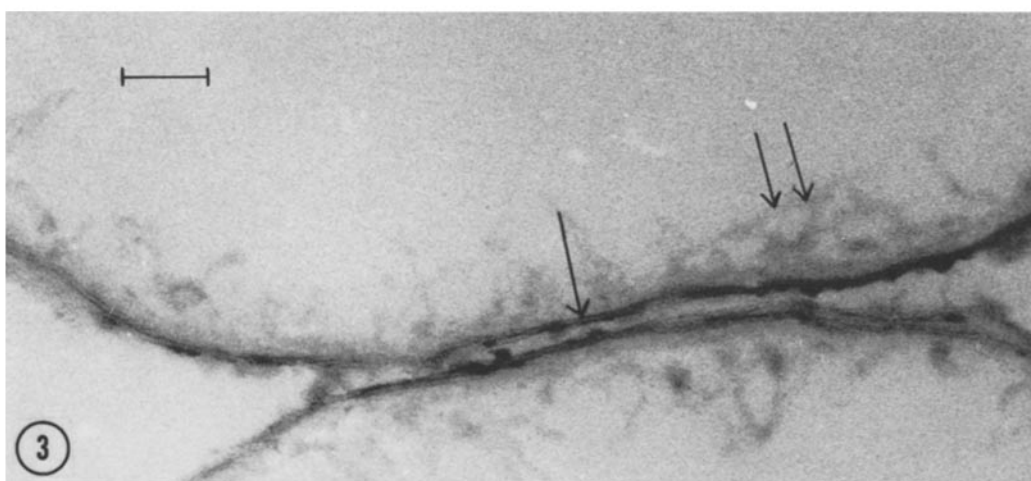
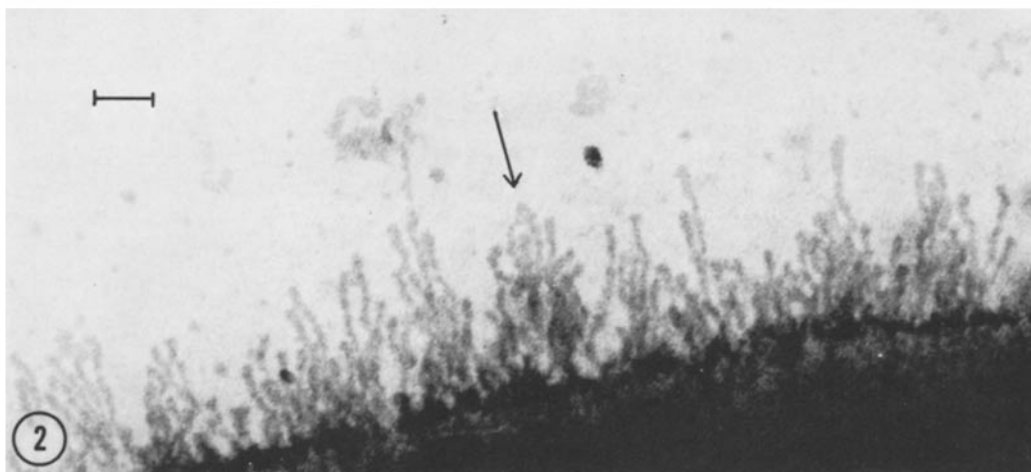


FIGURE 2 Electron micrograph of a negatively stained preparation of amoeba surface isolated from strain T₁D₁₃. Lyophilized cell surface was incubated for 30 min at room temperature in 1.0 ml of 1.0% sodium phosphotungstate at pH 7.0. After incubation, a drop of the cell surface suspension was placed on the surface of a carbon-coated Formvar grid, and the excess solution removed by touching the grid to filter paper. After drying, the grids were examined in a Zeiss Em-9A electron microscope. Note the presence of filaments (arrow). $\times 76,000$. Bar: 0.1 μm .

FIGURE 3 High power electron micrograph of a cell surface preparation from strain T₁D₁₃. Surface was fixed in unbuffered 1% osmium tetroxide at room temperature for 30 min, dehydrated in an ethanol series, and imbedded in Epon; thin sections were cut. The sections were stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope. Note the unit membrane (arrow) and filaments (double arrow). $\times 115,000$. Bar: 0.1 μm .

About half of the phosphate is very acid labile and is released as orthophosphate by hydrolysis with 0.5 N HCl or 0.5 N H₂SO₄ at 100°C for 15 min. The remaining phosphate is much more acid stable and is slowly released by hydrolysis with 3 N HCl or 3 N H₂SO₄ for 24 h at 100°C. Time-course for the hydrolysis of the labile and stable phosphate in 1 N H₂SO₄ is shown in Fig. 5. The

nature of the phosphopolymer is currently under study.

Table III presents the molar ratios of the neutral sugars in the cell surface fractions from three strains of *Amoeba*. In agreement with the observations of O'Neill (12), mannose is the major neutral sugar. Rhamnose, galactose, glucose, ribose, and an unidentified component designated

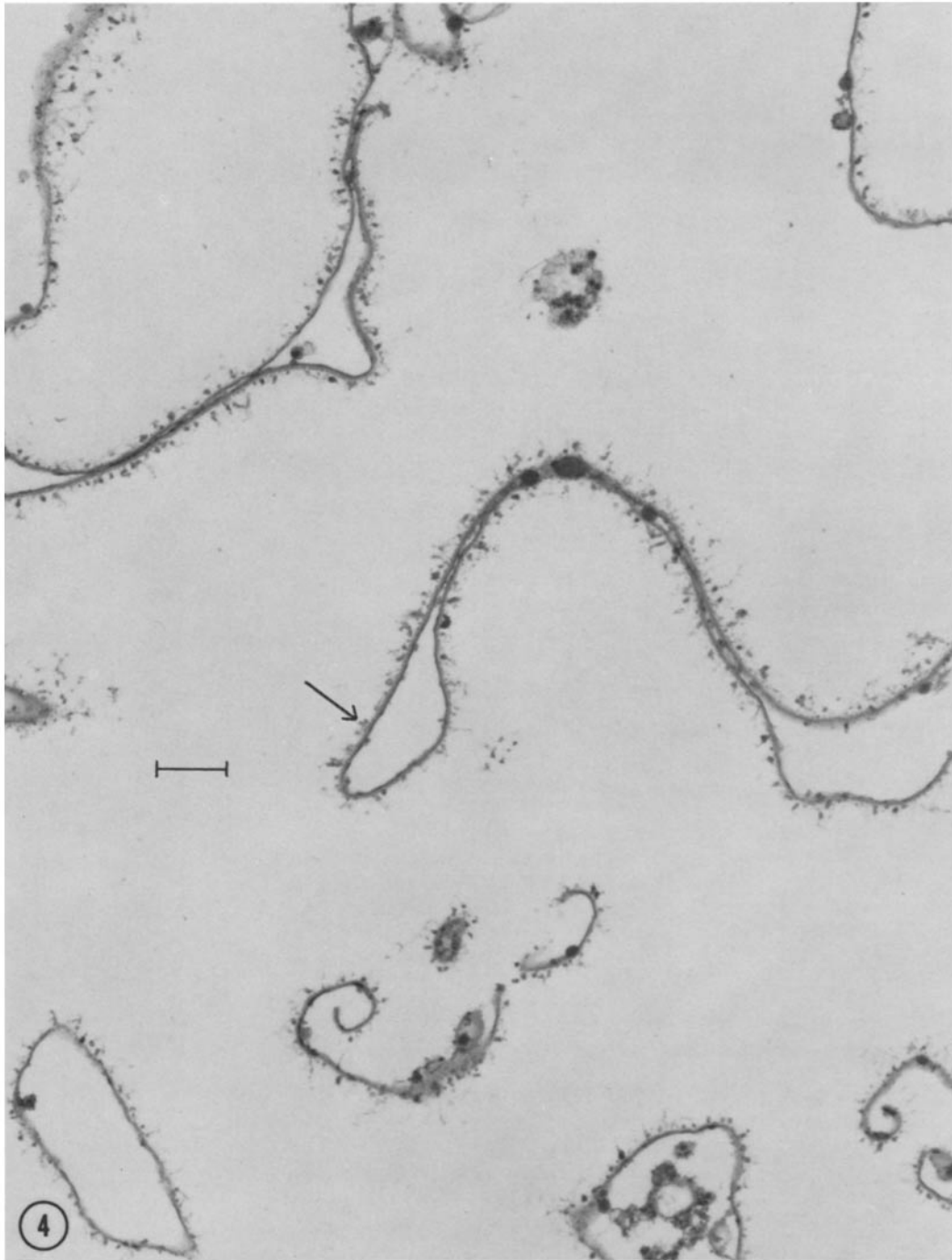


FIGURE 4 Low power electron micrograph of a cell surface preparation from strain T₁D₁₃. Surface was prepared as for Fig. 3. Note the surface filaments (arrow) and lack of nuclei, food vacuoles, mitochondria, and other particulates. $\times 21,956$. Bar: 0.5 μm .

TABLE I
Acid Phosphatase in Fractions and Glycosidases in
Cell Surface Fractions of *Amoeba T₁D₁₃*

Fraction	Total activity per fraction* · ‡	Specific activity§	
<i>Acid Phosphatase</i>			
Cell homogenate	1,770	10.9	
(A) Sucrose layer	704	9.0	
(B) 20% glycerol layer	164	14.4	
(C) 900 g pellet	456	10.9	
(D) CaCl ₂ washes	1,415	60.5	
(E) 110 g pellet	304	20.5	
(F) 10% glycerol layer	57	23.6	
(G) 20% plus 20-50%	149	14.0	
(H) 50% glycerol layer	29	5.3	
(I) CaCl ₂ washes	79	23.2	
(J) 110 g pellet	22	3.8	
<i>Glycosidases</i>			
Fraction	α -mannosidase	β -galactosidase	β -N-acetylglucosaminidase
Total activity per fraction¶			
Cell homogenate	490.4	102.8	560.4
	(100)**· ‡‡	(100)	(100)
Cell surface	2.4	0	6.8
	(0.5)	(0)	(1.2)

Enzymes in fractions of *Amoeba T₁D₁₃*. Aliquots of the various fractions (see Fig. 1) were analyzed for acid phosphatase, glycosidases, and protein as described in Materials and Methods.

*Expressed as change in optical density units per hour per 7.5 ml packed cells.

‡Calculation of initial and recovered activities complicated by apparent lysis of lysosomes.

§Expressed as change in optical density units per hour per milligram protein.

||Letters in parentheses refer to fractions in Fig. 1.

¶Expressed as change in optical density units per hour per 2.0 ml packed cells and as percent of original activity.

**Assuming all enzyme is accessible to substrate.

‡‡Numbers in parentheses express percentage.

premannose are present in similar amounts. Most of the ribose was a component of RNA and could be removed by ribonuclease. The glucose was not present as contaminating glycogen since it could not be digested by amylase. Significant differences between the three strains occurred only in their content of rhamnose and premannose.

The amino acid compositions of the cell surface

TABLE II
Gross Composition of Cell Surface from
Amoeba T₁D₁₃

Component	mg/100 mg dry weight*
Neutral sugar	24.9
Protein	34.2
Lipid	16.7
N-acetylhexosamine	4.1
Phosphate	11.4
Calcium	5.9
Sialic acid	ND‡
Uronic acid	ND
Muramic acid	ND
Sulfate	ND
Ribonucleic acid	<1
Glycogen	ND
Total	98.2

Gross composition of cell surface from *Amoeba T₁D₁₃*. All components were determined as described in Materials and Methods. Analyses were performed on many different preparations of cell surface.

*Expressed on a moisture-free basis.

‡Indicates not detected.

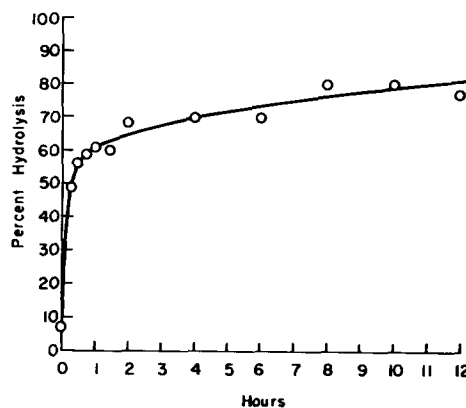


FIGURE 5 Acid hydrolysis of amoeba surface phosphate, strain *T₁D₁₃*. 77.4 mg amoeba surface was extracted with chloroform-methanol (2:1). Portions of the nonlipid residue were weighed and placed into screw-cap tubes. 1.5 ml of 1.0 N H₂SO₄ were added to each tube and the samples hydrolysed at 100°C for various times. Aliquots of the hydrolyzates were assayed for free phosphate.

fractions of the three strains of *Amoeba* are compared in Table IV. A striking similarity in the molar ratios of the amino acids is apparent.

The relatively high amounts of aspartic and glutamic acid residues in the cell surface protein

TABLE III
Individual Neutral Sugars (moles/100 moles)

Component	Strain T ₁ D ₁₃	Strain Q	Strain S
Mannose	61.3	63.6	63.2
Rhamnose	13.8	8.8	5.2
Galactose	9.6	7.9	6.5
Glucose	5.9	11.9	5.8
Ribose	3.7	3.1	5.2
Premannose*	5.8	4.7	14.1

Individual neutral sugars in amoeba cell surface from strains T₁D₁₃, Q, and S. The neutral sugars were determined by gas-liquid chromatography according to the method of Lehnhardt and Winzler (18).

*Unidentified component eluting just prior to mannose by gas-liquid chromatography calculated as a hexitol.

do not contribute to the anionic properties of the cell surface since they are primarily present as amides. This was shown by extensive Pronase digestion and the demonstration that free aspartic acid and glutamic acid were absent from the digest until hydrolysis in 2 N HCl for 30 min at 100°C. These conditions hydrolyzed the amide groups from asparagine and glutamine but did not cause significant hydrolysis of dipeptides.

Two approaches were made to determine the relation between protein, neutral sugar, and phosphate in the cell surface of strain T₁D₁₃. Sonication in the presence of EDTA (1 mg Na₄-EDTA:2 mg amoeba surface) proved very effective as a solubilizing procedure for the phosphate and neutral sugar components, 85-95% remaining in the supernate upon centrifugation at 27,000 g. Under these conditions, 20-50% of the protein was sedimented. Gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) (Fig. 6) showed that much of the phosphate was separated from the neutral sugar and protein components and emerged as a sharp peak just before the salt volume of the column. A small amount of free orthophosphate was hydrolyzed and emerged with the salt volume. The neutral sugar and the protein, however, eluted with the void volume. These results indicate that the phosphate is not associated with protein or neutral sugar.

A second approach to the study of the relation between protein, neutral sugar, and phosphate involved extensive digestion with Pronase followed by gel filtration on Sephadex G-200 and G-25.

TABLE IV
Amino Acid Composition of Amoeba Cell Surface (moles/100 moles)

Amino acid	Strain		
	T ₁ D ₁₃	Strain Q	Strain S
Lysine	4.8	4.3	4.7
Histidine	2.1	2.0	1.8
Arginine	3.6	3.4	3.8
Aspartic acid	12.0	11.5	10.9
Threonine	10.1	10.0	8.1
Serine	7.5	8.9	7.7
Glutamic acid	8.1	9.0	9.2
Proline	5.8	5.8	6.3
Glycine	10.8	10.7	13.8
Alanine	7.0	6.9	6.5
Half-cystine	4.5	4.2	3.3
Valine	6.2	5.8	5.1
Methionine	1.2	1.0	0.5
Isoleucine	4.0	4.0	4.3
Leucine	6.1	6.1	8.2
Tyrosine	2.6	2.5	1.9
Phenylalanine	3.1	3.1	3.9

Amino acid composition of amoeba cell surface from strains T₁D₁₃, Q, and S. The amino acids were determined on a Beckman amino acid analyzer after hydrolysis of samples in 6 N HCl at 110°C for 22 h. Destruction of serine and threonine was not corrected for. Glutamic acid and aspartic acid are present in the cell surface as the amides.

Cell surface material from T₁D₁₃ was sonicated, denatured at 100°C, digested with Pronase as described for Fig. 7, and chromatographed on a G-200 column. Distribution of neutral sugar, ninhydrin-reactive amino acids, and free and bound phosphate are shown in Fig. 7. It is clear that virtually all of the protein was digested to amino acids and small peptides whereas components containing the neutral sugar retain a high molecular weight and remain with the void volume. This suggests that the neutral sugar fraction consists of high molecular weight carbohydrate, which may or may not be linked to protein.

Fig. 7 also shows that the bound phosphate again emerges slightly before the salt volume, free of neutral sugar. Free orthophosphate was observed in the salt volume and it is likely that the labile phosphate previously discussed had been partly hydrolyzed during the lengthy digestion with Pronase. When the Pronase digest was subjected to chromatography on Sephadex G-25, the bound phosphate and neutral sugar emerged with the void volume (Fig. 8).

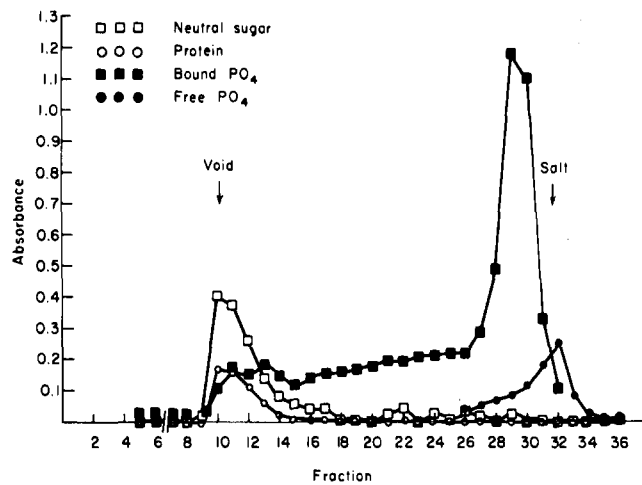


FIGURE 6 Sephadex G-200 chromatography of EDTA-solubilized fraction of amoeba surface from strain T₁D₁₃. 22.0 mg amoeba surface was sonicated in absolute methanol and centrifuged at 27,000 *g* for 15 min and the process was repeated on the sediment. To the nonlipid residue was added 11.0 mg EDTA plus 4.0 ml water and the mixture was sonicated. Three drops of toluene were added, and the suspension shaken at 37°C for 48 h. The suspension was then centrifuged at 27,000 *g* for 15 min, and the supernate applied to a 1.5 × 85-cm column of G-200 equilibrated with water. Aliquots of fractions were analyzed for total and free phosphate, neutral sugar, and protein. Flow rate = 3.5 ml/h; fraction volume = 4.3 ml; void volume = 45 ml; salt volume = 133 ml.

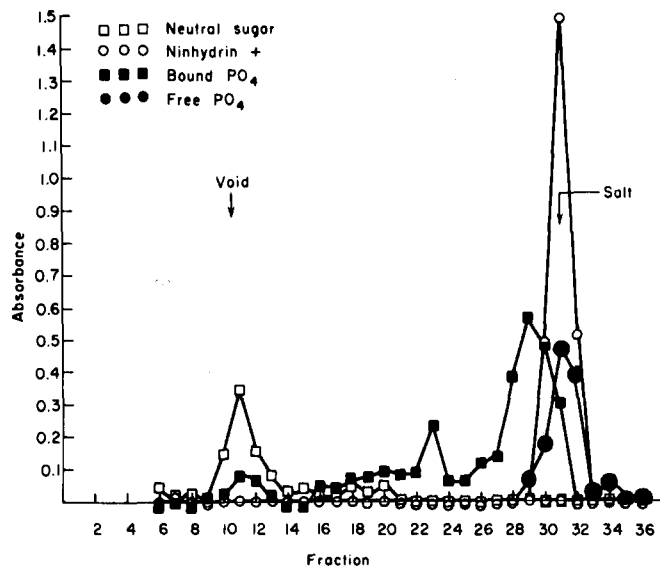


FIGURE 7 Sephadex G-200 chromatography of Pronase-digested amoeba surface, strain T₁D₁₃. 56.8-mg cell surface was extracted with methanol as for Fig. 6. 5.0 ml of 0.050 M maleate buffer, pH 7.2, containing 1 mM CaCl₂, was added to the nonlipid residue and the mixture heated at 100°C for 5 min and sonicated. 1.7 mg Pronase and three drops of toluene were added. The digest was incubated at 37°C with shaking for 24 h. After 24 h another 1.7 mg Pronase was added and digestion continued for another 24 h. 28.4 mg EDTA were added to the digest followed by centrifugation at 27,000 *g* for 15 min. A portion of the supernate was applied to a G-200 column as described for Fig. 6. Aliquots of fractions were analyzed for total and free phosphate, neutral sugar, and ninhydrin-positive material.

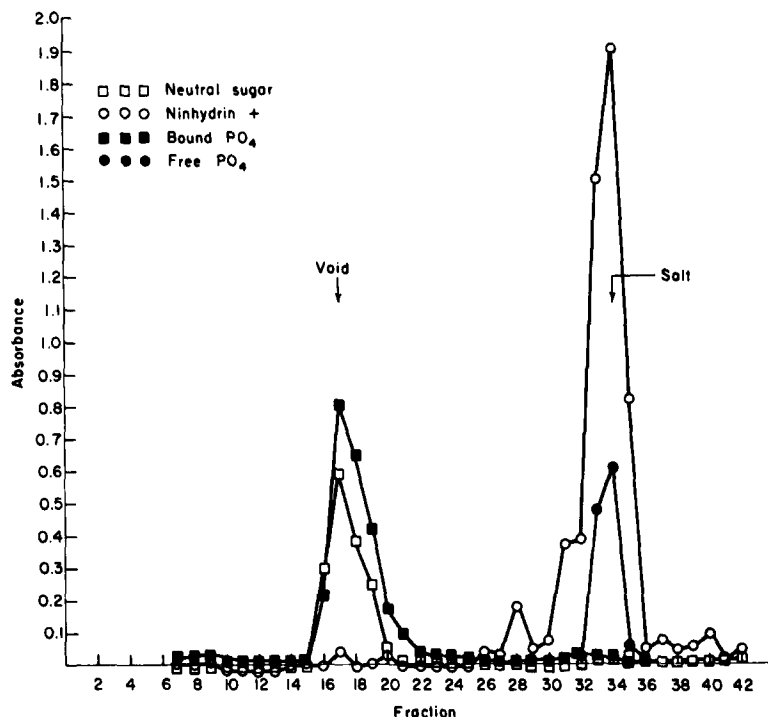


FIGURE 8 Sephadex G-25 chromatography of Pronase-digested amoeba cell surface, strain T₁D₁₃. To 25.4 mg surface was added 4.0 ml of 0.050 M maleate buffer, pH 7.2, containing 1 mM CaCl₂. The suspension was heated at 100°C for 5 min, sonicated, and then cooled; 0.40 mg Pronase was added plus three drops of toluene and the suspension shaken at room temperature for 45 h. An additional 0.40 mg Pronase was added at 45 and 73 h. After 144 h, 12.5 mg EDTA was added to the digest and centrifuged at 27,000 *g* for 15 min. The supernate was applied to a 1.8 × 75-cm G-25 column equilibrated with water. Aliquots of fractions were analyzed for free and total phosphate, neutral sugar, and ninhydrin-positive material. Flow rate = 15 ml/h; fraction volume = 5.4 ml; void volume = 93 ml; salt volume = 183 ml.

The approximate molecular weight of the calcium-free phosphopolymer was determined by gel filtration on Sephadex G-50 in 0.1 N acetic acid-2 M urea-0.01 M Na₄EDTA. It was eluted as a single peak (Fig. 9) at a volume corresponding to a molecular weight of about 2,500. Premannose was found to be the major sugar present in the phosphate peak with only traces of the other sugars present. Further studies on the nature of this phosphopolymer are in progress.

DISCUSSION

The method of isolation of amoeba cell surface reported here has advantages over a previously reported method (12) in that the calcium ions present in the media stabilize the cell surface, and the procedure can be completed in a few hours. The stabilizing effect of calcium ion is consistent with the observation that several strains of

Amoeba cytolyze rapidly in dilute EDTA solutions (28) and degenerate over a period of a few days in Ca-free medium (29).

Identification of the isolated preparation as the tripartite cell surface was confirmed by electron microscopy showing the presence of the unit membrane, the amorphous layer, and the surface filaments. However, it is recognized that some loss of surface filaments or their components could occur during the isolation procedure. It is known that amoebae contain several digestive enzymes and release of these enzymes may have degradative effects on the cell surface during its isolation. Such alteration does not appear too likely since quantitative chemical analyses on many different surface preparations gave consistent results. We are at present examining this point in more detail by preincubating surface samples with fractions containing potential enzymes followed by disk

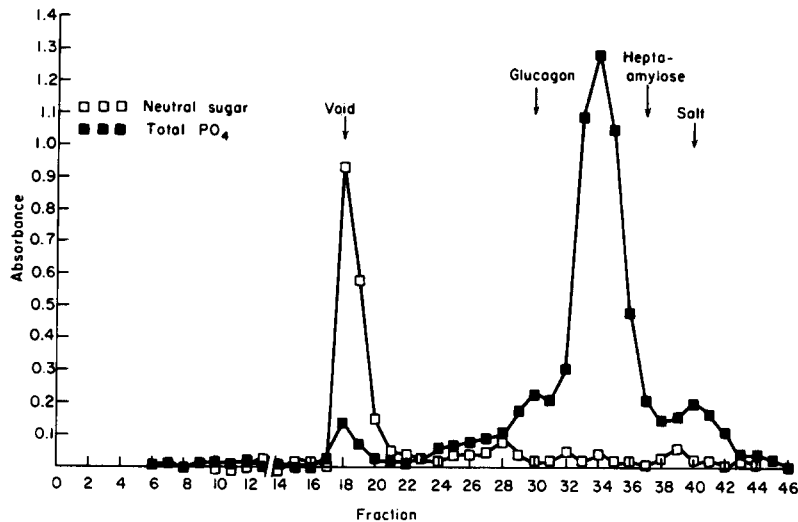


FIGURE 9 Sephadex G-50 chromatography of calcium-free amoeba surface from strain T₁D₁₃. 20.9 mg amoeba surface was extracted with methanol as for Fig. 6. 4.3 ml of 2 M urea was added to the nonlipid residue and the mixture sonicated. 1 ml of Dowex 50 H⁺ resin in 2 M urea was added and the suspension shaken overnight in the cold. The suspension was centrifuged at 37,000 *g* for 10 min, the supernate removed, the sediment washed twice with 1 ml of 2 M urea, and the supernates combined. The combined supernate was made to 0.1 N acetic acid–0.01 M Na₄EDTA, and an aliquot applied to a 1.5 × 86-cm G-50 column equilibrated with 2 M urea–0.1 N acetic acid–0.01 M Na₄EDTA. Aliquots of fractions were analyzed for total phosphate and neutral sugar. Flow rate = 15 ml/h; fraction volume = 3.3 ml; void volume = 59 ml; salt volume = 132 ml. The elution peaks of glucagon (MW 3600) and cyclohepta-amylose (MW 1135) are indicated. 0, 24, 59, and 85% of the initial calcium, protein, neutral sugar, and phosphate were present in the resin supernate.

acrylamide gel electrophoresis. To date, analysis of many different surface preparations not preincubated have given identical protein band patterns with constant relative amounts of the various proteins. Six major protein bands are observed but no glycoproteins. In addition, incubation of cell surface with an acid phosphatase-containing supernate of amoeba homogenate gave rise to no hydrolysis of surface ester phosphate.

In the absence of known chemical or enzymic markers for the amoeba cell surface, criteria to evaluate purity were necessarily arbitrary. Phase-contrast microscopy showed the absence of nuclei and food vacuoles. Small quantities of granules and crystals could not be completely eliminated because their density is similar to that of the surface, and they also tend to be trapped in surface sheets. No intact cellular particulates or organelles could be demonstrated by electron microscopy. Some unidentifiable material was occasionally seen. Succinic dehydrogenase and acid phosphatase assays showed the absence of functional mitochondria and lysosomal contamination. β -galac-

tosidase, β -*N*-acetylglucosaminidase, and α -mannosidase were found to be removed during the isolation of the cell surface. Glycogen was also removed while a small amount of RNA remained.

A special note should be made concerning amoeba enzyme assays. The amoeba is a highly compartmentalized organism with many subcellular organelles which undergo disruption upon cell lysis. The mitochondrion is one such organelle which disintegrates rapidly during the surface isolation procedure as viewed by phase-contrast microscopy. This observation agrees with the low recoveries of succinic dehydrogenase consistently obtained.

The phosphatase and glycosidase activities behave differently. In the absence of Triton X-100, these activities behave as though they are in osmotically sensitive particles, the particles lysing in 50 mM CaCl₂ and freeing enzyme (as expected if they are in lysosomes). This behavior gives rise to enzyme recoveries of 200% or greater (the enzymes in the lysosome are not accessible to added substrate). For example, fraction D in Table I

contains as much measurable phosphatase activity as the original homogenate. In addition to the behavior of the osmotically sensitive lysosomes, one must also consider the presence of endogeneous substrate which may compete with the *p*-nitrophenyl substrate added. Such a phenomenon may occur with acid phosphatase since the addition of Triton X-100 did not lower the recoveries to 100%. The more dilute fractions show more activity than the preceding fractions. In contrast, the glycosidases are apparently inhibited by Triton X-100. All of these phenomena make it impossible to perform quantitative enzymology during the isolation of the cell surface. An alternative would be to develop methods applicable to *Amoeba* for the preservation of functional subcellular organelles without regard to preserving the cell surface.

When the above observations are taken into consideration, we find that the specific activity of lysosomal enzymes is reduced by about sixfold in the surface fraction. This is compatible with the observation that the galactosamine to protein ratio increases by a factor of seven compared to the original homogenate. Galactosamine may be a component unique to the cell surface.

The chemical composition of all three strains of *Amoeba* were generally similar with respect to neutral sugars and amino acids. The only significant differences appeared to be in the levels of rhamnose and premannose for strains T₁D₁₃ and S. Mannose is the major neutral sugar in all three, in agreement with observations of O'Neill (12). All three contain an unidentified component designated premannose. This component is apparently a nonreducing polyol, since it is destroyed by periodate oxidation and is not modified by reduction with borohydride. Present evidence shows that it is eluted with the bound phosphate in EDTA or Pronase extracts of cell surface material subjected to gel filtration.

N-methylhistidine was not detected in the isolated surface, indicating the absence of actin microfilaments. However, more electron microscopy is required to definitely rule out their presence.

The isolated amoeba surface retains its capacity to bind Alcian blue and to stain metachromatically with toluidine blue. Therefore, one of the major aims of the present investigation was the identification of the anionic groups present in the isolated amoeba surface responsible for this binding. Rustad and Rustad (30) showed that the

amoeba cell surface stained metachromatically with toluidine blue, and the presence of acid mucopolysaccharide was postulated. Brewer and Bell (31) studied the interaction of quaternary ammonium compounds with *Amoeba proteus* and postulated the presence of carboxyl groups in the cell surface. Revel and Ito (32) and Stockem (33) showed that the anionic groups were strongly acidic, capable of binding thorium dioxide at pH 2. Marshall and Nachmias (34) reported the presence of sulfate in the cell surface of *Chaos carolinensis*; however, no experimental details were given. The only anionic group detected in the cell surface of *Amoeba* T₁D₁₃ was phosphate which accounted for over 10% of the isolated surface. Korn and Olivecrona (35) have found large amounts of nonlipid, nonnucleic acid, organic phosphate in the membranes of *Acanthamoeba castellanii*, an amoeba which has little detectable surface coat. Korn and Wright (36) reported that this organic phosphate is present as amino phosphonic acids, distinctly different from the phosphorylated polyol reported here. The nature of the phosphopolymer and its association with the filaments of the cell surface remain to be elucidated.¹

From adsorption isotherms, Hendil (37) estimated that the number of fixed external charges in *Chaos chaos* was 0.5 meq/liter of packed cells. Calculations based upon the phosphate content of *Amoeba* T₁D₁₃ cell surface presented in this study give a value of about 2 meq of negative charges/liter of packed cells. In view of the fact that *C. chaos* is considerably larger than *Amoeba* T₁D₁₃, the data are in reasonable good agreement.

This work is dedicated to the memory of Dr. Richard J. Winzler.

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¹ Gas-liquid chromatography and mass spectrometry indicate that premannose is in fact *neo*-inositol. M. Laird, H. J. Allen, and R. J. Winzler, unpublished observations.

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