COMPOSITION OF AN AMOEBA PLASMA MEMBRANE

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SUMMARY: The plasma membrane of Acanthamoeba castellanii contains per mg of protein 0.6-0.7 μmoles of phospholipid, 0.81-0.98 μmoles of sterol, 1 μmole of non-lipid, organic phosphorus, and 1 μmole of carbohydrate (as glucose). Solutions of the lipid-extracted plasma membrane in 8 M urea separate on Sephadex G-200 into a voided fraction which contains all of the carbohydrate and non-lipid P, and 35% of the protein, and an included fraction which contains only protein of apparently low molecular weight. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of solutions of fresh membranes or of lipid-extracted membranes reveals 2-3 protein-staining bands and two carbohydrate-staining bands all of which have mobilities approximately equal to that of lysozyme.

Acanthamoeba castellanii is a small, free-living soil amoeba that grows well on a soluble medium (1). Its ultrastructure and physiology make this organism an intriguing subject for studies of the plasma membrane. The amoeba does not, apparently, actively transport solute molecules and may depend on pinocytosis for uptake of nutrients under conditions of culture (2-4). Furthermore, the amoeba contains all of the usual cell organelles of typical eukaryotic cells (5). Thus, the functions of the plasma membrane of Acanthamoeba may be much less diversified than those of the plasma membranes of mammalian and bacterial cells and it might be supposed that the proteins of the amoeba plasma membrane may be correspondingly less complex. In contrast to many other amoebae, Acanthamoeba has no surface coat external to the plasma membrane detectable by light and electron microscopy (5).

Isolated amoeba plasma membranes (6-8) are homogeneous by electron microscopy with typical trilaminar appearance. The membrane preparations contain very little DNA (< 1 μg/mg protein) or RNA (5 μg/mg protein) and no detectable NADH-cytochrome c reductase, NADPH-cytochrome c reductase, or succinic

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Table I

Analysis of Membrane Fractions Prepared from Acanthamoeba Castellanii

Fraction	Total P	Lipid P	Sterol	Non-lipid P	Carbohydrate	Protein
			μmoles/mg protein			%
Membrane	1.7	0.7	0.6			100[a]
	1.6	0.6				
	1.5		0.7			
Lipid-extracted residue	1.3	0		1.3	1.0	95[a]
	1.1			1.1	1.1	
	1.1	0		1.1	0.9	
	1.3	0		1.3	1.3	
	1.2	0		1.2	1.1	
G-200 Voided fraction	4.1[b]	0		4.1[b]	3.8[b]	35[c]
	6.5	0		6.5	6.0	
	4.6	0		4.6		
	5.5	0		5.5	5.5	
	4.8	0		4.8	4.6	
G-200 Included fraction	0	0		0	0	65[c]

Amoebae were grown for 4-5 days in oxygenated 15 l carboys to a cell density of about 5 x 10^5/ml. The amoebae were collected by low speed centrifugation, washed in 0.01M Tris, pH 7.4 and homogenized, and plasma membranes were isolated by several steps including low speed and isopycnic centrifugations (7). Membranes were extracted overnight at room temperature with chloroform-methanol (2:1) and the residue was collected by centrifugation, washed with methanol and acetone, and then dried under reduced pressure at room temperature. The lipid-extracted residue was dissolved in 8M urea in 0.02M Tris chloride, pH 8 and separated into two fractions by chromatography on Sephadex G-200 as described in Fig. 2. Samples were analyzed for phosphorus (9), sterol (10) anthrone-positive carbohydrates as glucose equivalents (11) and protein.

[a]Lowry reaction (12). [b]Proteins were quantitated by the Lowry reaction (12). Variations in the phosphorus: protein and carbohydrate:protein ratios are probably due to difficulties in measuring small amounts of protein. The phosphorus:carbohydrate ratio was always nearly 1:1. [c]Calculated from the quantitative amino acid analyses (Table II). Values of 30% and 70% were obtained for the distribution of radioactivity when membranes were prepared from amoebae grown in the presence of tritiated amino acids, and values of 23% and 77% were obtained by the Lowry reaction.

dehydrogenase (7). The plasma membranes do contain alkaline phosphatase and 5'-nucleotidase activities (which may be the same enzyme) both enriched 15-20 fold over the whole homogenate (7,8). Many enzymatic activities found in mammalian cell plasma membranes are not present (7). The isolated amoeba plasma membranes have a high content of phospholipids (Table I) and a high molar ratio of sterol to phospholipid (Table I). The detailed lipid composition of the plasma membrane will be published elsewhere (7).

Methods and Results: Aqueous suspensions of freshly prepared or frozen plasma membranes can be extracted with 20 volumes of chloroform-methanol, 2:1, with no loss of protein (Table I). Analysis of the lipid-extracted residue shows a high content of non-lipid organic phosphorus and of carbohydrate (Table I). Less than 1% of the phosphorus in this residue could be accounted for as phospholipid, RNA and DNA. When plasma membranes were isolated from amoebae grown in the presence of ^{32}P-phosphate the non-lipid organic phosphorus had the same specific activity as the phospholipid phosphorus.

Gel electrophoresis of an SDS solution of the lipid-extracted residue shows the presence of only 2, or sometimes 3, bands when stained for protein in contrast to the multiple bands revealed when the whole amoeba homogenate is treated similarly (Fig. 1). Two intensely staining bands are seen when similar gels are stained for carbohydrate by the PAS reaction (Fig. 1). When lipid-extracted samples labeled with ^{32}P were electrophoresed all of the radioactivity was found in this same region. The positions of the PAS-positive bands may not correspond exactly to the positions of the amido black-positive bands but all of the bands have about the same mobility as lysozyme (Fig. 1). Identical results are obtained when the amoeba plasma membranes are dissolved directly into SDS without prior delipidation and when the preparations are reduced and alkylated.

Two well separated peaks are obtained when the lipid-extracted residue is dissolved in 8 M urea and fractionated on Sephadex G-200 (Fig. 2). The fraction that elutes in the void volume contains all of the phosphorus, all

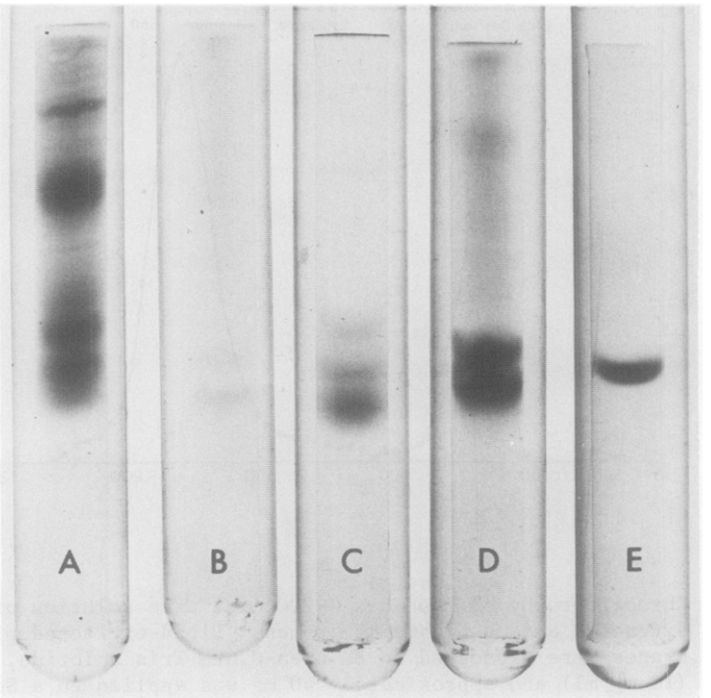

Figure 1. SDS-polyacrylamide gel electrophoresis of whole homogenates of Acanthamoeba castellanii (A and B) and of lipid-extracted amoeba plasma membranes (C and D). Amoeba homogenates and lipid-extracted plasma membranes (Table I) were dissolved in 0.5% sodium dodecylsulfate and electrophoresed in 12.5% polyacrylamide gels in 0.1% SDS-0.1 M Tris phosphate, pH 6.8. Electrophoresis was for about 2 hours with a constant current of 4 ma per gel. Gels A, C and E were stained for protein with amido black and gels B and D were stained for carbohydrate by the PAS reaction. Lysozyme (E) served as a standard.

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of the carbohydrate and about 35% of the recovered protein (Table I). The included fraction contains about 65% of the recovered protein and no detectable phosphorus or carbohydrate (Table I). The ratio of protein:P:carbohydrate (as glucose equivalents in the anthrone reaction) in the voided fraction is about 1:5:5 (mg:μmole:μmole) (Table I). The total recoveries of carbohydrate and phosphorus are essentially quantitative and the recovery of protein is at least 80%.

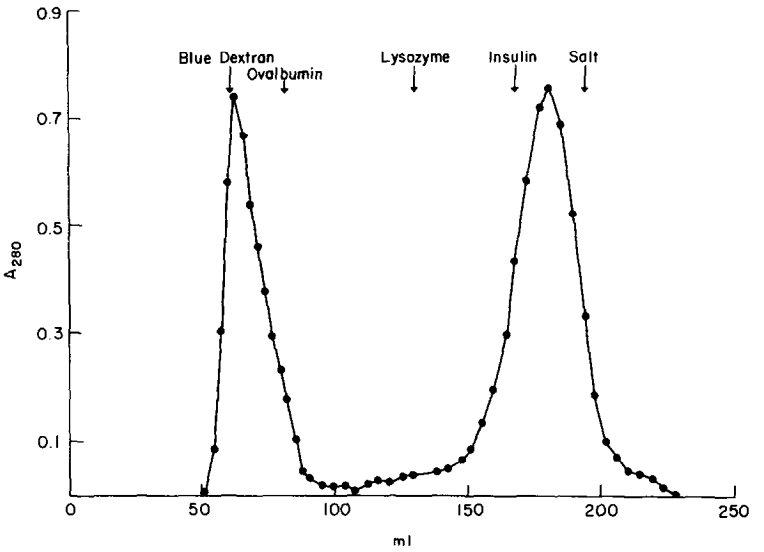

Figure 2. Chromatography on Sephadex G-200 of a urea solution of lipid-extracted amoeba plasma membranes. Lipid-extracted plasma membranes were dissolved in 8M urea-0.02M Tris chloride, pH 8, (10 mg/ml) and approximately 40 mg was applied to a Sephadex G-200 column (2.5 cm x 40 cm) that was equilibrated and eluted with the same solvent. The eluate was monitored by its absorption at 280 mμ. The voided and included fractions were individually pooled and analyzed for protein, carbohydrate and phosphate (Table I). At least six similar experiments have been performed, some of which were monitored for radioactive proteins and others by the Lowry reaction with essentially identical results. Standards were chromatographed on the same column under identical conditions. Ovalbumin and lysozyme were reduced and alkylated before the chromatography.

The voided fraction was dialyzed against water and hydrolyzed in 2N H_2SO_4. The sugars were separated by paper chromatography and analyzed by colorimetric reactions (13), by gas-liquid chromatography after reduction to sugar alcohols and acetylation (14) and by reaction with glucose oxidase. Glucose, mannose and xylose were present in approximately equimolar amounts. Ribose and deoxyribose were not found confirming the absence of RNA and DNA. The hydrolysate contained at least one additional sugar which showed some similarities to ketosugars in color tests and produced four GLC peaks after reduction and acetylation but was otherwise unidentified. The nature of the phosphorus-containing compound in the voided fraction is still unknown. The

phosphorus is not released as inorganic phosphate by hydrolysis in 1N NaOH at 100° for 2 hours or in 2N acid at 100° for 4 hours nor does such acid hydrolysis render the organic phosphorus susceptible to E. coli alkaline phosphatase. About 30% of the phosphorus is hydrolyzed to inorganic phosphate by 6N HCl at 105° for 16 hours and about 80% is hydrolyzed after 72 hours. The voided fraction contains the full complement of amino acids and two additional ninhydrin-positive components that are eluted well before aspartic acid (Table II). More than one protein may, of course, be present. Finally, the sizes of these components are not known; they behave as smaller molecules in SDS solutions (Fig. 1) than in urea solutions (Fig. 2).

The included fraction obtained by chromatography on Sephadex G-200 of solutions of the lipid-free residue of plasma membranes in 8 M urea (Fig. 2) contains about 65% of the recovered protein (Table I). As judged from the elution pattern this fraction contains polypeptides of low molecular weight (Fig. 2). The amino acid composition of the proteins in the included fraction differs from that of the proteins in the voided fraction (Table II). The proteins in both fractions are radioactive when they are prepared from plasma membranes isolated from amoebae grown in the presence of tritiated amino acids.

DISCUSSION: The amoeba plasma membrane has a rather high content of carbohydrate compared to many other plasma membranes. Of particular interest is the high concentration of organic phosphate that cannot be accounted for by phospholipid, RNA, DNA, or serine phosphate (as calculated from the amino acid composition of the 16 and 72 hour hydrolysates of the voided fraction). A phosphate-containing polymer of this kind has not previously been found in animal cell plasma membranes. The linkages, if any, between the phosphate, carbohydrate and protein of the voided fraction are not known but are being studied.

A large proportion of the proteins of the amoeba plasma membrane, as isolated, are of apparently low molecular weight but it is yet to be estab-

Table II
Amino Acid Analyses of the Proteins in the Voided and Included Fractions Obtained by Chromatography of Lipid-extracted Plasma Membranes on Sephadex G-200

Amino Acid	Voided Fraction Moles %	Included Fraction Moles %
A[a]	9	0
B[a]	1	1
CYS[b]	0	1
ASP	8	11
THR	5	6
SER	9	6
GLU	9	14
PRO	3	4
GLY	10	10
ALA	9	9
VAL[c]	6	9
MET	1	1
ILEU	5	4
LEU	10	7
TYR	3	3
PHE	5	2
LYS	4	3
HIS	1	4
ARG	3	3

The lipid-extracted residue of amoeba plasma membranes was separated into two fractions by chromatography on Sephadex G-200 in 8 M urea-0.02M Tris Cl, pH 8. (Fig. 2). The voided and included fractions were individually pooled and then reduced and alkylated (15). The voided fraction was freed of urea and reagents by extensive dialysis against deionized water and the included fraction by passing it through a Sephadex G-10 column in 25% HCOOH. Both fractions were lyophilized and dissolved in 6N HCl together with internal standards of hydroxylysine and norleucine. Hydrolyses were performed at 105° for 16 and 72 hours and the amino acids were analyzed on a Beckman automated amino acid analyzer. The composition of each fraction was calculated from the integrator values and color constants determined for each amino acid and were corrected for the recovery of hydroxylysine and norleucine. In two different experiments the results were very similar to the ones reported here. Amino acids whose concentrations differ in the two fractions are underlined.

[a]In addition to the normal amino acids the voided fraction contained a ninhydrin-positive compound that was eluted from the long column (neutral and acidic amino acids) in 8 minutes and both fractions contained another ninhydrin-positive compound that was eluted in 12 minutes. For comparison, ASP was eluted in 27 minutes. [b]Determined as S-carboxymethylcysteine. [c]The included fraction contained a third unknown compound that was eluted immediately before VAL and which accounts for about one third of the VAL value. VAL is probably present in equal concentrations in the two fractions.

lished that this is also true for the membrane *in situ*,; i.e., that degradative artifacts are not formed during the 6 hours it takes to isolate purified plasma membranes from the amoeba homogenate (7). Several experimental controls suggest, however, that extensive proteolysis does not occur during the

isolation procedure. (1) Alkaline phosphatase activity does not decrease when amoeba homogenates are incubated at 0° or at 24° for 6 hours and all of the alkaline phosphatase and 5'-nucleotidase activities of the homogenate are recovered in the sum of the several fractions obtained during the isolation of the plasma membrane. Thus, the two enzymatic activities known to be components of the plasma membrane survive the isolation procedure. (2) There is no increase in ninhydrin-reacting material when amoeba homogenates are maintained at 0° for 6 hours. (3) When whole amoebae are dissolved directly in 10% SDS and heated for 30 minutes at 100°, gel electrophoresis reveals only two PAS-positive bands which are at the same position as in electrophoretic gels of isolated plasma membranes. A broad unresolved band is seen in the same region when similar gels are stained for proteins.

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