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A NOVEL TECHNIQUE FOR GENTLE LYSIS OF EUCARYOTIC CELLS

ISOLATION OF PLASMA MEMBRANES FROM *DICTYOSTELIUM DISCOIDEUM*

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A new type of lysis technique for eucaryotic cells was used for the isolation of highly purified plasma membranes from *Dictyostelium discoideum*. Suspensions of amoebae (10 μm diameter) were lysed by forced passage through Nuclepore filters with pores of 5 μm diameter. Virtually complete lysis was effected with minimal fragmentation of lysosomes and mitochondria. By subsequent differential and isopycnic centrifugation, 25–35-fold purified plasma membranes were isolated in 35–50% yield for cells from vegetative through tip formation stages of development. Lysis, yield and purity were enhanced by use of slightly alkaline conditions. Contamination with other organelles and with soluble proteins was found to be minimal. At each developmental stage, the plasma membranes generated three closely-spaced, equally pure bands in a sucrose density gradient. Two-dimensional electrophoretic analysis of the individual bands showed that they were very similar to each other, indicating that the density differences are not due to gross differences in protein composition.

Introduction

Plasma membranes of nucleated, eucaryotic cells are complex organelles with physical and chemical properties which overlap those of other subcellular membranes. Hence, their isolation has proven difficult and many strategies have been applied (reviewed in Ref. 1). The problem is particularly acute for analysis of developmental systems where diverse cell types emerge which have significant differences in many properties, including plasma membrane constituents. In the face of these problems, differentiation of the amoebae of the cellular slime mold, *Dictyostelium discoideum*, provides a simplifying model system.

Exhaustion of the food supply in this organism triggers the onset of a developmental process consisting of aggregation of amoebae into multicellular masses by 10–12 h followed by emergence of a controlling tip region on cell mounds at 13–14 h. This multicellular aggregate subsequently undergoes morphological differentiation through various stages, culminating in the formation by 24 h of a fruiting body consisting of a spore sac supported by a slender cellular stalk (reviewed in Refs. 2 and 3). Since the cellular sensory processes which are involved in the above phenomena are mediated by the plasma membrane, significant changes might be expected in the biochemical properties of the plasma membrane during development. In order to study synchronous plasma membrane differentiation in a homogeneous cell population, we have studied cells from the vegetative through tip formation stages, after which two cell types are

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clearly present, i.e., pre-stalk and pre-spore cells.

A plasma membrane purification procedure was sought which would ideally include the following features: (a) a gentle cell lysis technique which would not extensively fragment the plasma membrane or the other cellular organelles, (b) a fractionation scheme which would give high purity as assayed by unequivocal plasma membrane markers, (c) high yield and evidence that the isolated material is representative of total plasma membrane, not a specialized subfraction, (d) ease and reproducibility of the methods and, finally, (e) applicability to cells at multiple developmental stages. This report describes a protocol for plasma membrane isolation from *D. discoideum* which satisfies these criteria to a large degree. Further, the success of the method is based predominantly on a cell lysis technique which should be applicable with very little modification to a wide variety of cell types.

Methods

Strains and growth conditions. *Dictyostelium discoideum* strain Ax-3 was grown in shaken suspension (120 rpm) at 22°C in HL-5 broth [4]. Strains V12 and NC4 were grown on *Escherichia coli* B/r suspended at a density of 10^{10}/ml in starvation buffer (20 mM potassium phosphate, 2 mM $MgSO_4$, pH 6.1).

Differentiation. Cells were harvested during late exponential growth at $(5–8)\cdot 10^6$/ml by centrifugation at $500 \times g$ for 5 min. They were washed and resuspended in starvation buffer, spread evenly on 2% agar plates at a density of $(1.5–4)\cdot 10^4/mm^2$ and incubated at 22°C. Cells started streaming towards aggregation centers after 7–8 h and formed loose mounds at 9 h. To obtain aggregation-competent amoebae, streaming cells were washed off the agar plates into ice-cold starvation buffer at 8–9 h and harvested. Sometimes, the agar plates were stored at 4°C overnight, and, on warming to 22°C, cells were seen to aggregate and develop normally. Unless otherwise indicated, the data in the tables and figures of this report were obtained from Ax-3 cells which were actively aggregating at the time of harvest.

Membrane preparation. Cells were washed and resuspended at $(3–5)\cdot 10^7$/ml in lysis buffer (5 mM glycine, pH 8.5 at 25°C, pH 8.95 at 2°C, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$) and allowed to warm to room temperature briefly (2–4 min). A 5 μm pore size Nuclepore polycarbonate filter (25 mm diameter) was placed in a Swin-Lok Holder (Nuclepore Corporation), and the holder was attached to a syringe. The cell suspension was placed in the syringe and lysed by forced passage through the filter. Lysis was always over 95%, usually complete. Occasional batches of filters gave less lysis. These had larger pores as seen through a microscope and were not used. There is eventual clogging of the filter after passage of 30–40 ml of vegetative cells or 20–30 ml of cells at later developmental stages, necessitating replacement of the filter. Also, when small numbers of cells are used ($< 1\cdot 10^8$), lysis is less than 95% complete. To avoid this problem, filters can be 'activated' by passage of $(3–5)\cdot 10^8$ cells and subsequent rinsing with buffer prior to lysis of the small sample. Since the filter does not adsorb significant amounts of material (see Results), this procedure does not contaminate the small sample.

The lysate was immediately chilled in an ice bath and centrifuged at $5000 \times g$ for 20 min. The crude membrane pellet was resuspended in a minimal volume of lysis buffer and layered over a 15 ml linear sucrose gradient (0.75 to 1.5 M sucrose in 50 mM glycine, pH 8.5 at 22°C) with a cushion of 2.5 M sucrose in 50 mM glycine. Pellets from $1.5\cdot 10^9$ cells or less were loaded per gradient. The gradient was centrifuged for 18 h at $100\,000 \times g$ at 2°C and fractions were collected. Membranes were harvested by dilution with 5–10 volumes of 20 mM Tris-HCl buffer (pH 7.4) and centrifugation at $20\,000 \times g$ for 90 min.

Sodium dodecyl sulfate (SDS) gel electrophoresis. Gradient fractions were harvested as above. The pellet was drained and resuspended in 15% glycerol, 5% β-mercaptoethanol, 50 mM Tris-HCl, pH 6.8, with vigorous vortexing, and 10% SDS was added to a final concentration of 3%. The samples were heated in a boiling water bath for 3 min. Slabs of 10% polyacrylamide, with 4% stacking gels, were run in 0.1% SDS using the Ornstein-Davis stacking system [5]. Fluorography was according to Laskey and Mills [6].

Two-dimensional gel electrophoresis. This was performed with modifications of the methods of

O'Farrell [7] and Ames and Nikaido [8]. Gradient fraction pellets were resuspended in 40 µl isoelectric focussing sample buffer (10 mM Tris-HCl, pH 6.8, 20% glycerol, 10% β-mercaptoethanol) with vigorous vortexing, and 10 µl 10% SDS were added. The samples were heated for 3 min in a boiling water bath, chilled and diluted with 50 µl of dilution buffer (16% Nonidet P-40, 9.5 M urea, 7.5% β-mercaptoethanol and 3% of a cocktail containing ampholytes of the pH ranges 4–6, 6–8 and 3.5–10 in the ratio 2:2:1). Each sample was saturated with crystals of urea and loaded into the isoelectric focusing tubes. The gels were poured as described [7] except that the ampholytes used were of the pH ranges 3–10, 4–6 and 7–9 in the ratio 7:3:3. The gels were run at 500 V for 18 h at room temperature and extruded into curved spatulas. To each spatula, 1 ml of SDS buffer (2% SDS, 25 mM Tris, pH 6.8, 5% β-mercaptoethanol) was added and the gel and buffer on the spatula were frozen at −70°C. When membrane samples were prepared from cells after long-term metabolic labelling with [^{35}S]methionine, at least 95% of the radioactivity entered the isoelectric focussing gel.

The second dimensions were run in 10% polyacrylamide slab gels with 4% polyacrylamide stacking gels containing 0.1% SDS. Prior to loading on the second dimension, the SDS-containing tip at the acid end of the tube gel was discarded. The tube gels were sealed in place with 0.5% agar containing 1% SDS. Molecular weight standards were loaded in slots cut at the edge of the agarose sealing gel. Gels were stained with Coomassie blue as in Fairbanks et al. [9].

Preparation of isethionyl acetimidate. Isethionyl acetimidate was synthesized as described by Whiteley and Berg [10]. The melting point was 3 deg C higher than the published value and the nuclear magnetic resonance spectrum in dimethylsulfoxide was consistent with the expected structure. Radioactive acetonitrile (C^3H_3CN) at 20.5 Ci/mmol was used for the preparation of labelled reagent. Competence of the radioactive reagent to label proteins was established by reaction with insulin. About 40% of the counts were precipitatble with trichloroacetic acid when [^{3}H]isethionyl acetimidate was incubated 30 min at room temperature, pH 8, at a 1:1 ratio of reagent to amino groups. The reagent was observed to be stable in water at pH 8.0 for at least one hour at 0°C.

Labelling with isethionyl acetimidate. Cells were washed and resuspended to $1 \cdot 10^8$/ml in 17 mM potassium phosphate, pH 8.0. [^{3}H]Isethionyl acetimidate was dissolved in the same ice-cold buffer and added to the cells to give approximately $1 \cdot 10^8$ cpm/ml. The suspension was shaken for an hour at 22°C. For the permeability control experiment, the reagent solution was added to ice cold cells. The suspension was divided into two equal parts. One part was allowed to react at 22°C; the other was lysed by filtration in the cold and then allowed to react at 22°C. For the prequenching experiment, the reagent solution (1 ml, $2 \cdot 10^8$ cpm) was incubated 60 min at 22° with 0.5 ml 0.5 M glycine (pH 8.5) before addition to cells.

Radioiodination. Cells ($5 \cdot 10^8$) were washed and resuspended in 4 ml of 20 mM potassium phosphate, pH 8.0. To 1 ml of the same buffer were added 500 µl of 100 mM glucose, 1 mCi carrier-free Na^{125}I (in 10–20 µl) and the cell suspension. The reaction was started [11] by adding 100 µl of the enzyme cocktail (1 mg/ml lactoperoxidase, 20 units/ml glucose oxidase). After 10 min at room temperature, the reaction was quenched by adding 10 ml of buffer containing 50 mM NaI. Cells were pelleted and washed twice with 10 ml each of phosphate buffer + NaI and twice with phosphate buffer.

Trichloroacetic acid-precipitation of labelled samples. Samples were loaded onto cellulose filter discs (Whatman) and dried. The dried discs were shaken for 10 minutes in 10% (w/v) trichloroacetic acid, washed twice with 10% acetic acid and once with 95% ethanol, all at room temperature. The discs were dried and radioactivity determined in 10 ml Scintiverse (Fisher).

Contamination by soluble proteins. Cells were labelled with [^{35}S]methionine by a modification of the method of Alton and Lodish [12]. Vegetative amoebae in starvation buffer were spread on three Nuclepore filters (47 mM diameter, 0.2 µm pore size) resting on agar plates. One filter was removed after 15 min and placed on a droplet of 100 µCi [^{35}S]methionine (500 Ci/mmol) in an empty petri dish; the others were placed in empty dishes. The dishes were covered with wet filter papers and incubated at 22°C for 9 h. The ^{35}S-labelled amoebae were washed, suspended in 6 ml ($3.3 \cdot$

10^7/ml) of lysis buffer and lysed as usual. The lysate was centrifuged for 2 h at $20000 \times g$ and the supernatant recovered. The 9 h non-radioactive cells were suspended in 10 ml lysis buffer and split into two equal aliquots. One batch was lysed directly into a tube containing 2.5 ml ^{35}S-labelled soluble fraction. The other batch was mixed with 2.5 ml ^{35}S-labelled soluble fraction prior to lysis. Plasma membranes were isolated as usual.

Enzyme assays. Succinate dehydrogenase was assayed by two methods. In the ferricyanide method [13] the assay tubes contained 100 mM potassium phosphate, pH 7.7, 40 mM sodium succinate, 500 μg bovine serum albumin and 0.05% potassium ferricyanide. Prior to assay, each fraction was sonicated three times 30 s in an ice-water bath on a Branson cell disruptor 350 at a setting of 5.

Succinate dehydrogenase was also assayed using the membrane-impermeable substrate phenazine methosulfate with dichlorophenol indophenol [14] as a test of the extent of mitochondrial rupture.

NADPH-dependent cytochrome *c* reductase was assayed as described [15] except that 10 mM KCN was present in both the assay and blank cuvettes, and the concentration of potassium phosphate was 50 mM.

N-Acetylglucosaminidase was assayed as described [16]. All assays were tested for linearity with increasing enzyme concentration.

Protein determination. Protein was assayed by the method of Bradford [17] as described in Bio-Rad Laboratories bulletin No. 1051.

Materials. Radioactive acetonitrile and carrier-free ^{125}I were obtained from New England Nuclear. Glucose oxidase was from Worthington Biochemicals and lactoperoxidase from Calbiochem. Nuclepore filters and Swin-Lok holders were purchased from VWR. Nonidet P-40 was from Shell. Ampholytes of 3–10 pH range were from Bio-Rad and narrow range ampholytes from LKB. Electrophoresis reagents were all from Bio-Rad except urea which was obtained from EM Biochemicals. Enzyme grade sucrose was from Schwartz-Mann. All other chemicals were of reagent grade.

Results

Cell lysis

Amoebae of *D. discoideum* are relatively resistant to shear forces and are difficult to lyse by conventional methods [16]. Therefore, a novel technique of lysing cells by forced filtration through Nuclepore filters was attempted. A filter with cylindrical pores of 5 μm diameter was chosen since this pore is smaller than a cell diameter but larger than the discrete subcellular organelles (lysosomes, nuclei, etc.) The protocol described in Methods was found to be optimal, yielding at least 95% lysis in a single pass through the filter. All the input protein is recovered in the lysate, and less than 1% of cell surface-specific radiolabel is found in the filter after lysis. Therefore, the filter does not trap any significant amounts of the cellular material. The technique is gentle as judged by two criteria. No activity of succinate dehydrogenase, an inner mitochondrial membrane enzyme, is found in the lysate in the absence of sonication when using a charged substrate (phenazine methosulfate) which should be membrane-impermeant. Activity is detected in the lysate when the sample is sonified prior to assay. Therefore, the mitochondria either are not lysed or they rapidly reseal with their original orientation. We have estimated the upper limit on lysosomal rupture by determining the fraction of a lysosomal marker enzyme [16], *N*-acetylglucosaminidase, which could not be sedimented in lysates (by centrifugation for 2 h at $100000 \times g$ over a cushion of 0.75 M sucrose). This value was 10.3% and suggests either some lysosomal breakage or some non-lysosomal contribution to the total activity in the crude lysate.

Cell surface labelling

We have used cell surface-specific, covalent radiolabels as markers to purify the plasma membrane. One reagent we have used, isethionyl acetimidate, has been shown by Whiteley and Berg [10] to be non-permeable in erythrocytes and specific for cell surface amino groups. To establish that the reagent is also specific for the cell surface in *D. discoideum* and does not penetrate into the cytoplasm, cells were suspended in buffer and [^{3}H]isethionyl acetimidate added in the cold. One half of the cells was lysed immediately by filtra-

tion; the other half was unlysed. Both samples were warmed to 22°C. When reaction occurred in the lysate, 35% ($7 \cdot 10^7$) of the input counts was found to be precipitable with trichloroacetic acid whereas only 0.1% ($2 \cdot 10^5$) was precipitable in unlysed cells. This 350-fold difference shows that the plasma membrane forms an effective barrier to reagent penetration into the cytoplasm. In addition, sucrose gradient fractionation of the 5000 × *g* pellet from the pre-lysed cells showed a radioactivity profile virtually identical to the protein profile (data not shown), as opposed to the surface labelled case discussed under Membrane isolation. Finally, SDS polyacrylamide gels of proteins labelled in intact cells versus lysates showed that different pools of proteins are detected. Major Coomassie blue-staining proteins (e.g., actin) are labelled easily in broken cell preparations but are not detectably labelled in unbroken cells (not shown). The same is true for radioiodination.

Label incorporation was confirmed to be amino group specific and covalent, rather than tight non-covalent adsorption, by incubation of cells with isethionyl acetimidate previously quenched by incubation with excess glycine. There was no incorporation into acid-precipitable material by intact cells nor was there acid-insoluble material present when sucrose gradient fractions of these cells were analyzed. Taken together, the control experiments establish that the reagent is membrane impermeant and that the labelling is covalent and specific for externally exposed amino groups.

Membrane isolation and characterization

When cells are labelled, lysed and centrifuged at 5000 × *g*, the pellet contains 70–80% of the acid-precipitable radioactivity. The remaining 20–30% is probably associated with small plasma membrane fragments since all of it can be sedimented at 25000 × *g*. The 20–30% of the label in the 5000 × *g* supernatant is discarded since the high centrifugal forces needed to sediment it also sediments other organelles (see below).

The 5000 × *g* pellet is resuspended, layered over a linear sucrose gradient and centrifuged to equi-

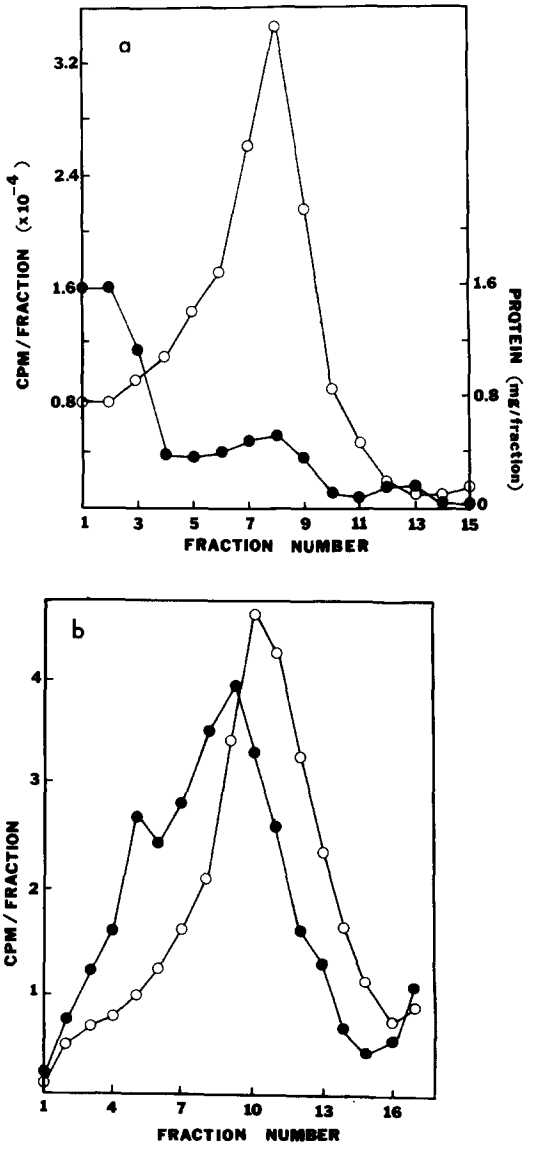
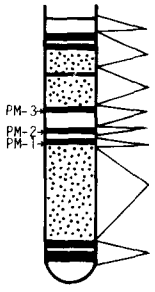


Fig. 1. Sucrose gradient profiles of protein and the cell surface radiolabel isethionyl acetimidate. In panel a, intact cells were reacted with [^{3}H]isethionyl acetimidate, washed and lysed as described in Methods. The lysate was centrifuged at 5000 × *g*, the pellet resuspended and applied to a sucrose gradient as described in Methods. Continuous, constant-volume fractions were collected, sedimented to remove the sucrose and assayed for the total protein (●———●) and trichloroacetic acid-precipitable radioactivity (○———○). In panel b, two batches of cells ($1.2 \cdot 10^9$ each) were labelled with [^{3}H]isethionyl acetimidate, lysed and centrifuged at 5000 × *g*. One pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.4) and layered over a linear 1.5–0.75 M sucrose gradient with a 2.5 M cushion containing 50 mM buffer at pH 7.4. The other was resuspended and layered over a similar gradient except that the buffer was glycine at pH 8.5 at the same molarities. Filled circles show the radioactivity profile at pH 7.4 ($9 \cdot 10^5$ cpm loaded on the gradient). Open circles show the radioactivity profile at pH 8.5 ($8 \cdot 10^5$ cpm loaded on the gradient).

TABLE I

BAND-WISE DISTRIBUTION OF RADIOACTIVITY AND PROTEIN FROM A SUCROSE GRADIENT

Cells were labelled with [^{3}H]isethionyl acetimidate, lysed and the 5000 × *g* pellet analyzed on a sucrose gradient as described in Methods. The sample applied to the gradient contained a total of 290850 tritium cpm and 4.89 mg of protein. Fractions were collected as indicated in the drawing.



Fraction #	% Input cpm	% Input Protein	Specific Activity (cpm/μg)
1	0	0	0
2	0.5	1.6	18
3	1.5	1.2	73
4	20.3	7.5	160
5	24.6	9.2	159
6	12.0	5.1	140
7	29.0	31.1	55
8	12.2	44.2	16

librium. A typical distribution of protein and acid-precipitable radioactivity from a gradient is shown in Fig. 1a. In this experiment, the peak fractions, 6–9, contained 60% of the radioactivity and centered at about 1.1 M sucrose. The protein profile shows that these fractions contained 20% of the protein on the gradient. This corresponds to 2% of the cellular protein and represents a purification of 30-fold of the label in the peak fractions as compared to whole cells. Essentially identical results have been obtained using radioiodination, as described in Methods, to label the cell surface.

Table I shows a schematic representation of a gradient and the typical distribution of radioactivity and protein when discrete bands rather than continuous fractions are collected. The fractions collected are shown in the schematic. The plasma membrane bands PM-1, PM-2 and PM-3 have almost the same specific radioactivity indicating that they are equally pure. Between them the bands contained 57% of the radioactivity on the gradient in this preparation, again, a typical result for cells at this stage.

Electron microscopy of wet mounts or thin sections using negative staining with uranyl acetate or osmium tetroxide showed the plasma membrane bands to be composed of smooth vesicular structures with the characteristic trilamellar structure of the plasma membrane (data not shown).

pH effects

We have observed that at pH values between 8.5 and 9, mutually cohesive, aggregated amoebae dissociate but retain impermeability to isethonyl acetimidate. Amoebae maintained thus as single cells in cold suspension for an hour will agglutinate when the pH is reduced to less than pH 8 and, if plated, will aggregate and proceed with normal development. On this basis, we perform cell lysis and subsequent gradient fractionation at elevated pH (8.5 at 25°C, 8.95 at 2°C). If lower pH is used, the clumped cells lyse less well, and the membranes aggregate and disperse more widely throughout the sucrose gradient reducing yield and purity (Fig. 1b).

TABLE II

BAND-WISE DISTRIBUTION OF MARKER ENZYMES

Cells were lysed and fractionated on a sucrose gradient as described in Methods. Bands were collected as shown, pelleted, resuspended and assayed. All enzyme activities are expressed in μmol of substrate utilized per min. Percent of the total lysate activity is shown in brackets.

	N-acetyl Glucosaminidase	Succinic Dehydrogenase	NADPH-Cytochrome c Reductase
Total Cell Lysate	1761 (100%)	466 (100%)	893 (100%)
5000xg Supernatant	1000 (57%)	204 (43.8%)	449 (50%)
Gradient Fractions			
4	463 (26%)	53 (11.4%)	275 (31%)
3	16 (0.9%)	4 (0.9%)	16 (1.8%)
2	56 (3.2%)	39 (8.3%)	172 (19.3%)
1	46 (2.6%)	223 (47.8%)	321 (36%)

Marker enzymes

Since there is currently no alternative such as exists for cell surface material, we have used marker enzymes to test the extent of contamination of the plasma membranes by other organelles. We have measured the activity of three marker enzymes as shown in Table II. *N*-Acetylglucosaminidase was used as a marker for lysosomes [16]. The recovery of the total activity in the various fractions was 90%, and only 0.9% of the activity was found in the plasma membranes. Succinate dehydrogenase has been used as an inner mitochondrial membrane marker. Recovery of activity was 112% with 0.9% present in the plasma membrane bands. There are no known perfect marker enzymes for the endoplasmic reticulum which might be expected to be the most abundant contaminant. Two commonly tested enzymes are glucose-6-phosphatase and NADPH-dependent cytochrome *c* reductase. We used the latter in keeping with previous reports for this organism [16,18]. The recovery of activity was greater than 100%, and 1.8% of the activity was present in the plasma membrane fraction. The marker enzymes for mitochondria and endoplasmic reticulum show a bimodal distribution in the sucrose gradients. This could be due to density differences between rough and smooth endoplasmic reticulum and between mitochondria with or lacking the outer membrane, but we have not tested these possibilities. While electron microscopy indicates that growing *D. discoideum* amoebae may have a rudimentary Golgi body which diminishes or disappears during development [19], marker enzymes have not been identified in this organism [16,19,20]. We have, therefore, not tested for this organelle.

Contamination by soluble proteins

Soluble proteins could easily contaminate a plasma membrane preparation by adsorption to the cell surface following lysis or by becoming trapped inside membrane vesicles if they seal following lysis. To test these possibilities, cells were lysed either after mixing with an ^{35}S-labelled, soluble protein fraction or were lysed directly into an aliquot of labelled soluble proteins as described in Methods. Plasma membranes, isolated as usual, contained only trace contamination in both cases as shown in Table III. Isolated plasma membranes contain 1.5 to 2% of the total input cell protein under these conditions. If 1.5% is assumed for this experiment, only 0.25% of the proteins in that fraction are due to contamination by soluble proteins. These controls may not measure contamination by soluble proteins that were actually inside the cells prior to lysis. Nonetheless, contamination due to adsorption to the cell surface is negligible. It is also possible that some of the radioactivity which cofractionates with the plasma membranes could be due to specific association by cytoplasmic peripheral proteins which are in equilibrium with membrane binding sites (actin for example, see Ref. 21).

Yield and protein composition of the plasma membranes

Based on cell surface radioactivity, the triplet bands PM-1, PM-2 and PM-3 give a range of

TABLE III

CONTAMINATION OF PURIFIED PLASMA MEMBRANES BY SOLUBLE PROTEINS

Nonradioactive amoebae were lysed (A) into or (B) in the presence of [^{35}S]methionine-labelled soluble proteins of amoebae at the same developmental stage. The plasma membrane fractions were isolated and freed of sucrose as described in Methods. Trichloroacetic acid precipitations were performed on the ^{35}S-labelled soluble fraction and on the isolated plasma membrane samples.

Amoebae lysed	Total input [^{35}S] cpm	Total cpm in membranes	% of input cpm	% of protein in membrane fractions
(A) into [^{35}S]	$3.5 \cdot 10^7$	500	0.0014	0.09 [a]
(B) In the presence of [^{35}S]	$3.5 \cdot 10^7$	1300	0.0037	0.25 [a]

[a] Assuming the isolated membranes to contain 1.5% of the total cell protein.

35–50% yield. However, even these fractions are of differing densities. Some (20–30%) plasma membrane material does not sediment efficiently after lysis and some (see Fraction 7 of Table I) has higher density. It is possible that the unrecovered portions of the plasma membrane have unique protein components not present in the purified samples. Two types of experiments have been performed to examine this possibility.

A sample of $4 \cdot 10^8$ cells was radioiodinated as described in Methods. Lactoperoxidase-catalyzed iodination has been used by several workers to selectively label the cell surface proteins of *D. discoideum* [22,23]. Controls similar to those described above for isethionyl acetimidate indicate that iodination is also cell surface-specific under our conditions. The labelled cells were lysed, the $5000 \times g$ pellet fractionated on a sucrose gradient and continuous fractions collected. Ten of these fractions, starting from the bottom, contained most of the acid-precipitable radioactivity. These were diluted, sedimented and the resuspended pellets loaded on an SDS slab gel for comparison with the surface proteins of whole cells. A fluorogram of the gel is shown in Fig. 2. Each lane contained 100000 cpm although the protein loads varied greatly for the gradient fractions (see Fig. 1a). For the whole cell sample it was very high and induced band spreading (right-hand lane of Fig. 2). Two conclusions can be drawn from Fig. 2. First, the same radioiodinated species appear to be present in each of the gradient fractions. Shorter fluorographic exposures of the gel and use of higher resolution two-dimensional gels (not shown) support this conclusion. Second, while the comparison is more difficult because of the band spreading, there are no species present in the whole cell sample (lane 11) which are obviously lacking in the gradient fractions (lanes 1–10). By this criterion, no unique species appear to be lost in the unrecovered plasma membrane containing fractions (supernatant from the $5000 \times g$ centrifuga-

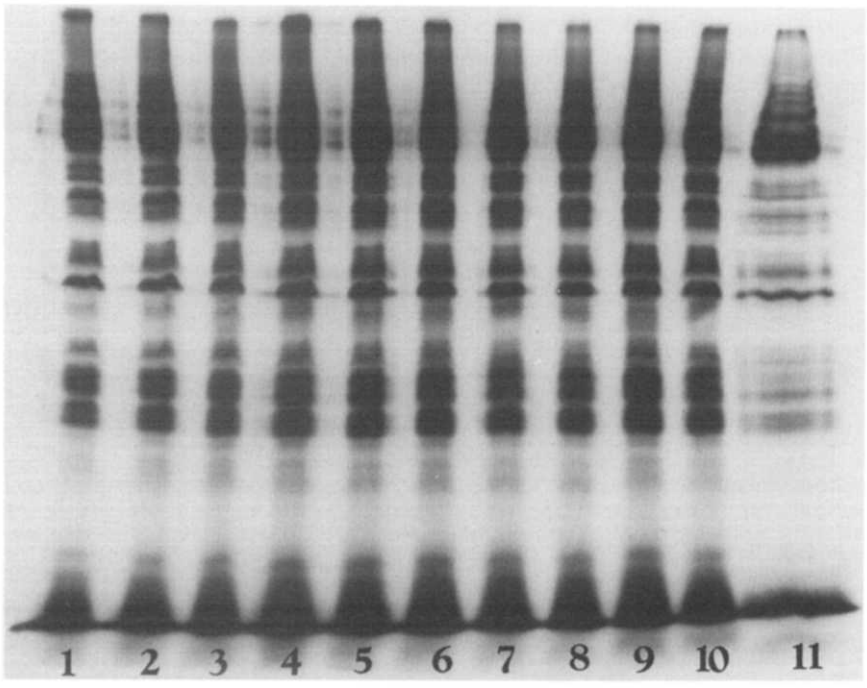


Fig. 2. Distribution of radioiodinated cell surface proteins in the sucrose gradient. Cells were labelled with ^{125}I, lysed and fractionated on a sucrose gradient as described in Methods. The gradient was collected into 16 equal fractions. Ten of these, numbered from the bottom, contained over 90% of the radioactivity in the gradient and were analyzed on the SDS gel shown. Lanes 1–10: Fractions 1–10 from the gradient. Lane 11: Whole cells radiolabelled and solublized in SDS after washing. Each lane has 100000 trichloroacetic acid-precipitable cpm.

tion and low purity sucrose gradient fractions).

It remains possible that unique proteins associated with the cytoplasmic face of the membrane could generate differing densities, and some of those proteins might be absent in the purified fractions. This issue cannot be addressed for the unrecovered fractions but can be tested for the differing density bands PM-1, PM-2 and PM-3 by two-dimensional electrophoresis. The Coomassie blue staining patterns of gels of these separate fractions are shown in Figs. 3a–c (most to least dense). The two most dense fractions have identical polypeptide composition, although the relative amounts of these polypeptides differ slightly. About 80 polypeptide species can be observed in the original gels. The least dense fraction (PM-3) does not contain detectable levels of six of those species (see arrows for examples) but is otherwise identical in composition to the more dense fractions. These tests show that different densities do not necessarily imply grossly differing polypeptide constituents and suggest that the purified membranes are at least largely representative of the total, native cellular plasma membrane. This heterogeneity may be due to variation in the extent of cofractionation of cytoskeletal components with the plasma membrane. For example, actin species (of heterogeneous isoelectric point and indicated by open arrows in Fig. 3) are more abundant in the more dense plasma membrane fractions.

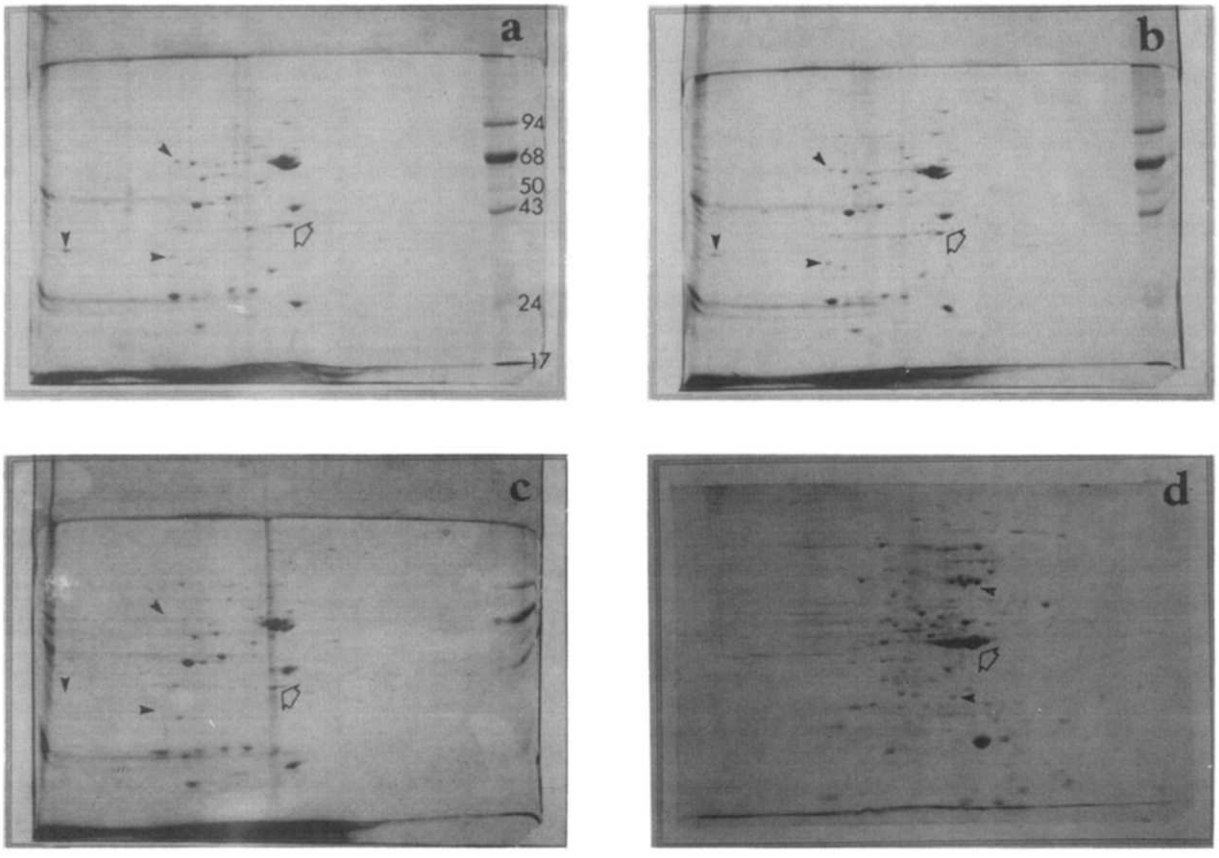


Fig. 3. Two-dimensional gels of plasma membrane and total cellular proteins. Plasma membrane bands PM-1, PM-2 and PM-3 and a whole cell protein sample were fractionated on two-dimensional gels a, b, c and d, respectively. Each contained 150 μg of protein. Isoelectric focusing is in the horizontal dimension (basic end to the left, acid end to the right) and SDS-polyacrylamide gel electrophoresis is in the vertical dimension. The open arrows in a–d indicate the position of actin. The closed arrows in a–c indicate polypeptides present in PM-1 and PM-2 (a and b) but absent in PM-3 (c).

Finally, a two-dimensional gel of whole cell proteins from amoebae at the same developmental stage is included (Fig. 3d). In general, only a few spots, including actin, are in common with plasma membranes (see arrows in Fig. 3d for examples). Note especially a tight cluster of spots above actin which are present in whole cells and some of which are enriched in the membranes. Otherwise, major whole cell components are absent, and this is compatible with the limited contamination of the preparation indicated by assays for marker enzymes and ^{35}S-labelled soluble proteins.

Discussion

There have been a number of methods reported for preparation of plasma membrane-enriched fractions from *D. discoideum* [16,18,20,24–28]. None of these preparations satisfy all the criteria enumerated in the Introduction. For certain types of studies, it is not necessary to satisfy all of these criteria, but for many studies, a method which can give plasma membranes of high yield and purity from a range of developmental stages is essential.

As noted earlier, *D. discoideum* amoebae are relatively resistant to shear forces. Since the amoebae are about 10 μm in diameter, it was possible that forced passage through a filter of smaller pore diameter would give efficient lysis. Nuclepore filters were selected because they are very thin, chemically inert, and their pores are virtually perfect cylinders, which should avoid extensive fragmentation of organelles. Filters with 5 μm pore diameter were found to be optimal, virtually complete lysis being effected with minimal fragmentation of the cellular organelles tested. Efficiency of lysis depends on use of a single-cell suspension, and it was observed that at pH > 8.5 cohesive, aggregated cells dissociated, though reversibly. Therefore, all manipulations were performed at pH 8.5.

Many workers have used marker enzyme enrichment as an estimate of plasma membrane purity, but there is disagreement as to which enzyme is a reliable marker in *D. discoideum* [16,18,20]. Enrichment of cell surface radiolabels was chosen for this reason and because, given recent advances in knowledge about membrane protein synthesis [29], it is possible that one would find significant amounts of an integral plasma membrane enzyme in intracellular membranes. There is also a potential caveat in the use of cell surface radiolabels as plasma membrane markers. While isethionyl acetimidate is membrane impermeant and therefore surface specific under our conditions, in this phago/pinocytotic organism, some plasma membrane internalization and recycling must occur during the 60 min labelling period. However, a 10 min radioiodination of cells generates an identical radioactivity profile in sucrose gradients and a similar enrichment in the bands collected. In 10 min, only a few percent of the cell surface is internalized by actively pinocytosing vegetative cells [30]. In addition, as shown by Coomassie blue staining of two-dimensional gels (Fig. 3), the protein composition of the three plasma membrane bands of the sucrose gradient is 90% identical. While the heterogenous densities of these bands could have originated from rapid membrane recycling, if the internalized plasma membrane fractions had undergone extensive fusion with intracellular membrane systems, this should have been reflected in greater diversity among polypeptide constituents. We conclude that the recovered plasma membrane bands cannot have undergone significant fusion with elements of non-plasma membrane origin. However, the fractions recovered should be defined to include cell surface membrane and those membranes which are in rapid equilibrium with it. It is quite possible that some of the cell surface radiolabel in the unrecovered fractions originates by fusions with internal membranes causing a reduction of label specific activity.

The plasma membrane fractions recovered from sucrose gradients are purified 25- to 35-fold with respect to surface radioactivity compared with starting cells and contain 1.5 to 2.0% of the total cellular protein. Based on marker enzymes, contamination by lysosomes, mitochondria and endoplasmic reticulum is very low. The latter test also argues against significant contamination by fragments of the nuclear envelope since this organelle is continuous with the endoplasmic reticulum and does not have known biochemical markers distinct from that organelle [31]. We have also not tested for the presence of the Golgi apparatus since this organelle is not well defined in *D. discoideum* and

markers have not been identified; nor have we tested for peroxisomes since these usually have a density similar to lysosomes in most cell types [1] as well as in *D. discoideum* on both sucrose and Renografin gradients [20]. Finally, it is not possible currently to test contamination by every membranous organelle, and this organism may contain vacuoles, etc., for which no markers are known. However, plasma membranes isolated from cells lysed in the presence of a metabolically-labelled soluble protein fraction contained very low radioactivity indicating, at least, that cytoplasmic proteins do not contaminate by adsorption to the external membrane surface.

The yield of plasma membrane is high, varying from 35 to 50%. (The lower yields are obtained from cells at or near the tip formation stage.) In addition, we present evidence that with respect to protein composition the isolated preparation largely represents the total plasma membrane of the cell and not a specialized subfraction. All radioiodinated cell surface proteins appear to be present. The two-dimensional gels show that differing equilibrium densities are not due to grossly differing protein composition. Consequently, within the detection limits, there is no reason to believe that the fractions lost during purification are substantially different in their polypeptide components. This is an important point in general for membrane isolations since heterogeneity of density is common for plasma [32,33] and subcellular [34] membranes. It is possible that density differences could be due to varying degrees of cytoskeletal association with the membranes or to variation in protein-to-lipid ratios in broken membranes [32]. As noted above, gross density differences which generate low specific activity of cell surface radiolabels could also be due to fusion of internalized plasma membrane with intracellular organelles.

Two-dimensional gels of the plasma membranes resolve about 80 different Coomassie blue-staining protein species. Many fewer species are resolved with a one-dimensional SDS gels. Examination of gels reveals that single bands in an SDS gel may actually be composed of two or more different proteins. The two-dimensional system is capable of resolving as many as 4000 different species [7]. With our samples, the upper limit is imposed by the sensitivity of detection. The number of detectable species on such gels is greatly increased when sensitivity is enhanced by fluorographic detection of proteins metabolically labelled with [^{35}S]methionine [35].

While most of the data in this report were obtained using cells which were actively aggregating at the time of harvest, the lysis and fractionation scheme described appears to be equally successful for amoebae from the vegetative stage through the stage of tip formation on cell aggregates; later stages have not been tested since two cell types are clearly present in late development. All the data in this report were obtained with a strain (Ax-3) grown on axenic media. In preliminary studies the method is also successful for non-axenic strains (NC4 and V12) grown on bacteria. Lysis of bacterially grown cells is, however, extremely difficult until the amoebae have been starved for a few hours, presumably because of the extensive intracellular membrane network in these growing cells. The method should be applicable to other cell types as well, provided filters of the appropriate pore sizes are chosen. In preliminary experiments with collaborators, we have obtained efficient lysis of fibroblasts, yeast spheroplasts and lymphocytes.

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