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# THE PURIFICATION AND CHARACTERIZATION OF *DICTYOSTELIUM DISCOIDEUM* PLASMA MEMBRANES

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## Summary

A new procedure for the purification of plasma membranes of *Dictyostelium discoideum* is described. Cells are broken by vigorously stirring in the presence of glass beads, and plasma membranes are isolated by equilibrium sucrose density centrifugation. The purified membranes are considerably enriched in alkaline phosphatase and 5′-nucleotidase and contain very low levels of succinate dehydrogenase and NADPH-cytochrome *c* reductase. The purified membranes contain relatively high levels of phospholipid, sterol and carbohydrate. They appear as a relatively homogeneous population of membrane vesicles in the electron microscope. This new method of purification is compared to previously published procedures which have been found to be unsuitable for our purposes.

## Introduction

During the differentiation of the cellular slime mould *Dictyostelium discoideum* from unicellular amoebae to a final fruiting body consisting of distinct stalk and spore cells, the establishment and maintenance of cell-cell contacts is essential [1]. The molecular basis of this cell-cell interaction is important, not only in its own right, but also for the insight it may provide into the nature of this process in more complex organisms.

It is known that changes in cell surface molecules do occur during differentiation. Gerisch et al. [2—4] in a very elegant series of papers have shown that new antigens are produced at the cell surface during *D. discoideum* aggregation. Furthermore, univalent antibodies to these antigens block aggregation suggesting that these surface antigens are contact sites. Smart and Hynes [5] have shown that the exposed proteins of the cell surface change during differentiation by labelling whole cells with $^{125}I$ in the presence of lactoperoxidase and $H_2O_2$ and analyzing the radioactive proteins by sodium dodecyl sulphate acrylamide elec-

trophoresis. Rosen et al. [6] have shown that the amount of a carbohydrate binding protein increases markedly during aggregation and have provided evidence that this protein resides at the cell surface; finally previous reports from this laboratory have shown that alterations in concanavalin A binding sites occur during aggregation [7-9].

A pure plasma membrane preparation is highly desirable to further investigate the molecular changes that occur at the cell surface during differentiation. Three methods of purifying plasma membranes have been published [10—12]. For our purposes these methods have certain disadvantages. This report describes a new procedure for the purification of plasma membranes from *D. discoideum*.

## Materials and Methods

### *Materials*

Bacto-peptone and yeast extract were obtained from Difco Laboratories. AMP, cytochrome *c*, digitonin, NADPH, phenylmethylsulphonyl flouride, *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine, *p*-nitrophenyl phosphate, rotenone and Triton X-100 were obtained from the Sigma Chemical Co. Amphotericin B, glucose-6-phosphate and thiobarbituric acid were from Calbiochem. [$^3$H]AMP was from Searle-Amersham and Liquifluor was from New England Nuclear. Sucrose was the special enzyme grade from Schwarz-Mann. Boron trifluoride/methanol was obtained from Supelco, Inc.

Standard reference compounds used in gas-liquid chromatography were obtained from the Sigma Chemical Co. and used without further purification. Phase-coated supports for gas-liquid chromatography were from Applied Science Laboratories.

All other reagents were the best available grade from Fisher Scientific Co. or Sigma Chemical Co. Chloroform was re-distilled prior to use.

Silicone-coated glass beads, 0.45—0.50 mm diameter (Braun) were obtained from Canlab.

### *Organism and growth conditions*

An axenic mutant (strain Ax-2) of *D. discoideum* [14] was obtained from Dr. J.M. Ashworth and was used throughout these studies. The strain was grown in HL5 medium as previously described [9] to a density of $5—8 \cdot 10^6$ cells/ml and harvested by centrifugation at $700 \times g$ for 7 min.

Cells were washed by resuspension and recentrifugation as indicated by the preparative procedure used.

### *Purification of plasma membranes*

In preliminary experiments, plasma membranes were prepared exactly as described by both Green and Newell [11] and by Rossomando and Cutler [12].

In addition, two novel purification procedures were developed. (a) Harvested cells were washed twice in 8.6% w/v sucrose/5 mM Tris · Cl (pH 7.4) (sucrose/Tris buffer) and resuspended to a density of $1 \cdot 10^9$ cells/ml in ice cold sucrose/Tris buffer, saturated with phenylmethylsulphonyl fluoride. Approximately 60% cell breakage was achieved by aspiration of this suspension through a stain-

less steel Swinney type filter holder, fitted with two 25 mm perforated filter support screens, separated by a 1 mm thick Teflon O ring [15]. The filter holder was fitted with a 10 cm 20 guage hypodermic needle and attached to a syringe. The cell suspension was drawn into a syringe and expelled rapidly six times. Cell breakage, assessed microscopically, was not increased by further aspiration. Unbroken cells were removed from the homogenate by centrifugation at 700 × *g* for 5 min. The supernatant was removed and centrifuged at 30 000 rev./min for 30 min in a Beckman-Spinco Type 30 rotor. The supernatant was removed and the pellet was resuspended in sucrose/Tris buffer by vortexing. The suspension was transferred to a clean centrifuge tube, leaving behind a lower hard clear pellet that contained no enzyme activity and very little protein. The suspension was re-centrifuged for 30 min at 30 000 rev./min. The pellet was resuspended in 20% w/v sucrose, 5 mM Tris · Cl (pH 7.4) by vortexing and transferred to a clean tube again discarding the residual clear pellet. Dispersion of the suspension was completed by aspiration through a 20 gauge hypodermic needle. A small aliquot was saved for enzyme determinations and then EDTA was added to the remainder to a final concentration of 0.1 mM. Aliquots (2.5 ml) were layered onto discontinous sucrose density gradients formed in 39 ml polyallomer tubes, (Beckman-Spinco), as shown in Fig. 1. All sucrose solutions used for the gradients contained 5 mM Tris · Cl (pH 7.4) and 0.1 mM EDTA. Routinely the crude membrane suspension derived from $2 \cdot 10^{10}$ cells was accomodated on six gradients. The gradients were centrifuged in a SW 27 rotor at 25 000 rev./min for 16—18 h.

Fractions were removed from the gradients by puncturing the sides of the tubes with a hypodermic needle fitted to a syringe. Membranous material was recovered by dilution of the fractions to 10% w/v sucrose with 5 mM Tris · Cl (pH 7.4) and centrifugation at 30 000 rev./min and 4° C for 30 min. Pellets were resuspended in small volumes of sucrose/Tris buffer for subsequent determinations. Preparations were stored at —70° C. All procedures were performed on ice or at 4° C.

(b) Harvested cells were washed in sucrose/Tris buffer as described above, and resuspended in cold sucrose/Tris buffer, saturated with phenylmethylsul-

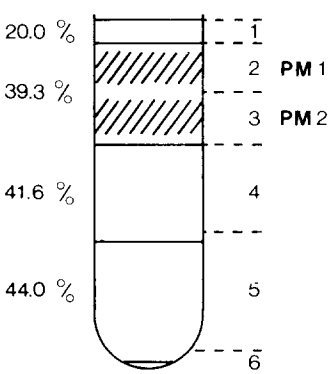


Fig. 1. The composition of the initial sucrose gradient (left hand side) and the membrane bands obtained after centrifugation of crude membranes at 25 000 rev./min for 16 h in a SW 27 rotor. The gradient was divided into six fractions, as denoted on the right hand side of the tube.

phonyl fluoride, to a cell density of $1 \cdot 10^8$ cells/ml. Cells were broken by vigorously stirring the suspension with glass beads. Routinely, 200 ml of cell suspension was divided equally into two portions and 30 g of beads were added to each in a 250 ml Erlenmeyer flask. The suspensions were stirred vigorously on a magnetic stirrer for 10 min. This procedure resulted in 80—85% cell breakage assessed microscopically. The homogenate was decanted and the beads rinsed with 20 ml of sucrose/Tris buffer. The combined homogenates and washes were centrifuged at $700 \times g$ to remove unbroken cells and the supernatant fraction was processed in an identical manner to that described in the previous procedure. Again all procedures were conducted at 4° C or on ice.

*Assay of marker enzymes*

Alkaline phosphatase, 5′-nucleotidase, succinate dehydrogenase and *N*-acetyl glucosaminidase were assayed as described previously [16]. NADPH-cytochrome *c* reductase was assayed as described by Scottocasa et al. [17], except that reaction volumes were 1 ml instead of 3 ml. Glucose-6-phosphatase was assayed as described by Hubscher and West [18] with inhibition of alkaline and acid phosphatase activities by 5 mM EDTA and 2 mM KF respectively. Liberated phosphate was determined by the method of Ames [19]. Cytochrome $P_{450}$ was measured as described by Omura and Sato [20] at room temperature in a Perkin Elmer 356 two wavelength double beam spectrophotometer.

*Other analytical procedures*

Protein was determined by the Folin procedure [21]. RNA was determined by the method of Fleck and Munro [22]. Neutral and amino sugars were assayed by the method of Porter [23]. Membrane fractions were dialysed for 48 h at 2° C against three changes of distilled water to remove sucrose. Acetylated sugars were analyzed by G-LC on a Perkin Elmer model 5750 gas-liquid chromatograph fitted with a 6 ft × 2 mm 3% ECNNS-M column. Analyses were performed isothermally at 170° C, with a 40 ml/min Helium gas flow. *myo*-Inostol was used as an internal standard. Sialic acid was assayed by the method of Warren [24] using a micro determination procedure [25].

Lipid extraction was performed by a modified Bligh and Dyer procedure [26], as described by Kates [27] for cell fractions. Lipid extracts in chloroform were assayed for fatty acids and sterols using the following scheme.

A suitable aliquot was evaporated to dryness under nitrogen and 1 ml of distilled water and 1 ml of 15% KOH in methanol added. Saponification was achieved by incubation at 70° C for 1 h under reflux. The solution was evaporated to a small volume (approx. 0.3 ml) and 1 ml of distilled water added. Non-saponifiable material was removed by extraction three times with *n*-pentane. Following acidification of the sample with 0.25 ml of 24 N $H_2SO_4$ the saponifiable fraction was removed by pentane extraction.

A sterol fraction was obtained from the non-saponifiable material by digitonin precipitation [28]. Digitonides were acetylated with acetic anhydride at 140° C for 30 min. Samples were analyzed directly by gas-liquid chromatography on both 3% OV17 (isothermally at 260° C, 40 ml/min Helium gas flow) and 3% SE 30 (isothermally at 245° C, 80 ml/min Helium gas flow). Cholesterol was employed as an internal standard.

The saponifiable fraction was evaporated to dryness and fatty acids methylated by addition of 1 ml of $BF_3$ in methanol and incubation at 37° C overnight. Samples were analyzed by gas-liquid chromatography on a 12% diethylene glycol succinate column at 150° C. The component methylated fatty acids were identified by comparison with the retention times of authentic standards, and by comparison with the previously published fatty acid composition of *D. discoideum* [29].

Lipid phosphorous in chloroform extracts was determined as described by Ames [19]. The amount of phospholipid was calculated assuming an average molecular weight of 730 for the phospholipid of *D. discoideum* [29,30].

*Electron microscopy*

Pellets of cell fractions obtained by centrifugation were fixed in 3% gluteraldehyde in 0.1 M sodium phosphate buffer (pH 7.0) and post-fixed in 1% $OsO_4$, 0.1 M sodium phosphate buffer (pH 7.0). The fixed pellets were washed in buffer and dehydrated in ethanol. Final dehydration and removal of ethanol was accomplished by propylene oxide infiltration. Pellets were embedded in Araldite 502/Epon 812 and thin sections cut with an LKB Ultrotome. Sections were stained with 1% lead citrate and 1% uranyl acetate and examined with a Phillips 300 electron microscope.

## Results

*Plasma membrane purification*

The previously described methods [10—12] for the purification of plasma membranes of *D. discoideum* were attempted during the course of this study but were found to be unsuitable for the particular needs of this laboratory.

The procedure described briefly by Riedel and Gerisch [10] employs digitonin to stabilize surface membranes which produce ghosts on cell lysis. However, we found that inclusion of digitonin in homogenization buffer leads to considerable solubilization of the lysosomal marker enzyme *N*-acetyl glucosaminidase [31] indicating disruption of the lysosomal vesicles (data not shown). Since the released lysosomal enzymes may degrade membrane molecular constituents, this procedure was abandoned.

Only limited success was obtained using the purification method described by Green and Newell [11]. Plasma membranes were detected by assaying the valid marker enzymes alkaline phosphatase [11,16] and 5′-nucleotidase [12,13, 16]. Succinate dehydrogenase and NADPH-cytochrome *c* reductase, employed by Green and Newell [11] as enzyme markers for mitochondria and endoplasmic reticulum respectively, were also assayed in the present study. The sucrose density gradient data shown in Fig. 2(a) is representative of several attempts at plasma membrane purification. Only a small portion of the alkaline phosphatase and 5′-nucleotidase activites was separated from the endoplasmic reticulum and mitochondrial markers on sucrose density gradients. Most of the plasma membrane material sedimented towards the bottom of the gradients along with the succinate dehydrogenase and NADPH-cytochrome *c* reductase. Furthermore, the purified plasma membrane fraction was considerably contaminated with RNA, an average preparation containing 200 μg RNA/mg protein. Since

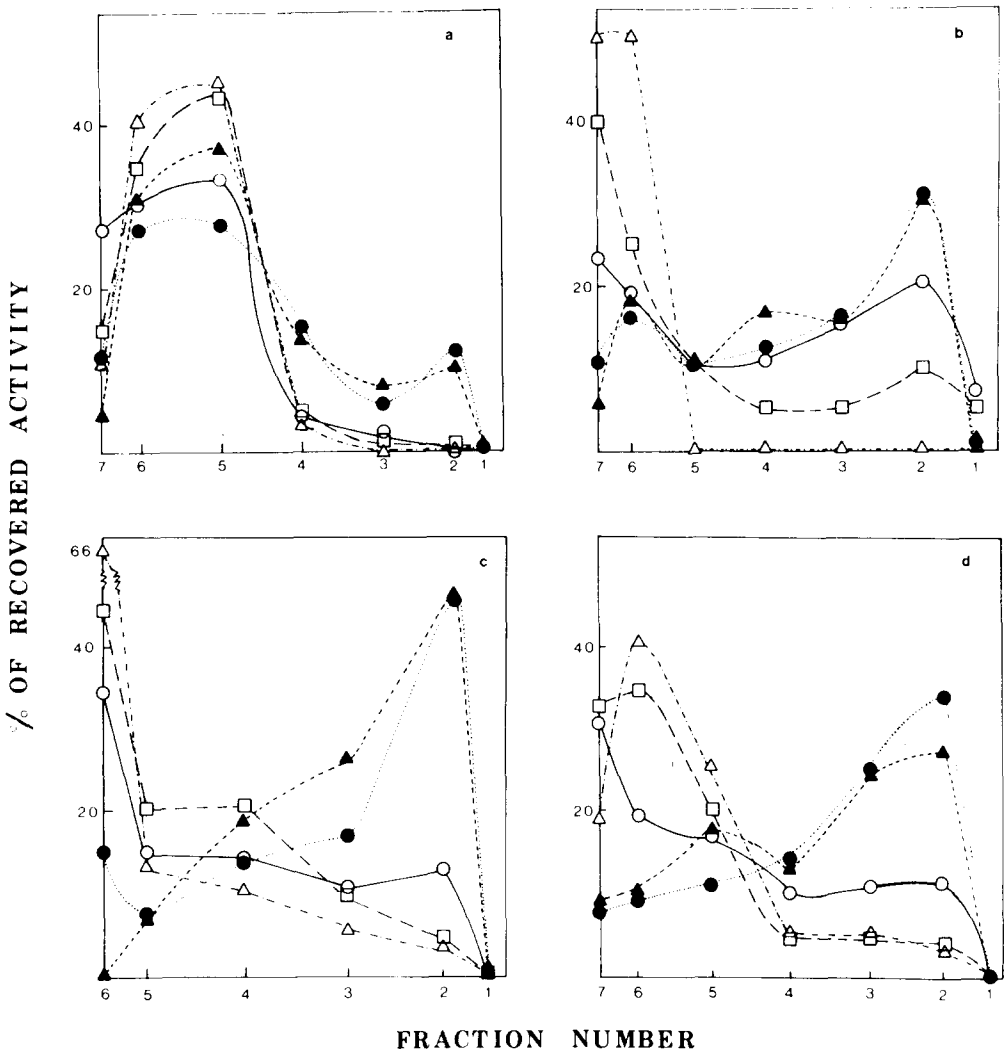


Fig. 2. Comparison of the different procedures for the purification of plasma membranes of *D. discoideum*. (a) Cell breakage, crude membrane preparation and sucrose density gradient were exactly as described by Green and Newell [11]. (b) Cell breakage, crude membrane preparation and sucrose density gradient were exactly as described by Cutler and Rossomando [12]. (c) Crude membrane preparation and sucrose gradient were as described under Materials and Methods, following cell breakage by the filter screen method. (d) Crude membrane preparation and sucrose gradient were as described under Materials and Methods, following cell breakage by vigorous stirring with glass beads. Following centrifugation, the gradients were separated into the indicated fractions. Each fraction was diluted to approx. 10% sucrose with water and the membranous material was pelleted by centrifugation at 30 000 rev./min for 30 min. The membrane pellets were resuspended in sucrose/Tris buffer and assayed for protein, (○- - - - -○); succinic dehydrogenase, (△- . - . - .-△); NADPH-cytochrome *c* reductase, (■- - - - -■); alkaline phosphatase, (●. . . . . ●); and 5′-nucleotidase, (▲- - - - -▲), as described under Materials and Methods. The amount of the components in each fraction is expressed as the percentage of the total material recovered from the gradient.

the plasma membrane yield was low and the material was heavily contaminated with RNA, attempts at further refining the procedure were abandoned.

The plasma membrane purification method described by Rossomando and

Cutler [12] was also attempted. Considerable amounts of alkaline phosphatase and 5′-nucleotidase were well separated from the bulk of the NADPH-cytochrome *c* reductase and succinate dehydrogenase on sucrose density gradients (Fig. 2b). Furthermore, the RNA contamination was considerably lower (≃ 50 μg RNA/mg protein) than that found using the Green and Newell [11] procedure. However, less than 20% of the alkaline phosphatase activity was membrane bound (data not shown). Since this enzyme is normally tightly bound to the plasma membrane [11,16] it is apparent that at least one plasma membrane component is partially solubilized during lysis of the cells with the antibiotic Amphotericin B [12], and this may be indicative of a more generalized dissociation. Consequently, while this procedure is suitable for the enzymatic studies of Rossomando and Cutler [12] it is unsuitable for our proposed investigation on the molecular composition of the plasma membrane. Accordingly, we proceeded no further with this method of purification.

In view of the unsatisfactory results obtained above, alternative preparative techniques were sought. Cell breakage by aspiration of cell suspensions through a finely perforated filter support screen [15] was therefore investigated. Crude membrane fractions were applied to a discontinuous sucrose gradient, similar to that previously described [12], and following centrifugation to equilibrium, two distinct membrane bands were observed near the top of the gradients (Fig. 1). Analysis of fractions taken from the gradient revealed that these two upper membrane bands (fractions 2 and 3 of Fig. 2(c)) contained large amounts of alkaline phosphatase and 5′-nucleotidase and only small amounts of NADPH-cytochrome *c* reductase and succinate dehydrogenase. These fractions were designated PM1 (upper band) and PM2 (lower band).

Both PM1 and PM2 fractions contained very low RNA contamination (≃ 20 μg RNA/mg protein). However, despite the excellent separation on the gradients the method has disadvantages for the purification of *D. discoideum* plasma membranes. Cell breakage is low (≃60%), and a large proportion of the potential plasma membrane yield is lost, (Table I). Even more serious, however, was the finding that considerable amounts of alkaline phosphatase did not sediment with the crude membrane pellet (Table I).

An alternative cell breakage method utilizing glass beads was therefore attempted. This method yielded higher cell breakage (≃80—85%) and also did not solubilize either alkaline phosphatase or 5′-nucleotidase from the membrane (Table II). Furthermore, sucrose gradients resolved these two enzymes from the NADPH-cytochrome *c* reductase and succinate dehydrogenase activities (Fig. 2(d)). Two distinct plasma membrane bands were again observed (PM1 and PM2) (Fig. 1).

*Purity and composition of the plasma membrane fractions*

The complete results of a representative purification procedure from the cells broken with glass beads are shown in Table II. Fractions PM1 and PM2 contain only small amounts of NADPH-cytochrome *c* reductase and succinate dehydrogenase activities, suggesting only low levels of contamination by endoplasmic reticulum and mitochondria (Table II). In this respect, PM1 is less contaminated than PM2. In this particular preparation (Table II) the alkaline phosphatase activity was enriched 18-fold in PM1 and 16-fold in PM2 compared

TABLE I

DISTRIBUTION OF ENZYME MARKERS FOLLOWING CELL BREAKAGE THROUGH FILTER SCREEN

The homogenate represents the total broken and unbroken cell population. The low speed pellet was obtained by centrifugation at 700 × *g* for 10 min and the high speed pellet and supernatants were obtained by centrifugation at 30 000 rev./min for 30 min as described under Materials and Methods. n.d., not detectable.

| Fraction | Protein | Alkaline phosphatase | | 5′-Nucleotidase | | Succinate dehydrogenase | | NADPH-cytochrome *c* reductase | |
|---|---|---|---|---|---|---|---|---|---|
| | (% yield) [a] | (spec. act.) [b] | (% yield) | (spec. act.) | (% yield) | (spec. act.) | (% yield) | (spec. act.) | (% yield) |
| Homogenate | 100.0 | 3.52 | 100.0 | 0.17 | 100.0 | 12.9 | 100.0 | 17.3 | 100.0 |
| Low speed pellet | 56.8 | 4.46 | 72.0 | 2.28 | 91.9 | 11.2 | 49.1 | 15.2 | 49.9 |
| Supernatant (i) | 28.2 | 3.54 | 28.4 | 0.05 | 3.7 | n.d. | — | 10.0 | 16.4 |
| Supernatant (ii) | 6.1 | 3.85 | 6.7 | n.d. | — | n.d. | — | 10.9 | 3.8 |
| High speed pellet | 5.5 | 5.99 | 9.3 | 0.89 | 28.4 | 17.7 | 7.5 | 43.8 | 13.9 |

[a] % yields are determined with respect to the homogenate.
[b] specific activities are given as nmol/min per mg protein.

TABLE II

DISTRIBUTION OF ENZYME MARKERS FOLLOWING CELL BREAKAGE WITH GLASS BEADS

The homogenate is the totel broken and unbroken cell population. The low speed pellet was obtained by centrifugation at 700 × *g* for 10 min and the high speed pellet and supernatants were obtained by centrifugation at 30 000 rev./min for 30 min as described under Materials and Methods. The gradient fractions were as shown in Fig. 1 and described under Materials and Methods.

| Fraction | Protein | Alkaline phosphatase | | 5′-Nucleotidase | | Succinate dehydrogenase | | NADPH-cytochrome *c* reductase | |
|---|---|---|---|---|---|---|---|---|---|
| | (% yield) [a] | (spec. act.) [b] | (% yield) | (spec. act.) | (% yield) | (spec. act.) | (% yield) | (spec. act.) | (% yield) |
| Homogenate | 100.0 | 1.22 | 100.0 | 0.33 | 100.0 | 4.39 | 100.0 | 4.85 | 100.0 |
| Low speed pellet | 17.0 | 1.30 | 17.3 | 0.45 | 25.7 | 3.53 | 13.6 | 11.25 | 39.2 |
| Supernatant (i) | 37.4 | 0.17 | 3.1 | 0.02 | 1.6 | n.d. | — | 2.17 | 10.2 |
| Supernatant (ii) | 3.7 | n.d. | — | n.d. | — | n.d. | — | 4.67 | 5.1 |
| High speed pellet | 11.7 | 3.44 | 42.8 | 0.61 | 28.4 | 6.89 | 23.9 | 10.87 | 34.1 |
| Gradient fraction | | | | | | | | | |
| 1 | — | n.d. | — | n.d. | — | n.d. | — | n.d. | — |
| 2 (PM1) | 0.2 | 22.15 | 3.74 | 8.5 | 5.35 | n.d. | — | 3.8 | 0.16 |
| 3 (PM2) | 0.2 | 19.81 | 3.01 | 7.7 | 4.35 | n.d. | — | 7.7 | 0.41 |
| 4 | 0.3 | 8.69 | 2.14 | 3.9 | 3.58 | n.d. | — | 7.6 | 0.48 |
| 5 | 0.5 | 5.30 | 2.18 | 2.2 | 3.30 | 3.5 | 0.41 | 11.1 | 1.16 |
| 6 | 2.7 | 1.24 | 2.79 | 0.2 | 1.82 | 5.7 | 3.55 | 15.9 | 9.00 |

[a] % yields are determined with respect to the homogenates.
[b] specific activities are given as nmol/min per mg protein.

with the crude homogenate. However, it should be noted that there is a considerable reduction in alkaline phosphatase activity following sucrose density centrifugation. This decreased activity is not due to removal of the enzyme from the membranes during the procedure and the 18-fold purification is therefore a minimal estimate of enrichment. The second plasma membrane marker enzyme, 5′-nucleotidase was enriched 26-fold in PM1 and 23-fold in PM2, relative to crude homogenates, in the preparation described in Table II. Again there is some inactivation of 5′-nucleotidase during the purification, but the inactivation is less pronounced than for the alkaline phosphatase, which probably accounts for the higher enrichment of this enzyme. The inactivation of alkaline phosphatase and 5′-nucleotidase is due in part to the inclusion of 0.1 ml EDTA in the sucrose density gradients. Omission of EDTA in the preparative procedure resulted in PM1 and PM2 fractions in which the plasma membrane enzymes were enriched 40—50-fold and yields of these enzymes were 20—25% (data not shown). However, the contaminating levels of NADPH-cytochrome *c* reductase and succinate dehydrogenase were increased under these conditions and therefore EDTA was routinely added. It is difficult to quantitate the contamination in our plasma membrane preparations. Succinate dehydrogenase activity of *D. discoideum* has been shown to be present in fractions that contain mitochondria [11,32] but there has been no proof that NADPH-cytochrome *c* reductase is a valid marker for the endoplasmic reticulum of this organism. In fact, Green and Newell [11] observed only a slight separation of succinate dehydrogenase and NADPH-cytochrome *c* reductase. Furthermore, in the gradients presented here, (Fig. 2, Table II) and in data presented previously [16] very little separation of succinate dehydrogenase and NADPH-cytochrome *c* reductase was observed. When differential centrifugation was employed over 95% of the succinic dehydrogenase and 80% of the NADPH-cytochrome *c* reductase were pelleted by centrifugation of crude homogenates at 8000 × *g* for 10 min (data not shown), suggesting that the majority of the NADPH-cytochrome *c* reductase might be mitochondrial. NADPH-cytochrome *c* reductase may therefore be a poor marker for the endoplasmic reticulum in *D. discoideum*. Glucose-6-phosphatase is also of little use in this context, since we have confirmed the observation of Green and Newell [11] that the majority of this enzyme does not sediment with the membranous material (data not shown). In view of the problems associated with the assessment of endoplasmic reticulum contamination, we analyzed the various fractions for cytochrome $P_{450}$ content. None of the fractions showed any indication of absorption at 450 nm in CO vs. reduced spectra (data not shown). Thus at present it is impossible to quantify the amount of endoplasmic reticulum contamination in our plasma membranes. It should be noted that the amount of RNA in the PM1 fraction was approximately 100 μg/ml. Assuming that this RNA is due to ribosomal contamination, the ribosomal protein contamination of the plasma membranes would be approximately 5%.

The plasma membrane fraction PM1 appeared as a relatively homogeneous population of membrane vesicles in the electron microscope (Fig. 3). The appearance of PM2 vesicles is identical (data not shown). The overall molecular composition of the plasma membrane fraction PM1 is shown in Table III. Considerable amounts of phospholipid, sterol and relatively high levels of carbo-

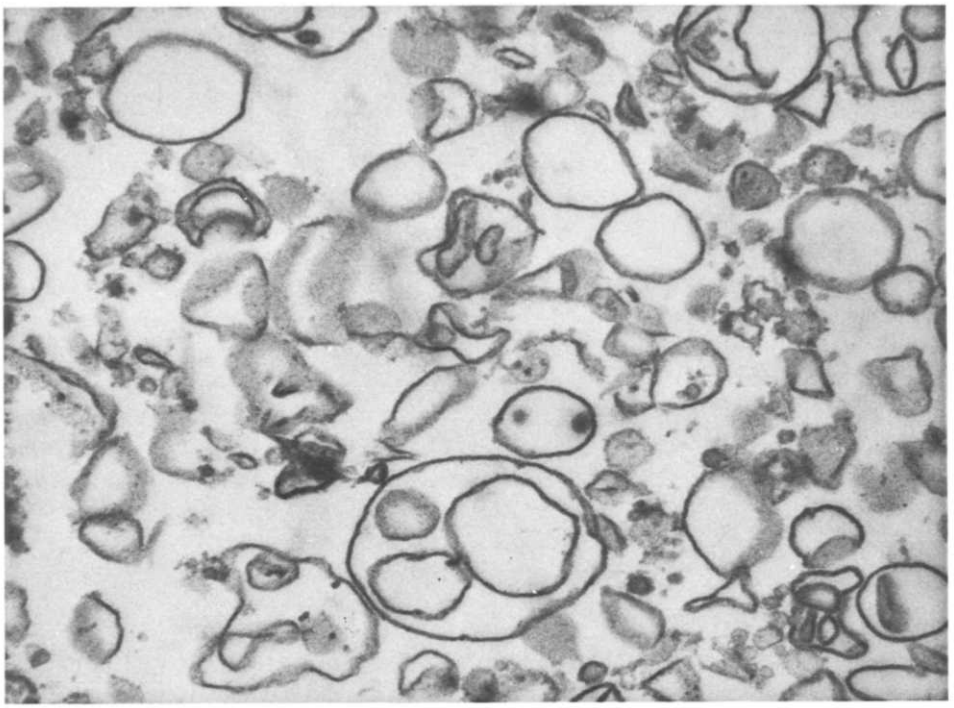

Fig. 3. Electron micrograph of the plasma membrane of *D. discoideum*, fraction PM1. Magnification × 40 800.

hydrate were detected. Gas chromatography of the sterol fraction on two different columns, revealed the presence of three components, one of which accounted for approx. 88% of the total sterol as determined by peak area. This major sterol constituent had retention times on the two columns that suggested its identity was $\Delta^{22}$-stigmasten-3$\beta$-ol [33], shown previously to be the major sterol in *D. discoideum* [34,35]. One of the minor components (approx. 5% of

TABLE III

COMPOSITION OF WHOLE CELL HOMOGENATE AND PLASMA FRACTION PM1

Values are given as mean ± S.D. n.d., not detectable.

| | Homogenate | PM1 |
|---|---|---|
| RNA (μg/mg protein) | 84.52 ± 14.08 | 115.95 ± 33.70 |
| Phospholipid (μg/mg protein) | 70.50 ± 17.76 | 431.41 ± 32.31 |
| Total sterol (μg/mg protein) | 13.60 ± 2.01 | 99.92 ± 11.38 |
| Sterol:phospholipid (mol/mol) | 0.342 | 0.410 |
| Total neutral and amino sugar (nmol/mg protein), excluding glucose and ribose | 124.50 ± 18.88 | 164.70 ± 21.83 |
| Ribose | 18.90 ± 4.33 | 9.49 ± 3.40 |
| Fucose | 15.77 ± 3.26 | 36.53 ± 6.33 |
| Glucosamine | 70.25 ± 15.18 | 74.21 ± 13.20 |
| Mannose | 38.48 ± 5.60 | 53.98 ± 11.70 |
| Galactose | <1.00 | <1.00 |
| Sialic acid | n.d. | n.d. |
| Glucose | 118.18 ± 43.12 | 47.36 ± 13.34 |

TABLE IV

FATTY ACID COMPOSITIONS OF WHOLE CELL HOMOGENATE AND PLASMA MEMBRANE FRACTION PM1

Values are the means of five determinations.

| | Homogenate | PM1 |
|---|---|---|
| Unknown | 2.43 | 4.99 |
| 14 : 0 | 1.02 | 1.17 |
| Palmitaldehyde | 4.83 | 5.12 |
| 16 : 0 | 7.11 | 8.42 |
| 16 : 1 $\Delta^9$ | 3.52 | 2.23 |
| 16 : 2 $\Delta^{5,9}$, 17 : 0 * | 2.36 | 0.96 |
| 18 : 0 | 1.67 | 0.99 |
| 18 : 1 $\Delta^9$ and 18 : 1 $\Delta^{11}$ * | 32.87 | 31.67 |
| 18 : 2 $\Delta^{5,9}$ and 18 : 2 $\Delta^{5,11}$ * | 41.87 | 41.25 |
| Others ** | 2.33 | 3.20 |

* These fatty acids are not separated under our conditions but were shown to be present by Davidoff and Korn [29].

** Several unidentified minor components.

the total sterol) had a retention time that was consistent with its identity being stigmastanol [33], which has been shown to be a minor sterol constituent in *D. discoideum* [35]. The remaining sterol component has yet to be identified.

The carbohydrate composition of PM1 indicated that the principal sugars were fucose, glucosamine, mannose and glucose. No sialic acid and only barely detectable levels of galactose were observed. Variability of the amount of glucose found in replicate determinations suggests that the presence of this component may be due to contamination. This may result from a failure to remove sucrose despite extensive dialysis following gradient centrifugation or be due to a small contamination of glycogen. Small amounts of ribose were also detected presumably arising from the RNA contamination. The current analysis did not determine the degree of *N*-acetylation of the amino sugar. Previous work has shown that at least part of the glucosamine in cell surface glycoprotein and glycolipid antigens from *D. discoideum* is present in the *N*-acetylated form [36]. Mannose and fucose are also components of these antigens [36]. The fatty acid composition of the PM1 fraction is similar to the fatty acid composition of whole cell homogenates (Table IV).

## Discussion

At present, membrane preparation procedures rely largely on monitoring the enrichment of various marker molecules thought to be associated with specific subcellular organelles. In order for purification data to be meaningful, criteria for selection of ideal organelle markers must be stringent and are not easily satisfied in practice. Ideally, a marker should be specifically related to a particular organelle, having properties which cannot be confused with similar molecular species present in the system. Moreover, it should remain stable and unaffected by manipulation during the purification procedure and be readily accessible to quantitative analysis. In mammalian systems, extensive investigations

by numerous workers have established a number of marker enzymes that satisfy the above mentioned conditions. There is far less accumulated information on the subcellular biochemistry of *D. discoideum*. While mammalian studies serve as a useful guide in the selection of organelle markers, their extrapolation to simpler eucaryotic organisms should be treated with caution.

In their report of plasma membrane purification in *D. discoideum*, Green and Newell [11] employed alkaline phosphatase as a marker for the plasma membrane. The validity of this selection is supported by coincident distribution of the enzyme with $^{125}I$ covalently bound only to components exposed at the cell surface [11,16]. Green and Newell [11] found that 5′-nucleotidase was predominantly solubilized by their methods of cell breakage. In contrast we have found that 5′-nucleotidase is tightly membrane bound (Tables I and II) and can be considered an excellent marker for *D. discoideum* plasma membranes [16]. In only one instance, when cells were inadvertently grown into stationary phase, did we find that 5′-nucleotidase was appreciably removed from the membranous fraction.

Analysis of the enrichment of plasma membrane marker enzymes can lead to problems in the assessment of plasma membrane purity. Since the proportion of whole cell protein that is localized in the plasma is unknown there is no known maximum enrichment value to be attained. Furthermore, partial plasma membrane dissociation without the loss of the marker enzyme will result in an over-estimate of the enrichment. An over-estimate will also result from the activation of the plasma membrane marker enzyme during the course of the purification procedure. Similarly, the inactivation of a plasma membrane marker enzyme during purification will lead to an under-estimate of the enrichment; this has occurred for alkaline phosphatase and 5′-nucleotidase in many of our preparations. It is difficult, therefore, to directly compare our procedure with that of previous workers [10—12].

We have subjected our PM1 fraction to a second sucrose density gradient and have obtained an increased enrichment of alkaline phosphatase and 5′-nucleotidase. Furthermore, the specific activity of the NADPH-cytochrome *c* reductase was reduced. However, there was a sizeable decrease in the yield of total protein, alkaline phosphatase and 5′-nucleotidase suggesting a possible dissociation of the plasma membrane during the second sucrose density centrifugation. This possibility is being further studied.

There is every indication that our purified plasma membrane preparations are at least equal in quality to those obtained by previously published procedures and, in our hands, are obtained in superior yields. Furthermore, the membranes obtained by Green and Newell [11] contained very little of the total cellular 5′-nucleotidase, while membranes prepared by the procedure of Rossomando and Cutler [12] are deficient in alkaline phosphatase. Since, for our research program, an undissociated plasma preparation is highly desirable, the previously described plasma membrane preparations [11,12] are unacceptable.

It should be reiterated, however, that NADPH-cytochrome *c* reductase may not be a good marker for endoplasmic reticulum in *D. discoideum*. Green and Newell provided no positive evidence for their assumption [11], and we obtain only marginal separation of the NADPH-cytochrome *c* reductase and the succinate dehydrogenase under a variety of equilibrium sedimentation conditions

[16] (Fig. 2). Furthermore, only a small proportion of the NADPH-cytochrome *c* reductase sediments with the properties of classical microsomes; the majority sediments with the succinate dehydrogenase. Thus a considerable portion of the NADPH-cytochrome *c* reductase may be of mitochondrial origin in *D. discoideum*. Rotenone sensitivity cannot be used to distinguish between the possible mitochondrial and microsomal components because the *D. discoideum* mitochondrial NADPH-cytochrome *c* reductase is insensitive to rotenone (data not shown), a situation similar to that observed in yeast [37].

Glucose-6-phosphatase has been used as a marker for endoplasmic reticulum contamination in a number of mammalian cells [38]. However, Green and Newell [11] showed that this enzyme is not membrane bound in *D. discoideum* and we have confirmed this observation. We have also attempted to measure cytochrome $P_{450}$, another potential endoplasmic reticulum marker [20], but find no characteristic absorption peak at 450 nm in any of our sub-cellular fractions. Consequently, we do not feel that investigations to date have adequately demonstrated the absence of contamination by endoplasmic reticulum in plasma membrane preparations obtained from *D. discoideum*. Until an adequate marker is found for this organelle the degree of contamination of such preparations continues to remain unknown.

While the activities of the plasma membrane marker enzymes were consistently found to be concentrated in the two membrane fractions equilibrated near the top of the gradients, some activity was always observed at higher sucrose concentrations. On the basis of this data, the occurrence of these enzymes on structures other than those derived from the plasma membrane cannot be excluded. Electron microscopy of the gradient pellet material, however, has revealed the presence of structures that resemble large plasma membrane vesicles, which may account for the marker activity. These plasma membrane vesicles do not seem to be associated physically with any of the organelles in this fraction, and we can offer no explanation as to why these vesicles sediment to such high density. The relative proportion of the high density plasma membrane markers does vary slightly from preparation to preparation (cf. Table II and Fig. 2(d)). Likewise some NADPH-cytochrome *c* reductase activity is always associated with PM1 and PM2. This may be a true contamination, or may reflect, at least in part, genuine plasma membrane associated activity. For example, it has been suggested that physical connections between the plasma membrane and the endoplasmic reticulum are a real aspect of membrane organization in vivo [39].

We consistently observe two plasma membrane fractions (designated PM1 and PM2). These two fractions appear as identical, relatively homogeneous vesicles in the electron microscope. Analysis of the two fractions reveals slight differences in phospholipid, sterol and carbohydrate content (data not shown). Furthermore, the specific activities of the 5′-nucleotidase and alkaline phosphatase are always higher for PM1 and PM2, whereas the levels of contaminants are always slightly higher for PM2. Thus PM2 may merely represent a more contaminated version of PM1. Alternatively the two fractions may represent areas of surface specialization. Green and Newell [11] achieved resolution of their plasma membranes into three distinct subfractions on a second sucrose density gradient and suggested that structural and functional specialization of different

areas of the plasma membrane might account for the phenomenon. However, on the basis of the available data, the nature of the distinction between the various plasma membrane fractions remains obscure.

In the purification of *D. discoideum* plasma membranes, the method of cell breakage is probably the most critical factor, since the pure plasma membranes have low buoyant density and are well separated from the majority of other subcellular organelles. Cell breakage by certain procedures probably leads to considerable artificial association of plasma membranes to other sub-cellular organelles and prevents their separation. Furthermore, some methods of breakage, such as the previously described Potter homogenization [16] lead to considerable quantities of RNA being associated with the plasma membranes, increasing their density and preventing their separation from other cell organelles. The critical nature of the cell breakage technique employed, probably explains our inability to reproduce Green and Newell's results [11]. Until homogenization techniques become more standardized there will be inevitable variation from one laboratory to the next.

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