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THE INVOLVEMENT O ` THE PLASMA MEMBRANE IN THE DEVELOPMENT OF *DICTYOSTELIUM DISCOIDEUM*

I. PURIFICATION OF THE PLASMA MEMBRANE

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Summary

A method for the isolation and purification of plasma membranes of *Dictyostelium discoideum* by equilibrium centrifugation on sucrose followed by Renografin continuous density gradients has been developed and monitored both with electron microscopy and a number of enzyme assays. On the basis of electron microscopy, the final plasma membrane fractions are judged to be free of nuclei, rough endoplasmic reticulum, lysosomes and peroxisomes. Some profiles of the mitochondrial inner membranes are found within the plasma membrane fractions, but this contamination has been estimated to be only 5%. On the basis on enzyme assays, the plasma membrane fractions contain all the 5'-nucleotidase activity in the final gradients and are free of catalase, acid phosphatase and malate dehydrogenase activity (markers for peroxisomes, lysosomes, soluble enzymes and the matrix of mitochondria). Their content of glucose-6-phosphatase is reduced by more than 70%. The large majority of RNA and DNA have been removed from the preparation.

Introduction

Specific sites on the plasma membrane are implicated in number of steps in the development of *Dictyostelium discoideum*. These include movement of the amoebae [1], sensation of chemotactic stimuli [2—5], cellular adhesion during aggregation [6,7] and cellular interactions regulating the biochemical differentiation of pseudoplasmodial cells [8—11]. To identify the components of the cell surface which participate in these events, we have developed a technique

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for isolating the plasma membrane under conditions which are gentle and avoiding extremes of ionic strength or pH or the removal of divalent cations from the isolation medium. We report here a method of isolating plasma membranes from *D. discoideum* amoebae during the log phase of growth which we have examined for purity both with electron microscopy and assays for enzyme markers for various cellular organelles. While we have confined our efforts primarily to the plasma membrane of log phase cells, we also provide some evidence that the isolation method is suitable for cells during aggregation and preculmination stages of development.

Materials and Methods

Culture of cells. Cells of *D. discoideum*, strain A3, were grown in HL-5 or 1.6X HL-5 medium at 22°C on rotary shakers (100 rev./min) and harvested at a concentration of $5 \cdot 10^6$—$1 \cdot 10^7$ per ml. Cells were either used immediately after harvest (as described below) for the preparation of membranes or were allowed to develop [10,12] and membranes were then prepared from the cells.

Chemicals. Biochemicals were the products of Worthington Biochemical Co., Sigma-Aldrich, or Calbiochem. Renografin-76 was the product of E.R. Squibb, Inc. Diphenylamine was recrystallized from boiling hexane, and orcinol recrystallized from boiling benzene or toluene before use. Glutaraldehyde was obtained from Traub Laboratories and diaminobenzidine from Sigma.

Biochemical assays. Glucose-6-phosphatase, adenosine-5′-monophosphatase (5′-nucleotidase) and thiamine pyrophosphatase were assayed by the method of Morre [13], malate dehydrogenase by the method of Ochoa [14], and catalase by the method of Baudhuin et al. [15]. Protein was measured by the Lowry et al. [16] method.

To measure the activity of acid phosphatase, 0.2 ml of enzyme suspension was added to 1 ml of substrate solution (5.5 M *p*-nitrophenylphosphate in 50 mM citrate buffer, pH 4.8) and after incubating the assay mixture for 30 min at 37°C, 4 ml of 0.1 M NaOH was added to terminate the reaction. The absorbance of the solution was measured at 400 nm and the absolute activity of the enzyme was calculated, using a standard of pure *p*-nitrophenol.

Cytochromes were measured by absorption spectroscopy of highly scattering suspensions, at the temperature of liquid N_2, by the method of Butler [17]. When the content of nucleic acids were measured, the plasma membranes or unfractionated cells were precipited and washed twice in 10% trichloroacetic acid. DNA was measured by the diphenylamine and RNA by the orcinol method using the hydrolysate [18].

Solutions. Tris/magnesium buffer is 20 mM Tris · HCl (pH 8.0), 5 mM $MgCl_2$. Homogenization buffer (10 mM Tris · HCl, pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 394 mM sucrose) was saturated with phenylmethylsulfonyl fluoride, an inhibitor of serine proteases, before use. Citrate buffer is 10.9 mM sodium citrate (pH 6.9), 1.2 mM EDTA, 5 mM $MgCl_2$.

Linear sucrose gradients were made by mixing 18 ml of 0.5 M sucrose in Tris/magnesium buffer with 18 ml of 2 M sucrose in the same buffer [19]. Linear Renografin gradients were made by mxing 18 ml of citrate buffer with 18 ml of a solution composed of equal parts citrate buffer, containing 10 mM $MgCl_2$, and Renografin-76.

Electron microscopy. Intact cells were prepared for electron microscopy by fixation in situ in plastic petri dishes with a solution containing 6.35% glutaraldehyde, 2% OsO_4 and 1% phosphotungstic acid at pH 7 [20]. Subcellular fractions after being centrifuged into hard pellets were fixed in 2.5% glutaraldehyde in a 0.1 M sodium phosphate or sodium cacodylate buffer (pH 7.4) for 2 h at 4°C and then in a solution of 1% OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.4, for 1—4 h at 4°C. In most instances the pellets of the various subcellular fractions were obtained by diluting the collected material with two volumes of Tris/magnesium buffer followed by centrifugation at 48000 × *g* for 10 min. The samples were dehydrated in ethanol, and embedded in Epon [21]. This sections were made through several areas in the samples with a diamond knife on a Reichert Ultrotome. The thin sections were picked up on Parlodion-coated grids, stained with both a 1% aqueous uranyl acetate solution and lead citrate [22], and examined in a Phillips 301 electron microscope.

Histochemical localization of cytochrome c oxidase activity. A modification of the diaminobenzidine hydrochloride method for localization of cytochrome *c* oxidase developed by Seligman et al. [23] was used to examine the plasma membrane fractions. The method used was adapted from a technique used on cells cultured in vitro [24]. Membrane fractions from the Renografin gradients were collected, rinsed in Tris/magnesium buffer and then in a solution of 0.44 M sucrose, 0.05 M sodium cacodylate (pH 7.4) and $2 \cdot 10^{-5}$ M EDTA. The membranes were pelleted each time by centrifugation at 48200 × *g* for 10 min and then incubated for 1 h at room temperature in a solution containing 0.5 mg/ml diaminobenzidine, 20 μg/ml catalase, 1 mg/ml cytochrome *c*, 0.44 M sucrose, 0.05 M Tris · HCl (pH 7.4) and 20 μM EDTA. Controls included samples to which 10 mM KCN was added before incubation and samples in which the diaminobenzidine was omitted.

Preparation of plasma membranes. All operations were done at 0—4°C. Cells, harvested by centrifugation for 1 min at 1465 × *g*, were resuspended in homogenization buffer and recentrifuged. The washed pellet of cells was suspended in homogenization buffer to a concentration of $5 \cdot 10^8$ cells/ml. After 10 min these cells were homogenized in a Ten Broeck homogenizer (Kontes Glass, Inc.). Log or aggregation phase cells required approx. 70 strokes to be 90—95% broken whereas cells from pseudoplasmodia required approx. 200—300 strokes for equivalent breakage.

5 ml of homogenate was layered onto a sucrose gradient and six gradients were centrifuged for 18 h at 23000 rev./min in a Beckman SW27 rotor. The major turbid band, centered about one-third of the way from the bottom of the centrifuge tube, was removed by puncturing the side of the tube with a syringe about 2—3 mm above the bottom of the band. The suspension was diluted with two volumes of Tris/magnesium buffer and the particulate material was pelleted by centrifugation for 10 min at 48200 × *g*. The pelleted membranes were resuspended in 0.5—1 ml of a mixture of equal volumes of citrate buffer and Renografin-76 per 2 ml of disrupted cell suspension. At this stage it is important to disrupt clumps of membranes by homogenizing the material with several strokes of a Duall Homogenizer with a Teflon pestle (Kontes Glass). Otherwise, the mixed clumps of plasma membranes and contaminants may band together.

5 ml of resuspended membranes were placed in the bottom of a centrifuge tube and a linear Renografin gradient was layered on top of the membrane suspension. After 2 h the gradients were centrifuged in the SW27 rotor for 12 h at 23000 rev./min. The major turbid band located about one-half of the way up the gradient was removed as described above, diluted with two volumes of Tris/magnesium buffer centrifuged for 10 min at 48200 × *g*. The pellet from six gradients was resuspended in 10 ml of Tris/magnesium buffer and recentrifuged. 25—30 mg of membrane protein were recovered from $1.5 \cdot 10^{10}$ cells. This comprises 1—2% of the initial protein content of the homogenate.

Results

A typical log phase cell, fixed while attached to a plastic petri dish is shown in Fig. 1. A nucleus with prominent peripheral nucleoli in the center of the cell, numerous mitochondria, and a number of vesicular organelles are obvious. The endoplasmic reticulum is masked by the dense cytoplasm, filled with ribosomes. No Golgi apparatus is apparent. Some suggestion of oriented microfilaments can be discerned at the bottom 10% of the cell where it is attached to the substratum (see also ref. 1).

The preparation of the plasma membranes from these cells was monitored with enzyme assays and using the electron microscope during their purification. Assays of marker enzymes were performed on serial 1.5-ml fractions of the entire gradient to follow the distribution and recovery of enzymes during purification. The pellet material was combined with the bottom 1.5 ml fraction before assay. It was not feasible to examine entire gradients in this much detail using the electron microscope, so after identifying the major regions of interest in the gradients by assay of marker enzymes, appropriate regions were pooled and these fractions examined in the electron microscope.

Appropriate enzyme markers for cellular organelles were chosen on the basis of previous investigations. Adenosine-5′-monophosphatase (AMPase) was chosen as a marker of the plasma membrane [25]. In order to support the presence of 5′-nucleotidase on the cell surface, the activity of intact cells in the assay was compared to that of an equal number of cells disrupted by sonication. The intact cells exhibited 50% of the activity of the disrupted cells (previously unpublished results). Cutler and Rossomando [26], using cytochemical assays, have shown that this enzyme is found on the external face of the plasma membrane. Although Green and Newell [27] have suggested that AMPase is not associated with the plasma membrane of *D. discoideum*, our results and those of Lee et al. [28] and Cutler and Rossomando [26] conflict with this suggestion. The absence of this enzyme from the preparation of Green and Newell [27] may reflect some degradation of their membranes since both Lee et al. [28] and Rossomando and Cutler [29] have shown that treatment of *D. discoideum* membranes with phospholipase C releases this enzyme into the supernatant. Acid phosphatase is a lysosomal enzyme in *D. discoideum* [30,31]. Catalase is a marker of peroxisomes [15,32] in the related genus *Acanthamoeba* [33], and also appears to the membrane bound in *D. discoideum* [34]. Glucose-6-phosphatase was used as a marker for endoplasmic reticulum [13, 27], and malate dehydrogenase was a marker for soluble enzymes and for the

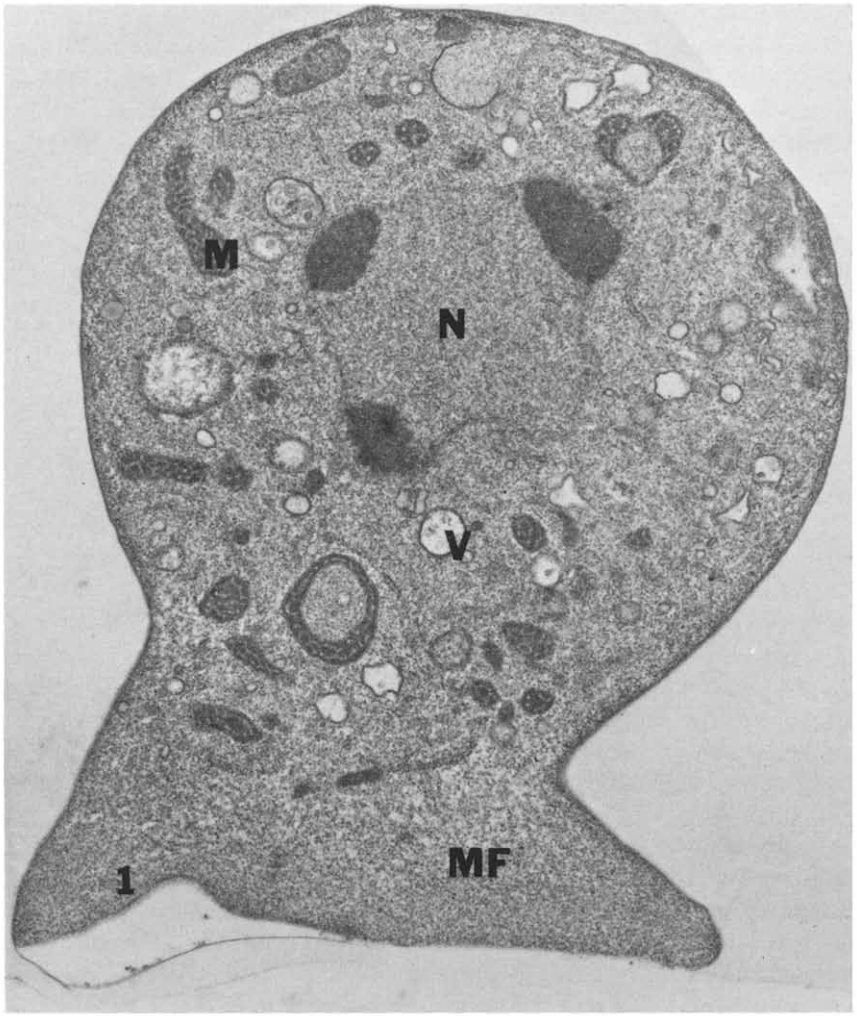

Fig. 1. An electron micrograph of a cell prepared for electron microscopy in situ on a plastic petri dish. The cells contain a nucleus (N) with prominent nucleoli adjacent to the nuclear membrane. The cytoplasm is filled with numerous mitochondria (M), other vesicular organelles (V), and many ribosomes. The basal portion of this cell has a thick subcortical microfilamentous layer (MF). Magnification, ×14 720.

matrix of the mitochondrion [14,35]. The suitability of the latter two enzymes was confirmed by the agreement between the results of biochemical and electron microscopic assays. No activity of thiamine pyrophosphatase, a marker for the Golgi apparatus [13] was detected in crude homogenates.

Fig. 2 summarizes the results of determinations of several parameters which were measured on a single sucrose gradient. Protein, as measured by the Lowry method, is distributed diffusely largely through the bottom half and upper third of the gradient. The major turbid region of the gradient, as measured using A_{400nm}, is localized in six fractions which are centered one-third of the way up the gradient. The enzyme, AMPase, the marker of the plasma membrane, was distributed in two peaks. One of these contained 17% of the total

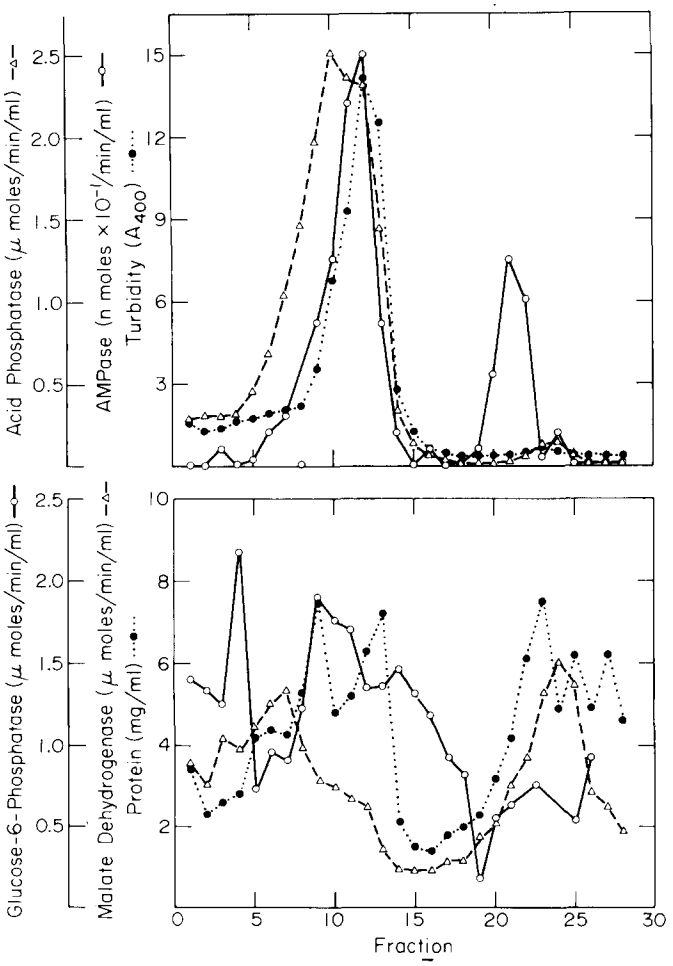

Fig. 2. Distribution of marker enzymes in a sucrose gradient. The cell homogenate was centrifuged in a sucrose gradient, as described in the text, and the turbidity, protein content and enzyme activity of 1.5-ml fractions collected from the entire gradient were determined. These determinations are divided among two panels for clarity. The gradient was centrifuged from right to left.

activity on the gradient and was found in the position expected for a soluble enzyme on the basis of sedimentation rate. Other methods of homogenizing *D. discoideum* have yielded an even greater fraction of this enzyme in the soluble fraction [28]. The remaining 83% of the activity was distributed in the crude plasma membrane (fractions 9—14). Malate dehydrogenase, a soluble enzyme of the cytoplasm and a marker for the matrix of the mitochondrion was also distributed primarily in two regions of the gradient. 39% was found in the position expected for a soluble enzyme whereas 47% was found in the lower half of the gradient. Approx. 13% of the total activity was distributed under the major turbid band. Although a small amount of glucose-6-phosphatase was found in the upper portion of the gradient, the bulk of this activity was broadly distributed in the lower half of the gradient. About one-third of the activity underlaid the major turbid band. The bulk of the acid phosphatase

activity was found in the turbid region of the gradient, less than 2% of the activity was detected in the region of the gradient which contains soluble proteins. Therefore, few of the lysosomes were disrupted during preparation. While the peak of acid phosphatase activity was displaced from the peak of turbidity and AMPase activity, 70% of the acid phosphatase activity was found under the major turbid band. In other experiments which are not presented here, approx. 70% of the catalase activity of the gradient was also found in the crude plasma membranes and in other experiments an enzymatic marker of the nucleus, α-amanitin-sensitive RNA polymerase, was found in the pellet (McMahon and Seed, previously unpublished results). Smart and Hynes [19] found that approx. 75% of the ^{125}I incorporated into intact cells with lactoperoxidase was in this band. These assays suggest that the major turbid band contains the cells' plasma membranes, that it is free of nuclei, but that it also contains about one-fourth of the mitochondria, up to one-half of the endoplasmic reticulum, and the majority of the lysosomes and peroxisomes.

Therefore, fractions from this gradient were examined by electron microscopy to determine whether the distribution of morphological components resembled that expected on the basis of the distribution of enzymatic activity. Fig. 3 presents a section of the material found in the crude homogenate. The single cell, which dominates this picture, whose plasma membrane is ripped on the periphery, demonstrates the appearance of cellular organelles when the cell has been disrupted. Especially noteworthy is the alteration in the appearance of the mitochondria, which become swollen. The microfilaments and the endoplasmic reticulum became somewhate more visible under the less compacted conditions of the disrupted cell.

Figs. 4 and 5 illustrate the composition of the crude plasma membrane fractions. In Fig. 4 the upper half of the band is seen to be composed almost entirely of membrane vesicles 0.5—1.0 μm in diameter. The bottom half of the band (Fig. 5) contains membrane vesicles and occasional sheets of membrane several microns in length. Occasional membrane profiles, 10 μm in length, were seen in this band. In agreement with the biochemical assays no nuclei are visible but occasional ribosomes and mitochondria are visible. The fractions below the turbid band (Figs. 6 and 7) are more enriched with mitochondria and rough endoplasmic reticulum. The pellet (Fig. 8) is almost entirely composed of rough endoplasmic reticulum (and ribosomes), mitochondria and nuclei. Thus the composition of various fractions as determined with the electron microscope agrees with that determined by enzymatic analysis.

We tried several techniques to reduce contamination of the plasma membranes with other membranous organelles, but only one was successful in removing much of the contaminating material while maintaining the activity of the marker enzymes. Fig. 9 shows the results of enzymatic assays of the partially purified plasma membrane preparation after flotation in a gradient of Renografin. During this centrifugation some of the 5′-nucleotidase activity is lost. We assume that this represents decay of the activity found only in the plasma membrane fraction since the enzyme is not found in any other fraction. The major turbid band, which contains the majority of protein, is free of both acid phosphatase and malate dehydrogenase. However, some of the glucose-6-phosphatase (29% of the activity on the gradient) remained in this band. The

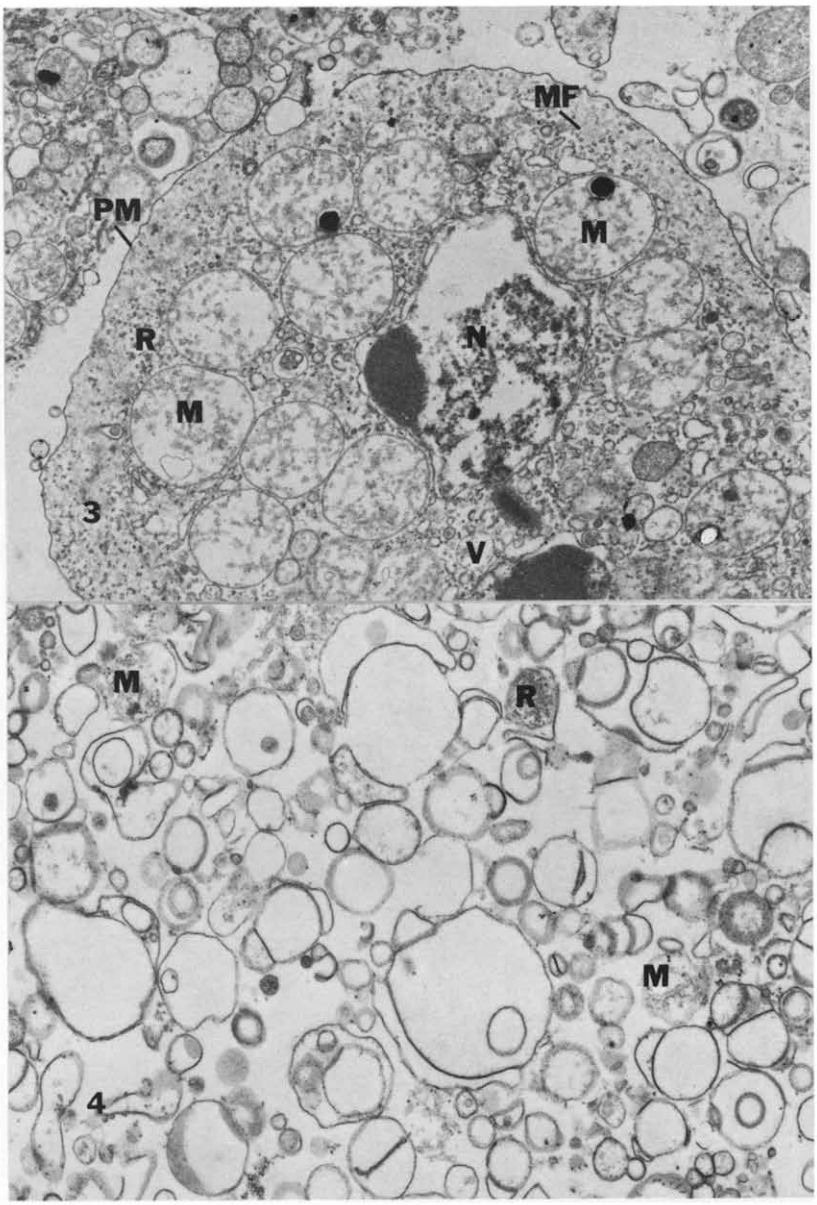

Fig. 3. An electron micrograph of a ruptured cell in the homogenate after disruption of the cells in the Ten Broeck homogenizer. The plasma membrane of this cell is ripped (upper center). All the cellular components, including nucleus (N), vesicular organelles (presumably including peroxisomes and lysosomes) (V), ribosomes (R), microfilaments (MF) and the plasma membrane (PM), are still recognizable. The mitochondria (M) are enlarged and dense material is present in many of these mitochondria. The subcortical microfilament system is more apparent after disruption of the cells. Magnification, ×16 500.

Fig. 4. An electron micrograph of the less dense half of the plasma membrane band in a sucrose gradient. This fraction contains large and small membrane vesicles (ranging to 1 μm in diameter), mitochondria (M) and a few ribosomes (R). This corresponds to fractions 12—15 in the sucrose gradient (Fig. 2). Magnification, ×22 500.

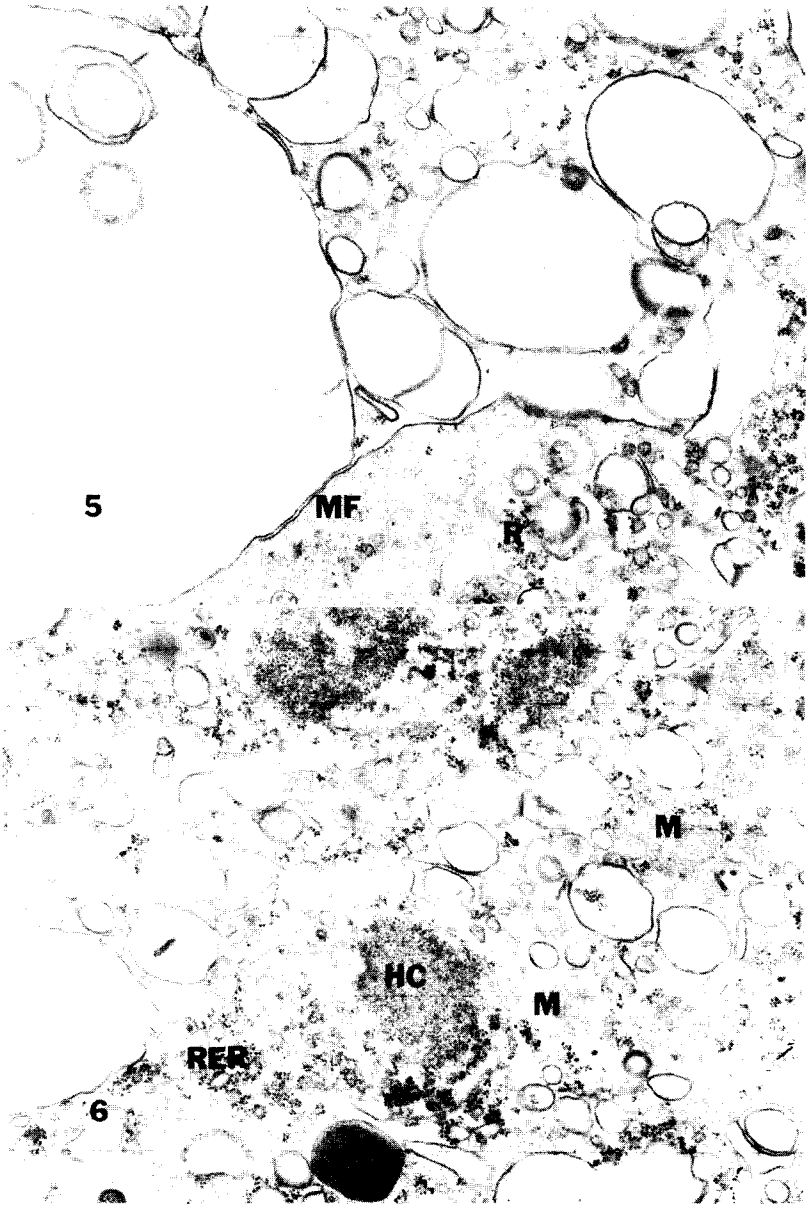

Fig. 5. The more dense fraction of the plasma membrane band contains long profiles of membrane and appears similar to the light fraction with the added presence of microfilaments (MF) and more frequent ribosomes (R). This corresponds to fractions 7—10 in Fig. 2. Magnification, X22500.

Fig. 6. A more dense fraction from the sucrose gradient above the pellet (fractions 3—6). It includes numerous mitochondria (M), rough endoplasmic reticulum (RER), material which resembles heterochromatin (HC) or clumps of ribosomes and membrane vesicles. Magnification, X22500.

turbid band contains all of the AMPase on the gradient but the activity is predominantly localized in the dense half of the band. The less dense half of the band is not free of enzymatic activity, however, for a Ca^{2+}-ATPase was found localized in the upper half of the band in other experiments (McMahon, D.,

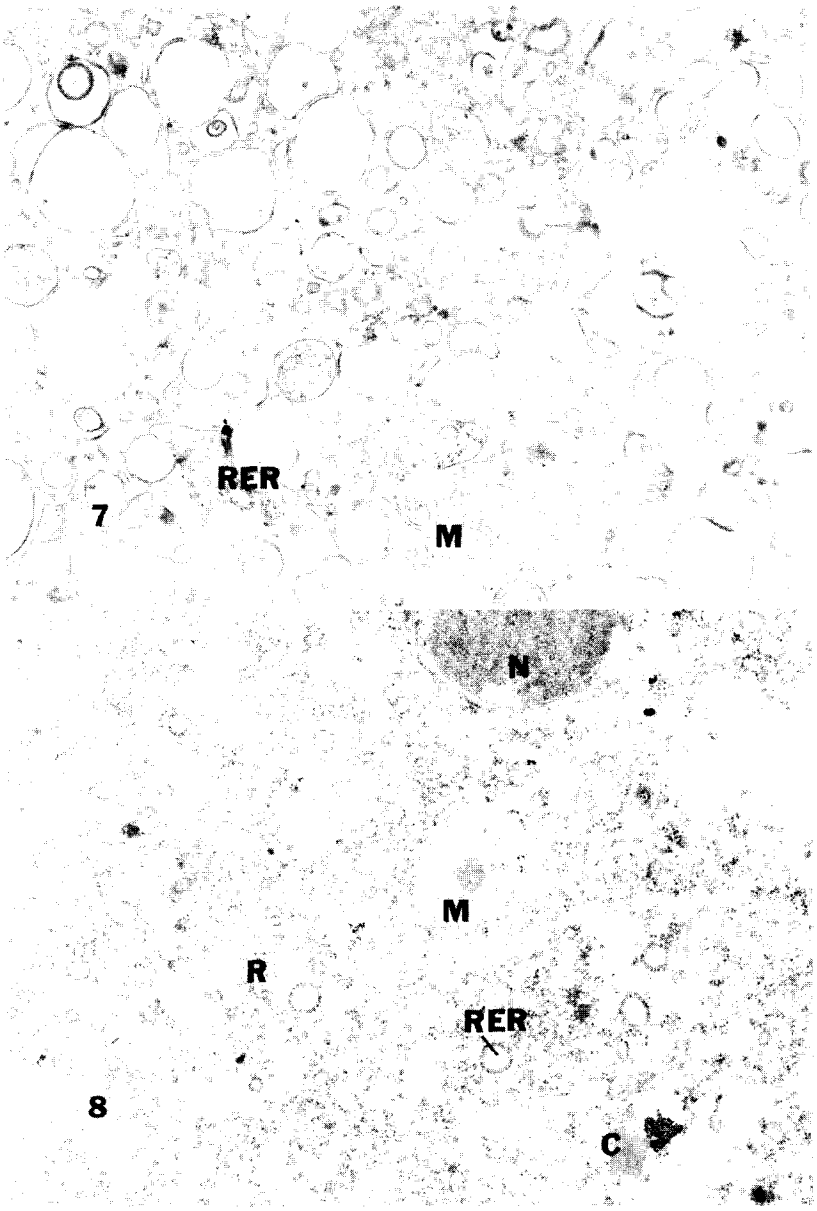

Fig. 7. The material above the pellet (fractions 1—2) in the sucrose gradient. It contains mitochondria (M), rough endoplasmic reticulum (RER), and membrane vesicles. Magnification, ×22500.

Fig. 8. The pellet of the sucrose density gradient is composed largely of ribosomes (R), remnants of nuclei (N), mitochondrial fragments (M), rough endoplasmic reticulum (RER), and occasional "crystals" (C). Magnification, ×22500.

previously unpublished results). Fig. 10 shows that catalase is also removed from the plasma membranes on the Renografin density gradient.

Figs. 11 and 12 illustrate the morphology of the membranes in the plasma membrane band. Both the upper and lower halves of the band are composed of smooth membrane vesicles (predominantly 0.2—1 μm in diameter). Occa-

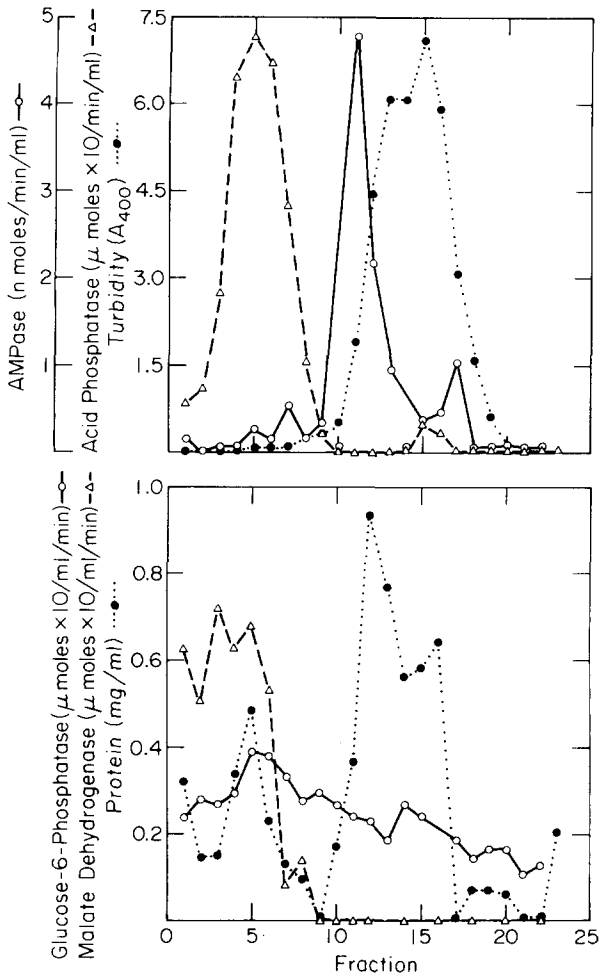

Fig. 9. Distribution of marker enzymes in a Renografin gradient. The partially purified band of plasma membranes was recentrifuged in a gradient of Renografin and fractions were assayed as described in the text. Centrifugation from right to left.

sionally sheets of membrane were found which ranged in length to 10—12 μm in contour length. No ribosomes were visible nor were any other non-membrane structures seen, although short fibers attached to the membrane were occasionally observed at high magnification (Luftig, R., personal communication). Preparations of plasma membrane from aggregation and pre-culmination phase cells appear similar in the electron microscope (Luftig, R., personal communication). Occasional membrane profiles were distinctly different in morphology, consisting of a cluster of small vesicles observed in the band. These structures (labeled M in Figs. 11 and 12) resemble the cristae of the mitochondria. The possibility that these were inner membranes of mitochondria was explored using a cytochemical assay for cytochrome oxidase. Fig. 13 presents the results of this assay on membranes from the Renografin gradient. When incubated with the complete assay mixture for cytochemical demonstration

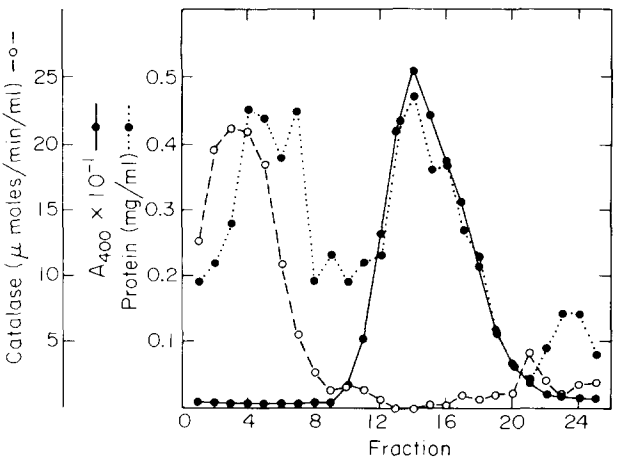

Fig. 10. Distribution of catalase in a Renografin gradient. The distribution of catalase, a marker for peroxisomes, was determined when partially purified plasma membranes were centrifuged in a Renografin gradient. Centrifugation from right to left.

of cytochrome *c* oxidase, the large membrane profiles did not stain, but the clusters of small vesicles did (Fig. 13a). If KCN was added to the incubation mixture (Fig. 13b) or diaminobenzidine was omitted from the incubation mixture (data not presented) the presumptive cristae no longer stained.

The content of mitochondrial membranes in the plasma membrane preparation was examined in another way. Difference spectra at the temperature of liquid N_2 were used to detect cytochromes. Fig. 14 shows the difference spectrum of a plasma membrane preparation containing 4.4 mg of membrane protein. The spectrum shows that membranes are free of cytochrome *c* but do contain cytochrome oxidase (peak centered at 598 nm) and cytochrome *c* (peak centered at 559 nm). Differentiation of the spectrum indicates that there are two cytochromes *b*, *b*-562 and *b*-559 (Poff, K., personal communication). These samples had been purposely made highly light-scattering by adding $CaCO_3$ to the cuvette. This enhances the sensitivity of detection of cytochromes but makes determination of the absolute content of cytochromes difficult. Therefore a known amount of cytochrome *c* was added to the preparation and its difference spectrum was determined again. On the basis of the absorption of the added cytochrome *c* and the extinction coefficients of cytochromes *c*, $a + a_3$, and *b* [36,37] it is possible to estimate the content of cytochrome oxidase at 0.09 nmol/mg protein and of cytochrome *b* at 0.07 nmol/mg protein.

The orcinol and diphenylamine methods were used to determine the RNA (on the basis of ribose content) and the DNA (on its deoxyribose content) of the preparation. Determinations made on preparations of plasma membranes and on whole cells from three stages of development are presented in Table I. These values themselves may over-estimate the contamination with nucleic acids since very small amounts of nucleic acid are being measured in the presence of very much greater amounts of protein, lipid and membrane-associated carbohydrate and, in the case of the measurements of DNA, these deter-

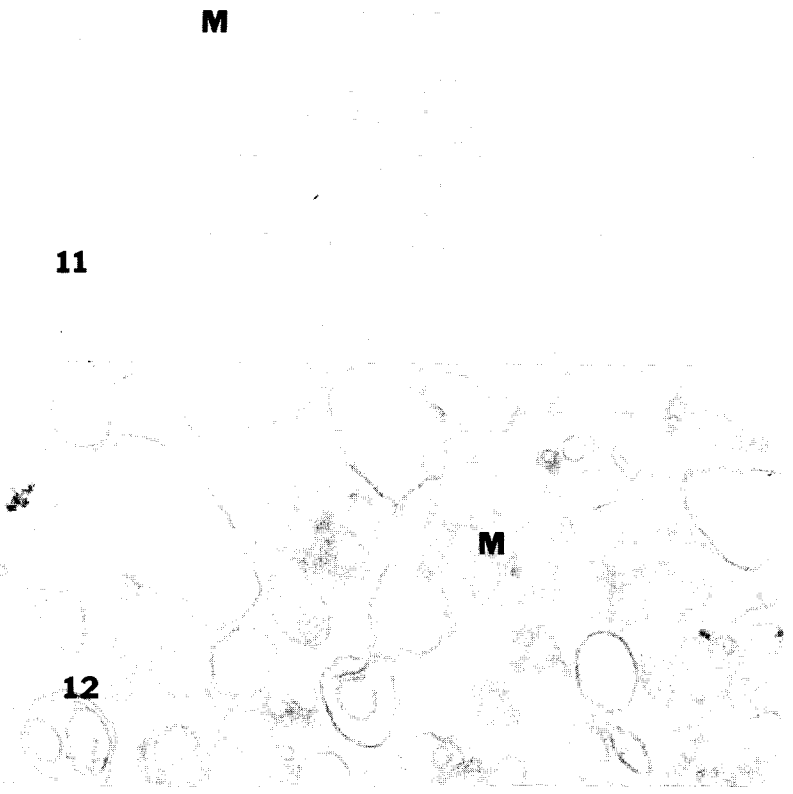

Fig. 11. The top fraction of the plasma membranes from a Renografin gradient contains only membrane vesicles. The predominant type of membrane stains more densely and is relatively large in profile. The minor component is a cluster of small vesicles reminiscent of the mitochondrial cristae (M). Magnification, X35440.

Fig. 12. The lower half of the plasma membrane fraction in the Renografin gradient is similar to the top half of the fraction. Both membrane configurations are present in this fraction as well. Note the small clusters of membranous vesicles (M). Magnification, X40500.

Fig. 13. (a and b) Electron micrographs of the diaminobenzidine histochemical assay for cytochrome *c* oxidase. Dense staining over portions of the membranes in a indicate the presence of cytochrome *c* oxidase. b is an electron micrograph of a control preparation containing 10 mM KCN, an inhibitor of cytochrome electron transport. The membrane fragments lack the dense stain seen in a. Magnification, X62400.

TABLE I

RATIOS OF NUCLEIC ACIDS TO PROTEIN IN WHOLE CELLS AND PLASMA MEMBRANES

The values for RNA and DNA content are the mean ± S.E. of two independent determinations of cells or of plasma membranes.

Development phase	μg RNA-ribose/mg protein			μg DNA-deoxyribose/mg protein		
	Cells	Plasma membranes	Plasma membranes/ cells	Cells	Plasma membranes	Plasma membranes/ cells
Vegetative	11.9 ± 0.3	0.96 ± 0.16	0.08	1.62 ± 0.28	0.141 ± 0.11	0.09
Aggregation	10.4 ± 0.3	0.40 ± 0.11	0.04	4.71 ± 1.4	0.200 ± 0.06	0.04
Preculmination	8.58 ± 0.15	0.42 ± 0.08	0.05	0.665 ± 0.12	0.375 ± 0.09	0.56

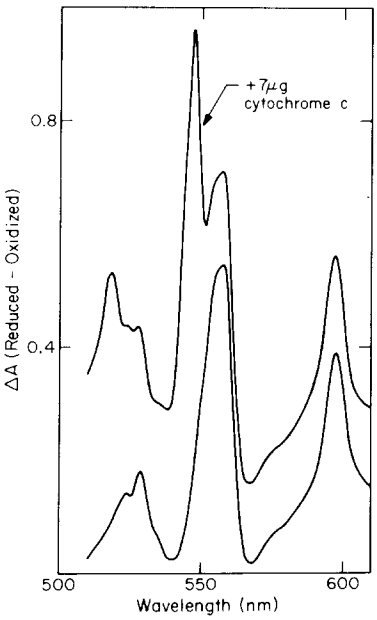

Fig. 14. Difference spectrum of the plasma membrane fraction. The lower curve is a difference spectrum of plasma membranes, made by the method of Butler [17], purified on Renografin gradients. The upper curve which is displaced upward by 0.13 A represents a spectrum of the same preparation after the addition of 7 μg of cytochrome c. 4.4 mg of membrane protein were used.

minations were close to the limits of detection. Nevertheless, the measured content of both RNA and DNA in these preparations is extremely low. The ratio of RNA content to protein content varies from 4 to 8% of that found in the whole cell. The ratio of DNA content to protein is 4—9% of that found in the whole cell, for vegetative and aggregation phase [38,39]. The relatively high DNA content of the pre-culmination phase membranes is partially due to a higher relative DNA content of the membranes and partially to an unexplained drop in the relative DNA content of the whole cells. Since only 1—2% of the total cellular protein is present in the plasma membrane fraction, 99—99.5% of the cellular DNA has been removed from the pre-culmination phase plasma membranes. These assays confirm the removal of nuclei and rough endoplasmic reticulum.

In other experiments we have examined purified plasma membranes from all three stages for their content of other enzymes of *D. discoideum*. The membranes contained no tyrosine transaminase, uridine-diphosphoglucose pyrophosphorylase or glycogen phosphorylase. They did contain alkaline phosphatase (McMahon, D., previously unpublished results). Other experiments have shown that the plasma membranes also contain discoidin [47] which has been identified by its molecular weight and sugar-specific binding properties [48].

Discussion

The method of preparation described here yields a preparation of plasma membranes which is free of several membranous cellular organelles. Biochem-

ical assays and/or electron microscopy indicate that the plasma membranes are free of nuclei, lysosomes, intact mitochondria, and peroxisomes. Attempts to detect and monitor the presence of a Golgi apparatus were unsuccessful. None of several soluble enzymes measured were found in these preparations. Nevertheless, a small fraction of the membrane preparation consists of the inner membrane of the mitochondrion. The extent of contamination of the preparations with the mitochondrial inner membrane cannot be determined with absolute accuracy since mitochondria of *D. discoideum* have not been purified to homogeneity and completely characterized. Nevertheless it is possible to estimate the extent of the contamination. Erickson and Ashworth [40] have determined that partially purified mitochondria of *D. discoideum*, strain Ax-2, contain 0.16 nmol cytochromes $a + a_3$, and 0.27 nmol cytochrome *b* per mg protein. These cytochromes are localized in the inner membrane of the mitochondrion [35,41]. If we assume that 20% of the mitochondrial protein is found in the inner membrane of *D. discoideum* mitochondria [35], then mitochondrial inner membranes constitute 11% of the protein in this preparation (i.e. 11% = content of cytochrome oxidase in these preparations/estimated content of cytochrome oxidase in the inner membrane of the mitochondrion of *D. discoideum*). If we use the content of cytochrome *b* of the preparation, our estimate of contamination with mitochondrial inner membrane is 5%.

In light of the difference between these estimates it is interesting that Stuchell et al. [34] have also partially purified a preparation of *D. discoideum* mitochondria from strain A3. Their measurement of the cytochrome *b* content of these mitochondria agrees with the measurement of Erickson and Ashworth [40] but they estimate a content of cytochrome oxidase 2.2 times greater than the previous estimate. Using this value the extent of contamination of our plasma membranes with mitochondrial inner membrane would be 5% on the basis of either cytochrome. The estimate of extent of contamination would be reduced if further purification increased the content of cytochromes measured by Erickson and Ashworth [40] or Stuchell et al. [34].

This question can be addressed in another way. The phospholipid content of plasma membrane preparations purified by our method has been measured by Dr. John Ellingson. The fraction of the total phospholipids which is cardiolipin (plus lysocardiolipin) is 3.1 and 1.5% of the phospholipid is phosphatidylcholine (and lysophosphatidylcholine) (Ellingson, J., unpublished results). If we assume that all of the cariolipin and phosphatidylcholine represent phospholipid contributed by mitochondrial contamination we can make maximal estimates of the content of mitochondria on the basis of the phospholipid measurements. Cariolipin is principally confined to the inner membrane of mammalian mitochondria where it composes 21—22% of the phospholipid [41, 42]. If cardiolipin content is similar for *D. discoideum* mitochondria, and all of the cariolipin comes from contaminating mitochondrial inner membrane, then the extent of contamination of the preparations with inner membrane of mitochondria is 14%. Phosphatidylcholine is partitioned relatively equally between the inner and outer membranes of those mitochondria where it has been measured and constitutes about 40—45% of the phospholipids [41—43]. Assuming that the total content of phosphatidylcholine was contributed by the sum of all inner and outer mitochondrial membranes contaminating the

preparation, they would compose 3—4% of the membrane in the preparation. It seems likely that the extent of contamination of this plasma membrane preparation with mitochondrial inner membrane is approx. 5%. Although no morphometric measurements have been made, this agrees with the general impression which is given by electron microscopy of the preparation.

Several of our observations indicate there is little, if any, endoplasmic reticulum in the plasma membrane preparation. The insignificant content of RNA and absence of ribosomes indicate that the preparations contain very little rough endoplasmic reticulum. However, this does not exclude the possibility of contamination with smooth endoplasmic reticulum. If the endoplasmic reticulum of *D. discoideum* has a phosphatidylcholine content of 55% similar to that of other endoplasmic reticula [44], an argument similar to that presented for mitochondria above suggests the plasma membranes contain slight contamination. Endoplasmic reticulum from both plants and mammals contains cytochrome b_5 with an A_{max} at 555 nm [45,46]. We did not detect a *b*-type cytochrome with this A_{max}. This also argues against possible contaimination with smooth endoplasmic reticulum. However, the plasma membrane band from the Renografin gradient contains 14% of the glucose-6-phosphatase activity recovered during preparation (i.e. 48% of the membrane-bound glucose-6-phosphatase was in the crude plasma membranes recovered from the sucrose gradient, 30% of the total glucose-6-phosphatase activity was recovered in the plasma membrane band on Renografin gradients). Because the measurements discussed above suggest that there is very little endoplasmic reticulum in this band, this may represent a glucose-6-phosphatase activity of the plasma membrane. Green and Newell [27] have also concluded that *D. discoideum* plasma membranes have an endogenous glucose-6-phosphatase activity.

Our identification of the plasma membrane fraction rests on the morphology of the membranes, the relatively large size of the membranous profiles (most easily explained as representing fragments of the cell surface) and the presence of all of the membrane-associated AMPase in the plasma membrane fraction. On the basis of recovery of 5′-nucleotidase activity, we can estimate that we have recovered about 90% of the plasma membranes in the original extract.

Although we have characterized the membrane isolated from vegetative cells relatively extensively, it has not been possible to examine those of aggregation and preculimination phases in as much detail. Their relative purity, however, is supported by their morphology in the electron microscope, by their low content of nucleic acids, and also by the fact that, in spite of a number of changes in composition associated with development, their composition of proteins and glycoproteins overlaps to a considerable extent with that of log phase plasma membranes as indicated by sodium dodecyl sulfate gel electrophoresis [12].

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