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The Isolation and Subfractionation of Plasma Membrane from the Cellular Slime Mould *Dictyostelium discoideum*

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A procedure for the isolation and separation of three different subfractions of plasma membrane from the cellular slime mould *Dictyostelium discoideum* is described. The cells were disrupted by freeze-thawing in liquid N_2 and plasma membranes were purified by equilibrium centrifugation in a sucrose gradient. The cell surface was labelled with radioactive iodide by using the lactoperoxidase iodination method. Alkaline phosphatase was identified as a plasma-membrane marker by its co-distribution with [^{125}I]iodide. 5′-Nucleotidase, which has been widely described as a plasma-membrane marker enzyme in mammalian tissues, was not localized to any marked extent in *D. discoideum* plasma membrane. The isolated plasma membranes showed a 24-fold enrichment of alkaline phosphatase specific activity relative to the homogenate and a yield of 50% of the total plasma membranes. Determination of succinate dehydrogenase and NADPH–cytochrome *c* reductase activities indicated that the preparation contained 2% of the total mitochondria and 3% of the endoplasmic reticulum. When the plasma-membrane preparation was further disrupted in a tight-fitting homogenizer, three plasma-membrane subfractions of different densities were obtained by isopycnic centrifugation. The enrichment of alkaline phosphatase was greatest in the subfraction with the lowest density. This fraction was enriched 36-fold relative to the homogenate and contained 19% of the total alkaline phosphatase activity but only 0.08% of the succinate dehydrogenase activity and 0.34% of the NADPH–cytochrome *c* reductase activity. Electron microscopy of this fraction showed it to consist of smooth membrane vesicles with no recognizable contaminants.

Dictyostelium discoideum is a unicellular phagocytic amoeba which can be cultured either on semi-defined synthetic media or on bacteria. Exhaustion of nutrient initiates a developmental phase in which amoebae, responding chemotactically to cyclic AMP, aggregate and form multicellular mounds. Cells within the aggregate then differentiate divergently along at least two distinct developmental pathways to form either spores or stalk cells. The actual differentiation pathway taken by any cell can be correlated with its position within the aggregate.

It is apparent that the cell surface must play an important role in the mechanism and regulation of this development. For example, a membrane-bound cyclic AMP phosphodiesterase has been suggested by Malchow *et al.* (1972) to be a component of the chemotactic receptor system. Investigations by Gerisch and co-workers (Beug *et al.*, 1973) have shown that the actual adhesion of cells within an aggregate is mediated by at least two immunologically distinct contact sites on the cell surface. The cell membrane is also implicated in the control of this differentiation process, since if aggregates are mechanically dispersed all development ceases. If allowed to re-aggregate, the cells cannot continue where they left off, but must rapidly recapitulate all previously undergone stages (Newell *et al.*, 1971).

We are particularly interested in the involvement of the surface membrane in the process of aggregation and subsequent differentiation and are investigating this problem from a combined genetic and biochemical approach. Many developmental mutants have been isolated and genetic analysis of these mutants is now possible by using improved genetic (Williams *et al.*, 1974) and cytological techniques. From a biochemical standpoint we hope to correlate changes in cell-surface properties with development events, both during differentiation and in developmentally deficient mutants. To this end we have developed a procedure for the isolation of *D. discoideum* plasma membranes.

The literature describing the isolation of plasma membranes from mammalian tissues is extensive (for review see Solyom & Trams, 1972; DePierre & Karnovsky, 1973). Despite this, no uniformly applicable method has emerged. It has been found necessary to vary the conditions of cell disruption or membrane purification as the source and type of tissue has varied. Reports on the isolation of plasma membranes from higher micro-organisms are few;

these include yeast (Schibeci *et al.*, 1973), *Amoeba proteus* (O'Neill, 1964) and *Acanthamoeba castellanii* (Schultz & Thompson, 1969; Ulsamer *et al.*, 1971). Of these reports only the plasma-membrane preparations of *A. castellanii* have been well characterized enzymically.

In preliminary investigations we found that techniques of homogenization applicable to mammalian cells were not necessarily effective in *D. discoideum*, possibly because it is an amoeboid organism and compared with mammalian cells it is relatively resistant to osmotic stress. Similarly we found that several enzymes that were marker enzymes for membranes of mammalian cells were not analogous markers for *D. discoideum* membranes. To approach the problem of identifying plasma-membrane marker enzymes in *D. discoideum* we introduced an external marker by labelling specifically the plasma membrane with radioactive iodide by using the lactoperoxidase iodination method described by Marchalonis *et al.* (1971). On the basis of these studies we report a method for the isolation of plasma membranes in both considerable yield and purity from *D. discoideum*.

Methods

Cell culture

Dictyostelium discoideum amoebae, strain AX3 (Loomis, 1971), were grown at 22°C in axenic suspension cultures and aerated by rotation at 150rev./min. The doubling time of amoebae was about 10h. At a cell density of between 5×10^6 and 1×10^7 cells/ml, amoebae were harvested by centrifugation at $300g_{av.}$ for 2min in an MSE 6L Mistral centrifuge. The cells were washed twice in cold 0.16M-sodium phosphate buffer, pH7.5 (phosphate buffer) before labelling and isolation of plasma membranes.

Optimal lactoperoxidase labelling conditions

The standard reaction mixture for labelling of cells consisted of phosphate buffer, 50μg of lactoperoxidase/ml, 2–5μCi of [^{125}I]iodide/ml, 2μM-NaI and cells at a concentration of 5×10^7/ml. The reaction was initiated by the addition of H_2O_2 (final concentration of 0.05mM). After incubation for 5 min at 20°C the reaction was stopped by the addition of 5 vol. of chilled 25mM-NaN_3 in phosphate buffer and the cells were collected by centrifugation. The cells were washed three times in 5vol. of cold phosphate buffer to remove unchanged [^{125}I]iodide and lactoperoxidase in preparation for the isolation of plasma membranes. Since *D. discoideum* is a phagocyte there is the possibility that some [^{125}I]iodide is withdrawn into the cell in vacuoles. Hence to ensure that all radioactivity measured was covalently bound to protein all samples were precipitated with cold 7% (w/v) trichloroacetic acid and the precipitate was washed once in 7% trichloroacetic acid before being dissolved in 1M-NaOH and counted for radioactivity in a Nuclear Enterprises gamma counter.

Solutions of H_2O_2 were prepared immediately before use by dilution in phosphate buffer of a stock solution. The concentration of H_2O_2 was determined spectrophotometrically at 230nm ($\varepsilon = 72.4$litre$\cdot$mol$^{-1}\cdot$cm^{-1}) because concentrated solutions of H_2O_2 are unstable. Lactoperoxidase solutions were prepared in phosphate buffer and their concentrations determined spectrophotometrically at 412nm, assuming $\varepsilon = 11.4\times10^4$litre$\cdot$mol$^{-1}\cdot$cm^{-1} (Morrison *et al.*, 1957).

Cell fractionation

Washed cells were resuspended in cold 10% (w/v) sucrose–10mM-*N*-tris(hydroxymethyl)methylglycine–NaOH buffer, pH7.5 (sucrose–Tricine buffer) at a concentration of 8×10^7cells/ml.

Portions (5ml) in plastic centrifuge tubes were frozen rapidly in liquid N_2 for 5min and then allowed to thaw at room temperature until the homogenate temperature reached 4°C. The homogenate was immediately centrifuged at $700g_{av.}$ for 20min in the swing-out head of a refrigerated MSE Multex centrifuge. This resulted in the formation of a very loose fluffy pellet (cell membranes) from which plasma membranes were purified, and a supernatant which was further fractionated into a 'microsomal' ($105000g_{av.}\times90$min in a Beckman 40 angle rotor) and a 'soluble' (post-microsomal supernatant) fraction.

For the isolation of plasma membrane, the low-speed pellet was resuspended in sucrose–Tricine buffer and homogenized with five up-and-down strokes in a tight-fitting glass homogenizer. The homogenate was then made 50% (w/v) in sucrose and overlaid with sucrose zones of decreasing density as described in the Results section. Centrifugation of the sucrose density gradients was carried out at 4°C in a Beckman SW25.1 rotor. Fractions were collected from the gradient by using a pasteur pipette with a bent tip. After dilution to 10% sucrose by addition of 10mM-*N*-tris(hydroxymethyl)methylglycine–NaOH buffer, pH7.5, (Tricine–NaOH) samples were collected by centrifugation and resuspended in sucrose–Tricine buffer before enzyme assay. Measurement of enzyme activities of samples of the low-speed pellet, suspended in both 10% and 50% sucrose showed that there was no effect on the various enzyme activities of simply exposing the preparation to high sucrose concentrations. All sucrose solutions contained 10mM-Tricine–NaOH buffer, pH7.5. The composition of all sucrose solutions are expressed as % (w/v).

Enzyme assays

All enzyme assays were done at 30°C. Continuous spectrophotometric assays were done by using a Pye-Unicam SP.1800 spectrophotometer, equipped with a multiple sample recorder.

Alkaline phosphatase (*EC* 3.1.3.1). This activity was determined as a Mg^{2+}-dependent alkaline *p*-nitrophenyl phosphatase activity. The assay system contained in the test cuvette: 40mM-ethanolamine–HCl, pH9.5, 5mM-$MgSO_4$, 2mM-KF, 0.1% (v/v) Triton X-100 and enzyme sample. $MgSO_4$ was replaced by 10mM-EDTA in the control cuvette. After 5min incubation the reaction was initiated by the addition of *p*-nitrophenyl phosphate to 10mM final concentration. The EDTA-sensitive hydrolysis was measured at 420nm. The inclusion of an EDTA control as suggested by Hübscher & West (1965) allows correction for hydrolysis by other phosphatases such as glucose 6-phosphatase, which do not require Mg^{2+} ions. The molar extinction coefficient for *p*-nitrophenol at 420nm was calculated to be 14000 litre·mol^{-1}·cm^{-1} from the data of Bessey & Love (1952).

Succinate dehydrogenase (*EC* 1.3.99.1). This was determined as described by Ells (1959), except that the concentrations of phenazine methosulphate and 2,6-dichlorophenol-indophenol in the assay were 0.43 and 0.018mg/ml respectively. Activity in the presence and absence of 20mM-sodium succinate was followed continuously at 600nm for approx. 2min. Solutions of KCN, 2,6-dichlorophenol-indophenol and phenazine methosulphate were made up immediately before use. A molar extinction coefficient of 21000litre·mol^{-1}·cm^{-1} for 2,6-dichlorophenol-indophenol was used (King, 1967).

NADPH–cytochrome c reductase (*EC* 1.6.2.4). This was assayed by the method of Duppel *et al.* (1973). The reaction was initiated by the addition of enzyme to a cuvette containing cytochrome *c* and NADPH and to a control cuvette containing cytochrome *c* only. Activity was measured at 550nm. A molar extinction coefficient (reduced minus oxidized) of 19600litre·mol^{-1}·cm^{-1} for cytochrome *c* was used (Yonetani, 1965).

5'-Nucleotidase (*EC* 3.1.3.5). This was determined as described by Michell & Hawthorne (1965) except that the assay mixture contained 20mM-sodium–potassium tartrate to inhibit lysosomal contributions to activity as suggested by the investigations of Kaulen *et al.* (1970). Phosphorus was determined by the method of Ames (1966).

Glucose 6-phosphatase (*EC* 3.1.3.9). This was assayed as described by Hubscher & West (1965). Activity was measured in the presence of 5mM-EDTA and 2mM-KF to inhibit alkaline and acid phosphatase respectively. Phosphorous was determined by the method of Ames (1966).

*Na^+,K^+-ATPase** (*EC* 3.6.1.3). This was assayed as described by Quigley & Gotterer (1969). Phosphorous was determined by the method of King (1932). Protein determinations for the determination of enzyme specific activities were done by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Electron microscopy

The plasma-membrane fraction was pelleted by centrifugation at 78000*g* for 15min. The pellet was fixed in 3% (v/v) glutaraldehyde in 0.1M-sodium cacodylate buffer, pH7.4, for 1h at room temperature, post-fixed with 1% osmium tetroxide also in cacodylate buffer for 2h, dehydrated through a graded series of alcohols and finally embedded in Spurr resin (Spurr, 1969). Thin sections were cut with a diamond knife on an LKB ultra-microtome, stained with a 25% (w/v) methanolic solution of uranyl acetate, and then with Sato's lead stain (Sato, 1968) and examined in an AEI 801 electron microscope.

Materials

All reagents used were of analytical grade. H_2O_2 was obtained from Fisons Ltd., Loughborough, Leics., U.K. Lactoperoxidase and substrates for enzyme assays were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Carrier-free [^{125}I]iodide was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., at a specific radioactivity of 14Ci/mg. The source of cytochrome *c* was Sigma (type III from horse heart).

Results and Discussion

An initial difficulty in isolating plasma membrane from a tissue that has not previously been investigated is that of recognition of the membranes during purification. One needs markers, either enzymic, chemical or morphological for both plasma membrane and for other subcellular organelles that may contaminate the preparation. Ideally these should be 'absolute' markers in that they are localized exclusively in only one type of organelle. Such enzymes are rather uncommon and in addition even enzymes that have been established as organelle markers in a particular tissue are not necessarily marker enzymes in other tissues.

Plasma-membrane markers

The most generally accepted plasma-membrane marker enzyme is Na^+,K^+-ATPase, which is thought

* Abbreviation: Na^+,K^+-ATPase, Na^+-plus-K^+-stimulated adenosine triphosphatase.

to be exclusively plasma-membrane bound. However, we were unable to observe consistently a stimulation by Na^+ plus K^+, or an inhibition by ouabain of the Mg^{2+}-dependent ATPase (adenosine triphosphatase) present in *D. discoideum*. The absence of a classical ouabain-inhibited Na^+,K^+-ATPase has been reported by Klein & Breland (1966) for *Acanthamoeba* sp. 5'-Nucleotidase and alkaline phosphatase are two enzymes which have been widely described as plasma-membrane markers (for review see Solyom & Trams, 1972; DePierre & Karnovsky, 1973). These enzymes are present in *D. doscoideum* and by analogy may also be bound to the plasma membrane.

To resolve the question of which enzymes are localized in the plasma membrane we included an independent marker by specifically labelling the cell surface with [^{125}I]iodide. The procedure is based on that described by Marchalonis *et al.* (1971), in which exposed tyrosine molecules on the cell surface are iodinated by the action of externally added lactoperoxidase and H_2O_2. The concentrations of H_2O_2 and lactoperoxidase were varied to obtain optimal labelling conditions for *D. discoideum* cells. Expt. A in Table 1 shows the dependence of the iodination reaction on the concentration of peroxide. Maximal labelling was obtained at a concentration of 0.05 μmol of H_2O_2/ml. At higher concentrations of H_2O_2 the apparent incorporation decreased as the amount of cell lysis increased. It is therefore crucial in the application of this technique to use a concentration of H_2O_2 great enough to initiate enzymic iodination yet low enough to prevent damage to the cell membrane. At a concentration of 0.05 μmol of H_2O_2/ml incorporation was nearly maximal with 50 μg of lactoperoxidase as shown in Expt. B of Table 1. The incorporation in the absence of either H_2O_2 or lactoperoxidase was only 0.01% of the input radioactivity; thus both enzyme and peroxide must be present for significant incorporation of radioactive iodide to occur. The reagents used in the experiments shown in Table 1 were incubated for 5min at 20°C, since investigation of the kinetics of cell labelling had shown that incorporation was almost completely saturated after 5min incubation.

Table 1. *Effect of reagent concentration on the binding of [^{125}I]iodide to D. discoideum cells*

Cells were labelled as described in the Methods section. The reaction mixture contained 5 μCi of [^{125}I]iodide/ml. Lactoperoxidase and H_2O_2 were varied as shown in the Table and all determinations were done in duplicate.

Expt.	Lactoperoxidase (μg/ml of reaction mixture)	H_2O_2 (μmol/ml of reaction mixture)	[^{125}I]iodide incorporated (% of added material)
A	50	0	0.01
	50	0.025	4.61
	50	0.05	5.10
	50	0.15	4.58
	50	0.30	3.96
	50	0.5	3.21
B	0	0.05	0.01
	12.5	0.05	2.68
	25	0.05	3.40
	50	0.05	4.71
	100	0.05	5.07

Markers of subcellular organelles

Mitochondria were located by assaying succinate dehydrogenase, an enzyme generally considered to be localized exclusively in the inner mitochondrial membrane (Schneider, 1946). Endoplasmic reticulum was located by determining both glucose 6-phosphatase, which has been considered as a microsomal marker for some mammalian cells (Goldfischer *et al.*, 1964), and NADPH–cytochrome *c* reductase, which was reported to be localized exclusively in rat liver endoplasmic reticulum (Phillips & Langdon, 1962).

Cell fractionation

In initial experiments we found that *D. discoideum* cells, whether suspended in iso-osmotic sucrose or hypo-osmotic Tricine buffers and in the presence or absence of EDTA or Mg^{2+} salts, were relatively resistant to osmotic or shearing stress, compared with mammalian cells. It was extremely difficult to obtain a reproducible and satisfactory breakage of cells by using conventional homogenization (Potter–Elvehjem) techniques. The eventual method of disruption chosen was freeze-thawing of cells, which resulted in a uniform breakage of all cells.

Table 2 shows the subcellular distribution of enzymes and radioactivity obtained when [^{125}I]-iodide-labelled *D. discoideum* cells were disrupted by freeze-thawing and the homogenate was fractionated by differential centrifugation into a low-speed (700*g* ×20min) cell-membranes fraction, a microsomal (105000*g*×90min) fraction and a 'soluble' (post-microsomal supernatant) fraction, as described in the Methods section. It is apparent from the results for [^{125}I]iodide, succinate dehydrogenase and NADPH–cytochrome *c* reductase that the low-speed pellet contained most of the plasma membrane, mitochondria and endoplasmic reticulum. Most of the alkaline phosphatase activity was also found in this fraction. Phase-contrast microscopy of the fraction showed the presence of cell-like structures in which the surface membrane had been punctured, but most of the particulate cell constituents had remained virtually enclosed.

Table 2. *Subcellular distribution of markers after fractionation of cell homogenate by differential centrifugation*

The results are expressed as a percentage of the total activity in the homogenate. Summation of the subcellular fractions gives the total recovery, which was always greater than 90%. Values are expressed as means. The S.E.M. values were in most cases between ±0.5 to ±1.5, and in no case greater than ±2.5.

Subcellular fraction	Protein (% yield)	[^{125}I]-iodide (% yield)	Alkaline phosphatase (% yield)	5′-Nucleotidase (% yield)	Succinate dehydrogenase (% yield)	NADPH–cytochrome *c* reductase (% yield)	Glucose 6-phosphatase (% yield)
No. of experiments	... 6	3	9	3	6	3	3
Homogenate	100	100	100	100	100	100	100
Cell membranes (700*g* × 20 min)	24.9	65.7	74.1	21.1	88.2	62.2	20.1
Microsomes (105000*g* × 90 min)	18.1	20.1	19.9	6.7	8.5	21.5	15.3
Soluble (post microsomal supernatant)	56.8	13.4	6.1	69.6	0	8.8	59.2

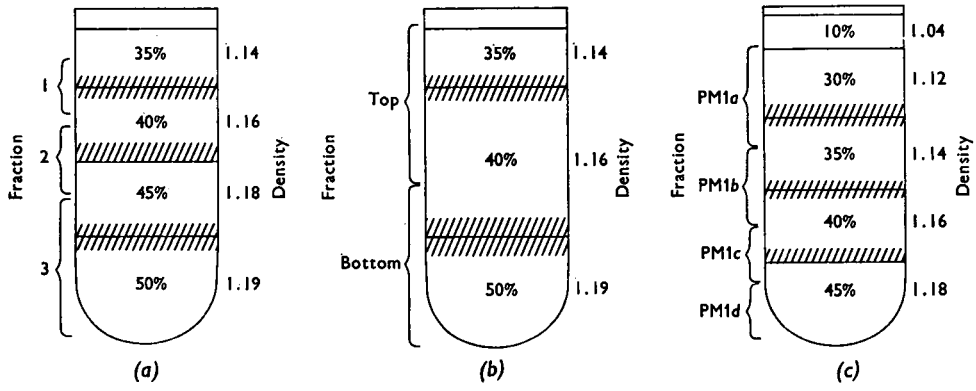


Fig. 1. *Composition of sucrose density gradients used for the purification of plasma membranes*

Tube (*a*) The cell membrane pellet was resuspended in 50% sucrose and a 10ml portion was layered beneath 8ml of 45% sucrose, 8ml of 40% sucrose and 4ml of 35% sucrose. After centrifugation the fractions 1, 2 and 3 were collected as shown. The volumes of fractions 1, 2 and 3 were, 5ml, 5ml, and 14ml respectively. Tube (*b*) The cell-membrane pellet was resuspended in 50% sucrose and a 10ml portion was layered beneath, 15ml of 40% sucrose and 5ml, of 35% sucrose. After centrifugation the top and bottom fractions were collected as shown. The volumes of the top and bottom fractions were, 14ml and 16ml respectively. Tube (*c*) Plasma membranes (PM1) were resuspended in 10% sucrose and layered on to 7ml each of 30% sucrose, 35% sucrose, 40% sucrose and 45% sucrose. After centrifugation the fractions PM1*a*, PM1*b*, PM1*c* and PM1*d* were collected as shown. The volumes of the fractions PM1*a*, PM1*b*, PM1*c* and PM1*d* were 11ml, 6ml, 7ml and 4ml respectively.

The 'soluble' post-microsomal supernatant contained about 60% of the total homogenate protein, indicating that most of the soluble cytoplasmic constituents had been extruded from the cell-like structures at the time of cell disruption. The soluble fraction also contained considerable amounts of the presumed membrane-bound enzymes 5′-nucleotidase and glucose 6-phosphatase. The appearance of these enzymes in the soluble fraction was not restricted to cells that had been disrupted by freeze-thawing, since similar results were obtained for cells disrupted by homogenization. These results indicated that at least 70% of the 5′-nucleotidase and 60% of the glucose 6-phosphatase of *D. discoideum* cells are either soluble or, if bound to membranes, are bound only weakly. Similar findings were reported in calf thymocytes by Van Blitterswijk *et al.* (1973) for 5′-nucleotidase and glucose 6-phosphatase and in pig lymphocytes by Allan & Crumpton (1970) for glucose 6-phosphatase.

Purification of plasma membranes

Since the low-speed fraction contained most of the cell membranes it was taken as the starting point for further purification, by sucrose-density-gradient centrifugation. The cell-membrane pellet was resuspended in 10% sucrose and homogenized with a tight-fitting glass homogenizer. Vigorous resuspension of the cell-membrane pellet was necessary to disperse fully the cell-like structures present and allow the purification of plasma membrane. The homogenate was then adjusted to 50% sucrose and layered beneath a discontinuous gradient consisting of 50, 45, 40 and 35% sucrose layers as shown in Fig. 1(*a*), and the gradient centrifuged for 2.5h at 64000g_{av}. Fractions were collected as shown in Fig. 1(*a*) diluted to 10% sucrose and sedimented by centrifugation at 105000*g* for 90min.

Marker enzyme activities, [^{125}I]iodide and protein content were determined on the pelleted fractions and the results are shown in Table 3. The high recoveries of the membrane-bound enzymes, alkaline phosphatase, succinate dehydrogenase and NADPH–cytochrome *c* reductase and of [^{125}I]iodide that were obtained in the fractions 1, 2 and 3 indicated that very little loss of membranes occurred during the sucrose gradient step. There was however, a considerable decrease of protein content in the pelleted fractions resulting from the release, on homogenization and gradient centrifugation, of soluble cell proteins and nucleoproteins which were trapped within the initial, crude cell-membrane fraction. The soluble cell proteins were recovered in the supernatant of fraction 3, and the nucleoproteins in a pellet at the bottom of the sucrose gradient.

The distribution of plasma membrane on the gradient is indicated by that of [^{125}I]iodide. The choice of alkaline phosphatase as a plasma-membrane marker enzyme was confirmed by the observation that its distribution followed closely that of the plasma membrane, as indicated by [^{125}I]iodide. The fraction most enriched in plasma membrane was fraction 1, which contained 30–40% of the total homogenate plasma membrane. The enrichment of [^{125}I]iodide specific radioactivity and alkaline phosphatase specific activity in this fraction, relative to the whole homogenate was 11- and 15-fold respectively.

The distribution of 5′-nucleotidase on the gradient is interesting in that it followed, at least in fractions 1 and 2, the trends of the plasma-membrane markers. For example, in the purification step from cell-membrane pellet to fraction 1 the 5′-nucleotidase specific activity was enriched to the same extent (four- to five-fold) as were the alkaline phosphatase specific activity and the [^{125}I]iodide specific radioactivity. In addition, all the 5′-nucleotidase applied to the gradient could be recovered by centrifugation;

Table 3. *Distribution of markers after sucrose-density-gradient purification of plasma membranes*

The composition of the sucrose density gradient and the positions of the fractions collected are shown in Fig. 1(*a*). The units of specific activity are: for [^{125}I]iodide, c.p.m./mg of protein; for alkaline phosphatase, succinate dehydrogenase and NADPH cytochrome *c* reductase, μmol of substrate utilized/min per mg of protein; for 5′-nucleotidase and glucose 6-phosphatase, nmol of substrate utilized/min per mg of protein. The yields are expressed as a percentage of the total homogenate. Values in parentheses represent the relative specific activities, which are the specific activities of the fraction divided by the specific activities of the homogenate. Results shown are for one experiment, but similar results were obtained in two other experiments.

Subcellular fraction	(Protein % yield)	[^{125}I]Iodide		Alkaline phosphatase		5′-Nucleotidase		Succinate dehydrogenase		NADPH–cytochrome *c* reductase		Glucose 6-phosphatase	
		Specific activity	% yield	Specific activity	% yield	Specific activity	% yield	Specific activity	% yield	Specific activity	% yield	Specific activity	% yield
Homogenate	100	7.8 (1.0	100	0.012 (1.0)	100	7.0 (1.0)	100	0.029 (1.0)	100	0.015 (1.0)	100	35.8 (1.0)	100
Cell membranes (700g×20min)	22.7	20.9 (2.7)	63.3	0.037 (3.1)	71.2	6.1 (0.9)	21.5	0.096 (3.3)	89.6	0.038 (2.5)	63.8	39.2 (1.1)	26.0
Sucrose gradient A Fraction													
1	2.5	87.5 (11.2)	28.6	0.178 (14.8)	37.2	27.5 (3.9)	10.5	0.043 (1.5)	4.3	0.025 (1.7)	4.6	20.8 (0.6)	1.4
2	2.6	51.6 (6.6)	17.6	0.100 (8.3)	21.7	12.2 (1.7)	4.9	0.102 (3.5)	10.8	0.067 (4.5)	12.7	13.5 (0.4)	1.0
3	4.4	13.4 (1.7)	7.6	0.02 (1.7)	7.3	9.0 (1.3)	6.1	0.395 (13.6)	70.6	0.138 (9.2)	44.3	1.5 (0.04)	0.2

thus no solubilization of activity had occurred. These results suggest a bimodal distribution of 5′-nucleotidase with approx. 30% at most, being strongly membrane bound (see Table 2; approx. 21% in the cell-membranes pellet and 7% in the microsomal fraction) and the remainder soluble, or very weakly bound to membranes. The distribution of the membrane bound 5′-nucleotidase on the gradient suggests that it may be localized in the plasma membrane. However, in view of the relatively greater distribution of membrane-bound 5′-nucleotidase in fraction 3 (fraction 3 contained one-third of the 5′-nucleotidase applied to the gradient, but only one-tenth of the applied alkaline phosphatase and one-eighth of the applied [^{125}I]iodide) one cannot exclude the possibility that some of the 5′-nucleotidase is is also bound to other cell membranes. 5′-Nucleotidase has been widely assumed by many workers to be exclusively plasma-membrane bound. Solyom & Trams (1972), however, after studying the results from a variety of mammalian tissues, concluded that its activity was derived from a group of isoenzymes, only one or two of which appeared to be intimately associated with the plasma membrane. Thus a lysosomal-membrane-bound (Kaulen *et al.*, 1970; Pletsch & Coffey, 1972), an endoplasmic-reticulum-bound (Segal & Brenner, 1960; Widnell, 1972; Song *et al.*, 1969) and a soluble (Fritzon, 1969; Van Blitterswijk *et al.*, 1973) 5′-nucleotidase have been described. The results described above show that in *D. discoideum* a large proportion of the 5′-nucleotidase is soluble and that even the remainder may not be bound exclusively to the plasma membranes. These findings underline the necessity for veryifying presumed plasma-membrane markers before attempting to isolate membranes.

In contrast with the results obtained for 5′-nucleotidase, only 2.6% of the total glucose 6-phosphatase was recovered from the gradient after collection by centrifugation, i.e. a recovery of only 10% of the activity layered on to the gradient in the cell membranes fraction. This was not due to a decrease in activity, as analysis of the supernatants formed after collection of the sucrose fractions by centrifugation revealed that the other 90% of the activity layered on to the gradient was found predominantly in the supernatant of fraction 3, with a small amount in the supernatant of fraction 2. Hence, approx. 90% of the applied glucose 6-phosphatase remained in the initial sucrose application layer, suggesting that this enzyme is either soluble, or extremely weakly bound to membranes of *D. discoideum*.

The low glucose 6-phosphatase activity present in fraction 1 probably represents an activity intrinsic to plasma membranes. This is suggested, not only by the observation that it is the only activity that did not become solubilized, but also by the finding that the specific activities of the glucose 6-phosphatase in fractions 1, 2 and 3 showed the same trends as those of alkaline phosphatase and [^{125}I]iodide, namely increasing to the lighter-density sub-fractions. The suggestion that plasma membranes may have a low glucose 6-phosphatase activity is supported by the observation that plasma-membrane preparations have always shown some glucose 6-phosphatase activity, the specific activity generally being of the order of that of the total homogenate (for review see Solyom & Trams, 1972). On the basis of the distribution of succinate dehydrogenase and NADPH–cytochrome *c* reductase the mitochondria and endoplasmic reticulum were both concentrated in the 45–50%-sucrose region (fraction 3). The specific activities in fraction 3 of succinate dehydrogenase and NADPH–cytochrome *c* reductase were enriched 14-fold and nine-fold respectively relative to the whole homogenate.

Despite the concentration of mitochondria and endoplasmic reticulum in fractions 2 and 3 it is apparent that the plasma-membrane preparation (fraction 1), although purified 11–15-fold, still contained measurable succinate dehydrogenase and NADPH–cytochrome *c* reductase activities. Therefore to improve the purity of the plasma membrane preparation further we developed a double-gradient procedure. The first gradient was a simplified version of that described above. The cell-membrane pellet was resuspended in 50% sucrose and layered beneath 40 and 35% sucrose layers as shown in Fig. 1(*b*). After centrifugation for 2.5h at 64000$g_{av.}$ the top 14ml was removed, diluted and membranes were collected by centrifugation at 78000$g_{av.}$ for 15min. Measurement of enzyme activities (Table 4) in sucrose fractions before sedimentation of membranes showed that the omission of a 45% sucrose layer resulted in a greater recovery (50% compared with 37%) of alkaline phosphatase in the 35–40% sucrose region, with no increase in the extent of contamination by succinate dehydrogenase of NADPH–cytochrome *c* reductase. In addition, sedimentation of membranes from the top fraction by centrifugation for only 15min at 78000*g* resulted in the complete recovery of alkaline phosphatase, but a further decrease in succinate dehydrogenase and NADPH–cytochrome *c* reductase activities.

The specific activities of succinate dehydrogenase and NADPH–cytochrome *c* reductase in the plasma-membrane preparation (PM1) were still comparable with those in the original homogenate; however, the purification of a plasma-membrane preparation should be correlated primarily with the enrichment of its marker enzyme. This is exemplified by the investigations of Korn and co-workers on *A. castellanii*, where in an initial report (Ulsamer *et al.*, 1971) a plasma-membrane preparation 13-fold enriched in alkaline phosphatase and with no detectable succinate dehydrogenase of NADPH–cytochrome *c* reduc-

tase activity was isolated. Further investigations, however, revealed that the preparation was contaminated with cytoplasmic actin filaments, which were not detectable by enzymic analysis, but were recognized by polyacrylamide-gel electrophoresis (Korn & Wright, 1973) and electron microscopy (Pollard & Korn, 1973). Thus the advantages of omitting the 45% sucrose layer are demonstrated by the observation that the specific activity of alkaline phosphatase in the plasma membrane preparation was thereby enriched 24-fold compared with the homogenate rather than 15-fold in the previous gradient. In addition the yield of plasma membrane increased from 37 to 50% of the total.

For the purposes of most investigations a 24-fold purification is quite adequate and a yield of 50% highly desirable. However, we were also interested in any possible heterogeneity of the plasma membrane, bearing in mind the numerous specialized functions in which it participates during development and so we fractionated the plasma membrane on a second gradient. The plasma membrane (PM1) was resuspended in 10% sucrose and layered on to a discontinuous gradient consisting of 45, 40, 35 and 30% sucrose layers (Fig. 1(*c*)). The gradient was centrifuged overnight at 40000g_{av}. Fractions were taken as shown in Fig. 1(*c*), diluted and membranes pelleted by centrifugation at 78000*g* for 15min. The distribution of enzymes between the fractions is shown in Table 4.

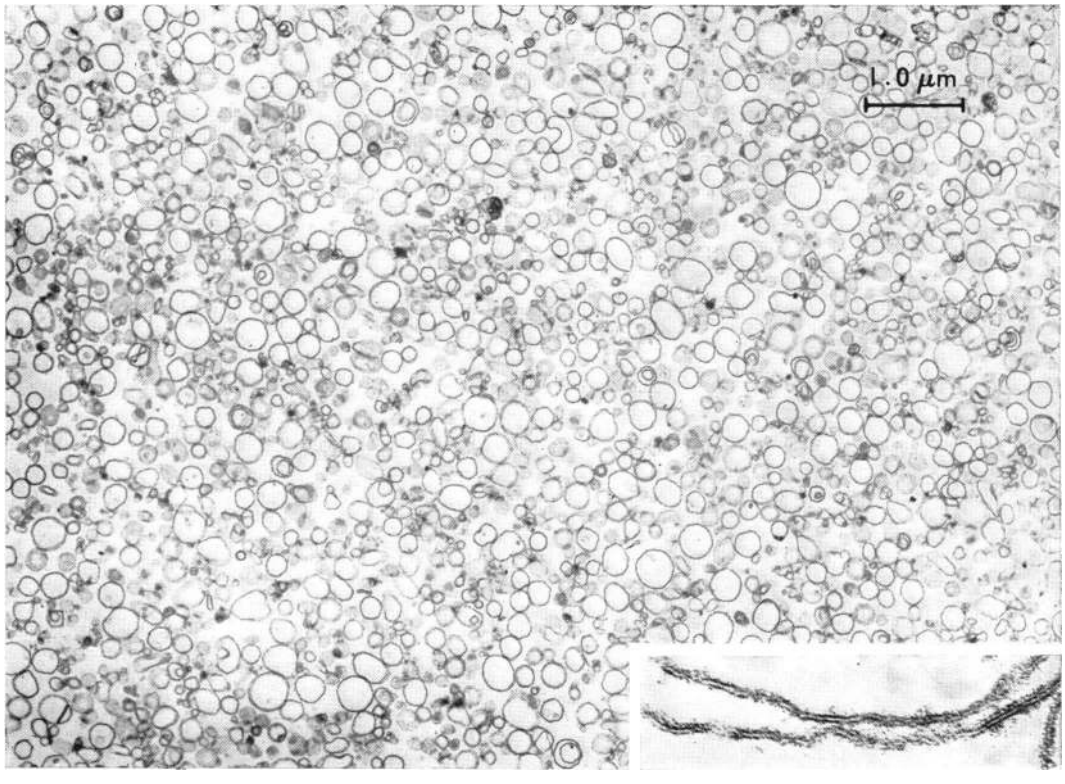
The results shown indicate that three major plasma-membrane subfractions were obtained: PM1*a*, PM1*b* and PM1*c*. The purest preparation obtained was PM1*a*, which based on alkaline phosphatase activity was 36-fold enriched compared with the original homogenate and had a yield of 19% of the total homogenate activity. Although we were able to detect succinate dehydrogenase and NADPH–cytochrome *c* reductase in this fraction we were not able to detect this contamination morphologically. Plate 1 shows a low-magnification electron micrograph of subfraction PM1*a* in which a relatively homogeneous population of membrane-limited vesicles can be seen and which reveals no structures recognizable as mitochondria or fragments of rough endoplasmic reticulum. Many such fields on sections cut from different parts of the plasma-membrane pellet were examined with the same result. Contaminants must therefore account for a negligible proportion of the protein of the preparation. At higher magnification (Plate 1 inset) the membrane profile features a 7.0nm thick unit-membrane structure which is typical of plasma membrane.

Subfraction PM1*b* is 28-fold purified with a yield of 17% of the total plasma membrane and subfraction PM1*c* 22-fold purified with a yield of 10%. It is possible that the relative amount of subfraction PM1*c* and possibly of subfraction PM1*d* in the total

Table 4. *Distribution of marker enzymes after subfractionation of the plasma membrane into regions of different density*

The compositions of the sucrose density gradients and the positions of fractions collected are shown in Figs 1(*b*) and 1(*c*). The numbers in parentheses are the relative specific activities. Specific activities are expressed in the units described in Table 3. Results shown are for one experiment, but similar results were obtained in three other experiments.

Subcellular fraction	Protein (% yield)	Alkaline phosphatase		Succinate dehydrogenase		NADPH–cytochrome *c* reductase	
		Specific activity	% yield	Specific activity	% yield	Specific activity	% yield
Homogenate	100	0.021 (1.0)	100	0.039 (1.0)	100	0.014 (1.0)	100
Cell membrane (700*g* × 20min)	26.2	0.060 (2.9)	74.6	0.137 (3.5)	91.8	0.031 (2.2)	60.9
Sucrose gradient B							
Fraction							
Top	—	—	49.5	—	4.1	—	4.3
Bottom	—	—	24.8	—	81.0	—	53.4
Plasma membrane 1 (PM1) (Top fraction sedimented at 78000*g* × 15min)	1.94	0.499 (23.8)	49.5	0.049 (1.3)	2.3	0.023 (1.6)	3.1
Sucrose gradient C							
Plasma membrane subfractions							
PM 1*a*	0.42	0.745 (35.5)	19.0	0.008 (0.2)	0.08	0.01 (0.8)	0.34
PM 1*b*	0.56	0.580 (27.6)	16.7	0.029 (0.7)	0.39	0.018 (1.3)	0.72
PM 1*c*	0.43	0.460 (21.9)	10.0	0.021 (0.5)	0.22	0.019 (1.4)	0.56
PM 1*d*	0.16	0.429 (20.4)	3.6	0.040 (1.0)	0.15	0.028 (2.0)	0.32



EXPLANATION OF PLATE 1

Electron micrograph of plasma-membrane subfraction PM 1a

The preparation consists of a uniform population of membranous vesicles. There are no structures recognisable as mitochondria or rough endoplasmic reticulum. Magnification × 13000. The inset shows the plasma membrane at higher magnification (×150000) where the typical unit membrane structure can be seen.

homogenate is greater than appears from these results, since in the first sucrose gradient stage plasma membrane present in the lower portion of the 40% sucrose layer and at the 40/50%-sucrose interface was not collected for further subfractionation. The total yield of protein in plasma-membrane subfractions 1*a*, 1*b*, plus 1*c* is 1.4% and the yield of alkaline phosphatase is 45.7%. If the alkaline phosphatase is localized virtually exclusively in the plasma membrane and if in addition it is homogeneously distributed throughout the plasma membrane one can calculate an upper limit of 2.8% for the proportion of the total cell protein present in the plasma membrane. If 2.8% of the total cell protein is plasma-membrane protein, the maximum possible enrichment of alkaline phosphatase activity would be 36-fold, which is the same as the enrichment found in subfraction PM1*a*, the purest fraction obtained.

Despite the increasing evidence for specialization of regions within a membrane the subfractionation of plasma membranes has not often been reported. A possible reason for this is that within any one homogenization technique many types of surface fragments may be produced which may be distributed by differential centrifugation into several subcellular fractions. Since each of the various methods purify the plasma membrane from only one subcellular cut considerable surface-specific material may be lost and the final product may not accurately reflect the constitution of the surface as a whole. This is particularly true for cells such as intestinal mucosa cells with surface specializations which, although readily isolated and identifiable, are poorly representative of the non-specialized surface regions (Forstner *et al.*, 1968; Fujita *et al.*, 1972). The subcellular cut from which we isolate *D. discoideum* plasma membranes contains 70%, and our unfractionated plasma membranes (PM1) 50%, of the total plasma membrane (on the basis of alkaline phosphatase and [^{125}I]iodide) and so presumably is fairly representative of the total surface material.

Subfractionation of plasma membrane has been described before in rat liver by House *et al.* (1972), who obtained three subfractions of buoyant densities 1.13, 1.16 and 1.18, and by Evans (1970), who obtained two subfractions of buoyant density 1.12 and 1.18. These densities are similar to the probable densities of the plasma-membrane subfractions of *D. discoideum* described above. Both authors observed a differentiation of structure and function between the two extreme-density fractions of rat liver. Enzyme activities such as 5′-nucleotidase, adenylate cyclase and cyclic AMP phosphodiesterase were concentrated in the low-density membranes. The high-density fraction appeared to consist of structural elements such as junctional complexes and bile canaliculi.

Electron microscopy of *D. discoideum* has not thus far revealed the presence of such obvious structural specializations in vegetative amobae; however, Mercer & Shaffer (1960) observed small areas of higher electron density in aggregating amobae, which they suggested resembled developing desmosomes. From observations of *D. discoideum* amoebae aggregating in streams Shaffer (1964) suggested that specially adhesive regions appeared to be localized at the fore and hind ends of the amoebae. Beug *et al.* (1973) have prepared antibodies to two adhesion sites, only one of which appears to be aggregation-specific. Reaction with antibody against the aggregation-specific site blocks the fore and hind adhesions, preventing the formation of streams. However, the site of action of antibody has not been localized to any specific region of the plasma membrane. Beug *et al.* (1973) have suggested that the adhesive sites may be spatially differentiated within the membrane or that polar sites may be preferentially activated. The isolation from aggregating amoebae of plasma-membrane subfractions of differing density may help to resolve this problem, the adhesive regions possibly being located exclusively in one fraction. Other problems that are possibly amenable to investigation in this way are the cyclic AMP receptor systems and the cyclic AMP pulse-propagation systems which may also be spatially differentiated within the plasma membrane.

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