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IMPROVED METHOD FOR ISOLATION OF PLASMA MEMBRANE ON CATIONIC BEADS

MEMBRANES FROM *DICTYOSTELIUM DISCOIDEUM*

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Summary

The plasma membrane from *Dictyostelium discoideum* was routinely purified 35-fold by an improved technique using beads coated with positively charged polymers. Cells were attached to the beads and bare regions between the cells were neutralized with a polyanion. The neutralization decreased contamination of the bare regions by intracellular proteins released when cells were disrupted to leave behind beads coated by plasma membrane. The neutralization increased the purification as measured by membrane-bound ^{125}I-labeled concanavalin A. Contamination by markers for various intracellular components was markedly decreased. Various bare-site neutralization reagents were evaluated and gave different results depending upon their charge density and molecular weight. The pH of the neutralization was critical. The optimum pH for cell attachment to beads, 5.0, had little effect as regards bare-site neutralization. A new procedure is given that optimizes the essential features for the plasma membrane isolation on beads.

Introduction

Techniques have been devised to expose the cytoplasmic surface of the plasma membrane for direct probing. However, they all have some inherent weaknesses. An example is the inside-out vesicles of erythrocytes [1]. Even in this case, where the plasma membrane is the only membrane present, the

Abbreviation: Mes, *N*-morpholinoethansulfonic acid.

inversion is not complete and the procedure results in the removal of unique peripheral proteins attached to the cytoplasmic side. Other methods have been developed which are based upon isolating a plasma membrane fraction by standard homogenization-centrifugation techniques and then separating the inside-out from the right-side out vesicles by sedimentation or binding to lectin coated polymers [2—4]. Unfortunately, in some of the cases the right-side out vesicles are removed, leaving behind the inside-out ones which have become enriched by contaminants [4]. Another inverting technique has been developed which entails the isolation of plasma membrane on beads coated with a polycation [5—9].

Isolation and inversion on polycation-coated beads is a technique whereby intact cells are attached by ionic interaction. The attachment is so tenacious that the cells can be gently disrupted and after rinsing away the cellular debris the beads are left coated by sheets of plasma membrane with the cytoplasmic surface exposed to the medium [6,10]. The advantages of the bead technique are its quickness and the fact that a variety of solutions can be used to disrupt attached cells so that, unlike the more time-consuming vesiculation procedures, peripheral proteins are not degraded or washed off. Furthermore, the total complement of membrane is isolated, not a selected region [6,9,10].

A weakness of the bead techniques as currently used is the incomplete coverage of the beads by attached cells, which leads to contamination by extraneous proteins. Uncovered or bare areas are formed when the space between attached cells is not large enough to accommodate another cell. The exposed positively charged polymer at these regions binds intracellular proteins which are released at the stage of the membrane isolation procedure when attached cells are deliberately disrupted to leave the adherent membrane. If the bare regions could be blocked or neutralized prior to the disruption step, the technique would be far more useful, not just to improve the purification of the attached membrane but to make feasible the study of the interaction of the inverted membranes with a variety of cytoplasmic components such as actin, secretory organelles, etc.

The rationale for the experiments reported here is the utilization of existing metabolically inert, non-proteinacious, polyanionic reagents to neutralize bare regions without dislodging attached cells or membranes or creating new surfaces to which other sets of contaminants could adhere. A single reagent that totally blocks the adsorption of all the intracellular proteins was not found and based upon theoretical considerations is non-existent; however, reagents were identified that blocked the bare regions and markedly improved membrane purification.

Materials and Methods

Membrane-isolated beads

The general scheme of isolating plasma membranes on cationic beads is depicted in Fig. 1. Two types of polyacrylamide bead were used, one coated with polylysine as previously described [7] and the other with polyethyleneimine (50 000—100 000, Polyscience). Polyethyleneimine coatings were made by procedures similar to those for polylysine [7]. Such beads are also

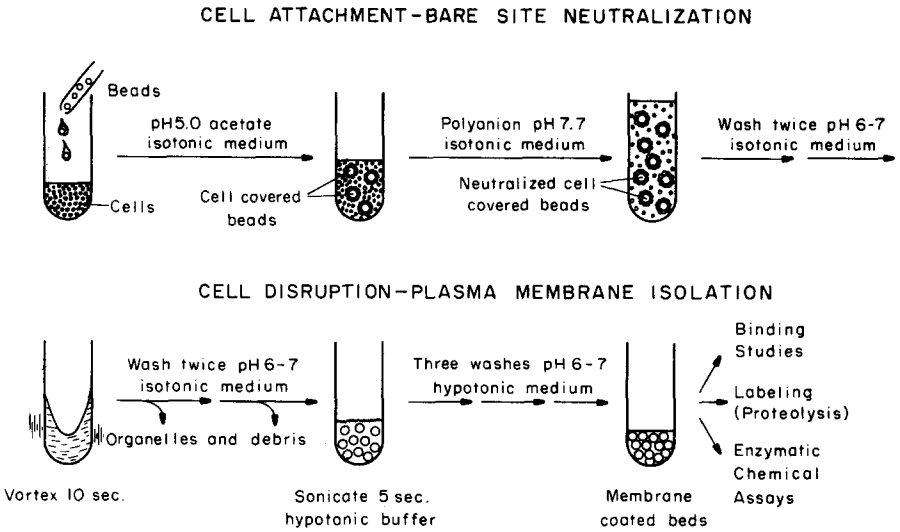

Fig. 1. Diagram of isolation of plasma membrane on polycation coated beads. The exact procedures are given in Materials and Methods. The isotonic buffer contained sorbitol as the osmoticum. The pH of the neutralization medium must be enough to raise the pH of the cell bead mixture above 5.7.

commercially available from Biorad Laboratories, Richmond, CA, sold under the trade name 'Affi-gel 731'. Both bead types worked similarly in all the procedures used. It was essential to wash the Affi-gel several times in 0.2 M NaCl before use. Routinely, beads were washed three times in 5—10 vols. of attachment buffer: 20 mM sodium acetate/140 mM sorbitol, pH 4.9—5.0. The beads were kept on ice and used as a 50% suspension.

Cell harvesting and stabilization

Cells from cultures of *Dictyostelium discoideum* AX-3 grown on HL5 medium as previously described [11] were washed three times in 20 vols. of ice-cold 40 mM KCl/50 mM KPO_4, pH 6.2. The methods of cell suspension and centrifugation were established so as to minimize cell damage. All procedures were done at 0°C to 4°C. Cells were sedimented in 50-ml tubes with a table-top clinical centrifuge run for 2—3 min at 650 × *g*. The supernatant was aspirated and the pellet gently agitated to resuspend the cells in the residual buffer. Rubber policemen, repeated pipetting or streams of buffer were not used for resuspension, since they ruptured many cells. After the final wash in the phosphate buffered KCl, the cells were stabilized (see Results) by suspending the pellet and diluting it with 20 vols. of 100 mM sorbitol/40 mM sodium acetate, pH 4.9—5.0. After 5 min the cells were washed two times in 140 mM sorbitol/20 mM sodium acetate, pH 4.9—5.0 and left as a 50% suspension in the same buffer.

Cell-bead attachment, neutralization and membrane isolation

The amounts of cells and beads and the tube size given below were found optimal for cell attachment and subsequent membrane isolation. They were also appropriate for all assays reported here. All procedures were carried out

on ice. A 50% bead suspension was vortexed and a 0.6 ml aliquot was quickly removed and added dropwise to 0.6 ml of 50% cells in a 13 × 100 mm culture tube. The tube was very gently agitated as the beads were added. Excess agitation results in premature cell rupture and contamination of bare sites with intracellular proteins. The bare sites were neutralized by adding immediately 1.0 ml of 1 mg/ml neutralization reagent in 100 mM sorbitol/20 mM Tris (Sigma), pH 7.7, and immediately but gently mixed. The quantity and pH of the neutralization buffer was sufficient to raise the pH of the cell-bead mixture from 5.0 to 6.5 — a pH value high enough to permit effective neutralization (see Results). If a higher pH is deisred, the pH of the neutralization solution should be raised, but not that of the solution in which the beads and cells are suspended. Following the addition of neutralization reagent, excess cells were removed by washing the beads three times with 130 mM sorbitol/10 mM Mes (Sigma), pH 6.0. After removing the supernatant the beads were vigorously vortexed at the maximum setting on a vortex mixer (Vortex-Genie, Scientific Industries, Bohemia, NY) for 5 s, at which time 1 ml of the pH 6.0 sorbitol solution was added and the sample was vortexed for a further 5 s. More sorbitol solution was added and the beads dispersed. After the beads settled, the supernatant containing intracellular organelles was aspirated and the above vortexing and washing procedure repeated again. To the beads was added 1 ml of 10 ml Mes, pH 6.0, and after the beads were dispersed they were sonicated in a well-type sonifier for 5 s. The beads were washed two more times with the same buffer. At this point the beads were coated by patches of plasma membrane, between which are areas coated by the neutralization reagent.

Protein extraction, quantitation and electrophoresis

A major problem with the bead technique has been the difficulty in concentrating the membrane proteins dissociated from the beads by sodium dodecyl sulfate (SDS) (Sigma) for polyacrylamide gel electrophoresis. Usually an excess solution of detergent is used followed by concentration by ultrafiltration [6,9]. This can be avoided by the procedure given below. After isolation of membranes on beads the tube in which they were suspended was centrifuged for 1 min at the maximum setting in a table-top clinical centrifuge and the supernatant was removed. The protein was removed from the beads by adding 35—70 μl of the following elution solution warmed to 70°C: 10% SDS/10 mM Na_2EDTA/80% glycerol/50 mM dithioerythritol (Sigma) and the tube was vortexed and immediately placed in a water bath at 70—80°C. After 3—5 min the tubes were vortexed and placed back in the water bath for an additional 4 min. The beads and the elution solution were transferred to the upper tube of a bead filter apparatus. The apparatus was made up of two 0.5-ml polyethylene tubes (Bolab, New Hampshire), one on top of the other. The bottom of the upper tube was cut off and covered by a piece of nylon monofilament net (5—7 μm mesh opening; Tetko, Inc., New York) and pressed into the top of the lower tube. They were held together by tape. The bead filter was centrifuged at maximum speed in a clinical table-top centrifuge for 1 min. Eluted proteins were recovered in the bottom tube. The solution was either frozen by immersing the tube into powdered solid CO_2 or was immediately used for electrophoresis. If the solution was frozen, the tubes were

incubated for 5 min in a water bath at 70°C and vortexed before electrophoresis. None of the polyanions used here interfered with the electrophoresis of proteins. Unless otherwise stated, 75 μl of solution was added to wells in the stacking gel. The wells were 6 × 25 mm and 1.2 mm thick. The slab gel system was identical to that previously used with *Dictyostelium* except in that the pH of the stacking gel was 6.0 and the running gel was 8—15% acrylamide [11].

The total protein on the beads was eluted and assayed by methods similar to those previously described [8,12]. Briefly, 0.8 ml of 5% SDS was added to 0.3 ml of beads and after heating for 5 min at 70—80°C the tubes were centrifuged for 1 min at the maximum speed with the clinical centrifuge. Beads interfere with the protein assay and were avoided by removing 0.5 ml of supernatant instead of the total amount. The supernatant was placed in 0.5 ml of 4% $NaCO_3$/2 N NaOH. Typically, from 60 to 70% of the protein is recovered. While vortexing the tube, 0.2 ml of 1 N phenol reagent was added to detect the tyrosine residues. Samples were read at 750 nm after 60 min at 20°C. Bovine serum albumin was used as the standard.

Enzymatic and chemical assays

Lipids were extracted and analyzed as previously described [6,12]. DNA and RNA were determined from their respective sugar contents [13,14]. Acid and alkaline phosphatases were assayed according to standard methods as modified for *D. discoideum* [15]. Cytochrome oxidase and NADH : cytochrome *c* oxidoreductase were measured as previously described [16]. Malate dehydrogenase was measured by following the disappearance of reduced NAD spectrophotometrically [17].

Results

Cell stabilization

A problem that has contributed to reduced membrane purification is the rupture of some of the cells during the first step of the bead membrane isolation procedure, which is the attachment of intact cells to the beads. The rupture occurs since attachment is so tight that the cells are stretched and distorted. The ruptured cells release intracellular debris which contaminates adjacent bead regions. Halting premature cell rupture is critical so that the bare regions between attached cells can be neutralized, thereby preventing contamination by intracellular debris released during the deliberate disruption of the cells.

Cell stabilization and plasma membrane purification from *D. discoideum* were improved by conducting cell attachment at pH 5.0 in acetate buffer rather than at higher pH values, e.g. pH 6 and 7. As with HeLa cells and platelets [8,9] the choice of buffer was critical. Phosphate could not be substituted for acetate in the above and the results with Mes were variable. The buffer that worked contained acetic acid, which has been shown to penetrate membranes readily [19,20]. The effect of the buffer on cells is depicted in Fig 2. Cells were treated with isotonic sorbitol buffered with 50 mM acetate. At pH 5, 6 or 7, cells appeared the same under phase contrast microscopy even

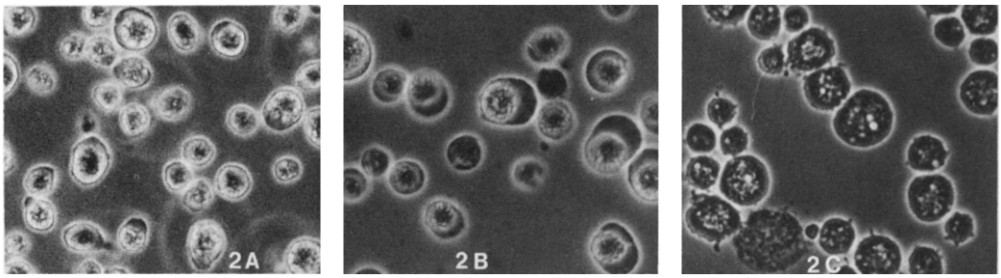

Fig. 2. Effect of low pH on cell stabilization as viewed with phase contrast microscopy. A, cells in isotonic sorbitol buffered to pH 5, with acetate. B, cells transferred from isotonic sorbitol, pH 5 to a hypotonic medium at pH 5.0. C as in B, but pH 7. Magnification 330X.

after 24 h on ice (Fig. 2A). After 90 min in the isotonic buffer, cells were either homogenized or transferred to a hypotonic medium (10 mM acetate). At pH 5.0 it was difficult to homogenize cells, while at pH 6 and 7 the cells disrupted easily. The effect of stabilization was better visualized when the cells were transferred to hypotonic media. At pH 5.0 the plasma membrane responds osmotically and swells away from a tightly congealed cytoplasm (Fig. 2B). At pH 6 or 7, the cells swell and break and the cytoplasm fills the swollen cells and flows out upon rupture (Fig. 2C: note the swollen intracellular organelles). The pH 5.0 treatment causes the cytoplasm to congeal and apparently holds the cell together during its attachment to beads, since more cells are seen to remain intact at this pH than after attachment at pH 6 or 7. At the higher pH values the cells break in response to attachment and the cytoplasm flows out contaminating adjacent bead surface. The congealing effect is reversible when the pH of the buffer is raised to 7.0. The critical or maximum pH was found to be 5.0 to 5.3 based upon titration of cells with acetic acid.

Bead neutralization

Various polyanions were screened for their ability to block or neutralize the cationic bead surface to prevent the attachment of intracellular proteins. It should be noted that the polyanions did not dislodge cells from the beads when *D. discoideum* was used, but erythrocytes were dislodged to the extent of 25% at 1 mg/ml polyanion.

The effect of pH on the adsorption of intracellular *D. discoideum* proteins to the surface of cationic beads neutralized with polyacrylate was determined by gel electrophoresis. The results are depicted in Fig. 3. A cell homogenate was made by shearing the cells in a mortar and pestle type homogenizer. This would best approximate the conditions of cell disruption used during the bead isolation scheme (Fig. 1). Untreated beads at all pH values bound high quantities of protein. The most intensely staining band comigrated with actin (Fig. 3, lanes 1, 2 and 3). Polyacrylate-treated beads at pH 5 bound about 60% the amount of protein bound by the untreated beads. However, the protein composition was distinctly different (compare lane 1 with 4). At pH 6 and 7 very

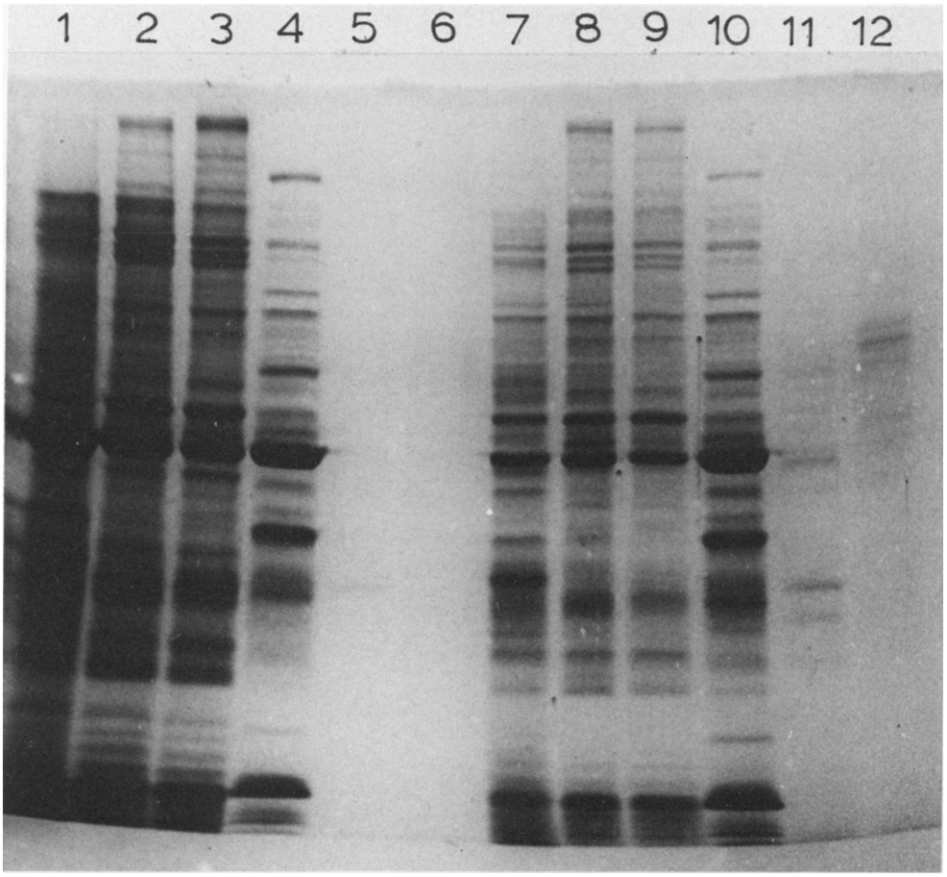

Fig. 3. Effect of pH on the binding of proteins from a total cell homogenate to cationic beads neutralized with polyglutamate. Lanes 1—3, polylysine-coated untreated beads; lanes 4—6, neutralized. Lanes 7—9, polyethyleneimine-coated untreated beads (Affi-Gel 731), lanes 10 to 12, neutralized. Lanes 1, 4, 7, 10, pH 5.0; 2, 5, 8, 11, pH 6.0; 3, 6, 9, 12, pH 7.0. Homogenate prepared in an isotonic solution with a Potter-Elvehjem tissue grinder. Homogenate supplied to beads was a solution of 2 mg/ml protein. Excess protein was washed away and the protein adsorbed to 0.3 ml of beads was eluted and the components separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Arrow indicates position of actin.

little protein bound to treated beads (Fig. 3, lanes 5 and 6). The conclusion from these experiments is that the blocking or neutralization step cannot be done at pH 5, where the cells are stabilized for attachment to beads. The procedure for blocking was, therefore, arranged such that the addition of polyanion was in solution with sufficient base capacity to raise the bead/cell/polyanion mixture to pH 6 to 7.

If cells are more extensively homogenized than described above, or are sonicated, the binding of intracellular protein occurs even to polyanion neutralized beads at pH 6 or 7, albeit to a much smaller extent than to the untreated beads. To test for contaminant protein binding, the untreated or neutralized beads were incubated in sonicates containing 4-fold higher protein concentra-

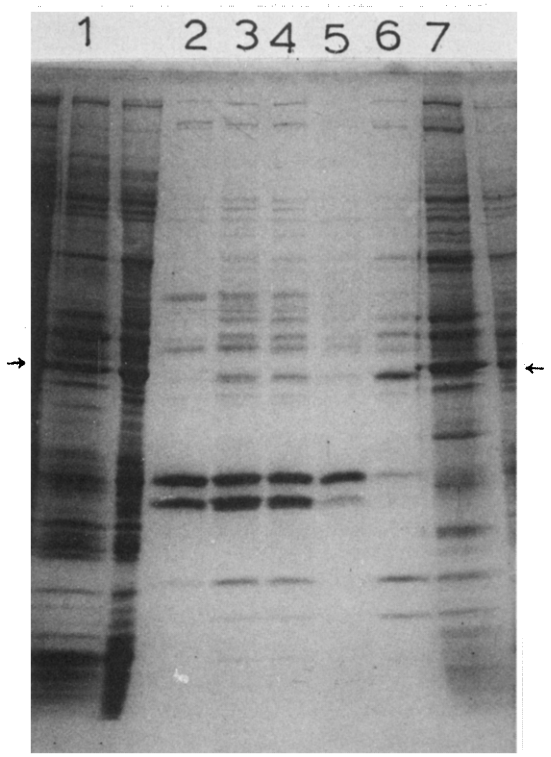

Fig. 4. Effect of various polyanions on preventing cellular proteins from binding to polylysine coated beads. Beads were treated with polyanion at 2 mg/ml in 10 mM Mes, pH 6.0. After the beads were washed free of excess polyanion, a cellular homogenate prepared by sonication in the same buffer without polyanion was added to the beads at 8 mg/ml protein. Each lane of the gel electropherogram is the total protein adsorbed to 0.3 ml beads. Lane 1, cell homogenate on polylysine coated beads; lanes 2, 3, 4, beads neutralized with polyacrylate with respective molecular weights of 5000, 90 000 and 300 000. Lanes 5, 6 and 7, neutralized respectively with polyglutamate, dextran sulfate and silica sol, 250 Å diameter. Arrow indicates position of actin.

tions than that obtained during the routine cell rupture procedure. Contaminant binding was determined by electrophoresis of bead extracts (Fig. 4). A comparison of bead neutralizing reagents indicated that silica, which has the greatest negative charge density, induces binding of almost as much protein as non-neutralized beads (Fig. 4, compare lane 1 with 7). Neutralization with polyglutamate (26 000) polyacrylate and dextran sulfate (40 000) prevented the binding of 70—80% of the protein, but of the protein that bound some bands constituted a larger percentage of the total protein than found with the untreated beads (Fig. 4, lanes 2 to 6). The amount and type of proteins that contaminate neutralized areas can be partly modulated by using different chain lengths of the same polymer or by carefully controlling the concentration of polyanion (Fig. 4, lanes 2, 3 and 4). Therefore, depending upon the types of experiment for which the bead-bound membranes are to be used, an evaluation of the potential blocking reagents should be made before selecting the conditions to be used.

Neutralization during membrane purification

Evidence supporting the improvement of plasma membrane isolation by the neutralization of the bare sites between bead-bound cells (Fig. 5A) was found in both morphological and biochemical studies. The neutralization reagents used in the studies are either low molecular weight polyacrylate or polyglutamate. Both polymers worked well at reducing contamination of neutralized beads by total cell protein.

Morphological evidence for bare-site neutralization was evaluated by scanning electron microscopy. Beads with attached cells were disrupted either before polyanion treatment or after it. If cell disruption was done in an isotonic buffer, non-neutralized bare sites became contaminated by intracellular organelles to the point at which the bare sites were indistinguishable from the inside of the ruptured cells (Fig. 5B); however, neutralization before cell

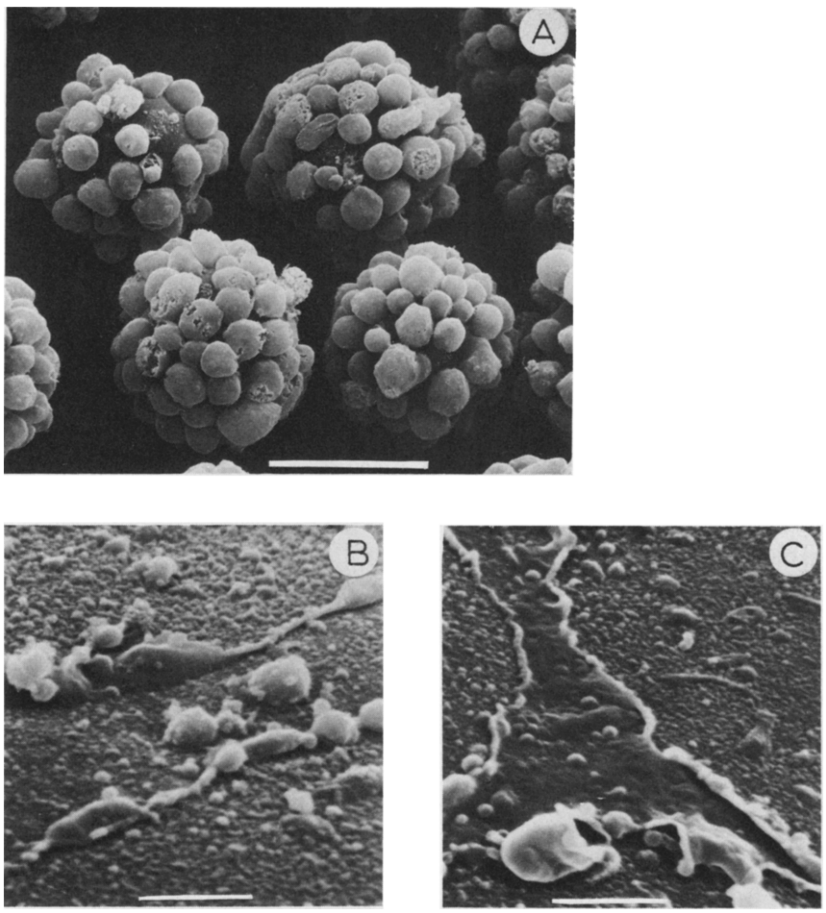

Fig. 5. Scanning electron micrographs of *Dictyostelium discoideum* bound to polylysine-coated beads. A, range of cell coatings of beads generally observed; note bare sites between cells. B, ruptured cells attached to beads showing intracellular organelles within the cell and contaminating the bare sites between the cells. C, as in B except that bare sites were neutralized with polyglutamate before cells were disrupted. The scale is 100 μm for A and 10 μm for B and C.

TABLE I

EFFECT OF NEUTRALIZATION ON BEAD-BOUND MARKERS DURING PLASMA MEMBRANE ISOLATION

The assays were devised such that the amount of protein from plasma membranes on the beads was enough to give at least 10% of the activity of an equivalent amount of protein in a total cell homogenate. Therefore, n.d., not detectable, indicates that the specific activity on the beads relative to the total cell homogenate was less than 0.1. All assays were corrected for the presence of beads and each value is an average of two experiments with triplicates and a S.E. of less than 10%.

Marker	Source of marker	Specific activity on beads relative to cell homogenate	
		No polyacrylate	Polyacrylate (1 mg/ml)
^{125}I-labeled concanavalin A	Plasma membrane	18	36
Alkaline phosphatase	Plasma membrane	7	—
Acid phosphatase	Lysosome	0.18	0.08
Cytochrome *c* reductase	Endoplasmic reticulum	0.1	n.d.
Cytochrome *c* oxidase	Mitochondria	n.d.	n.d.
Malate dehydrogenase	Soluble	0.4	0.1
Actin *	Soluble	3	n.d.

* Determined as percent of total present by scanning gel from electrophoresis.

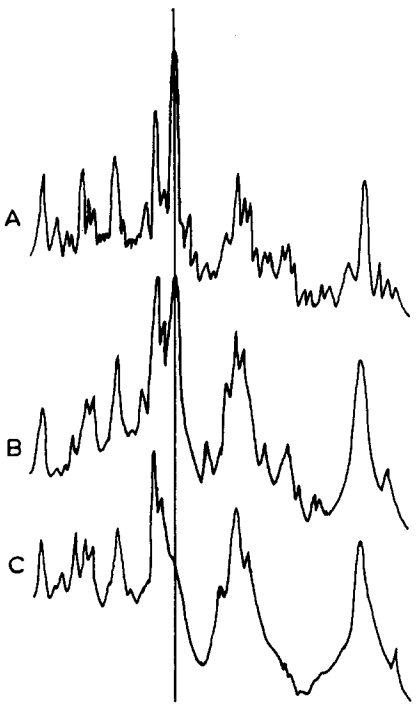

Fig. 6. Scans of gel electropherograms showing the effect of neutralization of bare sites during membrane isolation. Scan A, total cell homogenate. Scan B, plasma membrane isolated on beads without neutralization. Scan C, as B but bare sites neutralized with 1 mg/ml polyglutamate before cell disruption. Gels were scanned with a Gelman integrating scanner. Plasma membranes on beads gave 24 separate integrable peaks. Top of the gel is on left and line indicates position of actin.

TABLE II

EFFECT OF NEUTRALIZATION ON BEAD-ASSOCIATED CHEMICAL CONSTITUENTS DURING MEMBRANE ISOLATION

Each value is an average of three determinations with an S.E. of less than 10%.

Constituent (mg/ml bead)	Neutralization reagent	
	None	Polyacrylate (1 mg/ml)
Protein	450	315
Phospholipid	140	129
DNA	3.6	3.6
RNA	7.6	4.9

rupture markedly reduces bar-site contamination (Fig. 5C).

Biochemical evidence for bare-site neutralization can be most readily obtained be demonstrating an enhanced degree of purification of a plasma membrane marker and a decrease in purification of markers for intracellular components. Table I gives the data for a series of experiments using polyacrylate as the neutralizing reagent; polyglutamate gave similar results. All non-plasma membrane markers were decreased by neutralization whereas the purification of the plasma membrane as determined by labeling with ^{125}I-labeled concanavalin A was doubled. One of the most prevalent proteins in the cytoplasm of the cell is actin [18] and its contamination is markedly reduced by the polyacrylate treatment (Table I). In this connection, polyglutamate was more effective than polyacrylate at preventing actin binding to neutralized beads (Fig. 4).

The greatest effect of neutralizing the bare sites after cell attachment was the reduction of the total amount of bead-associated protein (Table II), much of which was most likely actin, as shown in Fig. 6. Generally, the amount of ^{125}I-labeled concanavalin A labeled plasma membrane was not reduced. As expected from these results, the amount of bead-bound phospholipid was not significantly reduced (Table II). DNA was not reduced by neutralization but RNA was. It was exceptionally difficult to quantitate both of these compounds, as the beads had a variable and marked effect on the respective assays. Contamination due to DNA was reduced by disrupting the cells in isotonic media. It should be noted that while RNA and DNA are determined by their respective sugars, the contamination could be a result of binding to attached membrane or neutralized bare sites by the associated proteins, most of which are cationic [21].

Discussion

Previous methods to isolate the plasma membrane of *D. discoideum* were lengthy and relied upon homogenization and centrifugation procedures [11, 15,22]. In faster procedures, ghost preparations made with detergents or by freeze-thawing in $ZnCl_2$ solutions were purified either by centrifugation or by partitioning between a two-phase aqueous polymer system [22—24]. Where

information could be obtained about the degree of plasma membrane purification, it was in the region of 20-fold [22] and some enzymes were lost of their activities decayed [15,22]. Ghost preparations gave the lowest degrees of purification; usually 4- to 10-fold. In all procedures, the sidedness or orientation of the membrane was not in question and the preparations were probably a mixed population of vesicles. In some cases, two to three populations of plasma membrane vesicles, as judged by presumed enzyme markers, have been isolated from one cellular homogenate [15,22]. The improved bead technique routinely provides membranes of 36-fold purification. The technique is quick, thus reducing the time the membrane contacts degradative lysosomal enzymes, and in addition, it maintains the cytoplasmic surface exposed while the external surface is shielded by apposition to the bead surface [6,10]. It should be pointed out that previous methods of plasma membrane preparation were for particular needs and the bead technique is not necessarily a replacement for all of them.

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