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PLASMA MEMBRANE ISOLATION ON DEAE-SEPHADEX BEADS

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Summary

A much-simplified method for the purification of plasma membranes of cultured cells is presented, based upon the attachment of viable cells to nitrocellulose-treated DEAE-Sephadex beads, and their subsequent shearing by hypotonic lysis, agitation on a vortex mixer and sonication. The method is suggested by an older procedure involving attachment to poly-(L-lysine)-coated glass or polyacrylamide beads; the preparation involved in the present method, however, is considerably easier, more rapid and less expensive. Recovery of L-cell plasma membrane marker enzyme activities is approx. 25%, while contamination by internal membrane markers is much less than 1%.

A number of techniques have recently been introduced for the isolation of cell plasma membranes based upon the general principle of adherence of whole cells to appropriately prepared solid supports, followed by a shearing away of the cell contents. While some of these methods involve a covalent bonding of the cell surface to another substrate [1], most take advantage of the tendency of the negatively charged cell to adhere tenaciously to some positively charged surfaces. The procedures of Branton and co-workers [2,3], in which cells are attached to glass [2] or polyacrylamide [3] beads that have been coated with poly-(L-lysine), have proved to be particularly useful. However, the preparation of the beads is relatively time-consuming and expensive, so much so that the beads are recycled for further use after each experiment [3]. Glass beads are successively silanized, succinylated and polylysinated [2], while polyacrylamide beads are carboxylated and treated with a carbodiimide reagent and poly-(L-lysine) [3]; both treatments require

several days. In the present report, we present an alternative method of plasma membrane isolation in which beads are attached to nitrocellulose-treated DEAE-Sephadex beads. Materials are inexpensive and preparation requires only a few hours. Purification and yield for plasma membranes of a cultured cell line compared favorably with values reported for other methods.

Beads were prepared by swelling 5 g of DEAE-Sephadex (Sigma A-50-120, particle size 40—120 μm) in 500 ml of 0.5 M NaCl, then washing successively with 0.5 M NaOH, 0.5 M NaCl, 0.5 M HCl, phosphate-buffered saline, and two changes of absolute CH_3OH. Beads were then suspended in 500 ml of CH_3OH with 0.1% (w/v) nitrocellulose (Parlodion strips, Mallinckrodt) for 30 min and this was followed by thorough washings with CH_3OH then phosphate-buffered saline. Beads were stored in the latter at 4°C with 1% antibiotic/antimycotic (Gibco).

LM cells (a gift of Dr. Richard Pagano) were maintained in Earle's minimum essential medium with 12% fetal bovine serum, 1% antibiotic/antimycotic, and 0.5 mg/ml supplemental glucose, and were routinely harvested with a mixture of trypsin (2 mg/ml), collagenase (0.02 mg/ml), and 2.5% chick serum in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salts solution.

Procedures for attachment of cells and membrane isolation closely followed those of the method of Cohen et al. [3]. Briefly, cells and beads were washed well in an attachment buffer consisting of 7 vols. of 310 mM sucrose and 3 vols. of 310 mosM acetate buffer, pH 5.0. (In common with published results [3], we found this buffer and pH to be optimal for attachment.) Cells were added to beads at a ratio of 20:1, in a final volume of about 20 ml for 10^7 cells, and this mixture was agitated gently on a rotary shaker or in a spinner flask for 30 min at 20°C. A maximal attachment of about 75% of cells added was attained in this time, at a rate that was independent of temperature between 2 and 37°C. Ionic strength was then partially restored with the addition of about 1/2 vol. of growth medium, and cells and beads were allowed to spread by incubating for 2 h at 37°C. Beads with attached cells were then transferred to a large conical tube, placed on ice, and allowed to settle for 5 min. Supernatants of this and each subsequent step were decanted and concentrated for enzyme analysis by ultrafiltration over an Amicon PM 10 filter. Approx. 30 ml of ice-cold distilled water were added to the settled beads and, after hypotonic lysis of the attached cells was observed (in about 10 min), the beads were washed twice in cold 10 mM Tris-HCl, pH 7.4. 10 ml of the same Tris-HCl buffer were then added and the beads were agitated on a vortex mixer for 10 s followed again by two washes. The beads were then sonicated for 10 s at 1.5 A in an MSE Ultrasonic, and washed twice again. This treatment left the beads undamaged, but completely denuded them of visible cellular debris; using an identical procedure with poly-(L-lysine)-coated beads, Cohen et al. [3] have observed adherent plasma membrane sheets by scanning electron microscopy.

A variety of membrane-associated enzymes were assayed directly on beads (with membranes attached) and on supernatants of successive steps in the purification procedure. Purifications and recoveries for these enzymes, and for DNA and phospholipid, are shown (Table I). A 12—16-fold enrich-

TABLE I

MEMBRANE-ASSOCIATED ENZYME ACTIVITIES OF BEAD-PURIFIED LM CELL MEMBRANES

All specific activities are expressed as μmol product/min per mg protein. Numbers in parentheses after assays indicate references for assay procedures. Numbers in parentheses after cellular components indicate references assigning given enzyme activities to those components. In phosphate-evolving assays, phosphate was assayed by using the method of Chen et al. [25]. To control for the effects of beads on enzyme activities, whole cell homogenates were assayed in the presence or absence of beads, and the final values corrected for any deviations (which in no instance exceeded 25%).

Assay	Cellular component	Specific activity			% recovery	
		Whole cell homogenate	Membranes on beads	Purification factor	On beads	Total
Leucine aminopeptidase (8)	plasma membrane (19)	1.7	27	16 X	27	78
5′-Nucleotidase (9)	plasma membrane (20)	8.8	105	12 X	19	70
$(Na^+ + K^+)$-ATPase (10)	plasma membrane (21)	3.1	41	13 X	20	70
Glucose-6-phosphatase (11)	endoplasmic reticulum (22)	1.2	0.31	0.25 X	0.5	63
Cytochrome *c* reductase (12)	endoplasmic reticulum (22)	0.26	0.08	0.31 X	0.2	68
Succinate dehydrogenase (13)	mitochondria (23)	6.1	0.22	0.04 X	0.01	87
Acid phosphatase (14)	lysosomes (24)	0.016	0.0022	0.14 X	0.2	89
DNA (μg/mg protein)(15)	—	85	22	0.25 X	0.2	88
Phospholipid (μmol/mg)(16, 17)	—	0.38	3.8	9.9 X	9	93
Protein (18)	—	—	—	—	0.3	79

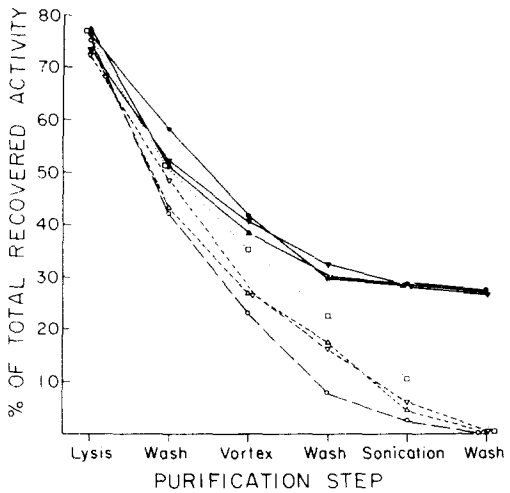

Fig. 1. Stepwise purification of membrane-associated enzyme activities on beads, expressed as percent of total activity recovered. These data are calculated from the cumulative recoveries of activities in supernatants; similar results were obtained when selected enzymes were assayed on beads at each step. Enzymes are leucine aminopeptidase (●), ouabain-sensitive ($Na^+ + K^+$)-ATPase (▼), 5′-nucleotidase (▲), succinate dehydrogenase (○), cytochrome *c* reductase (▽), glucose-6-phosphatase (△) and acid phosphatase (□).

ment was achieved for plasma membrane marker enzymes, leucine aminopeptidase, 5′-nucleotidase, and ouabain-sensitive ($Na^+ + K^+$)-ATPase. Endoplasmic reticulum markers, glucose-6-phosphatase and cytochrome *c* reductase, were depleted 3—4-fold, and even greater reductions were found with a mitochondrial marker, succinate dehydrogenase, and a lysosomal marker, acid phosphatase. About a quarter of the plasma membrane-associated activities were recovered on the beads, compared with less than 1% of internal membrane markers. Total recoveries of all enzyme activities, on beads and in washes, ranged from 60 to 90%.

Recoveries of these enzymatic activities on beads, expressed as a percent of total recovered activity, were also followed at each step of the purification procedure (Fig. 1). As can be seen, recoveries of plasma membrane markers decreased through several steps, then levelled off at about 25%. Internal membrane markers were ablated steadily, to less than 1% in the final step. The relative rates of disappearance would seem to indicate that mitochondria were removed most rapidly, followed by endoplasmic reticulum, then by lysosomes; these differences may be due to varying affinities of these organelles for either the cytoplasmic surfaces of the attached membranes or exposed areas of bead surfaces. The similar recoveries observed with three different plasma membrane markers suggest a uniform sampling of the cell surface. Nevertheless, with only a quarter of the membrane being recovered, the possibilities of selection of cell populations or of fractionation of membrane domains must be borne in mind. Even so, these yields compare favorably with other bead-purification techniques [3], as well as more conventional methods [4].

We have had similar success in isolating plasma membranes from a number of cell lines of varying morphology (Searls, D.B. and Edidin, M., un-

published data). Cholesterol-to-phospholipid ratios for these cells were enriched 2—3-fold by bead purification, a factor consistent with published results [3].

The function of the nitrocellulose treatment of the DEAE-Sephadex is apparently to decrease the surface charge of the beads [5]. Van Wezel [6] originated this method in order to reduce the toxicity to cells of beads used in 'microcarrier' culture techniques and Vosbeck and Roth [7] found that nitrocellulose treatment was necessary to obtain sufficient binding of cells to beads for adhesion studies (Ref. 7, and Roth, S., personal communication). Similarly, we have observed that untreated DEAE-Sephadex produced less efficient attachment with several cell types, including erythrocytes.

At the ratio of cells-to-beads (20:1) used in this study, cells did not completely cover the surfaces of the beads; nevertheless, this ratio could not be increased without a sharp drop in the number of cells attached, and no increase in total bead area covered. This, and the observation that some beads (or sections of beads) were more heavily coated with cells than others, suggest that the nitrocellulose coating may not be uniform. This would also help to explain why subcellular components apparently do not adhere after lysis, despite the large bead area not covered by cells; these areas may be more heavily coated with nitrocellulose, and thus be unable to bind either cells or contaminants. With this possibility in mind, we are currently investigating several new bead products designed specifically to carry uniform charges appropriate for microcarrier culture (Pharmacia Cytodex and Bio-Rad Biocarriers), in hopes of obtaining even higher efficiencies in this system. Work is also in progress to develop methods for removing membrane sheets from beads; the most promising possibilities thus far are increased ionic strength at high pH, and trypsinization.

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