MEMBRANE ISOLATION ON POLYLYSINE-COATED BEADS

Plasma Membrane from HeLa Cells

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ABSTRACT

HeLa cell plasma membranes have been purified after binding cells to polylysine-coated polyacrylamide beads. Cell attachment to beads and membrane recovery were maximal in a sucrose-acetate buffer, pH 5.0, at 25°C. Measurements of ouabain-sensitive NaK-adenosine triphosphatase, membrane-bound 125I-wheat germ agglutinin, and chemical analyses showed that membranes on beads were of comparable or greater purity than membranes isolated by conventional methods. Because the isolation procedure is rapid (~2.5 h), and produces membranes whose protoplasmic surfaces are fully exposed, it should be a useful supplement to standard isolation techniques.

KEY WORDS membrane isolation · polylysine-coated beads · plasma membrane · HeLa cell · membrane composition

Many cell types can be obtained in pure form and in large quantity, and in some cases, methods for the isolation of their plasma membranes have been described (13). However, the majority of membrane isolation procedures that are rapid (e.g. aqueous two-phase polymer separations) provide minimal purification, and many of those that are more reliable (e.g. cell fractionation by rate zonal and isopyknic density-gradient centrifugation) are time consuming.

We have devised a rapid method for the isolation of plasma membranes on polylysine-coated acrylamide beads (9, 23). Cells with a net-negative surface charge tenaciously bind to the beads which have a positive surface charge. When bound cells are sheared from the beads by vigorous vortexing, large patches of plasma membrane remain behind. Attachment to the surface of large acrylamide beads, an extension of density perturbation methods (37, 43, 46), facilitates membrane sedimentation, and under appropriate conditions, may provide access to the protoplasmic surface of the membrane.1 This paper shows that the method can be used to isolate and study HeLa cell plasma membranes, and compares bead-isolated membranes with those isolated by other techniques (3, 5, 6, 24).

MATERIALS AND METHODS

Materials

Poly-lysine (85,000 ≤ mol wt ≤ 250,000, used without further purification), crude wheat germ lipase (type I), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl (EDC), ouabain, and Tris-adenosine triphosphate (ATP) were obtained from Sigma Chemical Co., St. Louis, Mo. Carrier-free 125I was obtained from New

England Nuclear, Boston, Mass., and Bio-Gel P-2 (200-400 mesh) polyacrylamide beads were obtained from Bio-Rad Laboratories, Richmond, Calif.

**Derivatization of Polyacrylamide Beads**

All procedures were performed at room temperature unless otherwise indicated. 5 g of Bio-Gel P-2 was added to 125 ml of water and incubated in a 90°C water bath for 3 h with periodic stirring. After removal from the water bath, the beads were sonicated for 15 s, using the semimicroprobe of a Heat Systems sonifier model 185D (Heat Systems-Ultrasonics Inc., Plainview, N. Y.) set at 50 W. The beads were allowed to settle for ~10 min, and after aspiration of the supernate, they were washed twice in distilled water. After removal of the final supernate, 100 ml of 0.5 M Na2CO3 was added, and the beads were incubated with intermittent stirring in a 60°C water bath for 2 h. The beads were then washed six times in distilled water, each time allowing them to settle for 10 min at 1 g. The supernate of the final wash had a pH within 0.5 pH U of distilled water.

The beads were allowed to settle, and after careful removal of the supernate, 5 ml of 1 M pyridine containing 200 mg of dissolved polylysine was added. The vessel which held the polylysine-pyridine was rinsed with 1 ml of 1 M pyridine which was added to the beads. After covering the vessel tightly with parafilm and agitating on a rotary shaker (250-300 rpm) for 1.5 h, 6 ml of 2.6 M pyridine-HCl (pH 5.1) was added to bring the pH of the suspension to ~5.5. At 1-min intervals, four 0.5-ml aliquots of freshly prepared 1 M EDC were added, and the suspension was agitated on a rotary shaker (250-300 rpm) for 48 h. At the end of this time the beads were allowed to settle, and the pyridine supernate containing unbound polylysine was removed and saved. The beads were washed once with 10 ml of distilled water which was combined with the pyridine supernate. 10 ml of 1 M NH4Cl and 1 ml of 1 M EDC were added, and the beads were again agitated on a rotary shaker for 3-4 h. The beads were finally washed in six to seven changes of distilled water, and stored in 10 mM Tris, pH 7.4, with 0.02% Na azide at 4°C.

Reclaimed beads were used for cell attachment.

**Preparation and Use of °°I-WGA**

WGA was purified from crude wheat germ lipase and labeled to high specific activity (4-8 μCi/mg) by standard techniques (10, 11, 30). °°I-WGA was bound to cells by adding 125-250 ng of °°I-WGA (containing ~2.5 x 10^6 dpm) to each ml of a 50% cell suspension in PBS plus 0.5% (wt/vol) bovine serum albumin (BSA), and incubating for 15-30 min at room temperature. Cells were then washed four times in 10 vol of ice-cold PBS and twice in the buffer to be used for attachment to beads, whether or not they were labeled.

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HeLa Cells

HeLa-S3 cells, grown at the Cell Culture Center of the Massachusetts Institute of Technology, in spinner culture in Roswell Park Memorial Institute medium 1640 with 5% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin were harvested in the late log phase of growth at a cell density of ~8 x 10^5/ml. The cells were sedimented from their growth medium in a Sorvall GSA rotor (Du Pont Co., Instrument Products Div., Wilmington, Del.) at 3,000 rpm (1,000 g) for 10 min and resuspended in phosphate-buffered saline (PBS). The cells were then washed four times in 10 vol of ice-cold PBS, each time sedimenting them for 1,700 g-min in a clinical centrifuge at 4°C. Cells were then taken for labeling with °°I-WGA, if applicable. The cells were then washed an additional three times in PBS and twice in the buffer to be used for attachment to beads, whether or not they were labeled.

Reclaimed beads bound cells as well as freshly prepared beads. Membrane purification (wheat germ agglutinin [WGA] assay—to be discussed) of membranes isolated on reclaimed beads was the same as that on fresh beads, but we have not performed extensive analyses of membranes isolated on reclaimed beads.

Reclaiming Used Beads

The beads were pooled after use and stored in 10 mM Tris, pH 7.4 at 4°C with 0.02% azide. When enough beads were accumulated (10-50 ml), they were washed twice in 10-20 vol of distilled water, and then suspended in 20 vol of a 1% solution of sodium dodecyl sulfate (SDS). The suspension was agitated and vigorously shaken for ~1 min, followed by a 15-s sonication, using a Heat Systems sonifier with a semimicroprobe set at an output of 20 W. The beads were then allowed to settle, and, after removal of the supernate, were washed once more with 1% SDS as previously described. The beads were then washed six to eight times in 20 vol of distilled water, each time vigorously shaking them and then allowing them to settle at 1 g for 10 min. Finally the beads were given one wash in 20 vol of 10 mM Tris, pH 7.4, with 0.02% azide, and stored at 4°C in this medium.

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Sampling of Beads

Membranes on beads were always suspended to 50% vol/vol in 10 mM Tris, pH 7.4, and briefly vortexed before each sample was withdrawn. Samples were usually taken with a Selectapette (Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N.J.) equipped with a polyethylene tip.

Enzymatic and Chemical Assays

When membranes on beads were assayed for enzyme activity, beads were kept in suspension by vigorous agitation in a rotary shaker bath. Blank beads, or membranes on beads which had been dematured by heating to 70°C for 10 min, or by treatment with 0.1 N acetic acid, were used as controls for enzyme assays.

Ouabain-sensitive NaK-ATPase was assayed by the method of Wallach and Kamat (41), with the modification that ATP and MgCl₂ were added to a final concentration of 2.2 mM. Membranes on beads were assayed by adding 0.2-0.4 ml of a 50% bead suspension (containing 30-60 μg of protein) to a small polystyrene test tube, carefully removing the supernate after a brief centrifugation, and adding the components of the assay medium; the final volume was 1 ml. The tubes were incubated at 37°C for 0.5 h, after which they were placed on ice; the reaction was terminated by the addition of 0.3 ml of 30% TCA. Beads were sedimented by a brief centrifugation, and the supernate was measured.

Glucose-6-phosphatase (used as a marker for endoplasmic reticulum and outer mitochondrial membranes) and cytochrome c oxidase (an inner mitochondrial membrane marker) were measured by the method of Hodges and Leonard (19), and glyceraldehyde-3-phosphate dehydrogenase (a cytoplasmic marker) was measured as described by Steck and Kant (40). Membranes on beads (0.1-0.4 ml of packed beads) were incubated in the assay medium at 22°C for times ranging from 1 to 20 min (NADH cytochrome c oxidoreductase and cytochrome oxidase assays) or 2 min (glyceraldehyde-3-phosphate dehydrogenase assay), and the absorbance of the bead-free supernate at the appropriate wavelength was measured.

Acid phosphatase (used as a marker for lysosomes) was measured by the method of Hubser and West (21) with the modification that p-nitrophenylphosphate was used as the substrate. Membranes on beads (0.2 ml of packed beads) were incubated with the assay medium for times ranging from 0 to 30 min at 37°C. After termination of the reaction by addition of 0.2 ml of 0.1 N NaOH, the absorbance at 410 nm of the bead-free supernate was measured.

To assess the possibility that enzymes are inhibited by adsorption to the bead, small amounts of cell homogenate or individual enzymes were mixed with blank beads under conditions where more than 90% of the added protein bound to the bead. The percent enzyme inhibition was calculated by comparing the activity of homogenate or enzymes on beads to that in solution. Inhibition of ouabain-sensitive NaK-ATPase and NADH cytochrome c oxidoreductase was also determined by comparing the activities of HeLa cell plasma membranes (prepared by the method of Johnsen et al. [24]) and mitochondria (prepared by the method of Douce et al. [15]) in solution with membranes or mitochondria which were attached to beads, and vortexed and sonicated as described in Results.

RNA was measured by the orcinol method of Dische (14). Beads were thoroughly washed in 10 mM Tris or PBS to remove sucrose, which interferes with the assay. The orcinol reagent was then directly added to 0.5 ml of packed beads, and the mixture was placed in a boiling water bath for 10 min. The absorbance of the supernate was measured at 665 nm. Standard curves were determined by assaying known amounts of RNA in the presence of beads.

DNA was measured by a modification (27) of the method of Burton (7). Beads were carried through the entire assay procedure, and standard curves were determined by assaying known amounts of DNA in the presence of beads.

Phospholipid and cholesterol were extracted from membranes on beads by incubating 0.2-0.4 ml of beads in 10 ml of chloroform-methanol (2:1 vol/vol) overnight. After removal of the supernate, the beads were washed with 2 ml of chloroform-methanol which was added to the first supernate. 2.5 ml of 0.9% (wt/vol) NaCl was added to the pooled supernates, and after vortexing and centrifugation, the upper phase was aspirated. This procedure was repeated by adding 2 ml of water to the lower phase and again aspirating the resulting upper phase. The lower phase was then transferred to a new culture tube, dried under a stream of Ng, combusted, and analyzed for phosphate according to the method of Ames (2). Samples of blank beads were used as controls for the assay. Cell homogenates and membranes in solution (in final volume of 0.3 ml or less) were extracted three times with 4 ml chloroform-methanol 2:1, each time sedimenting particulate matter in a clinical centrifuge at maximum speed for 5 min. The pooled extracts were treated as described above. Cholesterol was measured by the method of Parekh and Jung (35), using crystalline cho-
lesterol in chloroform as a standard.

To assess the phospholipid extraction efficiency, sonicated dispersions of $^{14}$C phosphatidylcholine (PC) and phosphatidylinositol (PI) (PC:PS or PC:PI mole ratio = 9:1) were bound to beads under the same conditions as the cells. The recovery of bound $^{14}$CPC after extraction as described above, was 90-95%.

In all experiments, the same samples of beads which had been counted for $^{3}H$-WGA were used for protein measurements. To determine the amount of protein on beads, 0.15 ml of beads was vigorously vortexed with 0.5 ml of 3% SDS in glass-distilled water. The protein-containing supernate was removed, the extraction was repeated, and protein in the pooled SDS extract was measured by the method of Lowry et al. (29), except that CuSO$_4$ was not included, so that polylysine which might have been removed from the beads did not contribute to color formation. Standards were prepared from crystalline BSA. In all experiments, a sample of blank beads was carried through the same experimental procedure as beads with membranes on them, and extracted with SDS as previously described. The calculated protein from these blank samples was subtracted from that of the samples with membrane. Low blank values were ensured by washing all beads five times in 10 vol of 0.15 M Tris, pH 7.4 before they were used in any experiment.

The efficiency of protein removal from beads by the procedure described above was determined by adding known amounts of cell homogenate to blank beads in either pH 5.0 sucrose-acetate buffer (7 vol 310 mM sucrose + 3 vol 310 mosM Na acetate, pH 5.0) or 10 mM Tris, pH 7.4. The beads were then vortexed, sonicated, and washed as described for membrane isolation (see Results), and then extracted with SDS as previously described. Protein removal from beads was at least 75-90% complete. Similarly, removal of membrane-bound $^{3}H$-WGA of membranes on beads was 85-95% complete.

Preparations were electrophoresed in 5.0% acrylamide gels according to Fairbanks et al. (17). In some cases, proteins which were extracted from beads with SDS, as previously described, were dialyzed against distilled water for 2 days, lyophilized, and resuspended in the solution for gel samples described below. Alternatively, 0.4-0.8 ml of a 50% (vol/vol) suspension of membranes on beads was centrifuged in a small (4 ml) test tube at top speed in a clinical centrifuge for 30 s, the supernate was removed, and 0.05-0.1 ml of a solution containing 2% SDS, 6% (vol/vol) β-mercaptoethanol, 1 mM EDTA, 10 mM Tris (pH 8.0), 2% (vol/vol) glycerol and 0.005% (wt/vol) pyronin Y (tracking dye) was added. The beads were then heated to 60°C for 15 min with intermittent vigorous vortexing. After centrifugation as described above, the supernate was withdrawn and run directly on a gel, or stored at 4°C. Although not all of the protein was removed from beads by this procedure, repeated extraction yielded identical gel patterns of decreasing intensity.

**Electron Microscopy**

Suspensions of cells or beads were fixed for SEM by dropwise addition, over a period of 15 min, with an equal volume of 2% glutaraldehyde in the same medium in which the cells or beads had been suspended. After 0.5 h, the cells or beads were washed in distilled water, postfixed with 1% osmium tetroxide for 0.5 h, rinsed in distilled water, dehydrated in a graded series of ethanol, and critical point dried using CO$_2$ as the transition fluid. Dried samples were coated with carbon and gold-palladium, and examined in an AMR-1000 scanning electron microscope. (A detailed description of attachment of cells and beads to the substrate used in SEM is given by Kalish et al.)

**RESULTS**

**Attachment of Cells to Beads**

Cells and beads were washed twice in the buffer (to be described), and at the temperature to be used for cell attachment; each was then suspended to 50% vol/vol. The bead suspension was briefly vortexed, and 1-ml portions were withdrawn using a Selectapette with a polyethylene tip. Beads were added dropwise to the cells (Fig. 1) as the tube was very gently agitated. This procedure was repeated until a volume of the bead suspension equal to the volume of the cell suspension had been delivered. Generally, 5 ml of beads was added to an equal volume of cells in a 38-ml plastic tube. The cells and beads were incubated together and occasionally mixed by slowly inverting the tube. After 10 min, the tube was filled with buffer, and the beads were suspended by several gentle inversions and allowed to settle at 1 g for about 10 min, and, after removal of the supernate, the beads were washed twice more in the same way. At this point, most of the unbound cells had been removed, and observation in the phase-contrast microscope or SEM showed that the beads were covered with cells (Fig. 2). Some regions on the beads contained adherent sheets of membrane from cells which had already been torn away (arrows, Fig. 2), and other regions were apparently uncovered. We do not know why some regions of the bead did not bind cells even though the cells were present in excess.
Isolation of Membranes on Beads

After the final wash to remove unbound cells, the beads were allowed to settle and the supernate was aspirated. The tube was then vigorously vortexed for 5-10 s (Fig. 1), immediately filled with ice-cold 10 mM Tris, pH 7.4, and mixed by inversion. The beads were then washed four to five times in ice-cold 10 mM Tris, each time allowing them to sediment at 1 g until a distinct pellet was formed. (All beads, regardless of the temperature or the buffer in which cells were attached, were washed in ice-cold 10 mM Tris after vortexing.) If many bound cells were still apparent (by phase-contrast microscopy) after the second wash, the beads were vortexed again. After the fourth wash, few or no cells were bound to beads, and only a small number of unbound cells were present in the solution. If this was not the case, additional washing and vortexing was done. The circular patches of material on the bead surface (Fig. 3) at this stage of the procedure are adhering sheets of plasma membrane and intracellular debris left on the bead as the cells were torn away. Small (<1 μm) vesicular material on and around the patches (inset, Fig. 3) may be mitochondria, released from cells upon lysis or microvilli broken off from or trapped beneath the cell membrane.

The membrane-coated beads were then suspended in an equal volume of ice-cold 10 mM Tris, pH 7.4, and sonicated with the microtip of a Heat Systems sonifier set at 20 W (lowest setting) for 5 s. They were then washed three times in 10 mM Tris, or until all particulate material both on and off the beads, as seen in the light microscope, was removed. At this point, the membranes on the beads were used for the analyses discussed in the text. The time required for membrane isolation was 2.5-3 h, starting from the time the beads were added to the cells.

Examination of membranes on the beads in the SEM, subsequent to sonication, revealed a perfectly smooth surface (not shown), in contrast to the surface before sonication (Fig. 3). Plasma membranes which were left behind cannot be seen on the beads at the resolution of the SEM; the particulate material had either been removed by sonication or disrupted to the point where it could no longer be resolved. However, the continued presence of the membrane patches was demon-
strable by fluorescence microscopy if intact HeLa cells, which had been prelabeled with fluorescein-conjugated WGA, were used (not shown).

**Membrane Markers**

Because lipids are commonly thought to form the membrane continuum, the relative amounts of plasma membrane markers (enzymes and receptors) were correlated with lipid to ensure that selected regions or components of the membrane were not being isolated. The markers used were ouabain-sensitive NaK-ATPase, a well-established component of the HeLa cell plasma membrane (5, 24), and 

\[ ^{125}I \]-WGA which was added to intact cells before they were attached to beads. Chang et al. (8) have shown that WGA is a highly specific label for the plasma membrane. In experiments similar to those of Chang et al., we labeled both intact cells and cell homogenates with WGA, and fractionated them on continuous sucrose density gradients. In both cases, the WGA migrated as a single peak to the same point in the gradient, suggesting that no additional cellular components were labeled with WGA had access to more than just the plasma membrane. The distribution and peak of WGA in the density gradient almost always coincided with ouabain-sensitive NaK-ATPase activity, and was well separated from cytochrome oxidase or NADH cytochrome c oxidoreductase peaks. WGA is not taken into the cells under any of the conditions used, as 85–95% can be removed by 50 mM N-acetyl-d-glucosamine plus 5 mg/ml ovomucoid added to samples of cells taken just before attachment to beads (data not shown). These experiments provide evidence that the plasma membrane is the only cellular component labeled by WGA.
Variables Affecting Cell Attachment and Membrane Recovery

Low ionic strength, which apparently enhances the electrostatic attraction of the negatively charged cell surface to the positively charged beads, was essential to maximize the attachment of cells to beads. We routinely used a medium consisting of 7 vol of 310 mM sucrose plus 3 vol of 310 mosM buffer solution (phosphate, Tris, or acetate).

Observation in the light microscope showed that beads were better covered with cells at pH 5.0 in sucrose-acetate buffer than at pH 7.0. This resulted in more membrane being associated with beads (Fig. 4). The amounts of bead-associated phospholipid, WGA, and ATPase increased as the pH was lowered, as did membrane purification measured by WGA. (Membrane purification was calculated by dividing the \(^{125}\)I-WGA or ATPase specific activity on the beads by that of a sample of cells taken just before addition of beads). This demonstrates that the yield of plasma membrane, and not specific membrane components, was increased at low pH. However, the amount of protein on the beads was not greater at pH 5.0 than at pH 7.0. This is to be expected, as less extraneous or intracellular contaminants would be bound when the beads are well covered by plasma membrane. pH 5 was the value at which subsequent isolations were performed because it was clear that beads should be maximally covered with cells before the cells are broken by vortexing, and because the amount of bead-associated membrane was greater at pH 5 than at higher pH values.

The reason for the enhanced bead coverage at low pH is not immediately apparent. For instance, it is unlikely that the positive charge density of the bead was increased, because the pK of the e-amino group of lysine is 10 and that of the carboxyl group is 2. Further, HeLa cells have a less negative surface charge at low pH due to titratable
The observation that the pH dependence, shown in Fig. 4, was abolished when phosphate was used as the buffer instead of acetate (not shown), suggested that low pH alone was not the determining factor. It may be that undissociated acetate (36% of the total at pH 5.0) was taken up by HeLa cells more rapidly and efficiently than was the phosphate anion, leading to lowered intracellular pH. An additional observation, relevant to this interpretation, is that in pH 5.0 acetate, HeLa cells became extremely rigid and difficult to disrupt, whereas this effect was not seen in phosphate. We have observed that the acetate-treated cells, when torn from the beads by vortexing, remained morphologically intact. Although these cells left behind large regions of plasma membrane on the bead, their interior contents did not appear to escape. In contrast, cells attached to beads at neutral pH released their cytoplasm and nuclei upon vortexing; nuclei from these cells were frequently seen attached to beads. Therefore, the low pH acetate buffer may act in a manner similar to that of the membrane-toughening agents discussed by Warren and his colleagues (45).

With the exception of protein, the recovery of plasma membrane constituents and markers on beads was doubled when cells were attached at 25°C as compared to 4°C (Fig. 5). Phospholipid, cholesterol, WGA, and ATPase all increased by the same proportion, showing that the recovery of membrane, and not selected markers, was enhanced. In addition, membrane purification measured by WGA and ouabain-sensitive NaK-ATPase was also increased (Fig. 6). However, when cells attached at 37°C were compared to those attached at 25°C, the WGA increase was not matched by the phospholipid increase. Furthermore, cholesterol, ATPase, and protein decreased. Raising the attachment temperature from 25°C to 37°C had no effect upon membrane recovery if cells were attached to beads at a pH above 5.0, e.g., pH 6.0 (Fig. 5). Therefore, because temperatures above 25°C at pH 5.0 may foster the isolation of selected membrane components (leading, for example, to the anomalously high 80-fold WGA-measured membrane purification at 37°C, Fig. 6), we routinely attached HeLa cells to beads in sucrose-acetate buffer, pH 5.0, at 25°C.

Under the conditions previously described, ~50% of the added HeLa cells bound to the beads (as determined by membrane-bound WGA,
FIGURE 5 Temperature dependence of membrane recovery on beads as assessed by several membrane markers. 

\[ \text{\textsuperscript{125}I}-\text{WGA-labeled HeLa cells were divided into three aliquots and washed twice in sucrose-acetate buffer, pH 5.0 at the three temperatures indicated. Some samples (WGA, pH 6, in figure) were washed at pH 6.0. After attachment of cells to beads, the samples were washed with their respective buffers to remove unbound cells. Membrane isolation was carried out as described in the text, all samples being washed in 10 mM Tris, pH 7.4, after vortexing. All data points are the mean of three independent determinations on separate samples; scatter in the data points was generally <15% of the values shown.}\]

After sonication and washing, ~15% of the plasma membrane of the cells initially added was recovered on the beads (Fig. 7). Only 50% of the added cells bound because the cells were usually added in excess. The \textsuperscript{125}I-WGA specific activity of the cells remaining unbound was generally within 15% of the initial cell suspension, and, if these unbound cells were subsequently attached to a second batch of beads, membrane purification was nearly identical to that of the first batch. In experiments where only a small number of cells were added, all of the cells bound, however individual cells attached to more than one bead, leading to bead clumping and premature cell lysis.

**Experimental Variability and the Effect of HeLa Cell Growth Density**

From any given sample of membranes on beads, all of the chemical, enzymatic, and isotopic measurements described in this paper generally agreed to within 15% for triplicate measurements. However, there was more pronounced variability from one experiment to the next, the amount of protein on beads varying by as much as 50% from week to week. It may be that small differences in the number of cells which were not washed away before sonication could account for this, as the intracellular contents of cells which were disrupted during sonication may have bound to the beads.

Although a systematic investigation of the effect of cell-growth density on membrane isolation was not done, it was repeatedly observed that HeLa cells grown to low densities (less than \(~5 \times 10^9/\text{ml}\) do not bind to beads as well as those grown to densities close to \(10^9/\text{ml}\). It is noteworthy that the ability of polylysine to agglutinate HeLa cells increases with increasing cell-growth density (12). This phenomenon appears to be related to an increased negative surface charge of cells grown to...
Composition of Membranes on Beads

Membrane purification measured by WGA was consistently greater for bead-purified membranes than for gradient-purified membranes, whereas membrane purification measured by ouabain-sensitive NaK-ATPase was about the same for the two procedures (Table I). Phospholipid:protein and cholesterol:phospholipid ratios were within the ranges previously reported for HeLa and other cell membranes (Fig. 6, Table I).

~12-18% of the total ouabain-sensitive NaK-ATPase of the intact cells on beads remained on the beads after membrane isolation (Table II), whereas only small percentages of nonplasma membrane enzymes were found. Although several of these enzymes were found to be partially inhibited or activated when adsorbed to beads (Table II), correction for this effect does not significantly alter the findings described above. The two major enzymatic contaminants were glucose-6-phosphatase and acid phosphatase, which, to our knowledge, have not been measured in isolated HeLa cell plasma membranes. However, plasma membranes from several other cells have been reported to have specific activities of these enzymes close to or equal to that of the cell homogenate (1, 33, 34, 28), suggesting that these activities may not be unique to a single subcellular structure.

The major contaminant on the beads was DNA (Table I). Several observations have shown that DNA was probably bound to uncovered areas of the bead and not to attached membranes. First, DNA contamination was inversely proportional, from experiment to experiment, to the amount of membrane covering the bead (data not shown). Second, if blank beads were mixed with a cell homogenate, and were vortexed and sonicated as usual, they bound ~ 430 µg DNA/ml beads, and membrane-covered beads bound <13 µg DNA/ml beads under the same conditions. Finally, pretreatment of blank beads with trinitrobenzene sulfonic acid (which reacts with and neutralizes positively charged amino groups) reduced DNA binding from a cell homogenate more than 10-fold. DNA contamination could be substantially reduced by adding negatively charged liposomes to the beads just after cell attachment and before vortexing. These liposomes apparently bound to regions of the bead not occupied by cells, and inhibited the subsequent attachment of nuclear material.

The polypeptide composition of bead- and gra-
gradient-purified membranes differ in several respects (Fig. 8). The most notable differences are the depletion of a polypeptide of 150,000 mol wt and of other peptides of 215,000 mol wt and greater, and the enrichment of a protein of 87,000 mol wt in bead membranes compared to gradient membranes. Although the bead membranes possess fewer major bands than the gradient membranes, the phospholipid:protein ratio for the two types of membrane does not differ by a large amount (Table I). Protein elution studies suggest that the two major differences between bead- and gradient-purified membranes (i.e., the differential association of the 150,000- and the 87,000-dalton proteins) are the result of a gain or loss of peripheral proteins, and not due to accumulation or exclusion of integral membrane proteins. The diminution of high molecular-weight polypeptides on bead membranes was probably not due to proteolysis, because the gel patterns of membranes isolated on beads with 2 mM phenylmethyl sulfonyl fluoride present during the entire procedure were no different from that found in its absence.

**Removal of Membranes from Beads by High Salt Concentrations**

Membranes removed from beads by high salt concentrations appeared as small vesicles and convoluted sheets by phase-contrast or freeze-fracture electron microscopy (Fig. 9). Although only 40% of the attached membranes (as measured by phospholipid release) was removed from the beads, the polypeptide composition (Fig. 8) was almost indistinguishable from membranes solubilized from beads by SDS. Therefore, although small differences may be obscured by incomplete extraction by SDS (see Methods), the major polypeptide species must be bound to the membrane and not directly to the bead, as they all sediment with the eluted membranes.

The appearance of particle-free and particle-rich vesicles in freeze-fractured, bead-isolated membranes may be the result of the salt treatment used to release the membranes, or the result of a polylysine-induced particle aggregation of membranes on beads (16). Such heterogeneity was not characteristic of the intact cell (not shown).

**DISCUSSION**

Our results show that HeLa cell plasma membranes can be purified and isolated on polylysine-coated beads by a procedure which takes advantage of the fact that the plasma membrane is the outermost component of the cell. The bead-isolated membranes were comparable in composition to membranes isolated by conventional methods, and were of comparable or greater purity when assayed using ATPase activity or WGA as membrane markers.

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2 Cohen, C. M. Unpublished data.
TABLE I

<table>
<thead>
<tr>
<th>Plasma Membrane Chemical Composition and Purification</th>
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<td>Whole cell homogenate</td>
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* From reference 6.
‡ From reference 23.
§ Purification calculated as described in text.
|| Uncorrected for inhibition by beads (see Table II).

Cells were attached to beads in sucrose acetate buffer pH 5.0, 25°C, and membranes isolated as described in the text. Gradient membranes were prepared by the method of Johnsen et al. (24) or Atkinson and Summers (3). All values shown for bead-isolated membranes and cell homogenates are the means of at least three experiments; variability was <20% of the values shown.

TABLE II

<table>
<thead>
<tr>
<th>Marker Enzymes in Whole Cells and Plasma Membranes</th>
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</thead>
<tbody>
<tr>
<td>Relative activity of membranes on beads (ratio of specific activity to that in whole cells)</td>
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<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Ouabain-sensitive NaK-ATPase</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>NADH cytochrome c oxidoreductase</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
</tr>
<tr>
<td>Acid phosphatase</td>
</tr>
</tbody>
</table>

* Specific activity is expressed as micromoles of product per milligram of protein per hour.
† These values are not corrected for the measured inhibition given in column 3. Therefore, for example, the corrected relative activity and total activity recovered on beads of ouabain-sensitive NaK-ATPase would be 17.8 and 17–26%; for cytochrome c oxidase, 0.16 and ≤0.1%.
§ Measured as described in Materials and Methods.
|| Given as percent of total activity of cells on beads before vortexing.

We found that membrane purification and recovery, as measured by WGA, was consistently higher than when measured by ouabain-sensitive NaK-ATPase (Table II, Fig. 7). Furthermore, the cholesterol:phospholipid ratio of the isolated membranes changed when the temperature of cell attachment was changed (Fig. 6). There are three likely explanations for such results. First, enzymes (e.g., NaK-ATPase) assayed while the membranes were on beads may have been inhibited by their proximity to the positively charged bead surface. Second, WGA may have been released from the membrane and reassociated directly with the bead. Third, selected regions or constituents of the membrane may have been preferentially included or excluded from membranes on beads.

The first explanation could account for only part of the discrepancy between the ATPase- and WGA-measured purification, because the ATPase-specific activity of membranes prepared by the method of Johnsen et al. (24) was reduced only 30% when these membranes were adsorbed to beads (Table II). The second explanation could account for part of the high WGA recovery on beads at 37°C, but probably not at the lower temperatures. Bead-associated WGA from cells...
FmtrR~ 8 5.0% SDS-polyacrylamide gels of HeLa cell proteins, stained with Coomassie Blue. (A) HeLa cell plasma membranes isolated on polylysine-coated acrylamide beads at pH 5.0, by WGA assay, these membranes are 10-fold purified over the cell homogenate. (B) HeLa cell membranes eluted from beads by 0.6 M K2HPO4 (final concentration) and washed twice in ice-cold 10 mM Tris, pH 7.4 (see Fig. 9 for details). (C) HeLa cell plasma membranes prepared by the method of Atkinson and Summers (3), washed twice in ice-cold 10 mM Tris, pH 7.4, before electrophoresis; by WGA assay, these membranes have been purified 10-fold over a whole cell homogenate. (D) Whole HeLa cell homogenate.

attached at 4° and 25°C increased in exact proportion to the other membrane markers and constituents (Fig. 5), showing that WGA is a valid membrane marker at these temperatures. The most likely explanation for discordant purification or recovery of markers and components is preferential exclusion or inclusion of membrane constituents on the bead as the isolation conditions are varied. The conditions of cell lysis and the temperature of membrane isolation are recognized as being critical variables in the preparation of plasma membranes (Wallach and Lin [42]). Therefore, although we have shown that isolation of selected membrane components may occur, any membrane isolation technique which does not result in 100% membrane recovery may lead to selective isolation of membrane components.

Although membrane isolation on beads has several recommendable features, certain aspects of the method deserve critical evaluation before it is used. The high surface charge of the bead, and its effect upon local pH must be kept in mind when dealing with membrane-associated enzymes and pH-dependent membrane properties. Although we have shown these effects to be small or negligible for several enzymes (Table II), it is known that fixed surface charges and soluble polylysine at low concentrations can greatly alter cell morphology and membrane structure, and have profound effects upon membrane function (25, 26, 31, 32, 36). Also, the condition which we have chosen for optimal membrane isolation on beads (pH 5.0 in sucrose-acetate buffer) may adversely affect sensitive membrane functions. However, for certain applications, pH values closer to neutrality may prove suitable, and, in any case, other cells may require quite different conditions for optimal attachment.

Finally, the relatively harsh conditions required for the removal of membranes from beads may make direct study of the bead-associated membrane a more attractive approach than the use of eluted membranes. Although the polypeptide composition of membranes eluted from beads is similar to that of membranes on beads, subtle changes in protein conformation or distribution, induced by high salt concentrations, may limit the usefulness of eluted membranes for functional studies.

Although we do not propose that membrane isolation on beads can replace standard isolation methods, it may be valuable for certain applications. Membrane isolation on beads is rapid, requiring only 2.5 h from the time cells are added to beads. Furthermore, membranes on beads rapidly sediment (under 10 s in a clinical centrifuge), and are quickly separated from unbound components in the supernate. Rapid separation can be important in the study of weak or transient associations of peripheral proteins with the plasma membrane. Finally, the protoplasmic surface of the isolated membranes is fully exposed, because the use of intact cells necessarily places their extracellular surface against the bead. This conclusion has been
FIGURE 9 Freeze-fracture of HeLa cell plasma membranes eluted from beads. Membranes were isolated on beads, as described, and washed in 10 mM Tris, pH 7.4. After the beads settled, the supernate was removed and 1 ml of 1 M K₂HPO₄ was added to 1 ml of beads, which were then vigorously vortexed for 2 min. After a 10-s centrifugation in a clinical centrifuge, the supernate, containing the eluted membranes, was removed, 1 ml of 0.6 M K₂HPO₄ was added, and the beads were vortexed and spun, as described above. The supernates were combined, diluted with 10 ml of 10 mM Tris, pH 7.4, and centrifuged at 100,000 g for 40 min. The translucent pellet was then resuspended and washed once in 10 mM Tris in the same way. 5 μl of glycerol were added to the pellet, and samples were taken and immediately frozen in liquid N₂-cooled Freon 22 (E. I. Dupont de Nemours & Co., Inc., Wilmington, Del.) for freeze-fracture, or stored at -40°C for gel electrophoresis. Membranes prepared in the absence of glycerol had similar fracture faces, but less extensive fracture faces were visible. × 75,000. Bar, 0.2 μm.

verified by experiments with erythrocyte membranes, which have also shown that under appropriate conditions, the extracellular membrane surface is inaccessible to probing enzymes.

Although HeLa cell proteins which are exposed at the extracellular surface have been partially identified (20), no information is available on their transmembrane disposition. In the erythrocyte, studies of membrane asymmetry were facilitated by the availability of inside-out vesicles (39, 40) which have also been obtained from other

3 To make the extracellular surface inaccessible, the membrane must be tightly apposed to a bead which is impermeable to probing molecules. For such studies, glass beads, such as those used in Kalish et al. may be more appropriate than porous polyacrylamide beads.

cells (4, 22, 44, 46). Membranes isolated on beads can also be used in such studies, and labeling experiments using lactoperoxidase-catalyzed iodination of bead-isolated HeLa cell plasma membranes are in progress. This approach may be applicable to a variety of complex cells. In addition to this work on the HeLa cell, we have obtained significant purification of L-cell plasma membranes and have observed tenacious attachment of lymphocytes, epidermal cells, and Dicystostelium to polylysine-coated beads.

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REFERENCES

29. Lowry, O. H., N. J. Rosebrough, A. L. Farr,