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ISOLATION OF CHROMAFFIN CELL PLASMA MEMBRANES ON POLYCATIONIC BEADS

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Summary

We have tried to define which proteins of chromaffin cell plasma membranes are facing the cytoplasm by surface labelling a selectively oriented membrane preparation.

Viable chromaffin cells were isolated by collagenase treatment of bovine adrenals. Plasma membranes from these cells were isolated on polycationic beads by the method of Jacobson and Branton (Jacobson, B.S. and Branton, D. (1977) Science 195, 302—304). The purity and orientation of the membranes were defined by biochemical and morphological criteria. The membranes, with their external side apposed to the bead surface, were enriched about 10-fold with respect to a whole cell homogenate, and contained only small amounts of contaminating organelles. Surface specific iodination of membranes on beads with 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen), followed by polyacrylamide gel electrophoresis, allowed the identification of cytoplasmically exposed proteins. A different pattern was observed when intact cells were labelled prior to membrane isolation. The advantages and possible uses of this immobilized membrane preparation are discussed.

Introduction

Secretion of catecholamines by the adrenal medulla is widely believed to occur by exocytosis. In this process, a slight increase in the Ca^{2+} concentration

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; Iodogen, 1,3,4,6-tetrachloro 3α,6α-diphenylglycoluril; DIDS, 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid.

of the cytoplasm promotes the fusion of secretory granules with the plasma membrane, as evidenced by both transmission [2] and freeze etching [3] microscopy.

Finkelstein and coworkers [4,5] have recently used a system consisting of pure lipid vesicles and a planar bilayer to model the process of exocytosis. The fusion rates observed with this system are ostensibly much lower than those found in biological systems during exocytosis. For this reason, and because of the ion specificity observed in the biological [6] but not in the model [4,5] system, it must be assumed that components other than lipids are involved. These putative components, in all likelihood proteins or glycoproteins, would confer ion specificity and increased reactivity to the fusing membranes.

The proteins of chromaffin granules have been widely studied, and their disposition in the membrane extensively explored [7,8]. Adrenomedullary plasma membrane proteins, in contrast, have been studied comparatively little [9,11]. Due to methodological difficulties, their distribution in the membrane remains largely unknown. In particular, those proteins exposed to the cytoplasmic milieu (and therefore likely to participate in chromaffin granule recognition and fusion) have not been identified. The purpose of this investigation was to obtain a preparation of adrenomedullary plasma membranes in which the cytoplasmic side was preferentially exposed, allowing the study of internally facing proteins.

A novel procedure was recently described by Jacobson and coworkers [1,12] for the isolation of cellular plasma membranes on a solid polycationic support. The method is based on the firm attachment of the external face of the membrane to the polycation-coated beads, followed by shearing of the rest of the cell. Large patches of the plasma membrane (with their cytoplasmic side facing the incubation medium) are left on the beads. This method has been successfully employed for the preparation of plasma membranes from several types of isolated cells (human red cells [1], platelets [13] and HeLa cells [12]), but has no obvious application in the case of solid tissues, such as the adrenal medulla.

We have combined the use of polycationic beads with methods for the isolation and purification of chromaffin cells. This combined procedure allows one to study separately and sequentially both faces of the plasma membrane (the external side in isolated cells, and the cytoplasmic side in purified and immobilized membranes on beads). When combined with surface-specific labelling of proteins, this protocol can be used to elucidate the topology of plasma membrane proteins.

Materials and Methods

Collagenase Type I (Lot No. 49C-0024), DNAase II, acetylthiocholine, 5,5′-dithiobis(2-nitrobenzoic acid), phenylmethylsulfonyl fluoride, glucose 6-phosphate, cytochrome *c*, trypsin and chymotrypsin were all purchased from Sigma. Polyethyleneimine coated polyacrylamide beads (Affi-gel 731) from Bio-Rad; Percoll from Pharmacia; Trypan blue from Grand Island; 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen) was from Pierce. 4,4′-Diisothiocyano-2,2′-[$^3H_2$]stilbenedisulfonate ([$^3H_2$]DIDS) was synthesized by Dr. M. Ramjeesingh.

Isolation buffer contained 140 mM NaCl; 5 mM KCl; Hepes (sodium salt) buffer (pH 7.3) 5 mM; glucose 10 mM. In some experiments divalent cation-free Krebs-HCO_3^- buffer was used instead of isolation buffer, with similar results. Sucrose-acetate buffer contained 7 parts of 310 mM sucrose and 3 parts of 310 mosM sodium acetate buffer, pH 5.2. EDTA was added to a final concentration of 1 mM. Phosphate buffered saline contained 150 mM NaCl and 5 mM sodium phosphate buffer, pH 7.5.

A set of calibrated density marker beads (Pharmacia, Lot No. EB 12008) was used to determine the density distribution of self made Percoll gradients. The beads were hydrated overnight in saline and mixed with the Percoll-buffer mixture immediately before centrifugation. The density assigned to the different bands is that given by the manufacturer.

Cell isolation. Bovine adrenal glands were purchased from a local slaughterhouse and transported in ice-cold oxygenated isolation buffer within 2 h of killing. The medulla was freed from the cortex and coarsely minced with scissors. The minced tissue was rinsed with fresh buffer and weighed. After this stage no glassware was utilized. The minced tissue (usually 30 g wet weight) was incubated at 37°C in isolation buffer containing 1 mg collagenase per g wet weight under continuous oxygenation and stirring. The incubation was stopped after 45 min and the tissue fragments allowed to sediment at $1 \times g$. The supernatant containing isolated cells and cell debris was then aspirated and replaced with fresh isolation buffer containing collagenase. This cycle was repeated 4 to 6 times and all but the first supernatant were retained for cell harvesting. This was achieved by filtering the suspension through a fine nylon mesh (200 mesh, Sargent-Welch) and then centrifuging the filtrate at $100 \times g$ for 4 min at room temperature. The supernatant was discarded and the pellet (henceforth referred to as the crude fraction) was resuspended in about 10 vol. of isolation buffer. Occasional cell clumping was observed at this stage, particularly in the case of the initial two or three harvests. Clumping could be prevented or reversed by adding 0.1 mg/ml of DNAase I during resuspension.

Pure viable cells were isolated by density gradient centrifugation on Percoll [14]. One volume of resuspended crude fraction was mixed with one volume of Percoll previously made isotonic by the addition of 10-fold concentrated isolated buffer. This mixture was then centrifuged for 20 min at room temperature in an angle rotor at $46\,000 \times g$. Similar results were obtained when centrifugation was carried out at 5°C. The purified cells were obtained from the middle third of the self-formed gradient (see Results and Fig. 1). The cells were washed twice in 40 ml of isolation buffer. Samples of this suspension were used for cell count determination in a Coulter Counter Model ZB1. Their viability was checked by exclusion of trypan blue (0.1% in saline).

Iodination by the Iodogen method was performed in scintillation vials precoated with 100 μg Iodogen as described by Markwell and Fox [15]. The cells, resuspended in about 5 vol. of phosphate buffered saline, were introduced to the vial and the reaction was started by the addition of 200—500 μCi carrier-free $Na^{125}I$. After 20 min incubation at room temperature with gentle shaking, the reaction was terminated by withdrawing the suspension from the vial and washing the cells up to eight times with approx. 25 vol. phosphate-buffered saline. A final wash was performed in sucrose-acetate. A similar protocol was

used for labelling membranes on beads.

Membrane isolation. Purified cells were washed once in sucrose-acetate buffer and the pellet resuspended in a small volume of the same medium by repeated drawing into and out of a plastic pipette tip. This thick suspension contained $(6—9) \cdot 10^7$ cells/ml. One volume of 50% Affi-gel 731 beads (previously hydrated and equilibrated with sucrose-acetate buffer) was then added dropwise while gently stirring the mixture. Incubation was continued for 10 min with occasional shaking. At this point 20 vol. of sucrose-acetate buffer containing 40 U/ml heparin sulphate were added to the mixture in order to block those areas of the beads devoid of cells, thus preventing binding of debris upon cell shearing *. The beads were then washed twice more in sucrose-acetate buffer and resuspended in one volume of this medium.

Cells were sheared off the beads by repeated vortexing (maximum setting) or by sonication with the microtip of a W140 sonifier-cell disrupter (Heat Systems), followed by washing of the beads in 10 mM Tris-HCl, pH 7.4. The resulting membrane-coated beads were then used for chemical and morphological analyses. Marker enzyme assays of membranes and of cell homogenates were all performed in 0.1% Triton X-100 containing solutions. Dopamine hydroxylase was measured by the method of Nagatsu and Udenfriend adding *N*-ethylmaleimide [16]. Glucose-6-phosphate was assayed by the method of Hubscher and West [17]. Acetylcholinesterase was measured as described by Steck [18]. Cytochrome oxidase was assayed as described by Hodges and Leonard [19]. In all cases the assumption was made that binding to the beads did not affect enzyme activity.

Polyacrylamide (10%) gel electrophoresis in sodium dodecyl sulfate (0.1%) was performed as described by Laemmli [20]. Membrane samples were eluted from the beads by incubating packed beads with one volume of 'sample solubilizer' for 30 min at 60°C. The sample solubilizer contained 3% (w/v) sodium dodecyl sulfate, 10% (v/v) glycerol, 5% (v/v) mercaptoethanol and 50% (v/v) of stacking gel buffer (see Ref. 6). Bromphenol blue was used as tracking dye. Red blood cell ghosts, prepared by hypotonic lysis in 5 mM sodium phosphate, were used as molecular weight standards. The molecular weight of the major ghosts bands was that listed by Steck [21]. Kodak X R-Z film was used for autoradiography. Autoradiograms of individual lanes were scanned at 600 nm with a Gilson Spectrophotometer Model 250, adapted with a gel scanning unit Model 2520 using a 0.05 mm aperture plate.

Scanning electron microscopy was performed by Dr. M. Teitelmann, using the methods described by Cohen et al. [12]. When indicated, proteolysis of iso-

* A series of preliminary experiments were carried out, in search of a substance capable of blocking the charges of the polycation exposed in areas devoid of cells. For this purpose, beads were preincubated with the presumptive blockers, washed and then incubated with a chromaffin cell homogenate, purified chromaffin granules or a human red cell lysate prepared by hypotonic lysis, Following removal of the unbound material, the amount bound was determined by either protein determination of formic acid eluted material, or, in the case of chromaffin granules, by fluorometric determination of catecholamines. Heparin and dextran sulfate were found to prevent binding of both soluble proteins and organelles by over 90%. After submission of the manuscript, a paper by Jacobson [30] appeared in which heparin and other polyanions were also found to be effective blockers of protein binding to polycationic beads. Also, in agreement with Jacobson, we found that the polyanions do not displace previously bound material.

lated membranes was carried out at room temperature using 4 vol. of 50 μg/ml trypsin or chymotrypsin per volume of packed beads. The reaction was stopped at different intervals with a molar excess of phenylmethylsulfonyl fluoride (30 μg/ml). Protein was determined by the method of Bradford [22]. For this purpose, protein was eluted from the beads by adding known volumes of concentrated formic acid. This obviates problems of incomplete elution and large volumes of eluate when detergents are used for protein elution [12,13]. Formic acid does not interfere with protein determination by the method of Bradford [22]. Unless otherwise indicated, all values given are the mean ±1 S.E. of the number of determinations indicated in parentheses.

Results

Isolation of chromaffin cells

A phase contrast micrograph of the 'crude' cell preparation is shown in Fig. 2a. Some viable adrenal cells are observed together with red blood cells and a large amount of cellular debris. Purification of chromaffin cells by the method of Fenwick et al. [23] was found to be unsatisfactory, i.e. red blood cells were only imcompletely removed and more than 70% of the viable chromaffin cells were lost in the process.

In contrast, separation of the cells in Percoll gradients gave remarkably better results. One such gradient is illustrated in Fig. 1. Three fractions could be

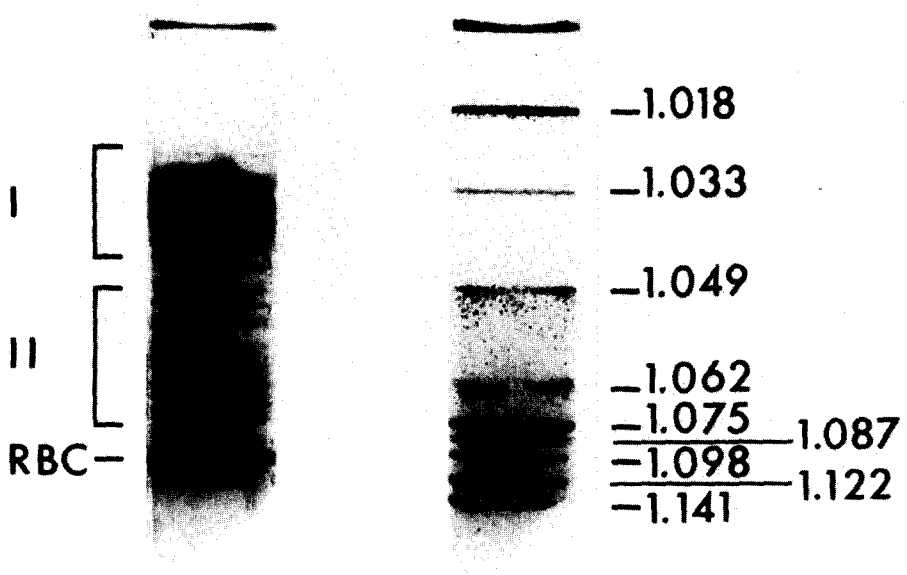

Fig. 1. Purification of chromaffin cells on Percoll gradients. Left: a crude fraction was mixed with Percoll and centrifuged as described in Materials and Methods. Fraction I contained mostly dead cells and debris. Fraction II contains essentially pure chromaffin cells. RBC, red blood cells. Right: density calibration. Marker Beads (Pharmacia, Lot No. 12008) were spun simultaneously with the gradient shown at left. The densities of the different bands (in g/ml) are those assigned by the manufacturer.

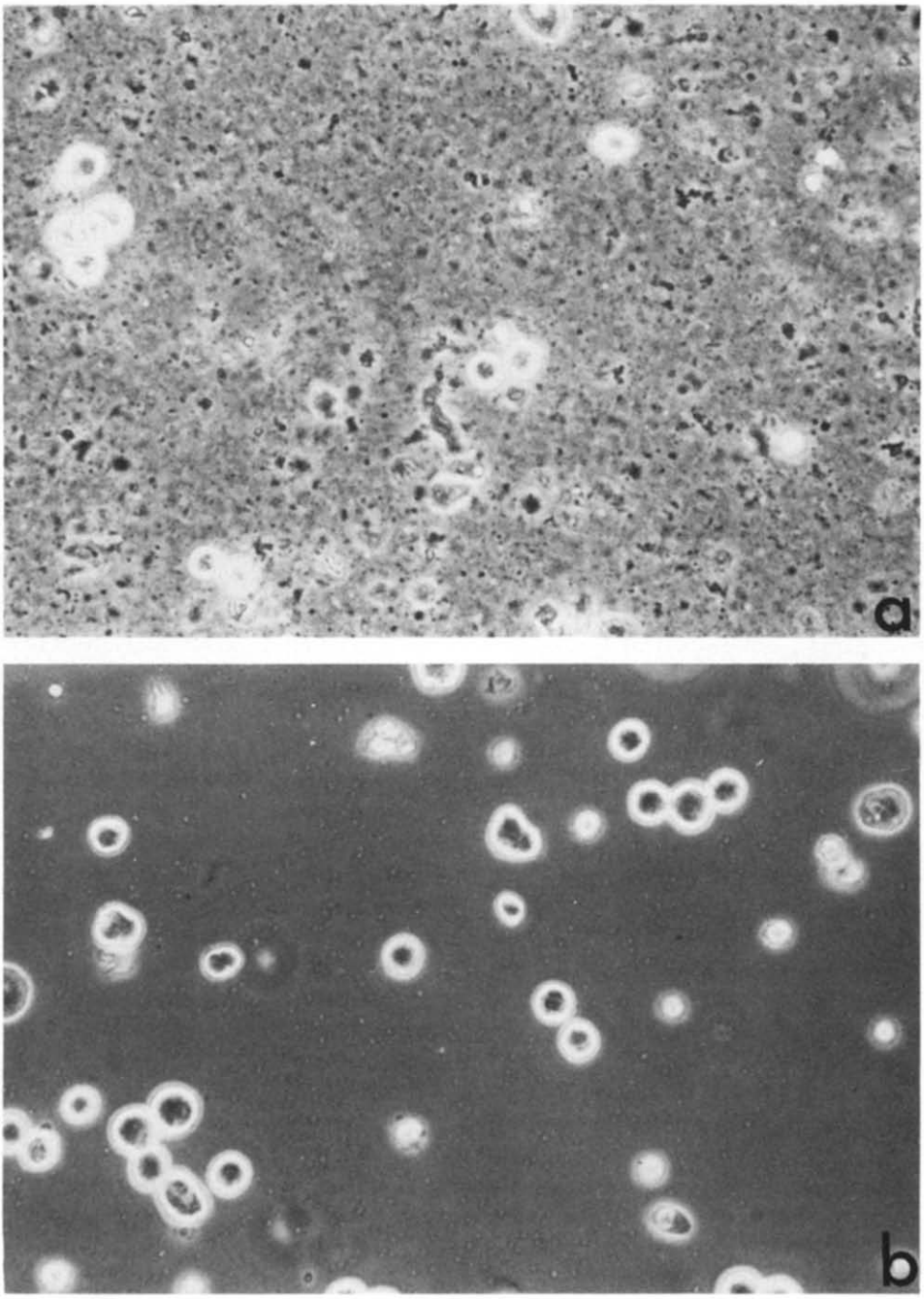

Fig. 2. Light micrographs of isolated chromaffin cells at different stages of the membrane purification process. (a) Crude cell fraction. Viable cells can be identified by a characteristic birefringent halo. Note the presence of large amounts of contaminating debris and fibres (×350). (b) Purified cell preparation obtained by density gradient centrifugation (×350). The apparently smaller cells are chromaffin cells lying below the focal plane. (c) Intact chromaffin cells attached to polycation-coated acrylamide beads. Unbound cells have been washed away (×350).

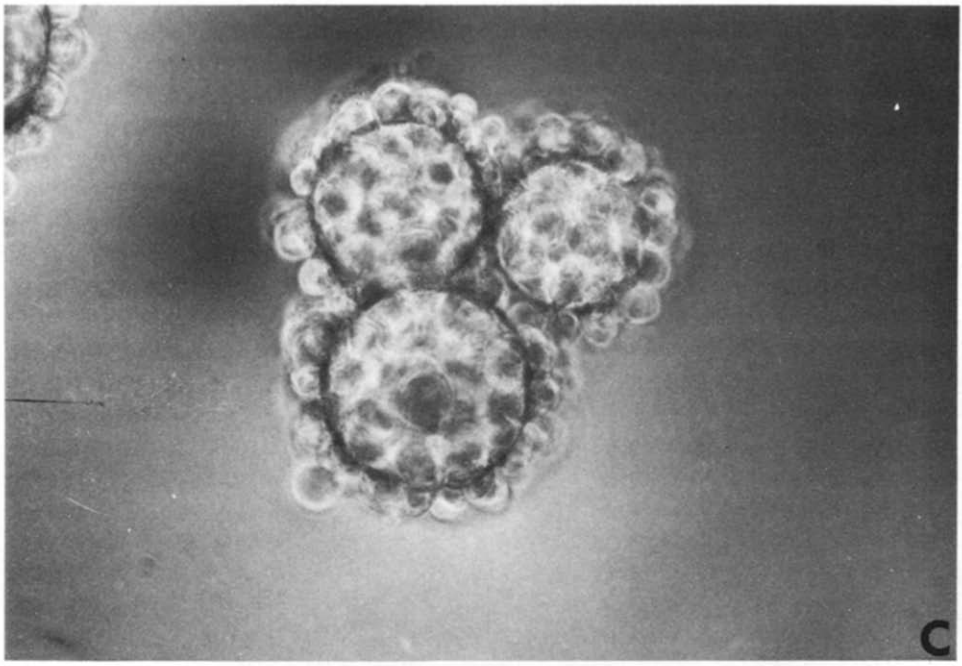

Fig. 2C.

separated as indicated on the figure: Fraction I (density 1.018 to 1.045) consisted largely of dead cells and cell debris. This fraction was routinely discarded. Fraction II contained essentially pure chromaffin cells with a viability of better than 96%. A typical field of cells isolated by this method as observed by phase contrast microscopy is shown in Fig. 2b. Viable cells were generally spherical and birefringent whereas cells permeable to trypan blue were frequently elongated and of irregular contour (Figs. 2 and 3). Viable fusiform cells were never observed in Fraction II, suggesting minimal contamination, if any, with fibroblasts. Red blood cells (RBC in Fig. 1) were always found in a very sharp band corresponding to a density of 1.098 g/ml. Contamination of the chromaffin cell population with cortical cells is unlikely to be significant because: (a) contaminating cortex represented less than 5% (judged visually) of the dissected material and (b) control experiments in which only cortex was subjected to the entire purification procedure showed that less than 10% (w/w) of the initial material was found in Fraction II of the Percoll gradient.

The yield of chromaffin cells was found to vary between $(7—10) \cdot 10^6$ cells per gram of wet medulla. This compares favourably with results obtained by differential centrifugation [23,24].

Isolation of plasma membranes

As is the case for both HeLa cells [2] and human platelets [13], only poor binding was observed when cells and beads were co-incubated under physiological conditions. Maximal binding of adrenal cells to beads was achieved instead in a medium of low ionic strength and a pH of 5.2 (i.e. sucrose-acetate

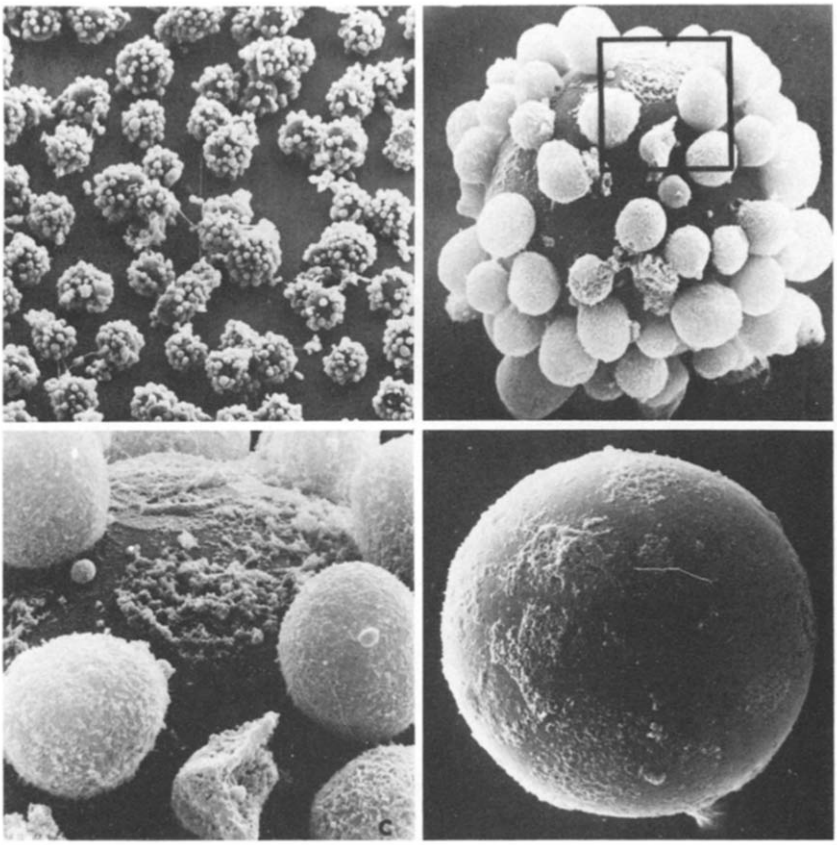

Fig. 3. Scanning electron micrographs of isolated chromaffin cells and their membranes on polycation-coated beads. Top left: Cells bound to beads. Unbound cells have been removed (×58). Top right: Higher magnification of a single bead coated with cells (×580). Note that some areas devoid of cells are actually covered by a portion of the membrane of a cell that was torn away during washing. Bottom left: Higher magnification of the area demarcated in the top right panel by a black square (×1500). Bottom right: Membrane-coated bead obtained by shearing away bound cells by vigorous vortexing (×600).

buffer). Incubation of the cells in this medium at room temperature for 10 min reduced the viability to about 87%.

Binding of cells to the beads could be demonstrated by both phase contrast (Fig. 2c) and scanning electron microscopy (Fig. 3). As observed by Cohen et al. [12] some cells appear to be bound to two beads, thus promoting the formation of small bead aggregates. Small cell clusters frequently observed bound to beads (Fig. 3) are the result of incomplete dispersion of cells following the final wash in sucrose-acetate buffer. A more thorough resuspension of the cell pellet in this buffer produced progressive cellular lysis, and the debris of the lysed cells reduced the yield and purity of the final membrane preparation. Cell viability also decreased upon binding to the beads. Only about 40% of the cells were impermeant to trypan blue 10 min after binding to the bead surface.

Even though an excess of cells was used for coating the beads, areas devoid of cells were usually observed on the bead surface (Fig. 3, top left panel). This is at least partly due to detachment of cells during the wash cycles the beads

are subjected to for the removal of unbound cells. This can be clearly seen in Fig. 3 (top right and bottom left panels), where a membrane 'print' demarcates the area previously occupied by a cell.

By phase contrast microscopy the beads appeared smooth and devoid of cells following either vortexing or sonication. However, membrane patches were seen by scanning electron microscopy to cover a substantial fraction of the bead surface (Fig. 3, bottom right). No significant differences were observed between vortexed (4 × 30 s) and mildly sonicated (2 × 5 s, 20% setting) beads, but a substantial fraction of the membranes (identified by the markers discussed below) was lost upon intense sonication (2 × 30 s, 50% setting).

Plasma membrane characterization

(A) Membrane enrichment. Three methods were used for the identification of the plasma membrane: (a) Covalent labelling of external proteins with the radiolabelled stilbene derivative, diisothiocyano [$^3H_2$]stilbene disulfonic acid [$^3H_2$]DIDS), which by virtue of its sulfonate moieties does not permeate membranes [25]. (b) Surface specific iodination of protein tyrosine residues with the sparingly soluble chloramide Iodogen [15]. (c) Measurement of the specific activity of acetylcholinesterase, an enzyme believed to be located exclusively on the outer face of the plasma membrane. The results of these experiments are compiled in Table I. About 8% of the original membrane added to the beads was recovered on their surface. The purified membranes were found to be enriched 9—10-fold in the markers listed above.

(B) Marker enzyme studies. The specific activities of marker enzymes characteristic of different cell organelles were compared in cell homogenates and isolated membranes. Acetylcholinesterase, glucose-6-phosphatase, dopamine β-hydroxylase and cytochrome *c* oxidase were used as markers of the plasma membrane, endoplasmic reticulum, chromaffin granules and mitochondria, respectively. The results are shown in Table II. Only small amounts of contaminating organelles could be found in the membrane fraction. However, caution must be exercised when considering the absolute values of some of the enzymes. This is particularly true for the least active ones (e.g. dopamine β-hydroxylase) in which case the small amounts of membrane material available for assay made the determinations somewhat unreliable.

(C) Polypeptide composition. The polypeptide composition of both the entire cell homogenate and the plasma membrane isolated on the beads are shown in Fig. 4. The pattern observed in eight preparations was very consistent with only minor differences in the relative intensity of some of the bands (com-

TABLE I

Enrichment was calculated as the ratio of the specific activities of the membranes and the isolated cells used for attachment to the beads. Yield is expressed as percent of the activity of the cells bound to the beads prior to disruption. Data are mean ±S.E. for the number of experiments given in parentheses.

Marker	(*n*)	Enrichment	Yield
Acetylcholinesterase	(5)	10.1 ± 0.9	6.7 ± 2.1
^{125}I + Iodogen	(4)	7.7 ± 1.4	8.0 ± 3.1
[$^3H_2$]DIDS	(4)	9.9 ± 1.8	8.6 ± 1.3

TABLE II

Specific activities are given in micromoles of substrate converted per hour per mg protein, except for dopamine β-hydroxylase (*) given in nmol/h per mg protein. The variation coefficients of the specific activities were less than 15% with the exception of dopamine β-hydroxylase, in which case the coefficients were 17 and 24% for the homogenate and membrane, respectively.

Enzyme	(n)	Homogenate specific activity	Membrane specific activity	Ratio
Acetylcholinesterase	(5)	0.59	5.96	10.10
Glucose-6-phosphatase	(5)	1.40	1.08	0.77
Dopamine β-hydroxylase	(3)	56.0 *	14.6 *	0.26
Cytochrome oxidase	(3)	26.7	14.9	0.56

pare Figs. 4 and 5). Moreover, iodination of the cells prior to membrane isolation, or of the membranes on the beads (see below) did not alter the polypeptide composition of the final membrane preparation. The membranes display major components with molecular weights of 43 000, 56 000, 68 000 and 82 000. A somewhat similar pattern has been reported by Meyer and Burger [26] and by Zinder et al. [11], using density gradients for membrane isolation. The membrane preparation contained very little material of low molecular weight (<35 000), suggesting that little proteolysis occurred during the isolation proce-

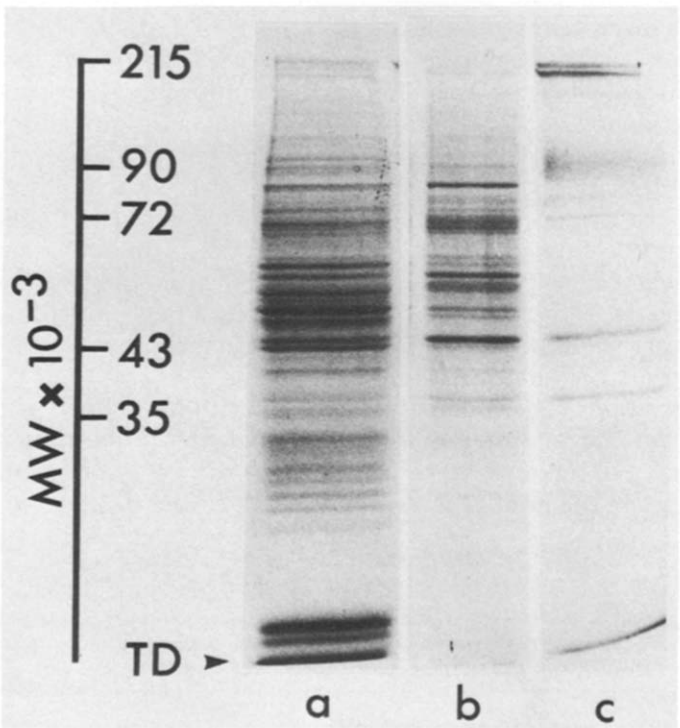

Fig. 4. Polypeptide composition of chromaffin cells and their membranes. Samples were reduced and electrophoresed in 10% polyacrylamide gels with 0.1% SDS. (a) Whole chromaffin cell homogenate; (b) chromaffin plasma membranes and (c) human red cell ghosts, used as molecular weight standards. TD, tracking dye.

dure. In addition, a similar pattern was observed when cell shearing and bead washing were performed in media containing phenylmethylsulfonyl fluoride (30 μg/ml).

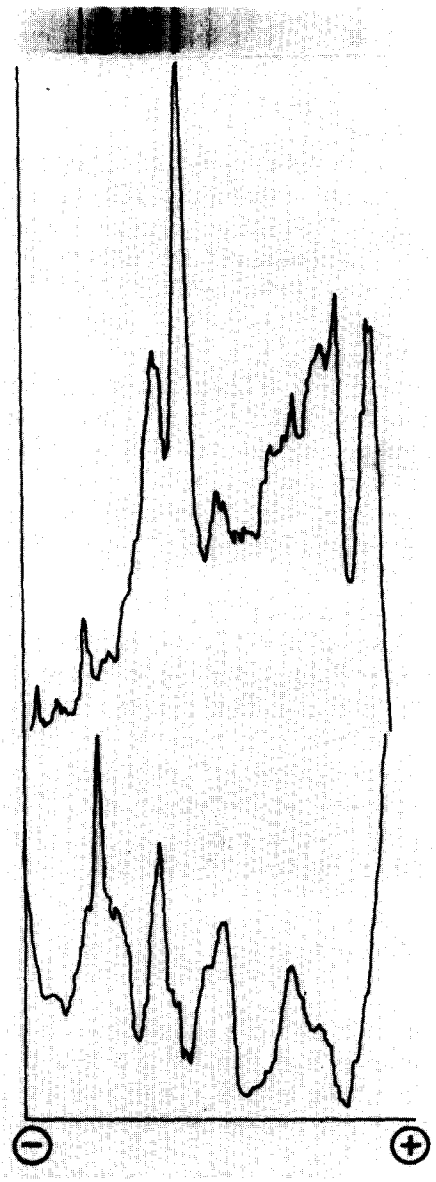

Fig. 5. Surface labelling of chromaffin cell plasma membranes. Top: Coomassie blue pattern of plasma membranes isolated on beads. Middle: Autoradiography of plasma membranes labelled with ^{125}I plus Iodogen while bound to the beads. The labelled membranes were eluted from beads and electrophoresed. A scan of a typical gel is shown. Bottom: Autoradiogram of membranes obtained from ^{125}I plus Iodogen labelled cells. The background, which was substantial in these samples, was subtracted by lowering the baseline. Both the autoradiography scans and the Coomassie blue stained gel were obtained from samples run in a single slab and were aligned so that direct comparison is possible.

(D) Membrane sidedness. In order to ascertain whether the cytoplasmic side of the isolated membranes was indeed exposed to the medium, the removal of membrane-bound actin by proteases was analysed. Even though actin itself is fairly refractory to proteolysis by most enzymes, the protein(s) that anchor actin to the membrane are markedly susceptible to proteolytic attack [27,28]. Incubation of the isolated membranes on the beads in solutions containing 50 μg/ml of either trypsin or chymotrypsin resulted in the progressive loss of most of the M_r 43 000 band (not illustrated). We interpret this as indicating that the majority of the membranes are neither sealed in a right side-out configuration nor apposing their cytoplasmic surface to the beads.

(E) Membrane labelling. The distribution of membrane proteins on both sides of the membrane was analysed by surface specific labelling of either intact cells or isolated membranes using ^{125}I and Iodogen [15]. Intact cells or isolated membranes on beads were reacted under mild conditions and the polypeptide profile resolved by polyacrylamide gel electrophoresis. The viability of the cells was only marginally affected by both labelling and the ensuing washes. The Coomassie blue pattern was not affected by labelling.

The pattern of ^{125}I incorporation into proteins from isolated membranes on beads is shown in Fig. 5. Inasmuch as Iodogen is thought to label only superficially exposed tyrosine groups [15] we conclude that the labelled bands are cytoplasmically exposed proteins. Indeed, the two major labelled bands (M_r 43 000 and 56 000) have been tentatively identified by Zinder et al. [11] as actin and tubulin, two proteins known to be on the cytoplasmic face of the membrane.

A much more efficient incorporation of ^{125}I was obtained by labelling intact cells than isolated membranes. The pattern of a typical gel is shown in Fig. 5. A large number of labelled bands, concentrated in four major regions, were observed in seven similar experiments. Unfortunately, a high background was consistently observed, and multiple attempts to reduce it were unsuccessful. These included repeated and prolonged washing of labelled cells; DNAase I treatment of labelled cells; DNAase II treatment of detergent solubilized labelled membranes; dialysis of the samples prior to electrophoresis; extensive leaching of fixed gels in destaining solution with or without 10 mM NaI.

Discussion

Plasma membrane isolation

Several groups [9—11,26] have reported the isolation of a plasma membrane fraction from adrenomedullar cells by differential and density gradient centrifugation. Although the density of intact chromaffin granules (the most abundant organelles) is very different from that of the plasma membrane, lysed granule membranes are only slightly less dense than plasma membranes. Unfortunately, some degree of granule lysis during homogenization is unavoidable. This makes the purification of plasma membranes by this method difficult, and contamination with dopamine β-hydroxylase containing membranes is commonly found [9,10]. Recentrifugation of this partially purified preparation on a second and shallower gradient increased the purity of the plasma membranes but reduced the yield dramatically [11].

Isolation of the plasma membrane on beads does not depend on the inherent density differences between the membranes but rather specifically isolates the plasma membrane because it is the only membrane exposed in the intact cell. Moreover, the randomness and unpredictability of the orientation of vesicles obtained by homogenization and centrifugation are not seen in membranes isolated on beads. The latter are always adsorbed to the bead surface so that their cytoplasmic side faces the incubation medium [1,12,29]. This is particularly important when studies on the sidedness of particular components are intended.

Another advantage of the method reported concerns the purity of the starting material. When homogenizing the adrenal medulla for fractionation, red cells, connective tissue, nerve endings and contaminating cortical cells are usually present. In our protocol, purification of adrenomedullar cells prior to cell disruption circumvents this problem. On the other hand, the polycationic bead method produces small yields (about 8% of the plasma membranes of the cells initially bound to beads) and is very laborious and time consuming (about 7 h).

Membrane markers

Individual membrane identification methods are prone to artefacts resulting from cell isolation or inherent to the fractionation procedure. For instance, acetylcholinesterase (an externally located enzyme) could be inactivated by the high charge density on the bead surface. Also labelling with either [$^3H_2$]DIDS or ^{125}I + Iodogen will be surface specific only in intact cells. Because a small fraction of nonviable cells is unavoidable, artefacts might arise particularly if many reactive groups become exposed upon cell death. This prompted us to compare results obtained by different methods. As shown in Table I, the data obtained by the three techniques agree reasonably well. A 10-fold purification of the memrbanes was obtained, a value that compares favourably with those obtained by other methods [9,10,26]. The comparison is not strictly valid because the reference material in previous reports was a whole medulla homogenate whereas in our studies it refers to a chromaffin cell lysate. The disagreement, however, is probably minor, since chromaffin cells constitute the vast majority of the cells in the adrenal medulla.

Labelling of membranes

Both sides of the membrane were selectively and separately labelled using ^{125}I and Iodogen. The stability and sparing solubility of the latter compound account for its specificity as a surface marker and for the minimal damage it causes to living cells [14]. Thus, the location of particular polypeptides can be inferred from their labelling properties (see Fig. 5). Bands labelled in isolated membranes, which are tightly bound to the beads exposing only their cytoplasmic surface, are internally facing proteins. The bands labelled in intact cells must be extracellularly exposed. Although some bands seem to be labelled from both sides of the membrane, this is not conclusive evidence of a transmembrane disposition since: (a) two different polypeptides with a similar molecular weight might have been labelled; (b) the extracellular side of membranes bound to the beads might not be entirely occluded by apposition against the bead surface. This possibility has been experimentally tested in red and

HeLa cells [29]. In these systems the extracellular side of membranes on beads was not accessible to either galactose oxidase or neuraminidase. In the case of human platelets, however, some labelling by galactose oxidase and NaB^3H_4 was observed [13], suggesting that loose membrane flaps might remain attached after cell disruption. This is probably related to the small diameter of platelets. Nevertheless the possibility that some of the bands labelled in isolated chromaffin membranes could have been iodinated from the outside, cannot be completely ruled out.

Use of membrane-coated beads as affinity matrix

The immobilized isolated membranes have an additional advantage over membrane suspensions prepared by conventional methods: the membrane-coated beads can be used as an affinity matrix for the identification and isolation of membrane binding proteins. Along this line, we are presently performing experiments aimed at the identification of those proteins on the surface of chromaffin granules that mediate the binding of these organelles to the plasma membrane during exocytosis. In this approach, plasma memrbanes covalently cross-linked to the beads are being tested as a matrix for the isolation of radioactively labelled membrane proteins from detergent solubilized granules (Emerson et al., unpublished observations). We found in preliminary experiments a large unspecific (i.e. calcium independent) binding of proteins to the membrane-covered beads. This unspecific component has so far precluded the determination of any calcium-dependent and tissue-specific binding.

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