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TRANSBILAYER MAPPING OF MEMBRANE PROTEINS USING MEMBRANES ISOLATED ON POLYLYSINE-COATED POLYACRYLAMIDE BEADS *

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Summary

Erythrocyte and HeLa cell plasma membranes were isolated on polylysine-coated polyacrylamide beads and the transbilayer disposition of their proteins was investigated.

When membranes of intact erythrocytes were isolated on beads and then labelled by lactoperoxidase-catalysed iodination, their labelling pattern was similar to that of inside-out vesicles in solution.

When the membranes of intact HeLa cells were isolated on beads and then labelled by galactose oxidase-[^{3}H]borohydride treatment, no glycoprotein or glycolipid sugars were accessible. On the other hand, when the HeLa cell membranes were isolated on beads and then labelled by the lactoperoxidase-catalysed iodination, all of the major membrane proteins were iodinated. These experiments confirmed for HeLa cell membranes what had previously been shown for erythrocyte membranes: when the membranes of intact cells are isolated on beads, the accessibility of their surfaces to enzymatic probes is the same as would be expected of inside-out vesicles in suspension. Double-label experiments, in which the HeLa cell membranes were labelled first on the intact HeLa cells and again after isolation on beads, identified several proteins which may span the membrane.

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* A preliminary report of some of this work has been presented [10].

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Introduction

Many investigators have successfully identified proteins exposed at the outer surface of a wide variety of cells by the use of membrane-impermeable agents [1—3] but the identification of cytoplasmic or transmembrane proteins has not been as easy because it is difficult to selectively probe or label the cytoplasmic surface of plasma membranes. The availability of sealed inside-out vesicles has facilitated the transmembrane mapping of the major proteins of the erythrocyte membrane [4]. More recently, latex-filled phagosomes have been used as a source of inside-out plasma membrane to identify transmembrane proteins in cultured mouse L cells [5,6].

Previous work from this laboratory has shown that selective exposure of the cytoplasmic surface of cell membranes can be achieved by isolating plasma membranes on polylysine-coated polyacrylamide beads [7,8]. Erythrocyte membranes isolated on polylysine-coated glass beads are, by a number of criteria, inside-out in that their cytoplasmic surface is fully exposed to a variety of probes while their extracellular surface is not [9]. Here, we show that erythrocyte membranes isolated on polylysine-coated polyacrylamide beads are also equivalent to inside-out vesicles with respect to the major transmembrane proteins. In addition, we provide evidence that HeLa cell plasma membranes isolated on such beads are in an inside-out configuration and show that lactoperoxidase-catalysed iodination of HeLa cell membranes on beads can provide a transmembrane map of the major proteins of this membrane. A preliminary report of some of this work has been presented [10].

Materials and Methods

Beads. Polylysine-coated polyacrylamide beads were prepared as described [8] and stored at 4°C in 10 mM Tris-HCl (pH 7.4)/0.02% NaN_3.

Isolation of membranes on beads. All procedures for erythrocyte membrane isolation were performed at 0—4°C. Erythrocytes from outdated storage bank blood were washed four times in 10 vols. phosphate-buffered saline (140 mM NaCl, 5 mM sodium phosphate, pH 7.6) and twice in a sucrose/phosphate buffer 310 mosM sucrose : 310 mosM sodium phosphate, pH 7.6 (7 : 3, v/v) in which the cells were finally suspended to approx. 50% (v/v). The beads were prepared for use by washing three times in 5—10 vols. of 0.15 M Tris-HCl (pH 7.4), twice in sucrose/phosphate buffer, and suspended to approx. 50% (v/v). The bead suspension was briefly vortexed and 1 ml was withdrawn and added dropwise to 1 ml of 50% (v/v) erythrocytes while gently mixing the cells and beads by rotating the tube. The tube was placed on ice for 2—5 min with intermittent gentle agitation, after which the suspension was diluted to 5 ml with sucrose/phosphate buffer and mixed by inversion. After 2—5 min, the beads and bound cells settled to the bottom of the tube and, after aspiration of the supernatant containing unattached cells, were washed twice more in the same way to remove unbound cells. The red pellet of beads covered with bound cells was then lysed in 5 ml of 20 mosM sodium phosphate buffer, pH 7.6, resuspended to 1 ml and sonicated for 5 s at 20 W (lowest settling) with the microtip of a Heat Systems Sonifier. The beads were then washed 2—3 times in 20

mosM sodium phosphate or until all visible traces of hemoglobin were removed.

Inside-out vesicles and resealed ghosts were prepared from erythrocyte membranes [11] and were suspended to approx. 1—2 mg/protein in 20 mosM sodium phosphate buffer for attachment to beads. Beads were washed as described above, except that the final two washes were in 20 mosM phosphate buffer. Membranes of ghosts or vesicles were isolated on beads as described for erythrocytes except that the attachment medium was 20 mosM phosphate buffer and that the beads were sonicated subsequent to the second post-attachment wash.

HeLa S_3 cells were grown in suspension culture and their membranes isolated on polylysine-coated polyacrylamide beads as using the pH 5.0 sucrose/acetate buffer described [8]. Use of this attachment medium is critical for the isolation of HeLa cell membranes, since significant or reproducible membrane purification has not been obtained at neutral pH or with other buffer systems tested.

Iodination of membranes. To 1 ml of erythrocyte ghosts or vesicles (0.2 mg protein/ml) in 20 mosM phosphate buffer was added: 3 μl 5 mg/ml lactoperoxidase (EC 1.11.1.7, 60—80 units/mg, from Sigma Chemical Co.) (approx. 1.0 unit, final concentration 0.15 μM), 200 μCi carrier-free $Na^{125}I$ (New England Nuclear), and at ten times 1 min intervals, 10 μl 2.5 μM H_2O_2 in 20 mosM phosphate buffer. The membranes were then washed twice in 20—40 vols. phosphate buffer and resuspended in 200 μl membrane-solubilizing solution (2% sodium dodecyl sulphate, 6% (v/v) β-mercaptoethanol, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0), 2% (v/v) glycerol and 0.005% pyronin-Y (tracking dye). 10-μl aliquots were electrophoresed on 7.5% acrylamide gels [12].

Intact HeLa cells were washed three times in phosphate-buffered saline, and 1 ml of packed cells were suspended in 9 ml of this buffer with the addition of 2 μg/ml butylated hydroxytoluene. To this was then added: 30 μl 5 mg/ml lactoperoxidase, 1—2 mCi $Na^{125}I$ (20 Ci/l) and, at ten times 1 min intervals, 100 μl 2.5 μM H_2O_2 in phosphate-buffered saline, with mixing by inversion after each addition. The labelled cells were then washed three times in phosphate-buffered saline and twice in sucrose/acetate buffer for attachment to beads [8].

To iodinate HeLa cell membranes, or erythrocyte membranes on beads, 0.2—0.5 ml packed beads containing approx. 70—170 μg membrane protein were suspended to 1.0 ml in either 20 mosM phosphate buffer (erythrocyte membranes) or 10 mM Tris-HCl (pH 7.4) (HeLa cell membranes), and labelled by addition of $Na^{125}I$, lactoperoxidase and H_2O_2 as described above for erythrocyte membranes in solution. Any modifications of this routine are specified in text. After each addition of H_2O_2, the tubes were agitated to resuspend the beads and after labelling they were washed four times in either phosphate or Tris-HCl buffer.

Membranes were solubilized off the beads for determination of protein content and analysis on 7.5 or 5.0% polyacrylamide sodium dodecyl sulphate gels [12] as described [8]. After electrophoresis, gels were either stained with Coomassie blue or frozen and sliced in 1.2-mm sections and counted for ^{125}I and ^{131}I or both.

Galactose oxidase-[^{3}H]borohydride labelling of membranes. Galactose oxidase from *Dactylum dendroides* (61 units/mg) was obtained from Worthing-

ton. The enzyme was dissolved in phosphate-buffered saline (100 units/ml) and was stored frozen after having been heated to 50°C for 30 min to inactivate proteases. NAB^3H_4 (242 Ci/mol, from New England Nuclear) was freshly prepared in 0.02 M NaOH for each experiment. Labelling of cell surface glycoproteins and glycolipids with galactose oxidase plus [^{3}H]borohydride was performed following the method of Steck and Dawson [13] using incubation conditions described for HeLa cells [14] modified as follows. After three washes in phosphate-buffered saline, 1 ml of packed cells was suspended in 10 ml of the same buffer (cell density approx. $4 \cdot 10^7$ cells/ml) containing [^{3}H]borohydride ($5 \cdot 10^7$ cpm/ml) and galactose oxidase (12.5 units/ml). After 30 min incubation at room temperature with slow tumbling, the cells were washed three times with phosphate-buffered saline and their plasma membranes isolated on 1 ml of beads as previously described [8]. To label membranes on beads, 1 ml of packed beads with attached membranes was washed twice with phosphate-buffered saline and then labelled with galactose oxidase plus [^{3}H]-borohydride using the same conditions as described above for intact cells. After two washes with 10 mM Tris-HCl (pH 7.4) the membranes were solubilized off the beads in sodium dodecyl sulphate and electrophoresed as described above. The gels were sliced into 1.2-mm sections for counting or stained with Coomassie blue. The gel slices were placed in scintillation vials and eluted with 5 ml of 3% Protosol (New England Nuclear)/4% Liquifluor (New England Nuclear) in toluene for 24 h and then counted for tritium. The electrophoretic patterns of the membrane polypeptides stained with Coomassie blue were indistinguishable for all HeLa cell membrane preparations. Thus neither proteolysis nor cross-linking was enhanced by treatments in the presence of galactose oxidase.

Results

Iodination of erythrocyte membranes

To demonstrate the usefulness of beads for studies of membrane asymmetry, we used the human erythrocyte membrane as a model system since its protein-labelling pattern has been well established [1,15–17]. When the membranes of intact erythrocytes were isolated on beads and then iodinated, the labelling pattern was similar to that of inside-out vesicles labelled in solution (Fig. 1). Conversely, when the membranes of inside-out vesicles were isolated on beads and then iodinated, the labelling pattern was similar to that of sealed ghosts labelled in suspension, although minor unexplained differences in the low molecular weight region of the gell were sometimes observed. Confirming earlier conclusions [9,18], these results show that when the membranes of intact cells are isolated on beads, these isolated membranes are inside out (cytoplasmic surfaces exposed).

The results also show that the iodination pattern of membranes labelled on beads depended only on which surface of the membranes was exposed to the lactoperoxidase in solution. While both band 3 and periodic acid-Schiff 1 span the erythrocyte membrane [19], periodic acid-Schiff 1 is poorly labelled by lactoperoxidase iodination from the cytoplasmic surface (Fig. 1b and c, and see also Ref. 15), presumably because there are few reactive tyrosines at the end of the molecule that protrudes from this surface. The incorporation of iodine into

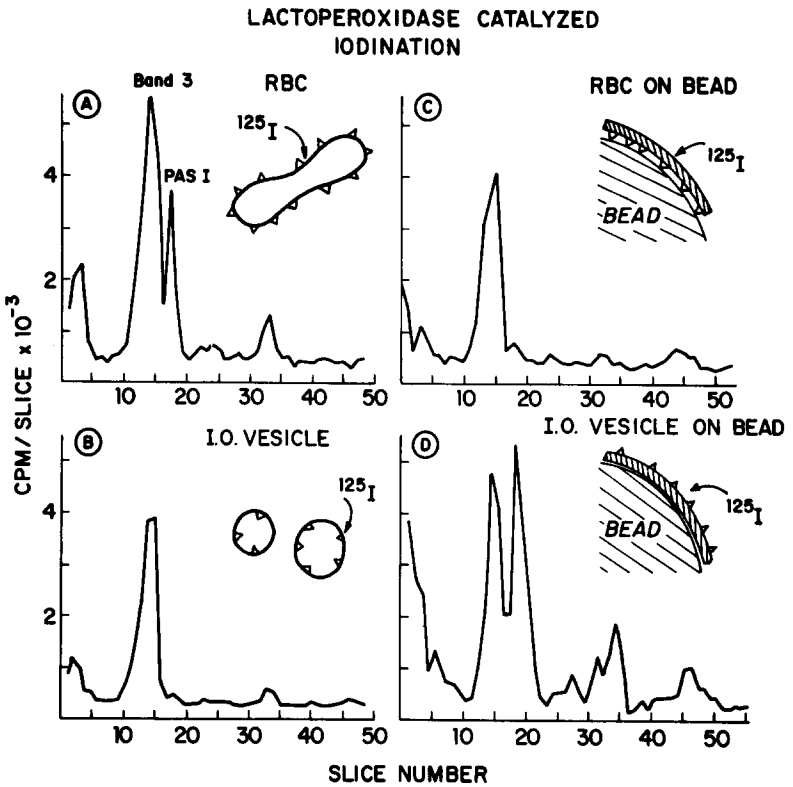

Fig. 1. Radioactivity in succesive slices of 7.5% polyacrylamide gels of lactoperoxidase-catalysed iodinated membranes of erythrocyte ghosts (RBC) and inside-out (I.O.) vesicles labelled in suspension and after isolation on polylysine-coated polyacrylamide beads. (a) Erythrocyte ghosts, incubated in 20 mosM phosphate buffer plus 1 mM $MgCl_2$ at 37°C to promote sealing [11], iodinated in solution. (b) Inside-out vesicles (85% inside out by acetylcholinesterase assay [11], iodonated in solution. (c) Erythrocyte membranes; iodinated after isolation on beads. (d) Inside-out vesicle membranes iodinated after isolation on beads. Some spectrin is labelled in (a) (highest molecular weight peaks) because not all of the ghosts were sealed to lactoperoxidase; spectrin appears poorly labelled in inside-out vesicles (b) because inside-out vesicles lose most of their spectrin during preparation. PAS, periodic acid-Schiff.

periodic acid-Schiff 1 can therefore be used as a sensitive indicator of the degree to which lactoperoxidase has functional access to the extracellular membrane surface once this surface is appressed to the bead. The poor iodination of periodic acid-Schiff 1 in membranes of intact cells which had been isolated on beads and then labelled (Fig. 1c) shows that lactoperoxidase does not have functional access to the membrane surface that is appressed to the bead. Controls show that neither the beads per se nor even the attachment of membranes to beads inhibited the lactoperoxidase labelling of membranes of inside-out vesicles isolated on beads (Fig. 1d). Thus, the membranes of intact erythrocytes isolated on beads are equivalent to inside-out vesicles in so far as the accessibility of macromolecules to the major membrane proteins is concerned. Their labelling patterns (Fig. 1) support the well-established identification of band 3 as the major transmembrane protein of the erythrocyte [19].

Galactose oxidase-[^{3}H]borohydride labelling of HeLa cell membranes

To see if our approach could be used to identify transmembrane proteins of complex cells, we determined the labelling pattern of HeLa cell membranes on beads. In separate experiments [8] we have shown that these HeLa cell mem-

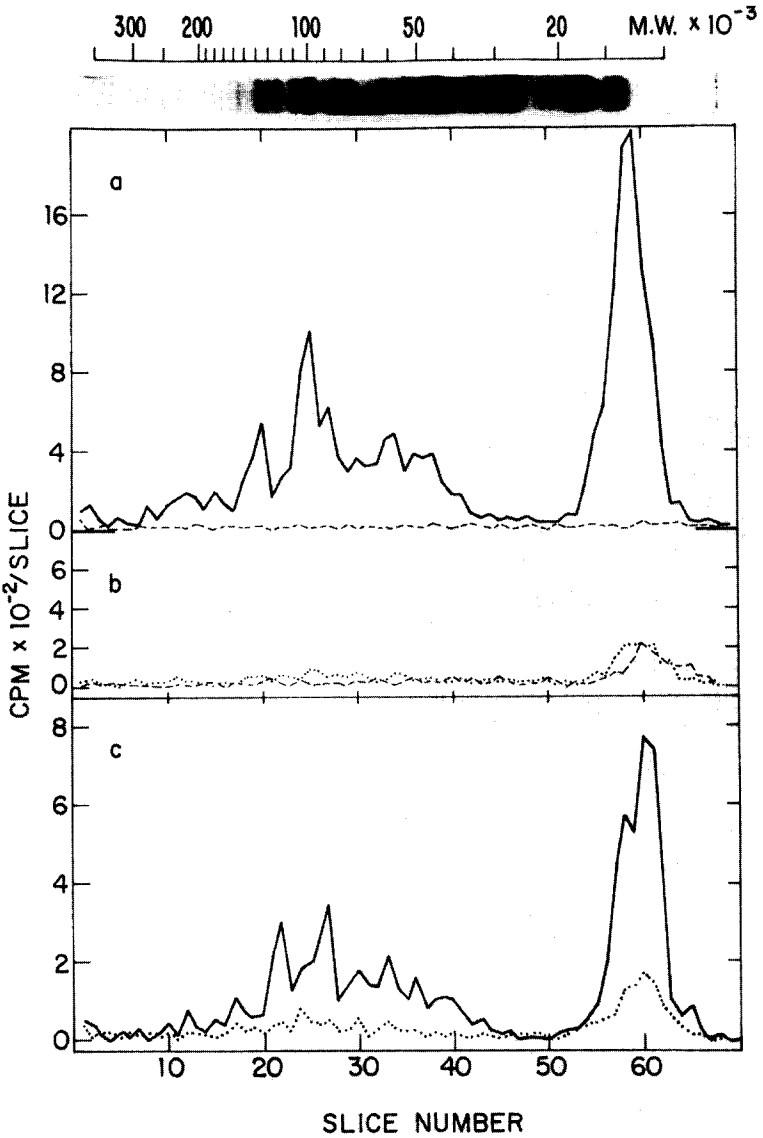

Fig. 2. Radioactivity in 5% polyacrylamide gels of galactose oxidase-[^{3}H]borohydride-reduced HeLa cell membranes labelled in suspension or after isolation on beads or both. Gels were stained with Coomassie blue to verify the recovery of proteins and sliced for counting. (a) Labelling of intact cells plus (———) and minus (- - - - - -) galactose oxidase; (b) labelling of membranes on beads plus (· · · · · ·) and minus (- - - - - -) galactose oxidase; (c) labelling of membranes of intact cells (———) and membranes on beads (· · · · · ·) coincubated in the presence of galactose oxidase. The apparent increase of tritium incorporated into membranes on beads in the coincubation experiment (c) was probably due to intact cells that adhered to empty patches on the beads. Phase contrast microscopy showed some intact cells did adhere to the membrane-covered beads.

branes on beads are substantially free of intracellular contaminants and are approx. 30-fold purified (measured by increased specific activity of membrane-bound ^{125}I-labelled wheat germ agglutinin) or 12.5-fold purified (measured by increased specific activity of ouabain-sensitive ($Na^+ + K^+$)-ATPase) over a cell homogenate.

When intact HeLa cells were labelled in suspension by galactose oxidase plus [^{3}H]borohydride and their plasma membranes subsequently isolated on beads, the membranes were heavily labelled (Table I), and nonspecific incorporation of tritium in the absence of galactose oxidase was slight. The labelling in the presence of galactose oxidase was concentrated in a series of polypeptides that migrated as discrete 50 000—150 000 dalton species upon electrophoresis (Fig. 2a). In addition, a very heavily labelled peak, attributable to glycolipid, migrated just behind the tracker dye. In contrast, when the plasma membranes were labelled after isolation on beads, labelling was slight (Table I) and the small amount of tritium incorporated migrated with the lipid peak upon electrophoresis (Fig. 2b). The significance of this tritium incorporation is doubtful because it is nearly equivalent to the amount of tritium incorporated in the absence of galactose oxidase. (Furthermore, enhanced tritium incorporation into glycolipids when isolated membranes, rather than intact cells, are incubated in the absence of galactose oxidase may be caused by the increase in reducible lipid components that occurs when cells are lysed [20].

To show that beads do not inactivate galactose oxidase, we labelled intact cells in the presence of membranes previously isolated on beads. The incorporation of tritium into the membranes of intact cells was reduced by only a third in the presence of membranes on beads (Table I). This apparent enzyme inactivation is too small to account for the large difference between tritium incorporated into membranes of intact cells and membranes isolated on beads. Furthermore, the labelling pattern was qualitatively the same when cells were labelled

TABLE I

LABELLING OF INTACT HeLa CELLS AND MEMBRANES ON BEADS WITH GALACTOSE OXIDASE-[^{3}H]BOROHYDRIDE

Intact cells, membranes on beads or mixtures were incubated under labelling conditions (see Materials and Methods) with or without galactose oxidase. ^{3}H labelling was determined by adding the total number of counts on polyacrylamide gels of membranes solubilized from beads by sodium dodecyl sulphate. Labelling of intact cells (a, c) was measured after their membranes were isolated on beads. In c and d, 1 ml of packed cells and 1 ml of membranes on beads were labelled together in a final volume of 10 ml using conditions described in Materials and Methods.

Labelling of:	^{3}H labelling (cpm × 10^3/mg protein)		Stimulation (+oxidase/ −oxidase)
	+Galactose oxidase	−Galactose oxidase	
a. Intact cells	245.1	16.6	14.8
b. Membranes on beads	31.3	24.1	1.3
c. Intact cells (labelled in presence of unlabelled membranes on beads)	125.7	12.1	10.4
d. Membranes on beads (labelled in presence of unlabelled cells)	31.6	22.9	1.4

in the presence or absence of membranes on beads (Fig. 2a and c). Thus, as with the erythrocyte membrane, experiments and controls show that when HeLa cell membranes are isolated on beads, the extracellular face of the membranes is inaccessible to macromolecular probes.

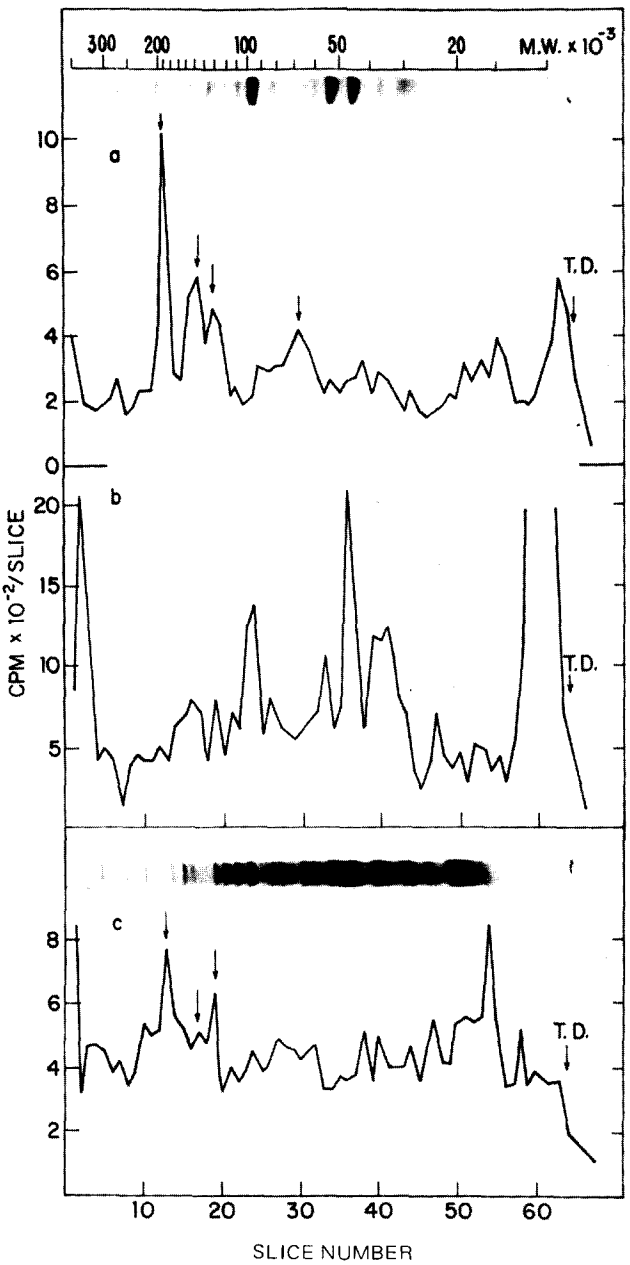

Fig. 3. Radioactivity in slices of 5% polyacrylamide gels of lactoperoxidase-catalysed iodinated HeLa cell plasma membranes. (a) HeLa cell plasma membranes isolated on beads after iodination of intact cells in solution. (b) HeLa cell plasma membranes isolated on beads and then labelled. Also shown are Coomassie blue-stained gels of (a) membranes isolated on beads and (c) a total cell homogenate. (c) Total cell homogenate obtained after iodination of intact HeLa cells in solution. T.D., tracker dye.

Iodination of HeLa cell membranes

When intact HeLa cells were labelled by lactoperoxidase-catalysed iodination and their membranes subsequently isolated on beads, four prominent proteins (Fig. 3a, arrows) of apparent molecular weights 180 000, 140 000, 120 000, and 62 000 (all ±10%, average of seven determinations from separate gel runs) and a large number of minor proteins were labelled. When membranes were labelled after isolation on beads, rather than before, a distinctly different labelling pattern was obtained (Fig. 3b). Comparison of the labelled proteins with the Coomassie blue-stained gel of the isolated membrane shows that nearly all of the major membrane proteins were labelled. All these proteins must be at the cytoplasmic surface, because we have shown (see above) that isolation on beads renders the extracellular face of the membrane inaccessible to macromolecular probes.

To examine the possibility that intracellular proteins were also labelled when intact cells are labelled by lactoperoxidase treatment, a homogenate of labelled intact cells was electrophoresed. The labelling pattern of the cell homogenate (Fig. 3c) was more complex than that of the plasma membrane isolated from those cells (Fig. 3a). Thus, the label was not completely restricted to the plasma membrane, possibly because of the presence of a small percentage of dead cells. Nevertheless, the major proteins of the isolated membrane appear prominently in the homogenate (arrows, Fig. 3c) and, relative to the Coomassie blue staining in this region of the homogenate gel, are heavily labelled. By comparison, those proteins in the lower molecular weight region of the gel where there is heavy Coomassie blue staining are only weakly labelled.

To obtain a more accurate comparison of the proteins labelled at the two membrane surfaces, intact cells were first labelled with ^{131}I, and their mem-

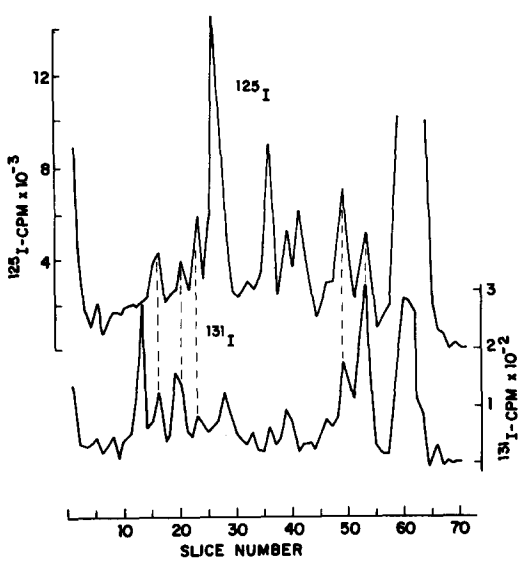

Fig. 4. Radioactivity in slices of 5% polyacrylamide gels of doubly labelled HeLa cell membranes. Intact cells were first labelled with ^{131}I, their membranes were isolated on beads, and the membranes on beads then labelled with ^{125}I.

branes subsequently isolated on beads and labelled with ^{125}I. Under a variety of labelling conditions in different experiments, five discretely labelled polypeptides (Fig. 4, dashed lines) of apparent molecular weight 140 000, 120 000, 100 000, 23 000, and 16 000 were consistently found to comigrate on such gels. Although in some experiments various other internally and externally labelled polypeptides appeared to comigrate, only these five did so consistently. That the externally labelled 180 000 dalton protein was not labelled at the cytoplasmic surface under the conditions of Fig. 3 argues against the possibility that lactoperoxidase leaks under the membranes previously isolated on beads. Rather, the results suggest that the five comigrating polypeptides span the membrane. The comigration of activity may also represent distinct proteins of identical molecular weight at each membrane surface.

Discussion

Our results show that membranes isolated on polylysine-coated polyacrylamide beads can provide important information about the transmembrane disposition of membrane proteins. Using membranes on beads, we have reproduced the well-established asymmetric labelling pattern of the major erythrocyte transmembrane proteins and have identified cytoplasmic and extracellular facing polypeptides of the HeLa cell plasma membrane as well as several proteins which may span its bilayer.

The results of our galactose oxidase-labelling experiments show that the extracellular surface of HeLa cell membranes isolated on beads is inaccessible to a macromolecular enzyme probe and that little or no disordering of glycoproteins across the membrane occurs during isolation. The nearly 10-fold reduction in tritium incorporation in membranes on beads relative to membranes of intact cells is particularly significant in the light of findings by other investigators that isolated membranes incorporate significantly more tritium by galactose oxidase labelling than an equivalent number of intact cells [13,20].

Lactoperoxidase-catalysed iodination has previously been used to identify HeLa cell membrane surface proteins [21] and it was found that 14 major polypeptides were labelled in intact cells [14]. They ranged in molecular weight from approximately 10 000 to 250 000, with a prominent ^{125}I-labelled band at 180 000 [14]. Comparison with our results shows that our labelled proteins of molecular weight 180 000, 140 000, and 120 000 (all ±10%) correspond well with three of the high molecular weight proteins found by Canellakis and coworkers [14,21], whereas we find no reproducible similarities with the smaller proteins. In any case, the labelling pattern in the lower molecular weight region of the gel was rather variable from experiment to experiment (compare Figs. 3a and 4). This variability may reflect variable contamination of the population with dead cells [3].

We consistently found that gels of HeLa cell membranes labelled after isolation on beads contained a large peak of radioactivity migrating just behind the pyronin-Y tracker dye (e.g. Fig. 3b). We have not characterized this peak, and no such peak was ever seen in erythrocyte membrane-labelling experiments. It seems unlikely that this peak corresponds to a heavily labelled low molecular weight protein, since little or no Coomassie blue stain was ever seen in this

region of the gel. Huang et al. [21] also observed such a peak when isolated HeLa cell membranes were labelled in solution, showing that its presence is not an artifact induced by the beads. Further, although Huang et al. show that this peak is extractable by alcohol/diethyl ether, arguments discussed by Hubbard and Cohn [1] suggest that it may be due to free iodide rather than to labelled lipids.

An important aspect of the asymmetry experiments presented here is that membranes were isolated on derivatized polyacrylamide beads, which are considerably easier to prepare than the derivatized glass beads used in previous asymmetry studies [9]. Another difference between the two bead types is that acrylamide beads are inherently porous, having a molecular weight cut-off of 1500 (which may be increased by the hydrolysis required for derivitization), while glass beads are non-porous. This difference in porosity can be demonstrated by examining the accessibility of acetylcholinesterase (an extracellular membrane marker [11]) of membranes on beads. On polyacrylamide beads, erythrocyte membrane acetylcholinesterase is 100% accessible; on glass beads it is only 50—60% accessible (Cohen, C.M., unpublished data; Ref. 9). We interpret this to mean that acetylcholine can diffuse into the polyacrylamide bead at regions unoccupied by membrane and can gain access to the extracellular membrane surface. While we have not characterized the porosity of the acrylamide beads, it is clear from the experiments described here that lactoperoxidase (78 000 daltons), galactose oxidase (75 000 daltons), and presumably other large molecules cannot penetrate the beads and are therefore appropriate probes of protein asymmetry in experiments using polyacrylamide beads. Even though the beads are not completely covered with membrane [8], the close appression of the membrane to the bead prevents functional access of macromolecules to extracellular facing membrane components. Because the membranes on beads are thus structurally equivalent to inside-out vesicles with respect to macromolecular probes, they should be useful in the study of membrane protein and lipid organization.

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