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ANALYSIS OF POLYPEPTIDE DISPOSITION IN HUMAN ERYTHROCYTE MEMBRANES EMPLOYING MEMBRANE INVERSION

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SUMMARY

High resolution segregation of erythrocyte membrane polypeptides achieved by isoelectric focusing in 8 M urea was employed in conjunction with surface-restricted radioiodination to analyze the disposition of polypeptides within the human erythrocyte membrane. Several membrane polypeptides showed significant uptake of radioiodine, with the principal labeled component migrating between pH values of 3.0 and 3.5. Two approaches were taken in examining membrane polypeptide disposition on both faces of the erythrocyte membrane. Saturation labeling of the outer face of the membrane with one iodine isotope followed by cell lysis and re-iodination with a second iodine isotope did not prove feasible and another procedure based on surface iodination with ^{125}I, formation of sealed inside-out vesicles and re-iodination with ^{131}I was adopted. Studies of sialic acid release from the membrane surface and trypsin cleavage of radioiodinated peptides indicated that selectively labeled, sealed inside-out vesicles had been formed. The ratio of ^{125}I to ^{131}I in membrane polypeptides separated by isoelectric focusing confirmed the existence of externally disposed, internally disposed and spanning proteins.

INTRODUCTION

The increasing emphasis placed on membrane-related phenomena in virtually all segments of cell biology has prompted comprehensive efforts to examine the topographic relationships among the chemically definable components of the cell membrane [1–3]. One particular area of membrane structure which has received considerable attention involves the nature and disposition of membrane-associated proteins and glycoproteins. Both surface-restricted chemical modification and enzymatic proteolysis have been employed to characterize membrane proteins disposed towards the cell exterior and, by simultaneously altering the permeability of the membrane, attempts have been made to examine the inner face of the membrane [4–10]. With similar intent, we have combined techniques of surface-restricted chemical modifi-

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cation, membrane inversion and high resolution segregation of membrane components to examine both faces of the human erythrocyte membrane. Our findings classify the polypeptides capable of iodination as to distribution within the erythrocyte membrane and illustrate several features of plasma membrane labeling systems which, if not recognized, can lead to misinterpretation of experimental results.

EXPERIMENTAL PROCEDURES

Isoelectric focusing

Analytical isoelectric focusing was performed as described [11] with the following modifications: the upper reservoir buffer contained 2 % ethanolamine (v/v) and a constant current of 0.5 mA/gel was maintained for 15 h followed by a constant voltage of 40 V/cm for 6.5 h. Gels were either fixed in 12 % trichloroacetic acid for 4 h at 37 °C prior to slicing or stained directly with 4.1 % trichloroacetic acid, 4.1 % sulfosalicylic acid, 16.5 % methanol and 0.05 % Coomassie Blue for 12 h at 37 °C. Gels were destained in acetic acid/methanol/distilled water (5:25:70, v/v), sliced into approx. 9.0-cm sections and scanned. Scanning was ordinarily preferred to direct photography as the 18 cm, 2.5 % polyacrylamide gels are extremely difficult to handle. Radioactivity distribution was determined on 0.2-cm slices; pH gradients were measured in 0.5 ml 0.01 M KCl washes of 0.2-cm slices.

Lactoperoxidase-catalyzed radioiodination of human erythrocytes and membrane vesicles

Catalytic iodination with lactoperoxidase was performed at 23 °C by a modification of the method of Phillips and Morrison [12]. To a 10-ml reaction vessel containing $3.3 \cdot 10^9$ Rh^+, Type O erythrocytes prepared as described [13], 0.1–0.4 mCi of $Na^{125}I$ (New England Nuclear, Boston, Mass.) was added. The following reagents contained in isotonic phosphate buffer [13] were introduced sequentially: (i) 5 μl of 0.1 M NaI, (ii) 250 μl of a $3.3 \cdot 10^{-6}$ M stock solution of lactoperoxidase (Calbiochem, San Diego, California), (iii) 10 μl of 0.84 mM H_2O_2. After 5 min, an additional 5 μl of 0.1 M NaI was added and the reaction was allowed to continue for 15 min. The final reaction volume was approx. 1.3 ml. 2.5 ml of 10 mM cysteine-HCl in isotonic phosphate buffer was quickly introduced and the tube placed on ice for 10 min. Iodinated intact erythrocytes were centrifuged at $1000 \times g$ for 5 min, washed three times in isotonic phosphate buffer, and supernates counted for estimation of relative uptake of radioiodine. Lysis was monitored by determination of hemoglobin [13].

For preparation of labeled ghosts used in vesicle formation, radioiodination was performed on 8–10 1.0-ml erythrocyte suspensions containing $3.3 \cdot 10^9$ cells. Following cysteine addition, 1.0 ml of packed erythrocytes ($1 \cdot 10^{10}$–$5 \cdot 10^{10}$ cells/ml) was added. Lysis in 15-fold volume excess of hypotonic buffer, 7 mM sodium hydrogen phosphate buffer, pH 7.4, was allowed to proceed for 1 h. The lysed erythrocytes were washed in minimal volumes of hypotonic buffer as described [13].

3–3.5 mg of isolated vesicles were resuspended in vesiculization buffer, 0.7 mM sodium hydrogen phosphate, pH 7.4, with a large bore pasteur pipette and between 0.1 and 1.0 μCi ^{125}I or ^{131}I was added. The reagents employed in labeling vesicles were at 20 % of the concentrations employed in labeling intact cells and were

contained in vesiculization buffer rather than isotonic phosphate buffer; reaction volumes were identical. Following addition of cysteine, the labeled vesicles first were placed on ice for 10 min and centrifuged at 12 000 $\times g$ for 15 min. The vesicles were washed free of unreacted iodine by addition of excess volumes of vesiculization buffer followed by centrifugation.

Preparation of sealed inside-out vesicles

"Inside-out" and "right-side-out" vesicles were prepared by an established [14, 15] or modified procedure. In the modified procedure cells were lysed for 1 h in hypotonic buffer and the membranes washed repeatedly with large volumes of cold hypotonic buffer until no residual hemoglobin was detected. The final pellet was resuspended in 25 volumes of ice-cold vesiculization buffer for 30 min to 12 h at 4 °C and sedimented for 40 min at 131 000 $\times g$ in a Beckman SW 27 rotor. The pellet was resuspended to 3.5 ml in vesiculization buffer and homogenized with four gentle strokes of a 7-ml Dounce homogenizer. Vesicles were separated on a five-step discontinuous gradient of Dextran T110 (Pharmacia, Piscataway, N.J.) (density = 1.01–1.07 g/cm^3) in vesiculization buffer containing 0.1 mM $MgSO_4$. Centrifugation, recovery of vesicles from gradients and washing of top zone, inside-out, and bottom zone, right-side-out, vesicles were as described [14].

Characterization of vesicles

Protein [16] and sialic acid [17] concentrations were determined as indicated. The ability of neuraminidase to liberate sialic acid from accessible sialoprotein was measured an by established procedure [14]. Cholesterol was determined as described [18] following extraction of lipid [19].

Trypsin cleavage of surface-disposed polypeptides

The concentrations of labeled vesicles was adjusted to 1.0 mg/ml protein in isotonic phosphate buffer or vesiculization buffer and L-(tosylamido-2-phenyl)-ethyl chloromethyl ketone trypsin (Worthington Biochemicals, Freehold, N.J.), 100 μg/ml, was added at a ratio of trypsin to protein of 1:100. The reaction was allowed to proceed at 37 °C and 100 μg aliquots of vesicle protein were removed at 15-min intervals, the aliquots were placed on ice and soybean trypsin inhibitor was added at a 3:1 molar ratio over trypsin. The digestion aliquot was diluted to 1.5 ml and centrifuged at 15 000 $\times g$ for 20 min, 1.2 ml of the resulting supernatant was removed, trichloroacetic acid added to a final concentration of 10 % and the resulting precipitate counted. A control at each time interval was provided by removing a sample of a reaction mixture treated in an identical fashion but containing no trypsin.

Removal of non-covalently bound radioiodine

Lyophilized membrane preparations were dissolved in denaturing reagent, 8 M urea (Heico, Inc., Delaware Water Gap, Pa.), 0.2 % 2-mercaptoethanol, 0.02 M EDTA for 2 h at 37 °C, placed into Spectrapor No. 3 dialysis tubing (molecular weight retention minimum of approx. 3500) (Spectrum Medical Industries, Los Angeles) and dialyzed twice against 500-fold excesses of denaturing reagent.

Removal of sodium dodecyl sulfate

Sodium dodecyl sulfate was removed from denatured membrane preparations by chromatography on AG 2-X10 (BioRad, Richmond, Calif.), as described [20].

RESULTS

Lactoperoxidase-catalyzed radioiodination

To ensure restriction of iodination to the cell surface, two alterations were made in the standard labeling method [12]. H_2O_2 was introduced only once, and its concentration in the final reaction mixture was reduced to 6.5 μM. With these modifications, a sufficient uptake of iodine (17–19 %) was obtained for our purposes. Cell lysis under these conditions, as indicated by liberation of hemoglobin into wash supernatants, was less than 0.5 %; a similar degree of lysis was encountered if non-treated cells were subjected to the same rigors of centrifugation and washing. During the latter phases of these studies, a modification of the lactoperoxidase method was introduced [21]. In this systems, a H_2O_2-generating system consisting of D-glucose and glucose oxidase was employed. Virtually identical polypeptide labeling patterns were obtained, although some variation in relative peak heights was noted. The results illustrated in the manuscript are based on our modification of the original procedure [12].

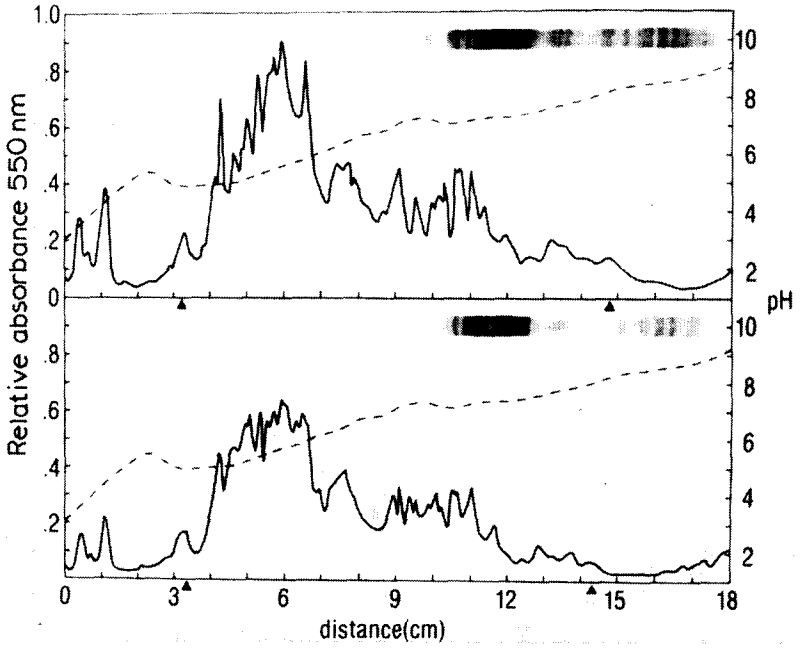

Fig. 1. Separation of human erythrocyte membrane polypeptides by isoelectric focusing in 8 M urea. Erythrocyte membranes prepared in 7 mM sodium hydrogen phosphate, pH 7.4 (top) and 5 mM sodium hydrogen phosphate, pH 8.0 (bottom). Relative absorbance at 550 nm (———) and slope of pH gradient (– – –). Area of densitometer tracing to which gel photographs correspond is indicated by ▲.

Isoelectric focusing of membrane polypeptides and distribution of covalently and non-covalently bound radioiodine

Fig. 1 illustrates the distribution of polypeptides in two different human erythrocyte membrane preparations subjected to isoelectric focusing in 8 M urea. Membrane preparations were consistently resolved into 35–40 components and run-to-run deviations in band position were insignificant when plots of band position vs relative pH profile were constructed; however, some variation in intensity of certain bands was noted on occasion. The slight discontinuity in the pH gradient was reproducible from run to run. Isoelectric focusing of a series of reference polypeptides with p*I* values in the range of 4.5–9.4 indicated that neither the slight discontinuity in pH gradient nor disproportionate heating in low conductivity regions of the ampholyte gradient was significantly altering the assignment of p*I* 0.2 units to membrane polypeptides. It should be noted that the use of a 5 mM sodium hydrogen phosphate buffer to prepare ghosts [14] consistently resulted in an overall diminution of several major protein peaks.

To ensure that maximum solubilization of the membranes was taking place in denaturing reagent, erythrocyte membranes at a protein concentration of 10 mg/ml were dissolved in 0.1 % sodium dodecyl sulfate. A portion of the resulting turbid solution was applied to an AG 2-X10 column equilibrated and developed with 8 M urea/0.01 M sodium hydrogen phosphate, pH 7.35. The effluent sample was subjected to isoelectric focusing in parallel with a membrane suspension dissolved in denaturing reagent. No discernible differences were noticeable when banding patterns were compared.

Isoelectric focusing of labeled membranes indicated approximately six major peaks of radioactivity corresponding to at least that many distinct labeled polypeptides (Fig. 2). By further reducing the H_2O_2 level over a 10-fold range, the labeling efficiency was correspondingly diminished, although the pattern of labeled polypeptides in the isoelectric focusing gels was not qualitatively altered. Increasing the concentration and number of additions of H_2O_2 to the maximum levels consistent with surface-restricted iodination similarly did not alter the distribution of labeled

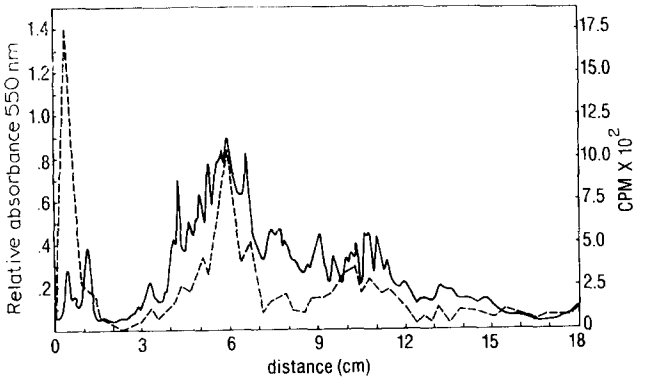

Fig. 2. Simultaneous analysis of Coomassie Blue-stained polypeptides and radioiodinated polypeptides of intact human erythrocyte membrane. Gel was first scanned at 550 nm (———) prior to slicing and ^{125}I determination (– – –).

polypeptides. The principal labeled component contained approx. 20 % of the total applied radioactivity and its position in the gel was equivalent to a polypeptide with a p*I* of 3.0–3.5. Dialysis of membrane derived from ^{125}I-labeled cells against denaturing reagent resulted in a selective depletion (15–18 %) of the major peak, indicating that free iodine which also migrates in the pH 3.0–3.5 range of the gel remains associated with the membrane.

Saturation double labeling

The concept of saturation labeling of the intact cell surface employing one isotope of iodine, inducing cell lysis and relabeling with a second iodine isotope, presented a feasible means for distinguishing polypeptides exposed on the exterior and/or interior surfaces of the cell membrane. An initial labeling with ^{127}I was performed employing the above protocol. The cells were washed free of reagents and relabeled with ^{125}I. Control cells were incubated with lactoperoxidase and H_2O_2, washed in an identical fashion to the test cells and labeled with ^{125}I. The double-labeled cells showed a 3.1-fold greater increase of ^{125}I over the control cells. Both sets of cells were lysed and the membranes subjected to isoelectric focusing (Fig. 3). A difference in labeling patterns can be noted; however, the labeling pattern of the control cells was indistinguishable from that of erythrocytes directly labeled with ^{125}I.

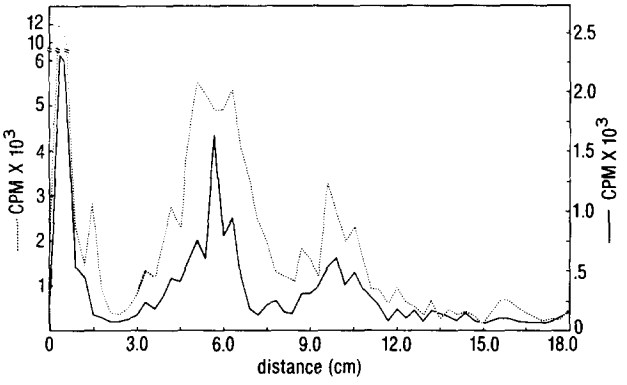

Fig. 3. Comparison of radio-iodine distribution in membranes of erythrocytes labeled with ^{125}I only (———) vs erythrocytes first labeled with ^{127}I, then washed and relabeled with ^{125}I (. . .).

Formation of sealed inside-out membrane vesicles

Two groups of erythrocytes containing an equal number of cells were subjected to lysis and vesiculization as described [14, 15] and by our modification of the above. Isoelectric focusing of the intact membranes prepared by both methods revealed a minor quantitative difference to exist in the overall banding profiles. The buffer system originally described consistently showed an overall diminution of several major protein peaks in the pH 5–7 region of the gel. After the intact membranes were subjected to vesiculization and density gradient ultracentrifugation, the various gradient fractions were removed, washed free of dextran, and subjected to either dry weight or protein analysis (Fig. 4). Using accepted convention, the top band was classified as containing vesicles of inside-out orientation and the material

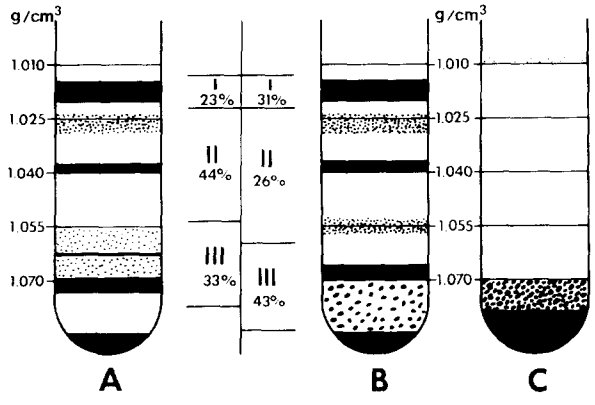

Fig. 4. Schematic representation of vesicle flotation in dextran density gradients. (A) Erythrocyte membranes vesiculized in 0.5 mM sodium hydrogen phosphate, pH 8.0. (B) Erythrocyte membranes vesiculized in 0.7 mM sodium hydrogen phosphate, pH 7.4. (C) Erythrocyte membranes subjected to vesiculization in 7 mM sodium hydrogen phosphate, pH 7.4. Percent contribution of single fractions to total protein recovered in (I), (II) and (III) is as indicated.

collected at the interface of the 1.05–1.07 g/cm^3 layer was classified as containing vesicles of right-side-out orientation. Vesiculization of ghosts in hypotonic buffer yielded mebrane fragments with densities of 1.07 g/cm^3 only.

Characterization of membrane vesicles

Membrane fractions prepared by our methods yielded ratios of sialic acid to protein of 76, 110 and 115 nmol/mg protein for the intact membrane, top zone and bottom zone vesicles, respectively. Neuraminidase treatment of both top and bottom zone fractions released 18 and 78 % of the membrane-associated sialic acid respectively. The relative inaccessibility of the protein-associated sialic acid in the 1.01 g/cm^3 zone can be taken to indicate that this fraction represents sealed vesicles in which the original surface disposition of sialoglycoprotein has been inverted. The ratio of cholesterol to protein in the top zone fraction was 670 nmol/mg protein. All of the above data are in accord with established values for the membranes and membrane vesicles [22]. Comparative isoelectric focusing of intact membranes and top zone vesicles indicated that the polypeptide composition of vesicles was deficient with regard to the parent membrane. The alteration could be accounted for by analyzing isoelectric focusing profiles of bottom zone vesicles and the concentrated supernatants of the vesiculization step.

Double labeling of inside-out vesicles

Intact erythrocytes were surface iodinated and subjected to hypotonic lysis as discussed above. During washing of the ghosts in the hypotonic media, approx. 2–3 % of the radioactivity associated with the membrane pellet could be recovered in the wash supernatant. The vesiculization step accounted for a further loss of 40 % of the membrane-associated radioactivity into the ultracentrifugation supernatant. The precipitate containing vesicles and membrane fragments was dissolved in a minimal volume of vesiculization medium and centrifuged to equilibrium. The dis-

tribution of ^{125}I-labeled polypeptides in the various gradient zones paralleled protein distribution. The top zone was harvested, washed free of dextran, and labeled with ^{131}I.

Characterization of the double-labeled inside-out vesicles

An assay for the accessibility of sialic acid to neuraminidase was performed immediately after the reactants of the second labeling were washed free of the vesicles. The neuraminidase continued to release only 18 % of the neuraminic acid for the vesicles occurring at the 1.01 g/cm^3 zone, consistent with the values determined for inside-out vesicles, vs the 78 % release for right-side-out vesicles run in parallel. A 5-fold greater release of trichloroacetic acid-precipitable ^{131}I-containing peptides as opposed to ^{125}I-containing peptides was observed 1.5 h after the addition of trypsin to the double-labeled vesicles. Fig. 5 illustrates the distribution of radioactivity associated with the polypeptides in the solubilized double-labeled vesicles. An aliquot of intact membrane derived from the labeled erythrocytes is included for reference. The difference in levels of ^{125}I present in these membranes and in the double-labeled vesicles parallelled, to an extent, observations regarding polypeptide distribution in the inside-out and intact vesicles. Identical labeling experiments with vesicles formed by an earlier procedure [14, 15] revealed a difference only in relative heights of the labeled peaks parallelling the minor differences in polypeptide composition between vesicles prepared by the two procedures.

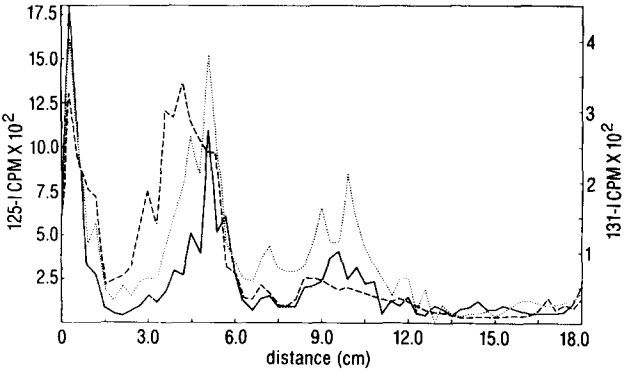

Fig. 5. Distribution of radioiodine in membranes of surface-labeled erythrocytes and in double-labeled inside-out vesicles. ^{125}I distribution in membrane of surface-labeled erythrocytes (———); ^{125}I distribution in inside-out vesicles derived from above surface-iodinated erythrocytes (· · · · ·); ^{131}I distribution in double-labeled inside-out vesicles (– – –). Labeling pattern of intact cell was determined in a parallel isoelectric focusing run with the double-labeled vesicles.

DISCUSSION

The intimate association of protein and lipid in the cell membrane poses a distinct problem for understanding cell surface topography, as detergents and other denaturants are required to maintain solubility of polypeptides following their separation from the lipid matrix. While the lipid component complicates analytical studies of membrane protein, it can be utilized advantageously, as polypeptides buried within

the lipid phase are not susceptible to surface-directed chemical modification [3–10, 21, 33]. If it were possible to achieve saturation labeling of the cell surface by a technique of surface-directed chemical modification, it should be possible to achieve selective labeling of the inner face of the membrane using an isotopically different modification reagent which had simultaneous access to both the exterior and interior of the cell. The results of the saturation labeling experiments indicate that the approach is not feasible. If near saturation labeling had been achieved at the maximum concentrations of H_2O_2 consistent with surface-directed modification, the secondary iodination with ^{125}I should have revealed less uptake of radioiodine than seen in the primary iodination. The finding of an approximately 3-fold increase in uptake of label suggested that either the reagents were gaining access to the cell interior or the initial labeling had induced an alteration in membrane structure exposing otherwise inaccessible regions of surface polypeptides. The minimal membrane damage as measured by insignificant hemoglobin release would tend to support the latter contention. The qualitative shifts in labeling patterns could arise from two sources: either the surface rearrangement allowed modification of other, isoelectrophoretically distinct polypeptides which may not have been noted in similar experiments employing conventional electrophoresis in sodium dodecyl sulfate or the polypeptide banding profiles are being influenced by p*I* shifts accompanying radioiodination (see below).

As there were no means to circumvent the problems in interpretation of saturation labeling experiments, an alternative approach relying on high resolution separation of membrane polypeptides by isoelectric focusing, lactoperoxidase-catalyzed radioiodination and vesiculization of membranes in hypotonic solution was adopted. Erythrocytes first would be surface iodinated with ^{125}I, induced to form sealed vesicles displaying inside-out orientation with regard to the parent membrane, and relabeled with ^{131}I. In this fashion, the two surfaces of the cell membrane would be independently exposed to a surface-directed labeling system. While the approach appears straightforward, considerable caution had to be exercised so that: (1) membrane polypeptides could be separated in a reproducible fashion, (2) labeling would remain surface restricted, and (3) orientation of the inside-out vesicles would be preserved.

As previously reported, analytical isoelectric focusing in polyacrylamide gels represents a useful alternative to the more conventional methods of membrane protein separation [11, 24]. Utilizing the above-described procedure, highly reproducible banding patterns were obtained for membranes derived from a single source. In evaluating the banding profiles of membrane polypeptides, consideration was given to artifactual banding introduced by degradation of polypeptides by proteolytic enzymes or by carbamylation of polypeptides owing to contamination of the urea by cyanate. Addition of protease inhibitors to membrane preparations prior to 37 °C incubation did not influence the electrofocusing distribution of proteins [7]. To minimize carbamylation effects, highly purified, cyanate-free urea was employed, although the possibility of cyanate formation at the acidic end of the pH gradient does exist, however, and should not be discounted. Shorter incubation periods (60–90 min) in denaturing reagent did not alter the polypeptide banding patterns.

The isoelectric focusing method proved superior to methods based exclusively on separation by molecular weight difference such as polyacrylamide gel electrop-

horesis in sodium dodecyl sulfate in resolving the radioiodinated, exterior-disposed human erythrocyte membrane polypeptides. An additional complication in interpretation of these isoelectric focusing profiles involves the shift in p*I* of the phenol group of tyrosine from 10.1 to 8.2 upon conversion to a monoiodotyrosine derivative (formation of diiodotyrosine apparently does not occur under lactoperoxidase labeling conditions similar to those employed in the majority of the experiments described [21]. Several lines of experimental evidence indicate that the effect on overall p*I* is not significant: (1) a p*I* shift of only $\approx$ 0.4 is observed in angiotensin I (molecular weight approx. 1400) following monoiodination of its single tyrosine [22]; (2) diminution of H_2O_2 concentration (10-fold range) with a subsequent reduction in extent of modification did not significantly alter the banding profiles, although the expected decreases in peak height were noted; (3) employing the amino acid composition of a membrane polypeptide [26], pK_a values of the ionizable amino acid side groups and constructing theoretical protein titration curves [27], it could be calculated that the monoiodination of all six tyrosines resulted in a shift in p*I* of the membrane polypeptide of less than 0.1 pH unit. Although the observed shifts in p*I* accompanying secondary iodination in the saturation labeling experiments (Fig. 3) were towards both more acidic and more basic p*I* values, it is possible that diiodination of tyrosine is influencing membrane polypeptide p*I*. While the above suggest minimal effects of trace iodination on the p*I* of polypeptides, we do not feel that assignment of p*I* values to labeled components is in order. Determination of such constants requires preparative focusing conditions where precise estimation of gradient equilibrium and pH measurements of discrete fractions can be made.

Although extensive analyses of top zone fractions indicated they are sealed and/or display inside-out orientation [14, 15, 28], consideration had to be given to the effect of iodination on the nature of the sealed inside-out vesicles. Labeling with ^{131}I on the exterior surface of ^{125}I-labeled inside-out vesicles was performed immediately after recovery from the density gradients; the significantly higher uptake (approx. 8–10-fold) of radioiodine/mg of membrane (vesicle) protein allowed for a concomitant reduction in the concentration of labeling reagents. In addition: (1) parallel determinations of radioactivity and protein in the various fractions of membrane vesicles derived from ^{125}I-labeled and unlabeled ghosts indicate that iodination did not significantly alter the flotation density of the vesicles; (2) the levels of the sialic acid released by neuraminidase from labeled and unlabeled inside-out vesicles were identical, although transmembrane re-orientation of sialic acid-containing glycoproteins has been suggested [29]; and (3) trypsin digestion studies suggest that ^{131}I-containing peptides were disposed towards the exterior of the inside-out vesicles (inner face of the parent membrane) and ^{125}I-containing peptides were sequestered on the interior face of the vesicles (outer face of the parent membrane), protected from the surface-restricted action of trypsin. In fully evaluating the significance of this last findings, attention should be given to the relative efficiency of trypsin in cleaving exterior- and interior-disposed polypeptides [30].

The simultaneous analysis of distribution of ^{131}I- and ^{125}I-labeled polypeptides provides insight into the disposition of proteins in the erythrocyte membrane. Several regions of the isoelectric focusing gel show significant uptakes of both the ^{125}I and ^{131}I labels; this observation could indicate that these polypeptides are exposed to both the interior and the exterior faces of the erythrocyte membrane.

Another class of polypeptides exhibits a greater ratio of ^{131}I and ^{125}I: this finding suggests disposition of these proteins predominantly towards the inner face of the cell membrane. A third group of polypeptides is labeled to a greater extent on the outer face of the erythrocyte membrane. In evaluating the labeling patterns, it must be remembered that : (1) ^{125}I-labeled polypeptides are exposed to rather extreme hypotonic conditions during the vesiculization step with concomitant extraction of surface-labeled polypeptides; (2) the overall polypeptide composition of inside-out and right-side-out vesicles is different from that of the parent membrane [22]; (3) the above mentioned reduction in the concentration of reagents for vesicle iodination may have resulted in iodination of only a fraction of the exposed polypeptides. Increasing the levels of labeling reagent/mg of protein to the same levels as employed in iodination of intact erythrocytes, however, did not result in qualitative alterations in the isoelectric profiles of the labeled polypeptides. Conclusions regarding the relative homogeneity of the various gel fractions containing varying amounts of ^{131}I and/or ^{125}I must be reserved until preparative isolation and re-electrofocusing or electrophoresis in sodium dodecyl sulfate-containing polyacrylamide gels can be achieved.

A previous analysis of membrane protein disposition utilized membrane inversion and concluded that membrane polypeptide orientation was asymmetric and that certain polypeptides spanned the membrane. These conclusions were questioned, however, when it was learned that premature Mg^{2+} addition resulted in contamination of inside-out vesicles by right-side-out vesicles [15]. Surface-restricted labeling of intact erythrocytes with formyl methionyl sulfone methyl phosphate resulted in modification of the two principal glycoproteins [7] Lactoperoxidase-catalyzed radioiodination primarily resulted in modification of one major size class of polypeptide [12]. In both cases the labeling of stroma revealed derivitization of most membrane proteins [5, 8]. Recently it has been demonstrated that glycophorin, the major glycoprotein of the human erythrocyte, could be iodinated on the cell exterior or on the interior if leaky ghosts were prepared [31]; localization of the iodinated residues in the primary structure suggests that glycophorin spans the membrane [32]. A variation in this approach involves sealing of lactoperoxidase into ghosts allowing for exclusive modification of the interior face of the membrane [30, 33, 34]. In two of the studies [33, 34], surface labeling was restricted to the major glycoprotein and the $\approx$ 100 000 molecular weight Component III. Interior labeling was directed at Component III and spectrin and not at the principal glycoprotein. Another study, utilizing the same technique did demonstrate, however, that the principal glycoprotein and two minor glycoproteins were labeled on both faces of the membrane [30]. These authors pointed out the importance of considering the relative extent of chemical modification in classifying membrane polypeptide organization, a fact which may explain the discrepancy between their work and the other studies.

The principal class of double-labeled polypeptide in our studies migrated in the pH 3.0–3.5 region of the gel. The reported p*I* of glycophorin is approx. 3.0 [35] and difficulties in making precise p*I* measurements at the lower end of the range of commercially available ampholytes could explain the discrepancy. In addition, this region of the isoelectric gel was devoid of other components, and abbreviated runs failed to show any material electrophoresing out of the gels.

Preliminary indications are that the principal labeled component and a preparation of lithium 3,5-diiodosalicylate-extracted glycophorin represent the same molecular entity. In this sense, the inversion technique we employed supports earlier conclusions [30, 31] regarding the spanning nature of glycophorin. The extraction during hypotonic vesiculization of polypeptide complexes such as spectrin which are ordinarily associated with the inner-membrane face may facilitate iodination of partially buried, interior-disposed regions of spanning polypeptides [3, 10, 30]. The identities of the other components separated by the isoelectric focusing technique are not clear at present and the recent introduction of techniques for analyzing charge heterogeneity of the major size classes of membrane polypeptide [24, 36] should aid in defining these species within the nomenclature previously utilized in characterizing erythrocyte membrane protein [10].

Unlike other methods previously utilized to analyze polypeptide disposition, the technique outlined in this manuscript does not require leaky or sealed ghosts which form only with the non-nucleated erythrocytes. The inversion technique adopted in these studies is applicable to any membrane capable of undergoing endocytosis and forming sealed vesicles and should be of use in examining those systems [37].

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REFERENCES

1 Singer, S. J. and Nicolson, G. L. (1972) Science 175, 720–731
2 Bretscher, M. S. (1973) Science 181, 622–629
3 Singer, S. J. (1974) Annu. Rev. Biochem. 43, 805–833
4 Phillips, D. R. and Morrison, M. (1971) Biochem. Biophys. Res. Commun. 45, 1103–1107
5 Phillips, D. R. and Morrison, M. (1971) Biochemistry 10, 1766–1771
6 Bender, W. W., Garan, H. and Berg, H. C. (1971) J. Mol. Biol. 58, 783–797
7 Bretscher, M. S. (1971) J. Mol. Biol. 58, 775–781
8 Bretscher, M. S. (1971) J. Mol. Biol. 59, 351–357
9 Wallach, D. F. H. (1972) Biochim. Biophys. Acta 265, 61–83
10 Steck, T. L. (1974) J. Cell Biol. 62, 1–19
11 Merz, D. C., Good, R. A. and Litman, G. W. (1972) Biochem. Biophys. Res. Commun. 49, 84–91
12 Phillips, D. R. and Morrison, M. (1970) Biochem. Biophys. Res. Commun. 40, 284–289
13 Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119–130
14 Steck, T. L., Weinstein, R. S., Straus, J. H. and Wallach, D. F. H. (1970) Science 168, 255–257
15 Kant, J. A. and Steck, T. L. (1972) Nat. New Biol. 240, 26–27
16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
17 Warren, L. (1959) J. Biol. Chem. 234, 1971–1975
18 Leffler, H. H. and McDougald, C. H. (1963) Am. J. Clin. Pathol. 39, 311–315
19 Albrink, M. J. (1959) J. Lipid Res. 1, 53–59
20 Lenard, J. (1971) Biochem. Biophys. Res. Commun. 45, 662–668
21 Hubbard, A. L. and Cohn, Z. A. (1972) J. Cell Biol. 55, 390–405

22 Steck, T. L., Fairbanks, J. and Wallach, D. F. H. (1971) Biochemistry 10, 2617–2624
23 Poduslo, J. F., Greenberg, C. S. and Glick, M. C. (1972) Biochemistry 11, 2616–2621
24 Bhakdi, S., Knüfermann, H. and Wallach, D. F. H. (1974) Biochim. Biophys. Acta 345, 448–457
25 Corvol, P., Rodbard, D., Drouet, J., Catt, K. and Menard, J. (1973) Biochim. Biophys. Acta 322, 392–400
26 Martin, R. B. (1964) in Introduction to Biophysical Chemistry, pp. 93–96, McGraw–Hill Book Co., New York
27 Marchesi, V. T., Tillack, T. W., Jackson, R. L., Segrest, J. P. and Scott, R. E. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1445–1449
28 Perrone, J. R. and Blostein, R. (1973) Biochim. Biophys. Acta 291, 680–689
29 Nicolson, G. L. (1973) J. Cell Biol. 57, 373–387
30 Morrison, M., Mueller, T. J. and Huber, T. (1974) J. Biol. Chem. 249, 2658–2660
31 Segrest, J. P., Kahane, I., Jackson, R. L. and Marchesi, V. T. (1973) Arch. Biochem. Biophys. 155, 167–183
32 Jackson, R. L., Segrest, J. P., Kahane, I. and Marchesi, V. T. (1973) Biochemistry 12, 3131–3138
33 Shin, B. C. and Carraway, K. L. (1974) Biochim. Biophys. Acta 345, 141–153
34 Reichstein, E. and Blostein, R. (1973) Biochem. Biophys. Res. Commun. 54, 494–500
35 Marchesi, V. T. (1972) Methods Enzymol. 28, 252–254
36 Knüfermann, H., Bhakdi, S., Schmidt Ullrich, R. and Wallach, D. F. H. (1973) Biochim. Biophys. Acta 330, 356–361
37 Bennett, V. and Cuatrecasas, P. (1973) Biochim. Biophys. Acta 311, 362–380