similar measurements on synthetic haptenic groups or to larger haptenic polypeptides which still retain some of their tertiary structure (Fujio *et al.*, 1968). These results are not surprising if one assumes that the molecular conformation of the hapten plays a role in its ability to combine specifically with the antibody. It has been suggested (Crumpton and Small, 1967) that in aqueous solution of a haptenic peptide, at any one time, only a small fraction of the molecules may possess the configuration they did in the whole antigen molecule. Thus, a large percentage of the hapten molecules (those not possessing the native configuration) are precluded from interacting with the antibody molecules.

The data from the equilibrium dialysis studies imply that antibody produced in rabbits to O-Fd is directed either to the COOH-terminal pentapeptide or the NH_2-terminal heptapeptide of the molecule, and that these two regions constitute the main antigenic determinants of O-Fd. It should be emphasized that these results apply only to the experimental conditions reported here, and it is probable that other antigenic determinants are present on the ferredoxin molecule, although under our laboratory conditions we have been unable to detect them.

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Exposed Protein on the Intact Human Erythrocyte*

David R. Phillips† and Martin Morrison‡

ABSTRACT: A method is described which shows that only one molecular weight class of proteins on the human erythrocyte membrane is in an exposed position on the outside of the cell. The method employs lactoperoxidase, a high molecular weight enzyme, to catalyze the iodination of membrane proteins. Since the enzyme iodinates by means of the usual enzyme–substrate complex and cannot diffuse through the membrane because of its size, only those proteins on the surface of the erythrocyte membrane are labeled. In these studies, three states of organization of the erythrocyte membrane have been employed: (1) the intact erythrocyte where only proteins on the surface of the cell were available to lactoperoxidase, (2) the isolated stroma, and (3) the solubilized membrane where all proteins were accessible. Membrane fractions, once iodinated, were solubilized in sodium dodecyl sulfate and fractionated by disc gel electrophoresis. Results showed that only one molecular weight class of proteins with a molecular size of 90,000 is exposed to the outside of the intact erythrocyte while in the conversion of erythrocytes to stroma most membrane proteins became exposed.

Proteins not labeled in these two membrane states could be labeled when free from the membrane indicating that some proteins are not exposed at either membrane surface.

One of the most widely studied membranes from mammalian sources is that of the human erythrocyte. Both qualitatively and quantitatively the lipids, carbohydrates, and proteins have been analyzed in this membrane (Bakermann and Wasemuller, 1967; Rosenberg and Guidotti, 1969; Maddy, 1966; Rouser *et al.*, 1968; Dodge *et al.*, 1963). Other work has evaluated the topography of the membrane with respect to the carbohydrate and lipid moieties (Murphy, 1965; Lenard and Singer, 1968; Eylar *et al.*, 1962; Winzler, 1969).

Although the number and type of peptide units present in the erythrocyte membrane have been defined, determinations of the protein distribution in the membrane has evolved very slowly. The primary reason for the limited amount of knowledge on the spatial arrangement of proteins in this membrane and all membranes is that no technique unequivocally establishes protein position in the membrane. Efforts have been made to determine the arrangement of proteins employing protein-labeling reagents; however, these reagents yield equiv-

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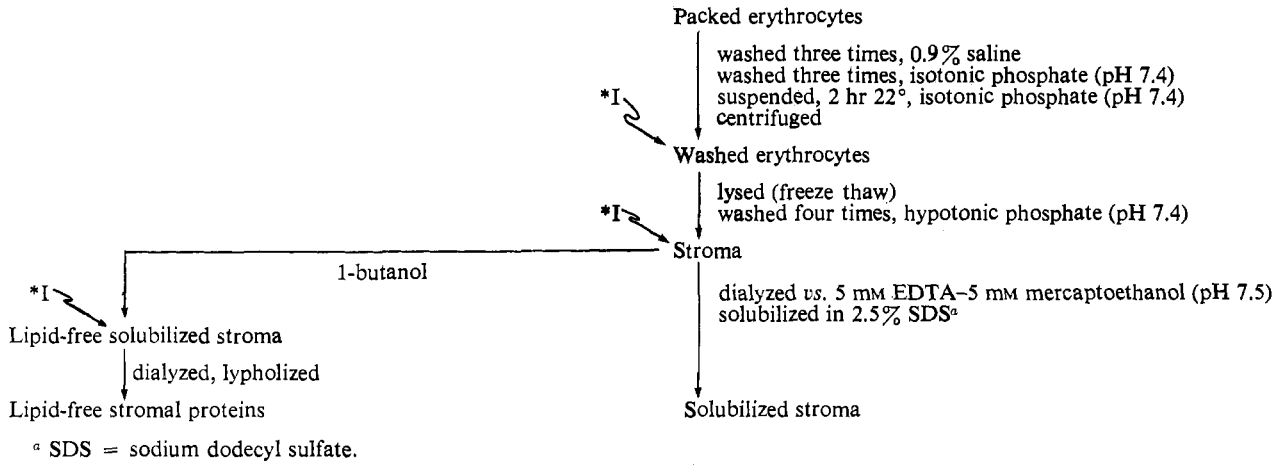

[a] SDS = sodium dodecyl sulfate.

FIGURE 1: Preparative scheme for the human erythrocyte fractions studied.

ocal results. They are relatively small in size and hence can pass into and through the membrane. They therefore do not give an exclusive vectorial label (Berg, 1969; Maddy, 1964). Many of those reagents will disrupt the membrane which also leads to results of questionable value (Berg, 1969). Enzymatic digestion of membranes removes groups from the surface of the membrane (Uhlenbruch, 1961); however, this approach has provided little reliable data on the protein distribution except when antigenic determinants have been altered (Winzler *et al.*, 1967). Antigen–antibody reactions clearly indicate the location of antigenic determinants on the surface of the membrane; however, use of this technique to determine protein distribution is limited since most of the red cell antigens are not proteins (Watkins, 1966). Finally, the activities of certain enzymes have suggested their location in the membrane (Schroter and Neuvians, 1970; Green *et al.*, 1965; Nilsson and Ronquist, 1969; Marchesi and Palade, 1967); however, diffusion of substrates and products of these enzymes have made positional interpretations difficult.

This report introduces a new method to determine the vectorial arrangement of membrane proteins. A general procedure is outlined making this method useful for all cell membrane systems. Using this method, we have been able to show that only one molecular weight class of proteins present in erythrocyte stroma is in an exposed position on the exterior of the human erythrocyte membrane.

Methods

All chemicals employed in these studies were reagent grade. Carrier-free ^{125}I was purchased from Schwarz Chemicals. Lactoperoxidase was isolated by the method of Morrison and Hultquist (1963).

Human erythrocytes were purified by a modification of the method of Dodge *et al.* (1963) as outlined in Figure 1. Normal A^+ blood was drawn into acid–citrate–dextrose and used in less than 5 days. All manipulations were carried out at 0–4° unless otherwise stated. Packed cells were washed three times in three volumes of 0.9% saline and once in 0.106 M sodium phosphate buffer (pH 7.4). The washed cells were then suspended in the 0.106 M sodium phosphate buffer for 2 hr at 22° to minimize the platelet count. The cells obtained from this suspension were the *intact erythrocytes*.

Erythrocytes introduced into media of a lower and higher osmotic strength cause the cells to swell and shrink, respectively (Seeman *et al.*, 1969). *Swollen erythrocytes* were obtained by suspending the intact erythrocytes in three volumes of 0.053 M sodium phosphate buffer (pH 7.4). The cells were centrifuged, and the packed cells were then suspended in an equal volume of 0.053 M sodium phosphate buffer. *Shrunken erythrocytes* were similarly prepared using 0.20 M sodium phosphate buffer (pH 7.4).

Stroma was isolated from intact, swollen, or shrunken erythrocytes as indicated in Figure 1. The packed, washed erythrocytes were lysed by freezing and thawing and diluted with four volumes of distilled water. The stroma was isolated by centrifugation at 35,000*g* for 20 min and washed three times by repeated suspension and centrifugation in 0.0068 M sodium phosphate buffer (pH 7.4) (Dodge *et al.*, 1963). The resulting pellet of milky-white *stroma* was used in the experiments outlined below.

Lipid-free *solubilized stroma* was obtained by extraction of the lipids from stroma with 1-butanol according to Rega *et al.* (1967). The protein solution was then dialyzed against water and lyophilization to dryness. The resulting powder was soluble in the 0.0068 M sodium phosphate buffer.

Iodination Procedure. Iodination of the washed, intact erythrocytes, the hemoglobin-free stroma, or the solubilized membrane proteins free of lipid was catalyzed by lactoperoxidase upon addition of hydrogen peroxide. The reaction mixture contained the membrane material, 3×10^{-7} M lactoperoxidase, and 10^{-5} M [^{125}I]NaI. The reactions were initiated by the addition of hydrogen peroxide. A Radelkis Type DP-711 iodide-sensitive electrode from Electrochemical Instruments, Budapest, Hungary, coupled to a Metrohm Impulsomat E473 Combititrator as described by Morrison *et al.* (1971) was employed to maintain the iodide concentration at 10^{-5} M during the course of the iodination of membrane proteins. The titrant contained 10^{-3} M KI with the same specificity activity ^{125}I as the reaction solution. After one aliquot of hydrogen peroxide was consumed, the amount of titrant added was recorded and an additional aliquot of hydrogen peroxide was added to again initiate the reaction. The sequential additions were employed to maintain low concentrations of hydrogen peroxide minimizing membrane oxidations, and at no time did the hydrogen peroxide concentration exceed 8 μM. At the termination of the reaction the mixture was chilled. Iodinated erythrocytes were washed twice with three volumes of 0.106 M sodium phosphate buffer (pH 7.4), lysed, and the stroma isolated as indicated above. The iodinated

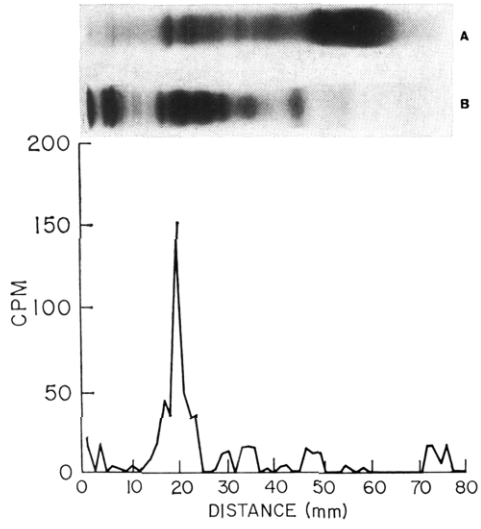

FIGURE 2: Intact erythrocyte iodination. Iodine distribution into the membrane proteins from the iodination of intact erythrocytes. The stroma from iodinated intact erythrocytes was isolated and fractionated by disc gel electrophoresis as described in Methods. Gel A is stained for carbohydrates, gel B is stained for protein, and the graph shows the radioactive distribution in the gel.

stroma were washed two times in 0.0068 M sodium phosphate buffer (pH 7.4) while the iodinated lipid-free solubilized stroma were exhaustively dialyzed against distilled water and lyophilized.

Gel Electrophoresis. The iodinated fractions were dialyzed against 5 mM mercaptoethanol–5 mM EDTA (pH 7.4) for 18 hr. All membrane fractions were then solubilized in 2–3% sodium dodecyl sulfate and electrophoresed on 5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate according to Lenard (1970a). Gels with identical samples were stained with coomassie blue to evaluate the protein distribution (Shapiro *et al.*, 1967) and stained for carbohydrate by the method of Clarke (1964). The distribution of radioactive iodide in the gel was determined by slicing the gels laterally to 1.5-mm sections with a Canalco slicer and counting the gamma emissions from each slice.

Other Procedures. Protein concentrations were determined by the method of Lowry *et al.* (1951). Hydrogen peroxide concentrations were determined from the optical density at 230 nm using a molar extinction coefficient of 72.4. The concentration of lactoperoxidase was determined from the millimolar extinction of 112 at 412 nm. The amount of lysis and exchange of K^+ for Na^+ of the iodinated erythrocytes was measured as reported by Berg (1969). Lipids were extracted from iodinated erythrocyte membranes by suspending the stroma in chloroform–methanol (1:1, v/v) at 22° for 10 min. The aqueous phase and organic phase were separated and the organic phase was taken to dryness in rotary evaporator. Chloroform–methanol was added to the powder obtained and the material which dissolved was taken as membrane lipid free of protein (Folch *et al.*, 1957).

Results

Membrane proteins in intact erythrocytes, hemoglobin-free stroma, and lipid-free solubilized stroma from human erythrocytes were iodinated. The proteins iodinated in these three states of the membrane varied and each state of the membrane will therefore be discussed separately.

TABLE I: Ion Retention and Susceptibility of Lysis of Iodinated Intact Erythrocytes.[a]

Time (min)	K^+ mequiv/l. of Packed Cells	% Lysis
0	69	0.19
75	70.7	0.08
135	57.8	0.11
255	58.1	0.14

[a] Washed erythrocytes were iodinated, suspended in three volumes of isotonic phosphate buffer (pH 7.4), and stored at 22°. At various time intervals an aliquot of the cell suspension was centrifuged and the concentration of Na^+ and K^+ in the packed cells as well as the per cent lysis was determined as described in Methods. Each value is an average of two determinations.

Intact Erythrocytes. Stroma has been isolated from iodinated intact erythrocytes which contained 30,000 iodine atoms/erythrocyte membrane. These membrane proteins were fractionated by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate and a typical gel stained for protein and carbohydrate is presented in Figure 2. The stroma proteins were clearly resolved into many molecular weight classes in agreement to the report of Lenard (1970a) and Bellhorn *et al.* (1970), and in contrast to the observations of Kiehn and Holland (1970). Complete resolution is dependent on prior treatment of the membrane with mercaptoethanol and EDTA as suggested by Lenard (1970a) and appears to be the source of the discrepancy. All blood types were electrophoresced in this system, and although the gross distribution pattern of stroma proteins was constant between the blood types, individual variations in certain molecular weight classes were observed. In Figure 2, the glycoprotein distribution in the gel indicates the presence of several glycoproteins, as indicated by Lenard (1970a). Two regions in the gel contain most of the carbohydrate and correspond to molecular weights of 18,000 and 15,000. In other molecular weight regions the carbohydrate stain was less intense. However, the stain pattern was reproducible. The membrane proteins from iodinated intact erythrocytes is also shown in Figure 2. The distribution of radioactive iodide in the gel indicates that only peptide chains in the 90,000 molecular weight group contain iodine. Further additions of hydrogen peroxide and prolonged incubation did not result in further incorporation of iodide into additional membrane proteins. The ionic strength of the iodinating media did not appear to effect this result since cells suspended in 0.20 M sodium phosphate buffer (pH 7.4) or 0.053 M sodium phosphate buffer (pH 7.4) yielded identical results.

Lipid extracted from these membranes contained no radioactivity indicating that membrane lipid is not iodinated. A nonenzymatic iodination procedure would have resulted in the iodination of lipid, primarily across isolated double bonds.

The membranes from iodinated erythrocytes appeared to be structurally similar to those from uniodinated erythrocytes since iodinated erythrocytes retained the normal resistance to lysis and ion selectivity as uniodinated erythrocytes. Table I shows that after 4-hr incubation at 22°, almost no lysis of the cells occurred (<1%) and the cells exchanged only 10% of the intracellular potassium ions for sodium ions.

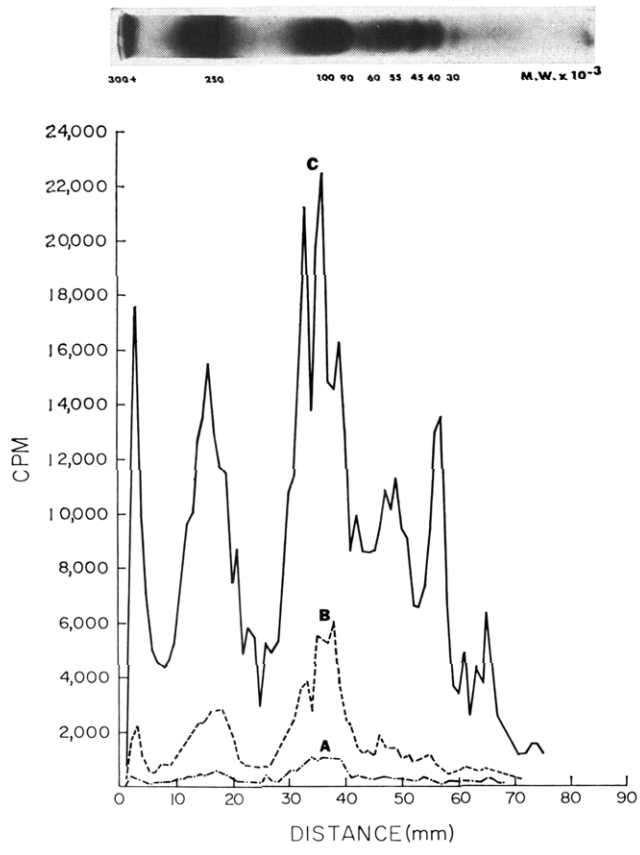

FIGURE 3: Stroma iodination. Iodine distribution into the membrane proteins from the iodination of hemoglobin-free stroma. The stroma was iodinated to the extent of 0.8×10^5, 3.1×10^5, and 13×10^5 atoms of iodide per stroma, curves A, B, and C, respectively.

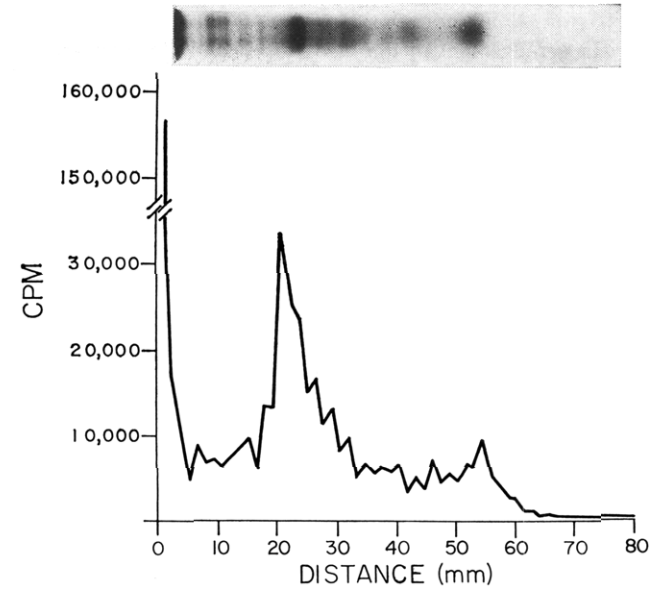

FIGURE 4: Membrane protein iodination. Iodine distribution into the membrane proteins from the iodination of lipid-free solubilized stroma. The membrane proteins, containing 0.25 μmole of iodine/mg of protein was fractionated by disc gel electrophoresis as described in Methods.

Stroma. In Figure 3, the results from the lactoperoxidase-catalyzed iodination of hemoglobin-free stroma is presented. Curves A, B, and C in Figure 3 correspond to 0.8×10^5, 3.1×10^5, and 13×10^5 atoms of iodine incorporated into the stroma per erythrocyte, respectively. The different amounts of iodination were obtained by varying the amount of hydrogen peroxide added to the iodinating system. Almost all molecular weight classes are labeled and appeared to be equally accessible to lactoperoxidase since the extent of iodination was proportionally increased in all groups iodinated. Two molecular weight classes, 45,000 and 30,000, do not contain a significant amount of label.

Solubilized Stroma. The lipid-free solubilized membrane proteins were also iodinated and the results are shown in Figure 4. This preparation readily incorporates iodine and, most important, all protein molecular weight classes are labeled with iodine as noted from the radioactive distribution in the gel. The resolution of membrane proteins in the absence of lipid is superior to that in the presence of lipid. This may account for the appearance of new protein bands in this gel. It may also be that lipoprotein complexes could persist in the presence of sodium dodecyl sulfate which would change the apparent molecular weight of the proteins.

Discussion

Lactoperoxidase has been employed to determine the vectorial arrangement of the proteins in the human erythrocyte membrane in a direction perpendicular to the surface of the membrane. This enzyme catalyzes iodination of exposed tyrosine and histidine groups on proteins with the predominant reaction occurring on tyrosine. The iodination of tyrosine takes place on the aromatic ring ortho to the hydroxyl group (Morrison *et al.*, 1970). This reaction has been shown to occur via an enzyme–substrate complex between the protein substrate and lactoperoxidase at the pH studied, 7.4 (Morrison *et al.*, 1970). The human erythrocyte has been shown to be impermeable to macromolecules such as lactoperoxidase (Phillips and Morrison, 1970). Therefore, lactoperoxidase will catalyze the iodination of only those proteins of the erythrocyte membrane which are exposed to the suspending media.

Other protein-labeling reagents previously employed for the determination of vectorial arrangement of proteins on the erythrocyte membrane are not as specific as the lactoperoxidase-iodinating system. Most of these reagents are small organic molecules which are permeable to the membrane such as N-substituted maleimides (Sandberg *et al.*, 1969; Lenard, 1970b), 1-fluoro-2,4-dinitrobenzene (Dodge *et al.*, 1963), stilbene isothiocyanate (Maddy, 1964), and mercury (Weed *et al.*, 1962). These reagents do not, therefore, label membrane components exclusively on the exterior of the membrane and therefore do not provide unequivocal data on the vectorial arrangement of protein in the membranes. Such reagents react with protein components on the basis of the chemical reactivity of groups on the protein but not necessarily on the basis of their position in the membrane. This is not the case for the lactoperoxidase-iodinating system, where the size of this enzyme limits its substrates to the outermost protein components of the membrane.

Treatment of red cell and stroma with hydrolytic enzymes has provided some evidence on the membrane structure. Thus, neuraminidase has been used to show that sialic acid is present on the outside of the red cell (Eylar *et al.*, 1962). Lipase has been employed to show that polar moieties of phospholipids are exposed to the outside of the cell membrane (Lenard and Singer, 1968). Similarly, the proteolytic enzymes, trypsin (Winzler *et al.*, 1967), and Pronase (Ohkuma and Shinohara, 1967) have been used to cleave off antigenic determi-

nants from the membrane. However, limited information on protein arrangement in the membrane has been obtained by these hydrolytic enzymes. The most obvious reason for this is that the use of these enzymes results in the removal of many layers from a membrane, releasing or digesting substrates not actually located on the surfaces. Moreover, the removal of membrane components might well result in alterations, destroying the spatial arrangement of membrane components.

The very interesting studies of Berg (1969) have shown that diazonium salt of sulfanilic acid will react with the erythrocyte to produce labeled membrane components. Berg has concluded that the lipids and proteins labeled are located outside the permeability barrier of the membrane. Although these studies were well conceived, the author did not show that a different labeling pattern would have resulted if the membrane was disorganized. In this type of study it is important to show that all the disorganized membrane proteins would react similarly, once exposed to the reagent. Figures 2, 3, and 4 do in fact provide such evidence for the present study. These figures show that all proteins have tyrosine residues which react at a similar rate when exposed to the labeling system. It should also be pointed out that treatment with the diazonium salt of sulfanilic acid results in loss of ion selectivity in the membrane within 10 min and can induce complete lysis of the cell. It is difficult, therefore, to determine whether the membranes were labeled after alteration. In contrast, cells labeled in the present study retain ion selectivity and show resistance to lysis, indicating that iodinated membranes retain their native configuration. The component with the highest specific activity labeled with the diazonium salt has a molecular weight of 140,000 and does not appear to correspond to the peptide chain labeled with iodine.

The data obtained with the iodinating systems for these studies clearly indicate that this system can be used to establish the arrangement of membrane protein. Thus, in the intact red cell only a single protein is iodinated even when the maximum amount of iodine is incorporated into the intact cell. In the stroma preparation, on the other hand, most of the membrane proteins are iodinated while the proteins of the completely disassociated membrane are all iodinated. This clearly indicates that the iodination procedure employed does reflect the state of the membrane proteins. In the stroma preparation at least two molecular weight classes of proteins are not labeled with radioactive iodine indicating that these proteins are not exposed to lactoperoxidase even when the cell is lysed prior to iodination and are probably covered by carbohydrates, lipids, or other proteins.

The qualitative and quantitative difference observed in the labeling pattern of intact cells and stroma is significant and reproducible. On one point the data are clear; only one molecular weight class of protein is exposed to lactoperoxidase on the outermost surface of the intact erythrocyte. This is shown by the observation that only this molecular weight class contains radioactivity even when all available sites on the exterior of the erythrocyte have been iodinated. It is possible that media different from isotonic could induce a conformational change in the membrane such that buried proteins of the membrane would be exposed to the exterior surface. The results show that changes in the ionic environment which stretch the membrane by swelling in low osmotic strength or shrink it in hypertonic solution (Seeman *et al.*, 1969) do not expose any additional proteins.

It is unlikely that proteins exist on the exposed exterior surface of the erythrocyte with all tyrosines buried. Crystallographic studies have indicated that hydrophylic groups in general are exposed to the exterior of proteins (Perutz, 1969). Indeed, all erythrocyte membrane proteins, once solubilized and free of lipid, can be iodinated by lactoperoxidase. The forces that hold membrane components together are believed to be primarily hydrophobic in nature (Lenard and Singer, 1968), with the polar components oriented to the aqueous environment which would be expected to contain the iodinatable tyrosine and histidine groups. These data suggest that any protein oriented on an exposed surface of a membrane should be iodinated by lactoperoxidase.

The difference in labeling between the stroma and the intact cell can be explained in a variety of ways. The stroma preparation used in these studies may be leaky, thus providing exposure of both the interior and exterior surfaces of the membrane to the iodinating system. If this is the case, and the exterior surface of the membrane has not been altered in the preparation of the stroma, the different patterns of labeling between the intact cell and stroma represents the proteins of the interior surface. Although much evidence suggests that the stroma are resealed, the methods of evaluating the extent of resealing depend on morphological observations or chemical methods incapable of establishing whether a small percentage of the stroma were open at any time. Hence, the above suggestion does not seem unlikely.

If the stroma are completely resealed, one must postulate a modification of the membrane surface. This could occur during the stroma preparation by either a rearrangement of membrane components or an elution of membrane components off the membrane. It is difficult to conceive of a large structural reorganization of the membrane which would be required to explain the observed results. The membrane must go from a conformation where only one molecular weight class of protein is exposed to one where almost all molecular weight classes are exposed. However, stroma preparations retain most of the functional and morphological properties of the intact cell which suggest major structural alterations of the membrane have not taken place. No iodinated proteins could be detected in solution when stroma was prepared from iodinated intact erythrocytes. This indicates that labeled proteins are not eluted off the outer membrane surface when the membrane is subjected to the hypotonic conditions. It is possible, however, that carbohydrates and/or lipids could be removed which either sterically or ionically blocked access of lactoperoxidase to most membrane proteins in the intact erythrocyte. If this occurred it would mean that most protein molecular weight classes of proteins are oriented on the outside of the membrane and are covered by carbohydrate and/or lipid molecules.

Thus, in the intact red cell only those proteins on the exterior of the red cell membrane are exposed to lactoperoxidase while in the stroma preparation the enzyme has access to many more proteins. It is interesting to note that stroma proteins are not sequentially iodinated but seem to be labeled to approximately the same extent independent of the degree of iodination as is shown in Figure 4. This is also true for the intact cells where only a single protein is iodinated.

Many investigators have suggested asymmetry in the erythrocyte membrane. The outer surface of the membrane is smooth (Tillack and Marchesi, 1970) while the inner surface of the membrane has many undulations and fibers (Marchesi and Palade, 1967) possessing a much larger surface area. Eylar *et al.* (1962) have demonstrated that all the sialic acid in the membrane, most of which is involved in antigenic determinants, is on the exterior of the membrane. Other antigenic determinants which are predominantly glycolipids and glyco-

peptides (Watkins, 1966) are on the cellular surface with the carbohydrate moieties exposed for antigenic recognition (Eylar *et al.*, 1962). Phospholipase C readily reacts with phospholipids in the membrane indicating that they are located on the outer aspect of the membrane (Lenard and Singer, 1968). Cholesterol, which is 15% by weight of the membrane (Rouser *et al.*, 1968), has been shown by radioautography to also exist in specific regions on the exterior of the erythrocyte membrane (Murphy, 1965).

At least 20 enzyme functions have been attributed to the erythrocyte membrane (Green *et al.*, 1965; Mitchell *et al.*, 1965; Schrier, 1963; Schroter and Neuvians, 1970) and the present data is consistant with an interior orientation of those proteins in order to participate in functions essential for maintenance of the erythrocyte. In at least one case the evidence is clear. Marchesi and Palade have shown that the ATPase activity of the erythrocyte membrane is on the interior of the cell by histochemical-staining procedure used with electron microscope (Marchesi and Palade, 1967). Also, glyceraldehyde 3-phosphate dehydrogenase has been demonstrated to be on the inner aspect of the erythrocyte membrane (Green *et al.*, 1965; Nilsson and Ronquist, 1969) and it is probable that other glycolytic enzymes are also oriented in the same way (Green *et al.*, 1965). Schroter and Neuvians (1970) have shown that 2,3-diphosphoglycerate phosphatase, which is believed to be directly involved in control of oxygen binding to hemoglobin (Benesch *et al.*, 1968), is also oriented to the inside of the membrane.

On the basis of the evidence one can conclude that only one molecular weight class of proteins with a molecular weight of 90,000 is exposed on the exterior of the intact cell. Furthermore, the evidence is also consistent with an asymmetric distribution of membrane proteins where most proteins are oriented to the inner surface of the membrane.

It should be emphasized that proteins on the outside of a membrane but covered by a layer of carbohydrates and glycolipids should still be considered exterior, but these are not necessarily exposed.

Acknowledgments

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