Biochimica et Biophysica Acta, 382 (1975) 621–633

BBA 76907

A COMPARISON OF INTACT HUMAN RED BLOOD CELLS AND RESEALED AND LEAKY GHOSTS WITH RESPECT TO THEIR INTERACTIONS WITH SURFACE LABELLING AGENTS AND PROTEOLYTIC ENZYMES*

Z. I. CABANTCHIK**, M. BALSHIN, W. BREUER, H. MARKUS and A. ROTHSTEIN

Research Institute, The Hospital for Sick Children, Toronto (Canada)

(Received August 19th, 1974)
(Revised manuscript received December 9th, 1974)

SUMMARY

Resealed ghosts and intact red blood cells were directly compared with respect to their interactions with surface probes and to digestion by pronase. The amount and pattern of labelling of surface proteins by 4.4′-diisothiocyano-2,2′-stilbene disulfonic acid (DIDS) and by pyridoxal phosphate-borohydride (as seen after sodium dodecylsulfate/acrylamide gel electrophoresis) was substantially the same in cells and resealed ghosts under conditions in which a relatively small change would be apparent. In each membrane system, DIDS labels a protein component of apparent molecular weight 95 000 and pyridoxal phosphate labels the same protein plus three glycoprotein components. The sensitivity of surface proteins and of DIDS and pyridoxal phosphate-labelled sites to pronase was also similar in the cells and resealed ghosts. The glycoproteins were digested, in each case, and the 95 000 (molecular weight) protein was largely split into two portions of apparent molecular weights 65 000 and 35 000, with both portions containing DIDS and pyridoxal phosphate binding sites. The pattern of labelling of "leaky" ghosts by pyridoxal phosphate in the presence of hemoglobin was similar to the labelling of intact cells, provided that the pyridoxal phosphate was present on both the outside and inside of the cells. Virtually all of the major protein components visible by staining on acrylamide gels were labelled. It is concluded that none of the probes could detect any substantial differences in reactivity of proteins of the outer surface of the membrane of the ghosts as compared to the cells and that no irreversible changes in membrane protein conformation or arrangement occur as a consequence of lysis and resealing of ghosts, that are detectable by the reported procedures.

Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′ stilbene disulfonic acid; HEPES, *N*-2-hydroxyethyl piperazine-*N*′-2-ethane sulfonic acid; NAP-taurine, *N*-(4-azido-2-nitrophenyl)-2-ethane sulfonate.

* Preliminary results were presented at the Biophysical Meetings held in Minneapolis, Minn. in June 1974 (Fed. Proc. 33, 1532).

** Dr Cabantchik's present address is the Biophysics Program, Institute of Life Sciences, The Hebrew University, Jerusalem, Israel.

INTRODUCTION

The action of non-penetrating chemical probes or proteolytic enzymes on intact cells can be used to identify the protein constituents of their surfaces [1–12] and in some cases they may indicate particular functions associated with them [1, 8, 11, 13, 14]. Those proteins that are located within the membrane structure or on the inner surface of the membrane cannot, however, be approached by such agents in the intact cell. It has, therefore, been necessary in determining the disposition of all the proteins in the membrane to use ghosts or membrane vesicles to overcome the accessibility problem.

In a number of studies the interactions of surface probes on intact cells have been compared with those on leaky ghosts [2, 3, 5, 9, 11, 12, 14, 15]. The conclusion, however, that the many additional proteinaceous sites that become accessible in the ghost are those that were exposed at the inner surface of the membrane, is subject to serious reservations [10, 16]. A major disruption of the membrane of sufficient magnitude to allow hemoglobin to escape [17] may have resulted in rearrangements of proteins in the membrane exposing additional agent-reactive sites, that may or may not have been accessible to the probes from either the inside or outside of the membrane of the intact cell. The interpretation of results is, therefore, ambiguous.

Other studies have involved the use of resealed ghosts, inside which a non-penetrating probe can be trapped [15, 18–20] or the comparison of the action of macromolecular probes on right side out or inside out vesicles [6]. These preparations more closely resemble the normal cell membrane, inasmuch as the permeability barrier is at least partially restored, but the arrangement and accessibility of proteins may have been disturbed irreversibly by the lysis procedure, exposure to low ionic strength or the resealing process. Furthermore, the membrane in the intact cell and that in ghosts or vesicles differ, inasmuch as the former is in contact, on its inner face, with high concentrations of hemoglobin, which might substantially influence the interactions of probes.

Because resealed ghosts are excellent membrane material for studying both the disposition of proteins and also the various related transport functions, it is important to know how closely they resemble the membrane in its native state in the intact cell. A comparison of the interaction of proteases (chymotrypsin and trypsin [9]), or lactoperoxidase [15, 18–20] with intact cells and resealed ghosts reveals no significant differences in acrylamide-gel profiles. Recently, however, it has been reported [21] that a low molecular weight, non-penetrating probe reveals certain differences in surface labelling of resealed ghosts as compared to intact cells.

This question was further examined with pyridoxal phosphate, a low molecular weight probe that can be fixed to proteins by reduction with $NaBH_4$. It can be used to detect proteins exposed either at the outer or inner faces of the membrane of the intact cell [22]. With this probe it is therefore possible to directly compare the labelling patterns in cells, resealed ghosts and leaky ghosts, and also to appraise the possible effects of hemoglobin on inside labelling. Comparisons were also made with a non-penetrating probe, 4,4′-diisothiocyano-2,2′-disulfonic acid (DIDS) which reacts specifically with a particular protein of 95 000 molecular weight [8, 11, 14], and with the redistribution of both labels after treatment of the cells or resealed ghosts with the proteolytic enzyme, pronase. It was expected that any irreversible changes in the

disposition or organization of proteins on the outer surface of the membrane resulting from the hemolysis and resealing process, would be reflected in an altered accessibility, and therefore, in an altered labelling pattern or susceptibility of labelled proteins to proteolytic attack.

MATERIALS AND METHODS

4,4′-diisothiocyano-2,2′-[$^3H_2$]stilbene disulfonate ([^{3}H]DIDS) (2.2 Ci/nmol) and 4,4′-diisothiocyano-2,2′-stilbene disulfonate (DIDS) were synthesized from their diamino analogs as previously described [8, 11]. Sodium borotritide (50 Ci/mol) was purchased from New England Nuclear, and dissolved just prior to its use. Pyridoxal phosphate and $NaBH_4$ were purchased from Sigma Chemical Company. Sodium dodecylsulfate was obtained from Matheson, Coleman and Bell Co. All other chemicals were of analytical or best available grade.

Human red blood cells from fresh or recently outdated blood were washed in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffered saline (HEPES (20 mM)/NaCl (145 mM)) pH 7.4 to remove white cells and contaminating supernatant. Isolated membranes (leaky or unsealed ghosts) were prepared by hypotonic lysis as described elsewhere [23]. Resealed ghosts were prepared by a modification of Bodeman and Passow's method [24]. A volume of 3.0 ml cells (60 % hematocrit) washed with NaCl (145 mM) was lysed with 10 vols of $MgSO_4$ 4 mM, HEPES 10 mM at 0 °C for 10 min. Isotonicity and desired pH conditions were restored by addition of a 20 × concentrated HEPES buffered saline solution. The membranes were subsequently resealed at 37 °C for 40 min. The ghosts were then centrifuged at 15 000 rev./min for 5 min to remove external hemoglobin, and washed twice with buffer at pH 7.4. The unsealed ghosts were then separated from resealed ghosts on a 43 % sucrose cushions as described elsewhere [24]. The contaminating sucrose was later removed by washing with buffer at pH 7.4.

In most experiments, the labelling of cells, leaky ghosts and resealed ghosts was performed on a 30 % suspension under the following conditions. [^{3}H]DIDS (1.5 μM) or DIDS (10 μM) were reacted at pH 7.4, 0 °C for 15 min. The cells or ghosts were subsequently washed with 10 vols HEPES buffered saline, pH 7.4. The reaction with pyridoxal phosphate (10 mM) was carried out at pH 8.0, 37 °C for 10 min, and then for 10 min at 0 °C. The cells or ghosts were then centrifuged, the supernatant removed and the samples resuspended to a 40 % hematocrit at 0 °C. NaB^3H_4 prepared in cold HEPES-buffered saline (0 °C) pH 11.0, was added in aliquots of 1/100 of the suspension's volume and the reduction carried out for 10 min at 0 °C. The cells or ghosts were then washed extensively with HEPES-buffered saline, pH 7.2, at room temperature until only trace amounts of ^{3}H were found in the supernatant. In a few experiments concerned with labelling of cells from the inside, they were exposed to pyridoxal phosphate (10 mM) for 12 h at pH 6.8, with replacement in fresh medium at 3 h intervals [22]. The washing and exposure to NaB^3H_4 were carried out as described above. All the steps involving work with pyridoxal phosphate or DIDS were carried out with minimal exposure to light. Normally, the chemical reactions were carried out before the separation on the sucrose cushion, but in some instances they were performed with resealed ghosts that had already been separated by the sucrose-cushion technique.

Isolated membranes (hemoglobin free) were prepared from resealed ghosts by hypotonic lysis as described before [23]. Proteins were measured by a modified micro-biuret technique [25]. Radioactivity was measured with toluene scintillation mixture containing 2,5-diphenyloxazole (PPO), 2,2'-paraphenylene-bis-5-phenyl-oxazole (POPOP) and 10 % protosol (New England Nuclear).

Sodium dodecylsulfate/acrylamide-gel electrophoresis, staining of gels for protein (Coomassie Blue) or for carbohydrates (periodic acid-Schiff) and analysis of radioactivity profiles of gels was performed as previously described [11, 26], acrylamide gels (7.5 %) were normally extruded with a Savant gel extruder; 3.3 % gels were first frozen and then sliced with a home-made slicer. The slices were placed in scintillation vials into which 0.1 ml water and then 10 ml of the protosol-based solvent was added. The vials were incubated for 72 h at 37 °C, then cooled to room temperature overnight in the dark and counted in a Packerd Liquid Scintillation Counter.

RESULTS

The labelling patterns of membrane proteins were appraised by electrophoretic separation on dodecylsulfate-acrylamide gels [11, 26]. The profiles of surface labelling

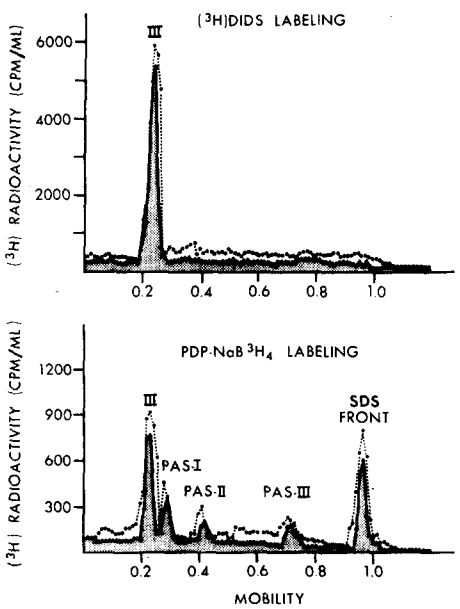

Fig. 1. Surface labelling of intact cells and resealed ghosts by DIDS and pyridoxal phosphate. Intact cells or resealed ghosts (30 % hematocrit) were reacted either with [^{3}H]DIDS (1.5 μM) in HEPES-buffered saline, pH 7.4, at 0 °C or with pyridoxal phosphate (PDP) (10 mM)/NaB^{3}H$_4$ (100 mM) in HEPES-buffered saline, pH 8.0, at 0 °C. Hemoglobin free membrane fractions containing 40 μg of protein were solubilized in 1.0 % sodium dodecylsulfate, subjected to electrophoresis on acrylamide gels (7.5 %), sliced and counted as described in Material and Methods. The mobility is given relative to the position of pyronin-y (sodium dodecylsulfate (SDS) front). Shaded areas (solid lines) correspond to labelling of cells, white areas (dotted lines) to resealed ghosts. PAS-I, II and III designate glycoproteins stainable with periodic acid-Schiff, and III designates a protein of 95 000 molecular weight stainable with Coomassie blue (see Fig. 3).

by [^{3}H]DIDS and by pyridoxal phosphate are essentially similar in the intact cell and in resealed ghosts (Fig. 1). In each preparation, the [^{3}H]DIDS labels only a single peak that corresponds to a protein of apparent molecular weight 95 000 (identified as band III). In each preparation, the pyridoxal phosphate labels the 95 000 (molecular weight) protein, and three additional bands corresponding to glycoproteins stainable for carbohydrate by periodic acid-Schiff reagent (PAS bands I, II and III). The large highly mobile band to the right in both preparations, is due to labelling of lipid by the tritiated borohydride used to fix pyridoxal phosphate to the proteins (borohydride alone does not label proteins [22]). The background labelling is definitely higher in the resealed ghosts than in the cells for both agents, attributable perhaps either to a small contamination with leaky ghosts, or inside-out vesicles. In the case of pyridoxal phosphate, for example, a small peak of very low mobility (0.08) corresponds to spectrin; a protein known to be located on the inner face of the membrane [27]. Thus, the labelling pattern in the resealed ghosts may represent the outside labelling superimposed on a small background of inside labelling. If the elevated background is taken into account, the outside labelling of the resealed ghosts not only resembles that of the cell in pattern, but also in the approximate amounts of labelling found in the various peaks.

Following the incubation period for resealing, the suspension of ghosts was layered on a 43 % sucrose cushion and centrifuged in order to separate resealed from unsealed ghosts [24]. It was observed that if the labelling with pyridoxal phosphate was performed after the exposure to sucrose rather than before, the amount of labelling was somewhat increased (Fig. 2). The exposure to sucrose apparently alters some

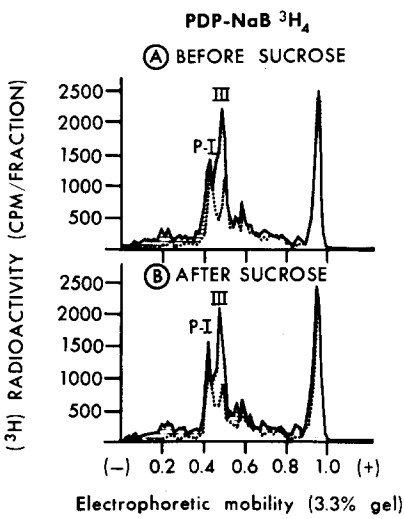

Fig. 2. The effect of sucrose on the surface labelling of resealed ghosts. Resealed ghosts prepared from normal cells (full lines-shaded areas) or from cells pretreated with 10 μM DIDS (broken lines-white areas) were subsequently labelled with pyridoxal phosphate (PDP)/[^{3}H]NaBH$_4$. The latter was performed either before (A) or after (B) the sucrose cushion separations. Isolated membranes (90 μg protein in A and 110 μg in B) were electrophoresed on 3.3 % acrylamide gels, sectioned and counted as described in Materials and Methods. P-I designates a glycoprotein stainable by periodic acid-Schiff (labelled PAS-I in Figs 1 and 3) and III, a protein of 95 000 molecular weight stainable with Coomassie blue [26].

properties of the surface, resulting in exposure of new sites to reaction with pyridoxal phosphate. For this reason, in all other experiments reported in this paper, the labelling of ghosts with pyridoxal phosphate and DIDS was done before the separation of resealed from leaky ghosts. It should be pointed out that the glycoprotein designated PAS-1 or P-1 is located to the left of band III in Fig. 2 and in Fig. 5, rather than to the right as in Fig. 1. The difference is due to the use of 3.3 % acrylamide in the former case and 7.5 % in the latter. The 3.3 % gels allow a greater dispersion of the higher molecular weight proteins.

Another method for investigating the proteins of the cell surface involves the use of proteolytic enzymes. Pronase was chosen because it produces substantial but specific effects. In the leaky ghosts, pronase rapidly digests all of the protein bands seen in acrylamide gels by Coomassie Blue staining and the glycoproteins identified by periodic acid-Schiff staining for sugars [2, 9]. In cells, on the other hand, the only effects of pronase are to eliminate the periodic acid-Schiff bands and to digest much of the 95 000 molecular weight protein to 65 000 molecular weight [2, 3, 6, 9, 14, 28]. When the proteolytic attack is stopped early*, a band at 35 000 molecular weight can

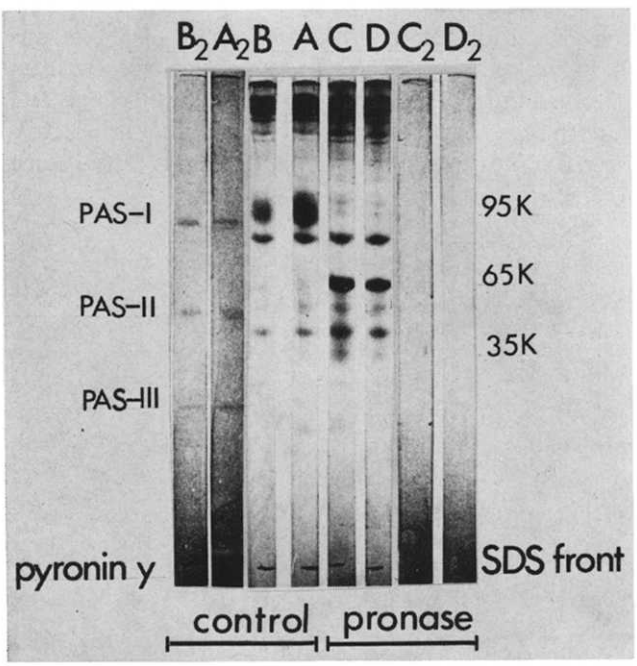

Fig. 3. The effect of pronase on the membrane protein and glycoprotein profiles of intact cells and resealed ghosts. Normal cells and resealed ghosts (30 % hematocrit) in HEPES-buffered saline, pH 7.4, (A and B, respectively) were treated with 0.2 mg/ml pronase at 37 °C for 20 min then with HEPES-buffered saline, pH 7.4, containing 0.5 % albumin (C and D). Membranes were isolated, subjected to electrophoresis on 7.5 % acrylamide gels and stained either for proteins with Coomassie Blue (A, B, C and D) or for glycoproteins with periodic acid-Schiff (A_2, B_2, C_2 and D_2). The designations of stained bands are from ref. 26. SDS, sodium dodecylsulfate.

* Pronase can be removed efficiently from the external solution by washing cells or resealed ghost with buffers containing 0.5–1.0 % bovine serum albumin [14].

also be observed so it has been concluded that the first attack of pronase splits the 95 000 molecular weight protein into two segments of 65 000 and 35 000 molecular weight [14]. In the resealed ghosts, the responses to pronase are the same as for cells in terms of gel patterns (Fig. 3).

Gel patterns would not be expected to detect more subtle, quantitative differences in pronase susceptibility of cells as compared to resealed ghosts. A more exact appraisal was achieved by determining the effects of pronase on the number and distribution of DIDS and pyridoxal phosphate binding sites. In the case of DIDS, the effect of pronase is unique. Instead of a single peak at 95 000 molecular weight, the label is redistributed into three peaks, a residual labelling at 95 000, and labelling at new locations equivalent to molecular weights of 65 000 and 35 000 [14]. Resealed ghosts labelled with DIDS and treated with pronase under the same conditions as the cells show virtually the same redistribution of label (compare Figs 4A and C). An even more sensitive test for possible differences between the resealed ghosts and cells is carried out in the following experiment. Cells are first treated with non-radioactive DIDS then with [^{3}H]DIDS. The 95 000 molecular weight band shows a peak of labelling that is considerably reduced in size, as expected (Fig. 4B). After treatment with pronase, three peaks are found as in the more heavily labelled cells (Fig. 4A). Another aliquot of cells pretreated with non-radioactive DIDS is lysed, and the ghosts separated and resealed. These are subsequently treated with [^{3}H]DIDS. A single peak is observed at 95 000 (Fig. 4D) similar in size to that found in the intact cells (Fig. 4B). Furthermore, treatment with pronase gives three bands similar in size to those found in cells. This experiment suggests that very few, if any, new sites could have been exposed to the action of DIDS or pronase as a consequence of the hemolysis and resealing process. Because over 95 % of the DIDS sites were blocked by non-radioactive DIDS, even a small increase in the amount or distribution of [^{3}H]-

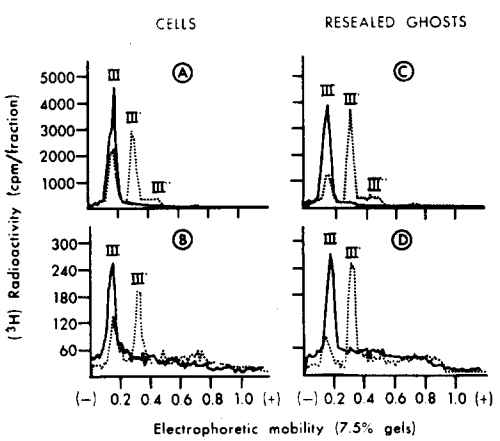

Fig. 4. The effect of pronase on the [^{3}H]DIDS distribution in membrane proteins from intact cells and resealed ghosts. Intact cells (A) or resealed ghosts (C) labelled with 1.4 μM [^{3}H]DIDS (solid lines) were digested with 0.02 mg/ml pronase in HEPES-buffered saline, pH 7.4, for 5 min at 37 °C (dotted lines). Membrane profiles of ^{3}H labelling were analysed as described in Fig. 1. Cells pretreated with 10 μM DIDS (B) or resealed ghosts prepared from them (D) were further reacted with [^{3}H]DIDS (solid lines) and subsequently digested with pronase as described above (dotted lines). ^{3}H distributions of membrane proteins were analysed as previously described.

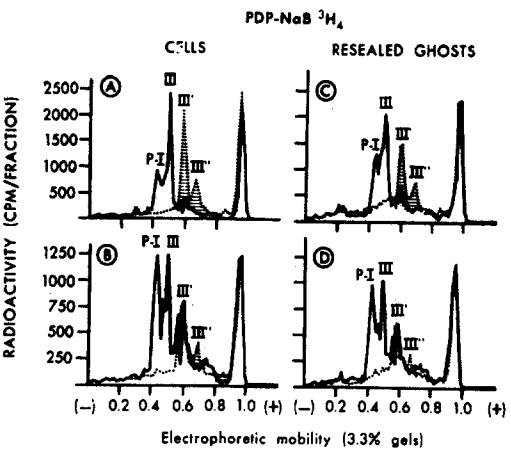

Fig. 5. The effect of pronase on the pyridoxal phosphate distribution in membrane proteins and glycoproteins from intact cells and resealed ghosts. Figure labels and treatments are the same as those described in Fig. 4, except that pyridoxal phosphate/NaB$^3H_4$ was used instead of [^{3}H]DIDS.

DIDS labelling would have been apparent in the ghosts.

In the case of pyridoxal phosphate, it has already been pointed out that glycoprotein bands, as well as the 95 000 molecular weight band, are labelled in intact cells (Fig. 1). The action of pronase with respect to the pyridoxal phosphate in the 95 000 molecular weight protein is somewhat similar to that found with DIDS labelling. The peak is diminished to an even greater degree (Fig. 5A, band III) and two new peaks appear (bands III′ and III″) that correspond to 65 000 and 35 000 molecular weights. Pronase eliminates all discrete labelled bands that correspond to the glycoproteins, in parallel to the elimination of periodic acid-Schiff stained bands (Fig. 3). The pattern of effects obtained with pronase is virtually the same in the case of resealed ghosts (Fig. 5C).

The potential sensitivity of the pyridoxal phosphate procedure for comparing cells and ghosts was increased by non-radioactive DIDS to block binding sites on the 95 000 molecular weight protein before labelling with pyridoxal phosphate. The amount of pyridoxal phosphate found in the 95 000 molecular weight protein and in the 65 000 and 35 000 molecular weight fragments resulting from pronase treatment is thereby reduced by at least 50–70 % (compare bands III, III′ and III″ in Figs 5A and 5B). However, no change was found in the amount of pyridoxal phosphate labelling of glycoproteins. The behaviour in resealed ghosts was substantially the same (Fig. 5D). Thus, the lysis and resealing process did not reveal any substantial number of new sites on the 95 000 molecular weight protein that become reactive to pyridoxal phosphate, nor were the existing sites differently affected by pronase.

The possible effects of hemoglobin on labelling patterns of membrane proteins was determined by the following procedures. Cells were allowed to equilibrate with pyridoxal phosphate for a long period of time under appropriate conditions, so that it penetrated substantially into the cell before fixation with tritiated borohydride. By this procedure membrane proteins on both the outside and inside of the membrane are labelled [22]. In parallel, an equal number of cells were lysed and the pyridoxal

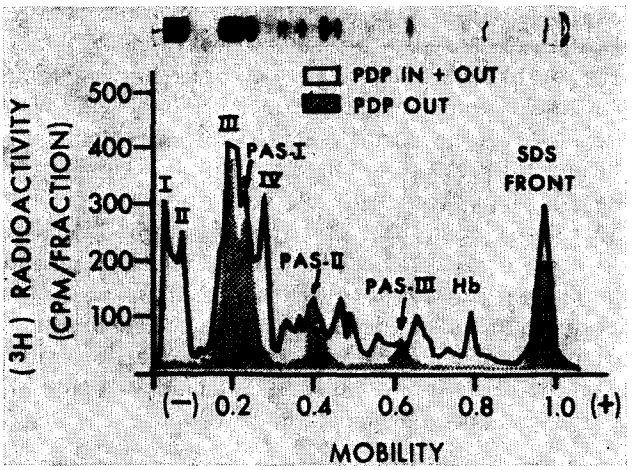

Fig. 6. The labelling of membrane proteins by pyridoxal phosphate present at the outside and the inside of intact cells. The shaded areas represent pyridoxal phosphate labelling from the outside as in Fig. 1. Red blood cells (30 % hematocrit) were incubated with 10 mM pyridoxal phosphate in HEPES buffered saline, pH 8.0, for 5 min at 37 °C, cooled to 0 °C for 10 min and then reacted with 100 mM NaB^3H_4. The white areas represent the additional labelling associated with pyridoxal phosphate (PDP) that diffuses into the cell upon prolonged exposure [22]. The exposure was 12 h at pH 6.8 with the external pyridoxal phosphate replaced at least 4 times (at 3 h intervals). The pH was then adjusted to 8.0 and the temperature to 0 °C and the reaction with NaB^3H_4 carried out as described above. SDS, sodium dodecylsulphate.

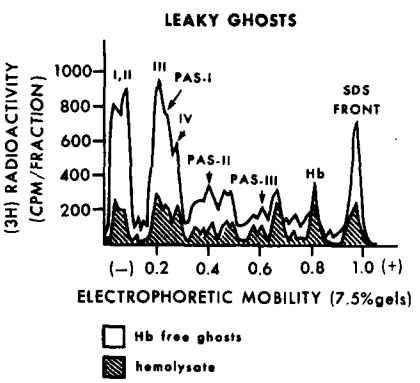

Fig. 7. The effect of hemoglobin on the labelling of membrane proteins of leaky ghosts by pyridoxal phosphate. The shaded area represents pyridoxal phosphate labelling in the presence of hemoglobin (Hb). 1 vol. of 0.3 ml packed cells was hemolysed in 10 vols of 10 mM NaCl, 5 mM NaH_2PO_4, pH 8.0 at 0 °C, centrifuged at 15 000 rev./min for 10 min and 2/3 of the supernatant removed. The ghosts were resuspended in the 1/3 vol. of hemolysate, reacted with (10 mM) pyridoxal phosphate/NaB^3H_4 (100 mM) and analysed as previously described. The white area represents additional labelling found in hemoglobin-free ghosts prepared from 0.3 ml packed cells which were reacted with pyridoxal phosphate/NaB^3H_4 and analysed under the same conditions as above. The designations are the same as in Fig. 6. SDS, sodium dodecylsulfate.

phosphate was added to the lysate before fixation with the borohydride. Finally, another aliquot of cells was used to prepare low-hemoglobin (leaky) ghosts which were also treated with pyridoxal phosphate/borohydride. In contrast to the specific labelling of the 95 000 molecular weight protein and glycoproteins in intact cells with the agent present only on the outside (Fig. 1), virtually every membrane protein band seen in gels is labelled when the agent is present on both sides (Fig. 6). The pattern and amount of labelling is very similar in ghosts with released hemoglobin present in the suspension to that in intact cells with pyridoxal phosphate present both outside and inside (compare Figs 6 and 7). If, on the other hand, the hemoglobin is largely removed, the amount of label in many peaks is four to five times as high (Fig. 7). It can be concluded that hemoglobin can modulate the binding of pyridoxal phosphate to membrane proteins by competing for reaction. Nevertheless the interaction of the agent with membrane proteins on the inside of the intact cell is similar to that with leaky ghosts provided that the same amount of hemoglobin is present.

DISCUSSION

The labelling of proteins by molecular probes depends on two factors, the specificity of the probe-ligand interactions and on the ability of the probe to reach the ligands. The latter depends in turn on the location of the ligands in the membrane and on the diffusibility of the probe [29]. The relative importance of specificity and of accessibility factors are illustrated by the fact that pyridoxal phosphate is highly specific for reaction with amino groups [30], whereas DIDS can potentially react with other ligands as well [8]. Yet in the surface-labelling studies, DIDS reacts only with the 95 000 molecular weight protein (Fig. 1) whereas pyridoxal phosphate reacts with the glycoproteins as well. In contrast, in leaky membranes, DIDS can reach and label virtually all of the membrane proteins [14].

The importance of accessibility is also indicated by the finding that the two probes react with a number of common sites in the 95 000 molecular weight protein, but pyridoxal phosphate can react with at least as many sites that are not labelled with DIDS (compare Figs 5A and 5B). The failure of DIDS to react with the latter sites can probably be attributed to lack of accessibility, because after trypsin treatment of cells to remove considerable sialoglycopeptides, the labelling of the 95 000 molecular weight protein by DIDS is increased by a factor of two [14]. After pronase treatment the labelling of the 65 000 and 35 000 molecular weight segments of the 95 000 molecular weight protein is also increased by a factor of more than two [14]. Thus the 95 000 molecular weight protein has substantial numbers of reactive sites that cannot normally be reached by DIDS. Trypsinization also reveals additional sites in the glycoproteins to other surface probes. Even a seemingly innocuous treatment such as exposure to hypertonic sucrose exposes additional sites to surface probes (Fig. 2).

From the foregoing, it might be expected that even relatively subtle changes in the configuration or arrangement of proteins in the membrane surface resulting from hemolysis and resealing might be reflected in exposure of additional reactive sites that could be readily detected by the surface probes or by the response of the labelled sites to proteolytic attack. No such changes were observed, even under conditions which would have allowed detection of a relatively small increase in the number

of accessible sites. Such conditions are present in the experiments depicted in Figs 4 and 5 in which many of the sites normally exposed to DIDS in the cell were first blocked with non-radioactive DIDS so that a small number of newly-exposed sites would be detectable. It can, therefore, be concluded that in terms of the particular tests applied the configuration and arrangement of the protein at the outside face of the resealed ghost are the same as in the intact cell.

In a recent paper [21] it has been reported that another low molecular weight surface probe, NAP-taurine, reveals differences in labelling between resealed ghosts and cells. The technique used, did not allow differences in band 3 (95 000 molecular weight) protein and PAS-I glycoprotein to be assessed, but the relative labelling in high molecular weight bands, 2.3 and 2.4 (nomenclature of Fairbanks et al. [26]), was increased in ghosts and band 4 was labelled in ghosts and not in cells. The degree of difference was dependent on the conditions at the time of lysis. It was concluded that the arrangement of surface proteins was not identical in the resealed ghosts as compared to cells. The differences in results obtained with NAP-taurine, pyridoxal phosphate and DIDS may be due to several factors. The latter probes react only with band 3 (DIDS) or band 3 plus glycoproteins (pyridoxal phosphate) and changes in these bands were not appraised with NAP-taurine. The latter, being a less specific reagent (the nitrene group can react with many ligands) may be detecting sites exposed in the ghost that are not reactive with the nucleophiles. Another possibility relates to possible effects of exposure to high concentrations of sucrose in the separation of resealed from unsealed ghosts. In the case of pyridoxal phosphate pre-exposure to sucrose gives a small but definite increase in labelling. The NAP-taurine labelling [21] was carried out after exposure to high concentrations of sucrose.

The question of possible rearrangements of the proteins of the inside face of the membrane is more difficult to answer. Although the inside, as well as the outside of the membrane of the intact red blood cell, can be labelled with pyridoxal phosphate [22], comparisons are complicated by the presence of large concentrations of hemoglobin inside the intact cell and its relative absence in resealed ghosts. Hemoglobin constitutes over 90 % of the red cell protein [32] and it has a high affinity for pyridoxal phosphate [33] (and for other protein reactive probes). As the probe penetrates into the cell, substantial amounts react with hemoglobin, reducing potential reactions with membrane proteins [22]. For this reason, under conditions of slow penetration (shorter exposure times and lower concentrations), the additional labelling over that associated with the outside face (95 000 molecular weight protein and glycoproteins) is restricted almost entirely to spectrin and to residual hemoglobin found in the membrane fractions. With longer times and larger concentrations of pyridoxal phosphate, however, peaks of labelling are found that correspond to virtually every stained band in the gels (Fig. 6). A comparable pattern and amount of labelling per band is found in leaky ghosts if the hemoglobin is not removed after lysis (Fig. 7). The protective effect of hemoglobin is evident, however, by comparing the results obtained with leaky ghosts in the presence and absence of hemoglobin. In the latter case, the amount of labelling in certain bands is increased considerably (Fig. 7). Thus, hemoglobin must be considered as an important modulating factor in the labelling of proteins from the inside of the intact cell. If sufficient probe is present inside the cell to substantially overcome the hemoglobin effect, then the same proteins seem to be accessible to pyridoxal phosphate to the same degree in the intact cell membrane

as in the leaky ghost, except that in the former, certain proteins are only exposed to the probe on the outside face and others on the inside face. In other words, no significant numbers of protein sites seem to be exposed to pyridoxal phosphate in the ghosts that were not exposed on one side or the other in the intact cell.

The results reported in this paper indicating normal protein reactivity and arrangement in the resealed ghosts is reinforced by the findings that they behave relatively normally in terms of permeability and transport functions [24, 34–36]. It is also germane that DIDS and pyridoxal phosphate, both specific inhibitors of anion permeability in intact cells [8, 11, 12], also inhibit the function in resealed ghosts (unpublished observations). It can be concluded that the resealed ghost is an appropriate model for studying the structure and function of the intact cell membrane with respect to certain components and functions, but that it cannot be taken for granted that the membrane of the resealed ghost is identical in all respects to the membrane of the intact cell.

ACKNOWLEDGEMENT

This work was supported by the Medical Research Council of Canada, Grant No.MA-4665.

REFERENCES

1 Berg, H. C. (1969) Biochim. Biophys. Acta 183, 65–78
2 Bender, W. W., Garan, H. and Berg, H. C. (1971) J. Mol. Biol. 58, 783–797
3 Bretscher, M. W. (1971) J. Mol. Biol. 59, 351–357
4 Bretscher, M. S. (1971) J. Mol. Biol. 58, 775–781
5 Phillips, D. R. and Morrison, M. (1971) Biochemistry 10, 1766–1771
6 Steck, T. L., Fairbanks, G. and Wallach, D. F. H. (1971) Biochemistry 10, 2617
7 Arroti, J. J. and Garvin, J. E. (1972) Biochim. Biophys. Acta 255, 79–90
8 Cabantchik, Z. I. and Rothstein, A. (1972) J. Membrane Biol. 10, 311–330
9 Triplett, R. B. and Carraway, K. L. (1972) Biochemistry 11, 2897–2903
10 Schmidt-Ullrich, R., Knuferman, H. and Wallach, D. F. H. (1973) Biochim. Biophys. Acta 307, 353–365
11 Cabantchik, Z. I. and Rothstein, A. (1974) J. Membrane Biol. 15, 207–226
12 Staros, J. W. and Richards, F. M. (1974) Biochemistry 13, 2720–2725
13 Passow, H. (1971) J. Membrane Biol. 6, 233–258
14 Cabantchik, Z. I. and Rothstein, A. (1974) J. Membrane Biol. 15, 227–248
15 Boxer, D. H., Jenkins, R. E. and Tanner, M. J. A. (1974) Biochem. J. 137, 531–534
16 Zwaal, R. F., Roelofsen, B. and Colley, C. M. (1973) Biochim. Biophys. Acta 300, 158–182
17 Seeman, P. (1967) J. Cell Biol. 32, 55–70
18 Morrison, M., Mueller, J. J. and Huber, C. T. (1974) J. Biol. Chem. 249, 2660–2758
19 Reichstein, E. and Blostein, R. (1973) Biochem. Biophys. Res. Commun. 54, 494–500
20 Shin, B. C. and Carraway, K. L. (1974) Biochim. Biophys. Acta 345, 141–153
21 Staros, J. V., Haley, B. E. and Richards, F. M. (1974) J. Biol. Chem. 249, 5004–5007
22 Cabantchik, Z. I., Balshin, M., Breuer, W. and Rothstein, A. (1975) J. Biol. Chem., in the press
23 Dodge, J. T., Mitchell, C. and Hanahan, D. (1963) Arch. Biochem. Biophys. 110, 119–130
24 Bodeman, H. and Passow, H. (1972) J. Membrane Biol. 8, 1–26
25 Itzhaki, R. F. and Gill, D. M. (1964) Anal. Biochem. 9, 401–410
26 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617
27 Nicolson, G. L., Marchesi, V. T. and Singer, S. J. (1971) J. Cell Biol. 51, 265–272
28 Phillips, D. R. and Morrison, M. (1971) Biochem. Biophys. Res. Commun. 45, 1103–1108

29 Rothstein, A., Knauf, P., Cabantchik, Z. I. and Balshin, M. (1974) in Drugs and Transport Processes (Callingham, B. A., ed.), pp. 54–72, MacMillan Press, London
30 Churchich, J. E. (1965) Biochemistry 4, 405–410
31 Phillips, D. R. and Morrison, M. (1973) Nat. New Biol. 242, 213–215
32 Hoogeveen, J. Th., Juliano, R., Coleman, J. and Rothstein, A. (1970) J. Membrane Biol. 3, 156–172
33 Benesch, R. E., Benesch, R., Renthal, R. D. and Maeda, N. (1972) Biochemistry 11, 3576–3582
34 Hoffman, J. F. (1962) J. Gen. Physiol. 45, 837–859
35 Jung, C. Y., Carlson, L. M. and Belzer, C. J. (1973) Biochim. Biophys. Acta 298, 101–107
36 Lepke, S. and Passow, H. (1973) Biochim. Biophys. Acta 298, 529–533