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The Organization of the Major Protein of the Human Erythrocyte Membrane

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The enzyme lactoperoxidase was used to catalyse the radioiodination of membrane proteins in intact human erythrocytes and in erythrocyte 'ghosts'. Two major proteins of the erythrocyte membrane were isolated after iodination of these two preparations, and the peptide 'maps' of each protein so labelled were compared. Peptides from both proteins are labelled in the intact cell. In addition, further mobile peptides derived from one of the proteins are labelled only in the 'ghost' preparation. Various sealed 'ghost' preparations were also iodinated, lactoperoxidase being present only at either the cytoplasmic or extracellular surface of the membrane. The peptide 'maps' of protein E (the major membrane protein) labelled in each case were compared. Two discrete sets of labelled peptides were consistently found. One group is obtained when lactoperoxidase is present at the extracellular surface and the other group is found when the enzyme is accessible only to the cytoplasmic surface of the membrane. The results support the assumption that the organization of protein E in the membrane of the intact erythrocyte is unaltered on making erythrocyte 'ghosts'. They also confirm previous suggestions that both the sialoglycoprotein and protein E extend through the human erythrocyte membrane.

The spatial organization of polypeptides within the erythrocyte membrane has recently been investigated. The use of non-penetrating radioactive protein-modifying agents has shown that two protein components are labelled on the external surface of the intact cell (Bretscher, 1971*a*; Phillips & Morrison, 1971*a*,*b*; Hubbard & Cohn, 1972). These proteins are the erythrocyte sialoglycoprotein and a protein with a subunit molecular weight of about 90000 (termed here protein E). Studies using controlled proteolysis of the erythrocyte surface and membrane preparations support these findings (Winzler, 1969; Bender *et al.*, 1971; Steck, 1972).

Bretscher (1971*b*,*c*) also labelled erythrocyte 'ghosts'. He assumed that, in this case, the reagent could modify proteins at both the cytoplasmic and external surfaces of the membrane. He compared the peptide 'maps' of the sialoglycoprotein and protein E so labelled with those of the same proteins labelled in the intact cell, and concluded that both proteins extend through the membrane.

The above conclusion was based on the assumption that the detailed organization of the proteins within the 'ghost' is the same as in the intact cell. Here we assess the validity of this premise, by investigating the lactoperoxidase catalysed iodination of various sealed and unsealed erythrocyte membrane preparations which enable us to selectively label either the cytoplasmic or extracellular surface of the membrane. The evidence we present strongly suggests that the organization of the major membrane protein does not differ in the intact erythrocyte, sealed or unsealed 'ghost' preparations, thus supporting the assumptions from which Bretscher (1971*c*) was able to draw his conclusions.

Methods

All blood used was freshly drawn from human volunteers, mixed with one-sixth of the volume of acid–citrate–dextrose anticoagulant, and used immediately for the preparation of 'ghosts' by the method of Fairbanks *et al.* (1971).

Enzymes

Lactoperoxidase was made by a modification of the method described by Morrison & Hultquist (1963), omitting the final gel-filtration stage. The preparation used for iodinations had an E_{412}/E_{280} of 0.84. D-Glyceraldehyde 3-phosphate dehydrogenase was assayed as described (Tanner & Gray, 1971) but the reaction was monitored by following NADH fluorescence at 420nm (excitation wavelength 340nm). Glucose oxidase [Sigma (London) Chemical Co., London S.W.6, U.K.; fraction V] was assayed by the method of Hubbard & Cohn (1972). One unit of enzyme activity is that amount of enzyme that produces 1 μmol of H_2O_2/min at 25°C. Acetylcholinesterase was assayed by the method of Ellman *et al.* (1961).

Iodination of membrane preparations

The procedure used was modified from Phillips and Morrison (1971*a*).

Intact and thermolysin-treated erythrocytes: Cells were washed twice with 0.15M-NaCl and three times with iso-osmotic sodium phosphate buffer, pH7.4, (0.155M-NaH_2PO_4 adjusted to pH7.4 with 0.103M-Na_2HPO_4). The iodination of 0.2ml of packed cells was done in a total volume of 0.4ml containing 10μM-KI, 0.4mg of lactoperoxidase/ml, 0.5mCi of $Na^{125}I$ (carrier-free, The Radiochemical Centre, Amersham, Bucks., U.K.), all in iso-osmotic sodium phosphate buffer, pH7.4. The reaction was done at room temperature and H_2O_2 (300nmol total) was added in 20 equal aliquots at 3min intervals. Later iodinations were modified by adding 1μg of butylated hydroxytoluene/ml (Welton & Aust, 1972), and replacing the direct addition of H_2O_2 by incubation of the reaction mixture at 25°C for 1h with 300nmol of glucose and 0.005 unit of glucose oxidase (Hubbard & Cohn, 1972). Identical peptide 'maps' were obtained whichever procedure was used. After iodination the cells were washed five times with iso-osmotic sodium phosphate buffer, pH7.4, and 'ghosts' were prepared by lysis into 15vol. of 5mM-sodium phosphate buffer, pH8.0, and centrifugation at 40000*g* for 20min.

Resealed 'ghosts' were iodinated in the same way as intact cells, but the cell-lysis step was omitted and washing was done at 40000*g* for 20min. When lactoperoxidase was sealed into resealed 'ghosts', no lactoperoxidase was added to the reaction mixture.

'Ghosts' and 'inside-out' vesicles: 'Ghosts' were washed once (at 40000*g* for 20min) with diluted sodium phosphate buffer, pH7.4, (iso-osmotic phosphate buffer, pH7.4, diluted with 19.5vol. of cold deionized water) at 4°C and iodinated in the same way as intact cells except that the reaction was done in diluted phosphate buffer, pH7.4, 'Inside-out' vesicles were iodinated as described for 'ghosts' but 0.5mM-sodium phosphate, pH8.0, was substituted throughout for diluted phosphate buffer, pH7.4.

Polyacrylamide-gel electrophoresis

Gel electrophoresis was done as described by Fairbanks *et al.* (1971) in the presence of 0.1% sodium dodecyl sulphate. Samples were prepared and the gels stained as described (Tanner & Boxer, 1972). Labelled proteins were isolated from 1.1cm diam. gels as described by Bretscher (1971*b*,*c*) except that the detergent was removed by dialysis against 50% (v/v) aq. methanol followed by exhaustive dialysis against distilled water. The sialoglycoprotein was purified as described by Bretscher (1971*b*). Protein E was purified in a single step by electrophoresis in an 8% (w/v) gel (Plate 1*c*).

Resealed 'ghosts'

'Ghosts' were resealed by the method of Passow (1969). Kinetically homogeneous populations of 'ghosts' (type II), prepared as described by Bodeman & Passow (1972), were used for iodination. Where lactoperoxidase was to be included inside the resealed ghosts, the enzyme (3mg/ml) was added after lysis of the cells into 4mM-$MgSO_4$ and the mixture was left at 0°C for 5min. The mixture was made 0.16M in NaCl, left for a further 5min, incubated at 37°C for 45min, and purified as described above. 'Inside-out' vesicles were prepared by the procedure of Kant & Steck (1972). The accessibility of marker enzymes to substrates in these preparations was measured by assay in the presence and absence of 0.1% Triton X-100.

Thermolysin treatment of intact erythrocytes

Packed, washed erythrocytes were diluted to a 20% suspension in 0.15M-NaCl containing 0.1M-ammonium acetate and 5mM-$CaCl_2$. The suspension was held at 37°C for 5min before the addition of 0.5mg of thermolysin/ml. Digestion was terminated after 30min at 37°C by washing three times with 0.15M-NaCl and twice with iso-osmotic sodium phosphate buffer, pH7.4, at 4°C.

Peptide 'maps'

Sialoglycoprotein. The freeze-dried protein (10^5–5×10^5c.p.m.) was dissolved in 0.4ml of 2M-NH_4HCO_3, boiled for 5min, and incubated at 37°C for 30min before the addition of 50μg of trypsin ('toluene-*p*-sulphonamidophenylalanine chloromethyl ketone'-treated) and 50μg of chymotrypsin (Worthington Biochemical Corp., Freehold 2, N.J., U.S.A.). After 10h a further 25μg of each enzyme was added. Digestion was terminated after 12–18h, the mixture centrifuged and the supernatant applied to the paper.

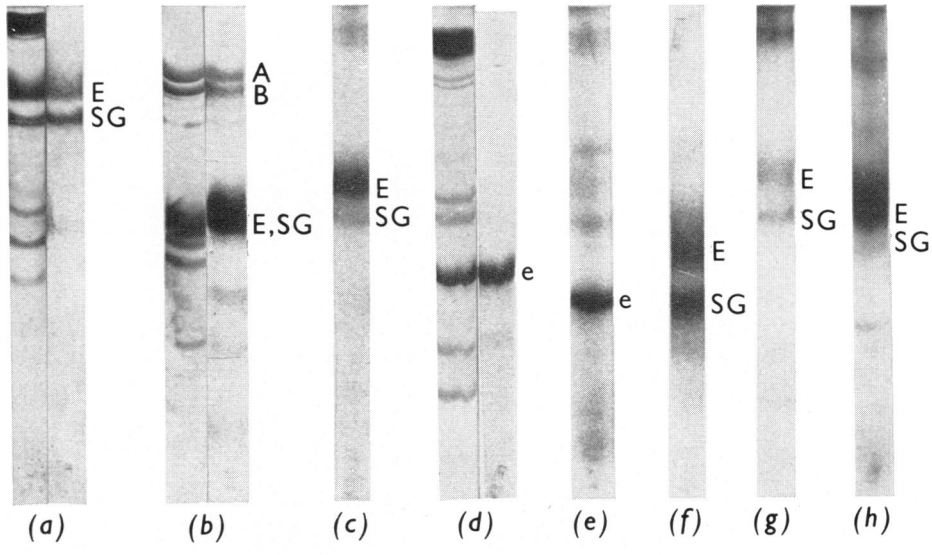
Protein E. The freeze-dried protein (10^5–10^6c.p.m.) was dissolved in 2ml of 0.2M-ammonium acetate containing 2mM-$CaCl_2$ and treated as for the sialoglycoprotein but with additions of 100μg of thermolysin (gift from Dr. D. M. Shotton) in place of trypsin and chymotrypsin. The total digest was applied to the paper.

Peptide 'maps' were obtained as described by Tanner & Gray (1971), and the radioactive peptides were located by radioautography. The identity of corresponding peptides was checked by elution and re-electrophoresis at pH6.5. The 'ghost' and intact erythrocyte peptides were also compared at pH1.65.

Results and Discussion

Iodination of intact erythrocytes and erythrocyte 'ghosts'

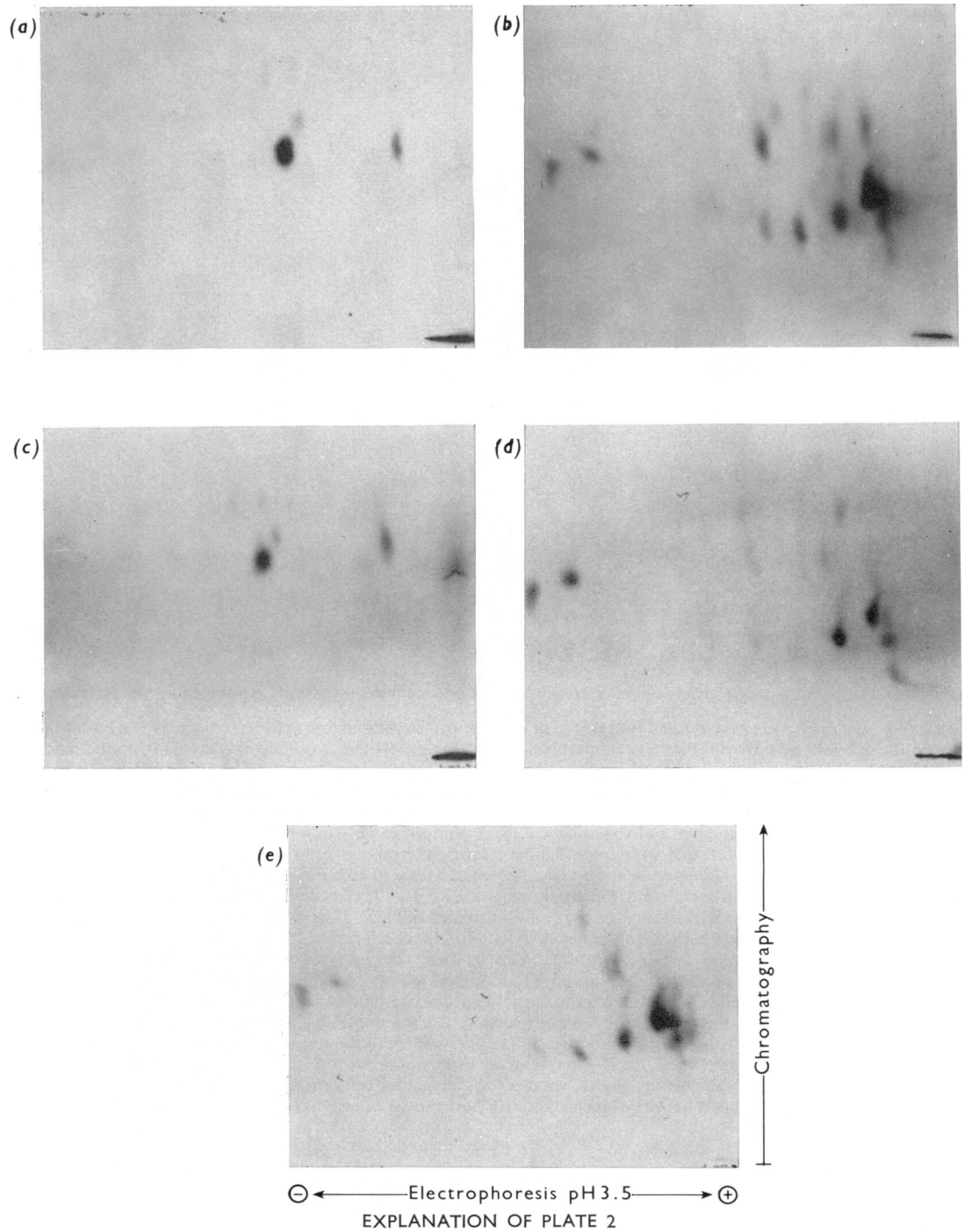
The distribution of protein and radioactivity obtained in 'ghosts' derived from labelled intact cells (Plate 1*a*) and labelled 'ghosts' (Plate 1*b*), is similar to



EXPLANATION OF PLATE 1

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of intact erythrocytes and erythrocyte membrane preparations

Sections of 1.1 cm diam. gels were stained for protein or dried down for radioautography as indicated. The mobilities of the bands on the different gels are not directly comparable. SG, Sialoglycoprotein. (*a*) 'Ghosts' derived from iodinated intact cells, labelled by the direct addition of H_2O_2, in the absence of butylated hydroxytoluene. Electrophoresis in 7.5% (w/v) gel containing 0.1% sodium dodecyl sulphate, 0.03 M-sodium phosphate buffer, pH 7.1 (Tanner & Boxer, 1972). Left, Coomassie Blue stain. Right, radioautograph. (*b*) 'Ghosts' iodinated by the direct addition of a total of 300 nmol of H_2O_2/0.2 ml of 'ghost' suspension, in the absence of butylated hydroxytoluene. Electrophoresis in a 5% (w/v) gel, under conditions described in (*a*). Left, Coomassie Blue stain. Right, radioautograph. Under these conditions protein E and the sialoglycoprotein co-migrate (Bretscher, 1971*b*). (*c*) 'Ghosts' iodinated by the direct addition of a total of 1000 nmol of H_2O_2/0.2 ml of 'ghost' suspension in the presence of butylated hydroxytoluene. Electrophoresis in a 8% (w/v) gel as described in the Methods section. Radioautograph. (*d*) 'Ghosts' derived from iodinated thermolysin-treated red cells. The cells were labelled by direct addition of H_2O_2 in the presence of butylated hydroxytoluene as described for intact cells in the Methods section. Left, Coomassie Blue stain. Right, radioautograph. (*e*) Iodinated 'ghosts' derived from thermolysin-treated erythrocytes. Iodination and electrophoresis conditions as in (*c*). Radioautograph. (*f*) Resealed 'ghosts', labelled with added external lactoperoxidase (iodination at extracellular surface). Iodination was done with glucose oxidase and butylated hydroxytoluene. Electrophoresis in an 8% (w/v) gel as described in the Methods section. Radioautograph. (*g*) 'Ghosts' resealed in the presence of lactoperoxidase and iodinated without further addition of lactoperoxidase (iodination at the cytoplasmic surface). Labelling was done with glucose oxidase and butylated hydroxytoluene. Electrophoresis in 8% (w/v) gel as described in the Methods section. Radioautograph. (*h*) Iodinated 'inside-out' vesicles (labelling at the cell cytoplasmic surface of the vesicles), labelled with glucose oxidase and butylated hydroxytoluene. Electrophoresis was in an 8% (w/v) gel, as described in the Methods section. Radioautograph.



EXPLANATION OF PLATE 2

Radioautographs of thermolysin peptide 'maps' of protein E labelled in various membrane preparations

(*a*) Protein E derived from iodinated erythrocytes, prepared as in Plate 1(*a*). (*b*) Protein E derived from iodinated erythrocyte 'ghosts', prepared as in Plate 1(*c*). (*c*) Protein E derived from resealed 'ghosts' iodinated with added external lactoperoxidase (labelling at the extracellular surface). Preparation as in Plate 1(*f*). (*d*) Protein E derived from 'ghosts' resealed in the presence of lactoperoxidase and iodinated without further addition of lactoperoxidase (labelling at the cytoplasmic surface). Preparation as in Plate 1(*g*). (*e*) Protein E derived from iodinated 'inside-out' vesicles (labelling at the cell cytoplasmic surface of the vesicles). Preparation as in Plate 1(*h*).

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that found by other workers (Phillips & Morrison, 1971*a*,*b*; Hubbard & Cohn, 1972).

Samples of the iodinated sialoglycoprotein and protein E labelled in each case were obtained by elution from gels. The peptide 'maps' obtained from the sialoglycoprotein iodinated in the intact cell and in the 'ghost' are identical (results not shown). However, as only two mobile labelled peptides are obtained, and an appreciable amount of radioactivity remains at the origin of the peptide 'maps', it is possible that a new site, which does not give rise to a mobile peptide, is labelled on the iodination of 'ghosts'.

Plates 2(*a*) and 2(*b*) show the peptide 'maps' of protein E derived from labelled intact cells and labelled 'ghosts'. Five peptides, iodinated in intact cells (Plate 2*a*), are also labelled in 'ghosts' (Plate 2*b*). In addition, ten strongly labelled, and several weakly labelled peptides are found only in the 'ghost' peptide 'map'.

When intact erythrocytes are treated with thermolysin, the membrane retains a fragment of protein E of mol. wt. 60000 (here designated 'e') but no identifiable fragments of the sialoglycoprotein remain. Triplett & Carraway (1972) and Bretscher (1971*c*) have noted similar results when erythrocytes are treated with other proteases. Erythrocytes were incubated with thermolysin and subsequently iodinated. The distribution of radioactivity and of protein is shown in Plate 1(*d*). 'Ghosts' derived from thermolysin-treated cells were also iodinated and the distribution of radioactivity is shown in Plate 1(*e*). The peptide 'maps' of iodinated 'e' are identical in both cases with the peptide 'maps' of unmodified protein E labelled under corresponding conditions (Plates 2*a* and 2*b*). Bretscher (1971*c*) has noted a similar phenomenon. This result eliminates the possibility that any of the labelled peptides are derived from another membrane component which co-migrates with protein E in polyacrylamide gels.

The above findings are in complete agreement with those of Bretscher (1971*c*), although a quite different labelling reagent was used. By assuming that the detailed organization of protein E in the membrane of the intact erythrocyte is the same as in the erythrocyte 'ghost', he concluded that the protein extends through the erythrocyte membrane. It is clear, however, that the erythrocyte 'ghost' is permeable even to rather large molecules such as ferritin (Nicolson *et al.*, 1971). This important difference between the membrane in the intact cell and in the 'ghost' could reflect a change in the arrangement of the membrane components.

Iodination of sealed membrane preparations

Further experiments were done to assess whether any such change occurred in the organization of protein E when 'ghosts' are made. It was necessary to establish that the 'ghost' specific peptides were derived from the cytoplasmic side of the membrane, and were outside the permeability barrier of the membrane. Two approaches were used: the iodination of resealed 'ghosts' (Passow, 1969), and the iodination of sealed 'inside-out' membrane vesicles (Kant & Steck, 1972). D-Glyceraldehyde 3-phosphate dehydrogenase and acetyl cholinesterase were used as markers for the cytoplasmic and extracellular surfaces of the erythrocyte membrane respectively, to check that the membranes were sealed and of the required orientation (Steck, 1972; Kant & Steck, 1972).

Resealed 'ghosts' were used in two ways. First, 'ghosts' were resealed and iodinated in the presence of externally added lactoperoxidase. In this case only the external membrane surface is accessible to lactoperoxidase. Before and after iodination, 0–1.5% of the membrane D-glyceraldehyde 3-phosphate dehydrogenase was accessible to substrate. The distribution of radioactivity is similar to that obtained when intact cells are labelled (Plate 1*f*). A peptide 'map' of protein E, thus labelled, is shown in Plate 2(*c*).

Secondly, 'ghosts' were resealed in the presence of lactoperoxidase, the external lactoperoxidase was removed from the 'ghosts' by washing, and the resealed 'ghosts' were iodinated without further addition of lactoperoxidase. In this case only the cytoplasmic membrane surface is in contact with lactoperoxidase. None of the membrane D-glyceraldehyde 3-phosphate dehydrogenase was accessible to its substrate, thus these 'ghosts' were completely sealed and had the same orientation as those described above. The pattern of radioactivity incorporated into the 'ghost' proteins under these conditions is shown in Plate 1(*g*), and Plate 2(*d*) shows a peptide 'map' of protein E so labelled.

'Inside-out' vesicles derived from erythrocyte 'ghosts' were iodinated in the presence of added lactoperoxidase. In this case, before and after iodination, D-glyceraldehyde 3-phosphate dehydrogenase was 100% accessible, and acetylcholinesterase 5–7% accessible, to their respective substrates. Thus the preparation was almost totally composed of sealed vesicles with an 'inside-out' orientation (having the cell cytoplasmic-membrane surface exposed to the exterior and to added lactoperoxidase), with a maximum contamination of 7% 'right-side-out' and unsealed vesicles. The distribution of radioactivity (Plate 1*h*) is similar to that of resealed ghosts iodinated only at the cytoplasmic membrane surface. In both these cases proteins A and B, protein E, and the sialoglycoprotein are labelled. The identity of the latter two bands was confirmed by comparison of their electrophoretic mobilities in gels with the bands obtained from the corresponding proteins iodinated in intact cells. A peptide 'map' of protein E labelled in 'inside-out' vesicles is shown in Plate 2(*e*).

In both situations where only the cell cytoplasmic surface is accessible to lactoperoxidase (Plates 2*d* and 2*e*) the putative 'cytoplasmic' labelled peptides of protein E are strongly iodinated. The peptides labelled in intact cells are absent, although residual traces of one peptide remain, perhaps owing to small amounts of contaminating unsealed 'ghosts' or oppositely orientated vesicles. It is also possible that an identical peptide is labelled at the cytoplasmic surface of the membrane.

When resealed 'ghosts' were labelled in the same way as intact cells the peptides labelled in protein E (Plate 2*c*) were also identical with those iodinated when intact cells are labelled (Plate 2*a*). The accessibility of the external sites of iodination on the protein is unchanged after 'ghost' formation and subsequent resealing, and the polarity of the iodination sites in the intact cell is retained in the resealed 'ghost'. It is therefore very likely that the sites on the protein which are at the cytoplasmic surface of the membrane in the intact cell are also unchanged in the resealed 'ghost'. It is not, however, possible to establish this unequivocally with the techniques used here.

It is noteworthy that the sites iodinated in unsealed 'ghosts' are equivalent to the sum of the sites labelled at the cytoplasmic and extracellular faces in resealed 'ghosts'. The leakiness of the 'ghost' is clearly not associated with any difference in the availability of sites on protein E and therefore it is unlikely that the organization of the protein in unsealed 'ghosts' differs greatly from that in resealed 'ghosts'. All the membrane preparations we have studied show the same division of iodinated sites into two distinct groups, each group being uniquely associated with one surface of the membrane. Within the limitations of the criteria used here, the arrangement of protein E appears to be the same in all these membrane preparations. Our results confirm that protein E certainly extends through the membrane of the erythrocyte 'ghost', and suggests that it is highly likely to be similarly arranged in the membrane of the intact erythrocyte.

Segrest *et al.* (1973) have been able to identify a peptide in the sialoglycoprotein which is only labelled when 'ghosts' are iodinated, thus confirming Bretscher's report (Bretscher, 1971*b*) that the sialoglycoprotein extends through the membrane. Although our peptide 'map' data are inconclusive on this point, the fact that the sialoglycoprotein is iodinated when lactoperoxidase is accessible only to the cytoplasmic surface of the membrane (Plates 1*g* and 1*h*) supports their conclusion.

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