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# Ionic-Strength-Dependent Changes in the Structure of the Major Protein of the Human Erythrocyte Membrane

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The effect of ionic strength on the proteolysis by trypsin of the major membrane-penetrating protein (polypeptide 3) in the erythrocyte membrane was studied. Both the intracellular and extracellular regions of the protein are susceptible to trypsin proteolysis under hypo-osmotic conditions, whereas under iso-osmotic conditions the extracellular region of the protein is resistant to trypsin, and the intracellular region yields only two cleavage products with trypsin. Studies of the fragments obtained from polypeptide 3 by trypsin digestion under iso-osmotic conditions of 'ghosts' radioiodinated with lactoperoxidase confirmed our earlier conclusions that the polypeptide chain of polypeptide 3 traverses the membrane twice. Ionic-strength-dependent changes were also observed in the incorporation of iodine by lactoperoxidase into the individual extracellular tyrosine sites of the protein. These results show that polypeptide 3 undergoes ionic-strength-dependent changes in structure.

The major protein (polypeptide 3) of the human erythrocyte membrane [band 3, Steck (1974); protein E, Jenkins & Tanner (1975)] is a glycoprotein with an apparent subunit mol.wt. of 90000–100000. The structure of this protein has become of particular interest since it has been shown to be involved in the transport of anions across the erythrocyte membrane (Cabantchik & Rothstein, 1974; Zaki *et al.*, 1975). Polypeptide 3 has been shown to traverse the membrane permeability barrier (Bretscher, 1971; Steck, 1972; Boxer *et al.*, 1974), and unique regions of the protein are accessible at each face of the erythrocyte membrane (Boxer *et al.*, 1974). Studies of the fragmentation of the native protein in the membrane by proteinases suggested that the protein has an S-shaped structure which contains a duplicated set of extracellular sites (Jenkins & Tanner, 1975). We have extended these studies and have found that both the extracellular and cytoplasmic regions of the protein undergo changes in structure in response to changes in the ionic strength of the medium.

## Methods

Trypsin digestion of intact erythrocytes, labelling of erythrocytes and erythrocyte 'ghosts', sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, isolation of labelled polypeptides and the preparation of peptide 'maps' have all been described previously (Boxer *et al.*, 1974; Jenkins & Tanner, 1975). Radioautographs were scanned in a model G scanner (Canalco, Rockville, MD, U.S.A.) by using a red filter. Coomassie Blue-stained gels were scanned in a linear transport (Gilford, Oberlin, OH, U.S.A.) attached to a Hilger–Gilford spectrophotometer.

### *Trypsin digestion of erythrocyte 'ghosts'*

(*a*) *Digestion under hypo-osmotic conditions.* 'Ghosts', in cold hypo-osmotic sodium phosphate buffer, pH 7.4 (0.155M-$NaH_2PO_4$ adjusted to pH 7.4 with 0.103M-$Na_2HPO_4$, and diluted with 19.5 vol. of deionized water), were digested for 20 min at 37°C with 30 $\mu$g of trypsin ('toluene-*p*-sulphonamidophenylalanine chloromethyl ketone'-treated)/mg of 'ghost' protein. The digestion was terminated by the addition of undiluted di-isopropyl phosphorofluoridate (2 $\mu$l/mg of trypsin) and/or soya-bean trypsin inhibitor (1 mg/mg of trypsin) [Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.]. The 'ghosts' were then washed once in hypo-osmotic sodium phosphate buffer, pH 7.4, centrifuged at 40000*g* for 20 min, and the pellet was prepared for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

(*b*) *Digestion under iso-osmotic conditions.* 'Ghosts' were prepared in cold hypo-osmotic sodium phosphate buffer, pH 7.4, as above, and kept on ice. The ionic strength was varied by the addition of small volumes of concentrated solutions to give the final concentration required (described in the text and Figure legends). Trypsin ('toluene-*p*-sulphonamidophenylalanine chloromethyl ketone'-treated; final concn. 30 $\mu$g/mg of 'ghost' protein) was added last as a solution in hypo-osmotic sodium phosphate buffer, pH 7.4. After addition of the enzyme, the 'ghosts' were transferred to a 37°C water bath for 20 min.

Digestion was terminated as described above, and the digested 'ghosts' were washed and prepared for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

## Results and Discussion

### *Trypsin digestion at the intracellular surface of the erythrocyte*

The digestion of erythrocyte 'ghosts' with low concentrations of trypsin results in cleavage at the intracellular regions of polypeptide 3 (Jenkins & Tanner, 1975). In the experiments referred to above, trypsin digestion was done under low-ionic-strength conditions (5mM-sodium phosphate buffer, pH8.0), and a number of partial-fragmentation products were obtained (TA to TG; Jenkins & Tanner, 1975). To obtain a more readily controlled and perhaps more specific cleavage of polypeptide 3, the effect of ionic strength on the trypsin digestion of erythrocyte 'ghosts' was investigated. To simplify the identification of the fragments derived from polypeptide 3, proteolysis experiments were done on 'ghosts' derived from trypsin-treated erythrocytes that had been radioiodinated with lactoperoxidase. Essentially the only protein labelled in these cells was polypeptide 3.

Fig. 1 shows the pattern of fragmentation produced by trypsin treatment under hypo- and iso-osmotic conditions. Under hypo-osmotic conditions (Fig. 1*b*), polypeptide 3 was completely degraded to two low-molecular-weight components (X and Y, mol.wt. 24000 and 20000). It should be noted that the concentration of trypsin used in this case was three times that used for our previous experiments, in which we obtained partial cleavage products (Jenkins & Tanner, 1975). By using similar high concentrations of trypsin, but in the presence of iso-osmotic (0.15M) KCl, two fragments (T1 and T2) were obtained. Fragment T1 was a broad diffuse band with mol.wt. 48000 at the leading edge and 62000 at the trailing edge. Fragment T2 was a much more sharply defined band of mol.wt. 24000 (Fig. 1*c*). The diffuse banding pattern found for fragment T1 was similar to that found on gel electrophoresis of intact polypeptide 3.

The pattern of cleavage under iso-osmotic conditions was not changed by substituting $Na^+$ or Tris for $K^+$, or by changing acetate or sulphate for chloride, or by the addition of either 5mM-magnesium acetate, 1mM-$CaCl_2$ or 5mM-$NaHCO_3$ to the iso-osmotic KCl reaction medium. Similarly, digestion in iso-osmotic KCl containing 5mM-magnesium acetate with any one of 2mM-ATP, 0.1mM-$NAD^+$, 0.1mM-NADH or 10mM-2,3-diphosphoglycerate gave the same cleavage pattern. However, when iso-osmotic KCl, 5mM-magnesium acetate, 2mM-ATP, 0.1mM-$NAD^+$, 0.1mM-NADH and 10mM-2,3-diphosphoglycerate were all present in the digestion medium, a radioactive band with mol.wt. 90000 remained resistant to proteolysis (band $3_A$, Fig. 1*d*). Band $3_A$ co-migrated under the polypeptide-3 band on gel electrophoresis of erythrocyte 'ghosts', but

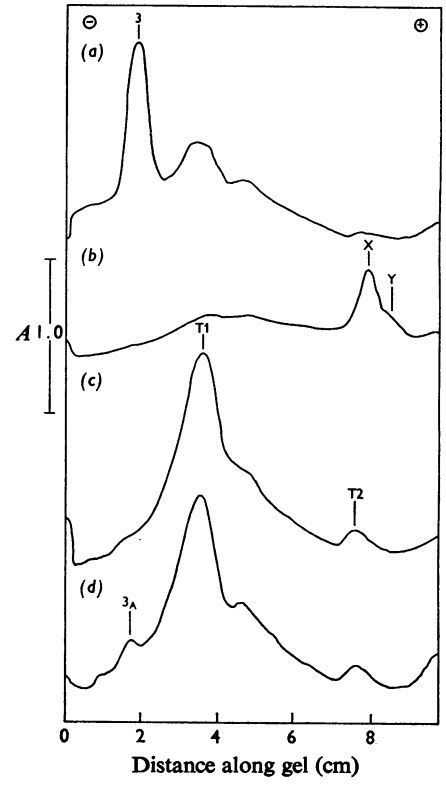



Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of 'ghosts' from labelled erythrocytes digested with trypsin under hypo- and iso-osmotic conditions*

Scans of radioautographs of dried longitudinal slices of 1.1cm-diameter gels containing 8% (w/v) acrylamide. (*a*) Untreated 'ghosts' from radioiodinated trypsin-treated human erythrocytes. (*b*) 'Ghosts' from radioiodinated trypsin-treated human erythrocytes that had been digested with trypsin under the hypo-osmotic conditions described in the Methods section. (*c*) 'Ghosts' from radioiodinated trypsin-treated human erythrocytes that had been digested with trypsin in an iso-osmotic reaction mixture containing 0.15M-KCl dissolved in hypo-osmotic sodium phosphate buffer, pH7.4, as described in the Methods section. (*d*) 'Ghosts' from radioiodinated trypsin-treated human erythrocytes that had been digested with trypsin in an iso-osmotic reaction mixture containing 0.15M-KCl, 5mM-magnesium acetate, 2mM-ATP, 0.1mM-$NAD^+$, 0.1mM-NADH and 10mM-2,3-diphosphoglycerate (disodium salt) dissolved in hypo-osmotic sodium phosphate buffer, pH7.4, as described in the Methods section. Trypsin-treated erythrocytes were radioiodinated with $Na^{125}I$ and lactoperoxidase as described by Jenkins & Tanner (1975).

carried only a small proportion of the total label found in polypeptide 3. When iso-osmotic KCl and 5mM-magnesium acetate were present, even more vigorous digestion conditions (50$\mu$g of trypsin/mg of 'ghosts' protein for 90min at 37°C, or 500$\mu$g of trypsin/mg of 'ghosts' protein for 60min at 37°C) failed to produce any further fragmentation of fragments T1 and T2. However, under the more vigorous digestion conditions, the relatively resistant 90000-mol.wt. band $3_A$ was degraded, despite the presence of 2mM-ATP, 0.1mM-$NAD^+$, 0.1mM-NADH and 10mM-2,3-diphosphoglycerate. As band $3_A$ was radioiodinated under the conditions used in these experiments, it must be available at the external surface of the intact erythrocyte membrane. Reichstein & Blostein (1975) noted a similar component in band 3 which was also found to be relatively resistant to proteolysis with trypsin at the cytoplasmic surface of the membrane.

These ionic-strength-dependent changes in the pattern of tryptic proteolysis probably reflect an ionic-strength-dependent alteration in the structure of the intracellular region of polypeptide 3. Our results suggest that the intracellular region of the protein adopts a more tightly folded conformation in an iso-osmotic environment than in an hypo-osmotic environment. This tightly folded conformation would be expected to prevail in the iso-osmotic environment of the intact erythrocyte.

The fragments T1 and T2 produced by proteolysis in iso-osmotic media were of a similar molecular weight to the fragments TA and TB, and TG, which we obtained from polypeptide 3 by using milder partial proteolysis under hypo-osmotic conditions (Jenkins & Tanner, 1975). Peptide 'maps' of both fragments TA and TG from erythrocyte 'ghosts' radioiodinated with lactoperoxidase (in which the extracellular and intracellular regions of polypeptide 3 were labelled) contained tyrosine peptides originating from both the inside and outside surfaces of the membrane. We concluded that each of these fragments penetrates the membrane, and that the polypeptide chain of polypeptide 3 therefore traverses the membrane at least twice. As trypsin cleavage under iso-osmotic conditions yielded a simpler and apparently more specific cleavage pattern, it was decided to repeat the above experiment by using these conditions of proteolysis.

'Ghosts' derived from trypsin-treated erythrocytes were radioiodinated with lactoperoxidase and digested with trypsin under iso-osmotic conditions. The fragments obtained were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 2*a*) and peptide 'maps' were prepared from the isolated fragments. The peptide 'maps' obtained from fragments T1 and T2 prepared in this way (see, for example, Plates 1*b* and 2*b*) were the same as those previously obtained from fragments TA and TG respectively (Jenkins & Tanner, 1975). This confirms our previous suggestion that the peptide chain of polypeptide 3 traverses the membrane at least twice.

In an earlier paper (Boxer *et al.*, 1974), we studied the lactoperoxidase labelling of the intracellular region of polypeptide 3 by labelling resealed 'ghosts' in an iso-osmotic medium and labelling 'inside-out' vesicles in a hypo-osmotic medium. In these cases, only the ten peptides which arise from the intracellular region of the protein were labelled. Inspection of these peptide 'maps' showed that the relative labelling of these peptides was not the same in each case. This suggests that the accessibility to lactoperoxidase of the tyrosine residues present in the intracellular region of the protein also depends on the ionic strength of the medium.

*Radioiodination of the extracellular regions of polypeptides*

In view of the ionic-strength-dependent structural changes in the intracellular regions of polypeptide 3, it was decided to determine whether similar ionic-strength-dependent conformational changes occurred

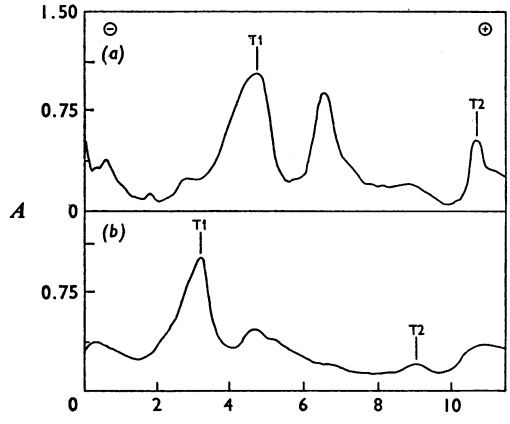
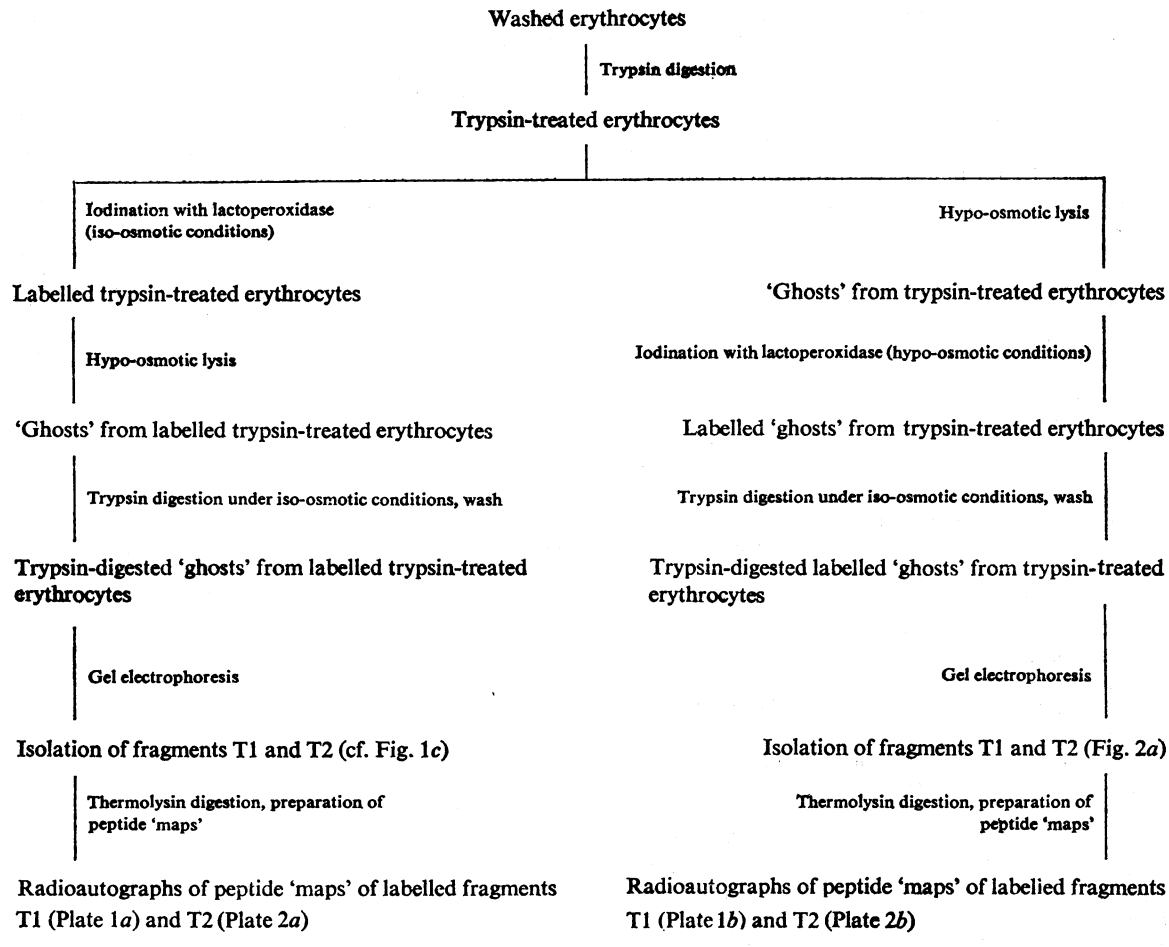



Fig. 2. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of trypsin-digested 'ghost' samples*

Scans of radioautographs of dried longitudinal slices of 1.1cm-diameter gels containing 10% (w/v) acrylamide. (*a*) Separation of fragments T1 and T2 from labelled 'ghosts' from trypsin-treated erythrocytes. The 'ghosts' were trypsin-digested under iso-osmotic conditions. Experimental details are given in the legend to Scheme 1. (*b*) Separation of fragments T1 and T2 from trypsin-treated erythrocytes that were labelled with $^{131}I$ and lactoperoxidase (under iso-osmotic conditions), lysed, and the 'ghosts' labelled with $Na^{125}I$ and lactoperoxidase (under hypo-osmotic conditions) before being digested with trypsin under iso-osmotic conditions. Experimental details are given in the legend to Scheme 2.

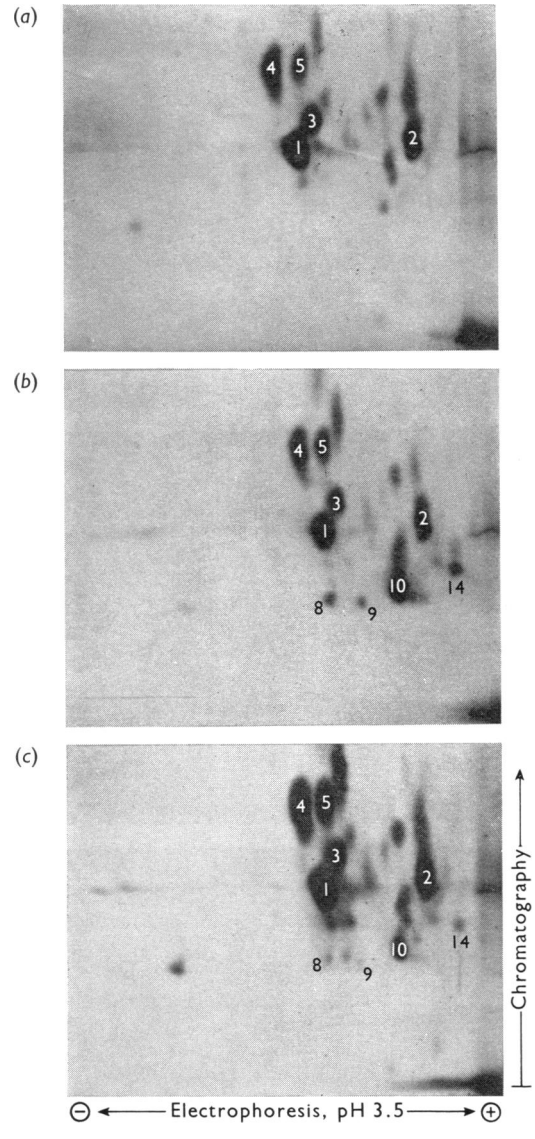



Scheme 1. *Isolation of fragments T1 and T2 after trypsin digestion under iso-osmotic conditions of 'ghosts' from labelled trypsin-treated erythrocytes and from labelled 'ghosts' derived from trypsin-treated erythrocytes*

Trypsin-treated human erythrocytes and 'ghosts' from trypsin-treated erythrocytes were radioiodinated with $Na^{125}I$ and lactoperoxidase under iso- and hypo-osmotic conditions respectively as described by Boxer *et al.* (1974) and Jenkins & Tanner (1975). Trypsin digestion of 'ghosts' was done in an iso-osmotic reaction mixture containing 0.15 M-KCl and 5 mM-magnesium acetate dissolved in hypo-osmotic sodium phosphate buffer, pH 7.4.

in the extracellular regions of the protein. Radioiodination with lactoperoxidase of the extracellular region of polypeptide 3 in the erythrocyte membrane gives rise to five distinct labelled peptides (peptides 1–5) on peptide 'maps' of thermolysin digests of the protein. At least two sets of peptides 1, 3, 4 and 5 are present in the intact protein, and these are distributed so that one set of peptides 1, 4 and 5 is present on each of the extracellular arms of the protein (Boxer *et al.*, 1974; Jenkins & Tanner, 1975).

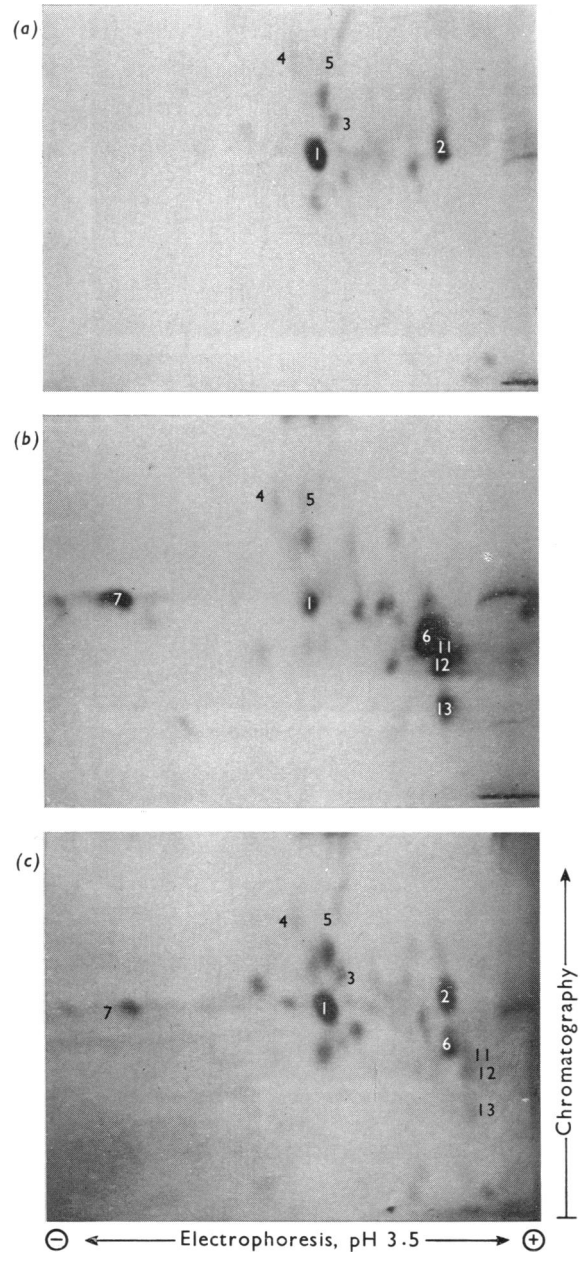
We have looked for ionic-strength-dependent changes in the extracellular regions of polypeptide 3 by comparing the incorporation of radioactive iodine into the extracellular tyrosine sites when intact erythrocytes were labelled by using lactoperoxidase (under iso-osmotic conditions) and when erythrocyte 'ghosts' were labelled (under hypo-osmotic conditions). The procedure used in these experiments is summarized in Scheme 1. Trypsin-treated erythrocytes were labelled with lactoperoxidase and the 'ghosts' derived from these cells were digested with trypsin under iso-osmotic conditions (cf. Fig. 1c). Peptide 'maps' of fragments T1 and T2 were then prepared (Plates 1a and 2a). In a parallel experiment, 'ghosts' derived from trypsin-treated erythrocytes were labelled with lactoperoxidase and digested with trypsin under iso-osmotic conditions (see Fig. 2a). The labelled fragments T1 and T2 obtained in this




## EXPLANATION OF PLATE 1

*Radioautographs of thermolysin peptide 'maps' of fragment T1 from labelled membrane preparations*

(*a*) Fragment T1 from 'ghosts' derived from labelled erythrocytes. 'Ghosts' from trypsin-treated erythrocytes that had been radioiodinated by using lactoperoxidase (under iso-osmotic conditions) were digested with trypsin under iso-osmotic conditions and fragment T1 was separated as described in Fig. 1(*c*). (*b*) Fragment T1 from labelled erythrocyte 'ghosts'. 'Ghosts' from trypsin-treated erythrocytes were labelled with lactoperoxidase (under hypo-osmotic conditions), digested with trypsin under iso-osmotic conditions, and fragment T1 was separated as described in Fig. 2(*a*). (*c*) Fragment T1 from erythrocytes that had been labelled with $Na^{132}I$, lysed and the 'ghosts' labelled with $Na^{125}I$. Trypsin-treated erythrocytes were labelled with $Na^{131}I$ and lactoperoxidase (under iso-osmotic conditions) and the 'ghosts' derived from these were labelled with $Na^{125}I$ and lactoperoxidase (under hypo-osmotic conditions). The 'ghosts' were digested with trypsin under iso-osmotic conditions and fragment T1 was separated as described in Fig. 2(*b*).




EXPLANATION OF PLATE 2

*Radioautographs of thermolysin peptide 'maps' of fragment T2 from labelled membrane preparations*

(*a*) Fragment T2 from 'ghosts' derived from labelled erythrocytes. (*b*) Fragment T2 from labelled erythrocyte 'ghosts'. (*c*) Fragment T2 from erythrocytes that had been labelled with $Na^{131}I$, lysed and the 'ghosts' labelled with $Na^{125}I$. Details are the same as for the parallel samples of fragment T1 described in the explanation to Plate 1.

Washed erythrocytes

↓ Trypsin digestion

Trypsin-treated erythrocytes

↓ Radioiodination with lactoperoxidase and $Na^{131}I$ (iso-osmotic conditions)

$^{131}I$-labelled trypsin-treated erythrocytes

↓ Hypo-osmotic lysis

'Ghosts' from $^{131}I$-labelled trypsin-treated erythrocytes

↓ Radioiodination with lactoperoxidase and $Na^{125}I$ (hypo-osmotic conditions)

$^{125}I$-labelled 'ghosts' from $^{131}I$-labelled trypsin-treated erythrocytes

↓ Trypsin digestion under iso-osmotic conditions, wash, gel electrophoresis

Isolation of labelled fragments T1 and T2 (Fig. 2*b*)

↓ Thermolysin digestion
Preparation of peptide 'maps'

Radioautographs of peptide 'maps' of labelled fragments T1 (Plate 1*c*) and T2 (Plate 2*c*). Measure ratio of $^{131}I/^{125}I$ incorporated into each peptide (Table 1).

Scheme 2. *Isolation of fragments T1 and T2 from membranes labelled with $Na^{131}I$ and lactoperoxidase under iso-osmotic conditions and labelled with $Na^{125}I$ and lactoperoxidase under hypo-osmotic conditions*

Washed trypsin-treated human erythrocytes (0.5ml) were labelled with 3mCi of $Na^{131}I$ as described by Jenkins & Tanner (1975). 'Ghosts' from these cells were labelled with 2mCi of $Na^{125}I$ as described by Boxer *et al.* (1974) before digestion with trypsin in an iso-osmotic reaction mixture containing 0.15M-KCl and 5mM-magnesium acetate dissolved in hypo-osmotic sodium phosphate buffer, pH7.4, as described in the Methods section.

case were also isolated and peptide 'maps' prepared (Plates 1*b* and 2*b*). The peptides numbered 1–5 are from the extracellular regions of the protein, and the remainder (6–15) are from the intracellular region of the protein (Boxer *et al.*, 1974; Jenkins & Tanner, 1975). Inspection of Plates 1(*a*) and 1(*b*) shows that there was little change in the relative labelling of the extracellular peptides (1–5) in fragment T1. However, the peptide 'maps' of fragment T2 show that, although no detectable labelling of peptides 2 and 3 was obtained when fragment T2 was prepared from radioiodinated erythrocyte 'ghosts' (Plate 2*b*, and Jenkins & Tanner, 1975), labelled copies of peptides 2 and 3 were apparent when the fragment was isolated from radioiodinated erythrocytes (Plate 2*a*).

To confirm these results, trypsin-treated erythrocytes were labelled with lactoperoxidase and $Na^{131}I$ (iso-osmotic conditions) and the 'ghosts' prepared from these $^{131}I$-labelled erythrocytes were then labelled with lactoperoxidase and $Na^{125}I$ (hypo-osmotic conditions). Scheme 2 shows an outline of the experiment. Only a small proportion of the total available tyrosine sites on the cell surface are iodinated (Hubbard & Cohn, 1972), and thus the relative incorporation of the two iodine isotopes into each of the labelled extracellular peptides should reflect the relative accessibility of each tyrosine site to lactoperoxidase under iso- and hypo-osmotic conditions. The 'ghosts' were trypsin-treated under iso-osmotic conditions, and fragments T1 and T2 were isolated by polyacrylamide-gel electrophoresis (Fig. 2*b*). The peptide 'maps' obtained in each case are shown in Plates 1(*c*) and 2(*c*). The extracellular peptides (1–5) were excised from both peptide 'maps' and counted for both $^{131}I$ and $^{125}I$ radioactivity. The ratio of $^{131}I/^{125}I$ incorporated into each peptide is shown in Table 1. The results show that the relative incorporation of radioactivity into each of the extracellular tyrosine sites of fragment T1 was similar whether labelling was done under hypo- or iso-osmotic conditions. However, changes did occur in the relative incorporation of iodine into the extracellular peptides of fragment T2 when labelling was done under hypo- and iso-osmotic conditions. Peptides

Table 1. *Ratio of $^{131}I/^{125}I$ incorporated into the extracellular tyrosine sites of fragments T1 and T2 from the experiment shown in Scheme 2*

The radioactive peptides (1–5) were cut out from the peptide 'maps' (Plates 1*c* and 2*c*) and counted for both $^{131}I$ and $^{125}I$ radioactivity as described by Jenkins & Tanner (1975). After correction for isotope cross-over, the ratio $^{131}I/^{125}I$ incorporated into each peptide was calculated. To facilitate comparisons between the labelling of the sites in fragments T1 and T2, the $^{131}I/^{125}I$ ratios obtained for the peptides from each peptide 'map' were calculated so that the $^{131}I/^{125}I$ ratio for peptide 5 = 1.0.

$$\frac{^{131}I \text{ (iso-osmotic conditions)}}{^{125}I \text{ (hypo-osmotic conditions)}}$$ ratio in sites from

| Site | Fragment T1 | Fragment T2 |
|---|---|---|
| 1 | 0.9 | 2.9 |
| 2 | 0.8 | 4.9 |
| 3 | 1.1 | 3.2 |
| 4 | 0.7 | 1.3 |
| 5 | 1.0 | 1.0 |

1, 2 and 3 of fragment T2 incorporated more radioactivity when polypeptide 3 was labelled under iso-osmotic conditions than when the protein was labelled under hypo-osmotic conditions.

This result suggests that the T2 arm of the extracellular region of polypeptide 3 undergoes an ionic-strength-dependent conformational change. Peptides 1, 2 and 3 in the T2 arm are more accessible to lactoperoxidase (and therefore probably less buried within the protein) in the structure taken up by the protein under isotonic conditions than in the structure adopted under low-ionic-strength conditions. The accessibility of the tyrosine residues in the T1 arm appears to be independent of the ionic strength of the environment.

It is clear that copies of peptides 2 and 3 are present in fragment T2. Our earlier report (Jenkins & Tanner, 1975) suggested that, although copies of peptides 2 and 3 were present in fragment TA (equivalent to T1), they were not present in fragment TG (equivalent to T2). Our inability to detect copies of peptides 2 and 3 in fragment T2 clearly resulted from the fact that these are very weakly labelled when erythrocyte 'ghosts' are labelled in the normal low-ionic-strength medium.

The experiments reported in the present paper show that polypeptide 3 contains two copies of each of the five tyrosine sites (1–5) in the extracellular region of the protein which can be labelled with lactoperoxidase. One set of these peptides is present on the 'T1 arm' of the protein, and another set on the 'T2 arm' of the protein (Fig. 3). Although the extracellular 'T1 arm' and 'T2 arm' of the protein are drawn separate in Fig. 3, it seems probable that the two arms of the protein will interact with each other.

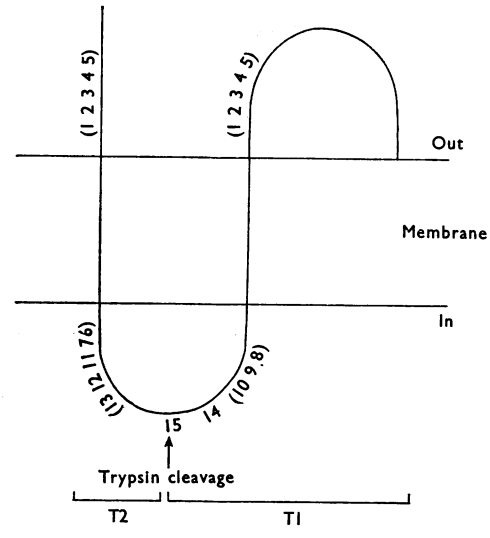



Fig. 3. *Location of labelled tyrosine sites on polypeptide* 3
The numbers represent the positions of the labelled tyrosine peptides obtained in peptide 'maps' of polypeptide 3.

Neither the extent of sequence duplication in the extracellular region of the protein nor the significance of this duplication is known. It may be associated with the involvement of the protein in erythrocyte anion transport and possibly reflect some sort of duplication of symmetry in the anion-binding sites of the protein.

The extracellular region of polypeptide 3 is resistant to trypsin when digestion is carried out under iso-osmotic ionic-strength conditions on intact erythrocytes (Fig. 4*a*; Steck *et al.*, 1971). When intact erythrocytes were digested by trypsin under the same conditions but at low ionic strength (in 300mM-sucrose containing 5mM-sodium phosphate buffer, pH8.0), 55% of the polypeptide 3 was cleaved to give a product of mol.wt. 60000 (Fig. 4*b*). This product has the same mobility as that obtained from polypeptide 3 by thermolysin (Fig. 4*c*) or Pronase treatment of erythrocytes under iso-osmotic conditions (Bender *et al.*, 1971; Bretscher, 1971; Boxer *et al.*, 1974). The pattern of digestion of intact erythrocytes by thermolysin was not affected by the ionic strength of the medium.

Our suggestion that polypeptide 3 traverses the membrane at least twice was based on the assumption that trypsin treatment of erythrocyte 'ghosts' (under low-ionic-strength conditions) results in cleavage only at the intracellular region of peptide 3 (Jenkins & Tanner, 1975). Although these earlier experiments use appreciably lower concentrations of trypsin than we have used here, the results shown in Fig. 4 suggest

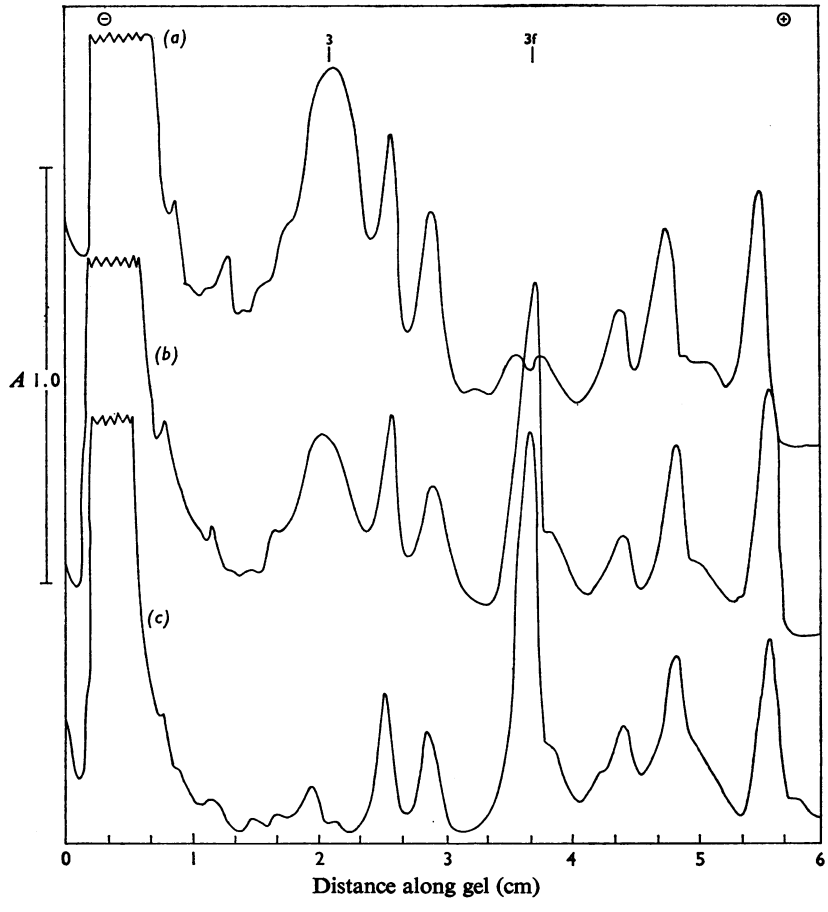



Fig. 4. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of 'ghosts' from erythrocytes that had been treated with trypsin under iso-osmotic and low-ionic-strength conditions*

Scans at 550nm of Coomassie Blue-stained gels containing 8% (w/v) acrylamide. (*a*) 'Ghosts' from erythrocytes that had been treated with trypsin under iso-osmotic ionic-strength conditions (in 0.1M-sodium phosphate buffer, pH8.0) as described by Jenkins & Tanner (1975). (*b*) 'Ghosts' from erythrocytes that had been treated with trypsin under low-ionic-strength conditions (in 300mM-sucrose containing 5mM-sodium phosphate buffer, pH8.0). Other conditions were as described for (*a*). (*c*) 'Ghosts' from erythrocytes that had been treated with thermolysin as described by Boxer *et al.* (1974). The pattern obtained from 'ghosts' from untreated erythrocytes was identical with (*a*).

that some digestion may have occurred in the extracellular region of polypeptide 3 under these conditions. However, the parallel experiments discussed above, in which trypsin digestion was done on labelled erythrocyte 'ghosts' in an iso-osmotic medium (a situation in which the extracellular region of polypeptide 3 is resistant to trypsin), completely confirm our earlier conclusions.

After these present experiments were completed, Steck *et al.* (1976) reported results that have led them to propose a model which differs in some respects from that shown in Fig. 3. The major point of difference between these two models is that, in the model of Steck *et al.* (1976), the region of the protein corresponding to T2 in Fig. 3 does not extend through the membrane to the extracellular surface but remains entirely on the cytoplasmic side of the membrane. [Steck *et al.* (1976) are incorrect in stating that we had suggested (Jenkins & Tanner, 1975) that the region corresponding to their 38000-dalton chymotrypsin fragment (the extracellular terminus of the T1 region of the protein in Fig. 3) extends to the cytoplasmic side of the membrane. We suggested, in fact, that this terminus may interact with the membrane but not

penetrate it through to the cytoplasmic side.] Comparison of our results with those of Steck *et al.* (1976) is difficult, since they used proteolysis under hypo-osmotic conditions, which, as discussed earlier (Jenkins & Tanner, 1975), and above, yields fragmentation patterns which contain overlapping peptides and are very sensitive to the digestion conditions. However, a major difference in interpretation is that Steck *et al.* (1976) do not consider our fragment T2 in aligning the protein sequence, although their data clearly show a fragment of mobility similar to T2 on electrophoresis of the membrane-associated residue remaining after trypsin digestion of 'ghost' preparations. The presence of the characteristic labelled peptides of polypeptide 3 in peptide 'maps' of fragment T2 and their known 'sidedness' with respect to the membrane allows us to unambiguously conclude that fragment T2 is a transmembrane segment, derived from polypeptide 3, that is distinct from fragment T1, the other transmembrane segment of the protein. This implies [a point also made by Steck *et al.* (1976)] that the intact polypeptide has an homogeneous primary structure [discussed by Jenkins & Tanner, 1975, 1977 (the following paper)]. The predicted presence of carbohydrate in fragment T2 (Jenkins & Tanner, 1977) is also consistent with the extracellular location of part of this fragment.

The peptide 'maps' of fragments T1 and T2 contain all the major labelled peptides found in peptide 'maps' of the intact protein, with the exception of peptide 15. The similar situation with respect to peptides TA and TG led to our earlier conclusion (Jenkins & Tanner, 1975) that these two peptides accounted for the entire protein, with the tryptic cleavage occurring at a basic residue in peptide 15. In this connexion the apparent molecular weight of fragment T1 obtained from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis is likely to be inaccurate, since the peptide is heavily glycosylated (Jenkins & Tanner, 1977), making the use of the molecular weights of the fragments unreliable. However, Steck *et al.* (1976) have shown that fragments, believed to be derived from polypeptide 3, are released from the membrane in their tryptic digests of erythrocyte-membrane preparations. We do not know whether such fragments are released from the membrane under our conditions of trypsin digestion, but, if they are present, they are likely to be derived from the region of site 15 in Fig. 3.

The present results show that both the extracellular and intracellular regions of band 3 have a tighter structure under iso-osmotic conditions than under hypo-osmotic conditions. The former conditions are likely to approximate more closely to the situation in the functioning erythrocyte. Although it is not known whether the structural changes that we have observed in band 3 at low ionic strengths have any effect on the functional properties of the protein, this possibility should be considered when the results from experiments carried out on erythrocyte 'ghosts' at low ionic strength are extrapolated to the situation in intact erythrocytes.

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