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The Isolation and Functional Identification of a Protein from the Human Erythrocyte 'Ghost'

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A protein, initially identified as a band on polyacrylamide-gel electrophoresis of erythrocyte 'ghosts', was isolated by selective extraction of 'ghosts' with EDTA solutions. The molecular weight of the polypeptide chain was estimated as 33000 and it represents approx. 5% of the membrane protein. The *N*-terminal sequence of the protein was established. Comparison with known protein sequences suggested that the protein might be the erythrocyte D-glyceraldehyde 3-phosphate dehydrogenase. This identification was confirmed by direct enzyme assay. It is suggested that this enzyme, which is strongly retained by erythrocyte 'ghosts' on haemolysis of erythrocytes, is unlikely to be an integral part of the structure of the erythrocyte membrane.

A number of workers have reported procedures for the separation, isolation and purification of proteins from the erythrocyte membrane (Rosenberg & Guidotti, 1969; Marchesi, Steers, Marchesi & Tillack, 1970; Blumenfeld, Gallop, Howe & Lee, 1970; Maddy & Kelly, 1970; Bakerman & Wasemiller, 1967; Schneiderman & Junga, 1968). In many cases, the conclusions of these reports are at variance with each other, and they show little agreement on questions, such as the extent of heterogeneity of the proteins in the erythrocyte membrane, that are basic to our understanding of the structure and function of the erythrocyte membrane and of membranes in general. This situation appears to stem partly from the general usage in studies of membrane proteins of criteria, such as molecular-weight estimation, that are not able to define uniquely the components of strongly aggregating membrane-protein mixtures, partly because of the intractable nature of membrane proteins themselves and partly because of the lack of specific functional assays for the proteins (either because the proteins themselves have no known functional activity that can be readily assayed or because of the loss of functional activity caused by the extensive use of denaturing solvents).

Our approach to this problem has been to attempt to characterize the erythrocyte membrane proteins in terms of their unique features, namely their amino acid sequences. The present report describes the isolation of a protein that constitutes about 5% of the protein present in the human erythrocyte 'ghost'. The *N*-terminal amino acid sequence of this protein showed a close resemblance to the known *N*-terminal sequence of a glycolytic enzyme. The identity of the protein was subsequently confirmed to be D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) by direct enzymic assay.

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MATERIALS AND METHODS

All chemicals were reagent grade when possible. Sodium dodecyl sulphate was recrystallized from 80% aqueous ethanol. Urea (reagent grade) was stored as a 10M solution and passed through a mixed-bed deionizer immediately before use. Guanidine–HCl was purified by stirring the technical-grade material overnight with water (3:2, w/w). The filtered solution was stirred with activated charcoal, filtered and the concentration of the solution was determined from its refractive index.

Preparation of erythrocyte 'ghosts'. Small-scale preparations of 'ghosts' were done with 50 ml of human blood (group O, Rh-positive) freshly drawn in acid citrate–dextrose medium. Haemoglobin-free 'ghosts' were obtained approx. 3–4h after lysis of the cells, by the method of Dodge, Mitchell & Hanahan (1963), and the preparation was done at 0–4°C.

Large-scale preparations of stroma used group O, Rh-positive human erythrocytes that had been stored under blood-bank conditions for less than 30 days after their collection. About 2 litres of packed erythrocytes were

processed as a batch by using a procedure similar to that of Dodge *et al.* (1963) by using 20 ideal milliosmolar phosphate buffer as the haemolysing medium, except that the Sorvall 'Szent-Gyorgi and Blum' continuous-flow centrifugation system was used to collect the stroma during the haemolysis and washing procedure. The stroma produced by this method tended to be pale pink in colour, and contained haemoglobin that could not be removed by further washing. The operation was done at 0–4°C and took approx. 36 h. The stroma were not stored, the relevant extractions being done as soon as possible after their preparation.

Polyacrylamide-gel electrophoresis. The reproducibility of the banding patterns was found to be critically dependent on the previous treatment of the samples. Freezing or freeze-drying changed the patterns markedly, and these procedures were avoided wherever possible. Several different systems were used.

Gel system A (5% acrylamide, 0.1% sodium dodecyl sulphate, pH 7.1; Shapiro, Vinuela & Maizel, 1967). Proteins or freshly prepared stroma were mixed with an equal volume of a buffer containing 2% sodium dodecyl sulphate, 2% 2-mercaptoethanol and 0.01 M-sodium phosphate buffer, pH 7.1. The stroma suspensions cleared immediately and the solution was warmed at 37°C for 3 h. Samples were then dialysed against 0.01 M-sodium phosphate buffer, pH 7.1, containing 0.1% sodium dodecyl sulphate, 0.1% 2-mercaptoethanol, 20% sucrose and 0.01% Bromophenol Blue. Electrophoresis was done at 2 V/cm for 16–17 h, until the Bromophenol Blue had migrated within 2–3 cm of the bottom of the tubes. Samples were fixed and stained with Coomassie Brilliant Blue R-250.

Gel system B (5% acrylamide, 0.1% sodium dodecyl sulphate, 8 M-urea, pH 7.1). A similar procedure was used, except that all buffers, including the sample buffers, contained 8 M-urea.

Gel system C (7.5% acrylamide, 9 M-urea, pH 2.7; Neville, 1967). Samples of protein were mixed with 2 vol. of a solution containing 9 M-urea, 10% 2-mercaptoethanol and 0.01 M-tris–HCl buffer, pH 7.0. After warming at 37°C for 3 h the sample was applied directly to the gel system. The gels were stained with Amido Black and destained by washing them in the presence of activated charcoal.

Specific staining for periodate-positive carbohydrate was done by cutting an unstained gel in half along its longitudinal axis, staining one piece with Amido Black and the other with the periodic acid–Schiff reagent stain (Zacharias, Zell, Morrison & Woodcock, 1969). Comparison of the stained pieces allowed confirmation that a particular Amido Black staining band was or was not periodate sensitive.

Reduction and S-aminoethylation. Reduction and *S*-aminoethylation were done with ethyleneimine (Cole, 1967). The sample was reduced with dithiothreitol in the presence of 5 M-guanidine–HCl under N_2 for 1 h at room temperature and pH 8.6 before aminoethylation under N_2.

Preparation of peptide 'maps'. A 3 mg sample of protein was mixed with trypsin (1:66, w/w; toluene-*p*-sulphonamidophenylethyl chloromethyl ketone-treated) in 0.2 ml of 0.4 M-NH_4HCO_3, and kept in a shaking incubator at 37°C for 4 h. Further trypsin (1:132, w/w) was added and digestion was continued at 37°C for 2 h. The samples were subjected to chromatography on Whatman no. 3 paper (with butanol–acetic acid–water; 4:1:5, by vol.) in one dimension and electrophoresis at pH 3.5 in the other direction by using the procedure of Katz, Dreyer & Anfinsen (1959). The 'fingerprints' were stained with the cadmium–ninhydrin stain (Dreyer & Bynum, 1967).

Large-scale purification of EDTA-extractable protein. All operations were done at 4°C. The freshly prepared packed stroma obtained from 2 litres of group O, Rh-positive erythrocytes were made up to 1500 ml with 0.1 M-EDTA (disodium salt) titrated to pH 7.4 with NaOH. After mixing, the suspension was allowed to stand at 4°C for 5 min and was then centrifuged in the Sorvall GSA rotor at 25000*g* for 45 min. The clear supernatant was aspirated and dialysed against three changes of 40 litres of deionized water over 3 days, and then freeze-dried.

The extracts from three 'ghost' preparations each derived from 2 litres of cells were combined at this stage, made up to about 300 ml with water and redialysed. A slight precipitate formed and was removed by centrifugation. The supernatant was made 0.01 M in tris–HCl, pH 7.0, to a total volume of 500 ml and then made 60% saturated with $(NH_4)_2SO_4$ at 4°C. After stirring at 4°C for 30 min the precipitate was centrifuged off. The supernatant was dialysed against deionized water at 4°C and freeze-dried. After this stage the material became very insoluble in aqueous buffers.

The protein was then dissolved in 15 ml of a solution containing 5 M-guanidine–HCl, 0.05 M-tris–HCl buffer, pH 7.0, 1 mM-EDTA (disodium salt) and 2% 2-mercaptoethanol. After warming it at 37°C for 2 h, it was applied to a column (5 cm × 90 cm) of Sephadex G-200 that was equilibrated with a solution of 5 M-guanidine–HCl, 0.05 M-tris–HCl, pH 7.0, 1 mM-EDTA (disodium salt) and 0.2% 2-mercaptoethanol. The major peak of material with absorption at 280 nm was pooled, dialysed exhaustively against deionized water and freeze-dried. Approx. 250 mg of protein was obtained from 6 litres of packed erythrocytes.

Amino acid analysis. Peptides or proteins were dried and hydrolysed with constant-boiling HCl under N_2 in sealed tubes or sealed desiccators (Dreyer & Bynum, 1967) at 105°C for 20 h. After being dried, the hydrolysed samples were dissolved in the starting buffer for the amino acid analyser.

Preparation of arginine peptides of EDTA-extractable protein. The procedure was done on 48 mg (1.6 μmol) of reduced and *S*-aminoethylated protein. The weight of protein was determined by amino acid analysis. The protein was trifluoroacetylated with 1.3 ml of *S*-ethyl trifluorothioacetate as described by Goldberger (1967), and subsequently digested at 37°C and pH 8.1 with a total of 0.9 mg of toluene-*p*-sulphonamidophenylethyl chloromethyl ketone-treated trypsin for 4 h, during which time 9.9 μmol of base was required/μmol of protein to keep the pH constant. Phosphorofluoridate (5 μl) was added to the mixture at the end of the reaction, at which point a clear solution was obtained. The solution was made 30% in acetic acid before application to the ion-exchange column and it had a final pH of 2.0. The peptides formed a clear solution under these conditions, with a tendency to gel.

The peptides were separated in a high-pressure chrom-

atographic system on a column (0.9 cm × 20 cm) of Technicon P resin at 50°C. The buffer system used was based on that of Schroeder, Jones, Cormick & McCalla (1962). The column was equilibrated with pH 2.8 buffer (1114 ml of acetic acid and 32.25 ml of pyridine made up to 4 litres with deionized water). After application of the sample, a gradient of 750 ml total volume (made up of equal volumes of pH 2.8, pH 3.1 and pH 5.0 buffers) was applied from a Varigrad. The latter two buffers were as described by Schroeder *et al.* (1962). This gradient was followed by a further gradient of 200 ml total volume, consisting of equal volumes of the pH 5.6 buffer of Schroeder *et al.* (1962) and 2 M-*N*-ethylmorpholinium acetate, pH 8.6. The column was finally washed with 0.5 M-triethylamine, pH 12.15. Peptides eluted with the first gradient were monitored on a continuous-flow ninhydrin-detection system, whereas peptides eluting later from the column were detected, after acid hydrolysis, by high-voltage paper electrophoresis (Dreyer & Bynum, 1967).

The relevant tubes were pooled, dried, and samples were removed for determination of the *N*-terminal residues of the peptides by the dansyl chloride technique (Gray, 1967). The remainder of each sample was treated with piperidine to remove the trifluoroacetyl blocking groups (Goldberger, 1967). At least 100 nmol of each peptide was digested with 40 μg of trypsin and all the samples were 'fingerprinted', together with a tryptic digest of the whole reduced and aminoethylated protein, to compare the 'daughter' peptides.

RESULTS

Polyacrylamide-gel electrophoresis of intact erythrocyte 'ghosts'. The pattern of protein migration obtained when freshly prepared human erythrocyte 'ghosts' were subjected to gel electrophoresis in 0.1% sodium dodecyl sulphate (gel system A) is shown in Plate 1(*a*). Similar patterns have been reported by Lenard (1970) and others.

When erythrocyte 'ghosts' were run in gels containing 0.1% sodium dodecyl sulphate and 8 M-urea (gel system B), the pattern shown in Plate 1(*b*) was obtained. The pattern is similar to that obtained when no urea is present but there appear to be fewer minor bands and band D moves to overlap band E. The patterns obtained with systems A and B are generally similar so it seems reasonable to assign the same nomenclature to the bands. A less satisfactory relationship between molecular weight and mobility for proteins of known molecular weights was found in system B than in system A, but the apparent molecular weights of the bands from erythrocyte 'ghosts' obtained from both systems agree within 10% in most cases. Application of the periodic acid–Schiff stain to a sample of 'ghosts' run in this gel system showed a periodate positive band in the region of bands E and F and a large amount of staining material in the region near the electrophoresis front.

Characterization of 30-day-old cells. The preceding experiments were done with 'ghosts' prepared within hours of drawing the blood sample. For the large-scale preparation of 'ghost' protein it was desirable to use erythrocytes outdated for transfusion purposes, as these can be obtained in large amounts. The gel patterns obtained from haemoglobin-free 'ghosts' prepared from erythrocytes freshly drawn on the day of preparation of the 'ghosts' (Plate 1*b*), and from 'ghosts' derived from erythrocytes that had been stored for 30 days (Plate 1*c*) in a blood bank are identical. It appears that no significant proteolysis or other change occurs in the membrane proteins of erythrocytes stored under blood-bank conditions for a period of 4 weeks.

In contrast, isolated 'ghosts' appear to be much more susceptible to degradation and cannot be kept free of proteolysis even when frozen. Sodium dodecyl sulphate-gel analysis of 'ghosts' that had been kept frozen for approx. 6 weeks showed a generally high degree of background staining, lack of sharpness of the bands and a considerable quantity of broadly dispersed material, clearly resulting from proteolysis. 'Ghosts' could, however, be kept frozen under liquid N_2 over a period of 6 months without any change in the banding pattern on the sodium dodecyl sulphate gels. Alternatively, freshly prepared 'ghosts' that had been mixed with the sodium dodecyl sulphate gel sample buffer could be stored frozen without having any effect on the banding pattern.

Selective extraction of proteins from erythrocyte 'ghosts'. Extraction with EDTA. When haemoglobin-free human erythrocyte 'ghosts' were washed with 0.1 M-EDTA, pH 7.9, and centrifuged, the only protein extracted into the supernatant was protein K (Plate 2*e*). Gel electrophoresis in urea at pH 2.7 showed that this protein was homogeneous (Plate 2*h*). The extent of extraction and the purity of the resulting product was found to be independent of pH of the extracting EDTA solution in the range pH 5–8.5. Concentrations of EDTA below 0.1 M were less efficient in solubilizing protein K, but when 0.1 M-EDTA was used the solubilization of the protein was complete as judged by the absence of the protein K band in the stromal residue (Plate 2*f*). The EDTA extract, however, also contained a large amount of non-protein material, as judged by the low recovery of amino acids after hydrolysis. Extraction of the membrane in the cold for a short time with EDTA did not result in extensive breakdown of the stroma as observed in the phase-contrast microscope. Extraction for a longer time caused the breakdown of the characteristic 'ghost' structure and the formation of small vesicles.

Extraction with urea. When 'ghosts' that had been extracted with EDTA (Plate 2*f*) were made up to a final concentration of 6 M-urea in the presence of 2-mercaptoethanol at pH 7.0, the

Table 1. *Amino acid analysis of human erythrocyte stroma and protein K*

Cysteine and cystine were estimated as *S*-aminoethylcysteine (AECys) after *S*-aminoethylation of the samples. The estimate of the number of residues/chain of protein K is based on the assumption of 9 residues of arginine/chain.

	Human stroma	Protein K	
	(mol/1000 mol of amino acids)	(mol/1000 mol of amino acids)	(residues/chain)
Asp	94	129	35
Thr	64	67	18
Ser	90	65	20
Glu	124	67	18
Pro	46	38	10
Gly	78	109	29
Ala	85	100	27
Val	56	107	29
Met	21	24	7
Ile	37	61	16–17
Leu	111	60	16–17
Tyr	17	17	5
Phe	36	42	11–12
His	39	25	7
Lys	58	78	21
Arg	47	30	9
AECys	8	13	4–5

Table 2. *Composition of tryptic peptides of protein K*

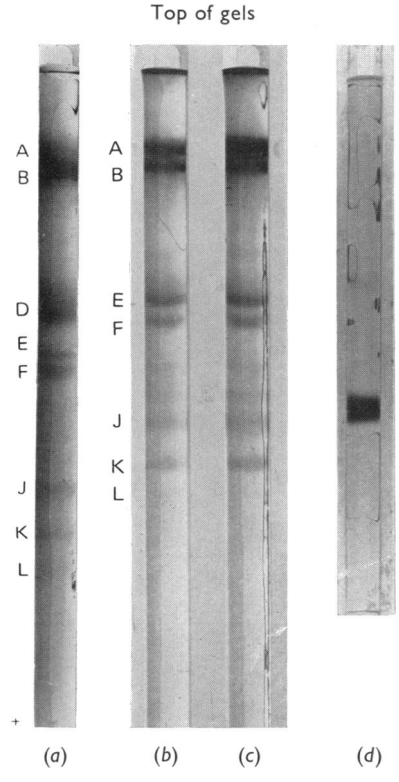
For key to peptides see Plate 3.

Peptide number	Composition
1	Lys
2	Arg
3	Val-Lys
4	Ile-Lys
5	(Thr,Gly,Val,Phe,His)Lys
6	Gly-Lys-Val-Lys
7	Gly-Lys
8	(Thr,Val,Leu)Arg
9	(Pro,Ala_2,Leu_2)Lys
10	(Thr,Gly,Ala,Met,Leu,Phe)Arg
11	(Glu,Pro,Ala,Leu,Lys_2)
12	(Glu,Gly_3,Ala_3,Leu,His)Lys
13	Asp-Gly-Arg
14	(Asp,Ser,Pro,Gly)Lys
15	(Asp,Glu,Gly,Ala)Lys
17	(Gly,Ala,Val)Lys
18	(Asp,Thr,Ser,Gly,Ala_2,Phe)Lys
19	Glu
20	Ile-Gly-Arg

centrifuged extract contained protein J as a major component. Protein L and large amounts of high-molecular-weight proteins were also solubilized (Plate 2*b*). When the extraction was done with the same solution at pH 4.7, proteins J and L were both selectively extracted (Plate 2*c*), although a trace of higher-molecular-weight material was also extracted. When the extraction was done at pH 4.8 in the absence of 2-mercaptoethanol, proteins J and L were still extracted selectively but the yield of protein J was lower. The extracts contained a large amount of non-protein material. On sodium dodecyl sulphate gels values of 38000 and 27000 were obtained for the mol.wt. of proteins J and L respectively.

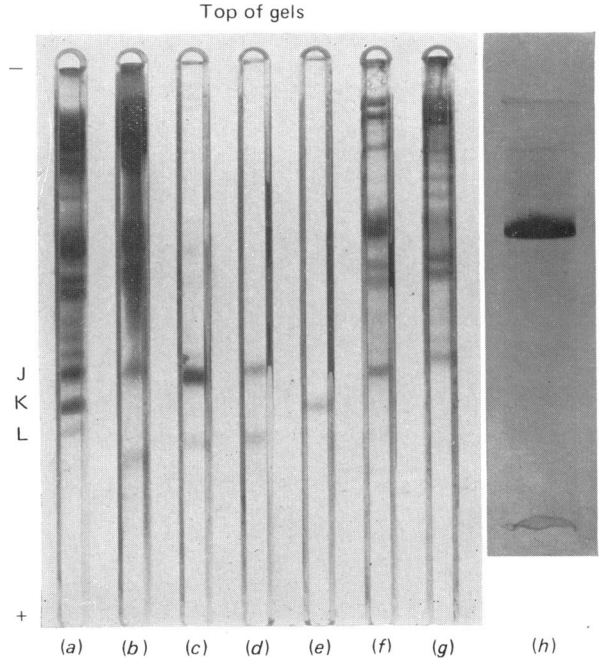
Large-scale preparation of protein K. The large-scale preparation of protein K, starting with 6 litres of packed cells, had to be done by a modified procedure. Preparation of the 'ghosts' took much longer, and they were not completely free of haemoglobin and non-'ghost' proteins. The EDTA extract of the 'ghosts' contained considerable amounts of material of molecular weights higher and lower than that of protein K. A homogeneous preparation was obtained by the following addition to the procedure.

The EDTA extract was dialysed against water, freeze-dried, and the water-soluble material was made 60% saturated with ammonium sulphate, a salt concentration at which protein K remains soluble. The 60% ammonium sulphate supernatant contained fairly pure protein K, but also contained a large amount of non-protein material. After dialysis and freeze-drying the protein became insoluble in aqueous buffers, but could be purified further by gel filtration on Sephadex G-200 in 5M-guanidine–HCl. Gel-electrophoretic analysis of the purified protein K in sodium dodecyl sulphate gels (Plate 1*d*) showed that this material was homogeneous.



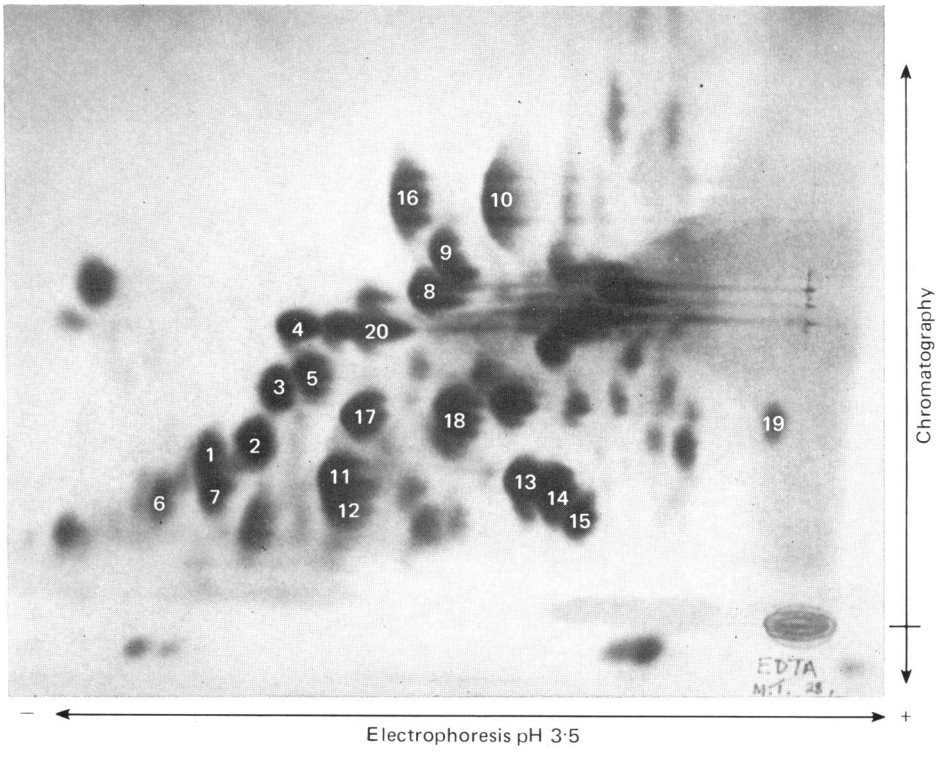
EXPLANATION OF PLATE 1

Sodium dodecyl sulphate polyacrylamide-gel electrophoresis of erythrocyte 'ghosts' and purified protein K. (*a*) Gel-electrophoresis pattern of intact erythrocyte 'ghosts' in gel system A (0.1% sodium dodecyl sulphate, pH 7.1). (*b*) Gel-electrophoresis pattern in gel system B (0.1% sodium dodecyl sulphate, 8M-urea, pH 7.1) of intact 'ghosts' from freshly obtained erythrocytes. (*c*) Gel-electrophoresis pattern in gel system B of intact 'ghosts' from erythrocytes stored for 30 days under blood-bank conditions. (*d*) Gel-electrophoresis pattern in gel system A of purified protein K. The mobility of the protein band in this gel is not directly comparable with the mobility of protein K in gel (*a*) as the gels were run on different occasions.



EXPLANATION OF PLATE 2

Gel-electrophoresis patterns of fractions obtained by selective extraction of erythrocyte 'ghosts'. (*a–g*) Sodium dodecyl sulphate gel-electrophoresis in gel system A of (*a*) intact 'ghosts'; (*b*) supernatant obtained when EDTA-extracted 'ghosts' (gel *f*) were mixed with 2 vol. of a solution containing 9M-urea, 10 mg of 2-mercaptoethanol/ml, 0.01M-tris–HCl buffer, pH 7.0, left for 1 h at 4°C and centrifuged at 35000*g* for 2 h, then dialysed and freeze-dried; (*c*) supernatant obtained when EDTA-extracted 'ghosts' (gel *f*) were mixed with 2 vol. of a solution containing 9M-urea, 10 mg of 2-mercaptoethanol/ml, 0.01M-tris–HCl buffer, pH 7.0, left at 4°C for 1 h, then titrated to pH 4.7, centrifuged at 35000*g* for 2 h, dialysed and freeze-dried; (*d*) supernatant obtained when EDTA-extracted 'ghosts' (gel *f*) were mixed with 2 vol. of a solution containing 9M-urea, 0.1M-sodium acetate buffer, pH 4.8, left for 5 min at 4°C, then centrifuged at 35000*g* for 2 h, dialysed and freeze-dried; (*e*) EDTA extract, the supernatant obtained when intact 'ghosts' were extracted with 17 vol. of cold 0.1M-EDTA, pH 7.9, left for 5 min at 4°C, then centrifuged at 30000*g* for 40 min, dialysed and freeze-dried; (*f*) EDTA-extracted 'ghosts'; pellet obtained on EDTA extraction (see details for gel *e*) of intact 'ghosts'; (*g*) pellet obtained after extraction of EDTA-extracted 'ghosts' with 6M-urea at pH 4.8 (see details for gel *d*); (*h*) gel electrophoresis in gel system C (9M-urea, pH 2.7) of EDTA extract (for method of preparation see details for gel *e*).



EXPLANATION OF PLATE 3

Tryptic peptide 'map' of protein K. The amino acid compositions of the numbered peptides are given in Table 2. For experimental details see the Materials and Methods section.

Characterization of protein K. Three separate determinations of the subunit molecular weight of protein K on sodium dodecyl sulphate gels gave values of 32000, 33000 and 32000. The amino acid composition of the protein is markedly different from that of whole 'ghosts' (Table 1), and is relatively high in aspartic acid (and/or asparagine), glycine, alanine and valine. There are about 9 arginine residues/chain based on a molecular weight of 33000. We were unable to detect any periodate-positive carbohydrate in this protein by specific staining on polyacrylamide gels by using the periodic acid–Schiff stain, under conditions that could clearly detect the carbohydrate present in the heavy chain of a γM macroglobulin. No amino sugars were found after amino acid analysis.

The *N*-terminal sequence was established by stepwise degradation for ten steps by using a 'protein sequenator' (Edman & Begg, 1967). Residues 1–9 were clearly identified as Gly-Lys-Val-Lys-Val-Gly-Val-Asn-Gly; the tenth residue was either Phe or Met.

The tryptic peptide 'map' shown in Plate 3 was obtained under conditions of rather high loading. Peptides eluted from a very lightly stained duplicate 'map' were analysed, and some of the results are given in Table 2. Not all peptides gave satisfactory analyses. Only peptide 16 contained tryptophan (detected by Ehrlich's reaction on the 'map'), but an insufficient amount was obtained by elution to give a satisfactory analysis. There was one other area stained rather diffusely by the Ehrlich's reagent.

Peptides were also produced by tryptic cleavage at arginine residues; the amount of acid liberated suggested that 9 arginyl bonds were split/mol of protein. These 'parent' peptides were chromatographed on a cation-exchange resin with their trifluoroacetyl blocking groups intact. Seven reasonably pure peptides were obtained, whereas three others (peptides A5, A9 and A10) were not obtained in a satisfactory state of purity. Peptide A5 was present as a broad streak, detected only by high-voltage electrophoresis of the hydrolysed

Table 3. *Arginine cleavage peptides obtained from aminoethylated protein K*

The results are not corrected for the destruction or slow release of any amino acids during hydrolysis. Peptide A2 was ninhydrin negative before unblocking and is the *N*-terminal peptide. The results for peptide A2 are corrected for contamination by 10 mol % of peptide A1; the results for peptide A3 are corrected for 20 mol % contamination by peptide A4. Peptide A4 is the *C*-terminal peptide, and the results are corrected for 15 mol % contamination by peptide A3. The results for peptide A5 are corrected for 20 mol % contamination by peptide A4, and the results for peptide A7 are corrected for the presence of 15 mol % of peptide A6. The results for peptides A1, A6 and A8 are not corrected for contaminants.

	Molar ratio							
Peptide ...	A1	A2	A3	A4	A5	A6	A7	A8
Asp†	2.9	1.2	2.0	1.1	1.8	1.0	0.1	—
Thr	2.0	—	1.9	—	1.0	0.1	1.0	—
Ser	1.5	—	0.8	0.8	0.8	0.1	0.1	—
Glu	3.0	—	0.1	1.1	1.0	0.1	0.1	—
Pro	2.1	—	1.1	—	—	—	—	—
Cys‡	—	—	0.9	—	—	—	—	—
Gly	4.0	3.9	0.5	0.3	6.4	1.0	0.2	0.9
Ala	5.0	0.2	1.0	1.9	2.1	0.2	1.0	0.1
Val	2.0	2.7	2.6	0.8	2.8	0.1	1.1	—
Met	0.7	0.8	—	1.8	—	—	—	—
Ile	1.8	—	0.2	—	—	0.1	—	0.9
Leu	2.6	0.1	0.9	0.9	1.0	0.1	—	0.1
Tyr	0.2	—	—	—	—	—	—	—
Phe	0.9	1.0	—	—	—	—	—	—
His	—	—	—	0.9	—	—	—	—
Lys	2.8	1.7	0.1	1.4	2.2	0.2	0.2	0.1
Arg	0.9	1.0	1.0	0.1	1.1	1.0	0.9	1.0
No. of residues	34	13	13	11–12	22	3	4	3
N-Terminal residue	—*	Gly	Val	Val	—*	Asp	Leu or Thr	Ile

* No derivative was obtained on *N*-terminal analysis, but these peptides are unlikely to have blocked *N*-termini.

† Also contains methionine sulphoxide.

‡ Estimated as *S*-aminoethylcysteine.

material. Peptides A9 and A10 are large peptides that eluted only at the high pH at the end of the gradient. They were not obtained free from each other, and probably were admixed with partially digested material. Compositions of the arginine peptides are given in Table 3, together with *N*-terminal results for some of the peptides.

Peptide A2 is clearly the *N*-terminal peptide of the protein. It was very poorly reactive toward ninhydrin when eluted from the column, and its *N*-terminus was glycine, after removal of the trifluoroacetyl blocking group. The tryptic 'fingerprint' of peptide A2 after the lysine residues were unblocked contained the 'daughter' peptides Gly-Lys and Val-Lys as well as Gly-Lys-Val-Lys, and the overall composition is compatible with the sequence established by degradation of the whole protein. It is thus Gly-Lys-Val-Lys-Val-Gly-Val-Asn-Gly(Phe,Met,Gly)Arg.

Peptide A4 contains no arginine, and is probably the *C*-terminal peptide. The tryptic 'fingerprint' of the unblocked peptide contained free glutamic acid, as did the tryptic 'map' of the whole protein. Its *C*-terminal sequence is thus ... Lys-Glu.

Identification of protein K. The *N*-terminal sequence of protein K was compared with the protein sequences that are collected in the *Atlas of Protein Sequence and Structure* (Dayhoff, 1969). It shows a striking resemblance to the *N*-terminal sequence of pig muscle D-glyceraldehyde 3-phosphate dehydrogenase (Harris & Perham, 1969). The resemblance can be seen to be more extensive when peptides A8 and A7 are added (Fig. 1). A similar close correspondence is found between the *C*-terminal peptides of both proteins (Fig. 2). The amino acid analysis of the peptide from protein K suggests that a valine residue may be deleted from this sequence. However, as the corresponding pig peptide contains a slowly hydrolysed Val-Val sequence, this conclusion requires confirmation by other methods. Several other tryptic peptides isolated from the peptide map could be assigned locations in the D-glyceraldehyde 3-phosphate dehydrogenase sequence.

The identification of protein K as erythrocyte D-glyceraldehyde 3-phosphate dehydrogenase has been confirmed by direct enzyme assay. The preparation used for protein sequence studies was unsuitable for enzyme activity measurements as the isolation procedure involved chromatography in 5M-guanidine–HCl. The distribution of D-glyceraldehyde 3-phosphate dehydrogenase activity of a small-scale preparation of 'ghosts' at the EDTA extraction step was measured (Table 4). Of the D-glyceraldehyde 3-phosphate activity, 97% was solubilized by EDTA, and the extract had a specific activity of 48units/mg of protein. Crystalline human erythrocyte D-glyceraldehyde 3-phosphate

Pig D-glyceraldehyde 3-phosphate dehydrogenase NH_2-Val-Lys-Val-Gly-Val-Asp-Gly-Phe-Gly - Arg-Ile-Gly-Arg-Leu-Val-Thr-Arg
Protein K NH_2-Gly-Lys-Val-Lys-Val-Gly-Val-Asn-Gly(Phe,Gly,Met)Arg Ile-Gly-Arg(Leu,Val,Thr)Arg
A2 A8 A7

Fig. 1. Comparison of the *N*-terminal sequences of pig muscle D-glyceraldehyde 3-phosphate dehydrogenase (Harris & Perham, 1969) and protein K.

Pig D-glyceraldehyde 3-phosphate dehydrogenase Arg-Val-Val-Asp-Leu-Met-Val-His-Met-Ala-Ser-Lys-Glu-CO_2H
Protein K Arg-Val(Asp,Leu,Met,Ala,His,Met,Ala,Ser)Lys-Glu-CO_2H

Fig. 2. Comparison of the *C*-terminal peptides of pig muscle D-glyceraldehyde 3-phosphate dehydrogenase (Harris & Perham, 1969) and protein K.

Table 4. *Distribution of enzyme activities on EDTA extraction of erythrocyte 'ghosts'*

The enzymes were assayed at 25°C, by using standard procedures. The D-glyceraldehyde 3-phosphate dehydrogenase assay mixture contained: 1.2mg of NAD^+/ml, 0.75mM-glyceraldehyde 3-phosphate, 8mM-sodium arsenate, 0.15mM-EDTA, 0.15M-triethanolamine–HCl buffer, pH 8.7. The initial rate of reduction of NAD^+ was measured. The phosphoglycerate kinase reaction mixture contained: 2mM-ATP, 30μg of D-glyceraldehyde 3-phosphate dehydrogenase/ml, 0.15mM-NADH, 6mM-3-phosphoglycerate, 20mM-$MgCl_2$, 50mM-KCl, 0.1M-triethanolamine–HCl buffer, pH 7.4. The initial rate of oxidation of NADH was measured. The adenylate kinase reaction mixture contained: 2mM-ATP, 2mM-AMP, 30μg of pyruvate kinase/ml, 30μg of lactate dehydrogenase/ml, 1.5mM-phosphoenolpyruvate, 20mM-$MgCl_2$, 50mM-KCl, 0.15mM-NADH, 0.1M-triethanolamine–HCl buffer, pH 7.4. The initial rate of oxidation of NADH was measured. Protein was determined by the biuret method (Beisenherz *et al.* 1953). A unit is the amount of enzyme that transforms substrate at the rate of 1μmol /min.

Enzyme	Specific activity (units/mg of protein) in: Intact 'ghosts'	EDTA extract	Extracted 'ghost' residue	% of enzyme solubilized by EDTA
D-Glyceraldehyde 3-phosphate dehydrogenase	2.6	48.0	0.1	97%
Phosphoglycerate kinase	0.043	0.8	0.014	78%
Adenylate kinase	0.02	0.39	0.0045	85%

dehydrogenase has been reported to have specific activities of 50units/mg of protein (Oguchi, Hashimoto, Minakami & Yoshikawa, 1966) and 64units/mg of protein (Wolny, Wolny & Baranowski, 1968). On the basis of these values 75–95% of the protein present in the EDTA extract is D-glyceraldehyde 3-phosphate dehydrogenase. On sodium dodecyl sulphate-gel electrophoresis of the EDTA extract a single band migrating in a position corresponding to protein K was obtained. The D-glyceraldehyde 3-phosphate dehydrogenase activity of intact 'ghosts' suggests that D-glyceraldehyde 3-phosphate dehydrogenase makes up 4–5% of the total membrane protein, a value that is consistent with the intensity of the protein K band on sodium dodecyl sulphate-gel electrophoresis of intact 'ghosts'. Similar measurements (Table 4) were done for two other enzymes reported to be closely associated with the erythrocyte membrane, 3-phosphoglycerate kinase (EC 2.7.2.3) and adenylate kinase (EC 2.7.4.3) (Nilsson & Ronquist, 1969). The specific activities of erythrocyte phosphoglycerate kinase and muscle adenylate kinase are 300units/mg of protein and 2000units/mg of protein respectively (Hashimoto & Yoshikawa, 1964; Noda & Kuby, 1963), showing that by contrast with D-glyceraldehyde 3-phosphate dehydrogenase these two enzymes are present at very low concentrations (less than 0.02% of the total 'ghost' protein) in our preparations of intact 'ghosts'. The substantial amount of D-glyceraldehyde 3-phosphate dehydrogenase present in the 'ghosts' suggested that the enzyme remained preferentially associated with the membrane on haemolysis of erythrocytes. Measurements of the recovery of D-glyceraldehyde 3-phosphate dehydrogenase in 'ghosts' through a typical large-scale 'ghost' preparation showed that 70% of the D-glyceraldehyde 3-phosphate dehydrogenase present in intact erythrocytes remained associated with the haemoglobin-free 'ghost' preparation.

DISCUSSION

This report describes the isolation of a protein initially characterized as a band obtained on polyacrylamide-gel electrophoresis of intact erythrocyte 'ghosts'. The protein (K) could be selectively extracted from intact 'ghosts' by 0.1M-EDTA solutions. The *N*-terminal sequence of this protein was determined and on comparison of this sequence with a collection of protein sequences a striking similarity with the *N*-terminal sequence of pig muscle D-glyceraldehyde 3-phosphate dehydrogenase was noted. The enzyme was positively identified as the erythrocyte D-glyceraldehyde 3-phosphate dehydrogenase by enzymic assay. To our knowledge this is the first instance of the functional identification of a protein by partial sequence analysis. However, as more protein sequences become available for reference, this method is likely to have increasing utility, particularly when the protein that has been isolated is denatured.

The retention of some glycolytic enzymes in erythrocyte 'ghosts' obtained from erythrocytes haemolysed under various conditions of pH and ionic strength has been reported by Mitchell, Mitchell & Hanahan (1965). These results suggest that under the conditions we use to prepare 'ghosts' D-glyceraldehyde 3-phosphate dehydrogenase would be the most strongly retained of the glycolytic enzymes, although they obtained a lower

degree of retention than we have found. However, sodium dodecyl sulphate-gel analysis of a large number of our 'ghost' preparations have reproducibly shown the presence of an estimated 5% of protein K in the membranes. Other workers (Green *et al.* 1965; Nilsson & Ronquist, 1969) have reported very firm retention of D-glyceraldehyde 3-phosphate dehydrogenase in the erythrocyte membrane, but as very different conditions were used to prepare the 'ghosts', direct comparison of their results with ours is difficult.

The large amount of D-glyceraldehyde 3-phosphate dehydrogenase found associated with 'ghosts' raises the question of whether it is an integral part of the membrane, or whether it associates strongly with the membrane under the conditions of haemolysis, this association being irrelevant to its situation in the intact cell. Mitchell *et al.* (1965) clearly showed that the content of haemoglobin and glycolytic enzymes in 'ghosts' prepared by osmotic haemolysis is extremely sensitive to the particular preparative procedure used. Because of this variability, 'ghosts' prepared by different methods, although clearly containing the membranes of the erythrocyte, cannot all be taken to represent the membrane structure accurately. The problems, partly semantic ones, resulting from the lack of a standard membrane preparation, make the link between the presence of an enzyme in a 'ghost' preparation and its situation in the membrane in the intact erythrocyte rather tenuous. Nilsson & Ronquist (1969) who used a carbon dioxide–water haemolytic procedure to prepare 'ghosts', consider that D-glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase and adenylate kinase form part of the structure of the erythrocyte membrane. Our 'ghost' preparations, which appear intact when viewed by phase-contrast microscopy, contain only trace amounts of the latter two enzymes. The 'ghosts' remain intact when viewed in the phase-contrast microscope after short exposures to 0.1M-EDTA in conditions that result in the quantitative solubilization of D-glyceraldehyde 3-phosphate dehydrogenase. It seems unlikely, therefore, that D-glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase or adenylate kinase form an integral part of the structure of the erythrocyte membrane.

The requirement for a minimum concentration of 0.1M-EDTA to obtain the maximum solubilization of D-glyceraldehyde 3-phosphate dehydrogenase suggests that, apart from any possible action of EDTA as a metal ion-chelating agent, the high ionic strength of the solution plays a part in dissociating the enzyme from the 'ghosts'. This would be consistent with the report of Green *et al.* (1965) who found that D-glyceraldehyde 3-phosphate dehydrogenase could be readily extracted from erythrocyte 'ghosts' by buffered saline solutions, and suggests that at salt concentrations comparable with that found in the intact erythrocyte D-glyceraldehyde 3-phosphate dehydrogenase shows little tendency to aggregate with the membrane, whereas at the low salt concentrations used for preparing 'ghosts' the enzyme is able to bind to the membrane by ionic interactions.

Although we have shown in the present study that it is possible to isolate proteins J and L on a small scale by selective extraction with urea at an acid pH after EDTA extraction, attempts to separate these proteins on a large scale by this procedure have been unsatisfactory. When analysed by polyacrylamide-gel electrophoresis the products have shown evidence of extensive proteolysis. Preparations of protein J that run as a single band on sodium dodecyl sulphate-gel electrophoresis yielded complex tryptic peptide 'maps' indicating that a large number of polypeptide chains were present in the preparation.

The stability of the membranes of intact erythrocytes under the conditions of storage in a blood-bank is not unexpected. Isolated 'ghosts', on the other hand, even if stored frozen at −20°C, underwent extensive proteolysis. There are proteases and peptidases in erythrocytes (Bishop, 1964; Lewis & Harris, 1967; Morrison & Neurath, 1953), and at least one protease is found associated with erythrocyte 'ghosts' and shows activity towards erythrocyte membrane protein (Moore, Kocholaty, Cooper, Gray & Robinson, 1970). It would seem that although these enzymes remain latent in the intact erythrocyte the haemolytic lesion occurring at the membrane during the process of making 'ghosts' activates them and leads to proteolysis of the membrane. This phenomenon may be connected with the mechanism of destruction of old or damaged erythrocytes *in vivo*.

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REFERENCES

Bakerman, S. & Wasemiller, G. (1967). *Biochemistry, Easton*, **6**, 1100.

Beisenherz, G., Boltze, H. J., Bucher, T., Czok, R., Garbade, K. A., Meyer-Arendt, E. & Pfleiderer, G. (1953). *Z. Naturf.* **8b**, 555.

Bishop, C. (1964). In *The Red Blood Cell*, p. 148. Ed. by Bishop, C. & Surgenor, D. New York: Academic Press Inc.

Blumenfeld, D. O., Gallop, P. M., Howe, C. & Lee, L. T. (1970). *Biochim. biophys. Acta*, **211**, 109.

Cole, R. D. (1967). In *Methods in Enzymology*, vol. 11, p. 315. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Dayhoff, M. D. (1969). *Atlas of Protein Sequence and Structure.* Silver Spring, Md.: National Biomedical Research Foundation.

Dodge, J. T., Mitchell, C. & Hanahan, D. J. (1963). *Archs Biochem. Biophys.* **100**, 119.

Dreyer, W. J. & Bynum, E. (1967). In *Methods in Enzymology*, vol. 11, p. 32. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Edman, P. & Begg, G. (1967). *Eur. J. Biochem.* **1**, 80.

Goldberger, R. F. (1967). In *Methods in Enzymology*, vol. 11, p. 317. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Gray, W. R. (1967). In *Methods in Enzymology*, vol. 11, p. 139. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Green, D. E., Murer, E., Hultin, H. O., Richardson, S. H., Salman, B., Brierley, G. P. & Baum, H. (1965). *Archs Biochem. Biophys.* **112**, 635.

Harris, J. I. & Perham, R. N. (1969). *Nature, Lond.*, **219**, 1025.

Hashimoto, T. & Yoshikawa, H. (1964). *J. Biochem., Tokyo*, **56**, 279.

Katz, A. M., Dreyer, W. J. & Anfinsen, C. B. (1959). *J. biol. Chem.* **234**, 2897.

Lenard, J. (1970). *Biochemistry, Easton*, **9**, 1129.

Lewis, W. H. P. & Harris, H. (1967). *Nature, Lond.*, **215**, 351.

Maddy, A. H. & Kelly, P. G. (1970). *FEBS Lett.* **8**, 1970.

Marchesi, S. L., Steers, E., Marchesi, V. T. & Tillack, T. W. (1970). *Biochemistry, Easton*, **9**, 50.

Mitchell, C. D., Mitchell, W. B. & Hanahan, D. J. (1965). *Biochim. biophys. Acta*, **104**, 348.

Moore, G. L., Kocholaty, W. F., Cooper, D. A., Gray, J. L. & Robinson, S. L. (1970). *Biochim. biophys. Acta*, **212**, 126.

Morrison, W. L. & Neurath, H. (1953). *J. biol. Chem.* **200**, 39.

Neville, D. M. (1967). *Biochim. biophys. Acta*, **133**, 168.

Nilsson, O. & Ronquist, G. (1969). *Biochim. biophys. Acta*, **183**, 1.

Noda, L. & Kuby, S. A. (1963). In *Methods in Enzymology*, vol. 6, p. 223. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Oguchi, M., Hashimoto, T., Minakami, S. & Yoshikawa, H. (1966). *J. Biochem., Tokyo*, **60**, 99.

Rosenberg, S. A. & Guidotti, G. (1969). *J. biol. Chem.* **244**, 5118.

Schneiderman, L. J. & Junga, J. G. (1968). *Biochemistry, Easton*, **7**, 2281.

Schroeder, W. A., Jones, R. T., Cormick, J. & McCalla, K. (1962). *Analyt. Chem.* **34**, 1570.

Shapiro, A. L., Vinuela, E. & Maizel, J. V. (1967). *Biochem. biophys. Res. Commun.* **28**, 815.

Wolny, M., Wolny, E. & Baranowski, T. (1968). *Bull. Acad. pol. Sci.* **16**, 13.

Zacharias, R. J., Zell, T. E., Morrison, J. H. & Woodcock, J. J. (1969). *Analyt. Biochem.* **30**, 148.