Biochem. J. (1972) **129**, 333–347
Printed in Great Britain

Separation and Some Properties of the Major Proteins of the Human Erythrocyte Membrane

By M. J. A. TANNER and D. H. BOXER
Molecular Enzymology Laboratory, Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

(*Received* 9 *February* 1972)

A fractionation procedure is described which allows the isolation of three major human erythrocyte membrane proteins. Their isolation involves three sequential extraction procedures followed by gel filtration in 1% sodium dodecyl sulphate and preparative gel electrophoresis. All three proteins can be isolated from a single preparation. One of the proteins is the erythrocyte sialoglycoprotein, for which no *C*- or *N*-terminal residues were found. The other two proteins, which have not previously been isolated, have subunit molecular weights of 74000 and 93000 and contain 9 and 7% carbohydrate respectively. These glycoproteins have blocked *N*-terminal residues and show similarities in their chemical properties. Preparations derived from blood-group O erythrocytes contain no *N*-acetylgalactosamine, but similar preparations from blood-group A erythrocytes do contain this sugar. These three proteins cannot easily be solubilized by gentle aqueous procedures and represent about half of the erythrocyte 'ghost' protein. They carry a large proportion of the cell-surface carbohydrate.

Although the proteins present in the erythrocyte membrane have been the subject of many reports over a long period, a clear picture of the properties of these proteins and their place in the organization of the erythrocyte membrane is only just beginning to emerge (Bretscher, 1971*b*,*c*; Phillips & Morrison, 1971*a*,*b*; Steck *et al.*, 1971). Most of these recent studies have been carried out on microgram amounts of proteins, which have been separated by analytical sodium dodecyl sulphate gel electrophoresis. This technique, although yielding valuable results, is difficult to adapt directly to the large-scale purification of the membrane proteins that is necessary to investigate their properties adequately.

Three well-characterized protein components have been isolated from human erythrocyte 'ghosts'. Marchesi *et al.* (1970) have isolated 'spectrin', a protein containing polypeptide chains of very high subunit molecular weights and representing a large proportion of the protein found in erythrocyte 'ghosts'. 'Ghost' preparations have also been shown to contain a large amount of the glycolytic enzyme, D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) (Tanner & Gray, 1971). The relevance of these two proteins to the structure of the erythrocyte membrane is uncertain. A sialoglycoprotein that contains about 60% carbohydrate and has a relatively low subunit molecular weight (30000) has also been isolated, and has been shown to carry the human erythrocyte M,N antigenic system (Winzler, 1969; Springer *et al.*, 1966). Bretscher (1971*b*) suggests that this protein extends through the membrane structure.

Our interest has centred around the major protein components, which cannot be easily solubilized by mild aqueous treatments from the human erythrocyte membrane. Our procedure has allowed the concurrent isolation of these proteins. One of these components is the membrane sialoglycoprotein. The other two proteins also contain carbohydrate, and some of their properties are described.

Methods

General methods

Analytical methods. Total phosphate was determined by the method of Bartlett (1959) and cholesterol was measured by the procedure of Zlatkis *et al.* (1952). Sialic acid was determined by the thiobarbituric acid method (Warren, 1959). Carbohydrates were determined by a g.l.c. method (Bhatti *et al.*, 1970). Amino acid compositions were measured after hydrolysis under N_2 with constant-boiling HCl at 105°C for 20–24h on a Technicon TSM-1 amino acid analyser, with norleucine as an internal standard. Performic acid oxidation was performed as described by Hirs (1967). D-Glyceraldehyde 3-phosphate dehydrogenase activity was assayed and peptide 'maps' were obtained as described previously (Tanner & Gray, 1971).

Electron microscopy. Freeze-etching of samples was done in a Balzers type BA 360M apparatus (Balzers A. G., Liechtenstein), by the method of Moor & Muhlethaler (1963). Replicas were viewed in an A.E.I. EM6B electron microscope.

Polyacrylamide-gel electrophoresis. Samples for gel electrophoresis were dissolved in a buffer containing 5% (w/v) sodium dodecyl sulphate, 10% (v/v)

glycerol, 1% 2-mercaptoethanol, 0.01M-sodium phosphate buffer, pH7.1, and 0.01% Bromophenol Blue, and immediately heated in boiling water for 3min or more. For the gel electrophoresis of various extracted 'ghost' preparations, 2mM-phenylmethanesulphonyl fluoride was included in the buffer (Bretscher, 1971*a*). Electrophoresis was performed on gels containing 5% polyacrylamide and 0.13% *NN'*-methylenebisacrylamide, in 0.1% sodium dodecyl sulphate and 0.1M-sodium phosphate buffer, pH7.1 (Shapiro *et al.*, 1967). For more concentrated gels a 30:1 (w/w) ratio of the gel monomers was used to prepare the gel. On occasions the gels contained less than 0.1M-sodium phosphate buffer, pH7.1, as indicated. Decreasing the sodium phosphate buffer concentration to 0.03M made no detectable difference to the banding patterns observed. Electrophoresis was done at 2V/cm for 16–17h, until the Bromophenol Blue migrated to within 2–3cm of the bottom of the tube. Gels were stained either for protein with Coomassie Brilliant Blue R-250, by the method of Berg (1969), or for carbohydrate with the periodic acid–Schiff's-base stain (Zacharias *et al.*, 1969). Molecular weights were determined by the use of a marker mixture of ovotransferrin (80000), a gift from Dr. J. Williams, bovine serum albumin (67000), from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., glutamate dehydrogenase (56000), a gift from Mr. R. B. Wallis, and erythrocyte D-glyceraldehyde 3-phosphate dehydrogenase (36000), prepared as described by Tanner & Gray (1971), run under identical conditions. Mobilities were referred to D-glyceraldehyde 3-phosphate dehydrogenase, which was added to the sample if this enzyme was not already present.

Erythrocyte 'ghosts'

Preparation. Freshly out-dated blood-group O,Rh+, or blood-group A, packed human erythrocytes were obtained from the blood bank. First 15–20 blood-bank donor units of cells were individually washed once with 250ml of cold 0.15M-NaCl. The cells were pooled, made up to 6 litres with cold 0.15M-NaCl and centrifuged at 2000*g* for 20min. The supernatant and buffy coat were carefully aspirated and the procedure was repeated for a total of four washes with 0.15M-NaCl and two washes with 0.103M-Na_2HPO_4 adjusted to pH7.4 with 0.155M-NaH_2PO_4 (iso-osmotic phosphate buffer, pH7.4); these and subsequent steps generally followed the procedure of Dodge *et al.* (1963). The cells were lysed into 37 litres of a stirred solution of iso-osmotic phosphate buffer, pH7.4, diluted with 19.5vol. of deionized water (diluted phosphate buffer, pH7.4) maintained near 0°C with a cooling coil connected to a circulating refrigeration bath, and a solution of 0.3ml of di-isopropyl phosphorofluoridate in 3ml of propanol was immediately added slowly to the lysate. After being stirred at 0°C for 20min, the lysate was made up to 90 litres with cold diluted phosphate buffer, pH7.4. The solution was pumped at 6–8 litres/h through a MSE continuous-flow system operating at 22000*g*. Stirring and cooling of the lysate was continued through the centrifugation, the lysate temperature remaining in the range 0–2°C. This operation could conveniently be done overnight. The sedimented 'ghosts' were dispersed in 30–40 litres of cold diluted phosphate buffer, pH7.4, and collected in a similar manner. The 'ghosts' were finally sedimented once or twice in 250ml bottles at 20000*g* for 40min, care being taken to discard the hard red pellet that formed under the lightly packed 'ghost' layer between washes. The preparation took approx. 24h. Subsequent extraction steps were performed immediately after the preparation of the 'ghosts'.

Low-ionic-strength extraction. The procedure follows that of Marchesi *et al.* (1970). All the operations were carried out at 0–4°C. The 'ghosts' from a large-scale preparation were made up to 600ml with a solution containing 5mM-EDTA and 5mM-2-mercaptoethanol, pH8.0, and dialysed overnight against 20 litres of the same solution. The solution was made up to 2400ml with the dialysis solution, stirred briefly and centrifuged at 20000*g* for 75min. The pellet was made up to 2400ml with a solution containing 5mM-glycine, 5mM-2-mercaptoethanol and 0.1mM-EDTA, titrated to pH 9.5 with NaOH, stirred in ice for 2h, and then centrifuged at 20000*g* for 75min. The pellet (low-ionic-strength-extracted 'ghosts') was resuspended in the minimum volume of cold deionized water.

Salt extraction. The low-ionic-strength-extracted 'ghosts' were diluted to 2400ml with an ice-cold solution containing 1M-NaCl, 5mM-glycine and 0.1mM-EDTA, titrated to pH9.5 with NaOH. A solution of 0.1ml of di-isopropyl phosphorofluoridate in 2ml of propanol was immediately added to the stirred mixture in ice, and the suspension was left in ice for 20min before centrifugation at 20000*g* for 100min. The pellet was washed twice with 2400ml of cold deionized water, the material being sedimented at 20000*g* for 120min each time. The final pellet (salt-extracted 'ghosts') was resuspended in the minimum volume of cold deionized water. In earlier preparations the salt extraction was carried out overnight and the extracting solution also contained 5mM-2-mercaptoethanol.

Pyridine extraction. The salt-extracted 'ghosts' were extracted with aqueous pyridine as described by Blumenfeld *et al.* (1970). The insoluble pellet obtained after this extraction was re-extracted by the same procedure and washed in the centrifuge with cold deionized water. The sediment was made up to 500ml with deionized water and poured into 9vol. of

acetone at 4°C. The mixture was stirred at 4°C for 30min and left at 4°C overnight. The precipitate was collected by centrifugation at 2000*g* for 60min, resuspended in 4 litres of acetone, stirred for 1h and left at 4°C overnight. The precipitate was again collected in the centrifuge and the procedure repeated with a further 4 litres of acetone. Finally, the pellet was suspended in a little water, the acetone was removed on a water pump, and the material was freeze-dried; 2–4g of this material, termed the pyridine precipitate, was obtained.

The combined supernatants and washings from the pyridine extraction were concentrated to about 40ml by pressure dialysis at 4°C under N_2 through an Amicon PM-10 membrane, to yield the material referred to as the pyridine supernatant.

Gel filtration of pyridine precipitate. The lipid-extracted pyridine precipitate (2–4g) was taken up in 100–200ml of a solution containing 10% (w/v) sodium dodecyl sulphate and 1% 2-mercaptoethanol. The solution was stirred, quickly titrated to pH8.5 with 5M-NaOH and heated in boiling water for 3min. The hot solution was stirred vigorously for about 30s and then replaced in the boiling water for a further 2min. The clear solution was cooled in a water bath to room temperature, and applied to a column of Sephadex G-150 (6cm × 150cm), equilibrated with unbuffered 1% sodium dodecyl sulphate. Protein was recovered by two methods. In the first (used for the fractionation described in Fig. 3), the samples were cooled to 8°C in a constant-temperature bath overnight. The precipitated sodium dodecyl sulphate was removed by centrifugation. The protein was precipitated by the addition of 10vol. of acetone and left overnight at 4°C. The protein was collected by centrifugation, mixed with a small volume of water and the acetone treatment was repeated before freeze-drying of the final precipitate. This method gave variable yields and the product tended to have a low protein content (typically about 25% dry wt.). Later preparations used a modified procedure, as follows. The fractions were dialysed for 24h against several changes of 8 litres of water. The diffusate was concentrated about 10-fold on the freeze-drier and dialysed for a further 24h against several changes of 8 litres of water, and the diffusate was treated twice with acetone as described above. This product contained about 70% dry wt. of protein.

Gel filtration of the pyridine supernatant. The supernatant (40ml) was made 5% in sodium dodecyl sulphate by addition of the solid, and 1% in 2-mercaptoethanol, and the pH was titrated, if necessary, to pH7.2–7.3 with 5M-NaOH. The resulting solution was heated at 50°C for 10min to ensure complete solubilization, and applied to a Sephadex G-150 column (4cm × 100cm) equilibrated with 1% sodium dodecyl sulphate. Protein was recovered as described above, except that the sialoglycoprotein-containing fraction was allowed to precipitate by standing in acetone at −20°C for 2 days.

Proteins

N-Terminal analysis. This was done on 3mg of the sialoglycoprotein and 7mg of the other proteins by the cyanate method of Stark (1967). The proteins were carbamoylated in the presence of 0.5% sodium dodecyl sulphate.

Chloroform–methanol extraction. Approx. 10–15mg of protein was suspended in 0.5ml of water. To this 17vol. of chloroform–methanol (2:1, v/v) was added and the mixture was homogenized for 3min in a Potter homogenizer, and left for 1h. The precipitate was sedimented in a bench centrifuge and the supernatant was carefully removed. The precipitate was resuspended in 15vol. of chloroform–methanol (2:1, v/v), left for 30min and the protein was again sedimented. The pellet was mixed with a little water and freeze-dried.

Butanol extraction. Protein (6mg) was dispersed in 0.6ml of water by brief sonication in ice with a Dawe Soniprobe type 1130A, to give a fine suspension. The suspension was extracted three times with 0.3ml of butan-1-ol, only the butanol layer (excluding the interface) being removed each time. The combined butanol layers were pooled and evaporated to dryness under vacuum.

Further purification of the sialoglycoprotein fraction. About 300mg of the freeze-dried sialoglycoprotein fraction was suspended in 30ml of cold deionized water, and 15ml of ice-cold pyridine was added with constant stirring. The mixture was dialysed against 10vol. of deionized water at 4°C and then centrifuged at 100000*g* for 2h. A large pellet was obtained. Ethanol precipitation of the supernatant was carried out as described by Zvilichovsky *et al.* (1971). A precipitate was obtained only in the second ethanol-precipitation step; 45mg dry wt. of the final dialysed and freeze-dried ethanol supernatant was obtained.

Reduction and S-aminoethylation. The protein, in a solution containing 5% (w/v) sodium dodecyl sulphate, 6M-urea, 0.1mM-EDTA and 1M-tris–HCl, pH8.6, was treated with ethyleneimine (Cole, 1967), after prior reduction with dithiothreitol under N_2 for 1h at 37°C. After the reaction, the protein was dialysed at 4°C for 6h against water, and precipitated by the addition of 10vol. of acetone in the cold. The precipitate was mixed with a little water and the protein was reprecipitated as described above.

Purification of protein E and protein F by preparative gel electrophoresis. The small amounts of protein that could be run on the gel and the dilution incurred on collecting the protein from the end of the gel caused difficulties in the detection of the protein in the eluate. Protein F usually gave a peak of absorbancy at 280nm. Protein E was found in the region after the

peak for protein F and often gave no detectable peak absorbing at 280nm. Protein was detected by pooling the eluate around and after the protein F peak into fractions, concentrating them by freeze-drying and then using analytical gel electrophoresis to determine the location of the two proteins. Pure proteins were obtained in the leading and trailing edges of the zone of eluted protein but recoveries of the pure proteins were low.

A portion (7mg; based on amino acid analysis) of the mixture of proteins (P3) was run on a Quickfit preparative gel-electrophoresis apparatus at 4V/cm for 48h. The protein was fractionated at 25°C on a 6% polyacrylamide gel (7.5cm long, 13cm² area) in 0.1% sodium dodecyl sulphate, 0.1M-sodium phosphate buffer, pH7.1. The fractions (7ml) were pooled, dialysed, concentrated by freeze-drying, mixed with 10 vol. of acetone and left at −20°C overnight. The precipitate was collected in the centrifuge, mixed with a small volume of water and the acetone precipitation was repeated. This gave 2.7mg (by amino acid analysis) of protein F. Samples of pure protein E and pure protein F were also obtained on the gel-electrophoresis apparatus of Brownstone (1969). A portion (200mg dry wt.) of a mixture of proteins E and F (containing an unknown amount of detergent) was applied to a 6% polyacrylamide gel (5cm length, 67cm² area) and run at 8V/cm for 36h. The gel contained 0.5% sodium dodecyl sulphate and 0.03M-sodium phosphate buffer, pH7.1. Of the recovered protein 90% remained on the surface of the gel at the conclusion of the run. The protein fractions were prepared as described above. This gave 1.3mg of protein F and 1.4mg of protein E (both values based on amino acid analysis).

Results

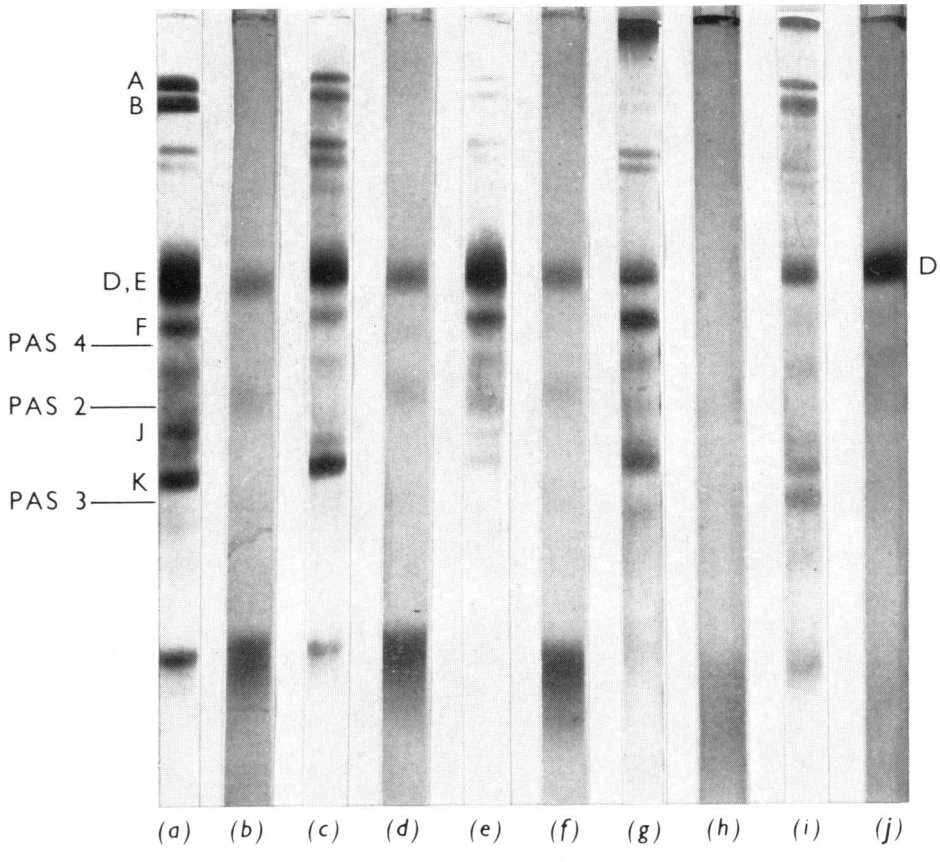
Large-scale fractionation of erythrocyte 'ghost' proteins

A convenient preliminary fractionation of the erythrocyte 'ghost' proteins may be effected by the sequential application of three selective extraction procedures (Scheme 1). Polyacrylamide-gel analysis of the stages in this procedure is shown in Plate 1. The first step, the procedure of Marchesi *et al.* (1970), selectively removes spectrin (bands A and B) and protein J; the second, extraction with solutions containing 1M-NaCl, primarily solubilizes the glycolytic enzyme D-glyceraldehyde 3-phosphate dehydrogenase (band K), and subsequent extraction with pyridine (Blumenfeld *et al.*, 1970) separates the sialoglycoprotein (D) from the two remaining major membrane protein components (E and F).

Measurements of D-glyceraldehyde 3-phosphate dehydrogenase activity indicated that 92% of the enzyme was solubilized on salt extraction. (No activity was solubilized by low-ionic-strength extraction.) Earlier preparations used a longer (overnight) salt-extraction procedure, which removed slightly more (96%) of the D-glyceraldehyde 3-phosphate dehydrogenase activity. Gels of salt-extracted 'ghosts' obtained by the extended extraction showed the additional presence of a diffuse zone of protein-staining material in the molecular-weight range 60000–40000, which we attribute to proteolysis. However, this has no effect on the subsequent purification of the proteins.

Treatment of the gels with the periodic acid–Schiff's-base carbohydrate stain showed that all the periodate-sensitive bands that are present in intact 'ghosts' are retained in the salt-extracted 'ghost' preparation (Plate 1*b,d,f*). The apparent molecular weights of these components on 5% and 10% polyacrylamide gels are shown in Table 1. The slow-moving major periodate-positive band is due to the sialoglycoprotein (Bender *et al.*, 1971; Bretscher, 1971*b*) and on 5% polyacrylamide gels its mobility is identical with that of the protein-staining band D,E (Plate 1). It has an increased relative mobility on 10% polyacrylamide gels. This anomalous mobility has been previously reported (Bretscher, 1971*b*). Faintly staining bands PAS 2 and PAS 3 have been reported (Fairbanks *et al.*, 1971) and we use the nomenclature of that report for these bands. However, we also detected another faintly staining band, PAS 4. (Bands PAS 3 and PAS 4 stain very faintly and do not show in Plate 1.) Bands PAS 2 and PAS 4 also show anomalous mobilities on 5 and 10% polyacrylamide gels. We cannot definitely assign these minor periodate-positive bands to corresponding protein-staining material. A further periodate-positive band, due to glycolipid, is found near the marker dye (Lenard, 1970) and this is also retained with the salt-extracted 'ghosts'. Pyridine treatment of the salt-extracted 'ghosts' results in the solubilization of all the periodate-positive material except for the glycolipids.

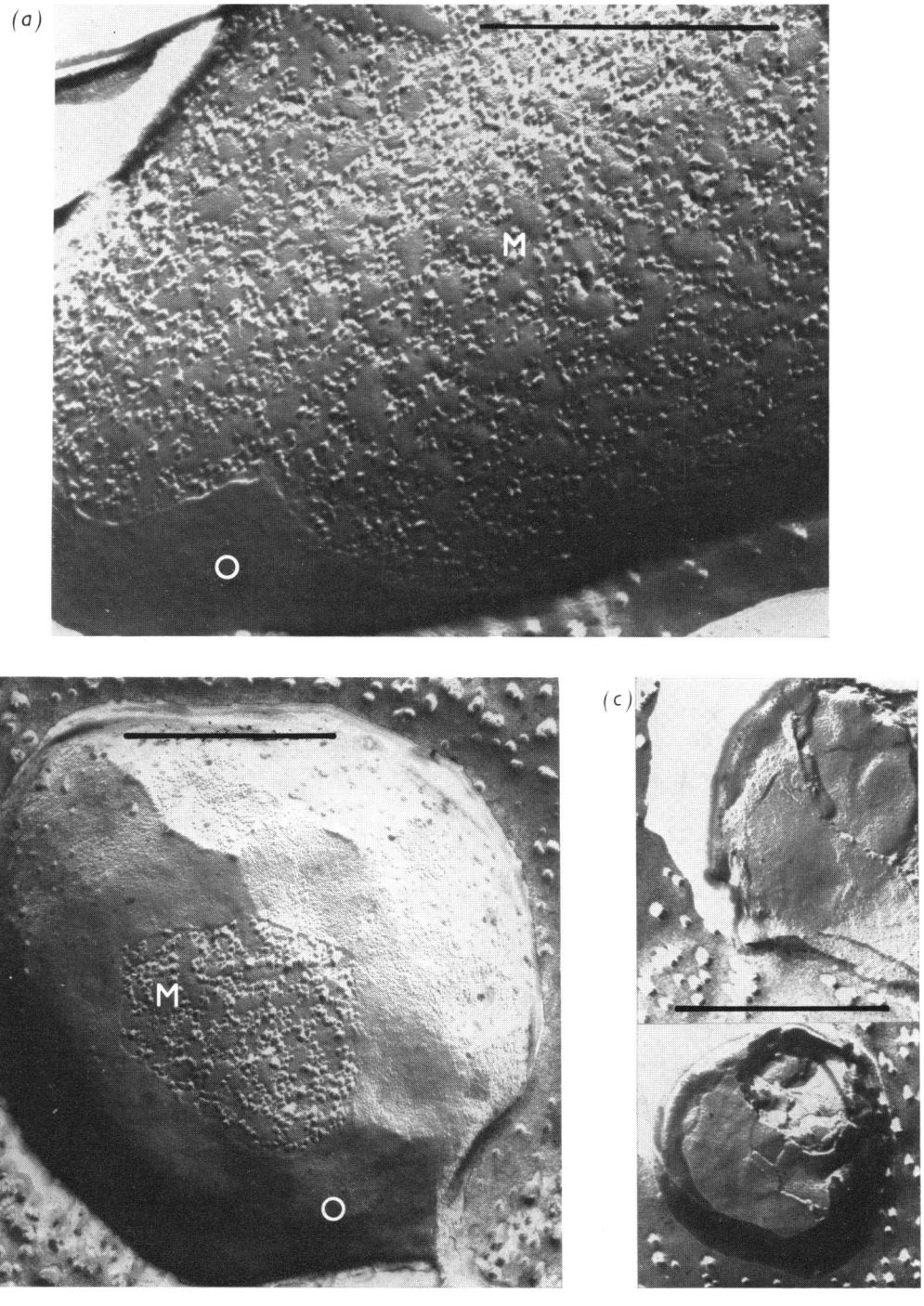
Polyacrylamide gels of the pyridine precipitate stained for protein show the presence of bands E and F and of highly aggregated material that only just enters the top of the gel. Residual D-glyceraldehyde 3-phosphate dehydrogenase is also present. When the overnight salt-extraction procedure was used the diffuse protein-staining band obtained in gels of salt-extracted 'ghosts' did not appear in the gels of either the pyridine precipitate or pyridine supernatant (Plate 3*a*). We presume that it forms part of the highly aggregated material that fails to migrate into the gel of the pyridine precipitate sample. This effect of pyridine treatment has proved to be useful, as it allows the purification of proteins E and F by gel filtration even if limited proteolysis occurs during the preparation.



EXPLANATION OF PLATE 1

Gel electrophoresis of fractions obtained during preliminary separation

The bands staining with Coomassie Blue are denoted by A–K. PAS 2, PAS 3 and PAS 4 denote the positions of the minor bands obtained with the periodic acid–Schiff's-base stain. The major carbohydrate-staining band is identical with band D. The fractions were prepared as described in the Methods section by using the short salt-extraction procedure, mixed with 2 vol. of gel sample buffer, heated in boiling water for 2min, and stored at −20°C until applied to the gel. Electrophoresis was in 5% polyacrylamide gels containing 0.1% sodium dodecyl sulphate and 0.03 M-sodium phosphate buffer, pH7.1. (*a*) Intact erythrocyte 'ghosts' (12μl), Coomassie Blue stain; (*b*) intact erythrocyte 'ghosts' (15μl), periodic acid–Schiff's-base stain; (*c*) low-ionic-strength-extracted 'ghosts' (15μl), Coomassie Blue stain; (*d*) low-ionic-strength-extracted 'ghosts' (20μl), periodic acid–Schiff's-base stain; (*e*) salt-extracted 'ghosts' (15μl), Coomassie Blue stain; (*f*) salt-extracted 'ghosts' (20μl), periodic acid–Schiff's-base stain; (*g*) pyridine precipitate (25μl), Coomassie Blue stain; (*h*) pyridine precipitate (30μl), periodic acid–Schiff's-base stain; (*i*) pyridine supernatant (20μl), Coomassie Blue stain; (*j*) pyridine supernatant (30μl), periodic acid–Schiff's-base stain.

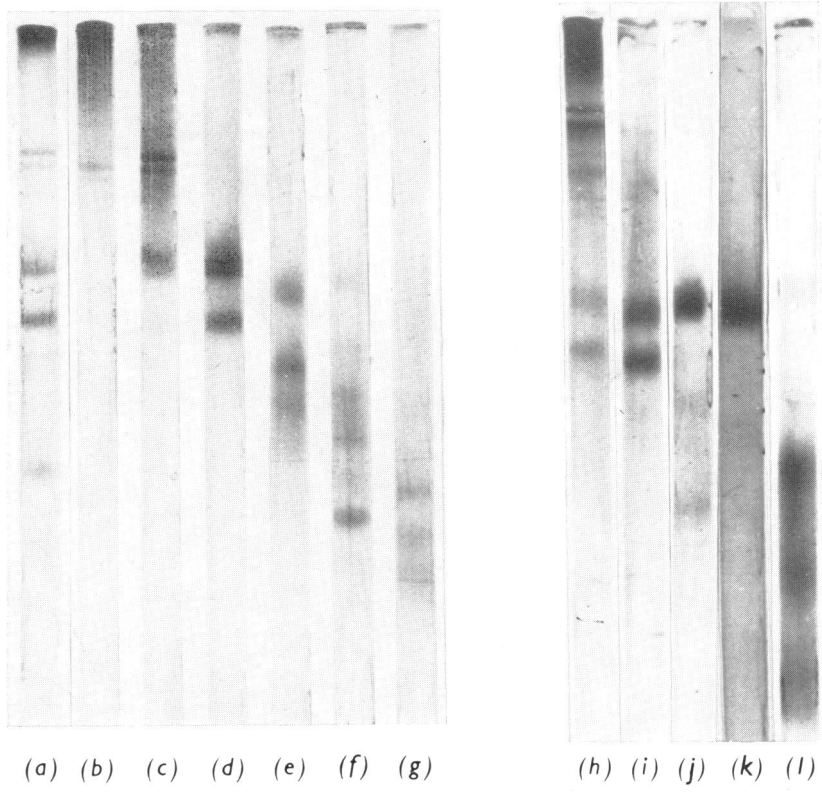


EXPLANATION OF PLATE 2

Replicas of freeze-etched erythrocyte 'ghost' fractions

The fractions were prepared as described in the Methods section by using overnight salt extraction. In (*a*) and (*b*), the outside surface of the membrane (O), and cleavage through the interior of the membrane with the characteristic particulate structure (M), are indicated. (*a*) Intact erythrocyte 'ghosts'; (*b*) low-ionic-strength-extracted 'ghosts'; (*c*) salt-extracted 'ghosts'. Two typical fragments from different portions of the replica are shown. The calibration bars represent 0.5 μm.

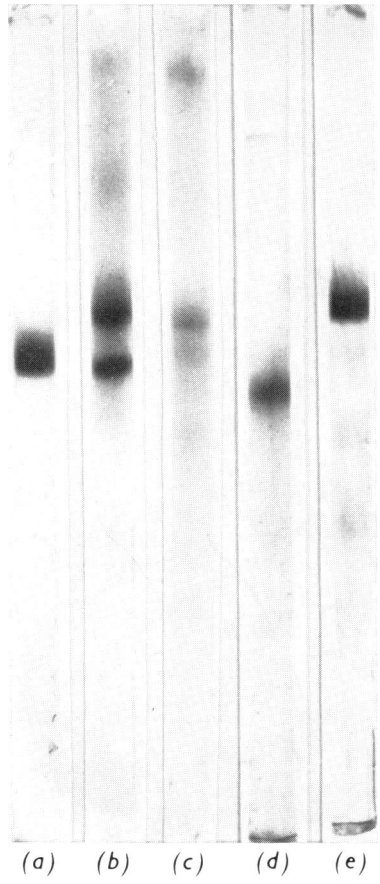
M. J. A. TANNER AND D. H. BOXER



EXPLANATION OF PLATE 3

Analytical gel electrophoresis of pyridine precipitate and pyridine supernatant column fractions

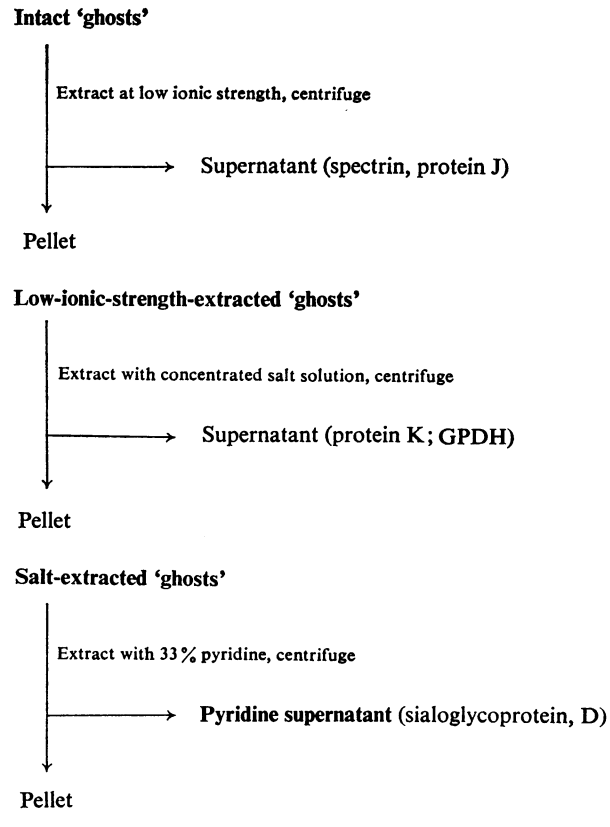
(*a*)–(*g*) Electrophoresis was done in 5% polyacrylamide gels containing 0.1% sodium dodecyl sulphate and 0.1 M-sodium phosphate buffer, pH 7.1. These gels monitor the column fractions obtained by chromatography of the pyridine precipitate (the same sample as that used for Fig. 2) and the fractions refer to those of Fig. 2. Each gel was stained with Coomassie Blue. (*a*) Unfractionated acetone-extracted pyridine precipitate (60 μg dry wt.); (*b*) fraction P1; (*c*) fraction P2; (*d*) fraction P3; (*e*) fraction P4; (*f*) fraction P5; (*g*) fraction P6. Samples (*b–f*) contained 5 μg of protein and sample (*g*) contained 12 μg of protein. For gels (*h*)–(*l*) electrophoresis was done as described above except that 0.03 M-sodium phosphate buffer was used. The gels refer to the chromatography of the pyridine supernatant and are of the samples obtained in Fig. 1. The gels were stained with Coomassie Blue except where indicated; 50 μg dry wt. of sample was applied to the gels. (*h*) Fraction S1; (*i*) fraction S2; (*j*) fraction S3; (*k*) fraction S3, periodic acid–Schiff's-base stain; (*l*) fraction S4.



EXPLANATION OF PLATE 4

Gel electrophoresis of purified proteins

Electrophoresis was done in 5% polyacrylamide gels containing 0.1% sodium dodecyl sulphate and the phosphate buffer indicated. The gels were stained with Coomassie Blue. The mobilities of the bands in the different gels are not directly comparable as they were run on different occasions. (*a*) Purified sialoglycoprotein (after additional ethanol procedure; 60μg dry wt.), electrophoresis in 0.03M-sodium phosphate buffer, pH7.1; (*b*) mixture of proteins E and F (fraction P3 from the separation described in Fig. 2) after reduction and *S*-aminoethylation (12μg of protein), electrophoresis in 0.1M-sodium phosphate buffer, pH7.1; (*c*) mixture of proteins E and F (fraction P3 from the separation described in Fig. 2) after performic acid oxidation (20μg of protein), electrophoresis in 0.1M-sodium phosphate buffer, pH7.1; (*d*) purified protein F (13μg of protein), electrophoresis in 0.03M-sodium phosphate buffer, pH 7.1; (*e*) purified protein E (30μg of protein), electrophoresis in 0.1M-phosphate buffer, pH7.1.



Scheme 1. *Scheme of preliminary fractionation procedure*

The nomenclature of individual protein bands is shown in Plate 1. GPDH, D-glyceraldehyde 3-phosphate dehydrogenase. For experimental details see the Methods section.

Composition of extracted 'ghost' fractions

Analytical results for the various 'ghost' fractions obtained during the preliminary fractionation of a typical large-scale preparation are given in Table 2. Up to the salt-extracted-'ghost' stage essentially only protein is solubilized, this preparation retaining about 80% of the lipid phosphorus, cholesterol and sugars initially present in the intact 'ghost'. Handling losses probably account for the small proportion of the latter group of components not recovered at this stage.

Appreciable amounts of carbohydrate are solubilized by pyridine, the lipids being retained in the precipitate. Individual sugars show significant differences in their distribution on pyridine extraction. Some 80% of the sialic acid and 50% of the *N*-acetylgalactosamine enter the supernatant, whereas *N*-acetylglucosamine and the neutral sugars predominantly remain in the precipitate. These results are in general agreement with those of Blumenfeld (1968), who used this procedure on intact 'ghosts'.

Freeze-etch electron microscopy of extracted 'ghosts'

Replicas obtained after freeze-etching of intact 'ghosts' show cleavages, which reveal characteristic particles that are believed to lie within the interior of the membrane (Pinto da Silva & Branton, 1970; Tillack & Marchesi, 1970). Replicas of our intact 'ghost' preparation showed a particle-size range of 110–140Å (0.011–0.014μm) and a particle concentration of $2100\pm30\%/\mu m^2$ in fractures which exposed the adjacent outer surface of the membrane on subsequent etching (Plate 2*a*). Low-ionic-strength-extracted 'ghosts' (Plate 2*b*) yielded corresponding cleavages, which revealed particles with a similar size range and concentration as the intact 'ghosts'.

Vesicularization of the 'ghosts' occurs during the low-ionic-strength extraction. Replicas of salt-extracted 'ghosts' did not show any cleavages that revealed a particulate structure and the fragments appeared to be further decreased in size (Plate 2*c*). We interpret these results as showing that the low-ionic-strength-extracted 'ghost' preparation retains the internal membrane structure of intact 'ghosts' as revealed by freeze-etching. The replicas obtained from salt-extracted 'ghosts' are difficult to interpret but some change appears to have occurred in the membrane structure on salt extraction.

Fractionation of pyridine supernatant

The pyridine supernatant can be fractionated by gel filtration on Sephadex G-150 in 1% sodium dodecyl sulphate (Fig. 1). A lower-molecular-weight shoulder is always found on the large sialic acid peak (S3): 56% of the dry weight and 60% of the protein recovered from the column was associated with peak S3. The high absorption at 280nm of peak S5 is probably due to residual pyridine present in the sample.

The 5% polyacrylamide gels of the column fractions stained for both protein and carbohydrate are shown in Plate 3(*h–l*). Fractions S1 and S2 contain high-molecular-weight material and contaminating proteins E and F. A very faint periodate-positive band with a mobility similar to that of protein E was also present in these fractions and corresponds to the small highest-molecular-weight peak of sialic acid (Fig. 1). This is probably due to a small amount of sialoglycoprotein, which is aggregated under the gel-filtration conditions. Fraction S3 contains the sialoglycoprotein and traces of component PAS 2. The latter is predominantly found in fraction S4 and is responsible for the sialic acid-containing shoulder to the main peak. The protein-stained gels of fraction S3 under normal loading give only a single band; however, on extremely high loading a lower-molecular-weight smear in a position similar to that of component PAS 2 is detectable. The gels in Plate 3 show that proteins E and F are clearly separated from the sialoglycoprotein by gel filtration; therefore these

Table 1. *Molecular weights of protein bands in gels of different concentrations of polyacrylamide*

For experimental details see the text.

Band	Apparent mol.wt. 5% gel with polyacrylamide	Apparent mol.wt. 10% gel with polyacrylamide
E	93000	92000
F	74000	75000
Sialoglycoprotein (D)	95000	70000
PAS 4	71000	56000
PAS 2	53000	40000
PAS 3	34000	32000

Table 2. *Composition of fractions obtained in preliminary separation*

The samples were derived from a preparation starting with 1200ml of packed, washed, blood-group O,Rh+ erythrocytes. For details of the preparation, which used overnight salt extraction, and for the analytical procedures used, see the Methods section. Sialic acid was measured by the method of Warren (1959). The phospholipid value is based on total phosphate and assumes that the mean molecular weight of the erythrocyte phospholipids is 700.

	Dry wt. (% of dry wt. of intact 'ghosts')	Composition (% of total present in intact 'ghosts') Salt-extracted 'ghosts'	Pyridine precipitate	Pyridine supernatant
Protein	42.5	49	36	7
Phospholipid	24.8	78	73	2.3
Cholesterol	12.1	83	60	2.5
Fucose	0.3	75	56	14
Mannose	0.5	79	30	19
Glucose	0.6	68	44	6
Galactose	2.9	83	35	16
GlcNAc	2.4	86	45	12
GalNAc	1.7	92	27	30
Sialic acid	1.9	88	12.5	45
Total dry wt. (mg)	4340	66	47	5

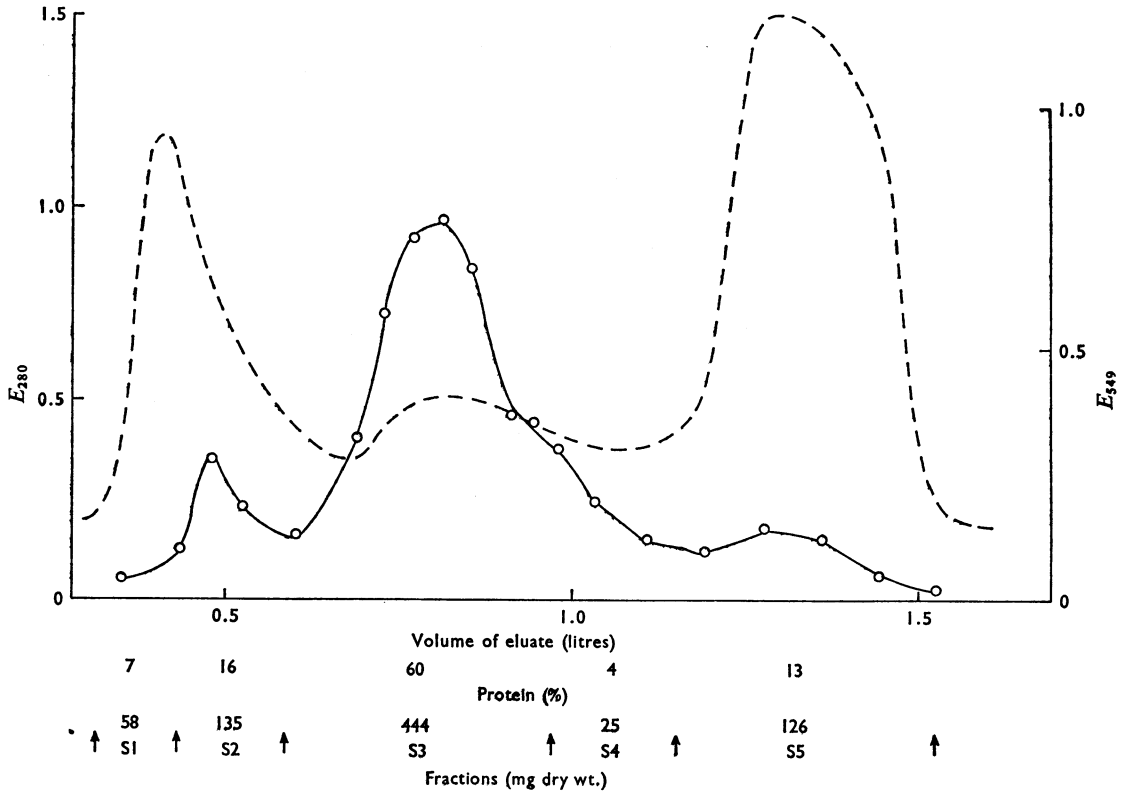


Fig. 1. *Gel filtration of the pyridine supernatant*

The pyridine supernatant, obtained from 18 blood-bank donor units of blood-group O erythrocytes, was applied to a Sephadex G-150 column equilibrated with 1% sodium dodecyl sulphate. Selected fractions were assayed for sialic acids by the method of Warren (1959). Tubes were pooled as indicated in the figure and the protein was recovered as described in the Methods section. The protein was determined by amino acid analysis after acid hydrolysis, and is expressed for each fraction as a percentage of the total protein recovered from the column. ——, E_{549}; – – – –, E_{280}.

proteins must have appreciably higher molecular weights than the sialoglycoprotein.

Characterization of the sialoglycoprotein

Fraction S3 (Fig. 1) was used to characterize the sialoglycoprotein further. Gel electrophoresis on 5 and 10% polyacrylamide gels of a mixture of the purified protein and intact 'ghosts' showed that the purified protein had a mobility identical with the major periodate-positive band present in intact 'ghosts'. The sample contained small amounts of cholesterol (0.4% dry wt.) and phospholipid (0.4% dry wt.). On haemagglutination assay a 30μg/ml solution of the protein inhibited three haemagglutinating doses of anti-(blood-group M) serum and a 500μg/ml solution of the protein inhibited a similar amount of anti-(blood-group N) serum.

C-Terminal analysis with carboxypeptidases A and B in the presence of 1% sodium dodecyl sulphate and the hydrazinolysis technique (Braun & Schroeder, 1967) failed to yield enough of any amino acid to account for the *C*-terminus of the protein. *N*-Terminal analysis, by the cyanate method, of the protein that had been extracted with chloroform–methanol (2:1, v/v) gave low yields of all the amino acids. Serine and leucine, the most significant, together accounted for 0.12mol/10000g of protein. If the lipid extraction is not done then a serine value approximately three times as high is obtained, presumably derived from serine lipids in the preparation. Assuming a molecular weight of 30000 (Morawiecki,

1964) and a 46.3% protein content, the molecular weight of the polypeptide chain can be estimated at 13900. The protein probably has a blocked *N*-terminus. All traces of component PAS 2 could be removed from the column fraction S3 by ethanol extraction (Zvilichovsky *et al.*, 1971). The material thus purified was entirely free of any other components, as shown by gel analysis run at a very high loading, stained for both protein (Plate 4*a*) and carbohydrate. Analytical results for the sialoglycoprotein are summarized in Table 3. The freely water-soluble protein contains very large amounts of serine and threonine, but no cystine or cysteine. Titration with 5,5′-dithiobis-(2-nitrobenzoic acid) in the presence of 10M-urea confirmed the absence of any free thiol groups. The proportion of hydrophobic amino acids in the protein is not unusually high. Sialic acids, galactose and *N*-acetylgalactosamine predominate among the carbohydrates, which account for 53.7% of the molecule. These analytical results, and in particular the carbohydrate composition, are in general agreement with results obtained by other workers (Winzler, 1969; Springer *et al.*, 1966) for the erythrocyte blood-group M,N sialoglycoprotein. Chromatography in 1% sodium dodecyl sulphate on a column of Sephadex G-150, which had been calibrated with proteins of known molecular weight, yielded an apparent subunit molecular weight of 46000 for the protein.

Fractionation of the pyridine precipitate

It was necessary to delipidate the pyridine precipitate with acetone to obtain the concentrated solutions in sodium dodecyl sulphate necessary for preparative separations by gel filtration. The extracted pyridine precipitate was fractionated by gel filtration on Sephadex G-150 in 1% sodium dodecyl sulphate (Fig. 2). Sodium dodecyl sulphate gel electrophoretograms of fractions P1 to P6 and of the material applied to the column are shown in Plate 3(*a–g*).

The excluded peak (P1) contains the aggregated

Table 3. *Analysis of sialoglycoprotein*

Amino acid analyses were carried out on non-oxidized material (hydrolysis for 26h at 105°C in constant-boiling HCl under N_2) and on performic acid-oxidized samples (26, 66 and 96h hydrolyses as described above). The results represent the maximum amounts obtained of each amino acid, except for serine and threonine, values for which were obtained by extrapolation to zero time. Analytical methods are described in the Methods section. Sialic acids were determined by the method of Warren (1959). All analyses were done on the purified sialoglycoprotein.

Amino acid	Content (mol/1000mol of amino acid)	Sugar	Content (mol/10000 g of protein)
Asp	58	Fucose	1.8
Thr	115	Mannose	2.4
Ser	146	Galactose	13.5
Glu	97	Glucose	1.2
Pro	75	GlcNAc	5.0
Gly	48	GalNAc	11.8
Ala	51	Sialic acid	13.5
Cys/2*	0	Carbohydrate (%)§	53.7
Val	77		
Met†	12		
Ile	74		
Leu	59		
Tyr	25		
Phe	19		
His‡	54		
Lys	37		
Arg	53		
Protein (% of dry wt.)	38.0		
Protein (%)§	46.3		

* Estimated as cysteic acid.
† Estimated as methionine sulphone.
‡ Subject to some uncertainty, because large amounts of galactosamine were present in the hydrolysates.
§ Based on the sum of the protein and carbohydrate contents.

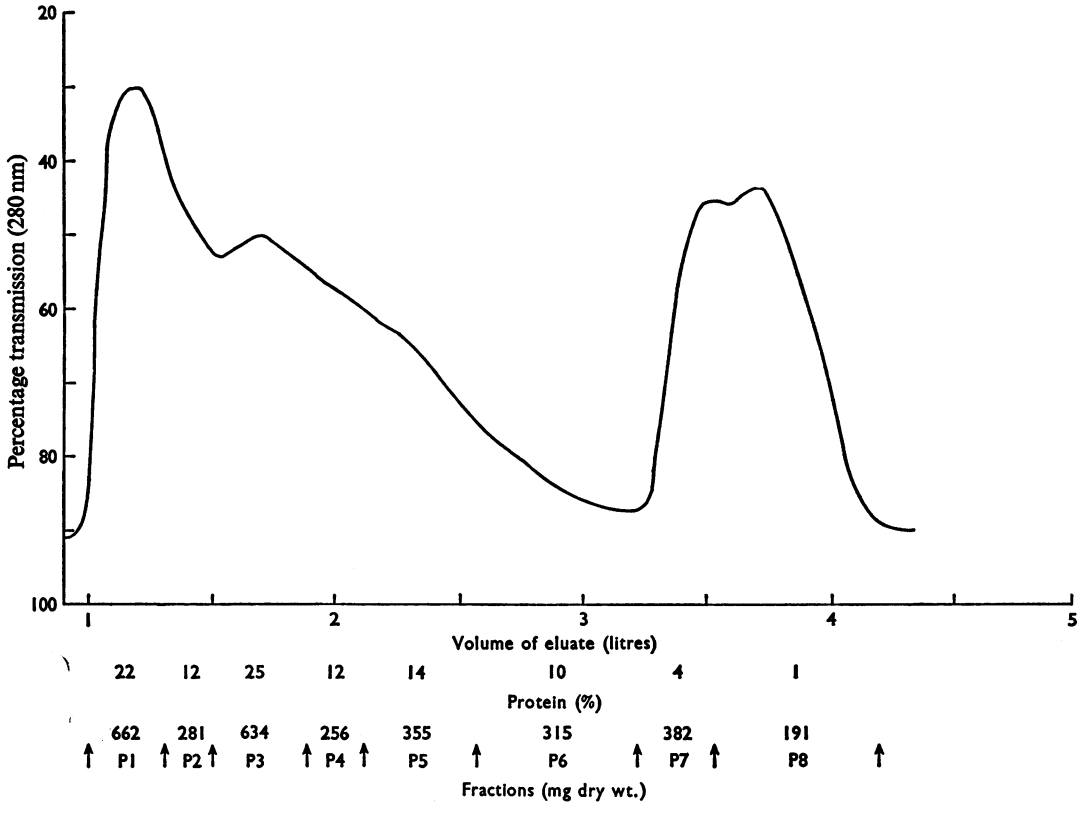


Fig. 2. *Gel filtration of acetone-extracted pyridine precipitate*

Acetone-extracted pyridine precipitate (4.4g dry wt.), prepared from 18 blood-bank donor units of blood-group O erythrocytes by overnight salt extraction, were applied to a Sephadex G-150 column equilibrated with 1% sodium dodecyl sulphate. For experimental details of the preparation and chromatography see the Methods section. Fractions were pooled as indicated in the figure and the protein was recovered from the column eluate by using cooling to precipitate excess of detergent (see the Methods section). The percentage of protein indicated for each fraction is the percentage of the total protein recovered from the column that was present in each fraction, based on amino acid analysis after acid hydrolysis of each fraction.

protein present in gels of the pyridine precipitate (Plate 1*g*). This aggregation appears to be irreversible, as sodium dodecyl sulphate gel electrophoresis of fraction P1 shows only traces of material which enters the gel. Fraction P3 contains a mixture of proteins E and F. A partial separation of protein E from protein F is obtained in fraction P2, but other higher-molecular-weight contaminants are also present in this material. We were not able to purify protein E by rechromatography of this fraction under similar conditions. Residual D-glyceraldehyde 3-phosphate dehydrogenase is present in fraction P5. The low-molecular-weight peaks, P7 and P8, contained only 5% of the protein recovered from the column, although they absorbed strongly at 280nm and represented nearly 20% of the recovered dry weight. Fraction P3 was used for the further study of the proteins E and F.

Characterization of fraction P3

Two determinations of the apparent subunit molecular weights of proteins E and F by sodium dodecyl sulphate gel electrophoresis in a 5% polyacrylamide gel gave values of 93000 and 94000 for the leading edge of the protein E band, and 74000 and 75000 for protein F. Similar values were obtained in a 10% polyacrylamide gel (Table 1). Electrophoresis of a mixture of fraction P3 and intact 'ghosts' showed that proteins E and F had electrophoretic mobilities on 5 and 10% polyacrylamide gels identical with those of the corresponding bands

in intact 'ghosts'. Protein F runs as a sharp band on gel electrophoresis, but band E is always obtained as a broader band with a sharp leading edge and diffuse trailing edge. Reduction and *S*-aminoethylation (Cole, 1967) of the mixture of proteins E and F had no effect on the banding pattern (Plate 4*b*). Performic acid oxidation of the protein mixture resulted in a general degradation of the banding pattern and the complete disappearance of band F (Plate 4*c*). Band E appears to be more resistant to this treatment. Treatment of the protein mixture with formic acid in the absence of oxidizing agent left the banding pattern unaffected. When relatively large amounts (15 μg) of the protein mixture were applied to the gel, a very faint broad periodic acid–Schiff's-base-staining zone could be detected in the region of proteins E and F, but the staining was so weak and diffuse that it was not possible to correlate it conclusively with either of the protein-staining bands E and F.

The protein content of samples from different preparations tended to be variable, probably because of contamination by residual detergent (ranging from 25 to 75% protein for different preparations). For this reason analyses were directly compared with the protein present in the samples, determined by amino acid analysis. Carbohydrate analyses for preparations from blood-group O and blood-group A_1 erythrocytes are shown in Table 4. Appreciable amounts of carbohydrate are present in these preparations. Analysis of several preparations from blood-group O erythrocytes showed the absence of *N*-acetylgalactosamine, although significant amounts of sialic acid were present. Preparations from blood-group A_1 erythrocytes consistently yielded *N*-acetylgalactosamine. The sugar composition of the proteins shows that these carbohydrates cannot be the result of contamination by traces of the sialoglycoprotein. To determine whether this carbohydrate was due to glycolipid contamination of the samples, the protein mixture was extracted with butan-1-ol. No carbohydrate was found in the organic phase.

One preparation was also analysed for cholesterol and total phosphate (Table 4). A surprisingly large amount of cholesterol was present (10.5 mol/100000 g of protein). Chromatography of a sample of cholesterol on Sephadex G-100 under conditions similar to those used in the preparation of the proteins indicated that cholesterol is eluted in the low-molecular-weight region of the column, none being found at an elution volume corresponding to that of the proteins. The sample also contained a small amount of phosphate (2.1 mol of phosphate/100000 g of protein).

N-Terminal analysis of the mixture of proteins E and F by the cyanate method gave very low yields of *N*-terminal amino acids. Serine, the amino acid found in highest yield, accounted for 0.125 mol/100000 g of protein after correction for losses during the

Table 4. *Analysis of mixture of proteins E and F*

Fraction P3 from the gel filtration of the pyridine precipitate (Fig. 2), derived from blood-group O,Rh+ erythrocytes, and a similar fraction derived from pooled blood-group A_1 erythrocytes were analysed as indicated in the Methods section. Sialic acid was measured by the method of Bhatti *et al.* (1970).

Erythrocyte blood group	Protein (% dry wt.)	Protein (%)†	Carbohydrate (%)†	Content (mol/100000 g of protein)							Cholesterol (%)†	Total phosphate (%)†
				Fuc	Man	Gal	Glc	GlcNAc	GalNAc	Sialic acids		
O	27	87	9.6	3	8	21	19	20	—	3	3.6	0.16
A_1	68	90	10.2	3	8	16	25	21	3	4	*	*

* Not assayed.

† Derived from the sum of the protein, carbohydrate (excluding glucose), cholesterol and total phosphate values for the blood-group O sample, and the sum of the protein and carbohydrate (excluding glucose) for the blood-group A sample.

procedure (Stark, 1967). This serine is not derived from contaminating serine lipids, as similar results were obtained after prior extraction of the proteins with chloroform–methanol (2:1, v/v). Proteins E and F probably have blocked *N*-terminal residues.

Separation of proteins E and F and characterization of the proteins

Preparative sodium dodecyl sulphate gel electrophoresis has allowed the purification of small amounts of each protein. Satisfactory results could be obtained only under conditions of very light loading, and only limited amounts of material could be separated even on rather large preparative gels. Plate 4(*d*,*e*) shows analytical gels of the purified proteins. Each of the purified proteins when added to a sample of the unfractionated mixture of proteins E and F co-migrated with the relevant band in the mixture.

Analytical results for the purified proteins from blood-group O erythrocytes are shown in Table 5. The amino acid analyses of the two proteins are strikingly similar. Appreciable amounts of half-cystine and methionine are present in both proteins, and they are rich in leucine, glutamic acid and/or glutamine and alanine, but contain relatively little tyrosine and histidine. The carbohydrates of the proteins are also similar both in composition and amount, but sialic acid is found only in protein F. The carbohydrate analyses are in general agreement with that of the mixture of proteins E and F, but the lower amounts of galactose found in the purified proteins suggest that a component rich in galactose is also present in the unfractionated mixture.

Column fraction of highest molecular weight

The large amount of material in fraction P1 (Fig. 2) was characterized to obtain some information on its origin. *N*-Terminal analysis by the cyanate procedure showed the presence of very small amounts of *N*-terminal amino acids. Serine was found at a concentration similar to that obtained from the mixture of proteins E and F. Carboxypeptidase A liberated

Table 5. *Analysis of protein E and protein F*

The preparations were derived from blood-group O,Rh+ erythrocytes. Amino acid analysis was performed on the performic acid-oxidized protein, after hydrolysis at 105°C for 24h under N_2 with constant-boiling HCl. The results are not corrected for the slow release or destruction of any amino acid during hydrolysis. Details of the analytical procedures are given in the Methods section. Sialic acids were measured by the method of Bhatti *et al.* (1970).

	Content (mol/1000mol of amino acid)	
	Protein E	Protein F
Asp	99	87
Thr	58	58
Ser	52	56
Glu	104	111
Pro	50	54
Gly	92	81
Ala	107	92
Cys/2*	13	20
Val	74	72
Met†	23	18
Ile	43	43
Leu	108	117
Tyr	14	15
Phe	41	37
His	32	25
Lys	57	52
Arg	57	61

	Content (mol/100000 g of protein)	
	Protein E	Protein F
Fucose	7	§
Mannose	8	6
Galactose	8	9
Glucose	||	32
GlcNAc	17	21
GalNAc	—	—
Sialic acid	—	4
Protein (% dry wt.)	28	18
Protein (%)‡	93	91
Carbohydrate (%)‡	7.2	9.1

* Estimated as cysteic acid.
† Estimated as methionine sulphone.
‡ Values based on the sum of protein and carbohydrate (excluding glucose).
§ Value uncertain.
|| Very high glucose value (200mol/100000g of protein) suggests considerable glucose contamination.

Table 6. *Release of amino acids by carboxypeptidase A from protein fractions*

Samples from the gel filtration of the pyridine precipitate (Fig. 2) were incubated at room temperature at a concentration of 19mg/ml (fraction P1) or 17mg/ml (fraction P3) with 0.5mg of bovine carboxypeptidase A [Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) type COA, treated with di-isopropyl phosphorofluoridate]/ml. The reaction mixture contained 1.4% sodium dodecyl sulphate (Guidotti, 1960), 1mM-norleucine and 0.3M-*N*-ethylmorpholine acetate buffer, pH8.6. After 80min a 0.25ml sample was removed, acidified with 0.5ml of 0.5M-HCl and kept at 4°C for 1h. After centrifugation, the clear supernatants were freeze-dried and applied to the amino acid analyser. The results are corrected for endogenous amino acids present in the samples, and for amino acid release from the carboxypeptidase itself, determined in a parallel experiment. Yields of the amino acids not listed below were less than 0.06mol/100000g of protein.

	Amino acid released (mol/100000g of protein)	
	Fraction P1	Fraction P3
Thr	0.22	0.18
Gly	0.28	0.03
Ala	0.49	0.68
Val	0.82	0.45
Leu	0.71	0.54
Tyr	0.19	0.15
Phe	0.28	0.12

the same three major amino acids, alanine, valine and leucine, as are obtained from proteins E and F (Table 6), although the relative amounts of each amino acid released were different. The amino acid composition, tryptic and thermolysin peptide 'maps' of this material were also very similar to those obtained from the unfractionated mixture of proteins E and F (results not shown). These results suggest that the material is derived from the aggregation of proteins E or F or both.

Discussion

The preparative method described here is based on a combination of several selective extraction procedures and subsequent column chromatography in the presence of sodium dodecyl sulphate. Low-ionic-strength extraction of 'ghosts', performed as described by Marchesi *et al.* (1970), results in the solubilization of spectrin (proteins A and B) and protein J. The extraction of these proteins under similar conditions has also been noted by Fairbanks *et al.* (1971). D-Glyceraldehyde 3-phosphate dehydrogenase (protein K) can be selectively solubilized from the residue by exposure to 1M-NaCl solution. Fairbanks *et al.* (1971) also describe the selective extraction from intact 'ghosts' by 0.5M-NaCl of a protein (their band VI), which is identical with this enzyme. Omission of the salt-extraction step from the procedure causes difficulties in the purification of the sialoglycoprotein, as it cannot easily be separated by gel filtration from D-glyceraldehyde 3-phosphate dehydrogenase. The results of pyridine extraction on the salt-extracted 'ghosts' are in general agreement with those of Blumenfeld *et al.* (1970), although they applied the procedure to intact 'ghosts'. It is possible, by using the procedure described here, to isolate the major protein components of the erythrocyte 'ghost' from a single preparation.

The sialoglycoprotein (D) is clearly similar to other preparations of the human erythrocyte blood-group M,N glycoprotein obtained by Springer *et al.* (1966), Winzler (1969) and Blumenfeld *et al.* (1970). We have been unable to find either an *N*-terminal residue or a *C*-terminal residue in this protein. Winzler (1969) also suggests that the *N*-terminus of this protein is blocked. It shows an anomalous mobility on sodium dodecyl sulphate gels of various polyacrylamide concentrations, as reported by Bretscher (1971*b*), emphasizing that the apparent subunit molecular weights obtained by this technique for the sialoglycoprotein are unreliable. Extrapolating from polyacrylamide-gel experiments, Segrest *et al.* (1971) have obtained a molecular weight of 59000 for this protein and they suggest that the anomalous electrophoretic behaviour of the protein is due to its carbohydrate content. Our results from gel filtration of the protein in sodium dodecyl sulphate yield a value of 46000 for the apparent subunit molecular weight. Ultracentrifugal analysis (Morawiecki, 1964) gave a value of 30000, and we have used this latter value in our calculations. The minor periodate-positive components (PAS 2 and PAS 4) show a similar anomalous mobility on sodium dodecyl sulphate gels, and their apparent molecular weights so obtained are equally unreliable. Component PAS 2, although it has an apparent molecular weight of 53000 on a 5% polyacrylamide gel, yields a value of about 30000 on gel filtration in 1% sodium dodecyl sulphate, suggesting that both this component and component PAS 4 have a carbohydrate content similar to that of the sialoglycoprotein.

Proteins E and F have been described as bands on analytical sodium dodecyl sulphate gels by several groups (Lenard, 1970; Fairbanks *et al.*, 1971). Bretscher (1971*c*) has described peptide 'maps' of a radioactively labelled protein that is identical with protein E. This is the first report of any analytical results for either of these proteins. The subunit

molecular weights of proteins E and F are 93000 and 74000 respectively as estimated by sodium dodecyl sulphate gel electrophoresis. In contrast to the sialoglycoprotein, consistent molecular weights are obtained from gels of different polyacrylamide concentrations, although they each contain about 8% of carbohydrate. We believe that these values are reasonable estimates of the subunit molecular weights of the proteins. No new components were detected on gel-electrophoretic analysis after reduction and *S*-aminoethylation, and it is probable that proteins E and F are true subunits. Fairbanks *et al.* (1971) applied several disaggregating procedures to intact 'ghosts' and their results support this conclusion. The loss of sharp bands on polyacrylamide gels after performic acid oxidation of these proteins is unexplained. However, as the proteins contain carbohydrate it is possible that this may result from complex reactions occurring subsequent to the oxidation of some of these sugar components. Protein E always migrates as a band with a sharp leading edge and diffuse trailing edge on gel electrophoresis of intact 'ghosts' and during all the stages of the purification. This may be an intrinsic characteristic of protein E, but we cannot eliminate the possibility that a further component is present. Fairbanks *et al.* (1971) also noted this phenomenon on gel electrophoresis of intact 'ghosts'. The two proteins have rather similar amino acid compositions and both contain significant amounts of half-cystine. A further feature of both of these proteins is their appreciable carbohydrate content (7–9%). The glycoprotein nature of these components has previosly escaped detection, probably because they stain very weakly with the periodate stain on polyacrylamide gels. By all the criteria that we have been able to apply the carbohydrate is covalently bound to the protein and is not due to the presence of contaminating glycolipids. The carbohydrate remains associated with the protein after gel filtration and gel electrophoresis in sodium dodecyl sulphate and cannot be removed by butanol extraction. The bound carbohydrate, which contains mannose, is quite different from that of the bulk of the erythrocyte glycolipids (Sweeley & Dawson, 1969), which are found in the lowest-molecular-weight region on gel filtration in sodium dodecyl sulphate, well separated from proteins E and F. It is noteworthy that the unfractionated mixture of proteins E and F derived from blood-group O erythrocytes does not contain any *N*-acetylgalactosamine, whereas this sugar is found in preparations from blood-group A_1 erythrocytes. The carbohydrate compositions of the purified proteins E and F are closely similar, the presence of sialic acid in protein F being the major difference between them. The lack of *N*-acetylgalactosamine in these proteins suggests that the carbohydrate may be linked to the polypeptide chain via an asparagine residue. As only limited amounts of the purified proteins were available, terminal-group analysis was performed on the unfractionated mixture. Both proteins appear to contain blocked *N*-termini. Our inability to detect any *N*-termini in the protein mixture confirms that the isolated proteins have not been degraded by 'ghost' proteases. The similarities in the amino acid and carbohydrate compositions of proteins E and F suggest that the two proteins may be related.

Pyridine treatment of the salt-extracted 'ghosts' causes the appearance of highly aggregated material, which does not enter a 5% polyacrylamide gel and which is found in the highest-molecular-weight region on gel filtration. This material yielded similar *N*- and *C*-termini and thermolytic and tryptic peptide 'maps' to those obtained from the mixture of proteins E and F. It appears that this material originated from protein E or F or both. Fairbanks *et al.* (1971) have noted a tendency of protein E (their band III) to form material that would not enter a polyacrylamide gel after treatment of intact 'ghosts' with organic solvents.

We have previously described the protein components obtained by using a procedure similar to the one described here, except that the pyridine treatment was omitted (Tanner & Owens, 1971). Further characterization of the components isolated in that study has shown that they contain large amounts of various *N*-terminal amino acids and gave broad diffuse bands on gel electrophoresis, which are not found in gels of intact 'ghosts'. We conclude that these components were proteolytic artifacts, since they must have originated from proteins D, E and F, none of which has detectable *N*-termini. Pyridine treatment before gel filtration has reproducibly eliminated the appearance of degradative products.

Fairbanks *et al.* (1971) distinguish two classes of protein in erythrocyte 'ghosts', the readily eluted group including spectrin, protein J and D-glyceraldehyde 3-phosphate dehydrogenase, and the tightly bound class comprising proteins E, F and the periodate-staining proteins. Our results support this classification. The removal of spectrin and protein J leaves unchanged the particulate fracture faces found on freeze-etch electron microscopy of intact 'ghosts'. The preparation obtained after removing the readily eluted proteins retains the 'ghost' lipid and carbohydrate in addition to the tightly bound proteins. It is noteworthy that two of the proteins in this preparation, the sialoglycoprotein and protein E, are accessible to impermeable radioactive reagents from both sides of the erythrocyte membrane (Bretscher, 1971*a*,*b*,*c*; Phillips & Morrison, 1971*a*,*b*). Reaction with protein F occurs only when the interior surface of the membrane is accessible to these reagents. This suggests that the carbohydrate carried by protein F is present at the cytoplasmic surface of the membrane. However, the possibility exists that protein F also extends through the membrane but is not reactive at

the outer surface for steric reasons or because of limited reagent specificity.

The amounts of the sialoglycoprotein and proteins E and F present in the membrane raises the question of their functional role. None of the enzymes known to be present in our erythrocyte 'ghost' preparations is found in sufficient quantities to account for any of these proteins (Mitchell *et al.*, 1965; Green *et al.*, 1965; Dunham & Hoffman, 1970; Belhorn *et al.*, 1970). In addition, the structure of the sialoglycoprotein appears to be quite different from that of known enzymes in both the extent and distribution of glycosylation of its polypeptide chain (Winzler, 1969). It is unlikely that these proteins are 'structural' proteins in the sense used by Green & Perdue (1966), as they are present in the membrane in amounts that would be insufficient to maintain the structure of the membrane by protein–protein contacts, unless they have highly extended structures within the plane of the membrane.

All three of the major tightly bound proteins are substantially glycosylated. Estimates of the proportion of 'ghost' sialic acid associated with the sialoglycoprotein range from 60% (Fairbanks *et al.*, 1971) to almost 100% (Winzler, 1971). Using this range and our analytical results we calculate that the sialoglycoprotein constitutes 5.0–8.5% of the 'ghost' dry weight and carries 30–45% of the total carbohydrate present in the erythrocyte 'ghost'. On the basis of the relative intensity of staining of the bands of intact 'ghosts', Fairbanks *et al.* (1971) estimated that protein E constitutes 30% and protein F 8% of the total 'ghost' protein. From our analytical results we estimate that together these proteins carry about 13% of the erythrocyte 'ghost' carbohydrate. The sialoglycoprotein and proteins E and F combined carry 45–60% of the total carbohydrate on the cell membrane. Each sialoglycoprotein chain carries about 65 sugar residues and a similar number are carried on the other two proteins (40 and 30 residues for proteins E and F respectively). This carbohydrate may serve to orientate the molecules in the membrane, or conversely it may be that one of the functions of the proteins is to act as carrier for carbohydrate. It is widely accepted that carbohydrate is responsible for cell-surface specificity. In addition the presence, at the cell surface, of quantities of hydrophilic carbohydrate closely bound to the membrane structure may contribute to the long-term stability that is characteristic of animal cell plasma membranes, by promoting extensive solvation of the membrane surface.

We thank Mrs. Judith Jefferies for her skilful assistance, Dr. J. R. Clamp for providing our carbohydrate analyses, Miss Geraldine Mumford for the electron micrographs, Mr. A. Brownstone for his help with preparative gel electrophoresis and the use of his apparatus, Mr. D. Anstee for performing the serological analysis, and the South West Regional Blood Transfusion Centre for supplies of out-dated erythrocytes. D. H. B. is the recipient of a Medical Research Council Research Studentship. The work was supported by the Medical Research Council.

References

Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466
Belhorn, M. B., Blumenfeld, O. O. & Gallop, P. M. (1970) *Biochem. Biophys. Res. Commun.* **39**, 267
Bender, W. W., Garan, H. & Berg, H. C. (1971) *J. Mol. Biol.* **58**, 783
Berg, H. C. (1969) *Biochim. Biophys. Acta* **183**, 65
Bhatti, T., Chambers, R. & Clamp, J. R. (1970) *Biochim. Biophys. Acta* **222**, 339
Blumenfeld, O. O. (1968) *Biochem. Biophys. Res. Commun.* **30**, 200
Blumenfeld, O. O., Gallop, P. M., Howe, C. & Lee, L. T. (1970) *Biochim. Biophys. Acta* **211**, 109
Braun, V. & Schroeder, W. H. (1967) *Arch. Biochem. Biophys.* **118**, 241
Bretscher, M. S. (1971*a*) *J. Mol. Biol.* **58**, 775
Bretscher, M. S. (1971*b*) *Nature New Biol. (London)* **231**, 229
Bretscher, M. S. (1971*c*) *J. Mol. Biol.* **59**, 351
Brownstone, A. (1969) *Anal. Biochem.* **27**, 25
Cole, R. D. (1967) *Methods Enzymol.* **11**, 315
Dodge, J. T., Mitchell, C. & Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* **100**, 119
Dunham, P. D. & Hoffman, J. F. (1970) *Proc. Nat. Acad. Sci. U.S.* **66**, 936
Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606
Green, D. E. & Perdue, J. F. (1966) *Proc. Nat. Acad. Sci. U.S.* **55**, 1295
Green, D. E., Murer, E., Hultin, H. O., Richardson, S. H., Salman, B., Brierley, G. P. & Baum, H. (1965) *Arch. Biochem. Biophys.* **112**, 635
Guidotti, G. (1960) *Biochim. Biophys. Acta* **42**, 177
Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 197
Lenard, J. (1970) *Biochemistry* **9**, 1129
Marchesi, S. L., Steers, E., Marchesi, V. T. & Tillack, T. W. (1970) *Biochemistry* **9**, 50
Mitchell, C. D., Mitchell, W. S. & Hanahan, D. J. (1965) *Biochim. Biophys. Acta* **104**, 348
Moor, H. & Muhlethaler, K. (1963) *J. Cell Biol.* **17**, 609
Morawiecki, A. (1964) *Biochim. Biophys. Acta* **83**, 339
Phillips, D. R. & Morrison, M. (1971*a*) *Biochemistry* **10**, 1766
Phillips, D. R. & Morrison, M. (1971*b*) *Biochem. Biophys. Res. Commun.* **45**, 1103
Pinto da Silva, P. G. & Branton, D. (1970) *J. Cell Biol.* **45**, 458
Segrest, J. P., Jackson, R. L., Andrews, E. P. & Marchesi, V. T. (1971) *Biochem. Biophys. Res. Commun.* **44**, 390
Shapiro, A. L., Vinuela, E. & Maizel, J. V. (1967) *Biochem. Biophys. Res. Commun.* **28**, 815
Springer, G. F., Nagai, Y. & Tegtmeyer, H. (1966) *Biochemistry* **5**, 3254
Stark, G. R. (1967) *Methods Enzymol.* **11**, 125
Steck, T. L., Fairbanks, G. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2617

Sweeley, C. C. & Dawson, G. (1969) in *Red Cell Membrane* (Jamieson, G. A. & Greenwalt, T. J., eds.), p. 172, J. B. Lippincott Co., Philadelphia
Tanner, M. J. A. & Gray, W. R. (1971) *Biochem. J.* **125**, 1109
Tanner, M. J. A. & Owens, D. (1971) *Biochem. J.* **122**, 40P
Tillack, T. W. & Marchesi, V. T. (1970) *J. Cell Biol.* **45**, 649
Warren, L. (1959) *J. Biol. Chem.* **234**, 1971
Winzler, R. J. (1969) in *Red Cell Membrane* (Jamieson, G. A. & Greenwalt, T. J., eds.), p. 157, J. B. Lippincott Co., Philadelphia
Winzler, R. J. (1971) *Int. Rev. Cytol.* **29**, 77
Zacharias, R. J., Zell, T. E., Morrison, J. H. & Woodcock, J. J. (1969) *Anal. Biochem.* **30**, 148
Zlatkis, A., Zak, B. & Boyle, A. J. (1952) *J. Lab. Clin. Med.* **41**, 486
Zvilichovsky, B., Gallop, P. M. & Blumenfeld, O. O. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1234