

## Original Article

# Electrophoretic Analysis of Erythrocyte Membrane Proteins and Glycoproteins from Different Species

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**Abstract.** The erythrocyte membrane proteins and glycoproteins of man, rat, mouse, sheep and dog were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using a discontinuous buffer system. Considerable similarities between the species were observed in the pattern of protein bands seen when gels were stained with Coomassie Blue. Equivalents to human Bands 1, 2, 3, 4.2, 5 and 8 appeared to be present in the rat, mouse, sheep and dog, and Band 4.1 was identified as a closely spaced doublet in all species except the rat.

When RBC membranes were stained with periodic acid-Schiff (PAS) after SDS-PAGE analysis, glycoproteins equivalent to human glycoporphins were identified in all the species studied. However, in contrast to the overall similarity of the protein patterns, the number, relative staining intensity and apparent molecular masses of the PAS-stained bands differed between species.

The silver stain was assessed in the detection of RBC membrane proteins in polyacrylamide gels, and found to be more sensitive than Coomassie Blue. The technique also stained many of the PAS-positive glycoporphins as diffuse orange zones, which could be distinguished from the darker protein bands by their differential colouration.

In view of the interspecies variation in the glycoporphins after SDS-PAGE, it is suggested that, unlike the membrane proteins, their functions do not require a conserved structure.

**Keywords:** Erythrocyte; Membrane proteins; Membrane glycoproteins; Comparative study; Gel electrophoresis

## Introduction

The structure of the red blood cell (RBC) membrane has been extensively studied for many years as a model for the membrane of more complex cells (reviewed by Bennett 1985). RBCs have proved attractive for such investigations, since they are readily available in large numbers, with membranes which are relatively simple and easily purified (Dodge *et al.* 1963). The erythrocyte membrane itself is also of considerable importance in the study of a number of medical conditions, including transfusion reactions, haemolytic disease of the newborn and autoimmune haemolytic anaemia.

Early attempt to analyse RBC membrane proteins were frustrated by their insolubility in aqueous media of physiological ionic strength (Maddy 1982). This problem was overcome by using the anionic detergent sodium dodecyl sulphate to achieve complete solubilisation of the membrane, enabling the proteins to be separated according to size by polyacrylamide gel electrophoresis (SDS-PAGE). Utilising this technique, the major membrane proteins of the human erythrocyte were identified (Fairbanks *et al.* 1971) and the RBC membrane components of a variety of other mammals were also analysed (Carraway and Kobylka 1970; Lenard 1970; Hamaguchi and Cleve 1972; Kobylka *et al.* 1972).

Since these early comparative studies, improved methods of SDS-PAGE with higher resolution have

become available, in which the apparent molecular mass of some human erythrocyte membrane components differs from that originally estimated (Thompson and Maddy 1982). Also, highly sensitive silver stains have been developed for the detection of proteins in polyacrylamide gels (Burk *et al.* 1983). The purposes of the current work were to analyse RBC membrane proteins and glycoproteins from a variety of different species, using the improved techniques of electrophoresis, and to compare silver staining with more conventional gel stains.

## Materials and Methods

### Blood Samples

Blood samples, collected in citrate anticoagulant, were obtained from normal animals maintained at the University of Bristol and from healthy human volunteers.

### Preparation of RBC Membranes

RBCs were washed six times in phosphate-buffered saline, pH 7.4 (PBS), with thorough aspiration of leucocytes in the buffy coat. Erythrocyte 'ghosts' (Dodge *et al.* 1963) were prepared by hypotonic lysis of 1 volume of washed RBC in 10 volumes of ice-cold 20 mM Tris, pH 7.6. After pelleting by centrifugation at 30 000 *g* for 30 min at 4°C, the membranes were retrieved and washed repeatedly in lysis buffer until the supernatant was clear of haemoglobin. Opaque buttons of residual leucocyte debris, which are a source of proteolytic activity (Fairbanks *et al.* 1971), were removed from translucent RBC membranes.

After preparation, RBC 'ghosts' were frozen immediately and stored at -70°C until use. The protein concentration of each sample was determined by the method of Bradford (1976).

### SDS-PAGE

RBC 'ghosts' were solubilised in gel sample buffer containing 8 M urea, 5% w/v SDS, 5% v/v 2-mercaptoethanol, 10% v/v glycerol and 0.00125% w/v bromophenol blue in 0.0625 M Tris-HCl, pH 6.8. SDS-PAGE was performed in 12% polyacrylamide gels according to the method of Laemmli (1970), using the Bio-Rad Mini Protean II. Apparent molecular masses were calculated from migration relative to the standard protein markers rabbit muscle myosin (205 kD), *Escherichia coli*  $\beta$  galactosidase (116 kD), rabbit muscle phosphorylase b (97.4 kD), bovine albumin (66 kD), egg albumin (45 kD) and bovine RBC carbonic anhydrase (29 kD) (Sigma SDS-6H).

### Coomassie Blue Stain

Polyacrylamide gels were stained for protein by rocking for 1 h at room temperature in a solution containing 0.5% w/v Coomassie Brilliant Blue R250 (Sigma), 45% v/v methanol and 10% v/v acetic acid. Gels were destained by constant agitation in several changes of 45% v/v methanol, 10% v/v acetic acid until maximum contrast was achieved between the stained bands and the background. After washing briefly in water, the gels were photographed.

### Periodic Acid-Schiff (PAS) Stain

The presence of glycoprotein bands in gels was revealed by the PAS stain (Fairbanks *et al.*, 1971). After fixing in 45% v/v methanol, 10% v/v acetic acid for 1 h, gels were washed 4  $\times$  10 min in 5% v/v acetic acid and incubated in 1% v/v periodic acid for 90 min. The washing step was repeated and the gels were developed overnight in Schiff's reagent (Sigma). The entire staining procedure was performed at room temperature, with continuous agitation. The gels were photographed after washing briefly with 0.1% w/v sodium metabisulphite in 0.01 M HCl, which minimises background staining and retards fading of the pale pink bands revealed by development.

### Silver Stain

Both protein and glycoprotein bands in polyacrylamide gels were developed using a silver stain (Burk *et al.* 1983). Gels were fixed in 27% v/v ethanol, 15% v/v formaldehyde for 1 h, washed in several changes of water overnight and incubated for 15 min in freshly prepared ammoniacal silver nitrate (0.8% w/v silver nitrate, 1.5% v/v concentrated ammonia, 0.084% w/v sodium hydroxide). Care was taken to dispose of the ammoniacal silver nitrate solution safely, since it can become explosive if allowed to dry. After three 5 min washes in water, gels were developed in 0.01% w/v citric acid, 0.05% v/v formaldehyde until the maximum contrast was seen between staining of the bands and the background. The reaction was stopped by washing the gels in water for 30 s and incubating in 0.1% w/v citric acid, 0.05% v/v methylamine for 15 min. All stages of the procedure were carried out with continuous agitation. Double-distilled water, or its equivalent, was used throughout. After washing several times in water, the gels were photographed immediately.

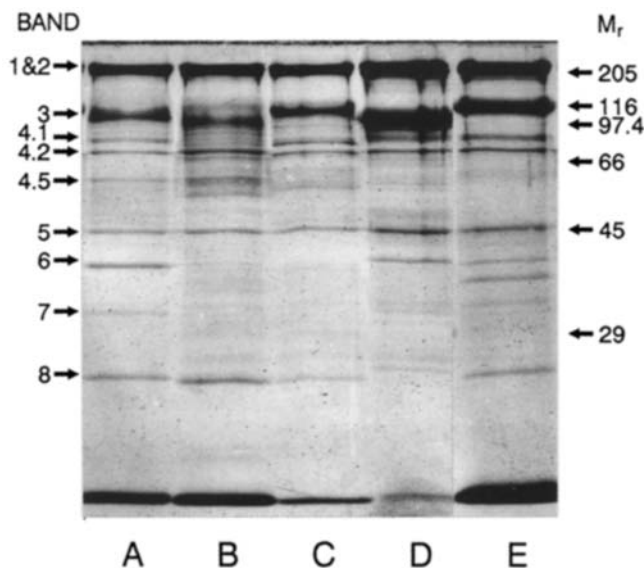
### Photography

Polyacrylamide gels were photographed in monochrome using Agfa 711p 4"  $\times$  5" film. Colour photographs were taken on Fujichrome 64T 35mm slide film.

## Results

### Major Proteins of the Erythrocyte Membrane

RBC membranes prepared from man, rat, mouse, sheep and dog were analysed by SDS-PAGE and stained with Coomassie Blue to reveal the major protein components. The results are shown in Fig. 1 and the apparent molecular masses of the major protein bands detected are summarised in Table 1. Up to nine distinct bands could be identified after analysis of the human erythrocyte membrane and these have been labelled according to the accepted nomenclature (Fairbanks *et al.* 1971; Steck 1974). It can be seen that the overall pattern of electrophoretic mobility and relative intensity of stained bands is similar in the species studied, particularly for the larger proteins. Equivalents to human Bands 1, 2, 3, 4.2, 5 and 8 appeared to be present in the rat, mouse, sheep and dog, although the apparent molecular mass of Band 3 showed some interspecies variation. In addition, Band 4.1 could be identified as a closely spaced doublet in all species except the rat, and a number of minor bands were stained between Bands 4.2 and 5 in all the samples analysed. The smaller proteins of the human RBC, Bands 6 and 7, appeared to have no clear equivalent in rat or mouse. However, additional proteins of similar size were present in sheep and dog erythrocytes.



**Fig. 1.** Coomassie Blue stain of RBC membrane proteins after SDS-PAGE. Erythrocyte 'ghosts' from different species were analysed at 20  $\mu$ g protein per lane as follows: Lane A, human; Lane B, rat; Lane C, mouse; Lane D, sheep; Lane E, dog. The major protein bands of the human RBC membrane are labelled using the nomenclature of Fairbanks *et al.* (1971) and Steck (1974). The molecular mass (kD) and the position of standard protein markers is indicated.

**Table 1.** Apparent molecular mass (kD) of the major protein bands stained with Coomassie Blue after SDS-PAGE analysis of RBC membranes from different species

RBC species of origin	Human	Rat	Mouse	Sheep	Dog
240 (Band 1 <sup>a</sup> )	240	240	240	240	240
220 (Band 2 <sup>a</sup> )	220	220	220	220	220
108 (Band 3 <sup>a</sup> )	100	110	110	104	117
83 (Band 4.1 <sup>a</sup> )	—	—	83	83	83
75 (Band 4.2 <sup>a</sup> )	75	75	75	75	75
44 (Band 5 <sup>a</sup> )	44	44	44	44	44
38 (Band 6 <sup>a</sup> )	—	—	—	38	38
	—	—	—	32	32
31 (Band 7 <sup>a</sup> )	—	—	—	31	—
25 (Band 8 <sup>a</sup> )	25	25	25	26	25

<sup>a</sup>Fairbanks *et al.* (1971), Steck (1974) Nomenclature for human RBC membrane proteins.

### Major Glycoproteins of the Erythrocyte Membrane

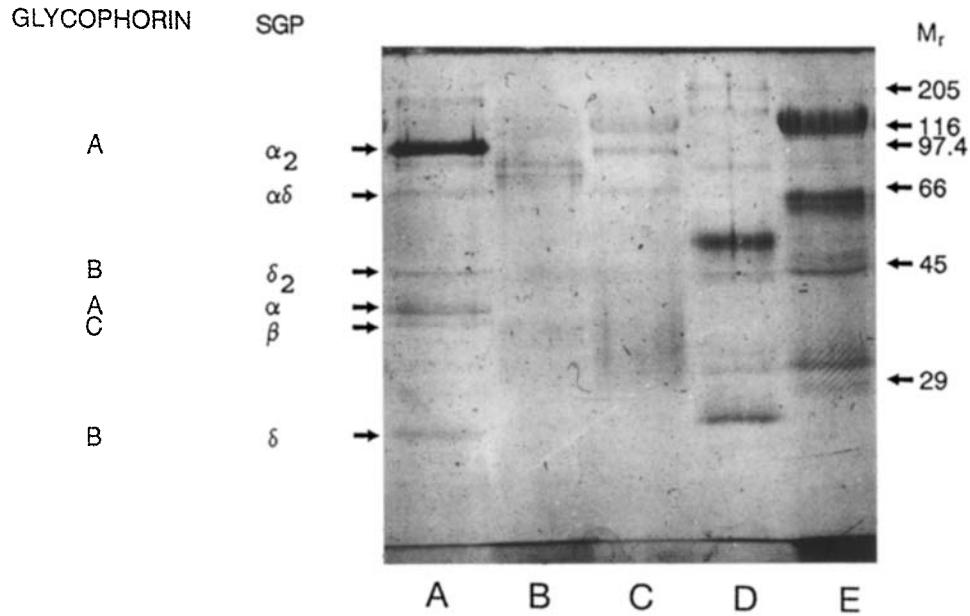
RBC membranes prepared from the same range of species were analysed by SDS-PAGE and stained with PAS to reveal the major glycoprotein components. The results are shown in Fig. 2 and the apparent molecular masses of the major bands stained are summarised in Table 2. The six major bands identified after analysis of human erythrocyte membranes have been labelled according to the nomenclature systems of both Marchesi *et al.* (1976) and Anstee *et al.* (1979). In contrast to the results seen after Coomassie Blue staining of the gel, the pattern of glycoprotein bands revealed by PAS showed very little similarity between species. The number, relative staining intensity and apparent molecular masses of bands differed in each of the species examined.

Apart from Band 3, which appeared to stain with PAS in the rat, mouse and dog, there was little correspondence between the RBC membrane components which were stained by PAS and those identified with Coomassie Blue.

In addition to the bands tabulated, PAS treatment of gels also revealed 2 bands of 130–200 kD in human and sheep erythrocytes, and a broad area of intense staining below the dye front in each lane.

### Silver Staining of RBC Membrane Components After SDS-PAGE

The results of silver staining of RBC membrane components after SDS-PAGE are shown in Fig. 3. It can be seen that this technique allows visualisation of the bands which stained with Coomassie Blue, together with numerous other minor protein components. The calculated molecular masses of the bands identified by both techniques were comparable.



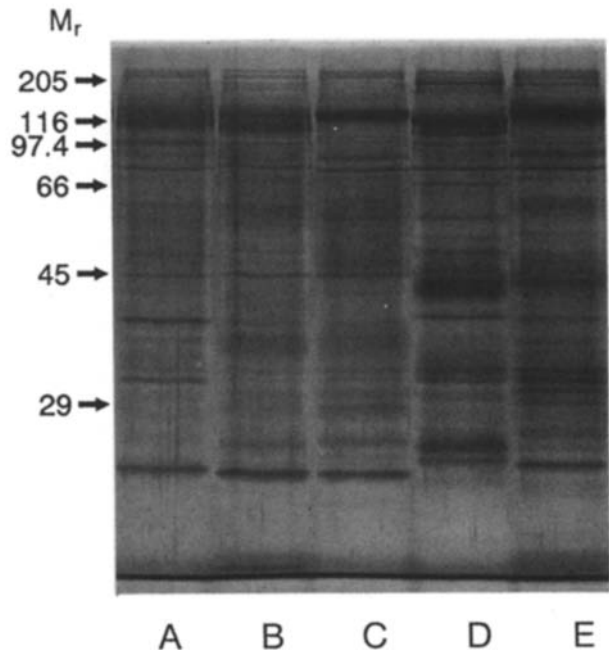
**Fig. 2.** PAS stain of RBC membrane glycoproteins after SDS-PAGE. Erythrocyte 'ghosts' from different species were analysed at 30  $\mu\text{g}$  per lane as follows: Lane A, human; Lane B, rat; Lane C, mouse; Lane D, sheep; Lane E, dog. The glycophorins, or sialoglycoproteins (SGPs), of the human RBC membrane are labelled using (from left to right) the nomenclature of Marchesi *et al.* (1976) and Anstee *et al.* (1979). The molecular mass (kD) and the position of standard protein markers in a Coomassie Blue stained strip of the same gel is indicated.

One striking feature of the silver stain was that, not only were proteins stained, but the position of glycoproteins in the gel was also revealed by areas of diffuse orange/brown staining which corresponded with the major PAS-positive bands. The glycoproteins of dog and sheep RBC could be identified particularly well with the silver stain, although the resolution was less clear than that achieved using PAS.

**Table 2.** Apparent molecular mass (kD) of the major glycoprotein bands stained with PAS after SDS-PAGE analysis of RBC membranes from different species

RBC species of origin				
Human	Rat	Mouse	Sheep	Dog
—	100 <sup>a</sup>	110 <sup>a</sup>	—	117 <sup>a</sup>
90	79	88	79	62
65	72	65	48	59
43	43	43	45	45
37	34	{35	43	43
36	30	}30	32	30
25			30	28
			26	

<sup>a</sup> Comigrates with corresponding Band 3 protein.  
{ Limits of diffuse band.



**Fig. 3.** Silver stain of RBC membrane proteins and glycoproteins after SDS-PAGE. Erythrocyte 'ghosts' from different species were analysed at 2  $\mu\text{g}$  protein per lane as follows: Lane A, human; Lane B, rat; Lane C, mouse; Lane D, sheep; Lane E, dog. The molecular mass (kD) and the position of standard protein markers is indicated.

## Discussion

The results of the current work illustrate that, when analysed by SDS-PAGE, the protein components of the erythrocyte membrane show a remarkable degree of similarity between different mammalian species. In an earlier comparative study, it was claimed that there was considerable interspecies variation in RBC membrane proteins (Carraway and Kobylka 1970), but many of these alleged differences were subsequently attributed to the effects of proteolysis on samples (Kobylka *et al.* 1972). Contaminating leucocytes were found to be the major source of proteases, which must be minimised by thorough aspiration of the buffy coat and removal of white cell debris during the production of erythrocyte 'ghosts' (Fairbanks *et al.* 1971; Kobylka *et al.* 1972). Later studies by Lenard (1970), Kobylka *et al.* (1972), Ralston (1975) and Sarris and Palade (1979) clearly demonstrated that erythrocyte membrane proteins from a wide variety of mammalian species generated very similar patterns when analysed by SDS-PAGE. That these proteins should appear to be conserved is not unexpected, since most are now known to have important functions in the RBC membrane. Thus, Bands 1 and 2, comprising the subunits of spectrin, are the major components of the erythrocyte cytoskeleton, which is essential for the maintenance of the characteristic RBC morphology. Band 5, now identified as actin, together with Band 4.1, also forms part of the cytoskeleton (reviewed by Bennett 1985). In addition, Band 3 functions as the erythrocyte membrane anion channel (Cabantchik *et al.* 1978).

In the present study, it was found that the pattern of smaller RBC membrane proteins in SDS-PAGE was not consistent between the species studied. Similar results were reported by Sarris and Palade (1979), who observed that murine erythrocyte membranes appear to lack an equivalent of Bands 7 and 8.

In man, two systems of nomenclature have been devised for the RBC membrane glycoproteins, designating them as glycophorins (Marchesi *et al.* 1976) or sialoglycoproteins (SGPs) (Anstee *et al.* 1979). In the current work, equivalent glycoproteins appeared to be present in the erythrocyte membranes of the other mammals studied, but there was marked variation in their number and molecular mass. It would, therefore, not be appropriate to extend either system of nomenclature directly from man to other species. Such variation in the pattern of RBC glycoproteins after SDS-PAGE analysis contrasts with the overall similarity of the proteins. Despite one early report which claimed that the erythrocyte glycoproteins of different mammals are similar (Lenard 1970), the results of most previous work support the current findings (Carraway and Kobylka 1970; Hamaguchi and Cleve, 1972; Kobylka *et al.* 1972; Sarris and Palade 1979). However, there is little consensus as to the number, or molecular mass of PAS-staining bands in species other than man. Thus, in the rat (Glossman and Neville 1971; Noeman *et al.* 1980; Edge and Webber 1981; Laing *et al.* 1988), the mouse (Sarris

and Palade, 1982; Hitsumoto *et al.* 1984), the sheep (Lenard 1970; Hamaguchi and Cleve 1972; Kobylka *et al.* 1972) and the dog (Lenard 1970) conflicting results have been reported. A number of factors may be responsible for these apparent discrepancies, including the effects of proteolysis (Sarris and Palade, 1982), acrylamide concentration (Kobylka *et al.* 1972) and the buffer system used in SDS-PAGE (Sarris and Palade 1982). In man, marked differences in the pattern of erythrocyte glycoproteins have been attributed to the use of the continuous (Fairbanks *et al.* 1971) or discontinuous (Laemmli 1970) buffer systems, such that the nomenclature of bands identified in the two systems is not interchangeable (Thompson and Maddy 1982). Glycoproteins are particularly prone to anomalous migration in SDS-PAGE, since uncharged carbohydrate can lead to overestimation of molecular mass (Segrest *et al.* 1971) and negatively charged sialic acid residues have the opposite effect (Brown *et al.* 1981). Furthermore, the glycophorins of man (Thompson and Maddy 1982) and the rat (Laing *et al.* 1988) have been shown to dimerise in SDS-PAGE, whereas other components are denatured. The high-molecular-mass PAS-positive bands from human and sheep RBC membranes in the current study may be large aggregates of glycophorins which are stable in SDS (Mueller *et al.* 1976).

The strong PAS-positive bands observed in the present study, running below the dye front after SDS-PAGE, represent lipids, which may stain intensely even if not glycosylated (Fairbanks *et al.* 1971). It is important to wash the SDS from gels thoroughly or proteins may also be inappropriately developed by PAS (Glossman and Neville 1971). The observed interspecies variation in PAS staining and apparent molecular mass of Band 3 may reflect differences in the glycosylation or sialation of this protein.

The silver stain used in the current work appears to have several advantages over more conventional methods for detecting RBC membrane components after SDS-PAGE. More protein bands can be identified than with Coomassie Blue, since not only is the technique more sensitive, but the use of formaldehyde fixation ensures that smaller peptides are not lost from the gel by diffusion (Burk *et al.* 1983). Also, PAS-positive glycoproteins are stained at the same time, with differential colouration which enables them to be distinguished from the proteins in a number of species. Similar use of silver staining to identify RBC glycoproteins in polyacrylamide gels has been reported previously in man (Dzandu *et al.* 1984) and the rat (Laing *et al.* 1987).

The physiological role of the glycoproteins is unclear, although they are known to carry lectin and virus binding sites and blood group determinants in man (reviewed by Marchesi *et al.* 1976) and have been implicated as autoantigens in canine autoimmune haemolytic anaemia (Barker *et al.* 1991). Their intense staining with PAS indicates a high degree of sialation, which clearly provides a strong negative charge on the surface of the erythrocyte membrane. It has been

suggested that the human glycoporphins play a role in erythropoiesis (reviewed by Fukada and Fukada 1984); however, patients who lack glycoporphin A show no signs of clinical disease (Gahmberg *et al.* 1976), and glycoporphin C appears to form an important link into the cytoskeleton via Band 4.1 (Mueller and Morrison 1980). Whatever the function of the glycoporphins, the current work demonstrates that it clearly does not require a highly conserved structure between different species.

*Acknowledgements.* The author wishes to thank Mr C. Jeal and Mrs P. Stirling for their photographic expertise. The work was supported by the Wellcome Trust.

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