Protein and Lipid Components of the Pigeon Erythrocyte Membrane

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The plasma membrane of the nucleated pigeon erythrocyte was isolated by a method that is simple, reproducible and minimally disruptive, the final preparation consisting of whole cell 'ghosts', recovered at over 40% yield. Alternative methods, which yield membrane fragments, were also tested and some of their possible disadvantages demonstrated. Analysis of the protein components of the isolated membranes by gel electrophoresis in the presence of sodium dodecyl sulphate revealed that their composition is very similar to that of the proteins of human erythrocyte membranes. However, two major proteins are unique to the nucleated cell membrane; these have apparent mol.wts. of 97000 and 57000. Also, the bands designated 4.2 (74500 mol.wt.) and 6 (35000 mol.wt.) by Steck [(1974) *J. Cell Biol.* **62**, 1–19] for the human cell membrane are absent from the pigeon cell membrane. Glycosylated membrane proteins could not be detected in gels stained with the periodate–Schiff–base procedure. Analysis of membrane phospholipids revealed the same components known to be present in mammalian erythrocytes, though in different proportions. These findings are discussed in the light of known physiological and biochemical differences between avian and mature mammalian erythrocytes.

Interest in the avian erythrocyte has developed during recent years, particularly because it possesses a catecholamine-sensitive adenylate cyclase (Davoren & Sutherland, 1963; Bilezikian & Aurbach, 1973) and therefore provides an unusually convenient and simple cell preparation for the study of this activity. Perhaps not so well known are the Na⁺-dependent transport systems for amino acids, which similarly distinguish the avian from the mature mammalian erythrocyte (Vidaver, 1964; Wheeler & Christensen, 1967; Eavenson & Christensen, 1967). Here again the avian erythrocyte is particularly convenient for the investigation of such transport processes and has provided considerable information about them. A complete understanding of the transport and the hormonal activities associated with these erythrocytes must await the elucidation of the fine structure of the membrane. The relatively recent developments in our understanding of membrane structure have depended to an overwhelming extent on the characterization of the mature mammalian erythrocyte membrane. The obvious simplicity of the cell, the ease of preparing pure plasma membranes from it and its ready availability have rendered it highly suitable as research material. The physiologically more complex nucleated avian erythrocyte might be expected to reflect its activities in its membrane

structure and it therefore provides a system of considerable interest, a 'step' more complex perhaps than its mature mammalian counterpart.

That this situation has been recognized is shown by recent publications describing the isolation and characterization of plasma membranes from both turkey (Caldwell, 1976) and chicken (Blanchet, 1974; Jackson, 1975; Weise & Ingram, 1976) erythrocytes. The other commonly used avian erythrocyte, particularly in connection with membrane transport studies, is that of the pigeon. In the present paper we have critically examined methods of isolating plasma membranes from these cells and present a characterization of the pigeon erythrocyte membrane in terms of its protein and lipid constituents.

Materials and Methods

Materials

Acrylamide and *NN'*-methylenebisacrylamide, especially purified for electrophoresis, were obtained from British Drug Houses, Poole, Dorset, U.K. Reagent-grade potassium tetrathionate was purchased from the same company. Phospholipid standards were obtained from Lipid Products, Redhill, Surrey, U.K., and ¹²⁵I was from The Radiochemical Centre, Amersham, Bucks., U.K., as carrier-free Na¹²⁵I. All other materials were of the highest grade available.

Abbreviation used: ATPase, adenosine triphosphatase.

Methods

Buffers. A combination electrode was used to adjust the pH of buffers at 4°C, hence both the glass and reference electrodes were at this temperature.

Preparation of pigeon erythrocyte plasma membranes. A feral pigeon was anaesthetized with ether and bled from a vein in the neck into 20 ml of 0.15M-NaCl/10mM-Tris/HCl (pH 7.4 at 4°C) containing approx. 500 USP units of heparin. All procedures were then carried out at 4°C unless stated otherwise. The blood was centrifuged at about 2000g for 5 min and the serum and 'buffy coat' were removed by aspiration. The cells were then washed three times by re-suspension and centrifugation in 0.15M-NaCl/10mM-Tris/HCl (pH 7.4 at 4°C). Approx. 10 ml of washed packed cells were obtained from a bird. The following procedures were modified from those of Ginsberg *et al.* (1976) and were performed in a Christ-UJIKS centrifuge unless stated otherwise.

The washed cells (1 vol.) were lysed with a buffer (10 vol.) containing 3mM-NaCl/2mM-MgCl₂/8.5mM-Tris/HCl (pH 7.8 at 4°C) (buffer 1) and were then centrifuged at 3100g for 7 min. The supernatant was removed and the nucleated 'ghosts' resuspended in buffer 1. The adherent small white pellet was discarded. The washing and centrifugation process was repeated until the pellet was creamy white (up to six washes). The 'ghosts' were washed once in 3mM-NaCl/0.2mM-MgCl₂/8.5mM-Tris/HCl (pH 7.8 at 4°C) (buffer 2), the final pellets pooled and made up to 15 ml with buffer 2 in a glass homogenizing vessel (capacity 20–25 ml). Extrusion of the nuclei was effected by ten vigorous passes of a tight-fitting motor-driven Teflon pestle operating at 600–700 rev./min (clearance 120 µm). The enucleation process was monitored by phase-contrast microscopy and homogenization continued (if necessary) until the extent of enucleation was approx. 60%. The homogenate was made up to about 80 ml (with buffer 2) and centrifuged at 300g for 10 min (the nuclei had already begun to aggregate and sedimented slowly at 1g). The nuclear pellet was gelatinous and could not be resuspended, whereas the supernatant was cloudy and rich in membranes and nucleated 'ghosts'. Centrifugation of this supernatant at 3100g for 10 min sedimented the nucleated 'ghosts', leaving the membranes in suspension. The supernatant was carefully removed with a pipette and centrifuged at 3100g for 10 min. The resulting supernatant was removed and centrifuged in an MSE 18 centrifuge operating at 38000g_{max.} for 20 min. The white pellet was taken up in 2–3 ml of buffer 2. The protein concentration was 2–5 mg/ml. The membranes were either stored at –20°C or taken up immediately in sample buffer for electrophoresis. They were visible as whole 'ghosts' under the phase-contrast microscope and neither nuclei or nucleated 'ghosts' were

detectable. Pigeon erythrocyte plasma membranes were also prepared by the method described by Caldwell (1976) for turkey erythrocytes and by the method described by Harris & Brown (1971) for the isolation of chicken erythrocyte nuclei.

Pigeon erythrocyte nuclear envelopes. Nuclei were prepared by the method of Harris & Brown (1971) and then sonicated on ice for 2 min at 8 µm amplitude in an MSE 150W ultrasonicator. The suspension was centrifuged at 38000g_{max.} for 20 min and the pellet washed three times with 1mM-NaHCO₃ (pH 7.2 at 4°C). This crude nuclear-envelope preparation was analysed by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulphate.

Human erythrocyte membranes. Human erythrocyte 'ghosts' were prepared as described by Fairbanks *et al.* (1971) and by a procedure similar to that described above for the pigeon erythrocyte membrane preparation.

Use of proteinase inhibitors. Tetrathionate (S₄O₆²⁻), a proteinase inhibitor shown to affect the preparation of chicken erythrocyte membranes (Jackson, 1975; Weise & Ingram, 1976) was used. Plasma membranes were prepared exactly as described above. The composition of buffer 1 was changed to 5mM-K₂S₄O₆/2mM-MgCl₂/7mM-Tris/HCl (pH 7.8 at 4°C) and that of buffer 2 was changed to 5mM-K₂S₄O₆/0.2mM-MgCl₂/7mM-Tris/HCl (pH 7.8 at 4°C) for these experiments.

Enzyme and chemical analyses. The (Na⁺ + K⁺)-dependent ATPase activities of whole cells, nucleated 'ghosts' and plasma membranes were measured as described by Wheeler *et al.* (1975), except that P_i was measured by the method of Baginski *et al.* (1967). Lactate dehydrogenase activity was assayed as described by Bergmeyer & Bernt (1974); the fall in NADH absorption at 340 nm was measured with a Cary 15 spectrophotometer. Succinate-cytochrome *c* reductase activity was assayed by the method of Lee *et al.* (1967). DNA was measured by the method of Kissane & Robbins (1958).

Iodination of whole cells. The procedure of Boxer *et al.* (1974) was adopted. Packed, washed whole cells (0.2 ml) were iodinated at 20°C for 30 min in a final volume of 0.4 ml of 310 mosM-sodium phosphate buffer, pH 7.4, containing 5 µM-KI, 8.5 mM-glucose, 0.4 mg of lactoperoxidase/ml, 1 µg of butylated hydroxytoluene/ml and 0.5 mCi of Na¹²⁵I (carrier-free). The reaction was initiated by the addition of 0.005 unit of glucose oxidase (Sigma). Afterwards the cells were washed six times by centrifugation (1500g, 2 min approx.) and resuspension in 0.15M-NaCl/5mM-Tris/HCl (pH 7.4) and then mixed with unlabelled cells. Membranes were prepared as described above and samples of material at various stages of the preparation were counted for radioactivity on a Nuclear-Chicago 1195 series gamma counter.

Lipid analyses. Phospholipids were extracted from

purified plasma membranes by the method of Folch *et al.* (1957), but omitting the 'salt-wash'. Two-dimensional t.l.c. and procedures for quantitative analysis were similar to those of Rouser *et al.* (1970). The 'activated' plates containing silica gel H (Merck, Darmstadt, Germany) magnesium acetate (40:1, w/w) were run in the solvents described (Rouser *et al.*, 1970). Phospholipid spots, revealed by exposure to iodine vapour, were scraped off the plate and each was made up to 50 mg by addition of silica gel from a blank area of the plate. Silica blanks and P_i standards were made up in the same way. After neutralization of silicate with HCl (Rouser *et al.*, 1970), 0.4 ml of 70% (v/v) $HClO_4$ was added and digestion of organic material effected by heating at 180°C for 2 h. After cooling, 1.7 ml of water, 0.6 ml of 1.25% (w/v) ammonium molybdate and 0.3 ml of 10% (w/v) ascorbic acid were added in turn, with mixing after each addition. After colour development at 100°C and sedimentation of the silica gel (1500 g for 5 min) the A_{797} of each tube was measured as previously described (Rouser *et al.*, 1970). The total phospholipid content of plasma membranes was measured as described by Wheeler *et al.* (1975), except that P_i was assayed by the method of Baginski *et al.* (1967). Cholesterol content of plasma membranes was determined by the method of Zak (1957).

Electron microscopy. Nucleated 'ghosts' and purified plasma membranes were fixed in 5% (v/v) glutaraldehyde for 1 h at 20°C, washed twice in 50 mM-sodium cacodylate buffer, pH 7.2, suspended in an agarose gel (about 2%, w/v) and stained with 1% OsO_4 in 50 mM-sodium cacodylate, pH 7.2, for 1.5 h at 20°C. The samples were then dehydrated by immersion in solutions of ethanol (10% to 100%, v/v) and embedded in Spurr's epoxy resin (Spurr, 1969). Finally, thin sections were cut on an LKB 4800A Ultratome and after double staining in uranyl acetate and lead citrate were examined in a JEOL JEM-100S microscope.

Polyacrylamide-gel electrophoresis. Sample preparation, electrophoresis and staining were carried out essentially as described by Fairbanks *et al.* (1971) in the presence of 0.1% sodium dodecyl sulphate.

Samples were solubilized at 100°C for 3 min in a buffer containing (final concentrations) 2.5% sodium dodecyl sulphate, 2% (v/v) 2-mercaptoethanol, 1 mM-EDTA, 10 mM-Tris/HCl (pH 8.0 at 20°C), 2 mM-phenylmethanesulphonyl fluoride (Bretscher, 1971), 5% (v/v) glycerol and 0.01% Bromophenol Blue. The protein concentration was 1–3 mg/ml. Gels (5.6% acrylamide/0.15% bisacrylamide) were polymerized in glass tubes (85 mm × 7 mm) and usually left under electrophoresis buffer overnight. Protein samples (50 µg) were applied to the tops of the gels and electrophoresis was carried out at 7–8 mA/cm of gel until the tracking dye was approx. 1 cm from the end of the gel. Fixing and staining was carried out in

methanol/acetic acid/water (5:1:5, by vol.) containing 0.25% Coomassie Brilliant Blue, and excess stain was removed into methanol/acetic acid/water (2:3:35, by vol.). Gels were stored in 5% (v/v) acetic acid and scanned on a Joyce-Loebl densitometer with the use of a red filter. Molecular weights were determined by the use of a marker mixture consisting of bovine serum albumin (67000) [from Sigma (London) Chemical Co., London S.W.6, U.K.] and catalase (60000) aldolase (40000) and chymotrypsinogen A (25000) [from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K.]. A molecular-weight marker kit supplied by British Drug Houses (mol. wt. range 53000–265000) was used on occasion, in conjunction with the phosphate-buffered system of Weber & Osborn (1969) to obtain better molecular-weight estimates for the slowly migrating polypeptides.

The procedures of Fairbanks *et al.* (1971) and Zacharias *et al.* (1969) were used in attempts to stain gels for carbohydrate.

Protein concentrations. Protein was measured by the method of Lowry *et al.* (1951) as modified by Miller (1959), with bovine serum albumin as a standard.

Results

Identification of plasma-membrane fraction

The plasma-membrane-associated enzyme (Na^+ + K^+)-dependent ATPase was used as a marker during the isolation procedure. Its specific activity was enriched over 50-fold in the plasma-membrane fraction relative to the whole cell, with a yield of 45% (Table 1). 5'-Nucleotidase was not detectable in freshly prepared plasma membranes when assayed by the methods of Belfield & Goldberg (1968) or Zentgraf *et al.* (1971). Caldwell (1976) similarly failed to detect this enzyme in turkey erythrocyte membranes. ^{125}I , covalently attached to cell-surface components, was chosen as a second plasma-membrane marker. The results (Table 1) show a 43-fold increase in specific radioactivity of plasma membranes relative to whole cells and a recovery of 32%. This is a lower recovery than that indicated by the (Na^+ + K^+)-dependent ATPase marker and is due, at least in part, to the persistence of radioactive iodide in the cytoplasm of the whole cells after six washes in iso-osmotic NaCl. This is shown by the recovery of 11% of the counts present in labelled and washed cells in the first supernatant after haemolysis. Yield of plasma membranes computed from recovery of counts in the latter fraction is thus inevitably underestimated.

The plasma-membrane fraction was deemed to be free of cytoplasmic contamination by virtue of the absence of visually detectable haemoglobin and of assayable lactate dehydrogenase. Over 90% of this

Table 1. *Purification of pigeon erythrocyte plasma membranes*

Data were obtained as described under 'Methods'. For ^{125}I the specific radioactivity (c.p.m./mg of protein) of whole cells was set to 1.0 and the specific radioactivity of other fractions adjusted accordingly. $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase activity is expressed as μmol of P_i/h per mg of protein and lactate dehydrogenase activity as nmol of NADH oxidized/min per mg of protein. DNA is expressed as $\mu\text{g}/\text{mg}$ of protein. The results are expressed as means $\pm$ s.e.m.; the numbers of preparations assayed are given in parentheses. Abbreviation: n.d., not determined.

Fraction	Protein Recovery (%)	^{125}I		$(\text{Na}^+ + \text{K}^+)$ -dependent ATPase		Lactate dehydrogenase		DNA	
		Relative specific radioactivity	Recovery (%)	Specific activity	Recovery (%)	Specific activity	Recovery (%)	Specific amount	Recovery (%)
Whole cells	100 (8)	1.0 (4)	100 (4)	0.019 ± 0.002 (3)	100 (3)	3.74 ± 0.14 (3)	100 (3)	42.8 ± 0.14 (4)	100 (4)
Supernatant 1	76.3 ± 2.2 (8)	0.150 ± 0.003 (4)	10.8 ± 0.4 (4)	0.0 (4)	0.0 (4)	4.4 ± 0.3 (3)	94 ± 8 (3)	3.4 ± 0.54 (4)	6.3 ± 0.9 (4)
Supernatant 2	8.5 ± 1.0 (8)	0.27 ± 0.04 (4)	2.2 ± 0.1 (4)	n.d. (4)	n.d. (4)	n.d. (4)	n.d. (4)	n.d. (4)	n.d. (4)
Nucleated 'ghosts'	10.8 ± 0.3 (8)	7.00 ± 0.15 (4)	72.0 ± 2.4 (4)	0.21 ± 0.03 (3)	108 ± 15 (3)	0.0 (3)	0.0 (3)	300 ± 27 (4)	78 ± 3 (4)
Plasma membranes	0.85 ± 0.05 (8)	42.8 ± 1.3 (4)	32.0 ± 2.4 (4)	1.11 ± 0.05 (6)	45 ± 2 (6)	0.0 (3)	0.0 (3)	6.7 ± 0.9 (4)	0.15 ± 0.02 (4)

enzyme was recovered in the first supernatant after haemolysis (Table 1). Electron micrographs (Harris & Brown, 1971; Harris, 1971) have indicated that chicken and turkey erythrocytes may contain a few mitochondria in each cell. Plate 1 shows that these organelles are also found in pigeon erythrocytes, but we were unable to find mitochondria in electron micrographs of the plasma-membrane fraction (Plate 2). Succinate-cytochrome *c* reductase activity was not detectable in any fraction, substantiating Caldwell's (1976) observation that if mitochondria were present in the whole cell they functioned poorly if at all. The possibility of mitochondrial contamination of the membranes was examined further by analysing the isolated plasma membranes for the presence of cytochromes by scanning between 600 and 400 nm in a Perkin-Elmer 356 spectrophotometer. Some samples tested showed a single absorption peak at 423 nm in a reduced-minus-oxidized scan. That this was probably due to residual haemoglobin contamination and not to cytochromes was shown by the absence of α and β peaks from the visible region of the spectrum. This interpretation was confirmed by examination of a carbon monoxide-minus-reduced spectrum of the same sample. This spectrum showed a peak at 419 nm and a trough at 432 nm, characteristic of a haemoglobin-carbon monoxide complex (Chance, 1957). Had there been significant contamination by mitochondrial membranes a peak at 429.5 nm and a trough at 445 nm (characteristic of a cytochrome a_3 -carbon monoxide complex) would have been observed (Chance, 1957).

Nucleated 'ghosts' and nuclei were undetectable in

the plasma-membrane fraction when it was examined by either phase-contrast or electron microscopy (Plate 2). Only 0.15% of whole cell DNA is recovered in the purified membranes (Table 1). Because it is an unusually striking example of the phenomenon, the close association between chromatin and the nuclear envelope has been well studied in avian erythrocytes (Davies, 1967; Everid *et al.*, 1970). The persistence of this association during isolation of the nuclear envelope is shown by the observation of several investigators (Zentgraf *et al.*, 1971; Jackson, 1976) that washes in buffers containing high salt concentrations and even treatments with deoxyribonuclease I fail to remove all the chromatin associated with the nuclear envelope. Indeed, histone proteins feature strongly on electrophoretic separations in polyacrylamide gels of nuclear-envelope preparations (Jackson, 1976). We have used this association as a measure of nuclear-envelope contamination of the plasma-membrane fraction. The results in Fig. 1 show that whereas crude nuclear-envelope preparations are dominated by histone proteins, they do not feature at all on plasma-membrane separations, i.e. there is clearly no gross co-purification of plasma and nuclear membranes.

Effects of different homogenization procedures

The delineating membrane of erythrocytes has, of necessity, to be particularly resistant to shear forces, and so conditions for plasma-membrane isolation from non-circulating cells (e.g. liver and kidney cells) are inadequate for the disruption of the nucleated

EXPLANATION OF PLATE I

Thin section of nucleated 'ghost' preparation

The thin sections were prepared and stained with both uranyl acetate and lead citrate as described in the text. Only plasma membrane is visible around the complete nucleus, but the membranous organelles adjacent to the portion of the other nucleus are assumed to be mitochondria. Scale bar represents 1 μm .

EXPLANATION OF PLATE 2

Thin section of purified plasma membrane preparation

The thin sections were prepared as described in the legend to Plate 1. Apparently intact plasma membranes, free from nuclei and mitochondria, characterize the preparation. Scale bar represents 1 μ m.

Fig. 1. *Electrophoretic profiles of pigeon and human erythrocyte membranes*

Plasma membranes from pigeon and human erythrocytes and a crude preparation of nuclear envelopes from pigeon erythrocytes were subjected to electrophoresis on polyacrylamide/sodium dodecyl sulphate gels, as described in the text. The separated protein bands were stained with Coomassie Blue and the gels scanned on a Joyce-Loebl double-beam densitometer. Bands unique to the avian profile and those common to both profiles are labelled above the scans, whereas those unique to the human profile are identified beneath the scans. The nomenclature used is based on that suggested by Steck (1974). The A_{620} scale refers to the human profile; the pigeon profile is shifted upwards by approx 0.1 A_{620} unit for clarity. (a) Superimposed scans of pigeon (—) and human (---) erythrocyte membrane preparations. (b) Scan of a crude preparation of pigeon nuclear envelopes.

avian erythrocyte. Thus the use of hypo-osmotic buffer solutions is essential, and under these osmotic conditions the inclusion of Mg^{2+} ions is required to maintain the integrity of the nuclei. Following the method described above we observed that insignificant extrusion of nuclei occurred at $2mM-Mg^{2+}$,

whereas in the presence of $0.2mM-Mg^{2+}$ enucleated 'ghost' membranes were visible under phase-contrast microscopy after homogenization (Ginsberg *et al.*, 1976). This method was both simple and reproducible. In addition we believe the isolation of whole plasma-membrane entities, which the method allows,

to be preferable to membrane fragmentation and subsequent collection of these fragments. Two other methods of cell disruption were tested.

The method of Caldwell (1976) was followed, with the use of a Sorvall blender, but the results obtained were variable. Thus although gel-electrophoretic profiles of membranes prepared in this fashion were identical with profiles of membranes prepared as described under 'Methods', yields of membrane material were found to vary. This is probably because [as Caldwell (1976) has pointed out] of individual machine characteristics and wear on the rotor shaft. This method was not therefore used as a routine.

Another method tested was that described by Harris & Brown (1971) for the isolation of chicken erythrocyte nuclei. The disruption method used was sonication and, because of the shear forces induced, membrane fragmentation occurred in iso-osmotic buffers. Electrophoretic separations on sodium dodecyl sulphate/polyacrylamide gels of plasma membranes derived from sonically disrupted cells revealed four additional faint bands compared with separations of preparations derived from the other two disruption procedures. These bands were in the 60000- (two bands), 53000- and 18000-mol.wt. regions of the gel: their origin is uncertain, but we believe them to be artifacts possibly derived from the outer nuclear envelope, which is disrupted by sonication (Harris & Brown, 1971). Alternatively they may be products of proteolytic degradation of the membrane (see below). Because of these anomalies sonication was not used again for the preparation of plasma membranes.

Membrane polypeptides resolved by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

The gel-electrophoretic profile of pigeon erythrocyte plasma membranes revealed by staining with Coomassie Brilliant Blue is shown in Fig. 1(a), together with the pattern obtained when human erythrocyte membranes were prepared in the same buffer solutions. Bands migrating to identical positions on profiles of human and pigeon membranes are considered to be identical or analogous membrane polypeptides. This stated, we can proceed to identify four major changes in the profile exhibited by the pigeon erythrocyte.

(1) The band-3 region (nomenclature of Steck, 1974) in the pigeon membrane profile resolved into two distinct bands. Although the leading edge of this band migrated to the same position in both species, the diffuse 'tail' characteristic of the human membrane profile was replaced in the pigeon preparation by a sharp band of apparent mol.wt. 97000 (band 3.1, Fig. 1a).

(2) Band 4.2 was not observed on the pigeon membrane profile.

(3) The pigeon membrane profile showed a major new band, labelled P4.51 (Fig. 1a), running in the 4.5 region equivalent of the human membrane profile. The apparent mol.wt. of this band is 57000.

(4) On the pigeon erythrocyte profile, band 6 (D-glyceraldehyde 3-phosphate dehydrogenase, Tanner & Gray, 1971) was not observed.

The banding pattern for the human cell membranes was not distinguishable from that obtained with human 'ghosts' prepared as described by Fairbanks *et al.* (1971), although the relative intensities of some bands were slightly different. Haemoglobin was clearly not efficiently removed from human erythrocytes by washing in either buffer 1 or buffer 2. Similarly band 8 (Fig. 1a) and a portion of the 4.5 region were more prominent in human erythrocyte 'ghosts' prepared by the scheme described under 'Methods'. The validity of a direct comparison between electrophoretic profiles of pigeon and human membranes was further checked by washing pigeon erythrocyte plasma membranes, prepared as described above, three times in the 5.0mM-sodium phosphate (pH 8.0 at 4°C) buffer normally used for the preparation of human 'ghosts' (Fairbanks *et al.*, 1971). No change in the banding pattern was observed. Attempts to increase the purity of the pigeon erythrocyte plasma membranes by centrifugation on discontinuous sucrose gradients did not alter the electrophoretic profiles obtained.

Glycoproteins

In common with the other electrophoretic studies on avian erythrocytes we have had great difficulty during this work in staining gels for carbohydrate by the periodic acid-Schiff procedure. The procedures of Zacharias *et al.* (1969) and Fairbanks *et al.* (1971) were tested on gels overloaded 7-8-fold with protein (350-400 µg per gel). Under these conditions all four of the bands characteristic of the human profile that react with the periodate-Schiff reagent were clearly visible. Electrophoretic separations of pigeon erythrocyte membranes stained at the same time, however, revealed only two very faint stained bands in the 70000- and 20000-mol.wt. regions of the gel. These values are unlikely to be reliable, since gels overloaded to this extent show an almost unrecognizable profile when overstained with Coomassie Blue. Clearly a more sensitive procedure, capable of detecting minor carbohydrate-containing components, will need to be used before the pigeon erythrocyte plasma membrane can be characterized in terms of its carbohydrate content.

Lipid composition

The phospholipid content of pigeon erythrocyte membranes is presented in Table 2. The total content was 0.63 ± 0.03 (mean $\pm$ s.e.m. of three determina-

Table 2. *Phospholipid content of pigeon erythrocyte plasma membranes*

Phospholipids were assayed quantitatively as described under 'Methods'. Results are expressed as means $\pm$ S.E.M. of six separate determinations. Recovery of lipid P from t.l.c. plates was $94 \pm 10\%$.

Phospholipid	% of total
Phosphatidylcholine	33 ± 3
Phosphatidylethanolamine	30 ± 1
Phosphatidylinositol	4 ± 1
Phosphatidylserine	8 ± 2
Sphingomyelin	19 ± 1
Unknown*	6 ± 1

* Material remaining at origin and running with the solvent front.

tions) μmol of lipid P/mg of protein and the cholesterol content was 0.51 ± 0.02 (mean $\pm$ S.E.M. of four determinations) $\mu\text{mol}/\text{mg}$ of protein. Hence, if an average mol.wt. of 750 is assumed for the phospholipids, the composition of the membrane by weight was 40% lipid and 60% protein.

We could find no previous report on avian erythrocyte membrane lipids except that of Kleinig *et al.* (1971) for the chicken erythrocyte plasma membrane. They found phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine in exactly the same proportions as we have measured for the pigeon erythrocyte plasma membrane (Table 2). They detected no phosphatidylserine, however, and sphingomyelin accounted for 31% of the total phospholipid. The approximately equimolar ratio of cholesterol and phospholipid observed in mammalian erythrocyte membranes seems also to exist in avian erythrocyte plasma membranes, at least in the chicken (Kleinig *et al.*, 1971) and the pigeon. The phospholipid composition of the pigeon erythrocyte plasma membrane does not correspond closely with any mammalian erythrocyte membrane studied so far. These themselves vary considerably in composition, particularly with respect to the relative proportions of phosphatidylcholine and sphingomyelin (Gurr & James, 1975).

Discussion

Comparison among different avian erythrocyte membranes

We believe this to be the first reported gel-electrophoretic study of the proteins of pigeon erythrocyte plasma membrane, but several reports have appeared for the chicken (Blanchet, 1974; Jackson, 1975; Weise & Ingram, 1976) and one for the turkey erythrocyte membrane (Caldwell, 1976). In those

studies various isolation procedures and different buffer solutions were used and the conditions under which gel electrophoresis was carried out differed from one study to another. However, provided that each gel system was calibrated with proteins of known molecular weight, anomalies should have been largely eliminated. On this basis a tentative comparison among these avian erythrocytes has been made (Table 3).

In general, although molecular-weight estimations differ slightly, the patterns shown by plasma membranes from the avian erythrocytes so far studied are similar. The apparent retention of band 6 (D-glyceraldehyde 3-phosphate dehydrogenase) by the turkey erythrocyte membrane is a curious exception. An explanation for this in terms of the conditions under which the turkey erythrocyte plasma membrane was isolated does not seem likely, since we obtained patterns identical with those in Fig. 1 when that method was used to isolate the plasma membrane from the pigeon erythrocyte.

Effects of membrane fragmentation

Inclusion of 5 mM-tetrathionate throughout our isolation procedure had no visible effect on the electrophoretic profiles obtained. This observation is in marked contrast with the findings of Jackson (1975) and Weise & Ingram (1976), who observed that the use of sodium tetrathionate in their preparative procedures for chicken erythrocyte membranes eliminated three or four bands prominent in a region of the gel profile between bands 1 and 2 and band 3. They concluded that tetrathionate was acting as a proteinase inhibitor and prevented the degradation of high-molecular-weight membrane polypeptides. Blanchet (1974) observed similar bands but did not test the effect of proteinase inhibitors.

A possible explanation of our result in the context of these observations may lie in the methods used to isolate membranes. Blanchet (1974) used a French pressure cell and Weise & Ingram (1976) nitrogen cavitation (2.76×10^6 Pa) to achieve disruption. The result of these 'shock' methods might be a perturbation of the membrane's inner surface architecture and the exposure of sites susceptible to proteinases that were previously protected. Our contention is that whole membrane 'ghosts' retain to a greater extent the architecture manifest in the state *in vivo*, which is presumably resistant to proteinases. (It is of course conceivable that the chicken erythrocyte contains an intrinsic proteinase activity which is higher than that in other avian erythrocytes.)

Differences between avian and mammalian erythrocyte membranes

Although it is possible that the major band (P4.51, Fig. 1a) characteristic of the pigeon membrane is

Table 3. *Comparison among avian erythrocyte membrane polypeptides*

The membranes are compared on the basis of their Coomassie Blue-staining profiles on sodium dodecyl sulphate/polyacrylamide gels. Band nomenclature is that of Steck (1974).

Aspect of membrane	Pigeon (the present paper)	Turkey (Caldwell, 1976)	Chicken	
			(Jackson, 1975)*	(Weise & Ingram, 1976)
Form of band 3, and mol.wts.	Doublet 97000 and 91500	Doublet 105000 and 90000	Doublet 105000 and 100000	Doublet 101000 and 92000
Band 4.2	Absent	Absent	Uncertain	Very faint
Band 6	Absent	Present	Absent	Faint
Major bands unique to avian profile (mol.wt.)	57000	50000–52000	Uncertain	48000–51000

* Jackson (1975) used a preparation composed of nuclear and plasma membranes; comparison with our results is therefore difficult in some regions of the profile.

associated with the established differences in membrane properties outlined in the introduction, there is no direct evidence for this. So far all membrane transport functions identified in mammalian erythrocytes seem to be associated with protein migrating in band 3 (Cabantchik & Rothstein, 1974; Ho & Guidotti, 1975; Lin & Spudich, 1974) and it is possible that polypeptides involved in amino acid transport are also to be found in this region of the gel profile. A protein of 57000 mol.wt. (P4.51, Fig. 1a) might fulfil the size requirement of these systems, but comparison with other studies of avian erythrocytes provides alternative possibilities. It could be tubulin, or a related protein (Zenner & Pfeuffer, 1976); or, as an oligomer, it could be the regulatory unit of adenylate cyclase (Pfeuffer & Helmreich, 1975; Caldwell, 1976).

We have shown that, under the same conditions of isolation, D-glyceraldehyde 3-phosphate dehydrogenase is retained by human but not by pigeon erythrocyte membranes. This does not prove, however, that the enzyme is unable to associate with the pigeon membrane in the intact cell. The observed difference after membrane isolation may be due simply to a different type of enzyme-membrane interaction in the pigeon erythrocyte. The absence of band 4.2 from the pigeon erythrocyte membrane preparation is also interesting, for this protein has been shown, like D-glyceraldehyde 3-phosphate dehydrogenase, to associate with band 3 in human erythrocytes (Yu & Steck, 1974).

Note Added in Proof (Received 19 June 1978)

Recent evidence indicates that the main protein associated with D-glucose transport in human erythrocytes has a molecular weight of 55000 (Kasahara & Hinkle, 1977).

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