Membrane Associated Proteins of Human Erythrocytes

by

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Dedication.

This thesis is dedicated to my three brothers and three sisters and especially to Angela and Horse of Astrakhan the potential megastars of the future.

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Abbreviations

The standard abbreviations, as recommended in the revised "Instructions to Authors" (Biochem. J. (1978) 145, 1-20) are used throughout this thesis with the following additions:-

ATPase	Adenosine Triphosphatase
PBS 7.4	Phosphate buffered saline pH 7.4
TBS 7.4	Tris buffered saline pH 7.4
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulphonylfluoride
DMSO	Dimethylsulphoxide
PCMB	Para-chloromercuribenzoate
EDTA	Ethylenediaminetetracetic acid
TLCK	N-Tosyl-L-lysylchloromethyl ketone
LDL	Low density lipoprotein
P/CL molar ratio	Phosopholipid/cholesterol molar ratio
2,3 DPG	2,3 Diphosphoglycerate
PNPP	Para-nitrophenyl phosphate
RBC	Red Blood Cell.

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SUMMARY

Phosphatase activities of normal mature human erythrocytes have been measured using p-nitrophenyl phosphate and a radio-phosphorylated casein substrate. Most of the p-nitrophenyl phosphate phosphatase activity (99.9%) could be removed from the membranes by repeated washing. The small amount of activity remaining associated with the membrane had similar properties (acidic pH optimum, inhibition by Pi, F^- and Ca^{2+}, and activation by EDTA) when compared to the soluble enzyme. Lysis of red cells in the presence of calcium ions (0.1 to 1.0mM) increased the phosphatase activity associated with the membranes, suggesting that calcium potentiates the binding of cytoplasmic phosphatases to the membrane.

Erythrocyte protease activities were measured using a radio-iodinated casein substrate. Approximately 18% of the total cellular proteolytic activity remained associated with the membranes after exhaustive washing of the ghosts. Of the membrane associated activity about 10% was detected on intact cells and is presumably located on the cell exterior. Proteolytic activities associated with the membrane and cytosolic fractions both showed two pH optima in the regions of pH 2.5 and 8.0. The activities measured at neutral pH, using the membrane and cytosolic fractions, were both inhibited by each of the following, PMSF, PCMB, DTT and EDTA, but to different degrees with respect to each inhibitor. Lysis of the cells in the presence of calcium ions (0.1 to 1.0mM) increased the level of the proteolytic activity associated with the membranes suggesting that calcium also potentiates the binding of cytoplasmic proteases to the membrane.

Changes in the binding pattern of erythrocyte membrane proteins were examined, using SDS-polyacrylamide gel electrophoresis, as an additional means of detecting proteolytic activity. Characteristic changes in the protein binding pattern were observed after the incubation of membrane preparations. These changes were accelerated by lysis of red cells in the presence of calcium and could largely be inhibited by the addition of PMSF (1mM).

Phosphatase and protease assays were performed on erythrocytes from a patient with spur cell anaemia. These samples showed elevated phosphatase and protease activities associated with the membranes when assayed using p-nitrophenyl phosphate and [^{125}I]-casein substrates respectively. Rapid degradation of the membrane proteins isolated from spur cells occurred following their isolation and could largely be inhibited by the addition of PMSF (1mM) at lysis.

The results obtained suggest that elevated intracellular calcium might be an important factor in the degradation of erythrocyte proteins. A possible role for elevated intracellular calcium in spur cell anaemia and in other disorders associated with altered erythrocyte morphology is discussed.

The possible contribution of contaminating leucocyte enzymes to the observed enzyme activites of erythrocytes was also examined. Leucocyte contamination had little effect on phosphatase assays, however the complete removal of white cells was shown to be an essential prerequisite for the assessment of erythrocyte membrane protease activites.

1. INTRODUCTION

1.1 Human Erythrocytes.

1.1.1 The circulatory survival of the erythrocyte.

The most immature form of the red blood cell that can be identified as belonging to the erythrocyte series is the proerythroblast. By an orderly irreversible process of multiplication and maturation this cell produces adult erythrocytes after approximately 6 days (Erslev & Silver, 1967). During this time the nucleus disappears from the cell by extrusion (Pease, 1956) and haemoglobin is synthesised (Stohlman, 1970). Very little is known concerning the mechanism whereby cells are released from the marrow to be delivered into the peripheral blood. However it is the circulating reticulocyte, which comprises about 1% of the total red cell number (Young & Lawrence, 1945), that finally differentiates to give the mature erythrocyte.

Haemoglobin constitutes almost 90% of the dry weight of the erythrocyte and functions as a carrier of oxygen. This high protein concentration also serves to buffer the blood against the variable pH changes encountered during the gas exchange process (Lehmann & Huntsman, 1961). The life span of haemoglobin is on average 120 days compared to the average 20 days for plasma proteins (White et al., 1973). The ^{51}Cr isotope labelling studies of Ebaugh (1953) revealed that the mean circulatory life span of the erythrocyte was 113 days, during which time the cells pass through the heart more than 500,000 times, making a 300 mile-long journey (Lux, 1979).

1.1.2 Erythrocyte Morphology.

Red cells are by far the most numerous of the blood cells; for every white cell there are around 500 erythrocytes and around 30

platelets. The mature erythrocyte measures approximately 7μm in diameter and appears as a biconcave disc with no nucleus (Richmond and Davies, 1968). The biconcave form of the erythrocyte is thought to allow optimum diffusion of gases by permitting a more uniform proximity of the surface membrane and the internal haemoglobin than would be possible in a spherical cell (Wintrobe, 1968).

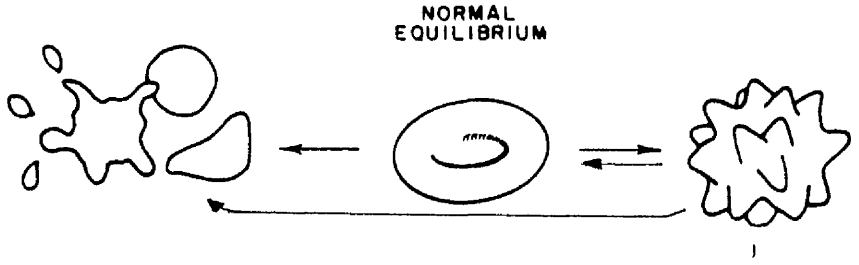
The erythrocyte requires ATP to maintain normal membrane shape and deformability *in vitro* (Nakao *et al.*, 1960; Weed *et al.*, 1969). Transformation of erythrocyte morphology from the biconcave disc to the echinocyte and then to the spherocyte occurs during ATP depletion (Figure 1). Discocytes have a flexible membrane which can accommodate changes in cell shape imposed on the cells during their circulatory journey. Echinocytes have a more spherical shape and are covered in regular thorny projections. Spherocytes are small spherical cells which have a reduced surface area to volume ratio, compared to discocytes and echinocytes, due to loss of the membrane in the form of vesicles (Lutz *et al.*, 1977a).

1.1.3 Energy Metabolism of the Human Erythrocyte.

The red blood cell is the site of numerous metabolic activities, most of which are concerned with maintaining the integrity of the cell membrane and preserving optimal osmotic conditions within the cell (Wintrobe, 1974).

The erythroblast is able to synthesise DNA, RNA, lipid, haem and protein; it has an electron transfer system, a functional citric acid cycle, a hexose monophosphate pathway and the Embden-Meyerhof pathway (Richmond & Davies, 1968). In the circulating non-nucleated erythrocyte most of these activities have been lost; the source of energy is glucose

FIGURE 1. MORPHOLOGICAL CHANGES OF THE ERYTHROCYTE DURING ATP DEPLETION.



SPHEROCYTE: fragile membrane which fragments easily.

DISCOCYTE: flexible, durable membrane.

ECHINOCYTE: rigid membrane covered in regular spikes.

which has to be transported across the membrane (Jung, 1975) and only two of the energy producing reactions are retained. Approximately 90% of the glucose utilized is degraded to lactate by the Embden-Meyerhof pathway and the remaining 10% is accounted for by the pentose phosphate pathway (Murphy, 1960). The energy liberated by anaerobic glycolysis in the erythrocyte is utilised to form the high energy phosphate bonds of ATP. Such energy is subsequently used for preserving the cell membrane and for the transport of ions and nutrients into and out of the cell (Harris & Kellermeyer, 1972). Reducing power is generated in the conversion of NAD^+ to NADH, the form of the coenzyme which reduces methaemoglobin to haemoglobin. 2,3 Diphosphoglycerate (2,3 DPG), an important modulator of haemoglobin affinity, is also synthesised in this pathway. The operation of the pentose phsophate pathway yields NADPH which is used to maintain a pool of reduced glutathione in the cell to protect against the effect of oxidants (Srivastava, 1977).

1.1.4 Erythrocyte Membrane composition.

1.1.4.1 Erythrocyte Membrane Isolation and characterisation.

Red cell membranes can be purified after osmotic haemolysis in dilute (hypotonic), mildly alkaline buffer by repeated washing until the ghosts or stroma are free of haemoglobin (Dodge *et al.*, 1963). The proteins can be separated from the lipids by extraction of the latter in polar organic solvents. A few percent of the protein is often lost to the organic phase, suggesting the presence of hydrophobic proteins or proteolipid complexes. Approximately 52% of the membrane mass is protein; 40% is lipid and 8% is carbohydrate (Dodge *et al.*, 1963). Only about 7% of the sugar is in the form of glycosphingolipids (Sweeley & Dawson, 1969); the rest appears as small oligosaccharides linked via O- and N-glycosidic bonds to serine (or threonine) and asparagine residues in the membrane glycoproteins (Winzler, 1969).

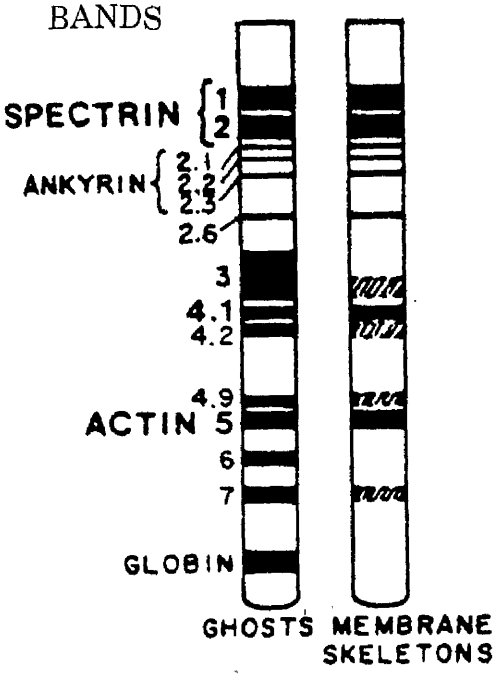
A simple, sensitive, high resolution technique for separating erythrocyte membrane proteins was introduced by Fairbanks et al., (1971). It involves the polyacrylamide gel electrophoresis (PAGE) of isolated erythrocyte membranes dissolved in sodium dodecyl sulphate (SDS). The anionic detergent denatures and solubilizes the membrane polypeptides. It binds in a constant weight ratio to the proteins and allows the resolution of molecular species which have different average molecular weights. However the molecular weight of glycoproteins cannot be accurately assessed using this system because they may bind less SDS per gram than proteins and hence migrate anomalously slowly.

The nomenclature, for red cell membrane protein banding patterns on SDS-polyacrylamide gels, established by Fairbanks et al., (1971) has been adopted universally (See Figure 2).

1.1.4.2 Asymmetry of the Membrane Structure.

The erythrocyte membrane is a well studied example of an asymmetrical membrane at which selective association reactions occur. Both membrane proteins and lipids are asymmetrically disposed across the membrane (reviewed Marchesi et al., 1976). Peripheral membrane proteins are defined as loosely-associated membrane components and may be identified using mild extraction techniques which do not disrupt the lipid bilayer. The relative ease of extracting spectrin from ghosts, using mildly alkaline low ionic strength buffers containing chelating agents, led Marchesi et al., (1969) to propose that spectrin was loosely associated with the erythrocyte membrane.

FIGURE 2. SDS-POLYACRYLAMIDE GEL PATTERN OF ERYTHROCYTE GHOSTS.



BAND	($x10^3$) MOL WGT
1	250
2	220
3	90
4.1	78
4.2	72
5	43
6	35
7	29

Only a few of the major protein components, associated with the membrane, actually span the membrane. Among these are the integral membrane proteins band 3 and glycophorin (Yu et al., 1973). These authors define an integral membrane protein as one which may be liberated from a membrane preparation only after the membrane lipids have been solubilized using detergents.

Most of the data in support of this general model of the membrane has been obtained using analytical techniques, such as the proteolytic treatment and radiolabelling of resealed right-side-out or inside-out erythrocyte ghosts (reviewed Steck, 1974). Studies of this nature using lectin molecules led to the discovery that all of the cellular carbohydrate on the membranes was located at the extracellular surface (Nicolson & Singer, 1971).

1.1.4.3 Membrane Lipids.

Lipids account for approximately 50% of the human erythrocyte membrane (Dodge et al., 1963). Phospholipids and small amounts of glycolipid constitute the polar lipid fraction and cholesterol is the other major lipid. The phospholipids which predominate in human erythrocytes are phosphatidylcholine (lecithin), phosphatidylserine, phosphatidylethanolamine and sphingomyelin. There are no triglycerides but there are a few free fatty acids (Cooper, 1978).

The turnover of phospholipid fatty acids in the erythrocyte membrane in situ is limited to deacylation of endogenous phospholipid and reacylation of the resulting lysophospholipids and/or exchange of intact phospholipid molecules with exogenous phospholipids (Oliveira & Vaughan, 1964). Erythrocytes cannot alter fatty acid chain length; degree of saturation or synthesise phospholipid de novo (Mulder & Van Deenen,

1965).

The close interposition and packing of sterols and phospholipids is the main contributing factor in determining the fluid quality of the membrane. Saturated acyl chains in phospholipids form ordered membranes in which fluidity is of low magnitude, conversely unsaturated acyl chains form membranes in which the fluidity is of high magnitude (Shinitzky & Henkart, 1979). The membrane lipid fluidity is described as the amount of molecular motion within the bilayer. This motion appears to be greatest within the hydrophobic core of the membrane and least near the hydrocarbon-water interface.

1.1.4.4 Membrane Cholesterol.

Gould et al., (1955) observed that only cholesterol and not cholesterol esters are present in erythrocytes. Cholesterol does not exist as a solute in water and cannot form micelles or liposomal membranes by itself, but it can be solubilized by association with plasma phospholipids and lipoproteins (Cooper, 1978). Cholesterol exchanges between membranes and lipoproteins through a simple equilibrium diffusion mechanism (London & Schwartz, 1953). Studies using whole plasma and isolated plasma lipoproteins both in vivo and in vitro have demonstrated that the equilibrium involves the entire free cholesterol pools of both plasma lipoproteins and erythrocyte membranes (Basford et al., 1964). This process of equilibrium has a half-time of 2 hours (Gould et al., 1955).

Lange and D'Alessandro (1980) were successful in determining the proportion of the membrane cholesterol which participates in the exchange diffusion process. Their study demonstrated that two distinct kinetic pools of cholesterol exist in erythrocyte membranes.

Approximately 10% of the cholesterol was found to exchange rapidly with that of the plasma, while the rest of the membrane cholesterol exchanged at a slower rate.

One constant feature of mammalian erythrocyte membranes is that the molecular ratio of cholesterol to phospholipid (C/PL mole ratio) is always between 0.9 to 1.0 (Rouser et al., 1968).

1.1.5 Erythrocyte Membrane Protein Components.

1.1.5.1 Band 3.

Band 3 is the major transmembrane glycoprotein of human erythrocytes and has been reported to mediate anion transport (reviewed Marchesi et al., 1976). On the basis of an assessment of published Coomassie blue-stained gel patterns (Jones & Nickson, 1980) band 3 consitutes 24% of the erythrocyte membrane polypeptides and has a molecular weight of 90,000. After SDS-polyacrylamide gel electrophoresis this protein component appears as a diffuse band. The broadness of the band has been largely attributed to the heterogeneity arising from varying degrees of band 3 glycosylation. Tanner and Boxer (1972) reported that band 3 contains approximately 10% carbohydrate and it is believed that there is a considerable degree of uniformity of the polypeptide chains in the region of 90% homogeneity (Steck, 1978). There is evidence, however, that there are multiple minor polypeptide components in band 3 which can be revealed by such techniques as isoelectric focusing (Bhakdi et al., 1976), two dimensional gel electrophoresis (Bhakdi et al., 1975) and immuno-electrophoresis (Golovtchenko-Matsumoto & Osawa, 1980).

Although the major function of the bulk of band 3 polypeptides is thought to be anion transport, numerous other functions have been

attributed to the minor components ranging from water transport (Brown et al., 1975), cation transport (Grinstein & Rothstein, 1978), ATPase activity (Avruch & Fairbanks, 1972) and cholinesterase activity (Bellhorn et al., 1970).

Band 3 has been cleaved, using specific proteases under controlled conditions, to elucidate the positioning of the molecule across the erythrocyte membrane (Jenkins & Tanner, 1975). The extracellular tyrosine residues of band 3 were radioiodinated using lactoperoxidase prior to proteolytic digestion of intact red cells. From the analysis of several radioautographs of peptide maps they deduced that band 3 traversed the membrane at least twice to form an 'S-shaped' structure. The C-terminal segment, which possesses the carbohydrate moieties, was found to extend into the extracellular milieu (Tanner & Anstee, 1976) and the N-terminal segment was found to extend intracellularly.

1.1.5.2 Sialoglycoproteins.

The major sialoglycoprotein of the human erythrocyte membrane is glycophorin A. There also exists a second component glycophorin B and although both polypeptides contain 60% carbohydrate they have different amino acid sequences. Sedimentation equilibrium studies gave the subunit molecular weight of glycophorin A to be 29,000 (Grefrath & Reynolds, 1974) which correlated well with the value obtained from sequencing studies carried out by Tomita et al., (1978).

Fairbanks et al., (1971) described three bands of membrane proteins (named PAS I, II and III) which stained with periodic acid-Schiff's (PAS) reagent after SDS-polyacrylamide gel electrophoresis. The staining property of these gel proteins was largely dependent on the presence of sialic acid. Gel filtration of the sialoglycoproteins showed that

glycophorin A corresponded to PAS-I and glycophorin B to PAS-III, while the proteins eluted from the PAS-II position appeared to be a mixture of both proteins (Janado et al., 1973). To explain the banding complexity of glycophorin, it is assumed that these sialoglycoproteins have a variety of association states which are not disrupted by weak detergents or even SDS except at high temperature.

Sialoglycoproteins comprise approximately 10% of the total membrane protein (Marchesi et al., 1972). The carbohydrate moieties of glycophorin A has MN blood group activity (Morton, 1972) and also acts as a receptor for viruses and plant agglutinins(Springer et al., 1966). The sialic acid residues of the molecule are responsible for most of the negative charge at the cell surface (Eylar et al., 1962).

Structural studies indicate that glycophorin A is made up of three domains composed of two hydrophilic segments which are separated by a region of 22 nonpolar amino acids. It is the N-terminal half of this molecule which contains the carbohydrate associated with this protein (reviewed Marchesi et al., 1976).

1.1.5.3 Spectrin.

The cytoskeleton is a complex of proteins associated with the cytoplasmic surface of the cell membrane. The most predominant constituent of this complex is the high molecular weight protein spectrin which accounts for approximately 75% of the protein mass (Lux et al., 1976). Spectrin is a large, asymmetric molecule composed of two nonidentical polypeptide chains, band 1 (α-subunit; 240,000) and band 2 (β-subunit; 220,000), which are similar in chemistry and structure (reviewed Kirkpatrick, 1976).

The protein is a tetramer composed of two heterodimers associated by end to end interactions and is thought to exist as a flexible rod in solution (Marchesi, 1979). Both subunits have compositions which are relatively high in acidic amino acids. The smaller subunit is multiply phosphorylated by an endogenous cAMP-independent protein kinase (Avruch & Fairbanks, 1974) at four closely spaced sites near the C-terminal end of the molecule.

1.2 Composition of the Erythrocyte Cytoskeleton.

1.2.1 Membrane Protein Organisation.

To survive in the circulation erythrocytes must be robust enough to endure the turbulent cardiac passage, entailing shear-stress forces, and flexible enough to negotiate the narrow portals of the spleen. These dual demands are met by the cytoskeletal protein network which functions as an interior scaffold to maintain red cell shape and deformability (Hainfeld & Steck, 1977).

Operationally the red cell cytoskeleton is defined as the insoluble residue which remains after the extraction of intact cells (Sheetz & Sawyer, 1978) or their isolated ghosts (Yu et al., 1973) with an aqueous solution of the non-ionic detergent Triton-X-100. Under the electron microscope the structure remaining appears as a cross-linked network of twisted irregularly orientated filaments dotted with globular protrusions (Lux et al., 1976). Analysis of this structure, using SDS-polyacrylamide gel electrophoresis, showed that it comprises about 60% of the membrane protein mass (reviewed Lux et al., 1979). It includes all of the spectrin (bands 1 and 2), actin (band 5), ankyrin (bands 2.1 to 2.6) and band 4.1; and a portion of the proteins designated bands 3, 4.2, 4.9 and 7 (Figure 2). Sheetz (1979) demonstrated that by washing the membrane cytoskeleton with hypertonic potassium chloride solution a

portion of the polypeptides were eluted leaving behind a residual structure. This structure was found to contain spectrin, actin, band 4.1 and band 4.9; they designated these as core cytoskeletal proteins.

1.2.2 Erythrocyte Cytoskeletal Protein Interactions.

The first indication that spectrin might interact with integral membrane proteins was built on the knowledge of the transmembrane nature of band 3 and glycophorin (Bretscher, 1971a; 1971b). Cells prepared for electron microscopy by freeze fracture revealed evidence that the lateral distribution of the intramembrane particles in the plane of the membrane was in some way influenced by the intact spectrin network (Elgsaeter & Branton, 1974; Elgsaeter et al., 1976). A direct and quantitative analysis of erythrocyte cytoskeletal protein-protein association was carried out by Bennett and Branton (1977) who measured the reassociation of [^{32}P]-spectrin with the cytoplasmic surface of spectrin-depleted membrane vesicles. Spectrin was found to be indirectly associated with band 3 in the membrane (Bennett & Stenbuck, 1980a). Proteolysis of inside-out vesicles abolished the high affinity binding of spectrin to the membrane. Analysis of the proteolytic fragments led to the identification of the receptor proteins. Bennett and Stenbuck (1980b) named this protein as ankyrin (band 2.1). They also demonstrated that this protein possessed a group of sequence related proteins, bands 2.2 to 2.6, which also contained high affinity binding sites for spectrin. All of these bands are now collectively called ankyrin and they were observed to form a 1:1 complex with band 3 in detergent extracts of spectrin-depleted vesicles (Hargreaves et al., 1980).

The self-association of spectrin into tetramers and interaction, via ankyrin, with band 3 explains how spectrin is anchored to the membrane of the intact erythrocyte, but it does not explain how the

spectrin maintains the rigidity of the interior of the cytoskeleton as for example in the case of the Triton extracted ghosts (Yu et al., 1973). Studies on the reassociation of purified cytoskeletal proteins in solution show that other interactions are involved. Actin and band 4.1 were found to play key roles in maintaining the integrity of the cytoskeleton (Pinder et al., 1979).

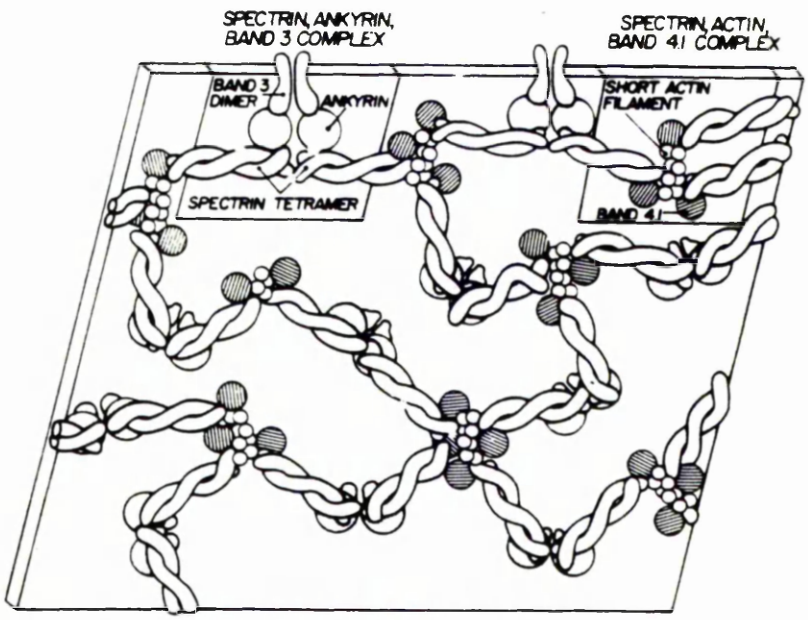
Ungewickell et al., (1979) observed that mixtures of purified spectrin tetramer, polymerised actin (F-actin) and band 4.1, combine at physiological ionic strength into pelletable ternary complexes, and in certain cases gel. No complexes were observed to form if non-polymerised actin (G-actin) was used. Electron micrographs of isolated complexes showed that F-actin filaments were bridged by fine curved fibrils, which were presumed to be spectrin tetramers (Shotton et al., 1979). This suggested that the actin binding regions of the spectrin tetramers were near the distal end of the molecule. Dimeric spectrin was also shown to attach to actin filaments but, being monofunctional, it could not cross-link them.

Figure 3 shows the postulated organisation of the major membrane skeletal polypeptides (not drawn to scale) proposed by Lux (1979). This model indicates the presence of the two main protein complexes which hold the cytoskeletal structure together.

1.2.3 Phosphorylation and Dephosphorylation of Spectrin.

Palmer and Verpoorte (1971) were the first to demonstrate the phosphorylation of proteins in the erythrocyte membrane. Protein bands 1 and 2 (spectrin) are the major extrinsic membrane polypeptides of the red cell cytoskeleton and are multiply phosphorylated (Harris et al., 1978). Spectrin appears to be involved in determining cell shape and

FIGURE 3. POSTULATED ORGANISATION OF THE MAJOR MEMBRANE SKELETAL PROTEINS.

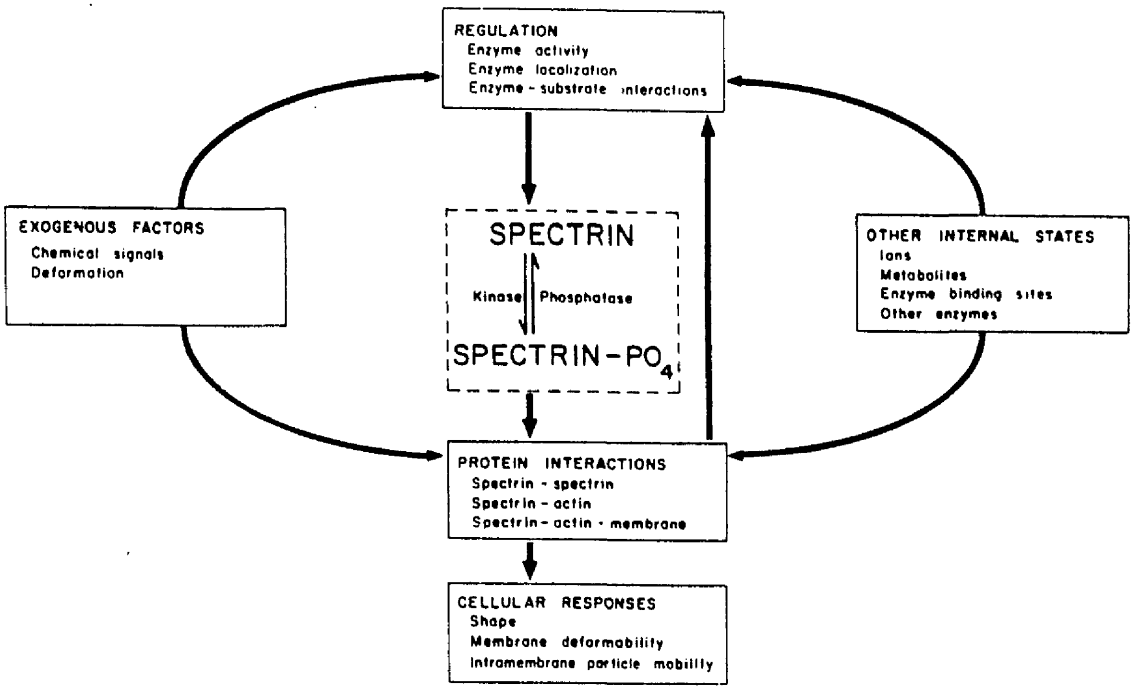


deformability (Lux, 1979). Dephosphorylation of spectrin has been correlated with red cell crenation either by metabolic ATP-depletion (Nakao et al., 1960) or by the incubation of red cells with low density lipoproteins (LDL). Hui and Harmony (1979) incubated intact erythrocytes with LDL and observed a transformation of the erythrocyte biconcave discs to echinocytes and spherocytes after 2 hours. LDL was found to decrease the content of radio-labelled phosphate in spectrin, relative to the control, and to stimulate by a factor of two the membrane-bound phosphatase activites as assayed using p-nitrophenylphosphate as the substrate. Conversely, magnesium ATP-dependent spectrin phosphorylation, in crenated ghosts, was correlated with the restoration of the discocyte morphology (Sheetz & Singer, 1977; Birchmeier & Singer, 1977).

Spectrin phosphorylation is accomplished by cytoplasmic (soluble) and membrane-bound (insoluble) protein kinases which are cyclic AMP-independent (Fairbanks & Avruch, 1974). Spectrin dephosphorylation is less well characterised. Crude cytoplasmic soluble and insoluble protein phosphatases have been identified in the human erythrocyte (Fairbanks et al., 1978), although the positive identification of a membrane-associated phosphatase activity was at one point disputed by Graham et al., (1976).

Since erythrocytes must be metabolically active to maintain their disc shape and deformability perhaps specific metabolic intermediates may indirectly influence cellular morphology. For example, Fairbanks et al., (1978) speculated that either internal signals and metabolic intermediates may modulate both enzyme activities or that the membrane localisation of each enzyme may govern the spectrin substrate availability. They constructed a diagram (Figure 4) to schematically represent the components and interactions of the spectrin

FIGURE 4. THE SPECTRIN PHOSPHORYLATION–DEPHOSPHORYLATION SYSTEM.



phosphorylation and dephosphorylation system.

1.2.4 Calcium and the Human Erythrocyte.

The cytoplasmic concentration of calcium in human erythrocytes is in the range 0.01 to 0.1μM in contrast to much higher calcium levels (10^{-3}M) in the extracellular fluids of the body (Schatzmann, 1975). The erythrocyte controls the calcium ion concentration by means of a membrane that is normally minimally permeable to calcium (Wiley & Shaller, 1977) and by an effective Ca-Mg-ATPase linked to a calcium pump (Schatzmann & Vincenzi, 1969) which in turn is controlled by calmodulin (Jarrett & Penniston, 1977). If a passive calcium influx is not entirely compensated for by active calcium efflux, increased intracellular calcium can lead to pathological states. In certain haemolytic disorders, notably sickle cell disease, calcium-induced cellular rigidity may contribute to the premature destruction of erythrocytes (Eaton et al., 1976).

Increasing the pH (Romero & Whittam, 1971) and the osmotic strength of the medium (Plishker & Gitelman, 1976), exposure of the cells to membrane-active drugs such as para-chloromercuribenzene-sulphonic acid (Garrahan & Rega, 1967), propanolol (Szasz et al., 1977) or the calcium ionophore A23187 (Reed, 1973) also enhance the calcium influx into erythrocytes.

Intracellular calcium is currently thought to exert its effects primarily through the activation of calcium-binding proteins, rather than acting directly in a free state (Jarrett & Penniston, 1977). Calmodulin was shown by Marcum et al., (1978) to catalyze microtubule disassembly and he suggested that it may cause also the disassembly of the spectrin-actin cytoskeleton. Eaton et al., (1976) observed that

calcium introduced into erythrocytes caused them to shrink. Inhibition of intrinsic calmodulin, using trifluoroperazine or phenothiazine, resulted in the swelling of the cell from the shrunken state. Brewer (1980) made the observation that the cell shrinkage was energy-independent and that the cell expansion was energy-dependent. He proposed that the ATP-dependent step involved the phosphorylation of spectrin to allow the assembly of normal cytoskeleton to support the membrane. The role of contractile proteins of the spectrin-actin network in relation to the intracellular concentration of calcium is not clear. However, Palek et al., (1978) and Seigfring & Lorand (1978) have observed that sickle cells or calcium-loaded normal cells exhibited membrane protein aggregation which these authors have suggested was mediated through the calcium-activated transglutaminase in the erythrocyte. Therefore calcium dependent polymerisation of the spectrin proteins by the transglutaminase may contribute to the shrinkage and stiffening of normal red cell membranes under conditions which lead to the intracellular accumulation of this ion (Lorand et al., 1976).

1.3 The Role of Erythrocyte Proteases.

1.3.1 Erythrocyte Proteolytic Activities.

Morrison and Neurath (1953) discovered membrane-associated proteolytic enzymes with optimal activities at pH 3 and pH 8 in human erythrocytes. These two enzymes were recovered in the aqueous phase after n-butanol extraction of ghosts. A third enzyme, which was optimally active at neutral pH, was released from the membranes using 1M potassium thiocyanate.

Moore et al., (1970) extracted a protease from human erythrocyte membranes using 1M salt solutions and purified the enzyme using column chromatography. The proteolytic activity was routinely assayed at pH 7.4

and was found to be active against a wide variety of protein substrates.

Tökés and Chambers (1974) reported the presence of two types of membrane-associated proteases which could digest erythrocyte proteins. The protease which was most active at pH 3.4 was found to digest the membrane to small fragmented peptides. Another protease which had a pH optimum at 8.6 showed a limited digestive capacity on membrane proteins.

Scott (1977) found that diisopropylfluorophosphate (DFP) partially inhibited the immune haemolysis and autolysis of sheep erythrocytes, suggesting that these processes were protease mediated. Scott and Kee (1979) demonstrated that both human and sheep neutral membrane proteases were DFP-inhibitable; had similar molecular weights (25,000); but different specificities towards protein substrates. On the basis of these findings they suggested possible roles for the neutral and acidic membrane proteolytic activities in the autolysis of human erythrocytes.

The identification of specific proteolytic activities associated with human erythrocyte membranes was achieved by Pontremoli et al., (1979). They isolated only proteases having acidic pH optima and the molecular weight values of the enzymes obtained were I 80,000; II 40,000 and III 30,000. To explain the lack of a neutral proteolytic activity on erythrocyte membranes they suggested that this arose from contaminating leucocyte membranes, which they carefully removed from erythrocyte samples. Pontremoli et al., (1980) went on to identify proteolytic activities of the erythrocyte cytoplasm using proteins and various peptide derivatives as substrates. One neutral protease (110,000) and three acidic proteases were isolated which had the same molecular weight values as the membrane-associated proteases. They also identified peptidylamino-peptidase activities in the soluble fraction of human

erythrocytes.

However, Tarone et al., (1979) found a neutral proteolytic activity which was tightly associated with the inner surface of the erythrocyte membrane. This enzyme was found to produce the degradation of band 3 when normal erythrocyte ghosts were incubated for several days at 4°C. This same enzyme, which hydrolyses band 3, was isolated and purified by Golovtchenko-Matsumoto et al., (1982) and was found to have an inactive precursor form (200,000) which was converted to the active form (50,000) via a complex and as yet unidentified process involving calcium. This enzyme was present in erythrocytes from which the leucocytes had been carefully removed. Furthermore Siegel et al., (1980) also identified a neutral proteolytic activity associated with the inner surface of erythrocyte membranes after the red cells had been freed of leucocytes. However, they found that other membrane proteins, in addition to band 3, were susceptible to proteolytic digestion at physiological pH.

1.3.2 The Degradation Pattern of Erythrocyte Membrane Proteins.

Examination of the nature and pattern of proteolysis by enzymes located on human erythrocyte membranes was carried out by Tökés and Chambers (1975). Digestion of the membranes at pH 8.0, after an incubation period of 5 hours at 37°C, indicated that there were two main regions which underwent proteolytic modification. These were the ankyrin band region and the band 4 to band 5 region with the major products appearing as band 2.3 and band 3'. Moreover they reported that band 3 remained unchanged as judged by SDS-polyacrylamide gel scans. However, this does not rule out the possibility that the composition of band 3 may be a mixture of both itself and proteolytic fragments after incubation.

Siegel et al., (1980) demonstrated that the proteins which were most rapidly degraded by neutral membrane proteases when ghosts were incubated for 24-48 hours at 4°C, were the spectrin-binding polypeptides. There are six of these proteins, collectively known as ankyrin (bands 2.1 to 2.6), which anchor spectrin molecules to band 3 in the membrane. By studying the pH optimum, cofactor requirements and inhibitor sensitivity of the membrane proteolytic activity, two enzymatic pathways for the cleavage of the ankyrin band 2.1 were established.

The first pathway involved a sequence of consecutive proteolytic cleavages of band 2.1 to produce band 3', a fragment which has a mobility in between bands 3 and 4. This pathway was found to be inhibited by 1mM phenylmethylsulphonyl fluoride (PMSF). The second pathway was composed of two major steps. The first step cleaved band 2.1 to produce a major fragment which had a mobility equal to that of band 2.3 thus leading to an increase in the staining intensity of ankyrin band 2.3. Step two of this second pathway involved the cleavage of the proteolytic fragment, at the band 2.3 position, to produce band 3'. Calcium was found to stimulate the first step (PMSF-insensitive) and EDTA produced inhibition; the second step was inhibited by 1mM PMSF. This process would be consistent with the apparent induction of membrane protein degradation by calcium ions as observed by Golovtchenko-Matsumoto et al., (1982).

The effect of calcium ions on human erythrocyte membranes is of considerable interest because of their possible involvement in producing changes in erythrocyte morphology (LaCelle, 1970) and rate of cell destruction (Haradin et al., 1969). Carraway et al., (1975) observed that haemolysing erythrocytes in the presence of 1mM calcium caused the

formation of an aggregate which was highly resistant to the disruption by SDS and other denaturing agents. King and Morrison (1977), using SDS-polyacrylamide gel electrophoresis, demonstrated that lysis of human erythrocytes in the presence of 1mM calcium produced a non-irreversible aggregate formed from several membrane proteins; affected the binding of cytoplasmic proteins to the membrane; and activated proteolysis of membrane proteins. Allan and Cadman (1979) found that these calcium induced changes could also be produced by overloading intact red cells with calcium. Moreover, they found that the changes observed in the erythrocyte membrane proteins, from patients with sickle cell anaemia and hereditary spherocytosis, could be reproduced in normal cells using calcium.

1.4 Changes in Erythrocyte Morphology.

1.4.1 Erythrocyte Aging In Vivo.

Senescent erythrocytes amounting to approximately 1% ($2x10^{11}$ cells) of the total red cell population are sequestered daily mainly in the spleen. The exact nature of the aging mechanism is not fully understood, however, the complexity of the system has been recognised by comparing both the chemical and physical properties of mature and effete cells.

Old erythrocytes exhibit alterations in cellular morphology with consequent reduction in their deformability (Figure 1). *In vivo* aged red cells show losses of membrane phospholipids (Prankerd, 1958) and cholesterol (Phillips *et al.*, 1969). Borun *et al.*, (1957) demonstrated that the density of human erythrocytes increases with age because there is less membrane area to contain the same mass of haemoglobin. On the basis of cells having different specific gravities erythrocytes can be separated from a population of cells into groups differing in mean age.

A relationship exists between the fragility of erythrocytes in hypotonic solutions and the shape of the cell. A normal red cell can accommodate changes in osmotic pressure whereas effete cells are more sensitive to hypotonic medium which causes them to lyse (Simon & Topper, 1957).

Decreased deformability in old red cells was observed by LaCelle and Arkin (1970). Deformability properties were measured as the negative pressure at which the erythrocytes entered and traversed calibrated micropipettes ranging in diameter between 2.6 and 3.2μ.

Bernstein (1959) reported that erythrocytes which had a higher than normal density (old cells) had a less than normal rate of glycolysis. This was found to be accompanied by a reduction in the high energy phosphate content and levels of enzyme activites in old cells. Rose and O'Connell (1964) found that the glucose-6-phosphate dehydrogenase, which catalyzes the initial step in the pentose phosphate pathway, 6-phosphogluconic dehydrogenase and phosphohexose isomerase were all present in relatively small amounts in old cells compared to young cells. These enzymes are often used as markers in the age fractionation of cells by density centrifugation.

The reduced level of glycolysis in old cells also leads to the accumulation of sodium ions (Bernstein, 1959) and calcium (LaCelle et al., 1972) because there is not enough ATP to fuel their respective Na/K ATPase and Ca/Mg ATPase active transport mechanisms. A summary of the changes reported to occur on in vivo aging of erythrocytes are in Table 1.

Table 1. Erythrocyte Aging In Vivo

OBSERVED CHANGE	REFERENCE
Loss of phospholipid	Prankerd, 1958
Loss of cholesterol	Phillips et al., 1969
Increased specific gravity	Borun et al., 1957
Increased osmotic fragility	Simon & Topper, 1957
Decreased deformability	LaCelle & Arkin, 1970
Decreased activity of specific enzymes	Rose & O'Connell, 1964
Increased sodium content	Prankerd, 1958
Increased calcium content	LaCelle et al., 1972

The alteration of the erythrocyte membrane with cellular aging is believed to occur by a complex mechanism which is influenced both by intrinsic erythrocyte factors and factors of the vascular system.

Kay (1975) observed that isolated macrophages could differentiate between mature and senescent erythrocytes obtained through the selective binding of immunoglobulin G molecules to old erythrocytes. This antibody binding was further demonstrated by Tannert et al., (1977) who observed the agglutination, by antiglobulin antiserum, of enriched senescent erythrocytes aged in vivo. Kay (1980) isolated an antigen from the surface of erythrocytes which was only exhibited on senescent erythrocytes. The mechanism of the autoantibody recognition of the senescent antigen has been shown to be specifically directed through the Fab region (Kay, 1979). The macrophage subsequently binds to the Fc region, exposed on the coated red cells, to initiate phagocytosis (Berken & Benacerraf, 1966). Exposure of this cryptic antigen, during in vivo aging of the erythrocytes may involve the release of sialic acid, glycopeptides, membrane vesicles or result from changes in the cytoskeleton. Initially the overall changes in composition of the membrane components during in vivo aging were thought to be confined to

the action of a neuraminidase (Danon et al., 1971). However, Baxter & Beeley (1978) observed the loss of other carbohydrates, including sialic acid, from the glycoprotein and glycolipid components of the erythrocyte membranes from old erythrocytes. Sialic acid was also observed to be lost in the form of glycopeptides (Balduini et al., 1974). Therefore it would seem likely to suggest that either the proteolysis of the erythrocyte glycocalyx reveals a hitherto unexposed antigen or direct hydrolysis of one membrane component produces the senescence antigen.

Alternatively, Lutz et al., (1977a) suggested that changes in the structure of the cytoskeleton produced after ATP-depletion alters the association of band 3 in the membrane. Freeze-fracture electron micrographs revealed pairing of the intramembrane particles in the vicinity of vesicle protrusions suggesting major cytoskeletal protein rearrangements in the redistribution of intramembrane particles during vesiculation (Lutz et al., 1977b). Intramembrane particles are believed to be multimeric forms of band 3 which may be associated with other membrane proteins (Elgsaeter & Branton, 1974).

1.4.2 Erythrocyte Aging In Vitro.

Erythrocytes depleted of glucose in vitro undergo changes in their cellular morphology from normal discocytes to echinocytes and then to spherocytes. Weed et al., (1969) established that erythrocytes in vitro require ATP to maintain normal shape and deformability. They observed that effete erythrocytes leak potassium, accumulate sodium and calcium ions, and release membrane vesicles during their shape change transformation to echinocytes and then spherocytes. These vesicles were found to be deficient in spectrin, suggesting a decrease in the interaction between cytoskeletal spectrin and the integral membrane protein band 3 (Lutz et al., 1977a). Moreover Palek et al., (1976)

reported the occurrence of spectrin aggregates in spherocytes obtained *in vitro*.

There has been no direct evidence of vesiculation occurring during the *in vivo* shape transformation of erythrocytes which would account for the decrease in membrane lipid of senescent cells. However, a recent study by Bocci *et al.*, (1980) indicated that the transfusion of rat and human erythrocyte vesicles into rats resulted in their clearance from the circulation (half-life 2 to 8 min.). This suggested that the fast sequestration rates may account for the lack of the identification of erythrocytes vesicles occurring *in vivo*.

ATP-depletion of blood stored for transfusion purposes is associated with disc-sphere transformation, loss of membrane, decrease in critical haemolytic volume and a striking increase in cellular rigidity which is reflected by increased viscosity and decreased cell filterability (Haradin *et al.*, 1969). Improved survival after restoration of cellular ATP, which results from incubation with adenosine, is not associated with any reversal of the lipid loss or increased critical haemolytic volume. Therefore the chances of the cells surviving after transfusion is greatly decreased after prolonged storage because of the increased occurrence of these irreversible changes.

1.4.3 Haemolytic Syndromes Associated with Liver Disease.

In patients with liver disease red cells become target-like in appearance due to the acquisition of and excess of both cholesterol and phospholipid into their membranes from plasma lipoproteins (Cooper *et al.*, 1972). Target erythrocytes are defined as corpuscles with a central rounded area of pigmented material, surrounded by a clear ring lacking pigment, outside of which is the pigmented border of the cell. This

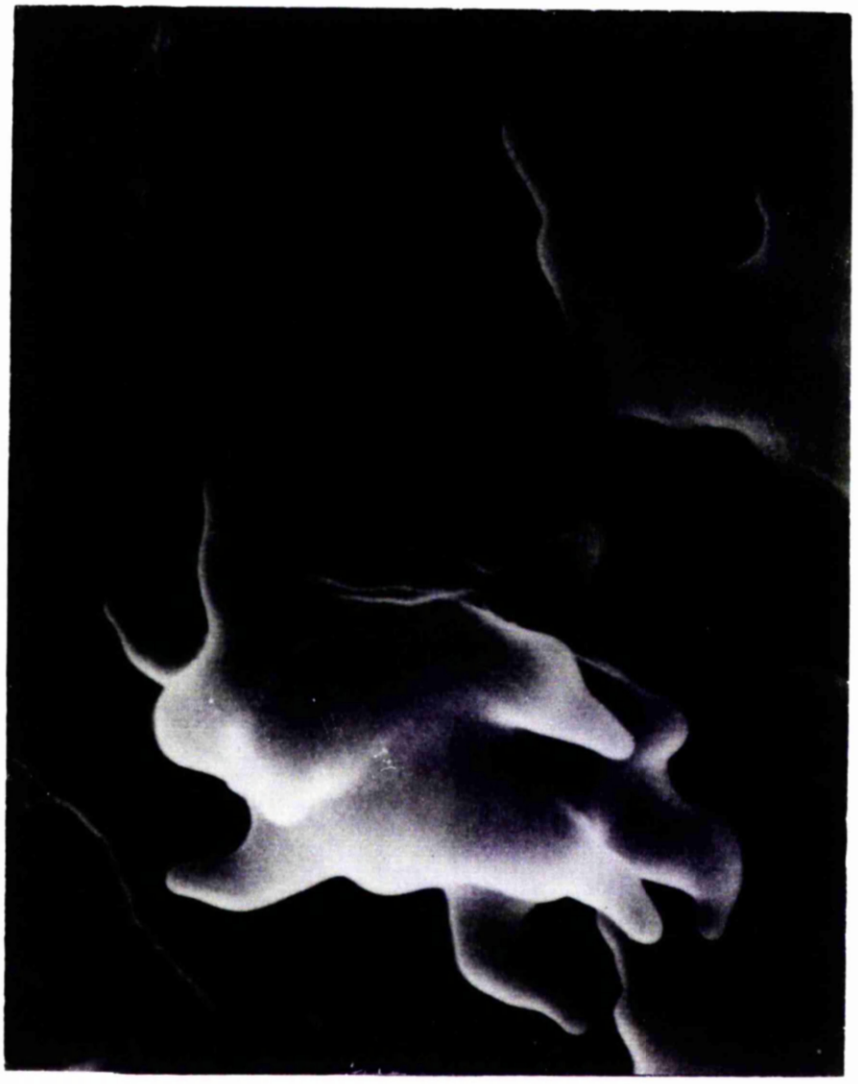
phenomenon occurs in patients with various forms of liver diseases including hepatitis, cirrhosis and obstructive jaundice. The phospholipid increase is largely confined to lecithin. In several patients studied to date membrane fluidity was found to be normal as a result of the acquisition of both cholesterol, which decreases fluidity, and lecithin, which increases fluidity (Neerhout, 1968).

Increases in lipid correlate with increases in membrane surface area as measured by a concomitant increased resistance to osmotic shock (Cooper, 1972). In the absence of congestive splenomelagy target cells survive normally (Cooper & Jandl , 1968).

Spur cell anaemia stems from a primary disorder of plasma lipoproteins and results in the increased cholesterol content of erythrocyte membranes (Cooper, 1969). It occurs in some patients with severe liver disease, usually alcoholic cirrhosis (Smith et al., 1964), but it has also been observed in neonatal hepatitis (Balistreri et al., 1980). Most patients show no symptoms of their anaemia unless haematocrit values are less than 20% (v/v). Reticulocytes increase from the normal 1% (v/v) of the total erythrocyte count, to a maximum of 15%. The disease is generally chronic and fatal but spontaneous remission has been known. Salvioli et al., (1978) reversed the abnormal morphology of target and spur cell erythrocytes after the infusion of polyunsaturated phosphatidylcholine into patients.

The spur cell erythrocytes are bizarrely spiculated and undergo premature destruction primarily in the spleen. Figure 5 shows an electron micrograph of erythrocytes, with the acanthocyte morphology, taken from a patient with abetalipoproteinaemia . It demonstrates the occurrence of irregular thorny projections on the cells giving them an

FIGURE 5. ERYTHROCYTE ACANTHOCYTE MORPHOLOGY.



abnormal morphology which is identical to spur cells. Spur cells have a ^{51}Cr half-life of approximately 6 days (Cooper et al., 1974). Membrane phospholipids are normal and cholesterol is increased by 25 to 65%, however, there is a disproportionate increase in the membrane lecithin content. This change in membrane cholesterol results in an increase of the cholesterol/phospholipid molar ratio to a maximum value of 1.60.

Clinical and experimental observations have evaluated spur cell anaemia as an acquired erythrocyte membrane phenomenon since normal transfused erythrocytes develop this abnormality (Silber et al., 1966). The acquisition of cholesterol by these cells is thought to take place via the exchange diffusion from plasma low density lipoproteins (LDL) which possess the abnormally high cholesterol/phospholipid molar ratio. This phenomenon has been reproduced in vitro by Cooper (1969) when he incubated normal compatible erythrocytes with serum from a patient with spur cell anaemia and found that the membrane cholesterol/phospholipid molar ratio increased. In addition, cholesterol-rich liposomes, prepared by sonication of large amounts of cholesterol and lecithin, have an effect on normal cells which is similar to the spur cell serum. Under all these conditions, increases in the cholesterol/phospholipid molar ratio of red cell membranes led to a decrease in membrane fluidity. However, the complete morphological transformation of normal cells to spur cells is reported only to take place in the spleen. Only echinocytic erythrocytes were observed by Cooper (1969) when normal erythrocytes were incubated with spur cell plasma. Evidence to the contrary was reported by Smith et al., (1964) who formed spur cells in vitro using normal red cells and spur cell plasma.

Thus cholesterol and lecithin, involved in the previously mentioned membrane disorders, are not only the most exchangeable of the major red

cell lipids; but these membrane components also undergo the greatest quantitative change in liver disease.

1.4.4 Haemolytic Syndromes not associated with Liver Disease.

Haemolytic anaemias can be classified as congenital or acquired; they may also be distinguished according to whether the corpuscular destruction occurs as a function of cell age or regardless of cell age.

Congenital abetalipoproteinaemia produces erythrocytes which are morphologically identical to spur cells and are termed acanthocytes. The term refers specifically to the thorny-shaped red cells found in patients with a hereditary absence of low density (beta) lipoprotein, as first described by Bassen and Kornzweig (1950). However the acanthocyte chemistry is distinctively different from that of spur cells. Their cholesterol/phospholipid molar ratio is more or less normal but they contain more sphingomyelin and less lecithin in their membranes. Enrichment of acanthocytes with sphingomyelin at the expense of lecithin results in an overall decrease in membrane fluidity of a magnitude similar to that of spur cell membranes. Moreover, the circulatory survival of acanthocytes is almost normal.

A variety of lipid abnormalities, arising from an increase of cholesterol in the red cell membrane, have been described. In particular, a deficiency in the lecithin:cholesterol acetyltransferase leads to disorders of the membrane cholesterol. The lack of this enzyme, which esterifies plasma cholesterol, leads to the accumulation of free cholesterol in the circulation (Godin et al., 1978).

Hereditary spherocytosis is probably the most common example of a congenital haemolytic disorder (Dacie, 1954). It is a form of jaundice

characterised by spherocytosis of the cells which fragment more easily than normal when they are directly (Smith et al., 1975) or metabolically stressed (Cooper & Jandl, 1969). Investigation into the molecular basis of spherocyte membrane instability has concentrated on three major areas; calcium metabolism; protein phosphorylation and the molecular interactions between the structural proteins of the membranes (Valentine, 1977; Zail, 1977).

Sickle cell anaemia arises from defects in the molecular structure of haemoglobin (Pauling et al., 1949). However, in sickle cell anaemia, it is clear that membrane damage plays an important role in the pathology resulting from this genetic defect. When sickle cells become deoxygenated they assume bizarre shapes caused by the crystallisation of the internal haemoglobin. Upon reoxygenation, the haemoglobin redissolves and most of the cells resume a normal shape but 5 to 50% may remain irreversibly sickled, rigid and incur permanent alterations of their membranes (Brewer, 1980). It is thought that the circulation of membrane-damaged, rigid and irregular shaped erythrocytes results in vascular occlusion. These events are directly related to the discomfort and pain, involving all parts of the body, in individuals with this anaemia (Chien et al., 1970).

The nature and damage to the membranes in the irreversibly sickled cells may involve abnormalities of calcium metabolism. It has been shown that during sickling the normal relative impermeability of the erythrocyte membrane to calcium changes to let calcium into the cells (Eaton et al., 1973) and more haemoglobin binds to the inner surface of the membrane (Palek et al., 1971).

1.4.5 Immunoglobulin G Binding to Erythrocytes.

Immune haemolytic anaemia is defined as an acquired anaemia resulting from premature destruction of red cells by the presence of antibody and/or complement on the red cell surface. Classification schemes have generally centred around the etiology or pathophysiology of the disorder (Wintrobe, 1974). Secondary cases may arise from a variety of underlying disorders of the erythrocyte membrane and ultimately result in the accelerated senescence and premature destruction of the erythrocyte (Engelfriet et al., 1974). One point of interest, which arises from the binding of antibody and complement to erythrocytes, was discovered by Campbell et al., (1979). They demonstrated an increse of the intracellular free calcium ions, from the resting level of 0.1μM to 25μM, when re-sealed pigeon erythrocyte ghosts were incubated with rabbit antibody and guinea-pig complement. Whether the complement induced increase in intracellular calcium concentration mediates other cellular changes, with eventual cell lysis, is unknown.

As discussed in section 1.4.1 the immune-mediated clearance of erythrocytes may also be involved in the sequestration of senescent erythrocytes which have aged normally in vivo.

1.5 AIMS OF THE PRESENT STUDY.

The aim of this study was to investigate the properties of enzymes in human erythrocytes which might be involved in producing changes of the red cell morphology. Attention was focused on the phosphatases and proteases together with the role of calcium in modulating both the activities of these enzymes and their association with the membrane.

Abnormally shaped erythrocytes, from an individual with spur cell anaemia, were studied in parallel with the investigations carried out on normal erythrocytes.

2. MATERIALS

Diethylaminoethyl cellulose (DEAE) was obtained from Whatman Biochemical Company Ltd., Maidstone, Kent.

Bovine serum albumin protein standard solution was obtained from Armour Pharmaceutical Company, Phoenix, Arizona, U.S.A.

Carrier free [^{125}I]-iodine was obtained from the Radiochemical Centre, Amersham.

Heparin, the type used for Rubella Haematology tests (5000 units/ml) was obtained from Flow Laboratories, U.K.

Sodium phenobarbitone (sagatal) was obtained from May and Baker Ltd., Dagenham, England.

High and low molecular weight calibration kits for gel electrophoresis were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Triton-X-114, 2,5-diphenyloxazole, dimethylsulphoxide and ortho-phenanthroline hydrate were obtained from Koch Light Laboratories Ltd., Bucks, Kent.

Ultrafiltration membranes (U.M.10) were obtained from Amicon , Lexington, Mass., U.S.A.

Casein, iodoacetamide, acrylamide and N,N^{1}-methylenebis-acrylamide were obtained from BDH Laboratory Chemical Devision, Poole, Dorset, U.K.

Pyronin Y was obtained from G.T. Gurr, London, U.K.

Phenylmethylsuphonylfluoride, para-chloromercuribenzoate, N-α-p-tosyl-L-lysine chloromethyl ketone, 2,3-dihydroxy-1,4,-dithiobutane, trypsin (EC.3.4.21.4), bovine serum albumin (fraction V), N-chloro-p-toluene-sulphonamide (chloramine T), p-nitrophenylphosphate, 2[N-morpholino]ethane sulphonic acid, adenosine 5'-triphosphate, adenosine 2,5 diphosphate, 2,3 diphosphoglycerate, streptomycin sulphate, benzyl penicillin, haemoglobin (fraction IV), Δ^5-cholesten-3-ol, Coomassie brilliant blue R, Triton-X-100, cellulose, Sigmacell (type 50), Tween-20, human immunoglobin G and horse radish peroxidase (type VI) were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

Micro-Elisa plates (MIC-2000) were obtained from Dynatech Laboratories Incorporated, Alexandria, Virginia, U.S.A.

Other reagents were of Analar grade and obtained from BDH, Laboratory Chemical Division, Poole, Dorset, U.K.

2.1 METHODS

2.2 Separation and characterisation of Erythrocytes.

Normal human erythrocytes were obtained from individual volunteers by venepuncture. Spur cell human erythrocytes were provided from a patient by Dr. P. Mills at the Gastroenterology Unit, Glasgow Royal Infirmary. Rat blood was withdrawn from the anterior vena cava after intraperitoneal injection of heparin (100 units/ml blood). The rats were previously anaesthetised in an ether jar to allow the administration of heparin and a lethal dosage of sagatal (1.0ml for 250-300g of body weight).

Heparinisation (100 units/ml blood) or defibrination was used to prevent blood coagulation. Defibrination was accomplished using one 5mm glass bead per millilitre of blood. The sample was shaken continuously, but gently until the fibrin clot formed on the beads (5 min.). The erythrocytes and serum were decanted from the glass beads.

The removal of leucocytes from blood samples was achieved by one of the following three methods. The efficiency of each method was determined by counting the remaining white cells after staining in 0.1% crystal violet solution (weight dissolved in 1.5% acetic acid) to lyse the red cells. The blood was diluted 1 in 20 using a Thoma red cell pipette and the white cells were counted in an improved Neubauer haemocytometer.

The plasma, buffy coat of white cells and platelets were removed by aspiration after centrifugation of the blood sample at 800g for 10 min. at 4°C. The erythrocytes were resuspended in a buffer which contained 5mM phosphate pH 7.4 and 150mM sodium chloride (PBS 7.4) for a total of four washes. After each centrifugation the surface cells of the packed erythrocytes were also aspirated. This procedure was based on that of Fairbanks et al., (1971).

The method of Tökés and Chambers (1975) was employed in the majority of the erythrocyte experiments which required the efficient removal of leucocytes since less of the white cells remained after using this method. A 30ml volume of erythrocytes (50% v/v) in PBS 7.4 was poured into a 50ml sterile syringe (with a close end) containing loosely packed cotton wool and glass wool up to the 50ml mark. The syringe was sealed at the top end and incubated in a water bath for 30min at 37°C.

The erythrocyte suspension was emptied under gravity and the remaining cells were washed out using PBS 7.4 (50ml) at 37°C. The red cells were then centrifuged and the top layer of packed cells was removed by aspiration.

The method of Beutler et al., (1976) was adopted for some of the experiments. A sample of freshly drawn human blood was defibrinated and washed four times in isotonic sodium chloride solution (150mM) after aspiration of the serum. The erythrocytes were then resuspended in the isotonic solution (50% v/v). A mixture of microcrystalline cellulose and α-cellulose (equal parts by dry weight) was made up in isotonic saline solution and poured into a 5ml plastic syringe with a closed end. The syringe was plugged with a small amount of cotton wool to hold in the cellulose and the dimension of the column was 1.6cm x 1.2cm (i.d.). The erythrocyte suspension (up to 20ml) was passed through the column with a flow rate of approximately 0.8ml/min. The remaining red cells were washed out with the isotonic saline solution (10ml). The cells were then centrifuged and the top layer of packed cells was removed by aspiration.

2.3 Preparation of Erythrocyte Ghosts.

Erythrocyte ghosts were prepared according to the method of Steck et al., (1970). Packed red cells (1.5ml) were lysed in 25ml of 5mM sodium dihydrogen orthophosphate buffer adjusted with sodium hydroxide to pH 8.0, maintained at 4°C in centrifuge tubes. Samples were centrifuged at 17,000g for 20 min. at 4°C. This process was repeated for a minimum of five washes. The ghosts were made up to 3ml in lysis buffer, giving a suspension of approximately 3mg protein/ml.

The pellet of red cell membranes can be divided into two distinct fractions. The top fraction of ghosts are less densely packed than the

bottom hard "button" fraction. Although Fairbanks et al., (1971) suggested that the "button" originated from the membranes of leucocytes, Baxter (1975) found that the "button" also contained membranes of aged erythrocytes. In some experiments the top and "button" ghost sub-fractions were isolated as separate samples. Fairbanks et al., (1971) recommended that the first "button", which formed in the erythrocyte membrane pellet, be discarded to remove leucocyte membranes.

Phenylmethylsulphonylfluoride (PMSF) was used at a final concentration of 1mM to isolate ghosts for gel electrophoresis studies. The serine protease inhibitor was dissolved in dimethylsulphoxide (DMSO) to give a stock solution of 100mM which was aliquoted and stored at -20°C.

2.4 Erythrocyte Phosphatase Assays.

2.4.1 Para-Nitrophenyl Phosphate substrate.

Assays were carried out in a final volume of 1.5ml in a reaction mixture containing 5mM para-nitrophenylphosphate (PNPP), 0.5mM magnesium chloride and 30mM Tris-hydrochloric acid buffer pH 7.4 (Tris-HCl, 7.4) along with 100µl of enzyme solution. Blanks without enzyme or substrate and a zero time control were employed.

The reaction was initiated by the addition of 200µl of 37.5mM PNPP to the samples which were equilibrated to 37°C in a heated water bath. After a 1h incubation period the reaction was terminated by the addition of 200µl of trichloroacetic acid solution (33% w/v). The contents of the tubes were vigorously mixed and centrifuged at 800g for 10 min at 4°C. An aliquot of 1.0ml of the supernatant was mixed with 2.0ml of 0.2M sodium hydroxide solution to develop the colour. Values were recorded as a change in absorbance at 418nm. After the deduction of appropriate

control values the results were calculated as specific activities (change in absorbance/h/mg protein). These and all subsequent colorimetric assays were performed in duplicate and measured in a Beckman D.B. spectrophotometer. The specific activities in some cases were converted to nanomoles of PNP/min/mg protein using the molar extinction coefficient of PNP (ϵ =18000).

2.4.2 [^{32}P]-Casein Substrate

Phosphatase assays were also performed using [^{32}P]-labelled casein as the substrate. This material had a specific activity of430,000 cpm per mg of protein and was stored as a solution in 50mM Tris-HCl pH 7.5 buffer containing 60mM ethylenediaminetetracetic acid (EDTA). Assays were carried out in Eppendorf tubes in a final volume of 60µl. The reaction mixture containing Tris-HCl pH 7.5 (50mM), magnesium chloride (8mM) and substrate (20µl) was allowed to equilibrate at 37°C, in a heated water bath, before the addition of the enzyme solution (40µl).

Incubation was for 30 min. at 37°C and the reaction was stopped by the addition of 100µl of cold trichloroacetic acid solution (15% w/v) and 100µl of cold bovine serum albumin solution (0.6% w/v). The tube contents were vigorously mixed and placed in ice for 10 min. to maximise precipitation. After centrifugation of the samples in an Eppendorf microfuge at 15,000g for 2 min., an aliquot (200µl) of the supernatant was added to 2.0ml of the Triton:toluene (1 volume of Triton-X-114 to 2 volumes of toluene) scintillator containing 4mg of 2,5-diphenyloxazole per litre. A blank without enzyme and a zero time control were employed and all samples were assayed in duplicate. Values were recorded as counts per minutes (cpm) using an Intertechnique S.L.30 liquid scintillation spectrophotometer. After the deduction of appropriate control values the results were calculated as specific activities

(cpm/mg protein). This method is an adaptation from the work of Antoniw et al., (1971).

2.5 Assessment of Phosphatase Activities in Erythrocytes

Both of the phosphatase assay systems were used to measure the activities of erythrocyte membrane and cytosolic enzymes. Aliquots of the erythrocyte phosphatases were used to asses the linearity of product release as a function of time and of enzyme concentration.

The pH optima were estimated for human and rat erythrocyte PNPP phosphatase activities using the following buffers at a 20mM concentration, with a 0.5pH unit interval. The buffer systems employed were:-

pH	4.0-5.5	Acetic acid/sodium acetate.
	6.0-6.5	Morpholinoethanesulphonic acid (MES)/sodium hydroxide.
	7.0-9.0	Tris/hydrochloric acid.

Inhibition studies were also performed on membrane and cytoplasmic PNPP phosphatases of normal and spur cell human erythrocytes. This involved the use of a variety of compounds known to modulate the activity of phosphatases. For salts occurring in erythrocytes, for example ATP and ADP, the concentrations employed were in the range known to occur in normal human erythrocytes.

The membrane PNPP phosphatases were compared between normal cells, spur cells and normal cells which had previously been incubated in normal or spur cell plasma. Normal compatible erythrocytes were incubated with the plasma (50% v/v) in a heated water bath for 24h at 37°C. Aseptic techniques were used to avoid bacterial contamination.

2.6 Erythrocyte Neutral Protease Assay.

Protease assays were performed using [^{125}I]-labelled casein as the substrate which was stored as a solution (5mg protein/ml) in a pH 7.4 buffer containing 50mM Tris-HCl and 100mM sodium chloride (TBS 7.4). The reaction mixture was in a final volume of 100µl with 50mM TBS 7.4 (containing penicillin 10,000 units/ml and streptomycin 10mg/ml), 10µl of substrate (specific activity $2x10^6$cpm/mg protein) and 20µl of enzyme solution.

The reaction was initiated by the addition of the substrate and the incubation was carried out in a heated water bath for 1h at 37°C. Termination of the protein hydrolysis was achieved by the addition of 100µl of cold trichloroacetic acid solution (15% w/v) and 100µl of cold bovine serum albumin solution (0.6% w/v). The tube contents were mixed thoroughly and placed in ice for 10 min.. After centrifugation in an Eppendorf microfuge at 15,000g for 2 min., each of the total supernatants were transferred into counting vials with caps and counted using an L.K.B. Wallac 1275 Minigamma counter. A blank enzyme and a zero time control were used in each assay and all samples were examined in triplicate. Values were recorded as counts per minute (cpm). After the subtraction of the appropriate controls the values were expressed as a specific activity either as cpm/h/mg protein or ng trypsin activity/mg protein. A trypsin standard curve of 0,10,20,50 and 100ng of trypsin per assay tube was employed in each assay.

2.7 Assessment of Proteolytic Activities in Erythrocytes.

The assay system was used to measure the activities of whole erythrocytes, erythrocyte membranes and cytoplasmic enzymes. Aliquots of the erythrocyte proteases were also used to assess the linearity of the

product release with respect to time and enzyme concentration.

Intact erythrocytes (10^8 cells) were used to determine the proteolytic activity located on the outside of the membranes. The control involved the incubation of 10^8 erythrocytes, under the normal assay conditions, for 1h at 37°C in the absence of the substrate. After centrifugation, at 800g for 10 min. at 4°C, the supernatant from this red cell incubation was assayed and the value was subtracted from the proteolytic activity measurement made on 10^8 cells incubated with the substrate. This control value accounted for any leakage of cytoplasmic proteases from cells which may have lysed.

Variation of activity with pH was examined in human erythrocyte membranes and cytoplasm. The pH profile for human leucocyte membrane was also measured. This was achieved by assaying (a) mixed erythrocyte and leucocyte membranes, and (b) homogeneous erythrocyte membranes, through a range of 50mM buffers (containing 100mM sodium chloride) covering a wide pH range. Subtraction of the pH curves, (a)-(b) gave the pH activity profile of leucocyte membrane proteases. The buffer systems employed were:

pH	2.5-3.0	Citric acid/Disodium hydrogen orthophosphate.
	4.0-5.0	Acetic acid/sodium acetate.
	6.0-	Succinic acid/sodium hydroxide.
	7.0-8.0	Tris/hydrochloric acid.
	9.0	Boric acid/sodium hydroxide.

Inhibition studies were carried out on erythrocyte membrane and cytosolic proteases. Various substrates, known to effect the catalytic activity of proteases, were tested in the assay solution. All of the test substances were prepared as concentrated stock solutions and were

pre-incubated in the assay system with the enzyme for 30 min. at 22°C before the addition of the substrate.

2.8 Isolation and Purification of Erythrocyte Cytosolic Proteases.

This method is an abbreviated version of that devised by Pontremoli et al., (1980) to identify the proteolytic activities located in the cytosolic compartment of mature human erythrocytes. The radio-labelled [^{125}I]-casein substrate was used to assay the proteolytic activity in this system.

A 20ml sample of freshly drawn human blood was defibrinated and made free of leucocytes using the method of Beutler et al., (1976). The cells were lysed by three cycles of freezing and thawing. After centrifugation at 10,000g in a 8x50ml fixed angle rotor for 1h at 5°C, the fraction (containing about 2.8g of haemoglobin) was diluted with 25mM sodium phosphate buffer pH 6.5 to reach a final concentration of 28mg of haemoglobin/ml. After the dilution the pH was adjusted back to 6.5. Diethylaminoethyl cellulose (DEAE), which had been previously equilibrated in 25mM sodium phosphate buffer pH 6.5 (column buffer), was added (10g wet weight/g haemoglobin) with constant stirring at 5°C. The suspension was then filtered on a Büchner funnel and the ion exchanger was transferred onto a column 10cmx2.2cm (i.d.) and submitted to extensive washing in column buffer. Elution was obtained with 0.5M sodium chloride solution in column buffer (50ml). The effluent containing all of the proteolytic activity was pooled and concentrated to 10ml by ultrafiltration on an Amicon Diaflow UM.10 membrane and dialysed extensively against 50mM sodium phosphate buffer pH 6.5.

The proteolytic activity and protein concentration was assayed at various stages throughout the purification procedure. The specific activity of the proteases was expressed as ng trypsin activity/mg protein.

2.9 Protein estimation.

All protein determination of erythrocyte ghosts were carried out by a slight modification of Lowry et al., (1951), using bovine serum albumin as a standard. The procedure was modified using the anionic detergent sodium dodecyl sulphate (SDS), in a final concentration of 0.5% (w/v) in the 1.0ml sample volume. This detergent was included to enhance the solubilisation of erythrocyte membrane proteins.

2.10 Phosphorylation of Casein.

[^{32}P]-labelled casein was a gift from Dr. H.G. Nimmo. It was prepared using the method of Antoniw et al., (1971). The specific activity of the substrate was approximately 420,000 cpm/mg protein(6mg/ml).

2.11 Chloramine-T Iodination of Casein.

The chloramine-T method was used to iodinate the casein substrate according to the details published by Hunter and Greenwood (1962). A 200µl aliquot of casein solution (5mg protein/ml) and 100µl of chloramine-T solution (1mg/ml) were transferred to a small tube and the iodination was immediately initiated by the addition of 400µCi of [^{125}I]-iodine (40µl). All of the chemical solutions used in this reaction were dissolved in PBS 7.4. The reaction was allowed to proceed for 1 min. at 22^{o}C, after thorough mixing, and was terminated by the addition of 100µl of sodium metabisulphite (1mg/ml) solution dissolved in PBS 7.4. The mixture was then passed down a G-25 sephadex column (25cm x 1.5cm), equilibrated in PBS 7.4, to remove the unreacted [^{125}I]-

iodine. The first [^{125}I]-iodine peak was pooled and dialysed against 50mM TBS 7.4 and diluted with cold casein solution to obtain a ratio of 1:10 of radio-labelled to non-labelled casein. The substrate was heat denatured (10 min/100°C), rapidly cooled and aliquoted for storage at -20°C.

Traces of contaminating proteases present in the casein which survived the heat denaturation could be inactivated by the addition of iodoacetamide. After the radio-labelled substrate was dialysed and diluted with cold casein, the solution was made 50mM with iodoacetamide and left in the dark for 1h at 22°C. The sample was again dialysed, to remove the excess iodoacetamide, and heat denatured, made into aliquots and stored frozen (-20°C). This modification was found to reduce the background of the substrate material. The specific activity of the [^{125}I]-casein substrate, after dilution with cold casein, was approximately $2x10^6$ cpm/mg protein.

2.12 Determination of Cholesterol and Phospholipid in Erythrocyte Membranes.

Cholesterol and phospholipid were extracted from intact erythrocytes according to the method of Rose and Oklander (1965). Packed erythrocytes (0.5ml), after thorough washing, were mixed with 0.5ml of cold water to achieve lysis. Isopropanol (0.5ml) was added to the sample and left for 1h at 22°C, after which chloroform (3.5ml) was added and left for a further hour at 22°C. The following samples were aliquoted after the mixture was centrifuged to remove the insoluble material:

1. Cholesterol determination 2.0ml in triplicate.
2. Lipid Phosphorus determination 1.5ml in duplicate.

Acid treated test tubes were used for the lipid phosphorus assay and all the samples were dried down under reduced pressure.

All cholesterol determinations of erythrocyte membranes were made according to the method of Zlatkis et al., (1953). Lipid phosphorus determinations were carried out according to the method of Bartlett (1959).

2.13 Flat Bed Polyacrylamide Gel Electrophoresis.

Flad bed gel electrophoresis, using the discontinuous buffer system of Laemmli (1970), was employed to analyse the protein banding patterns of erythrocyte membranes. Electrophoresis was carried out in the presence of 0.1% (w/v) sodium dodecyl sulphate (SDS) and two types of gel apparatus were employed. The first apparatus had a gel dimension of 20x20x0.1cm with a capacity for twelve samples. The second smaller apparatus of Amos (1976) was adopted because it produced a much sharper resolution of the protein bands. The gel dimension was 7x7x0.15cm with the capacity of seven tracks for sample loading. The electrophoresis conditions and method of sample preparation were identical for each type of gel apparatus. The membrane sample (40µg protein) was in a final volume of approximately 30µl containing 50mM Tris-HCl pH 8.8 buffer, 15% (w/v) sucrose, 2% (w/v) SDS, 50mM dithiothreitol and 100ng of pyronin Y, as final concentrations. The samples were heated for 2 min. at 100°C followed by 30 min. at 37°C.

The stacker gel was a 5% acrylamide solution which contained 0.1 M Tris-HCl pH 6.8 buffer and the main separation gel was a 9% acrylamide solution containing 0.4M Tris-HCl buffer. The ratio of N,N^1-methylenebisacrylamide to acrylamide in the stock solution was 1 to 38. The reservoir buffer was 25mM Tris-glycine pH 8.8 solution containing 0.1% SDS, and the gels were run at a constant current of 15mA at 22°C.

Electrophoresis calibration kits of high (330,000 to 18,500) and low (94,000 to 14,400) molecular weight ranges were used to calibrate the gel systems. By plotting the relative mobility of each protein (with respect to the dye front) against the logarithm of molecular weight, molecular weight values for other proteins were obtained.

High Molecular Weight Calibration Kit

PROTEIN	MOLECULAR WEIGHT (subunit)
Thyroglobulin	669,000 (330,000)
Ferritin	440,000 (18,500)
Catalase	232,000 (60,000)
Lactate Dehydrogenase	140,000 (36,000)
Albumin	67,000 (67,000)

Low Molecular Weight Calibration Kit

PROTEIN	MOLECULAR WEIGHT
Phosphorylase b	94,000
Albumin	67,000
Ovalbumin	43,000
Carbonic anhydrase	30,000
Trypsin inhibitor	20,100
∝-Lactalbumin	14,400

Staining and fixing of the proteins into the polyacrylamide gel were achieved simultaneously using an overnight soaking in a solution containing 0.05% (w/v) Coomassie blue in acetic acid:isopropanol:water mixture (1:25:1.5 by volume). This was followed by a second stain of 0.005% (w/v) Coomassie blue in acetic acid:isopropanol:water mixture (1:1:3 by volume) which involved soaking the gel for 6 to 9 hours. Destaining was accomplished using several changes of a 10% (v/v) acetic acid solution.

2.14 Isolation of Antiserum.

Antiserum to human immunoglobin light chains was obtained from

Stuart Clark of the Biochemistry Department at the University of Strathclyde, Glasgow. The serum was precipitated with 14% (w/v) sodium sulphate added in the solid form. The serum (2ml) was centrifuged at 17,000g for 10 min. at 22°C. The pellet was resuspended in a solution of 14% (w/v) sodium sulphate and again centrifuged. The second pellet was resuspended in 0.9% (w/v) sodium chloride solution. The concentration of the antiserum solution was adjusted to 15mg protein/ml by relating the absorbance of the solution at 280nm to a standard solution of human immunoglobin (1mg protein/ml). This preparation was used in an assay to detect immunoglobin molecules. The antibody molecules were coupled to Horse radish peroxidase to quantitate the measurement of human immunoglobin-G using the enzyme-linked immuno-sorbent assay (ELISA) according to the method of Engvall and Perlmann (1971).

2.15 Enzyme Linked Immuno-sorbent Assay (ELISA).

All of the immunological reagent used in this assay was provided by Stuart Clark of the Biochemistry Department at the University of Strathclyde in Glasgow. He established this assay for the specific detection of human antibody light chain molecules (lambda and kappa): he followed the method of Avrameas (1969) for conjugating the enzyme to the antibody molecules and the method of Engvall and Perlmann (1971) to quantitate the assay. However it was necessary to adapt this technique to detect antibody molecules in the presence of the non-ionic detergent Triton-X-100. The detergent (0.1% v/v) was used to solubilize antibody molecules from erythrocyte membranes.

An aliquot of 100µl of coating antibody solution (15mg/ml), diluted 1:400 in 0.2M Tris-HCl pH 9.0 buffer, was added to each well of a micro-Elisa plate and incubated overnight at 4°C. The plate was then washed twice with 0.2M Tris-HCl pH 7.4 buffer containing 0.19M sodium chloride

(Tris-wash buffer); once with Tris-wash buffer containing 0.05% Tween-20 (Tris-Tween buffer); once with Tris-wash buffer and dried thoroughly by shaking.

The sample (100µl) dissolved in PBS 7.4 was added to each well in triplicate and incubated for 30 min. at 22°C. The tray was then emptied and washed four times in Tris-Tween buffer and dried thoroughly. Then 100µl of the conjugated antibody (Horse radish peroxidase) solution (12.5µg protein/ml) was added to each well and incubated to 30 min. at 22°C. The tray was emptied and washed four times, using the Tris-Tween buffer, and dried thoroughly. An aliquot (100µl) of the substrate solution of ortho-phenylene diamine (0.4mg/ml of 0.2M phosphate citrate buffer pH 6.0) containing 0.0075% (v/v) hydrogen peroxide, was added to each well. The reaction was allowed to proceed in the dark for 30 min. at 22°C and was stopped by the addition of 50µl of 2M sulphuric acid. The absorbance of each well sample was measured at 492nm using a Flow Titertek Multiskan spectrophotometer.

A standard curve was included using 0 to 1000ng of human IgG solution following serial dilution of a stock solution (1mg protein/ml). The Flow Multiskan spectrophotometer was blanked against empty wells. All erythrocyte membrane samples were in a final concentration of 0.1% (v/v) Triton-X-100 dissolved in PBS 7.4. They were initially dissolved in 1.0% (v/v) Triton-X-100 and left for 30 min. at 22°C and then centrifuged to remove any debris. The supernatants were removed and diluted one in ten to reduce the detergent concentration. Standard curves were prepared in the presence and absence of the detergent and the values were compared.

3. RESULTS

3.1 Separation of Leucocytes from Erythrocytes.

The efficiency of the removal of leucocytes was assessed by counting the white cells remaining in erythrocytes isolated by three methods. The technique of Fairbanks et al., (1971), which involved the removal of the buffy coat by aspiration, left approximately 18% of the leucocytes; the cotton/glass wool adsorption technique of Tökés and Chambers (1975) left approximately 5% of the leucocytes; and the ion exchange chromatography technique of Beutler et al., (1976) left less than 0.5% of the leucocytes in the blood samples. Recovery of erythrocytes from each of the three methods was not less than 95%.

To investigate the possible influence of leucocytes in the phosphatase and protease assay systems the buffy coat of white cells were left in some of the blood samples to serve as experimental controls.

3.2 Spur Cell Erythrocytes.

Spur cell anaemia stems from a disorder of plasma lipoprotein which gives rise to increased membrane cholesterol content causing a spiculated (acanthocyte) appearance of the erythrocytes (Cooper, 1978). The term spur cell is derived from the presence of the irregular thorny projections or spurs which occur on these cells.

Analytical results provided by the Haematology department in the Glasgow Royal Infirmary quoted the patient as having a haemoglobin level of 6.86% (w/v); a reticulocyte count of 3% and a haematocrit of approximately 30%. The patient was a male Caucasian, aged 57. He was an alcoholic but stopped drinking after he became jaundiced. A liver biopsy

confirmed the severe cirrhotic condition of his tissue, and the onset of spur cell anaemia was identified in January 1980. The patient also suffered from splenomelagy and hepatomelagy and liver failure was diagnosed on the death of the patient seven months later.

The results of the cholesterol/phospholipid molar ratio determinations using normal and spur cell erythrocytes are shown in Table 2. Spur cell erythrocytes had an increased cholesterol content (146%) compared to control levels. This resulted in a cholesterol/phospholipid molar ratio of 1.26 (154% of the controls). The mean value of the cholesterol/phospholipid molar ratio found for four normal individuals was 0.82. This compares with the normal range of the cholesterol/phospholipid molar ratio reported by Cooper (1978) to be 0.9 to 1.0.

Table 2. Measurement of the Cholesterol/Phospholipid Molar Ratio.

Leucocytes were removed from blood samples using the method of Tökés and Chambers (1975). The values in parenthesis denote the number of subjects used. The difference between duplicate analyses was less than +5%. An average molecular weight of 750 was employed to determine phospholipid concentration.

Membrane Sample	CHOLESTEROL(±SD) (umoles/ml packed cells)	PHOSPHOLIPID(±SD) (umoles/ml packed cells)	C/PL Molar ratio
Normal (4)	3.48 (±0.05)	4.23 (±0.20)	0.82
Spur Cell (1)*	5.00	3.98	1.26

* average of two determinations in duplicate

The membranes isolated from spur cell erythrocytes always contained a larger "button" fraction of densely packed ghosts compared to normal erythrocyte membranes. Analysis of the protein content of membrane sub-fractions, prepared from normal and spur cell erythrocytes, are shown in

Table. 3. Almost four times as much of the total membrane protein was present in the spur cell "button" as that found in the normal "button".

Table 3. Protein Distribution in Erythrocyte Membrane Sub-Fractions.

Leucocytes were removed from erythrocyte blood samples using the method of Tökés and Chambers (1975). The total number of subjects used are quoted in parenthesis. The error between duplicates in the assay was less than ±5%.

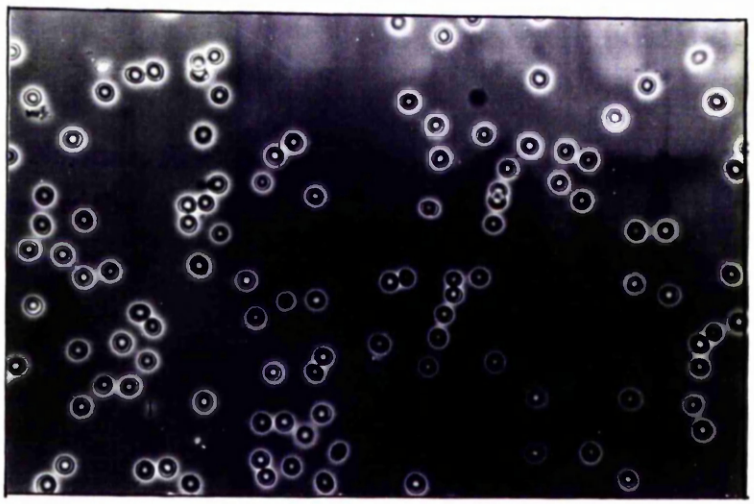
Blood Sample	Membrane Fraction	PERCENTAGE WEIGHT OF TOTAL MEMBRANE PROTEIN			
Normal Cells (2)	Top	Subject 1	73	Subject 2	84
	"Button"		27		16
Spur Cells (1)	Top		16		40
	"Button"		84		60

3.3 Spur Cell Serum Incubation Experiment.

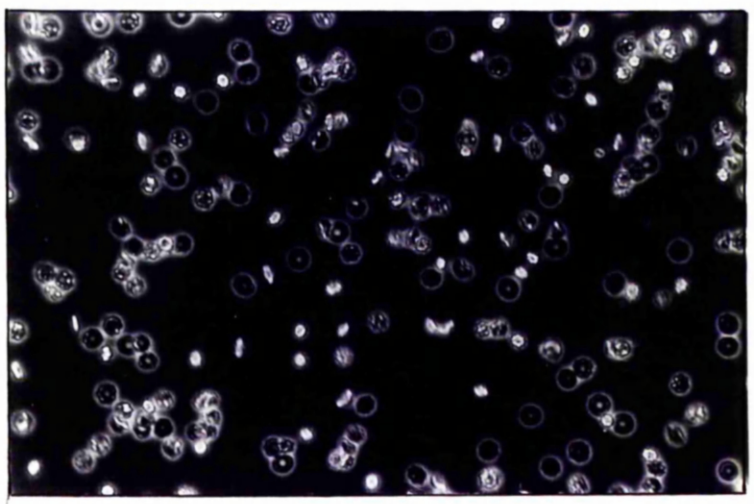
An experiment was carried out to determine whether incubation of spur cell serum and normal (B, Rh +ve) compatible erythrocytes produced a change in cell shape. Periodic examination of the erythrocytes, using phase contrast microscopy, revealed a change in the morphology of the erythrocytes which was not observed in the control after 4h (Figure 6). At this stage the cells in spur cell serum were predominantly echinocytes, but the normal serum contained only a few echinocytes. After 9h the normal serum contained mainly echinocytes. Normally erythrocytes have a smooth biconcave disc shape; echinocytes are rounded cells with small regular spikey protrusions on their surface and spur cells have large irregular thorny projections on their surface. This spur cell serum failed to convert normal erythrocytes to spur cells but it did accelerate the formation of echinocytes.

FIGURE 6. MORPOLOGICAL TRANSFORMATION OF NORMAL ERYTHROCYTES INCUBATED IN SPUR CELL SERUM.

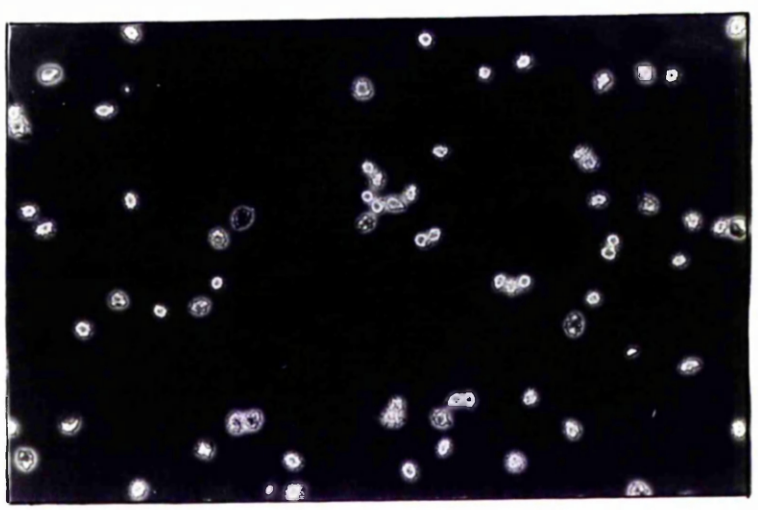
Control – Normal RBC incubated with normal serum.



Sample 1 – Normal RBC incubated with spur cell serum.



Sample 2 – Spur cell RBC incubated with spur cell serum.



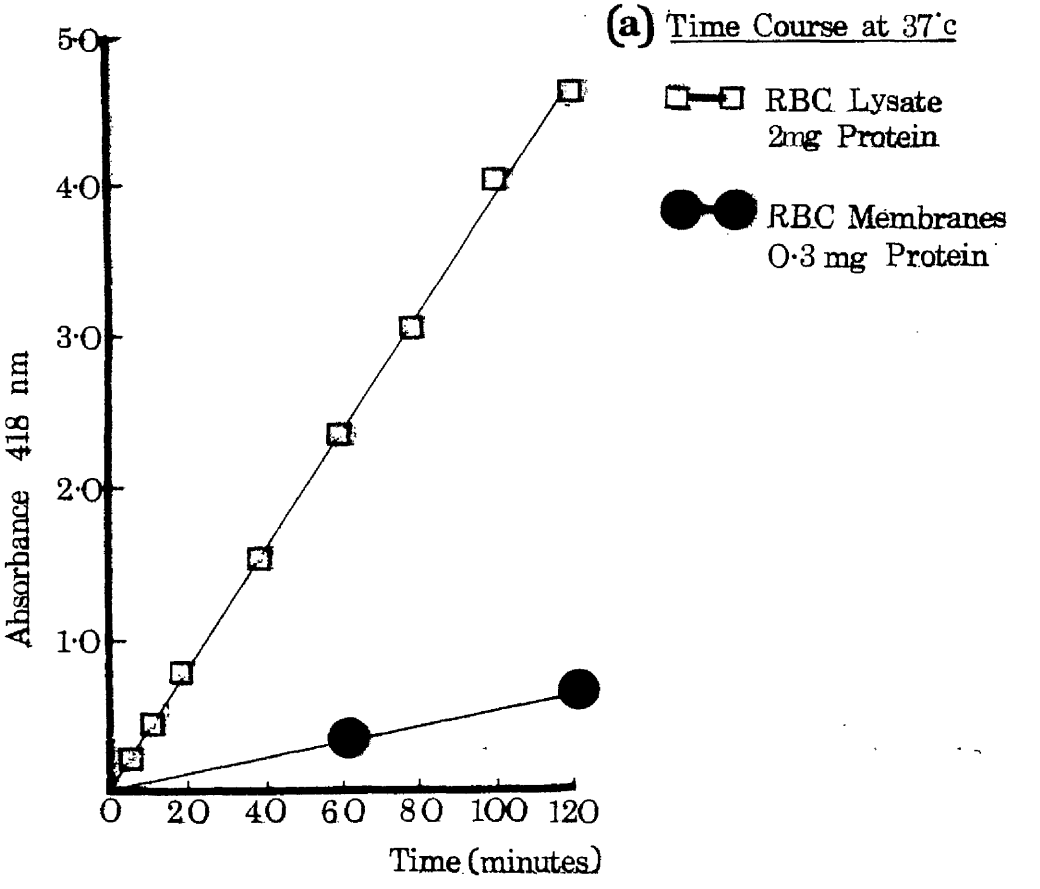
magnification x 40.

3.4 Phosphatase Activities in Erythrocytes.

The substrate p-nitrophenyl phosphate (PNPP) was employed to measure the properties of erythrocyte membrane-associated and cytoplasmic phosphatases. Figure 7(a) shows that the reaction kinetics of both the membrane and cytosolic enzymes are first order over a period of 2h at 37°C. Figure 7(b) depicts the change in the rate of PNPP hydrolysis with increasing amounts of enzyme for both the membrane and cytosolic preparations. Release of PNP was a linear function of the amounts of erythrocyte lysate (up to 2mg protein) and membrane dispersion (up to 0.3mg protein) used when incubated for 1h at 37°C. Similar conditions were employed for all PNPP phosphatase determinations carried out on human or rat erythrocytes. Rat erythrocytes had higher phosphatase activities and were used in the initial characterisation of the system.

A [^{32}P]-casein substrate was used in an attempt to measure the properties of the phosphoprotein phosphatases of erythrocyte membranes and lysates. Figure 7(c) shows the kinetics of hydrolysis of the phosphorylated casein by the membrane phosphatase to be first order over a period of 30 min at 37°C. The reaction was also shown to be linear for two amounts of the membrane dispersion (60µg and 120µg protein). Figure 7(d) shows the kinetics of the phosphorylated casein hydrolysis, by the lysate phosphatases, to be first order over a period of 30 min at 37°C. The reaction was also shown to be linear for two amounts of the lysate (40µg and 80µg protein). Release of labelled trichloroacetic acid soluble material was found to be a linear function of the amounts of erythrocyte lysate (up to 80µg protein) and membrane dispersion (up to 120µg protein). Similar conditions were employed in the determination of phosphoprotein activities in human erythrocytes.

FIGURE 7. p-NITROPHENYL PHOSPHATASE ACTIVITIES IN ERYTHROCYTES (R.B.C).



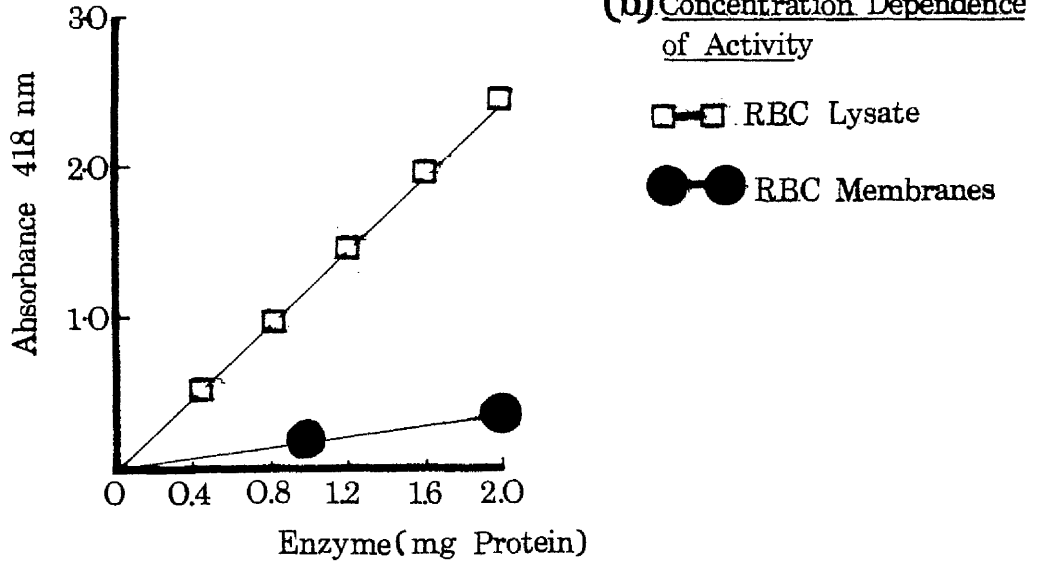
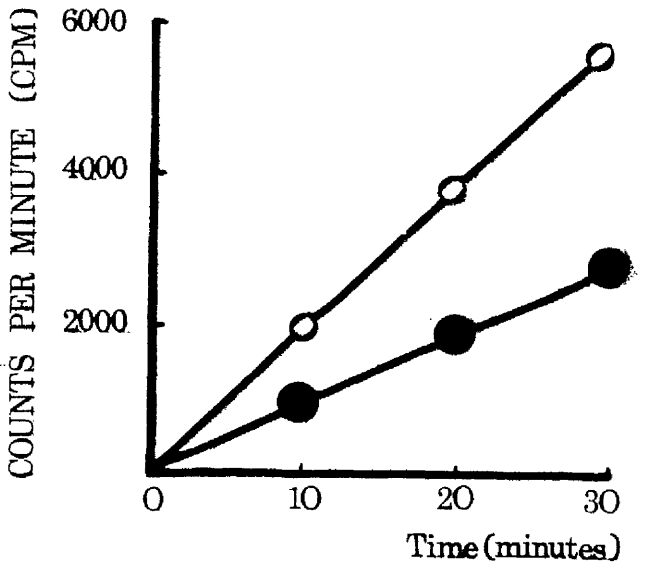


FIGURE 7. PHOSPHOPROTEIN PHOSPHATASE ACTIVITIES IN ERYTHROCYTES.

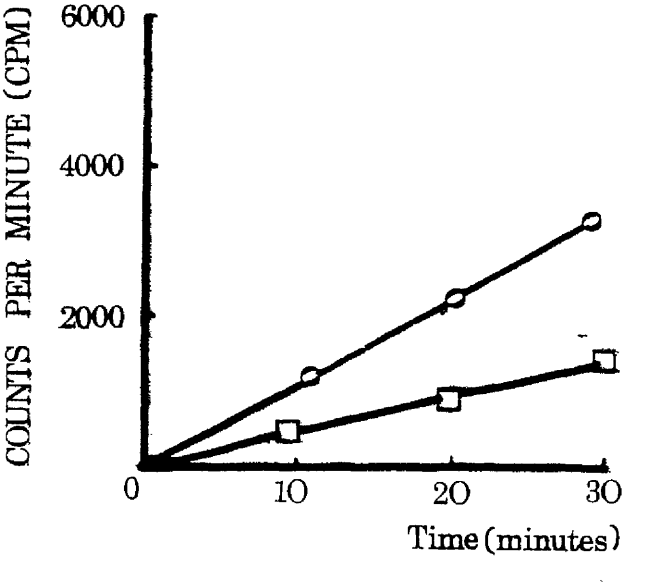


(c) Time Course at 37°c

RBC Membranes

60 µg Protein

120 µg Protein



(d) Time Course at 37°c

RBC Lysate

80 µg Protein

40 µg Protein

3.5 pH Dependence of Erythrocyte Phosphatases.

The activity profile of the PNPP phosphatases were measured over a wide range of pH values using different buffering systems. Both the membrane and cytosolic phosphatases of rat, human and spur cell erythrocytes had pH optima of 5.5 (Figure 8). There was an apparent shoulder in the activity profile in the pH 7.0 region. This effect may have been produced as a result of the change in the type of buffer used at pH 7.0. Alternatively it could be an indication that more than one p-nitrophenyl phosphatase activity is present in erythrocytes.

Although the phosphatase activity was predominantly acidic in nature the assay was carried out at neutral pH to measure the enzyme which would be active inside the erythrocyte at physiological pH. However it was essential to control the assay pH with particular care since the value chosen did not correspond to the optimum value for the phosphatases.

3.6 Distribution of Erythrocyte Phosphatase Activities.

The distribution of the PNPP phosphatase activities was assessed using rat erythrocytes, normal and abnormal (spur cell) human erythrocytes (Table 4). The [^{32}P]-casein activity distribution was measured only using normal human red cells. In all cases studied, the cytoplasmic PNPP activity comprised greater than 95% of the total cellular activity. Spur cell membranes possessed almost four times as much membrane-associated phosphatase activity as normal controls. Rat erythrocytes contained almost five times as much membrane-associated and cytoplasmic activity compared to human erythrocytes but were not used for further experiments.

FIGURE 8. HUMAN ERYTHROCYTIC PHOSPHATASE ACTIVITY DEPENDENCE ON pH.

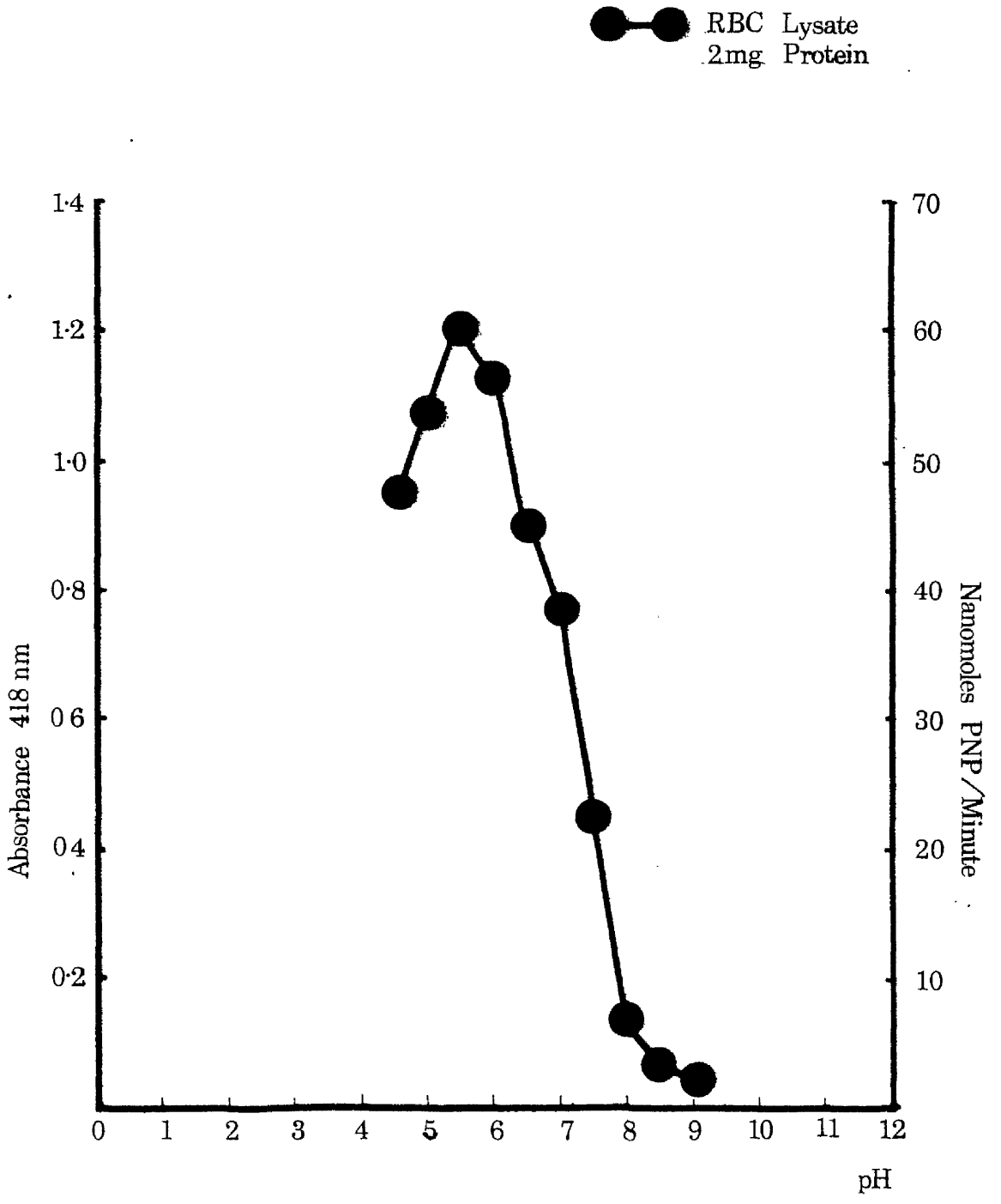


Table 4. Distribution of PNP Phosphatase Activities in Erythrocytes.

Leucocytes were removed from blood samples using the method of Tökés and Chambers (1975). The difference between duplicate analyses of both assays was below ±5%. The standard deviation was calculated for human erythrocyte PNPP phosphatase activities where five subjects were used. The values in parenthesis indicate the percentage of the total activity.

BLOOD SAMPLE	PNPP-PHOSPHATASE*		[^{32}P] PHOSPHATASE+	
RAT‡ Membranes	600	(1.3%)	ND	
Lysates	44,000	(97.7%)	ND	
HUMAN Membranes	100±30	(0.1%)	30,000	(0.2%)
Lysates	9,000±2000	(99.9%)	14,700,000	(99.8%)
SPUR CELL‡ Membranes	±00	(4.3%)	ND	
Lysates	9,000	(95.7%)	ND	

* Results expressed as nanomoles PNP/min/ml packed cells
\+ Results expressed as cpm/30min/ml packed cells
‡ Average of two determinations in duplicate
ND Not determined.

The substrate PNPP is hydrolysed by a variety phosphoprotein phosphatases, ATPases and other phosphatases (Hui & Harmony, 1979) which are present in the erythrocyte. For a more specific assay of phosphoprotein phosphatases, which may be responsible for spectrin dephosphorylation, the [^{32}P]-casein substrate was employed. Although this substrate was a more specific phosphoprotein phosphatase substrate it was also susceptible to proteolytic degradation by the numerous membrane and cytosolic proteases (Section 3.12). When this became apparent the use of this substrate was curtailed. The PNPP substrate, although less specific than the phosphorylated casein, was not affected by proteases.

3.7 Membrane Association of Phosphatase Activities.

To investigate the stability of the association between phosphatase activity and erythrocyte membranes the enzyme specific activity (nanomoles PNP/min/mg protein) was monitored after repeated washing of

the ghosts. The study was carried out on three membrane samples prepared from normal erythrocytes free of leucocytes and on erythrocytes lysed in the presence of 0.1mM and 1.0mM calcium ions.

Table 5 contains the results obtained from the phosphatase determination carried out on the three samples after five hypotonic washes and after three additional hypotonic or isotonic washes. They indicated that the specific activity of the erythrocyte membrane decreased only slightly with repeated hypotonic (6%) and isotonic (21%) washes. Prior to these investigations information concerning the stability of the membrane phosphatase activity was obtained for hypotonic buffer. The membranes were resuspended and centrifuged for a maximum of eight times and the elution of the loosely associated cytoplasmic activity was followed by assaying the membrane activity after each wash. The specific activity of the membrane phosphatases was found to decline rapidly over the first three to four washes and remain relatively constant with successive washing. Therefore to ensure the complete removal of the loosely bound cytoplasmic phosphatases from ghosts the membrane samples were routinely washed five times in hypotonic lysis buffer and these were used as the controls for further washing experiments involving high ionic strength media.

Table 5. Membrane Association of Erythrocyte Phosphatase Activity.

Leucocytes were removed from blood samples using the method of Beutler et al. (1976). The values in parenthesis express each figure as a percentage of the control, taken as 25. The difference between duplicate analyses in the assay was less than ±5%.

PNPP PHOSPHATASE* ACTIVITY	NUMBER OF MEMBRANE WASHES			
MEMBRANE SAMPLE+	5xHYPOTONIC	5xHYPOTONIC 3xHYPOTONIC	5xHYPOTONIC 3xISOTONIC	LYSATE
Erythrocytes without leucocytes	25 (100)	23 (94)	20 (79)	48
Erythrocytes lysed in 0.1mM calcium ions	33 (130)	22 (88)	20 (81)	50
Erythrocytes lysed in 1.0mM calcium ions	46 (186)	26 (104)	22 (90)	49

* Results expressed as nanomoles PNP/min/mg protein
\+ Mean values taken from two subjects (less than 10% variation)

By including two concentrations of calcium ions (0.1mM and 1.0mM) in the lysis buffer throughout the isolation procedure, the specific activity of the phosphatases associated with the erythrocyte membranes increased. It also appeared greater when more calcium ions were employed, however, there was no appreciable difference in the phosphatase activities observed for the cell lysates. This increased activity observed, in the cases of calcium lysis, on the membranes was only loosely associated since it could be eluted using repeated hypotonic and isotonic washes.

These results showed that there are two types of membrane phosphatase activities. One type of activity can be removed efficiently by isotonic washing and less efficiently by hypotonic washing. The second type of phosphatase activity resists elution from the membranes by washing and therefore may be an integral or tightly associated membrane activity possessing a specific function.

3.8 Effect of Ions and Metabolites on Erythrocyte Phosphatases.

Human erythrocyte phosphatases of the membrane and cytoplasm were characterised by incorporating a variety of ions and metabolites into the assay system. The effects of these compounds on the PNPP phosphatase activities are shown in Table 6.

Table 6. Modulation of Erythrocyte PNP Phosphatase Activity.

Each test compound was included in the assay at the concentration indicated. Stock solutions of all the test compounds were adjusted to pH 7.4. Leucocytes were removed from blood samples using the method of Beutler et al., (1976).

Modifier Concentration (mM)		% PHOSPHATASE ACTIVITY REMAINING (±SD) Membranes* %	Lysate+ %
0.1	ATP	84 ± 18	114 ± 8
1.0	ADP	66 ± 17	123 ± 25
10.0	NaP	54 ± 24	47 ± 16
10.0	NaF	28 ± 25	70 ± 13
100.0	KCl	120 ± 2	86 ± 10
10.0	$CaCl_2$	60 ± 5	48 ± 15
5.0	2,3 DPG	45 ± 17	155 ± 38
1.0	EDTA	182 ± 20	238 ± 45
	-$MgCl_2$	39 ± 13	82 ± 21
10.0	$MgCl_2$	64 ± 9	86 ± 10
0.1%(v/v)	Triton-X-100	151 ± 20	109 ± 9

* control activity 12 nmoles/min/mg protein

\+ control activity 18 nmoles/min/mg protein

This experiment was carried out on blood samples from three individuals. The difference between duplicate analyses was less than ±5%.

Lysate phosphatase activity was slightly decreased to 82% in the absence of magnesium and to 86% using a higher magnesium ion concentration than was present in the normal assay system (0.5mM Mg^{2+}). The activity was stimulated by the addition of 1mM EDTA to the assay system containing 0.5mM Mg^{2+}. This effect presumably was a result of activation of the enzyme by the removal of inhibitory divalent cations.

Therefore the lysate phosphatases required the presence of magnesium ions for their activity in the concentration region of 0.5mM. Physiological concentrations of ATP, ADP and 2,3 diphosphoglycerate stimulated the activity of the lysate phosphatases. All of the other compounds tested inhibited the levels of the lysate phosphatase activities with the most effective being inorganic phosphate (47%) and calcium (48%). The non-ionic detergent Triton-X-100 did not significantly effect the lysate phosphatase activity (109%).

The response of the membrane associated phosphatase activities to these ions and metabolites varied from the profile of the lysate phosphatases. Magnesium ions produced more inhibition of the membrane phosphatases (64% activity remaining) and in the absence of magnesium the activity was reduced further to 39% of the control activity. Again these phosphatases may have a requirement for magnesium below the concentration of 10mM. Non-ionic detergent stimulated the membrane phosphatase activity to 151% of the control, indicating that all of the enzyme was not freely accessible to the assay substrate. Unlike the lysate phosphatases the membrane phosphatase were inhibited by physiological concentration of ATP, ADP and 2,3 diphosphoglycerate, although both the insoluble and soluble enzymes were similarly inhibited by inorganic phosphate. EDTA also stimulated the insoluble phosphatases (182%) and potassium ions stimulated the activity to 120% of the control value. The fluoride ion was a very effective inhibitor of the insoluble phosphatases (28%) and calcium ions inhibited the activity to 60% of the control.

Therefore the similarities which exist between the soluble and insoluble phosphatases are that they are both inhibited by inorganic phosphate (10mM), fluoride ions (10mM) and calcium ions (10mM). Moreover

they are both stimulated by EDTA (1mM) and are most active in the presence of low concentrations of magnesium. However, the insoluble phosphatases were more inhibited by fluoride ions and less stimulated by EDTA compared to the soluble phosphatases.

However, when interpreting these results, the limitations imposed by both the impure state of the enzymes and the latent nature of the membrane associated phosphatase activity, must be taken into account.

3.9 Phosphatase Activities in Leucocytes and Serum.

The level of PNPP phosphatase activity was compared in normal human erythrocyte membranes with and without leucocytes present and the enzyme activity of normal human serum was also measured.

Table 7 shows the results which indicate that the specific activity for the erythrocyte membrane sample was almost 50 percent greater when leucocyte membranes were present. This high value was not observed to decrease on additional hypotonic washing but almost half of the activity could be eluted using additional isotonic washing (results not shown). The leucocytes only contributed to the membrane-associated phosphatase activity and did not significantly elevate the activity measured in the cell lystates. These results demonstrate the importance of the removal of leucocytes from blood samples before isolating erythrocyte membranes to be used in the determination of phosphatase activity.

Table 7. Phosphatase Activities Measured on Leucocytes and in Serum.

Leucocytes were removed from blood samples using the method of Beutler et al., (1976). The values were taken as the mean of two sets of data from two normal donors. The difference between duplicate analyses was less than ±5% for both assays.

MEMBRANE SAMPLE	PNPP Phosphatase Activity nanomoles/min/mg protein	
	MEMBRANES	LYSATE
Erythrocytes without leucocytes	25 ± 6	48 ± 5
Erythrocytes with leucocytes	38 ± 4	52 ± 6
Erythrocyte serum*	14 ± 2	

* Results expressed as nanomoles/min/ml serum

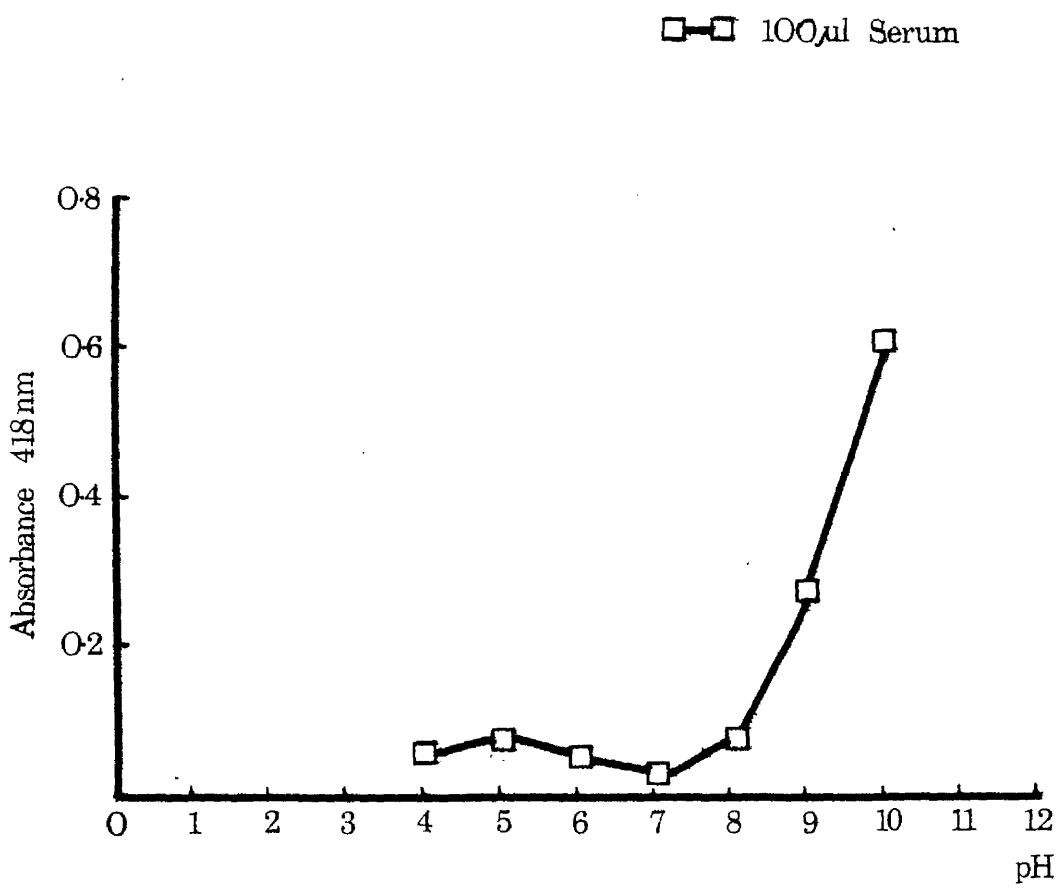
The serum phosphatase activity was relatively low compared to the activity of the membranes even though this activity was expressed per millilitre of serum (Table 7). The phosphatase activity present in the serum would therefore not be expected to interfere in the assay of the erythrocyte membrane phosphatase activity. Figure 9 shows that the human serum predominantly contains an alkaline phosphatase with a pH optimum greater than pH 9.0. This is in agreement to the observations made by Boyer (1961).

3.10 The Effect of Spur Cell Serum on Normal Erythrocyte Membrane Phosphatases.

Normal erythrocytes were incubated with spur cell serum for a total of 18h at 37°C. A control incubation was also carried out and the membranes of the erythrocytes were isolated and used in the phosphatase assay.

Table 8 shows the results which indicate that spur cells contain at least four times as much phosphatase activity associated with their membranes compared to normal erythrocyte membranes (both samples were

FIGURE 9. HUMAN SERUM PHOSPHATASE DEPENDENCE ON pH.



not incubated). Incubation of normal cells in normal serum slightly decreased the membrane phosphatase activity. However incubation of normal cells in spur cell serum did not alter the phosphatase activity associated with the membranes.

Table 8. Erythrocyte Membrane Phosphatase Activities.

Leucocytes were removed from blood samples using the method of Tökés and Chambers (1975). The difference between duplicate analyses was less than ±5%.

Membrane Sample	PNPP PHOSPHATASE ACTIVITY nanomoles PNP/min/mg protein
Normal Cells	16
Spur Cells	55
Normal Cells Incubated with Normal Serum	14
Normal Cells Incubated with Spur Cell Serum	16

3.11 Effect of Ions and Metabolites on Spur Cell Erythrocyte Phosphatases.

Human spur cell erythrocyte phosphatases of the membrane and cytoplasm were characterised by incorporating a variety of ions and metabolites into the assay system.

The effect of these compounds on the PNP phosphatase activities are shown in Table 9. Both the membrane and lysate phosphatases of spur cell erythrocytes followed generally the same trend of inhibition and stimulation as the normal erythrocyte phosphatases. Inorganic phosphate and fluoride ions inhibited both activities. However, EDTA had little effect on both soluble and insoluble spur cell phosphatase activites, in addition to this 2,3 diphosphoglycerate and calcium had no inhibitory effect on the insoluble phosphatases. The non-ionic detergent Triton-X-100 more than doubled the spur cell membrane phosphatase activity (231%) indicating that more than half of the insoluble phosphatase activity was

latent. This fact might explain some of the differences observed between the extent of inhibition produced by the test compounds on spur cell phosphatases compared to normal erythrocyte phosphatases.

Table 9. Modulation of Spur Cell Erythrocyte PNP Phosphatases.

Leucocytes were removed from blood samples using the method of Tökés and Chambers (1975). The difference between duplicate analyses was less than ±10%.

Modifier Concentration (mM)		% PHOSPHATASE ACTIVITY REMAINING Membranes* %	Lysate+ %
0.1	ATP	86	75
1.0	ADP	135	113
10.0	NaP	84	42
10.0	NaF	70	79
100.0	KCl	70	34
10.0	$CaCl_2$	102	64
5.0	2,3 DPG	107	56
1.0	EDTA	126	92
	$-MgCl_2$	121	77
0.1%(v/v)	Triton-X-100	231	79

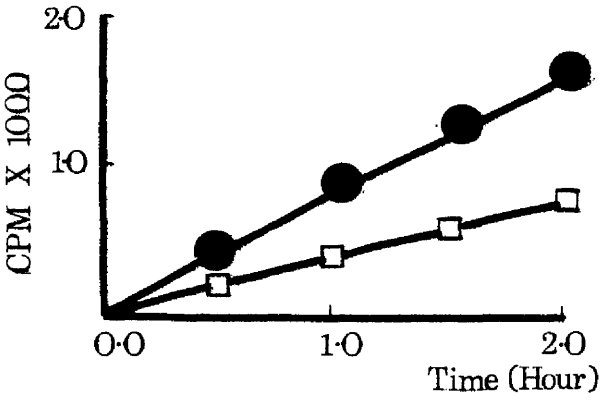
* control activity 30 nmoles PNP/min/mg protein
\+ control activity 10 nmoles PNP/min/mg protein

3.12 Proteolytic Activities in Human Erythrocytes.

The properties of membrane and lysate neutral proteolytic activities in normal human erythrocytes were measured with [^{125}I]-casein as the substrate.

Figure 10(a) shows the release of product using membrane and lysate proteases. The reaction time course for both were linear over a period of 2h at 37°C. Figures 10(b) and 10(c) show the rate of product release with increasing amounts of erythrocyte lysate and membranes respectively. The release of labelled trichloroacetic acid soluble material was found to be linear during a 1h incubation period at 37°C for amounts of lysate (up to 500µg protein) and membrane dispersion (up

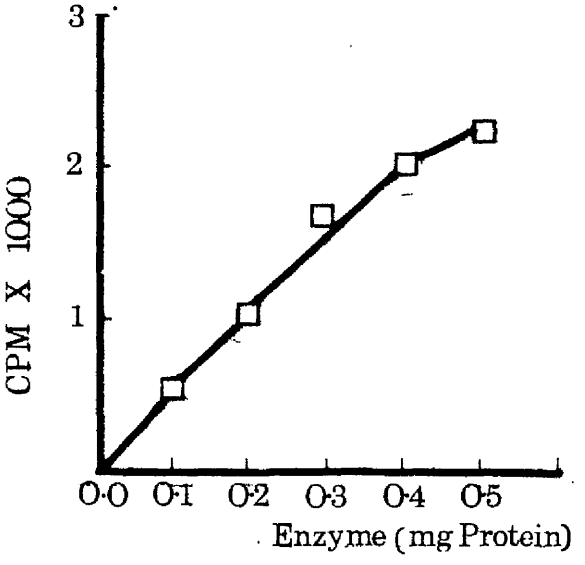
FIGURE 10. ERYTHROCYTE PROTEOLYTIC ACTIVITIES.



(a) Time Course at 37°c

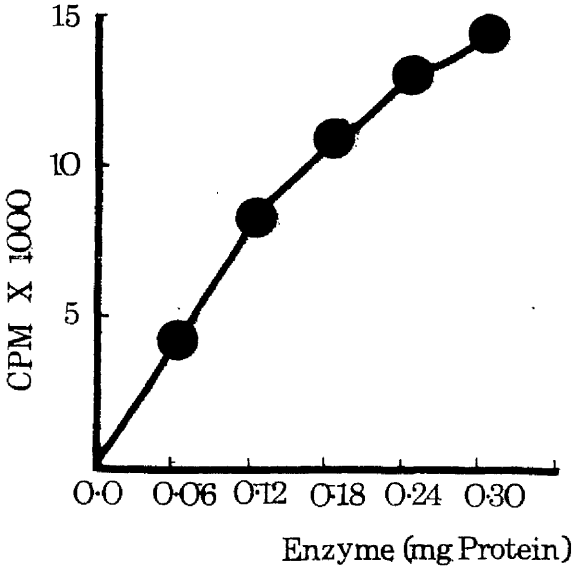
RBC Lysate 800 µg Protein

RBC Membranes 300 µg Protein



(b) Concentration Dependence of Activity

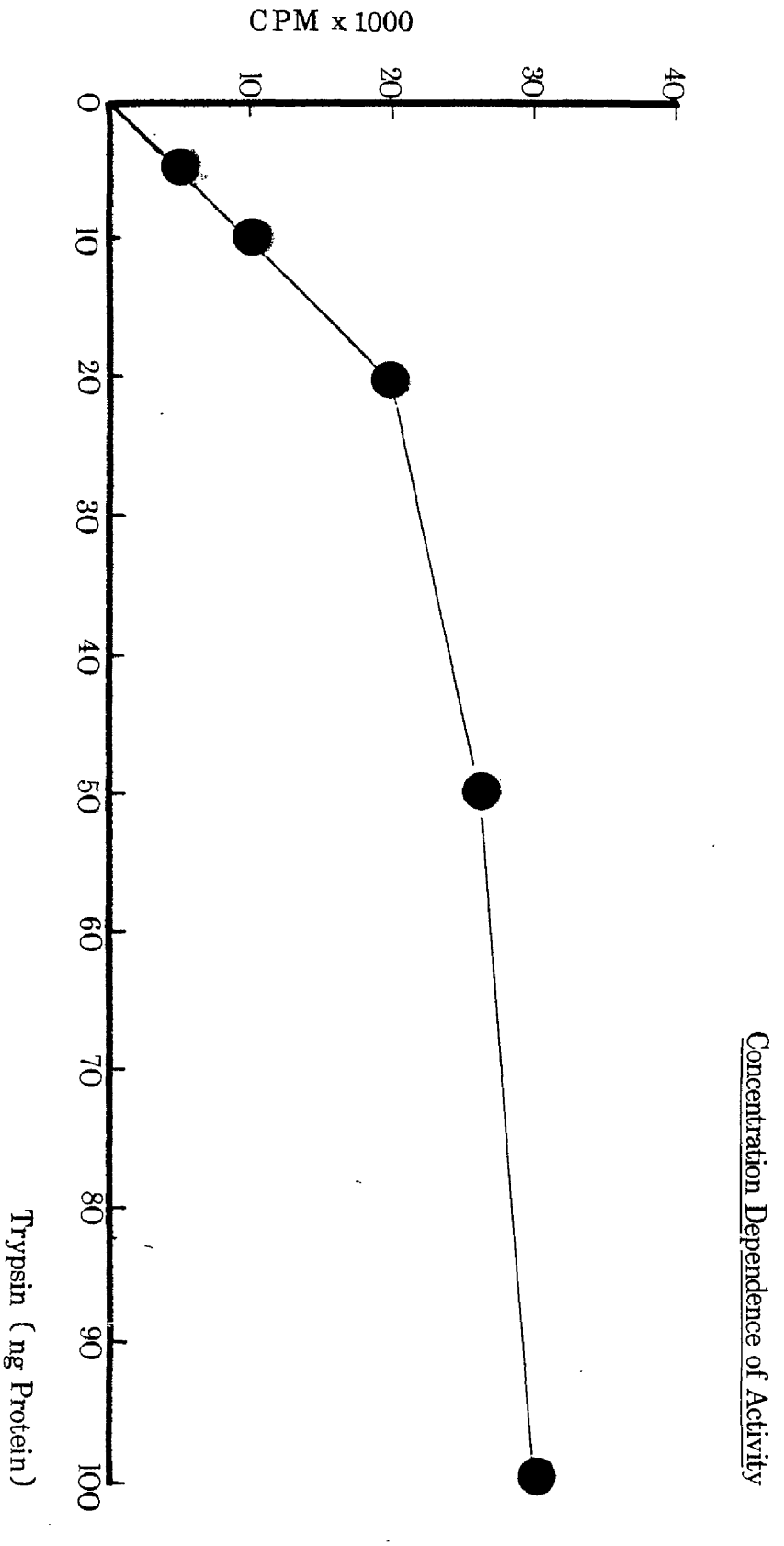
RBC Lysate



(c)

RBC Membranes

FIGURE 11. TRYPSIN STANDARD CURVE.



to 120µg protein). Similar conditions were employed for all proteolytic determinations made on human erythrocytes.

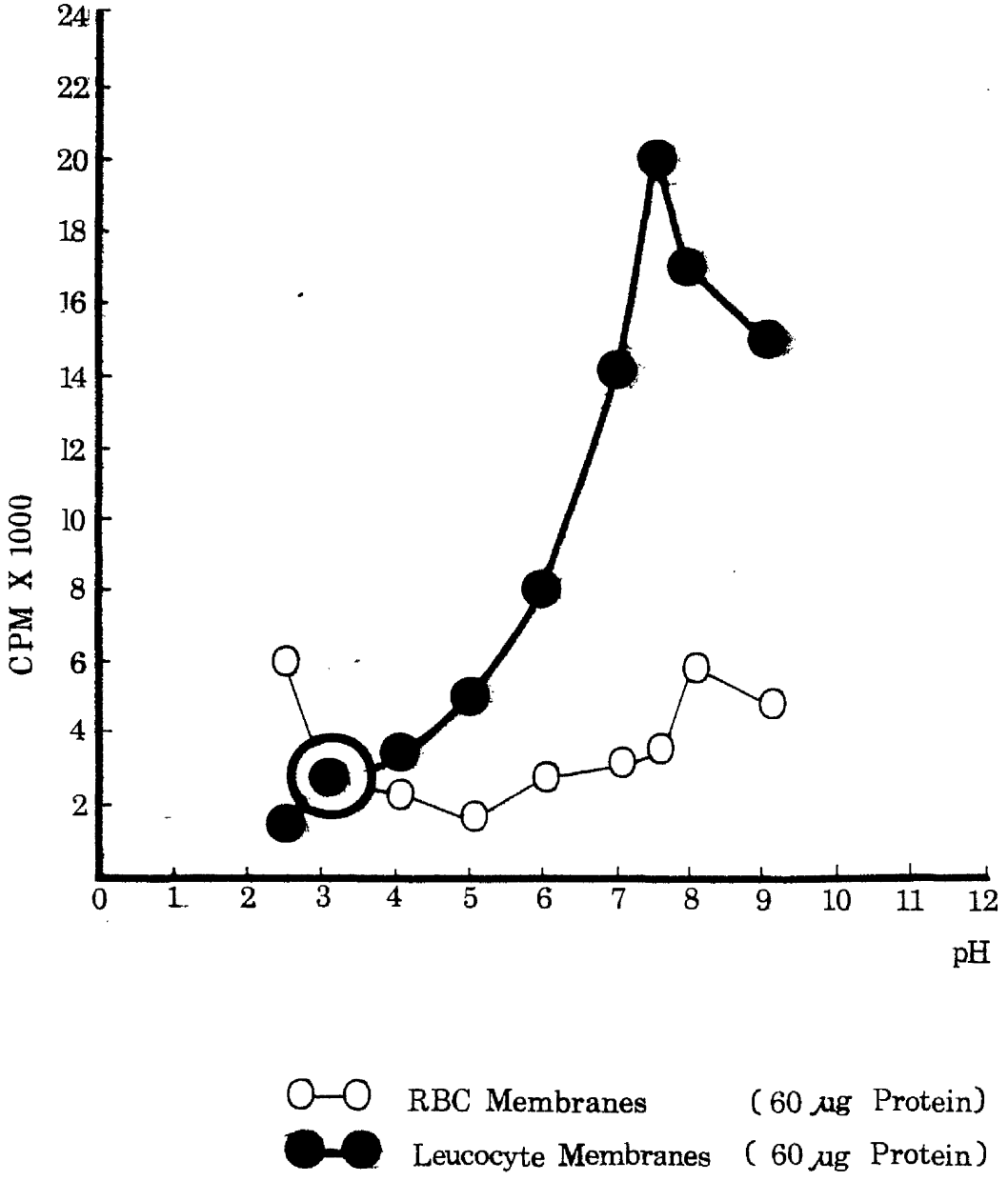
A trypsin standard curve, covering the range of 5 to 100ng, was included in each assay and the erythrocyte proteolytic activites were measured using this curve. Figure 11 shows the rate of hydrolysis of the casein substrate with increasing amounts of trypsin over a period of 1h at 37°C. The release of product was found to be a linear function of the amount of trypsin below 20ng. Validity in using the trypsin standard curve was tested using purified cytoplasmic proteases (section 3.16, figure 13). Hydrolysis of casein by trypsin and erythrocyte proteases is shown to be identical.

3.13 The Effect of pH on Erythrocyte Proteolytic Activites.

Normal human erythrocytes were used to examine the effect of pH on the membrane proteolytic activity. Two erythrocyte membrane samples were prepared in the presence and absence of leucocytes. The values obtained for the erythrocyte membrane activities were subtracted from the values obtained with the leucocytes present to obtain the pH profile for leucocytes (Figure 12).

The results indicate that two widely spread pH optima peaks for erythrocyte membranes were obtained. The acidic (pH 2.5) activity was slightly greater than the basic activity at pH 8.0. The leucocyte membrane proteolytic activities were also a mixture of two activity peaks, where the neutral activity maximum at pH 7.5 was of a much higher magnitude than the acidic activity peak at pH 3.0. Leucocyte membranes also had a much higher activity in the neutral pH range compared to erythrocyte membrane protease levels in that pH region. Since the physiological pH was again chosen for the measurement of erythrocyte

FIGURE 12. ERYTHROCYTE AND LEUCOCYTE PROTEOLYTIC ACTIVITY DEPENDENCE ON pH.



protease activities, as in the case of the phosphatase assay, the pH also favoured the detection of neutral proteases in leucocytes. These results emphasised the importance of the removal of white cells from erythrocytes to be used in this assay system.

3.14 Distribution of Erythrocyte Proteolytic Activities in Normal and Abnormal Cells.

Proteolytic activity measurements, using intact cells, membranes, and lysates, were carried out to establish the distribution of the proteases in human erythrocytes. Normal cells and spur cells were used in this study, the results of which are shown in Table 10.

Table 10. Distribution of Proteolytic Activities in Erythrocytes.

Leucocytes were removed from blood samples using the method of Beutler et al., (1976). Section 2.6 contains the details for this experiment. The difference between duplicate analyses was below ±5%.

Blood sample	PROTEOLYTIC ACTIVITY ng trypsin/ml packed cells Intact Cells	Membranes	Membrane-Free Lysate
Control cells*	4 ± 1	34 ± 10	157 ± 20
Control cells with leucocytes present+	4 ± 1	264 ± 20	156 ± 20
Spur Cells‡	10 ± 2	85 ± 15	190 ± 20

* mean value from two individuals
\+ mean value from two individuals
‡ mean of two separate estimates on one individual

Approximately 18% of the total cellular activity (soluble and insoluble proteases) in normal human erythrocytes was found to be membrane-associated. Of the membrane-associated activity 10% was detected on intact cells and hence presumably this activity was present on the outside of the erythrocyte since controls for cell lysis were included. However spur cell erythrocytes had approximately 31% of the total

cellular activity located on their membranes and 11% of the membrane-associated activity was on the outside of the erythrocytes.

When the buffy coat of white cells was not removed from erythrocytes the proteolytic activity measurement for the intact red cells did not change, therefore leucocytes do not possess any proteases on the external surfaces of their cell membranes which is assayable with the radiolabelled casein.

3.15 Membrane Association of Proteolytic Activities.

The association of proteases with membranes was examined by repeated washing of erythrocyte ghosts. Table 11 shows the results of the membrane washing experiment carried out using human erythrocytes. Calcium ions (0.1mM) were included in the lysis buffer of one sample and all of the membrane proteolytic activities were assayed in the presence and absence of calcium ions (0.1mM).

The results indicated that after five hypotonic washes the proteolytic activity associated with erythrocyte membranes did not alter with further hypotonic washes. The value for the membrane activity slightly decreased after further isotonic and hypertonic washes. Lysis of the erythrocytes in the presence of 0.1mM Ca^{2+} increased the specific activity of the proteases to 133% of control values. However this activity returned to the control level on further isotonic washing. Calcium ions added to the proteolytic assay decreased casein hydrolysis. Pre-incubation of calcium ions with the proteases before the addition of the substrate also decreased casein hydrolysis. Therefore this suggested that calcium does not activate the erythrocyte proteases.

The results showed that there are two types of membrane proteolytic activities, those that can be removed efficiently by isotonic and hypertonic washing compared to hypotonic washing. This type of activity also appears to be greater when calcium ions are used in the isolation buffer. The second type of the proteases resists elution from the erythrocyte membranes and may therefore be an integral or firmly associated membrane activity, situated on the internal and/or external surface perhaps possessing a specific function.

Table 11. Membrane Associated Proteolytic Activities.

Leucocytes were removed from blood samples using the method of Tökés and Chambers (1975). Membrane proteolytic activities were compared after their isolation using five hypotonic washes and three further washes of (a) hypotonic buffer, (b) isotonic salt solution (0.15M NaCl), (c) hypertonic solution (1.0M NaCl) or (d) isotonic salt solution containing 0.1mM Ca^{2+} employed in the total eight washes. The difference between duplicate analyses was below ±5%.

Membrane Sample	PROTEOLYTIC ACTIVITY nanograms trypsin/mg protein			Assay+Ca^{2+}
(a) 5 hypotonic washes	4.4	3 hypotonic	4.4	2.2
(b) 5 hypotonic washes		3 isotonic	3.3	1.8
(c) 5 hypotonic washes		3 hypertonic	3.4	3.0
(d) 5 hypotonic washes+Ca^{2+}	5.7	3 isotonic+Ca^{2+}	4.5	2.2

Another experiment was constructed to examine in more detail the effects of calcium ions on the proteolytic activity associated with erythrocyte membranes. Membranes were also prepared from one sample of red cells, in which the leucocytes had not been removed, and the proteolytic activity was measured.

Table 12. Effect of Calcium Ions and Leucocytes on the Proteolytic Activity of Erythrocyte Membranes.

Leucocytes were removed from blood samples using the method of Beutler et al., (1976). The difference between duplicate analyses was less than ±5%.

MEMBRANE SAMPLES*	PROTEOLYTIC ACTIVITY nanograms trypsin/mg protein MEMBRANES(±SD)	LYSATES(±SD)
Erythrocytes without leucocytes	4 ± 2	1.6 ± 0.2
Erythrocytes lysed in 0.1mM Ca^{2+}	6 ± 2	1.6 ± 0.3
Erythrocytes lysed in 1.0mM Ca_{2+}	13 ± 5	1.6 ± 0.2
Erythrocytes with leucocytes	44 ± 30	1.6 ± 0.4

* average results estimated from four different individuals.

Calcium ions, at a concentration of 0.1mM, increased the specific activity by one and a half times; 1.0mM calcium ions increased the specific activity by three times the activity measured for homogeneous erythrocyte membranes (Table 12). The calcium effect on the proteolytic activity of the membranes was concentration dependent and no change in the respective lysates occurred. The results indicated that calcium ions did not activate the soluble proteases. As found in the previous section (Table 11) extra hypotonic washing of the membranes did not change the specific activities of the membrane-associated proteases but further isotonic and hypertonic washing decreased the activity of the membranes isolated in the presence of calcium (values not shown).

Leucocyte membranes elevated the normal membrane proteolytic activity by approximately ten times the control values and the activity only slightly decreased with further isotonic washing (values not shown).

3.16 Purification of Erythrocyte Cytosolic Proteases.

The aim of the study was to remove the haemoglobin from the human

erythrocyte cytosolic (soluble) proteases and then to carry out proteolytic activity measurements. Throughout the purification protocol of the soluble proteases their specific activity was determined after each step (Table 13).

The results indicated that after the removal of haemoglobin, by ion exchange chromatography, the proteases were purified twenty-five fold. Concentration of the proteases, by ultrafiltration, gave an increase in the specific activity to forty three fold. Speed in carrying out the purfication procedure was necessary to maintain a high specific activity for the proteases. The concentrated enzyme, after dialysis, was aliquoted and stored frozen (-20°C) without significant loss of activity.

Table 13. Purification of Erythrocyte Cytosolic Proteases.

Human blood (10ml packed cells) was used for the preparation of soluble proteases after removing leucocytes using the method of Beutler et al., (1976). The purification levels are expressed in parenthesis and the total trypsin activities are expressed for the total volume of each protein solution. The difference between duplicates was less than ±5%.

Purification step (volume)	TOTAL PROTEIN (mg)	TOTAL TRYPSIN ACTIVITY (ng)	ng TRYPSIN ACTIVITY per mg PROTEIN
1. Membrane free lysate (100 ml)	3200	8447	3
2. Büchner funnel filtrate (100ml)	2850		
3. DEAE column pooled fractions (52ml)	68	5115	75 (X25)
4. Ultrafiltration concentrate (10ml)	41	5311	130 (X43)
5. Ultrafiltration filtrate (42ml)	0.42		
6. Dialysed ultrafiltration concentrate (10ml)	36	4302	120 (X40)

Figure 13 shows the dependence of the purified cytosolic proteases, expressed as nanograms of trypsin read on a standard curve, on the amount of enzyme assayed. The linearity of the graph indicated that the soluble erythrocyte proteases and trypsin have identical calibration curves with the range up to 100µg protein.

3.17 The pH Dependence of Erythrocyte Purified Cytosolic Proteases.

Figure 14 shows the pH profile obtained when the haemoglobin-free erythrocyte cytosol was used to examine the effect of pH on the proteolytic activity. The purified enzyme preparation was found to contain two major activity peaks, one in the acidic region (approximately pH 2.5) and the other in the neutral pH region (pH 8.0). These two activity peaks were at the same pH values determined for the membrane associated proteolytic activity of erythrocytes (Section 3.13). However the level of the proteolytic activity in the acidic pH region is slightly higher than the activity at pH 8.0 for the purified soluble proteases.

3.18 Haemoglobin Effect on the Assay of Cytosolic Proteases.

During the erythrocyte lysis step, referred to in section 3.16, the cytosolic proteases were in a solution containing 2.0% (w/v) haemoglobin. The final concentrations of haemoglobin in the [^{125}I]-casein assay decreased to 0.2% (w/v). An experiment was carried out to investigate the effect of haemoglobin on the assay of proteolytic activities. A range of haemoglobin concentrations were employed in the assay of trypsin and purified cytosolic proteases. Haemoglobin solutions (0.05% to 2.0% w/v) were used in the presence of (a) 5ng trypsin and (b) erythrocyte purified cytosolic proteases which had a level of activity approximately equivalent to the amount of trypsin chosen.

FIGURE 13. CONCENTRATION DEPENDENCE OF ERYTHROCYTE CYTOSOLIC PROTEASES.

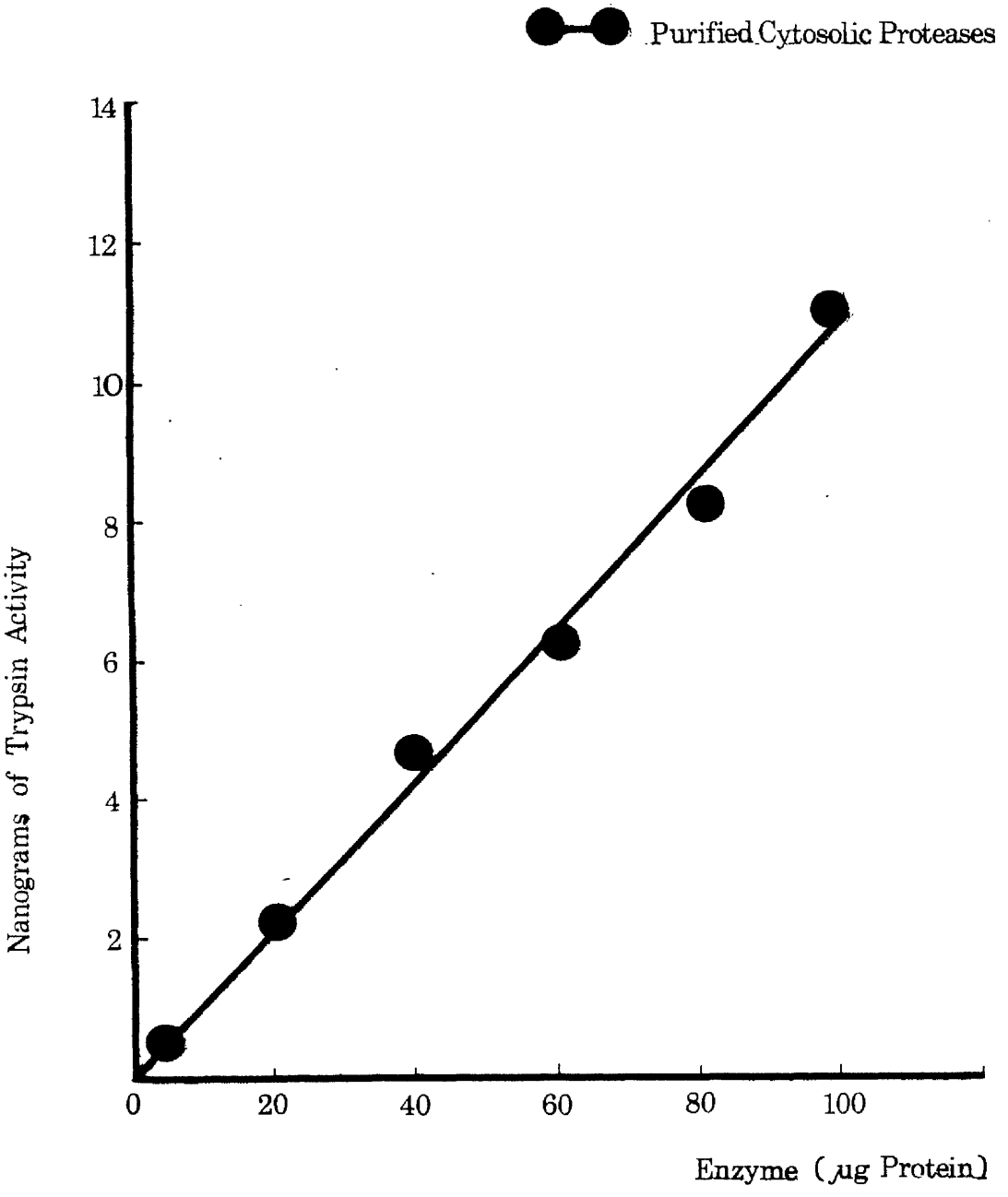
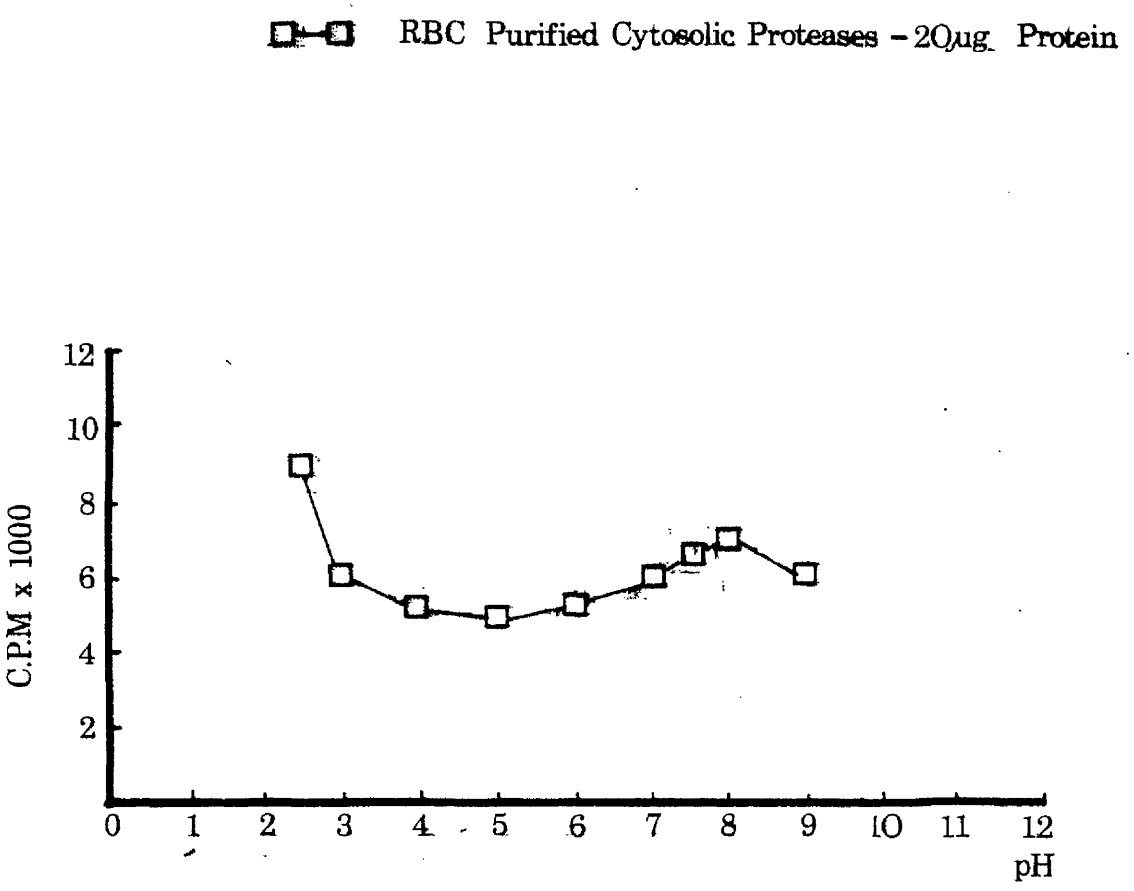


FIGURE 14. CYTOSOLIC PROTEOLYTIC ACTIVITY DEPENDENCE ON pH.



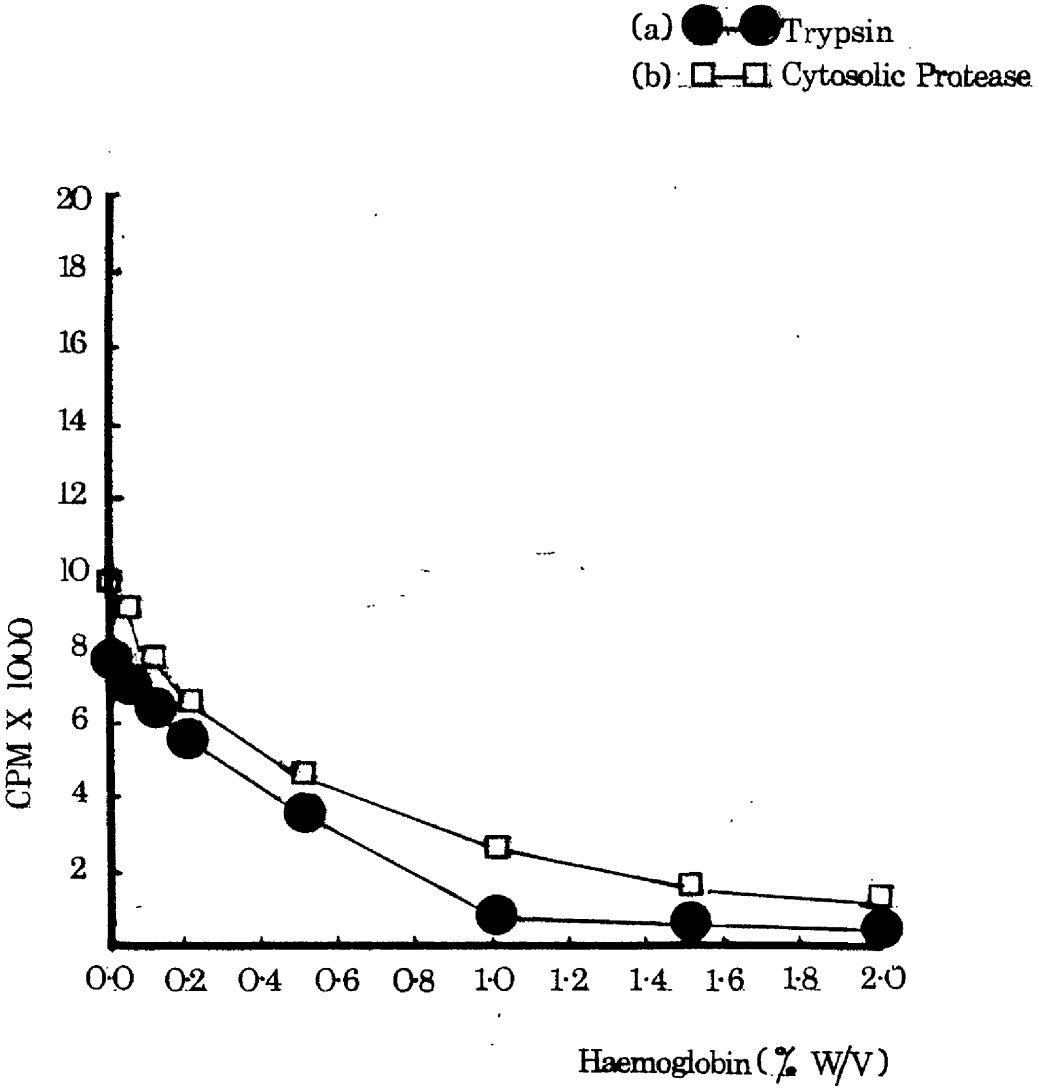
The results can be seen in Figure 15. Haemoglobin at a final concentration of 0.2% (w/v) partially inhibited the proteolytic activity of the cytosol giving only 64% of the original value estimated in the absence of haemoglobin. Thus the specific activity of the membrane free lysate was under-estimated by 36%. Applying this correction, the true value for the specific activity of the neutral proteases in the lysate would be 4ng trypsin/mg of protein (section 3.16).

3.19 Effect of Inhibitors on Erythrocyte Proteases.

The erythrocyte membrane and purified cytosolic proteases were characterised using a variety of substances known to modify proteolytic activities. The buffy coat of white cells was retained in one erythrocyte sample and the membranes were also used in the study to examine the response of leucocyte membrane proteases to the inhibitory compounds.

Table 14 shows the results obtained. The irreversible serine-protease inhibitor PMSF (1mM) almost totally abolished the proteolytic activity associated with erythrocyte membranes and was not observed to be more inhibitory at higher concentrations. PMSF (10mM) also inhibited the proteolytic activity associated with erythrocyte membranes containing leucocyte membranes. The soluble proteases were inhibited by 10mM PMSF to 54% of the control containing DMSO which was used as a solvent for the inhibitor. The thiol protease inhibitor PCMB, at a concentration of 1mM, decreased the membrane activity to 73% and to 3% at ten times the inhibitor concentration. The sample containing leucocyte membrane proteases was inhibited to 5% at 10mM PCMB concentration and the cytosolic proteases were greatly inhibited, down to 4% of the control activity, using 1mM PCMB. Dithiothreitol (1mM) stimulated all three proteolytic activities indicating that these

FIGURE 15. EFFECT OF HAEMOGLOBIN ON THE ASSAY OF R.B.C CYTOSOLIC PROTEASES.



proteases possibly required thiol groups for activity. The trypsin inhibitor (TLCK) at 1mM and 10mM concentrations inhibited the soluble proteases, down to 38% and 19% respectively, more than the membrane associated activity, down to 50% and 34% respectively. At the 10mM concentration TLCK decreased the erythrocyte and leucocyte membrane proteolytic activity down to 57%. Calcium ions (1mM), ortho phenanthroline (1mM) and ethylenediamine tetracetic acid (1mM), in general caused greater inhibition of the erythrocyte membrane associated proteases.

The results indicated that there is more than one type of proteolytic activity in human erythrocytes. All of the membrane associated proteolytic activity can be inhibited by PMSF (10mM) whereas a second type of protease, not inhibited by PMSF, accounts for about 50% of the cytosolic activity. The PMSF inhibitable activities of cytosol and membrane may arise from more than one enzyme. Both membrane and cytosolic proteases could be inhibited partially by PCMB (1mM), however the soluble activity appears to be more sensitive to this compound. The leucocyte proteases were found to be totally inhibited by PMSF (10mM).

Table 14. Erythrocyte Proteolytic Activity Inhibition.

Leucocytes were removed from blood samples using the method of Beutler et al., (1976). The values in parenthesis indicate the percentage of the activity remaining using the appropriate solvent controls. Details of the experimental procedure are in section 2.7. Both PMSF and TLCK were dissolved in DMSO. The difference between duplicate analyses was less than ±10%

Modifier	Concentration (mM)	% PROTEOLYTIC ACTIVITY REMAINING Membranes of Erythrocytes*	Membranes of Erythrocytes and Leucocytes+	Purified Cytosolic Proteases‡
Phenylmethylsulphonyl fluoride (PMSF)	1.0	6 (10)	ND	79 (79) ± 24
	5.0	4 (7)	ND	50 (66) ± 19
	10.0	2 (3)	0	28 (54) ± 16
Dimethylsulphoxide (DMSO)	1.0ul	58	ND	100 ± 1
	5.0ul	60	ND	73 ± 16
	10.0ul	65	79	46 ± 20
Para-chloromercuri benzoate (PCMB)	1.0	73	ND	4 ± 4
	10.0	3	5	ND
DL-Dithiothreitol (DTT)	1.0	125	188	121 ± 24
Ethylenediamine tetracetic acid (EDTA)	1.0	56	ND	38 ± 20
	10.0	51	59	ND
Ortho-Phenanthroline	1.0	7	ND	36 ± 9
	10.0	5	15	9 ± 5
N-Tosyl-L-lysylchloro methyl ketone (TLCK)	1.0	50 (86)	ND	38 (38) ± 5
	10.0	34 (52)	57 (72)	19 (37) ± 6
Calcium chloride ($CaCl_2$)	1.0	56	ND	116 ± 45
	10.0	39	76	ND

* control activity 8ng trypsin (one experiment in duplicate)
\+ control activity 13ng trypsin (one experiment in duplicate)
‡ control activity 10ng trypsin (mean of three estimations in duplicate from two individuals)
ND not determined

3.20 Proteolytic Activities of Normal and Spur Cell Erythrocyte Membranes.

The membrane associated proteolytic activity was measured using normal red cells and spur cell erythrocytes. Normal erythrocyte membranes were also divided into subfractions as top and "bottom" ghosts and their respective proteolytic activites were determined (Table 15). The proteolytic activity of spur cell membranes was found to be almost

four times the activity obtained for normal erythrocyte membranes. Fractionation of the normal erythrocyte membranes revealed that the "button" membrane fraction, which contained approximately 20% of the total membrane protein, had the highest proteolytic activity compared to the top ghost fraction.

Table 15. Normal and Spur Cell Erythrocyte Membrane Proteolytic Activities.

Leucocytes were removed from blood samples according to the method of Tökés and Chambers (1975). The numbers in parenthesis indicate the number of subjects used. The error of estimation between duplicates was less than ±5%.

Blood Sample	Membrane Fraction	Percentage Weight of total Membranes	Proteolytic Activity ng Trypsin/mg protein
Normal cells (2)	Total ghosts		4.8 ± 1
	Top ghosts	80 ± 8	3.3 ± 1
	Button ghosts	20 ± 8	11.3 ± 2
Spur cells (2)	Total ghosts		13.5 ± 2

3.21 Erythrocyte Membrane Protein Banding Pattern.

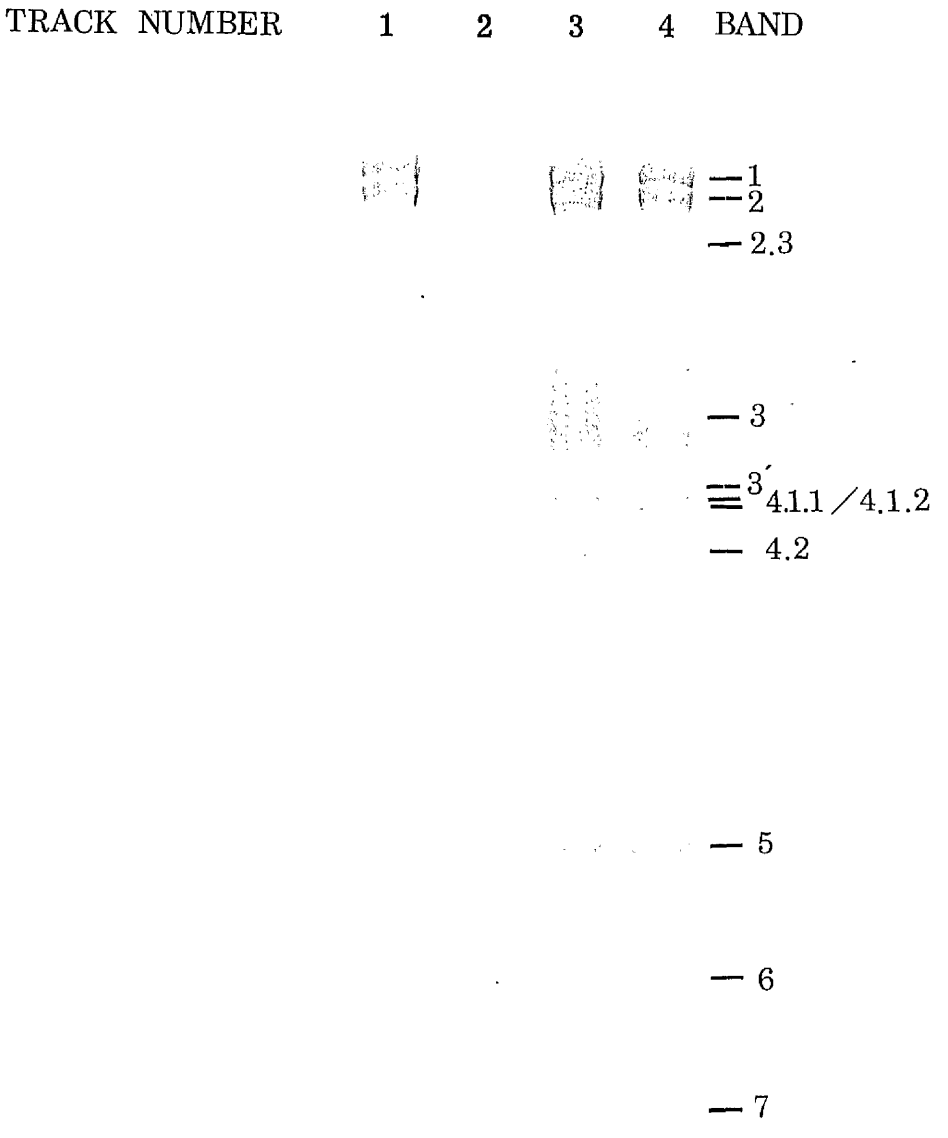
Membranes from normal and spur cell human erythrocytes were isolated according to the method of Steck et al., (1970). In addition to the usual ghost preparation the "button" fraction appearing as a packed pellet in the membrane preparation was examined. The membrane proteins were examined by polyacrylamide gel electrophoresis in the presence of 0.1% SDS.

Analysis of the results (Figure 16) indicated that the normal erythrocyte total membranes displayed the presence of the expected complement of eight major polypeptides, identified by the system of

FIGURE LEGEND.

Normal and abnormal (spur cell) erythrocyte membrane polypeptides are shown in this photograph of a slab gel employing the discontinuous buffer system of Laemmli (1969). Band 4.1 which appears homogeneous by phosphate gels (Fairbanks et al. 1971) is actually a mixture of two protein components, namely 4.1.1 and 4.1.2.

FIGURE 16. NORMAL AND ABNORMAL ERYTHROCYTE PROTEIN GEL BANDING PATTERNS.



1. Spur cell total ghosts.
2. Spur cell button ghosts.
3. Normal total ghosts.
4. Normal button ghosts.

Fairbanks et. al., (1971). However the "button" membrane fraction of normal erythrocytes contained less of bands 2.3 and 3 with the appearance of a new component, between bands 3 and 4, which will be designated hereafter as band 3' (approximate mol. wt. 80,000).

Spur cell membranes, on the other hand, had a protein banding pattern almost identical to that obtained for normal erythrocyte "button" ghosts, with one exception demonstrated by the absence of band 4.1.1 in spur cell total membranes. The corresponding "button" membrane fraction was completely lacking in the major protein bands showing only a slight staining intensity in the region of the dye front. The tracker dye was accidentally allowed to pass out of this particular gel.

The presence of these new lower molecular weight peptides, coupled with the partial or complete loss of major polypeptide bands, suggests the occurrence of proteolytic degradation in erythrocyte membranes. The proteolysis was apparently more pronounced in the spur cell erythrocyte membrane preparation, particularly in the "button" ghost fraction.

3.22 Isolation of Erythrocyte Membranes in the Presence and Absence of PMSF.

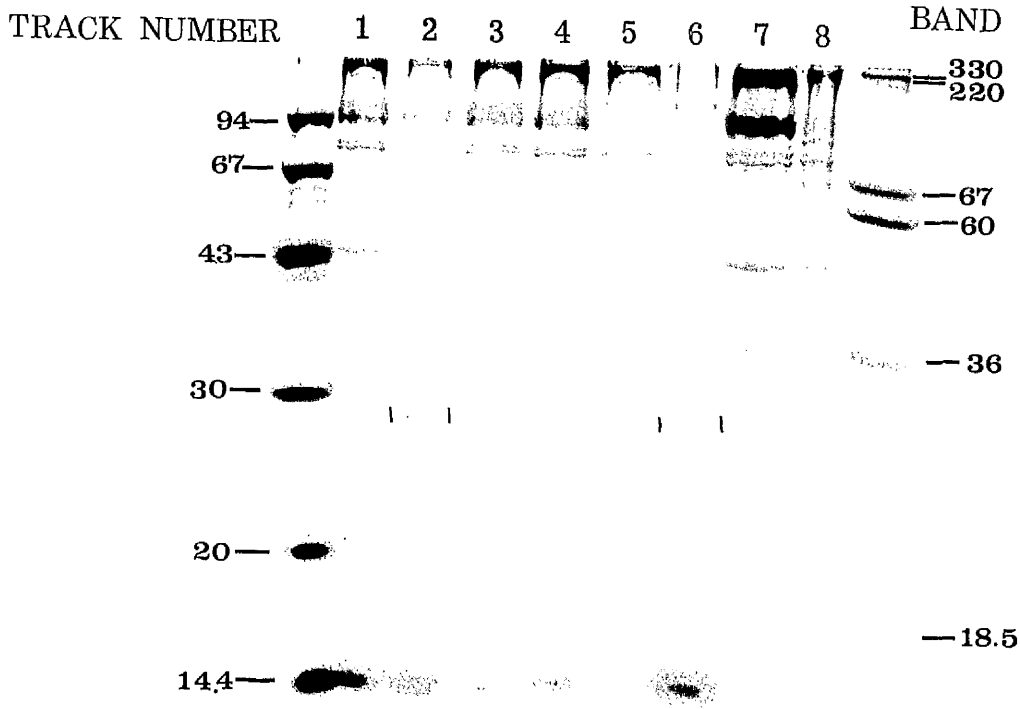
To examine the occurrence of proteolytic degradation normal human erythrocytes and spur cell erythrocytes were lysed and isolated in the presence or absence of PMSF (1mM). An inhibitor would be expected to prevent degradation of membrane proteins by membrane associated proteases (section 3.19). The ghosts were divided into top and "button" ghosts after the last wash and examined on SDS-polyacrylamide gels.

Figure 17 depicts the protein banding patterns obtained after the electrophoresis of the membrane samples. Normal erythrocyte membranes

FIGURE LEGEND.

Tracks 1 and 2 contain membranes of spur cell erythrocyte top and button fractions isolated in the presence of the serine protease inhibitor. Tracks 5 and 6 contain the same samples without inhibitor. Tracks 3 and 4 contain membrane samples from normal erythrocyte top and button fractions isolated with inhibitor and tracks 7 and 8 are the same samples isolated with no inhibitor present. The outer two tracks contain low and high molecular weight standards respectively. Leucocytes were removed from blood samples according to the method of Tökés and Chambers (1975).

FIGURE 17. PROTEIN BANDING PATTERNS OF MEMBRANES ISOLATED IN THE PRESENCE AND ABSENCE OF PMSF.



isolated in the presence of 1mM PMSF showed no signs of degradation in either the top or "button" sample. However, the "button" membrane samples showed evidence of mild proteolytic degradation having taken place when no PMSF was employed.

Spur cell erythrocyte top and "button" membranes were both degraded in the absence of PMSF with the "button" again exhibiting the most evidence of degradation. Moreover the "button" membrane sample derived from spur cells continued to display evidence of proteolysis even in the presence of PMSF, although the degradation was limited to a few proteins. Spur cell erythrocytes also contained more haemoglobin associated with their membranes.

3.23 Degradation Pattern produced by Membrane Associated Proteases.

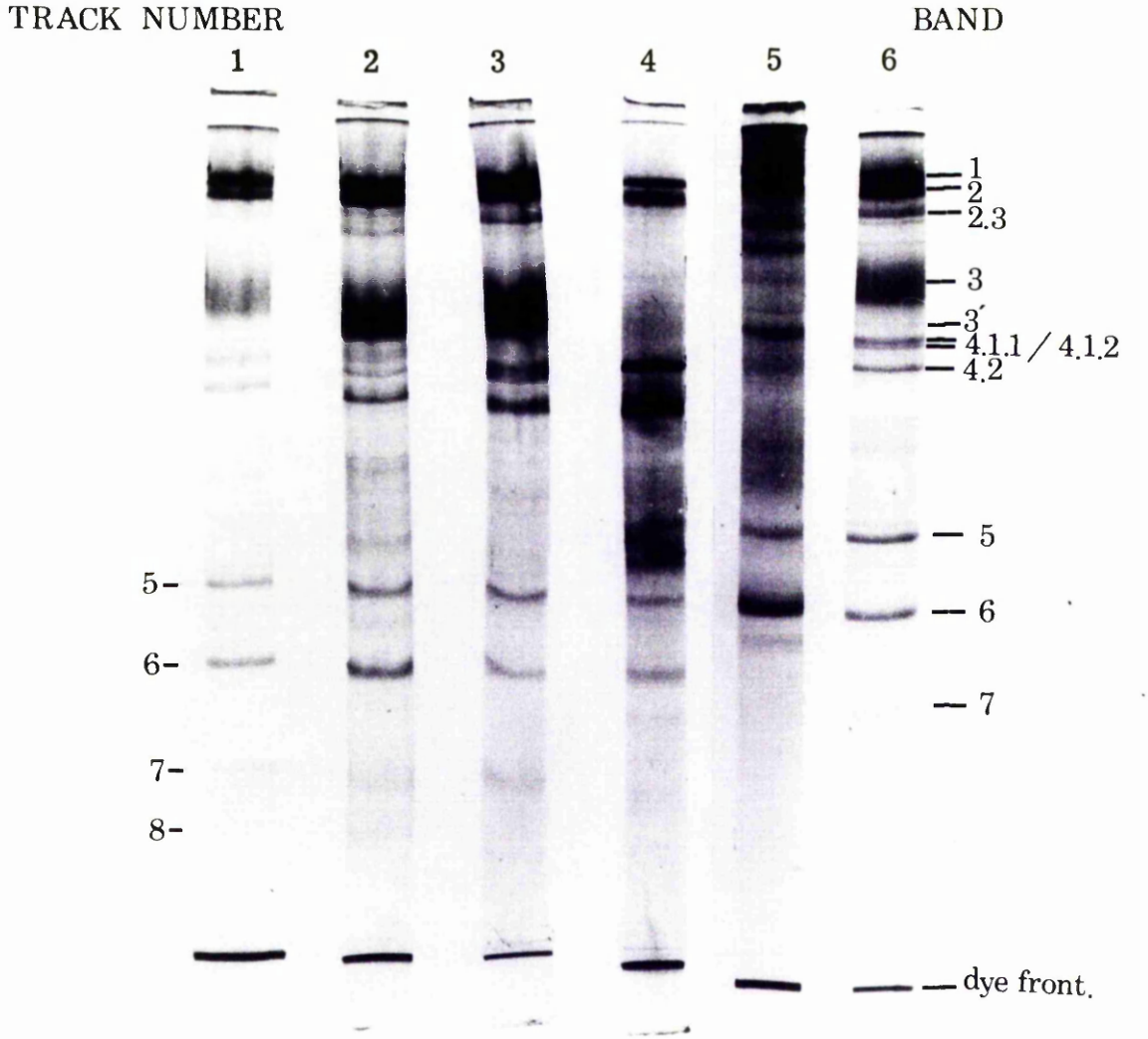
Normal and spur cell erythrocyte membranes were isolated in the presence or absence of 1mM PMSF. The ghosts isolated in the absence of the serine protease inhibitor were then incubated for 24h at 4°C to allow proteolytic degradation to take place. The purpose of this experiment was to examine the pattern of degradation of the erythrocyte membrane proteins. The samples were run on SDS-polyacrylamide gels using the micro-gel electrophoresis apparatus.

Figure 18 shows the protein banding patterns obtained from each of the samples. Control erythrocyte membranes isolated in the presence of PMSF had a normal protein banding pattern. Unfortunately this gel track was slightly under loaded. Without the serine protease inhibitor, the protein banding pattern shows evidence of mild degradation having taken place. Band 2.3 increased in staining intensity and band 3' appeared. Incubation of these membranes (24h/4°C) produced more of bands 3' and 4.2 with some diffuse stained areas appearing between bands 4 and 5 and

FIGURE LEGEND.

Leucocytes were removed from the blood samples according to the method of Tökés and Chambers (1975). Membranes were resuspended in lysis buffer (3mg/ml) and incubated in an equal volume of the same buffer. Tracks 1 and 2 contain normal and spur cell membranes which were isolated in the presence of PMSF. Tracks 3 and 4 contain the same samples isolated without PMSF and incubated (24h/4°C). Spur cell and normal membranes isolated in the absence of PMSF are contained in tracks 5 and 6.

FIGURE 18. THE PATTERN OF PROTEOLYTIC DEGRADATION ON RBC MEMBRANE PROTEINS.



below band 7.

Spur cell erythrocyte membranes isolated using PMSF show signs of limited degradation having taken place. The staining of band 2.3 increased in intensity, band 3' appeared along with band 8 (mol. wt. 24,000) and increased staining was observed between bands 4 and 5. However most of the details have not been clearly reproduced on this picture of the gel photograph. In the absence of PMSF these membrane proteins were extensively degraded and only spectrin (bands 1 and 2), actin (band 5) and band 6 were identified as being relatively resistant to degradation. Many other new bands appeared as a result of the degradation. Incubation of the membranes (24h/4°C) led to losses of more protein bands which gave rise to small polypeptide fragments. Again spectrin, actin and band 6 apparently remained intact, although this does not rule out the possibility that bands 5 and 6 are non-homogeneous and contain degradation products from proteins with very high molecular weight values.

The pattern of degradation produced by the insoluble proteases on the membrane-associated polypeptides followed the same pattern in both membrane samples except that the occurrence of this phenomenon in spur cell membrane proteins was more rapid and therefore more extensive after incubation. From the results it can be seen that the first indication of proteolysis was observed in the ankyrin band region (track 6) where band 2.3 increased in intensity whilst the other ankyrin bands are relatively unchanged and band 3' appeared. However after 24 hours incubation at 4°C normal erythrocyte ghosts showed an increase in the amount of band 3' present on the gel (track 3) and the staining intensity of band 4.2 had become more diffuse. Moreover these alterations in the banding pattern occurred with spur cell membrane proteins which had been isolated in the

presence of PMSF (track 2). Spur cell membranes isolated in the absence of PMSF (track 5) showed definite losses in the protein banding pattern where many degradation products appreared as a diffuse stain spanning the top region of the gel. Track 4 showed the progression of the spur cell membrane protein degradation after incubation at 4°C where the degradation products were observed to spread down towards the dye front into the middle and bottom sections of the gel.

It is possible that the rate of degradation of spur cell membrane proteins results from either an increased susceptibility of the membrane polypeptides to hydrolysis or the presence of a greater quantity of the protease in the membranes or cytosol. The results from the radio-casein assay showed that there is an increased level of proteolytic activity on these membranes.

3.24 The Effect of Calcium Ions on Erythrocyte Membrane Proteins.

Calcium ions were previously shown to increase the association of proteases with erythrocyte membranes (section 3.15) when included in the erythrocyte lysis buffer. Normal erythrocyte membranes, isolated in the presence of calcium ions, were examined using SDS-polyacrylamide gel electrophoresis to study the banding pattern of erythrocyte membrane polypeptides.

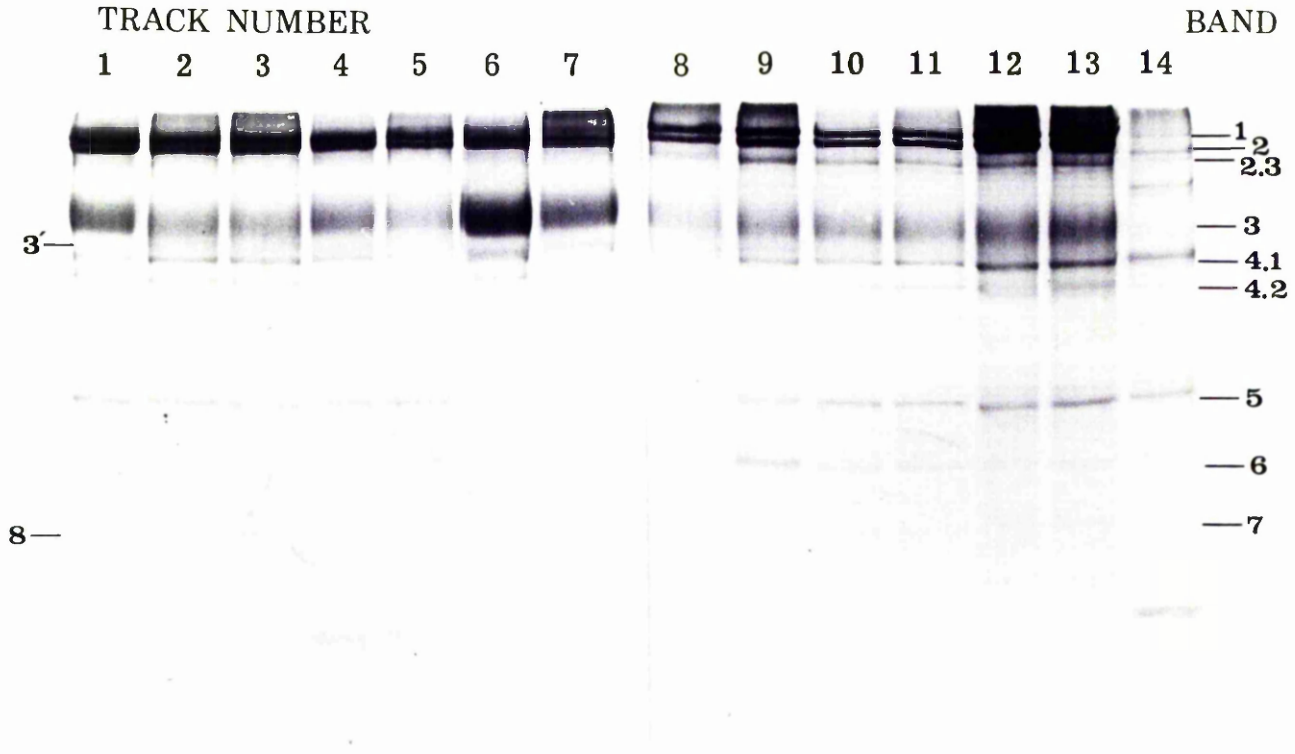
The results from the membrane isolation and incubation experiment are shown in Figure 19. Human erythrocytes were lysed and washed either in the usual way or in the presence of 1.0mM calcium ions. The erythrocyte lysate and purified cytosolic protease were used in membrane incubation experiments carried out in the presence or absence of 1.0mM calcium ions. The results of the incubation experiments were obtained by examining the samples using SDS-polyacrylamide gels on the micro-gel

FIGURE LEGEND.

Leucocytes were removed from blood samples according to the method of Beutler et al.,(1976). Two types of membranes were prepared 1. normal, and 2. calcium (1mM) isolated ghosts. The two membrane samples were used in the following way and the protein banding patterns were examined.

Track.	Sample.	Treatment.
1	1	Control.
2	1	Incubated 1h / 37°C.
3	1	Incubated with Ca 1h / 37°C.
4	2	Control.
5	2	Incubated 1h / 37°C.
8	1	Preincubated with Ca and lysate 1h / 4°C; washed; incubated 1h / 37°C.
9	1	Preincubated with lysate 1h/4°C; washed; incubated 1h/37°C.
10	1	Preincubated with Ca and purified proteases 1h/4°C; washed; incubated 1h / 37°C.
11	1	Preincubated with purified proteases 1h/4°C; washed; incubated 1h/37°C.
12	1	Incubated with Ca and purified proteases 1h/37°C.
13	1	Incubated with purified proteases 1h / 37°C.
14	1	Incubated with Trypsin 1h / 37°C.

FIGURE 19. EFFECT OF CALCIUM IONS ON RBC MEMBRANE PROTEINS.



apparatus. Again the reproduction of the gel photograph does not ideally show all of the changes originally observed.

Normal erythrocyte membranes displayed a total complement of eight major bands (track 1), band 2.3 seemed to be more intensely stained than the rest of the ankyrin bands and a faintly stained band 3' appeared. This sample was used as the control. Incubation of this membrane sample (1h/37°C) in the absence (track 2) or presence (track 3) of calcium ions (1mM) brought about very little change in either case.

Erythrocytes lysed and isolated in the presence of calcium ions (1mM) displayed changes in the protein banding pattern which were not observed in the control. There was a decrease in the staining intensities of band 3, 4.1 and 4.2, and increase in the intensities of bands 3' and 4.5, and band 8 (24,000) appeared (track 4). Incubation of these calcium lysed membranes (track 5) produced decreases in bands 1,2,3, 4.1 and 4.5. Low molecular weight products were diffusely stained at the dye front of tracks 4 and 5.

Pre-incubation of the erythrocyte membranes (1h and 4°C) in the presence of lysate with and without calcium ions (1.0mM) produced more degradation of the membrane proteins when calcium ions were included in the first incubation. Track 9 showed an increase in the density of band 2.3; band 3' was present; band 4.2 decreased and there was a new band observed between bands 5 and 6. Track 8, which had the calcium-lysate treatment, showed decreases in bands 2.3, 3, 4.1, 4.2 and 5 and both tracks 8 and 9 had an increased staining intensity at their respective dye fronts.

Erythrocyte membranes which had been pre-incubated with the

purified cytosolic proteases (tracks 10 and 11) both showed an increase in band 2.3; the presence of band 3' and a loss of band 3 staining. However, these degradation effects were not apparently increased when calcium was present in the pre-incubation sample.

The direct incubation of membranes and purified cytosolic proteases in the presence (track 12) and absence (track 13) of calcium ions showed the same extent of degradation of the erythrocyte proteins. Track 14 shows the degradation produced by trypsin on the polypeptides of the erythrocyte membranes. These last tracks demonstrated the ability of both trypsin and cytosolic protease to degrade the membrane proteins.

The main conclusion to be drawn from this experiment was that calcium ions promoted the degradation of erythrocyte membrane polypeptides either when the cells were lysed in the presence of calcium ions or when the ghosts were incubated with the lysate in the presence of calcium. For this to occur calcium most probably promoted the adsorption of cytosolic proteases onto the membranes. However in purifying the lysate proteases their ability to be adsorbed onto erythrocyte membranes by calcium was apparently lost.

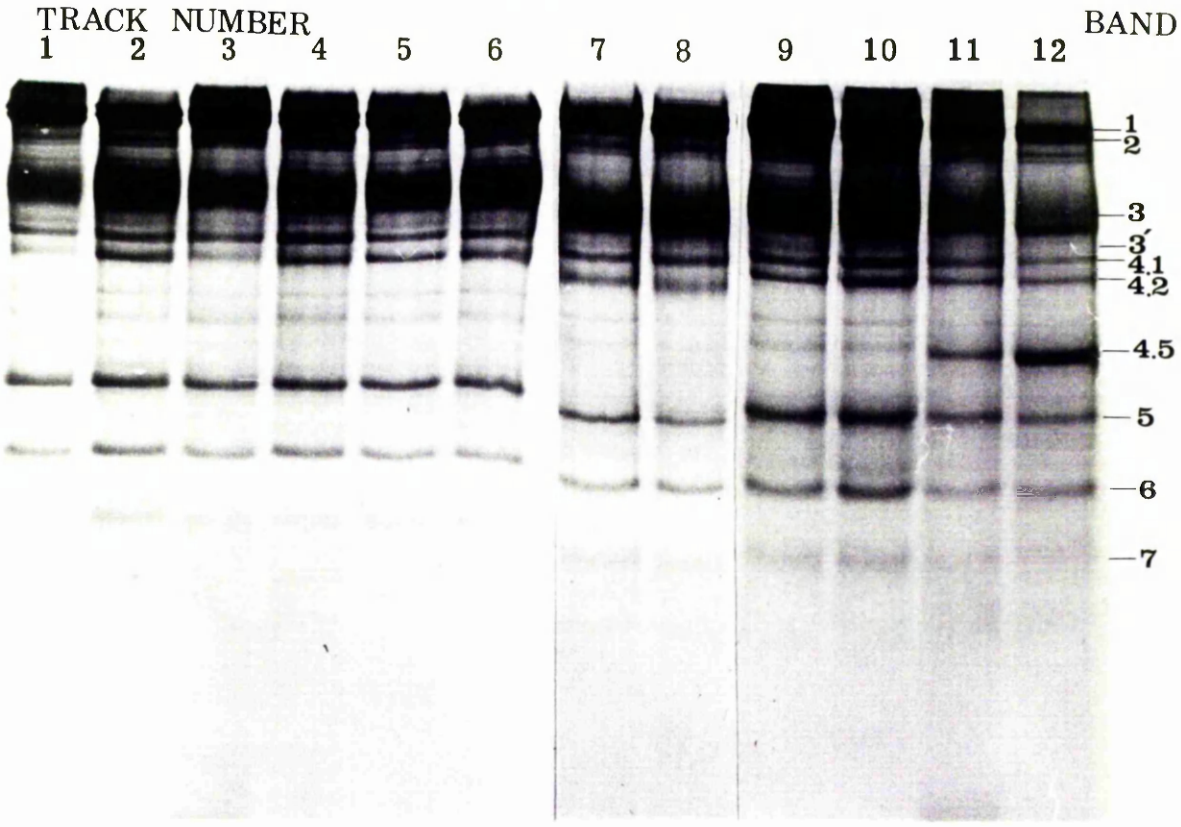
3.25 The Effect of leucocytes on Erythrocyte Membrane Proteins.

Normal human erythrocytes were used in the preparation of total, top and "button" ghost fractions. Only half of the blood sample was made free of leucocytes and the method of Beutler *et al.* (1976) was employed to achieve this. Each of the samples was divided into two aliquots, one of which was incubated for 24h at 4°C. All the samples were compared on SDS-polyacrylamide gels using the micro-gel apparatus. The aim of the experiment was to examine the degradation potential of leucocyte membrane proteases on erythrocyte membrane polypeptides.

FIGURE LEGEND.

Leucocytes were removed from blood samples using the method of Beutler et al.,(1976).Membrane samples were resuspended in lysis buffer (3mg / ml) and incubated in the same buffer(1.5mg / ml). Tracks 1,3 and 5 contain the total, top and button membranes respectively of homogeneous erythrocytes. Tracks 7,9 and 11 contain the total, top and button membranes respectively of erythrocytes which were not made free of leucocytes. Each of the samples were incubated for 24 h / 4°C and the proteins were examined in gel tracks parallel to the original sample (which was not incubated).

FIGURE 20. THE EFFECT OF LEUCOCYTES ON THE DEGRADATION PATTERN OF RBC MEMBRANE PROTEINS.



From the results (Figure 20) the only tracks which exhibited evidence of a change in the membrane protein banding patterns through degradation were tracks 11 and 12. The proteins originated from erythrocyte and leucocyte membrane "button" fraction. Protein band 3 decreased in intensity; band 3' appeared and band 4.5 increased in intensity in track 11. After incubation of the membrane suspension, spectrin and more of band 3 were degraded, and the intensity of band 4.5 increased.

All of the other tracks showed some increase in the staining intensity of band 2.3, also band 3' appeared, however, no changes were observed after incubation of the samples represented in tracks 1,3,5,7 and 9.

3.26 The Detection of Antibodies on Erythrocyte Membranes using the ELISA Assay.

In one experiment normal and spur cell erythrocyte membranes were prepared in the presence of 1.0mM PMSF and fractionated into total, top and "button" ghosts. The membranes were examined on SDS-polyacrylamide gels (Figure 21). However, prior to the membrane isolation procedure the spur cell erythrocytes (90% v/v in homologous plasma) were used to determine their concentrations of phosphorus cations by employing Nuclear Magnetic Resonance (NMR) spectroscopy. This technique involved spinning the cells suspended in plasma for 3h at 4^{o}C within the NMR instrument.

Figure 22 shows the plot of the relative mobilities of the erythrocyte proteins against the logarithm of their molecular weights. Two new polypeptide bands, designated x and y, appeared on the gel

FIGURE LEGEND.

Leucocytes were removed from blood samples using the method of Tökés and Chambers (1975). Tracks 1,2 and 3 contain total, top and button ghosts isolated from normal erythrocytes in the presence of PMSF. Tracks 4,5 and 6 contain total, top and button ghosts isolated from spur cell erythrocytes in the presence of PMSF.

FIGURE 21. IMMUNOGLOBULIN BINDING TO SPUR CELL RBC MEMBRANES.

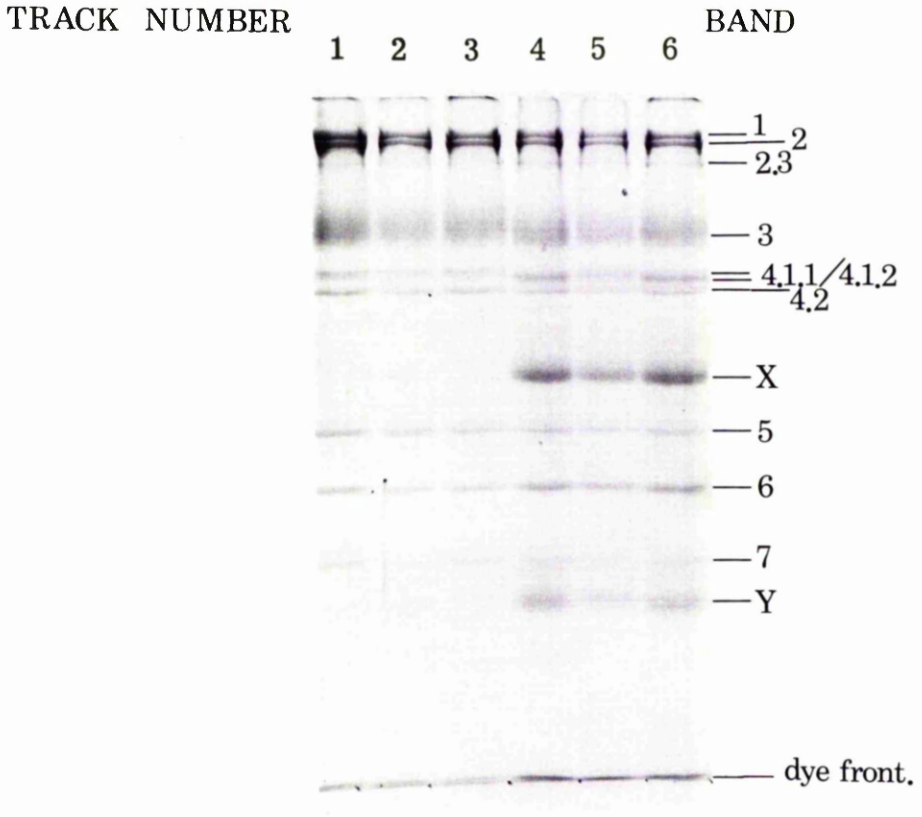
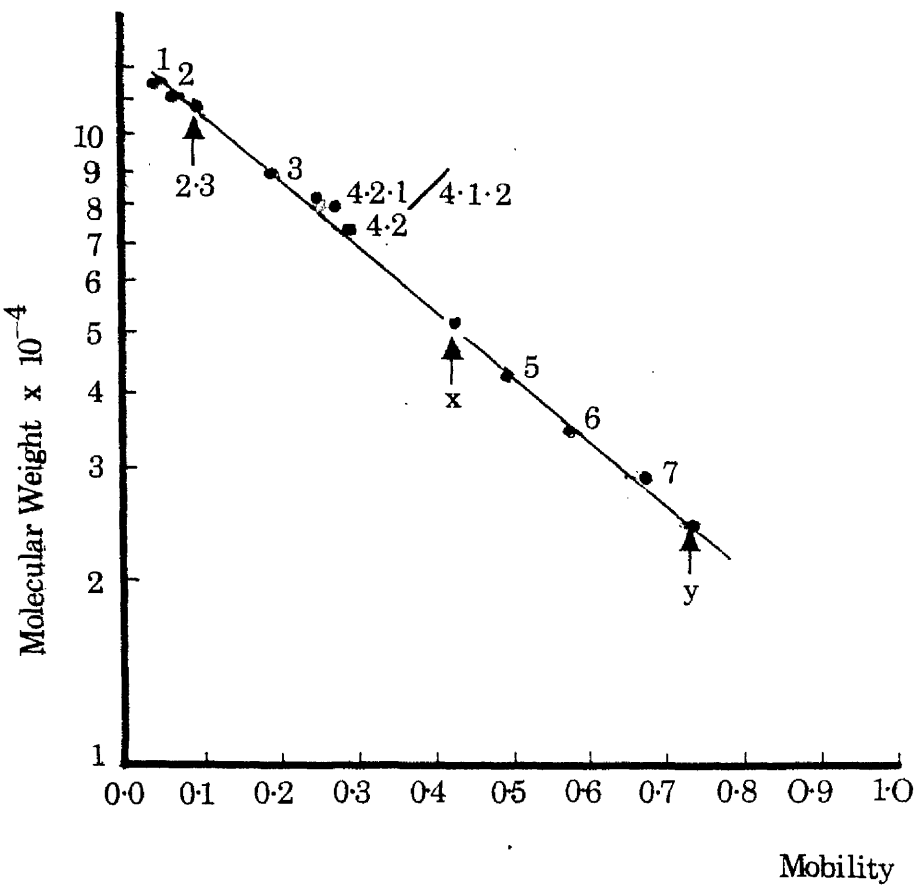


FIGURE 22. MOLECULAR WEIGHT DETERMINATIONS OF ERYTHROCYTE POLYPEPTIDES BY S.D.S – POLYACRYLAMIDE GEL ELECTROPHORESIS.



tracks containing spur cell membrane proteins. These were identified as having molecular weights of approximately 50,000 and 25,000 respectively. The two polypeptides were not degradation products but were suspected to represent the heavy and light chains of the immunoglobulin and antibody molecule.

To quantitate the binding of antibody molecules to erythrocyte membranes the enzyme-linked immunosorbent assay (ELISA) was carried out. Triton-X-100 (0.1% v/v) extracts of erythrocyte membranes were assayed. Figure 23 shows the standard curves obtained for human IgG in the presence and absence of Triton-X-100 (0.1% v/v). The reduction in the sensitivity of the standard curve in the presence of detergent was found to result from the elution of coating antibody molecules from the sample wells. Higher concentration of the detergent (1%) totally reduced binding. However it was desirable to use this detergent concentration to bring about solubilisation of membrane bound IgG molecules from erythrocyte ghosts.

Table 16 shows the results of the assay which signifies the presence of large amounts of antibody molecules on spur cell membranes derived from cells used in the NMR study. It also indicates that spur cell erythrocytes do not normally possess large amounts of IgG bound to the membranes, compared to the control levels on normal erythrocyte membranes. This rules out the possibility of spur cell anaemia being an autoimmune disease with respect to IgG molecules.

FIGURE 23. ELISA STANDARD CURVE FOR HUMAN Ig G.

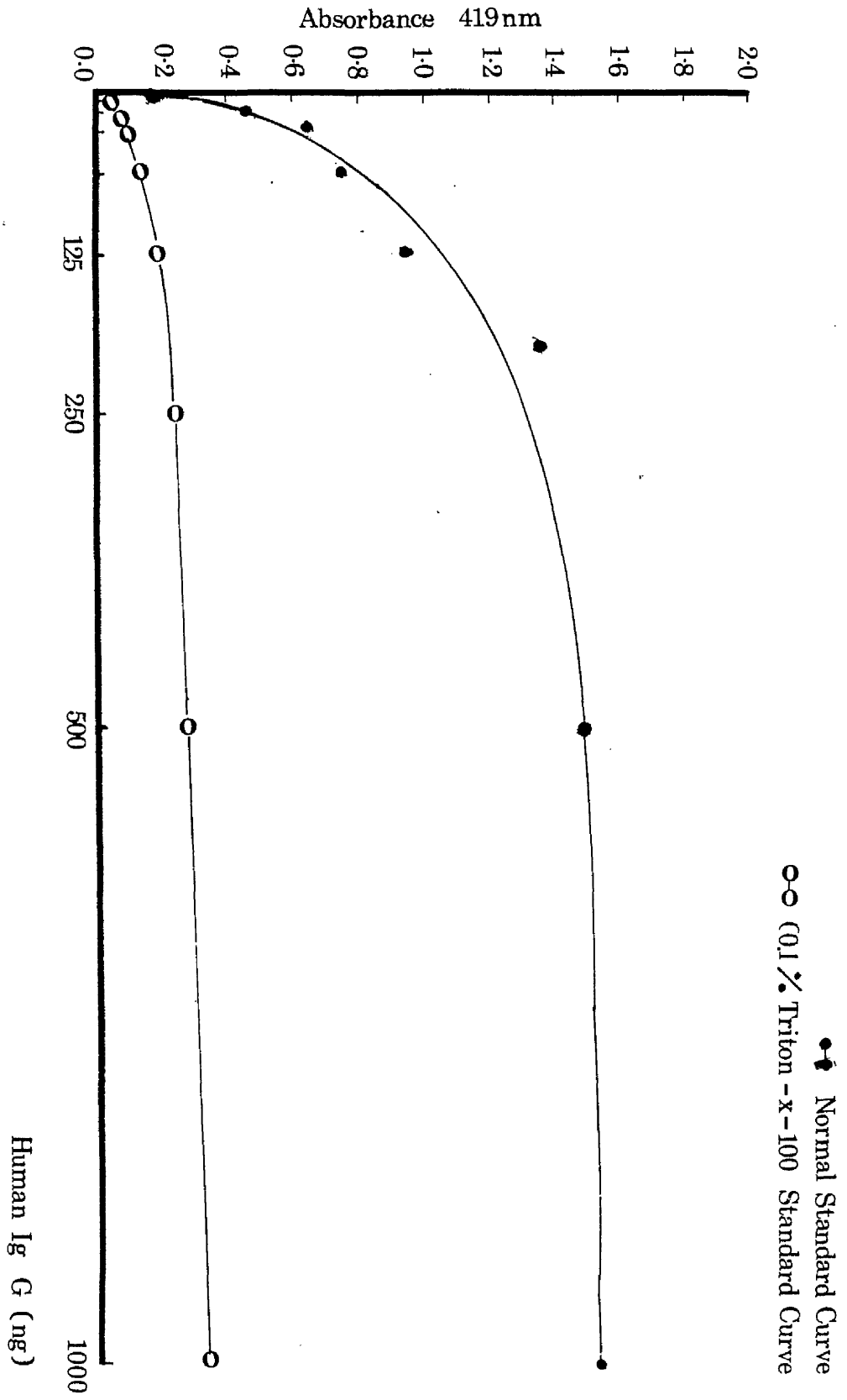


Table 16. IgG Binding to Erythrocyte Membranes.

Leucocytes were removed from blood samples using the method of Tökés and Chambers (1975). The difference between duplicate analyses was below ±10%.

Erythrocytes Membrane Sample	ELISA ASSAY ng human IgG/mg protein
Spur Cell (Control)	111
Cirrhotic	432
Cirrhotic	79
Normal	254
Spur Cell (NMR Study)	7143

The NMR study itself was not pursued further because there was no evidence to suggest that the phosphorus cation content of spur cells was abnormal. However the fortuitous observation made concerning the binding of antibodies to spur cells was of interest. This effect could have been a manifestation of the shear stress forces experienced by the red cells whilst they were being spun in the magnetic field, or it could have been a result of the magnetic field itself. Moreover, it would be useful to discover whether this same enhancement of IgG binding will occur when normal human erythrocytes are used in the NMR spectrophotometer.

4. DISCUSSION

Erythrocyte phosphatase activities of membranes and cytoplasm were measured using two substrates. p-Nitrophenyl phosphate was employed as a convenient general substrate for the detection of phosphatases. Radiophosphorylated casein was used as a specific substrate for phosphoprotein phosphatases. Hui and Harmony (1979) had previously employed these substrates to measure changes in the levels of membrane phosphatase activities which were shown to be associated with erythrocyte shape changes. The protein phosphatase assay is designed to measure the release of inorganic phosphate from the [^{32}P]-labelled phosphocasein in the form of trichloroacetic acid soluble counts. However it was found during the course of this work that acid soluble peptides could be liberated from the phosphorylated casein by erythrocyte proteases. This information therefore questions the validity of applying protein phosphatase substrates in the detection of erythrocyte phosphatase activities unless careful efforts are made to inhibit the proteases. Because of this problem the protein phosphatase assay was not used extensively in these studies.

Hui and Harmony (1979) suggested that the p-nitrophenyl phosphatase activity of erythrocyte membranes, measured in the presence of ATP to inhibit membrane ATPases, reflected membrane protein phosphatase activity. Other workers have used specific phosphorylated proteins to examine the soluble spectrin phosphatase (Graham *et al.*, 1976) and membrane protein phosphatase activities (Fischer *et al.*, 1980) of human erythrocytes. However neither of these authors examined the nature of the products release by the phosphatases to exclude the possibility of proteolytic cleavage of their substrates.

Erythrocyte proteolytic activities were measured using radio-iodinated casein as substrate. Assay methods for proteolytic enzymes which have been used by other workers have employed substrates such as casein and urea-denatured haemoglobin (Morrison & Neurath, 1953); casein, native haemoglobin and erythrocyte stroma (Moore et al., 1970); human globin (Pontremoli et al., 1979); and tritiated acetylated haemoglobin (Bernacki & Bosmann, 1972). Different radio-iodinated proteins including human immunoglobulin, horse heart cytochrome c and casein covalently linked to plastic beads were used as substrates by Tökés and Chambers (1975) to avoid the necessity for trichloroacetic acid precipitation. The use of radio-labelled proteins has the advantage of high sensitivity; protease activity equivalent to 5ng trypsin (Figure 11) could be measured with the [^{125}I]-casein assay developed in this work for the detection of erythrocyte membrane proteolytic activities. In later experiments casein was pretreated with iodoacetamide to reduce the background proteolysis due to endogenous proteases in the casein preparation.

One essential factor to be taken into consideration when studying erythrocytes is their degree of purity and the extent of interference produced by non-erythrocytic cells in enzyme assays. For example it has been shown that leucocytes have a proteolytic activity which is several orders of magnitude higher than that of erythrocytes (Haschen & Krug, 1966). Therefore several methods were examined to reduce or exclude these cells from blood samples. The most effective method was found to be that of Beutler et al., (1976). Some earlier experiments were carried out using the method of Fairbanks et al., (1971) and Tökés & Chambers (1975) which both gave a less complete removal of leucocytes from red cells. The effect of leucocytes in the phosphatase and protease assays was examined, however, to determine the degree of their interference in

each assay. Leucocytes were found to elevate the erythrocyte membrane phosphatase activity only slightly (Table 7) and they had no detectable effect on the soluble cytoplasmic activity. In contrast the proteolytic activity measured in the membrane sample from unfractionated blood cells was elevated eleven-fold (Table 12) compared to erythrocytes. The leucocyte proteolytic activity was located exclusively in the membrane fraction and could not be eluted by repeated washing. Two pH optima were identified for the leucocyte proteolytic activities with the activity at pH 7.0 being four times greated than the activity at pH 3.0 (Figure 12). The serine protease inhibitor PMSF, at a 10mM concentration, totally inhibited the leucocyte proteases at physiological pH. The results emphasised the need to remove leucocytes from blood samples before carrying out phosphatase and protease determinations on red cells. Erythrocytes prepared by the method of Beutler et al., (1976) however had less than one leucocyte per 200 erythrocytes.

Erythrocyte membrane associated phosphatases were detected using p-nitrophenyl phosphate as substrate (Table 4). This enzyme, which had the same pH optimum as the soluble enzyme (Figure 8), remained bound to the red cell membrane after exhaustive washing with buffers of varied ionic strength (Table 5) suggesting that it might be associated with the membrane. Such an association could occur directly as in the case of an integral membrane protein or indirectly via associations with components of the membrane, for example intramembrane particles (IMP).

Characterisation of the erythrocyte membrane associated and cytoplasmic phosphatase activities was carried out at neutral pH using various metabolites in the p-nitrophenyl phosphatase assay system. The membrane associated phosphatase activity only comprised 0.1% of the total cellular phosphatase activity (Table 4). However from the pH

optima values and inhibition studies the soluble and membrane associated p-nitrophenyl phosphatase activities appeared to be very similar or identical. Both phosphatases were inhibited by inorganic phosphate, fluoride ions, calcium ions and stimulated in the presence of EDTA (Table 6). Minor quantitative differences in inhibition and activation may be due to the crude nature of the enzyme preparations. Both phosphatases required magnesium ions for their activity. The stimulation of the membrane phosphatase activity by Triton-X-100 could have resulted from the release of enzyme from resealed ghosts or the cryptic nature of the insoluble enzyme which would limit substrate accessibility. The former explanation is less likely because red cells have little tendency to reseal in hypotonic phosphate buffers. A positive indication of this occurrence would have been the presence of haemoglobin in the ghost membrane pellets, however this phenomenon was not observed.

Phosphatase activities of human erythrocytes appear to be almost totally soluble. The membrane associated acitivity may be a small fraction of this activity rather firmly associated with the membrane, through a limited number of specific binding sites, where the binding affinity is unaffected by high ionic strength media. The regulation of the phosphatase binding to the membrane could perhaps be mediated by erythrocyte metabolites since some of these compounds, in the phosphatase assay system, were observed to alter the levels of phosphatase activity. Examples of such enzymes have been found in erythrocytes. They can exist in both the soluble and membrane bound states and are hence defined as being "ambiquitous enzymes". In vitro it has been shown that glucose-3-phosphate dehydrogenase (Yu & Steck, 1975), aldolase (Strapazon & Steck, 1976; 1977) and phosphofructokinase (Richards et al., 1979) bind to band 3 in the erythrocyte membrane. Binding was reported to be reversible and in most cases the activity of

the bound form was significantly different to the soluble form of the enzyme. In addition the *in vivo* binding of glucose-3-phosphate dehydrogenase to the membrane via band 3 was observed (Kliman & Steck, 1980) and it could be displaced from the membrane *in vitro* using low concentrations of metabolites such as Pi and NAD. Such phenomenon led to the suggestion by these authors that the regulation of the activity of these enzymes could be achieved by intracellular metabolites.

Graham *et al.*, (1976) identified a phosphoprotein phosphatase activity in human erythrocytes by measuring the release of ^{32}Pi from partially purified [^{32}P]-spectrin. They reported that the phosphatase activity was entirely cytoplasmic and detectable at very high dilutions. Fischer *et al.*, (1980) found that human erythrocytes contained a membrane-associated phosphoprotein phosphatase activity capable of dephosphorylating membrane proteins which had previously been phosphorylated by an endogenous protein kinase. Both of the aforementioned authors characterised their respective phosphoprotein phosphatases using erythrocyte ions and metabolites and their results are quoted in Table 17. The phosphatase assay employed by Graham *et al.*, (1976) did not include magnesium ions and those authors found that only 58% of the control activity remained in the presence of 10mM magnesium ions. Fischer *et al.*, (1980), on the other hand, routinely used 10mM magnesium ions and they reported that the amount of protein dephosphorylation could be correlated directly with the magnesium ion concentration. In the absence of magnesium , they observed virtually no dephosphorylation during an incubation period of one hour. The concentration of magnesium was found to influence the specificity of the enzyme in the range of 3 to 20mM. The physiological concentration of magnesium in the erythrocyte appears to be in the region of $10^{-3}M$ (Weed *et al.*, 1969).

Table 17. Erythrocyte Phosphatase Inhibition.

The values in parenthesis are quoted from Table 6.

METABOLITE	CONCENTRATION(mM)	PERCENTAGE PHOSPHATASE CONCENTRATION REMAINING Fischer et al., (1980) MEMBRANE	Graham et al., (1976) LYSATE
Mg^{2+}	10.0	40 (64)	58 (86)
Pi	10.0	67 (54)	2 (47)
F^-	10.0	67 (28)	10 (70)
pPi	1.0	80	0
ATP	1.0	77 (84; 0.1mM)	2(114; 0.1mM)
ADP	1.0	77	12 (123)
2,3 DGP	5.0	61 (45)	12 (155)

Because of the effect of magnesium ions it is difficult to compare the results of these authors for the membrane and cytoplasmic phosphoprotein phosphatase activities. Comparison of the erythrocyte protein phosphatase activities with the p-nitrophenyl phosphatase activities obtained in this thesis (Table 17) indicates little similarity in the lysate activity with the results of Graham et al., (1976). But some general similarity exists concerning the inhibitory effects of inorganic phosphate and the phosphorylated metabolites reported by Fischer et al., (1980). In contrast to Graham et al., (1976), Fischer et al., (1980) reported that the addition of large amounts of cytoplasm to their membrane preparations did not significantly increase the extent of dephosphorylation of the [^{32}-P]-labelled membrane proteins. This suggests that either these are different enzymes or that phosphorylated membrane proteins are not accessible to soluble phosphatases. Perhaps only membrane-associated phosphatases and kinases control spectrin

phosphorylation and their action may be restricted to the protein substrates which are in the closest proximity to the enzymes. It seems probable that the p-nitrophenyl phosphatase activity of the erythrocyte represents a mixture of phosphatases which may include the enzymes which dephosphorylate spectrin.

To determine the quantitative distribution of the proteolytic activities in mature human erythrocytes the radio-labelled casein substrate was incubated with the membranes, lysates and intact red cells. Approximately 18% of the total cellular proteolytic activity remained on the membranes. Repeated washing of the erythrocyte ghosts, using buffers of increasing ionic strength, failed to significantly elute the residual proteolytic activity (Table 11). Therefore this enzyme activity was identified as being associated with the membranes. Tökés and Chambers (1975) used similar criteria when they identified the presence of erythrocyte membrane proteolytic activity. About 10% of the membrane proteolytic activity was detected on the outside of intact cells and thus represents 10% of the total membrane activity if the proteolytic activity is expressed to the same extent on intact cells as in red cell ghosts. This estimation of the proportion of activity on the exterior of the cells involves the assumption that the assay substrate is impermeable to intact cells and completely permeable to cell ghosts. As an example trypsin (24,000) can degrade proteins located on the cytoplasmic surface of ghosts but is restricted to degrading only the proteins on the cell surface when incubated with intact red cells (Steck, 1972). The molecular weight of casein is approximately 70,000 and therefore a similar accessibility behaviour might be anticipated from a protein of this size. However a substrate protein attached to particles such as the latex beads used by Tökés and Chambers (1975) may not possess these properties. Insoluble substrate assay systems may have

an improved sensitivity as a result of eliminating the need for trichloroacetic acid precipitation but these substrates may also have limitations of accessibility where membrane proteases are concerned. Radio-iodinated casein was chosen to satisfy these criteria. Therefore the activities measured on the outside and inside of the erythrocyte membrane using casein may be considered as being representative of the total membrane proteolytic activities.

The erythrocyte membrane proteases had two pH optima in the regions of pH 2.5 and pH 8.0 (Figure 12). Purified cytosolic proteases also showed the presence of the same acidic and basic proteolytic pH maxima and the ratio of the acidic to basic activity levels were approximately equal in both the soluble and insoluble protease fractions of erythrocytes. Tökés and Chambers (1975) reported the presence of proteolytic activity peaks in the acidic and basic pH regions for erythrocyte membranes. The pH optima reported in this work differ slightly from those reported by these authors however their assay system employed the use of radio-labelled protein substrates coupled to latex beads thus making the substrate insoluble. They also reported that the ratio of acidic to basic proteolytic activities on erythrocyte membranes varied between 2:1 and 5:1 when different iodinated protein substrates such as casein, human immunoglobulin and horse heart cytochrome c were used. Bernacki & Bosmann (1972) compared the levels of the proteolytic activity in the acidic and basic pH regions using the substrate tritiated acetylated haemolgobin and reported the presence of 75 times more proteolytic activity in the acid pH region for erythrocyte membrane proteases.

However the erythrocyte membrane associated proteolytic activities measured using the soluble iodinated casein, where the activity peaks at

high and low pH were equal, were found to be reproducible and consistent using this assay system.

Pontremoli et al., (1979) did not find a neutral proteolytic activity associated with erythrocyte membranes however they identified three proteolytic activities in the acidic pH region. Their assay system involved the measurement of the α-NH_2 groups, liberated from acid-denatured globin, using the reaction of fluorescamine with acid soluble degradation products. They suggested that the neutral proteolytic activity found to be associated with erythrocyte membrane was in fact due to the presence of residual leucocyte membranes. To prepare homogeneous erythrocyte membranes Pontremoli et al., (1979) used the ion exchange chromatography technique of Beutler et al., (1976). Other workers in this field, however (Tarone et al., 1979; Siegel et al., 1980; Golovtchenko-Matsumoto et al., 1982), have identified a proteolytic activity with a neutral pH optimum in homogeneous erythrocyte membranes. The work of Siegel et al., (1980) and Golovtchenko-Matsumoto et al., (1982) involved the use of the ion exchange chromatography column technique of Beutler et al., (1976) to remove leucocytes; this technique was also employed in the work described in this thesis. In view of these findings it would seem possible that the sensitivity of the fluorescent assay system used by Pontremoli et al., (1979) may not have been sufficient to enable the detection of the neutral proteolytic activity associated with erythrocyte membranes since large polypeptide fragments and amino acids each give the same amount of fluorescence. This system will not therefore be able to distinguish between endo- and exo-peptidases whereas assay systems using radio-labelled protein substrates will be more effective in detecting endopeptidase proteolytic activities.

Characterisation of the erythrocyte proteolytic activities was carried out by pretreating the enzymes with several protease inhibitors before incubation with the radio-labelled casein substrate. Only the neutral proteolytic activities of the erythrocyte membrane and cytosolic fractions were examined because of the interest in discovering the status of these enzymes in the intact cell at physiological pH.

The purified cytosolic proteases were found to be partially inhibited by phenylmethylsulphonylfluoride (5mM) and almost totally inhibited by p-chloromercuribenzoate (1mM). Dithiothreitol (1mM) slightly stimulated the activity and ethylenediaminetetracetic acid (1mM) inhibited the soluble proteases to 40% activity at neutral pH. This inhibition pattern was found to be almost identical to the results obtained by Pontremoli et al. (1980) for the purified cytosolic proteases with the exception that 1mM EDTA was reported to stimulate the activity to 150% of the control. The reason for this latter difference is unknown.

The membrane associated neutral proteolytic activity was found to inhibited more by phenylmethylsulphonyl fluoride (5mM) and less by p-chloromercuribenzoate (1mM) compared to the purified soluble proteases. However it was found that EDTA (1mM) inhibited the insoluble proteases to approximately 60% of the control compared to 40% for the soluble proteases although both enzymes were similarly stimulated by dithiothreitol (1mM). Therefore, in general, the erythrocyte appears to have a mixture of proteolytic enzymes which have some characteristics of serine and thiol proteases there may also be metallo-proteases present. The properties of the soluble and insoluble neutral proteases, with respect to their inhibition pattern, are apparently different although this may be a manifestation of an uneven quantitative distribution of

the different types of proteases in the two erythrocyte fractions.

Calcium ions were observed to elevate the level of the proteolytic activity associated with human erythrocyte membranes when they were employed in the hypotonic lysis buffer (Table 12). The erythrocyte p-nitrophenyl phosphatase activities were also observed to be elevated in similarly treated membrane samples (Table 5). However, unlike phosphatases, the increased proteolytic activity could not be eluted by repeated washing of the membranes which were lysed in the presence of calcium. Increasing the calcium ion concentration of the erythrocyte lysis buffer, from one to ten millimolar, increased the protease and phosphatase activity levels of the ghosts further, indicating that the effect of this cation was concentration dependent. The stimulatory effect of this cation on these membrane-associated enzyme activities did not appear to be a direct effect on enzyme activity. The presence of this ion either with or without a pre-incubatory period in the phosphatase assay (Table 6) or protease assay (Tables 11 & 14) did not produce activation of either enzyme activity. Therefore calcium appears to promote the adsorption of cytosolic constituents onto the membranes thereby increasing the amount of phosphatases and proteases detectable on the erythrocyte ghosts.

Tökés and Chambers (1975) initially observed an effect of calcium upon the amount of proteases on erythrocyte membranes. They found that the proteolytic activities of the membranes, with the acidic and basic pH optima, were both elevated when erythrocytes were lysed in the presence of 0.1mM calcium.

The membrane associated neutral proteases were also found to be active against the polypeptides of the erythrocyte membranes. The

progression of the proteolytic degradation upon the membrane substrates could be followed by the periodic examination of ghost samples which were incubated at 4°C. SDS-polyacrylamide gel electrophoresis of the samples revealed that the ankyrin (bands 2.1 to 2.6) were degraded rapidly whereas the other membrane protein components were degraded more slowly.

Degradation of the membrane polypeptides by the membrane associated proteases besides providing a qualitative method for the detection of proteases, in conjunction with the quantitative radio-casein assay, was envisaged as a means of elucidating the possible physiological role of the enzymes in the red cell. The technique of examining membrane protein proteolysis, using SDS-polyacrylamide gel electrophoresis, was also employed to further investigate the calcium ion absorption effect on erythrocyte membranes. Erythrocyte ghosts, isolated in the presence of calcium (1mM) showed evidence of mild degradation having occurred at 4°C during the isolation procedure (Figure 19). Incubation of these ghosts, for 18h at 4°C, promoted further degradation of the membrane associated polypeptides. Pre-incubation of washed ghosts with calcium ions and red cell lysate caused the adsorption of proteases and proteins (haemoglobin and band 8) onto the membranes. However after the purification of the lysate proteases the calcium adsorption phenomenon ceased to occur. The most likely explanation for this latter observation is that the proteases most susceptible to the calcium adsorption effect were lost or denatured during the purification process. The controls to examine the possibility that calcium was directly stimulating or activating the membrane proteases, involved the incubation of washed ghosts with and without calcium ions. However, no increase in the amount of degradation was observed in either sample. Thus it was found that calcium ions were required in the incubation of the ghosts and red cell lysate at 4°C to

promote the accelerated degradation of membrane proteins compared to the controls. The pattern of the membrane protein degradation produced by these adsorbed proteases followed the same specific sequence observed for the membrane-associated proteases (Figure 18). Two main areas of the protein banding pattern were observed to change with the largest polypeptides in the ankyrin band region being the most rapidly hydrolysed components. However the membrane-associated proteases were only observed to produce the same degree of proteolytic change upon the membrane proteins after longer incubation periods at 4°C.

King and Morrison (1977) examined proteins from erythrocyte membranes on SDS-polyacrylamide gels after incubating samples, for 45h at 37°C, which had been isolated in the presence and absence of calcium ions. Only membrane samples which were isolated in the presence of 1mM calcium ions showed evidence of membrane protein degradation. High molecular weight aggregates, shown by Carraway et al., (1975) to result from a calcium-stimulated transglutaminase, were also visible at the interface of the separation and stacker gels. In addition the gel tracks containing the calcium isolated erythrocyte membrane proteins showed the presence of the cytosolic proteins band 8 (24,000) and globin.

Allan and Cadman (1979) subsequently found that incubating erythrocytes either for a 24-48h, without glucose and with calcium (1mM) or for 1h with the ionophore A23187 and calcium (1mM) produced the same alterations of the protein banding pattern as those observed after haemolysing red cells in hypotonic buffers containing 1mM calcium ions. These changes produced by calcium on erythrocyte membrane proteins were similar to the observations reported by King and Morrison (1977) and in this thesis (section 3.24).

Calcium ion adsorption effects may also be significant in the erythrocytes from individuals with sickle cell anaemia which have a characteristic morphology. This condition is associated with increased intracellular calcium (Eaton et al., 1973).

Observations made on the erythrocytes obtained from one individual with spur cell anaemia indicated that the abnormal ghost membranes had at least a four fold increase in both the p-nitrophenyl phosphatase activity (Table 4) and the neutral proteolytic activity (Table 15) compared to normal membranes. Increased proteolytic activity levels were detected both on the outside and inside of the abnormal spur cell membranes and was manifest after examining these ghost polypeptides on SDS-polyacrylamide gels (Figure 16). The normal protein banding pattern was virtually undetectable. However loss of the membrane protein could be almost completely prevented by using PMSF (1mM) at lysis (Figure 17).

The comparative examination of significant differences between the abnormal and normal erythrocytes may not have been completely ideal because of the difference between the ages of the spur cell individual and the control subjects; and the restricted number of the controls tested. The limiting factors which prevented the complete sampling of appropriate controls were in the lack of available donors who were of similar age to the spur cell individual and the time factor involved in assaying many samples. However the normal control subjects were from an age group, covering a span of approximately 15 years, and they were younger than the patient by at least twenty years and more.

It might be argued that some of the increased membrane protease activity of spur cell erythrocytes could be attributed to the presence of leucocyte membranes as in these experiments membranes were isolated

by the method of Fairbanks et al., (1971) or Tökés and Chambers (1975). However the extent of degradation observed with spur cell membranes far exceeded the amount of degradation of normal erythrocyte membrane proteins prepared from unfractionated red cells. It is therefore very unlikely that the elevated proteolytic activity associated with spur cell membranes, indicated by both the iodinated casein assay and their protein banding pattern on gels, could arise from leucocytes.

The possibility of reticulocytes contributing to the elevated protease activity level estimated for spur cells must be considered. While the mature erythrocyte has little or no protein turnover the immature red cell shows the occurrence of extensive protein breakdown (Rapoport et al., 1974). However most of the reticulocyte proteolytic activity is apparently soluble (Etlinger & Goldberg, 1977) and the membrane associated proteases appear to be acidic endopeptidases (Melloni et al., 1981). This assessment may not be completely accurate since Pontremoli has contributed to these findings and the same fluorimetric assay system was used to detect membrane associated proteolytic activity. Reticulocytes have a specific gravity which is lower than adult corpuscles (Key, 1921) and thus tend to collect in the upper portion of the red cell suspension which is aspirated after blood centrifugation to remove leucocytes and platelets (Fairbanks et al., 1971). Blood from the spur cell patient had a relatively low reticulocyte count of only 3% for this disorder (normally 1%) which suggests that these cells would have been almost completely removed by this method.

Centrifugation of erythrocyte ghosts normally produces a small tightly packed button of membranes below the pellet of ghosts. An interesting feature of this case study of spur cell anaemia was that these membranes

had approximately 80% of the total ghost protein present in the "button" fraction compared to normal erythrocyte "button" ghosts which contained 20% of the total protein (Table 3). Baxter (1975) reported that when erythrocytes were fractionated into populations of different in vivo age that old cells contained a larger than normal "button" pellet upon lysis. Other ways of promoting the occurrence of large "button" pellets in erythrocyte ghosts have been reported. The enzymic reduction of the cell surface charge using neuraminidase enhances the association of red cells to produce aggregated membranes on lysis (Baxter, 1975). This "button" ghost effect might also result from cytoskeletal changes within these spur cells or from the increased membrane cholesterol acquired by spur cells. Lux et al., (1978) found that the ability of extracting spectrin from erythrocytes dimninished with changes in the cell shape and deformability. The severe deterioration of the spur cell morphology, which seems to indicate extensive membrane fragmentation, may suggest that the cytoskeleton has undergone molecular rearrangement. Alternatively a direct effect of the increased membrane cholesterol may be that it produces large sections of the lipid bilayer which are devoid of intramembrane particles. Decreasing the distribution of the cell surface glycoproteins could effectively reduce the cell surface charge over small areas in the lipid bilayer and allow the ghosts to pack more tightly.

The increased association of phosphatases, proteases, band 8 and haemoglobin with spur cell membranes were observed to parallel the changes induced in normal erythrocyte membranes lysed in the presence of calcium ions (Tables 5 & 12). In addition to this, normal erythrocytes, which were lysed in the presence of calcium and incubated, showed a similar membrane protein degradation pattern to that obtained for freshly isolated spur cell membrane proteins. This then raised the

question of whether spur cell erythrocytes have an increased concentration of intracellular calcium. This might occur if the increased cholesterol of spur cell membranes directly or indirectly perturbed the normal impermeable nature of the erythrocyte membrane to calcium ions. However the intracellular accumulation of calcium does not apparently reach the high concentration necessary to stimulate the transglutaminase (0.5mM) because the majority of the spur cell membrane proteins were soluble in SDS.

The rationale for measuring the quantitative association of immunoglobulin molecules to spur cell membranes was based upon an observation made on an SDS-polyacrylamide gel (Figure 21). Spur cell erythrocytes, which had been used as a sample for NMR spectroscopy, showed the presence of large amounts of IgG molecules on their gel tracks after their membrane proteins were electrophoresed. However spur cell anaemia was not found to be a characteristic example of an autoimmune disease (Table 16). The presence of the IgG molecules in the NMR sample was probably an artefact of the technique which involved spinning the red cells rapidly in plasma for a prolonged period.

It is interesting to note that not all individuals who are alcoholic cirrhotics acquire spur cell anaemia and that not all lipid abormalities of red cells invoke the same extreme cellular changes as those documented for spur cells. Target cells which accumulate both additional membrane cholesterol and phospholipid and acanthocytes which have membranes enriched in sphingomyelin at the expense of lecithin survive normally in the circulation. The aetiology of spur cell anaemia remains unresolved. However Borochov and Shinitzky (1976) suggested that in general the effect of an excess of cholesterol in erythrocyte membranes might result in changes of the transport properties and enzyme

activities due to increased exposure of the lipid bilayer to the aqueous environment. Hui and Harmony (1979) are investigating the theory that low density lipoproteins (LDL) affect the intracellular cation concentration of erythrocytes thus enhancing the membrane phosphatase activity. They have ruled out the main plausible effects of LDL interacting with other membrane constituents and have shown that the LDL is not internalized by the erythrocyte. Therefore this may be in accordance with the results of the work in this thesis

which apparently indicate the presence of elevated concentrations of calcium in spur cells.

Fractionation of normal erythrocyte membranes into top and "button" ghosts indicated that the highest assayable neutral proteolytic activity was contained in the "button" membrane fraction (Table 15). This increased activity was also apparent upon examination of these ghost protein fractions on SDS-polyacrylamide gels (Figures 16 and 17). There was evidence to suggest that the "button" membranes had undergone degradation. The ankyrin protein bands 2.1 to 2.6 and some of band 3 was degraded. Band 2.3, however had increased in staining intensity. This evidence seemed to suggest that the ghost membranes which pelleted into the densely packed "button" fraction had more proteases associated with their membranes. Leucocytes may partially be responsible for this observation, however the evidence which relates the origin of the "button" ghosts to old erythrocytes has not been thoroughly investigated.

Density fractionated red cells were isolated by Baxter (1975) on discontinuous density gradients of iso-osmotic bovine albumin. By this method he obtained six fractions of erythrocytes of different <u>in vivo</u> age and examined their membrane polypeptides using SDS-polyacrylamide

gel electrophoresis. Two tracks containing the membranes from the oldest erythrocytes showed changes in the membrane protein banding pattern in two main areas. Ankyrin bands 2.1 to 2.6 and band 3 were reduced in staining intensity and bands 2.3 and band 4.5 had an increased staining intensity. Band 8 (24,000) and globin were also visible on these gel tracks containing these samples. The alterations in the membrane protein banding pattern were identical to the changes observed by King and Morrison (1977) and in this thesis for erythrocyte membranes lysed in the presence of calcium. This suggests that increased levels of proteases may be associated with the membranes of old erythrocytes.

LaCelle et al., (1972) and Schatzmann (1975) have both reported that the extrusion of calcium by senescent erythrocytes progressively declines as the intracellular ATP concentration decreases. Therefore calcium accumulation during red cell senescence may increase the association of cytosolic proteases with the membrane causing proteolysis of the membrane associated polypeptides. If a relationship does exist between the level of proteases associated with erythrocyte membranes and the cell age then it may give credence to the suggestion, made by Scott and Kee (1979), of these proteases being involved in erythrocyte autolysis. However the information which has shown that a decrease in the ATP content of erythrocytes may be one of the causative factors controlling red cell senescence (Lichtman, 1975) must be considered with some reservation because of a recent report of Kirkpatrick et al., (1979) which showed no decrease in the ATP concentration for old erythrocytes.

The problem of comparing the ATP concentration in red cells of different age seems to be in the expression of the results. Kirkpatrick et al., (1979), using density fractionated human erythrocytes, showed

that the ATP content (mole/cell x 10^{17}) of dense (old) cells is less than that of light (young) cells. Also the mmole of ATP/gram of haemoglobin values indicated that old cells contained less ATP. However they corrected the ATP values by taking into account the decreased volume and increased haemoglobin concentration of the dense cells and obtained a value for intracellular ATP which does not differ from young or control (unfractionated) cells.

In conclusion, normal human erythrocytes possess membrane associated proteases and phosphatases which are active at neutral pH. Hypotonic lysis of erythrocytes in the presence of 1mM calcium ions resulted in the adsorption of cytosolic proteins onto the membranes as indicated by the increased levels of assayable p-nitrophenyl phosphatase and [^{125}I]-casein protease activities on these membranes compared to controls. The SDS-polyacrylamide gel protein patterns of erythrocyte ghosts, isolated in the presence of calcium, provided evidence which confirmed the calcium adsorption effect during red cell lysis. The degradation of membrane proteins was observed to be accelerated and two new protein bands (globin and band 8) of cytosolic origin were present in these gel tracks compared to the controls. These calcium effects of normal erythrocyte membranes were found to parallel the changes observed for spur cell membrane phosphatase activities, protease activities and gel patterns of the ghost proteins obtained from the red cells of one individual with this anaemia. This prompted the suggestion that these changes in spur cells could have been produced through an increase in the intracellular calcium ion concentration.

The calcium ion accumulation by senescent erythrocytes aged *in vivo* may also be responsible for the changes observed in these cells. The protein degradation of ghosts isolated from old erythrocytes seemed to

indicate an increased association of proteases with the membranes. Moreover the general effect of both the membrane associated and calcium adsorbed proteases was in the degradation of the cytoskeletal proteins which are in closest proximity to the membrane bilayer. These proteins which function to anchor the cytoskeleton to the membrane are possibly the most crucial of the red cell cytoskeletal architecture. A relationship may exist between the integrity of the membrane-cytoskeletal association and the shape and deformability properties of the erythrocyte.

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