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CHANGES IN MEMBRANE PROTEIN AND CARBOHYDRATE
ASSOCIATED WITH ERYTHROCYTE AGING

by

Allan Baxter

A thesis presented for the degree of
Doctor of Philosophy,
Faculty of Science,
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To my parents, with thanks for encouragement and
financial support.

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ABBREVIATIONS

The standard abbreviations, as recommended in the revised "Instructions to Authors" (Biochem. J. (1975) 145, 1-20) are used throughout this thesis with the following additions:-

ATP-ase	adenosine triphosphatase
OM II	ovomucoid, fraction II
PBS 7.0	phosphate buffered saline, pH 7.0
PBS 7.4	phosphate buffered saline, pH 7.4
SDS	sodium dodecyl sulphate
WGA	wheat-germ agglutinin

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PROLOGUE

Now it is a strange thing, but things that are good to have and days that are good to spend are soon told about, and not much to listen to; while things that are uncomfortable, palpitating and even gruesome, may make a good tale, and take a deal of telling anyway.

J.R.R. TOLKIEN

SUMMARY

The in vivo aging of human erythrocytes is accompanied by a progressive increase in red cell specific gravity. Use has been made of this fact to obtain erythrocytes of different in vivo age by ultracentrifugation of washed red cells on discontinuous density gradients of iso-osmotic bovine albumin.

The red cells of two individuals exhibited an age-related decrease of about 20% in the cell surface N-acetylneuraminic acid. This change was shown to be due to a generalised loss of sialic acid from all of the major membrane sialoglycoproteins. Membranes were isolated from cell fractions of different age and subjected to carbohydrate analysis. The finding for N-acetylneuraminic acid was confirmed and additional decreases were observed in galactose, glucose, N-acetylgalactosamine and N-acetylglucosamine of old cell membranes. While some of these decreases are probably attributable to glycolipid losses, which are known to accompany aging, part of the galactose/N-acetylgalactosamine depletion has been related to the sialoglycoproteins of the membrane.

Changes in cell surface carbohydrates have also been detected by studying the relative susceptibility to agglutination of erythrocytes of different in vivo age using a number of agglutinins. Old cells were consistently more agglutinable and in the case of one of these agglutinins, wheat-germ agglutinin, the increased agglutinability was shown not to be due to an increase in the number of lectin binding sites, nor to any marked redistribution of intramembraneous particles of old cells. Moreover, the interaction of in vivo aged cells with agglutinins could be mimicked by neuraminidase or trypsin treated unfractionated erythrocytes.

Analysis of the major protein components of the membranes of fractionated cells by SDS-polyacrylamide gel electrophoresis indicated that aging was accompanied by the appearance of two new polypeptides of approximate molecular weights 63,000 and 25,000, with probable degradation of component 3 - an extracellular surface membrane glycoprotein.

Treatment of intact red cells with a variety of proteolytic enzymes produced a new component of molecular weight 61-63,000 and also degraded component 3 and the membrane sialoglycoproteins. Since trypsin treatment of erythrocytes produced cells which mimicked in vivo aged cells in their behaviour with agglutinins, the findings presented here are consistent with erythrocyte in vivo aging being accompanied by proteolysis of the external membrane surface of the red cell. The relevance of these observations to the sequestration of senescent erythrocytes is discussed.

INTRODUCTION

1.1 The human Erythrocyte

1.1.1 Erythrocyte Function and Lifespan

The human erythrocyte is a highly specialised enucleate cell whose function is the carriage of haemoglobin in the circulatory system. Sequestration of haemoglobin within a biological membrane has a number of advantages, the most important of which is that haemoglobin can be isolated from the changing environment of the plasma and kept in a regulated cytoplasmic environment. The significance of this is borne out by the fact that haemoglobin has a lifespan of 120 days, compared to an average half-life of 10 days for most plasma proteins (White et al., 1973). Denaturation of haemoglobin is prevented by various enzymes within the red cell (Allen, 1964; Jaffé, 1964).

The lifespan of the human erythrocyte has been shown to be 120 days, and as there is little random destruction, an aging process has been invoked (Berlin, 1964). Therefore, a circulating red cell sample will contain erythrocytes of different in vivo age and in order to investigate the changes occurring on aging, it is necessary to separate these cells. A number of techniques have been used which make use of a property of the red cell shown to change on in vivo aging. The most widely used methods are those of centrifugation and differential lysis. Labelling studies have shown that old human erythrocytes are more dense than young cells (Borun et al., 1957) and that old cells are more easily lysed than their younger counterparts (Simon & Topper, 1957). The separation by centrifugation can be improved by use of iso-osmotic density gradients of bovine albumin and this has the advantage of yielding intact cells for study (Dishop & Prentice, 1966; Pionelli et al., 1967).

Changes shown to occur on in vivo aging are summarised in

Table 1.

Table 1 Changes Occurring in Erythrocytes During In Vivo Aging

Change Observed	Reference
Increased specific gravity	Borun <u>et al.</u> , 1957; Piomelli <u>et al.</u> , 1967
Loss of lipid	Prankerd, 1957; Westerman <u>et al.</u> , 1963
Decreased potassium content	Prankerd, 1958
Increased sodium content	
Increased osmotic fragility	Simon & Topper, 1957
Decreased activity of specific enzymes	Pennell, 1964
Decreased deformability	La Celle & Arkin, 1970
Decreased cell surface charge	Danon <u>et al.</u> , 1971

It is evident that fairly complex changes take place in the erythrocyte during its lifespan. Any or all of these may dictate that a given cell is too old to remain in the circulation.

1.1.2 Erythrocyte Metabolism and Aging

1.1.2.1 Lipids

The mature erythrocyte is unable to carry out the synthesis of fatty acids from labelled acetate (Marks et al., 1960), to incorporate fatty acids into neutral lipids (Mulder & Van Deenen, 1965), or to incorporate glycerol into neutral lipids (Sloviter & Bose, 1966). Similarly London & Schwarz (1953) have shown that cholesterol is not synthesised by erythrocytes. Red cell membrane cholesterol does, however, exchange with plasma cholesterol (Murphy, 1962).

Little, if any de novo synthesis of phospholipids takes place in the mature erythrocyte (Van Deenen & de Gier, 1964), although in vitro studies indicate the presence of acyltransferases which can incorporate specific fatty acids into phospholipids (Oliveira & Vaughan, 1964; Mulder & Van Deenen, 1965). It is possible that a similar mechanism operates in vivo since membrane phospholipid fatty acids are subject to dietary fatty acid composition at a turnover rate greater than that of the red cells (Farquar & Ahrens, 1963). Reed (1959) has shown that another mechanism of membrane phospholipid renewal may be exchange with plasma phospholipids. In spite of this Frankerd (1958) and Westerman et al., (1963) have shown that in vivo aged erythrocytes are deficient in cholesterol and phospholipid.

In human erythrocytes, the glycosphingolipids constitute less than 5% of the total lipid. The structures of glycolipids have been studied because of their antigenic activity. However, one group of glycolipids whose catabolism has been studied is globoside and its analogues. These have been implicated in a number of diseases, where lack of a glycosidase has been shown to prevent their degradation. Studies, on their normal catabolism, by Sweeley & Dawson (1969) have provided evidence that these glycolipids are lost intact from the red cell directly into the plasma, just before red cell destruction.

1.1.2.2 Proteins

The maturation of a reticulocyte to an erythrocyte, with the resultant loss of polysomes, terminates protein synthesis in the red cell. Bishop (1971) maintains that the mature erythrocyte retains the ability to synthesise the tripeptide glutathione, implying that uptake of amino acids is not defective.

Using rabbit reticulocytes, Lodish & Deslaur (1973) have shown that these cells are capable of haemoglobin synthesis. This has proved useful as a means of cohort labelling red cells for studies on cell survival. Although haemoglobin is the major protein being synthesised by reticulocytes, Lodish (1973) also reports the synthesis of two major membrane components. One of these proteins may be lost during reticulocyte maturation (Koch et al., 1973).

However, it would appear that the majority of intracellular and membrane proteins and enzymes are synthesised by an erythropoietic precursor of the reticulocyte. This inability to carry out protein synthesis means that the erythrocyte cannot, for example, replace defective enzymes. A number of important enzymes have been shown to lose activity as the red cell ages (Pennell, 1964).

The presence of proteolytic enzymes in erythrocytes was demonstrated by Morrison & Neurath (1953). They identified three proteases, two of which were active at physiological pH. Conditions required to solubilise the activity suggested that the enzymes were tightly bound to the membrane. A more recent study by Moore et al. (1970) indicated that erythrocyte membrane proteases could degrade haemoglobin, membrane proteins and the isolated membrane sialoglycoprotein. It was not established however, whether the membrane location of these enzymes was cytoplasmic or extracellular.

1.1.2.3 Carbohydrate

Metabolism of carbohydrate within the erythrocyte is limited but sufficient to meet its requirements for ATP, NADH and NADPH.

Glycolysis takes place in the mature red cell but in the absence of mitochondria, there is no citric acid cycle activity. However, glycolysis yields sufficient ATP to maintain the active transport of K^+ and Na^+ (Konsek & Bishop, 1962). Bishop (1971a) points out that the ATP/ADP levels could affect the glycolytic rate of erythrocytes by their respective inhibitory and activating influence on phosphofructokinase. Glycolysis also produces NADH by the oxidation of glyceraldehyde-3-phosphate. This reaction is generally coupled to the reduction of pyruvate to lactate, but can also be coupled to the reduction of methaemoglobin and is mediated by the enzyme NADH-methaemoglobin reductase (Scott & McGraw, 1962).

Glucose can also be metabolised by the hexose monophosphate pathway. The first two steps in this pathway give rise to NADPH. De Loecker & Frankerd (1961) have shown that if red cells are supplied with a mediator for NADPH oxidation, like methylene blue, their oxygen uptake and metabolism of glucose by the hexose monophosphate shunt are greatly increased. NADPH is used by the cell to maintain glutathione in a reduced state (Rall & Lehninger, 1952). This in turn is required for the stabilisation of sulphhydryl containing proteins and for mopping up peroxides (Mills, 1959).

A number of enzymes, involved in carbohydrate metabolism, have been shown to decrease in activity during in vivo aging (Pennell, 1964). It can be seen from the foregoing description that this will, in turn, lead to further deteriorative changes.

1.1.2.4 Nucleotides

In the absence of cell division or protein synthesis, with their consequent requirements for nucleotides, by far the most

abundant nucleotide in human red cells is ATP. Other purine and pyrimidine nucleotides have been reported (reviewed in Bishop & Surgenor, 1964). Bishop (1960) showed that mature human erythrocytes would not incorporate [^{14}C]-glycine into nucleotides and that de novo purine synthesis was not carried out. Preformed purines were, however, shown to be incorporated into red cell nucleotides (Bishop, 1960; Lowy et al., 1962), implying that these nucleotides are being constantly metabolised and that red cells require an external source of preformed purines, which carbohydrate metabolism can use to form ATP.

It was not at all clear why the red blood cell required so much ATP. It is the substrate for the Na^+/K^+ active transport mechanism (Hoffman, 1962), but this only accounts for at most 20% of the ATP output of the cell (Whittam & Ager, 1965). However, from in vitro aging studies, Weed & La Celle (1969) have shown that ATP is required for the structural integrity of the red blood cell and for its subsequent in vivo survival in the circulation. Schatzmann & Vincenzi (1969) have demonstrated a $\text{Ca}^{2+}/\text{Mg}^{2+}$ dependent ATP-ase which is thought to be responsible for maintaining Ca^{2+} at a low level in red cells. Palek et al. (1971) present evidence that such an ATP-ase is involved in membrane conformation.

Since ATP levels and carbohydrate metabolism are closely linked, and since carbohydrate metabolism is reduced in in vivo aged cells, the old red cells' requirements for ATP cannot be met.

1.2 The Erythrocyte Membrane

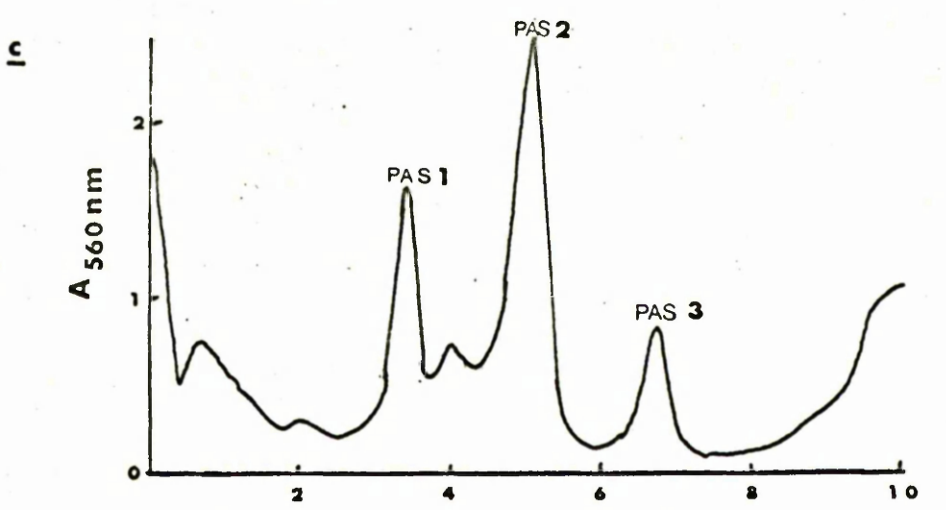
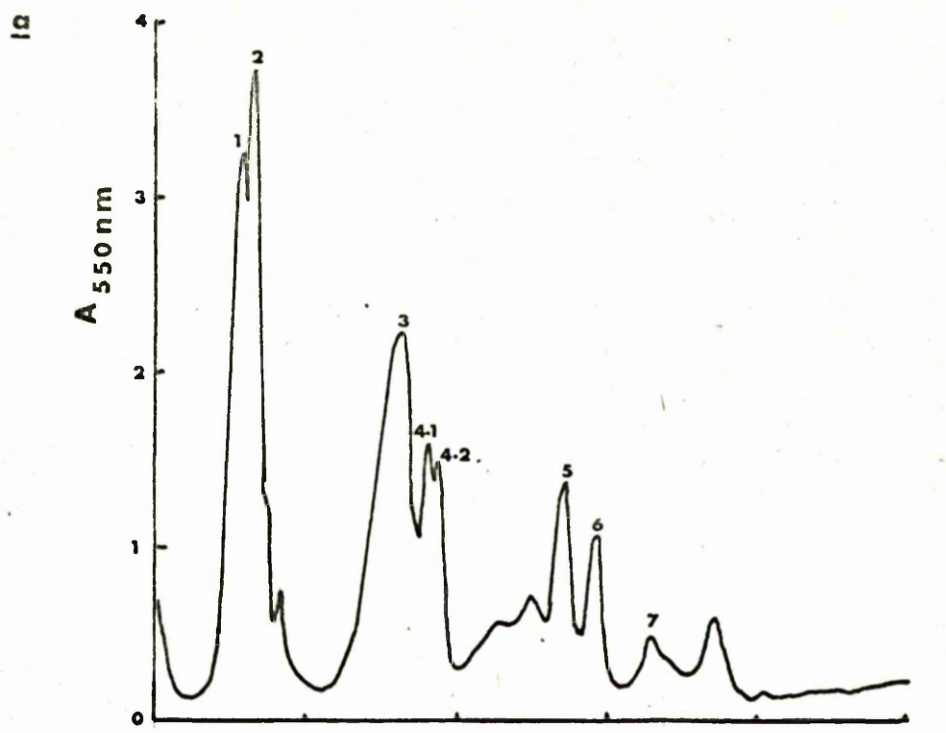
1.2.1 Isolation and Characterisation

In the absence of subcellular organelles, haemoglobin-free

Figure 1 Sodium Dodecyl Sulphate Polyacrylamide Gel

Electrophoresis of Human Erythrocyte Membranes

Ghosts were solubilised at 100°C in 1% SDS and reducing agent prior to electrophoresis. (a) Densitometric scan at 550nm of a gel, shown in (b), stained for protein with Coomassie blue. (c) Densitometric scan at 560nm of a gel, shown in (d), stained for carbohydrate with periodic acid/Schiff reagent. Bands are referred to by the nomenclature of Fairbanks et al. (1971).



erythrocyte membranes can be prepared by hypotonic lysis, centrifugation and washing (Dodge et al., 1963). These authors have shown that essentially all the lipid remains associated with the ghosts. However, it has been shown that associations of proteins with the membrane can be affected by ionic strength. For example, Kant & Steck (1973) have shown that about 50% of the glyceraldehyde-3-phosphate dehydrogenase of ghosts prepared by hypotonic lysis is released at physiological ionic strength. This raises the question of what constitutes a membrane component.

However, using a standard isolation procedure, it has been possible to characterise the erythrocyte membrane. Rosenberg & Guidotti (1968) have shown that the erythrocyte membrane consists of 49.2% protein, 43.6% lipid and 7.2% carbohydrate. By solubilising membranes in sodium dodecyl sulphate and treating with a disulphide reducing agent, Fairbanks et al. (1971) were able to fractionate the membrane polypeptides by polyacrylamide gel electrophoresis. The major proteins were stained with Coomassie blue (Figs. 1a & 1b) and are referred to throughout this thesis by these authors' nomenclature. The carbohydrate stain periodic acid/Schiff reagent demonstrates the presence of several glycoproteins (Figs. 1c & 1d), all of which appear to be sialoglycoproteins as they can be labelled by the sialic acid specific periodate oxidation/tritiated borohydride reduction technique of Blumenfeld et al. (1972). Comparison of Figs. 1a & 1c shows that none of the Coomassie stained components correspond to Schiff stained bands. The sialoglycoproteins are very rich in carbohydrate and so do not adsorb the protein stain. On the other hand, component 3, shown by Tanner & Boxer (1972) to be a glycoprotein containing 10% carbohydrate, is not stained by

Schiff stain.

Glycoproteins containing a large amount of carbohydrate, such as the sialoglycoproteins, do not bind sodium dodecyl sulphate to the same extent as globular proteins and so exhibit anomalous electrophoretic mobilities on polyacrylamide gels. This leads to difficulty in assigning a molecular weight to these components (Segrest et al., 1971). A further caution in considering electrophoresis is that sodium dodecyl sulphate has been shown to be unable to disrupt completely all protein/protein interactions. A prime example of this is the interconversion of PAS 1 and PAS 2 (Marton & Garvin, 1973).

1.2.2 Techniques for Studying Membranes

1.2.2.1 Chemical and Enzymatic Modification

The ease of isolating erythrocyte ghosts has led to their extensive use as a model membrane system. Fractionation of the polypeptides of the membrane on sodium dodecyl sulphate/polyacrylamide gels (Fairbanks et al., 1971) has led to the identification of a number of distinct membrane protein components. Initial studies were directed at determining how individual membrane proteins were distributed with respect to the cytoplasmic and extracellular membrane surfaces. This was done by comparing the reactivity of a component in the intact cell with its reactivity in isolated, unsealed ghosts. Steck (1974) used impermeable right-side-out and inside-out membrane vesicles to investigate the same phenomenon. The probe molecules used were considered to be unable to penetrate the membrane and so reacted at a defined membrane surface.

The probes used were proteolytic enzymes (Steck et al., 1971), lactoperoxidase catalysed radioiodination in the presence

of hydrogen peroxide (Phillips & Morrison, 1971) and non-penetrating anionic chemical labelling reagents such as [³⁵S]-formylmethionyl sulphone methylphosphate (Bretscher, 1971).

The labelling of membrane carbohydrate can be carried out using two fairly specific methods. Gahmberg & Hakomori (1975) labelled cell surface galactose/galactosamine using galactose oxidase oxidation and tritiated sodium borohydride reduction. Blumenfeld *et al.* (1972) labelled membrane *N*-acetylneuraminic acid by sequential sodium metaperiodate oxidation and tritiated sodium borohydride reduction.

1.2.2.2 Use of Lectins

Lectins are plant proteins possessing the ability to agglutinate erythrocytes by binding specifically to cell surface carbohydrates. Their properties are well reviewed by Lis & Sharon (1973). Lectins were found to be able to discriminate between normal and transformed cells in that transformed cells showed an increased susceptibility to agglutination by lectins. Attempts to relate this to an increase in the numbers of lectin binding sites, using radioactively labelled lectins, gave conflicting results. Electron microscopy studies, to show how the distribution of receptor sites on the cell surface determined its agglutinability, were equivocal. However, it was observed that treatment of normal cells with proteolytic enzymes often resulted in an increased susceptibility to agglutination like that of transformed cells. This suggested that there may be some form of electrostatic or steric effect involved in agglutinability. Receptors, on the erythrocyte membrane, for a large number of lectins, have been shown to be associated with the major glycoproteins (Fukuda & Osawa, 1973; Pinto da Silva & Nicolson,

1974). Proteolytic cleavage of these receptors has been shown to increase erythrocyte agglutinability and studies by Pollack (1965) show the importance of surface charge and steric effects in agglutination. Moreover, there is evidence that the distribution of glycoprotein receptors in the red cell membrane may be restricted by interaction of the receptors with the fibrous protein spectrin on the cytoplasmic membrane surface (Nicolson & Painter, 1973; Elgsaeter & Branton, 1974).

The use of lectins, which could be conjugated with an electron dense molecule like ferritin, enabled the study of the distribution of carbohydrate on the membrane surface by electron microscopy (Nicolson & Singer, 1971). In conjunction with freeze-cleaving and freeze-etching, this technique allowed comparisons of surface topography and intramembranous organisation to be made (Tillack et al., 1972).

1.2.2.3 Freeze-Fracture Electron Microscopy

Freeze-fracture of erythrocyte membranes, within the lipid bilayer, exposes globular particles of approximate diameter 8.5nm (Pinto da Silva & Branton, 1970). These intramembranous particles have been associated with glycoproteins believed to span the membrane (Pinto da Silva et al., 1971; Pinto da Silva & Nicolson, 1974). In freshly prepared ghosts, the particles are not easily aggregated without a pretreatment which removes a substantial amount of the fibrous protein spectrin from the cytoplasmic membrane surface (Elgsaeter & Branton, 1974). Particles can then be aggregated by changes in pH or ionic strength or by treatment of the membranes with trypsin or neuraminidase.

Similar properties have not been described for intact red

cells (Elgsaeter et al., 1973), again suggesting that spectrin places a constraint on the mobility, within the membrane, of intramembraneous particles.

1.2.3 Organisation of the Membrane

Extensive studies on the intact erythrocyte and its isolated membrane suggest that there is no protein component which is symmetrically distributed at both the cytoplasmic and extracellular membrane surfaces. For example, Marchesi & Palade (1967) and Schatzmann & Vincenzi (1969) assigned the ATP-ases of the red cell to the cytoplasmic surface. Marchesi et al. (1969) suggest that spectrin is also on the cytoplasmic surface. In contrast, sugars have been shown to be exclusively located on the extracellular surface. Eylar et al. (1962) were able to release all the N-acetylneuraminic acid from intact human erythrocytes using neuraminidase. Nicolson and Singer (1971) showed that the carbohydrate binding sites of lectins were exclusively located at the extracellular surface.

A third possibility is that components may be exposed at both surfaces, either by spanning the membrane or by being orientated differently at each surface. The components involved in this type of distribution are the sialoglycoproteins and component 3 (Figs. 1a & 1c). Evidence for this is that, while carbohydrates have been exclusively assigned to the extracellular surface, Reichstein & Blostein (1973) and Morrison et al. (1974) have shown that the polypeptide chains of these components can be labelled at both the cytoplasmic and extracellular membrane surfaces. Steck (1974) reviews evidence for the theory that component 3 spans the membrane, based on differences in reactivity of this component, at the two membrane

surfaces, towards enzymatic and chemical modification. More compelling evidence is available for the major sialoglycoprotein. Sequence data on this component shows it to be an amphipathic molecule with carbohydrate located at the amino terminus (Segrest *et al.*, 1973). It also possesses a hydrophobic domain between the amino terminus and the hydrophilic carboxy terminus. These authors, using enzymic radioiodination, have shown that the amino terminus is exposed at the extracellular surface, while the carboxy terminus is exposed at the cytoplasmic surface. Moreover, the size of the hydrophobic domain is sufficient to span the membrane. These studies, together with the observations of Steck & Yu (1973) and Yu *et al.* (1973) on the conditions required to solubilise component 3 and the sialoglycoproteins from the membrane, suggest that these components do in fact span the membrane.

1.2.4 Properties of Specific Erythrocyte Membrane Proteins

Specific solubilisation procedures and specific labelling techniques in conjunction with sodium dodecyl sulphate/polyacrylamide gel electrophoresis and electron microscopy have enabled detailed studies of the properties of individual membrane protein components to be carried out.

1.2.4.1 Spectrin

Components 1, 2 & 5 (Fig. 1a), collectively known as spectrin (Marchesi & Steers, 1968), can be isolated from erythrocyte ghosts by exposing them to mildly alkaline, low ionic strength buffers, especially in the presence of chelating agents. These authors suggested that these proteins are structurally significant in that their removal results in the breakdown and vesiculation of the membrane. The spectrin complex is responsible for the

fibrillar structure observed at the cytoplasmic surface of ghosts (Marchesi et al., 1969). Moreover, Yu et al. (1973) have shown that extraction of ghosts with non-ionic detergent solubilises the glycoproteins and leaves behind a ghost-shaped reticular structure, consisting largely of the spectrin polypeptides. These authors suggest that these polypeptides self-associate to form a submembranous continuum rather than being specifically bound to the membrane.

A number of lines of evidence have implicated the spectrin complex in an erythrocyte contractile system for maintaining red cell shape and deformability. Weed & La Celle (1969) have shown that such properties of the erythrocyte are dependent on ATP and divalent metal ions. Avissar et al. (1975) have isolated spectrin from human erythrocytes and demonstrated Ca^{2+} -ATP-ase activity and Mg^{2+} -ATP-ase activity in the presence of skeletal muscle actin. This suggests the presence of a myosin like protein in the red cell. These authors have also shown that component 5 of the spectrin complex co-electrophoreses with rabbit muscle actin and Dunn & Maddy (1973) showed that this component is capable of forming aggregates.

Apart from the structural or the actomyosin-like roles described above, spectrin has been implicated in another membrane function. Nicolson (1973) has shown that aggregation of spectrin on the cytoplasmic side of the membrane, by sequestration of antispectrin γ -globulin inside resealed ghosts, produced aggregation of the sialoglycoproteins at the extracellular membrane surface. Thus peripheral proteins on the internal surface of the cell may, under certain conditions, exert a form of control on the external topography of the membrane receptor sites.

1.2.4.2 Component 3

The major Coomassie stained band of the erythrocyte membrane, component 3 (Fig. 1a), is a component of molecular weight 88,000, and accounts for approximately 24% of the membrane protein (Steck, 1972). It is an integral membrane protein in that it can only be solubilised from the membrane by detergent disruption of the lipids (Yu *et al.*, 1973). Tanner & Boxer (1972) have shown it to be a glycoprotein containing about 10% carbohydrate. Steck (1974) has reviewed evidence that this glycoprotein spans the membrane and Pinto da Silva & Nicolson (1974) have shown that component 3 is involved in intramembranous particles.

Moreover, a number of studies have implicated component 3 in facilitating the transport of various solutes across the membrane. Cabantchik and Rothstein (1972, 1974a, 1974b) have shown that the permeability of red cells to the anions sulphate and chloride can be blocked by compounds which are themselves anionic and bind component 3. Taverna & Langdon (1973) have also implicated this component in the glucose transport mechanism. Avruch & Fairbanks (1972) have shown it to be involved in a Mg^{2+} dependent Na^+/K^+ ATP-ase. Some reservation should be expressed concerning these observations since component 3 on electrophoresis is a very diffuse band and may consist of several co-migrating species.

Steck (1972) has suggested that component 3 polypeptides may self-associate within the membrane, as they can be cross-linked to form dimers by sulphhydryl oxidation both in the membrane and when solubilised by detergent. Wang & Richards (1975) suggest that component 3 may exist in a tetrameric form, on the

basis of their cross-linking studies. Although such tetrameric units would correspond to the number of anionic inhibitor sites, suggesting the possibility of anion channels, there are not enough component 3 tetramers to account for all the intramembraneous particles.

1.2.4.3 Sialoglycoproteins

The major glycoproteins of the erythrocyte membrane, the sialoglycoproteins shown in Fig. 1c, can be solubilised only by detergents or organic solvents (Juliano, 1973). They are therefore integral membrane components and evidence was presented in 1.2.3 that they span the membrane. The component PAS 1 has been generally referred to as the major sialoglycoprotein since, after reduction and detergent solubilisation of ghosts at 37°C prior to electrophoresis, this component is the major staining band. Fig. 1c shows PAS 2 to be the major stained band and in this case solubilisation was carried out at 100°C. These observations were first made by Marton & Garvin (1973) and have been extended by Tuech & Morrison (1974), who have shown that the interconversion of PAS 1 and PAS 2 is reversible and takes place in Tris but not phosphate buffers. Marton & Garvin (1973) have proposed that PAS 1 is a dimer of PAS 2. However, Mueller & Morrison (1974) have shown that enzymatic radioiodination of PAS 1, but not PAS 2, can take place at the extracellular membrane surface. Since PAS 2 has been shown to be accessible to proteolytic degradation at the extracellular surface (Steck *et al.*, 1971), this data suggests that PAS 2 can exist in different conformations in the membrane. Slutzky & Ji (1974) claim to have evidence for the identity of PAS 1 and PAS 2 from peptide mapping experiments. The same authors, studying the different

response of PAS 1 and PAS 2 to proteolysis, propose a model in which PAS 2 can exist as tight or loose dimers within the membrane. Although Steck (1972) was unsuccessful in cross-linking the sialoglycoproteins, using aldehydes or sulphhydryl oxidising agents, Ji (1974) has shown that PAS 1 and PAS 2 can be cross-linked by dimethyladipimate dihydrochloride. This provides some evidence of association within the membrane.

This affinity for aggregation may account for the variation in molecular weights which have been reported (reviewed in Segrest et al., 1971). These authors propose a molecular weight of 55,000 from gel electrophoresis, but this is probably for PAS 1. Grefrath & Reynolds (1974) report a molecular weight of 29,000 which agrees well with 31,000 reported for PAS 2 by Marton & Garvin (1973). Ultracentrifugation studies of Kathan et al. (1961) and Morawiecki (1964) give subunit molecular weights of 31,400 and 30,000 respectively.

The composition and structure of "The major sialoglycoprotein" has been studied by Winzler (1969) and Marchesi et al. (1972). The molecule is 60% carbohydrate and 40% protein. The major sugars are N-acetylneuraminic acid, N-acetylgalactosamine and galactose, with a significant amount of N-acetylglucosamine, some fucose and mannose. Winzler (1969) has shown that there are at least two types of oligosaccharide chain. The simplest is alkali-labile and contains N-acetylneuraminic acid, galactose and N-acetylgalactosamine.

Marchesi's group have investigated the structure of the protein moiety and have shown it to be an amphipathic molecule with carbohydrate at the amino terminus, a hydrophilic carboxy terminus and a hydrophobic core region (Segrest et al., 1972; Segrest

et al., 1973). This hydrophobic domain of the polypeptide chain may be responsible for the aggregation discussed above. Recently Lea et al. (1975) have shown that the hydrophobic domain, which can be isolated as a tryptic peptide, can interact with lipid bilayers and affect their permeability properties. Segrest et al. (1974) have also shown that, at a given concentration, these tryptic peptides will aggregate to form structures similar to intramembranous particles. It is doubtful if this could happen under physiological conditions. However, the sialoglycoproteins have been implicated in intramembranous particle formation by Pinto da Silva et al. (1971), Tillack et al. (1972) and Nicolson (1973). These workers altered the distribution of sialoglycoprotein surface receptors and showed a parallel alteration in the distribution of particles.

A number of functions have been attributed to the sialoglycoproteins. Kathan & Winzler (1963) showed that PAS 2 is the receptor for influenza virus and myxoviruses. The major sialoglycoprotein has been purified and shown to possess MN antigenic activity (Cleve et al., 1972). It is not clear however, if AB blood group activity can be attributed to glycoprotein or to slight glycolipid contamination (Gardas & Kościelak, 1971; Hamaguchi & Cleve, 1972). The glycoprotein does, however, possess the receptors for a number of plant agglutinins (Fukuda & Osawa, 1973).

1.3 Biological Significance of Glycoprotein Carbohydrate

1.3.1 Possible Role of Carbohydrate in Cellular Recognition

A proposal by Roseman (1970), that cells may adhere to one another by forming an enzyme/substrate complex between glycosyltransferases on one cell and suitable glycoprotein acceptors on

a complementary cell, has led to a search for plasma membrane-bound glycosyltransferases in a number of cells.

Bosmann (1971) described four such glycosyltransferases in platelets. Two of these were very specific for transfer of glucose and galactose to collagen. As platelets do not synthesise collagen, Bosmann suggested that these transferases were involved in the adherence of platelets to collagen in haemostasis. He also suggested that the other two glycoprotein: glycosyltransferases might be involved in platelet-platelet adhesion, although no endogenous acceptors were found. Jamieson et al. (1971) reported similar findings in their study of a platelet membrane collagen-specific glycosyltransferase. They showed that inhibitors of the collagen glycosyltransferase would also inhibit adhesion of platelets to collagen.

Similarly, Roth et al. (1971) have shown the presence of a galactosyltransferase on the surface of chicken embryo neural retina cells. The adhesion specificity of these cells could be interfered with by introduction of a suitable exogenous glycopeptide substrate for the glycosyltransferase.

Roth & White (1972), using cultured Balb/c3T3 cells, provided evidence for membrane glycosyltransferases which could transfer sugar to endogenous acceptors on adjacent cells. Their results were dependent on the degree of contact between cells and may suggest a role for glycosyltransferases in contact inhibition of growth. Bosmann (1972) found similar results for normal and oncogenic virus transformed fibroblasts.

Although these studies support the hypothesis that inter-cellular adhesion, cell-glycoprotein adhesion and contact inhibition may be mediated by a glycosyltransferase/glycoprotein

interaction, the generality of the phenomenon is not established. Indeed Evans & Wisher (1975) were unable to demonstrate glycosyltransferase activity in the plasma membrane between adjacent hepatocytes. They did in fact find a potent nucleotide pyrophosphatase on the extracellular membrane surface. This would hydrolyse sugar nucleotides and so inhibit glycosylation reactions.

1.3.2 The Role of Carbohydrate in Circulatory Survival

The importance of the carbohydrate moiety to the circulatory survival of plasma glycoproteins was suggested by the observation of Morell et al. (1966) that desialylated caeruloplasmin, on injection into rabbits, was rapidly removed from the circulation. Morell et al. (1968) subsequently showed that the galactose residues, exposed on removal of sialic acid, were responsible for recognition of the desialylated glycoprotein, since their removal, modification or resialylation enabled the glycoprotein to manifest its normal survival time. The site of uptake of asialocaeruloplasmin was found to be the liver and shown to be exclusively in the parenchymal cells. Further study with this same system (Wickman et al., 1970; Van Den Hamer et al., 1970) suggested that only a very limited removal of sialic acid was required for exhibition of this uptake phenomenon. Subsequently, Morell et al. (1971) carried out similar studies on a number of plasma glycoproteins and the observed phenomenon was found to be generally applicable, with the exception of transferrin.

Hudgin et al. (1974) have isolated the binding protein from rabbit liver which specifically binds desialylated plasma glycoproteins. The binding protein is a sialoglycoprotein whose sialic acid is required for binding. There is also a requirement

for calcium. Since desialylated glycoproteins are natural substrates for a sialyltransferase, Hudgin & Ashwell (1974) looked for glycosyltransferase activity in their purified binding protein. It was shown to be absent.

It would appear, therefore, that removal of desialylated glycoproteins from the circulation is not mediated by a glycosyltransferase/glycoprotein interaction but rather that there is some complementary recognition between two glycoproteins.

Despite the generality of this process and the elucidation of the uptake mechanism, the significance of desialylation in the catabolism of plasma glycoproteins has not been established. Ashwell and Morell (1974) suggest that demonstration of partially desialylated glycoproteins in vivo and the locus of the neuraminidase responsible for desialylation would be required, in order to establish a physiological role. Evidence for such a neuraminidase is equivocal (Schengrund et al., 1972; Bernacki & Bosmann, 1973).

Further evidence for a role for sialic acids in circulatory survival comes from the work of Woodruff and Gesner (1969), who showed that treatment of rat lymphocytes with neuraminidase caused them to be temporarily sequestered in the liver before recirculating normally in the blood and lymph after twenty-four hours. These authors did not quantitate the minimum loss of sialic acid necessary to observe this phenomenon, nor did they indicate whether exposure of an underlying carbohydrate determinant was necessary for this sequestration. Since other non-specific treatments, such as exposure to glycosidases (Gesner & Ginsburg, 1964) or to trypsin (Woodruff & Gesner, 1968) gave similar effects, again the physiological significance of these observations is difficult to assess. Jancik & Schauer (1974)

have suggested that the twenty-four hour delay in recirculation of neuraminidase treated lymphocytes may be the time required to resynthesize cell surface sialic acids, in which case it would be interesting to know if trypsin treated lymphocytes recirculate.

Jancik & Schauer (1974) have demonstrated that cell surface sialic acids are necessary for the in vivo survival of rabbit erythrocytes. They suggested that, in contrast to plasma glycoproteins, the sugar residue exposed on removal of sialic acid does not affect removal from the circulation. Moreover, in contrast to the finding with lymphocytes, erythrocytes did not appear to re-enter the circulation and Jancik & Schauer were unable to demonstrate the presence of enzymes which could regenerate or transfer sialic acids.

These observations, in conjunction with those on plasma glycoproteins and lymphocytes, suggest the indispensability of sialic acids for normal circulatory survival. They do not, however, prove that loss of sialic acid is a process normally taking place in vivo and leading to removal from the circulation.

However, Danon et al. (1971) have proposed that removal of sialic acid in vivo is a major factor in the elimination of old erythrocytes from the circulation. They have obtained erythrocytes of different in vivo age and have shown that old erythrocytes exhibit a decreased electrophoretic mobility and a decreased density of staining of the cell membrane with a positively charged colloidal iron suspension.

Since the surface charge of erythrocytes is largely attributable to sialic acid (Eylar et al., 1962), these results suggest that in vivo erythrocyte aging is accompanied by loss of sialic acid.

1.4 Aims of the Present Study

Studies on the in vivo aging of red blood cells have shown that a number of degradative and metabolic changes occur, for which the erythrocyte, as an enucleate cell, cannot compensate. Any or all of these changes, outlined in 1.1, might dictate that a cell is too old to remain in the circulation.

It might be anticipated that the recognition of such effete cells would be mediated by the surface membrane characteristics of the senescent erythrocytes. With the advent of new techniques for studying membranes (1.2), it was decided to characterise the membrane surface properties of old and young erythrocytes in anticipation of detecting changes which might be recognised in vivo and lead to the removal of effete cells from the circulation. Since there was some tentative evidence that carbohydrate might be involved in cell recognition processes (1.3), these studies concentrated on membrane glycoproteins.

MATERIALS AND METHODS

2.1 Separation and Characterisation of Erythrocytes

Fresh human blood was obtained by venepuncture and prevented from coagulating by immediate suspension in one tenth volume of Alsever's solution (Bukantz et al., 1946). Plasma and the buffy coat of white cells and platelets were aspirated after centrifugation at 800g for 10min and resuspension in a buffer of pH 7.4, which contained 5mM sodium dihydrogen orthophosphate and 0.15M sodium chloride (PBS 7.4), for a total of four washes. Contamination with white cells was assessed by staining a sample of cells with Giemsa stain (British Drug Houses Chemicals Ltd., Poole, England). Cell counting was performed in an improved Neubauer haemocytometer. Determination of the ABH blood group of a donor was carried out using human antisera obtained from Hyland Div. Travenol Laboratories Inc., Costa Mesa, California, U.S.A.

2.2 Preparation of Erythrocyte Membranes

Haemoglobin free erythrocyte membranes (ghosts) were prepared by lysis and washing in 5mM sodium dihydrogen orthophosphate, essentially according to the method of Steck et al. (1970). These authors advise the discarding of a hard-packed pellet of material allegedly from white cells and possessing proteolytic activity. If care is taken to remove white cells during erythrocyte washing, this step is unnecessary.

2.3 Preparation of Iso-osmotic Bovine Albumin Solutions

Bovine albumin, Fraction V, (Sigma Chemical Co., St Louis, Missouri, U.S.A.) was dissolved at 4°C in deionised distilled water to yield a 20% (w/w) solution, which was deionised by passage through a column of analytical grade Amberlite monobed resin MB3 (British Drug Houses Chemicals Ltd., Poole, England).

The resulting solution was diluted to 5% (w/w) and lyophilised. The protein was powdered and dissolved in a buffer of composition 153mM sodium hydrogen carbonate, 5.2mM magnesium chloride and 5mM potassium chloride (Bishop & Prentice, 1966), containing 0.1mg/ml streptomycin sulphate and 0.6mg/ml penicillin (both obtained from Glaxo Laboratories, Greenford, England). The desired protein concentration was approximately 42% (w/w).

The osmotic activity of an albumin solution was determined on an Advanced Digimatic Osmometer, model 3D (Advanced Instruments Inc., Needham Heights, Massachusetts, U.S.A.), employing the method of Piomelli *et al.* (1967). The desired osmolality of the albumin solution was 291 milliosmoles per kg. The osmolality of the concentrated albumin solution was determined by measurement at twofold dilution in a solution of composition 148mM sodium chloride, 5.2mM magnesium chloride and 5mM potassium chloride (diluent solution), which was 291 milliosmolar. Adjustments of the osmolality of the concentrated albumin solution were achieved by the addition of solid sodium chloride until a twofold dilution in the diluent solution left the osmolality unchanged at 291 milliosmoles/kg.

The specific gravity of the concentrated solution was determined by weighing in a 100ml volumetric flask at 20°C on a Stanton Unimatic balance (Stanton Instruments Ltd., London, England). Fractions of required specific gravity were prepared by dilution of the concentrated albumin with the diluent solution and yielded solutions of pH 7.4.

2.4 Fractionation of Human Erythrocytes

Washed, packed erythrocytes (1ml) were applied to the top of a discontinuous density gradient of iso-osmotic bovine albumin.

The gradient consisted of five 2.3ml fractions layered in $3\frac{3}{4}$ " x 9/16" cellulose nitrate centrifuge tubes. The specific gravity range of the fractions for blood group O erythrocytes, from donor A.B., was 1.095-1.115g/ml by steps of 0.005, while for blood group B erythrocytes, from donor J.G.B., the range was 1.090-1.114g/ml by steps of 0.006. Gradients were centrifuged at 4°C for 40min in a Beckman SW40 rotor at 40,000 r.p.m. in a Beckman L2-65B ultracentrifuge. The resulting six fractions of cells were obtained by slicing the tubes using a Beckman tube slicer and washing the cells free of albumin by four washes in PBS 7.4. The distribution of cells among the fractions, was obtained by measuring the haemoglobin content of each fraction.

2.5 Determination of Haemoglobin

Haemoglobin was measured by the method of Van Kampen & Zijlstra (1961). Sodium dodecyl sulphate (British Drug Houses Chemicals Ltd., Poole, England) replaced Sterox SE in the assay solution. It was present at 0.05% (w/w) and did not affect the colorimetric assay. This and all subsequent colorimetric assays were performed in a Beckman DB spectrophotometer. Human haemoglobin, type IV, obtained from Sigma Chemical Company, St Louis, Missouri, U.S.A., was used to prepare standard curves.

2.6 Measurement of Erythrocyte Glucose-6-phosphate

Dehydrogenase Activity

The assay of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was based on the method of Piomelli *et al.* (1968). Packed red cells were lysed by 250-fold dilution in distilled water. Lysate (1ml) was made up, in the substrate-containing assay medium, to a volume of 2.9ml to which was added 0.1ml of 0.03M disodium NADP, obtained from Boehringer Mannheim GmbH, Mannheim, Germany, to

start the reaction. A blank, from which the substrate sodium D-glucose-6-phosphate (Sigma Chemical Co., St Louis, Missouri, U.S.A.) was omitted, was used. The assay was allowed to proceed for 20 min at 25°C in a Cary Model 15 recording spectrophotometer (Cary Instruments, Monrovia, California, U.S.A.). The rate of change of absorbance at 340nm was measured and related to the formation of NADPH using the extinction coefficient of 6200M⁻¹cm⁻¹. The enzyme activity was related to the haemoglobin present by assaying an aliquot of lysate for haemoglobin.

2.7 Chemical and Enzymatic Hydrolysis of Erythrocyte

N-Acetylneuraminic Acid

N-Acetylneuraminic acid was assayed by the method of Aminoff (1961), which requires the presence of free N-acetylneuraminic acid. The free acid was released from intact cells by Clostridium perfringens neuraminidase (EC 3.2.1.18) or by acid hydrolysis. The neuraminidase (Type VI) was obtained from Sigma Chemical Co., St Louis, Missouri, U.S.A. Enzymatic hydrolysis was carried out by incubating cells equivalent to 46.9mg haemoglobin in a total volume of 1ml of buffer of composition 5mM sodium acetate and 0.15M sodium chloride at pH 5 containing 0.03 mg enzyme protein. Incubation was for 1h at 37°C with shaking.

Conditions of acid hydrolysis were standardised to allow for the buffering effect of haemoglobin. A sample of cells, equivalent to 46.9mg haemoglobin, was made up to 1ml in 0.05M sulphuric acid. Hydrolysis was carried out at 80°C for 1h.

Samples from enzymatic and acid hydrolysis were treated with an equal volume of 5% (w/v) dodeca-tungstophosphoric acid (British Drug Houses Chemicals Ltd., Poole, England) and the

precipitate was centrifuged at 20,000g for 10min. The precipitate was washed with 1ml of 2.5% (w/v) dodeca-tungstophosphoric acid and the N-acetylneuraminic acid content of the combined supernatants was determined.

2.8 Hydrolysis and Purification of Erythrocyte Membrane

N-Acetylneuraminic Acid

N-Acetylneuraminic acid was released from erythrocyte ghosts by hydrolysis in 0.025M sulphuric acid at 80°C for 1h. Substances which interfere in the assay were removed by binding the N-acetylneuraminic acid to an ion exchange resin. Hydrolysates were treated with an equal volume of 0.025M barium hydroxide, pH 6, to precipitate sulphate ions. After centrifugation at 20,000g for 10min, the supernatant was passed through a column of Dowex 1X8-100 formate. The column was washed with 10 volumes of water after which the N-acetylneuraminic acid was eluted in 18 volumes of 0.3M formic acid. Samples were lyophilised and redissolved in a small volume of water for assay. The recovery of N-acetylneuraminic acid by this procedure was determined by running standards simultaneously under identical conditions. Recoveries were routinely 76%.

Dowex 1X8-100 formate was prepared by washing the chloride form of the resin, obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A., with acetone, water, 3M hydrochloric acid, water, 3M sodium hydroxide, water and 2M sodium formate.

2.9 The Thiobarbituric Acid Assay of N-Acetylneuraminic Acid

An aqueous sample (0.5ml) containing N-acetylneuraminic acid was treated with 0.25ml of 25mM sodium metaperiodate pH 1.2, and incubated at 37°C for 30min. This was followed by the addition of 0.2ml of 2% (w/v) sodium arsenite in 0.5M hydrochloric acid.

Blanks were obtained by addition of arsenite prior to periodate. After the disappearance of the yellow colour, 0.1M 2-thioarbituric acid (2ml), obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England, was added and the sample boiled for 7.5min. The sample was cooled in ice and the colour was extracted into 5ml of butan-1-ol containing 5% (v/v) hydrochloric acid. The colour developed was determined at 549nm and compared to that of N-acetylneuraminic acid standards. N-acetylneuraminic acid was a product of Koch-Light Laboratories Ltd., Colnbrook, Bucks., England. The assay is that of Aminoff (1961).

A number of aqueous solutions, potentially capable of interfering with the assay, were subjected to the conditions of the assay both as samples and as blanks. These were unhydrolysed ghost supernatant, aqueous solutions of malonaldehyde bis - (dimethyl acetal), obtained from Ralph N. Emanuel Ltd., Wembley, England, and the supernatant of a linseed oil emulsion. Linseed oil was obtained from Affchem Ltd., Falkirk, Scotland. Spectra of the colour developed by all these substances were obtained on a Pye-Unicam SP8000 ultraviolet recording spectrophotometer (Pye-Unicam Ltd., Cambridge, England).

2.10 Determination of Amino Sugars

The release of N-acetylglucosamine and N-acetylgalactosamine from ghosts was carried out in 4M methane-sulphonic acid (Koch-Light Laboratories Ltd., Colnbrook, Bucks., England) at 100°C for 24h in evacuated, sealed pyrex tubes.

Ghost suspension (0.2ml), containing approximately 0.3mg protein, was made up to a volume of 0.5ml in 4M methane-sulphonic acid. DL-norleucine (50nmol), obtained from Sigma Chemical Co.,

St. Louis, Missouri, U.S.A., was added as an internal standard. After hydrolysis, 3.5M sodium hydroxide (0.5ml) and 1M sodium hydroxide (0.2ml) were added. This was followed by further small additions of alkali until a pH of 2-2.5 was achieved. The resulting solution was analysed in an amino acid analyser JLC-5AM of the Japanese Electron Optics Laboratory Company Ltd., Tokyo, Japan, using 10cm and 50cm columns of LCR1 resin.

2.11 Determination of Galactose and Glucose

Ghost suspension (0.3ml), containing approximately 0.45mg protein, was dried down over phosphorus pentoxide under vacuum. Samples were then prepared for gas chromatographic analysis by the method of Clamp *et al.* (1971). Sugars were run isothermally at 165°C in a Pye series 104 gas chromatograph with a flame-ionisation detector. Mannitol was used as an internal standard. Determination of the sugar of interest was carried out by measurement of peak areas and relating these to a series of standard curves derived from sugar standards covering the expected concentration range.

2.12 Determination of Protein

Erythrocyte membrane protein was estimated by the method of Lowry *et al.* (1951), using deionised, lyophilised bovine albumin as standard.

2.13 Incorporation of a Tritium Label into N-Acetylneuraminic of Erythrocyte Membrane Glycoproteins

The labelling procedure is based on that of Blumenfeld *et al.* (1972). A 50% (v/v) suspension of packed, washed erythrocytes in PBS 7.4, containing sufficient sodium metaperiodate to give a tenfold molar excess of periodate to N-acetylneuraminic, was shaken for 10min at 20°C. The oxidation was terminated by the

addition of cold PBS 7.4 and centrifugation at 800g for 10min for a total of three washes. The packed red cells were resuspended in an equal volume of PBS 7.4 and a quantity of tritiated sodium borohydride (130 mCi/mmol) in 0.01M sodium hydroxide was added to yield a twelvefold molar excess of borohydride to N-acetylneuraminic acid. After 30min at 20°C with frequent shaking, the reaction was terminated by the addition of cold PBS 7.4, with centrifugation and washing as above, until the supernatant was essentially free of radioactivity. All radiochemicals were obtained from the Radiochemical Centre Ltd., Amersham, England.

2.14 Incorporation of a Tritium Label into Galactose/ Galactosamine Residues of the Erythrocyte Membrane

The labelling procedure was based on that of Gahnberg & Hakomori (1973). Packed erythrocytes (0.1ml) were suspended in 2 volumes of PBS 7.0 containing 80 units of galactose oxidase (EC 1.1.3.9), and the suspension was incubated at 37°C for 1h with gentle shaking. Galactose oxidase was obtained from Worthington Biochemical Corp., Freehold, New Jersey, U.S.A. The reaction was terminated by the addition of cold PBS 7.4, with centrifugation and washing for a total of three washes. The packed cells were resuspended in an equal volume of PBS 7.4 and 0.8mCi of tritiated potassium borohydride (130mCi/mmol) in 0.01M sodium hydroxide was added. After 30min at 20°C with shaking, the reaction was terminated by the addition of ice cold PBS 7.4, with centrifugation and washing until the supernatant was essentially free of radioactivity.

2.15 Sodium Dodecyl Sulphate Gel Electrophoresis

Electrophoresis of erythrocyte membranes was performed as described by Fairbanks et al. (1971). Sodium dodecyl sulphate,

ammonium persulphate, N,N¹-methylenebisacrylamide, dithiothreitol and N,N,N¹,N¹-tetraethylenediamine were obtained from British Drug Houses Chemicals Ltd., Poole, England. Acrylamide was a product of Koch-Light Laboratories Ltd., Colnbrook, Bucks., England. Pyronin Y was obtained from George T. Gurr, London, England. Coomassie brilliant blue R and basic fuchsin were obtained from Gurr/Searle Diagnostic, High Wycombe, Bucks., England.

Erythrocyte membrane samples, containing approximately 0.1mg protein, were incubated, in buffer containing reducing agent and sodium dodecyl sulphate, at 100°C for 3min, before application to 10cm x 0.6cm polyacrylamide gels.

Gel densitometry was carried out using a Gilford spectrophotometer model 240 with a linear transport attachment model 2410 (Gilford Instruments Ltd., Teddington, Middlesex, England). Coomassie stained gels were scanned at 550nm; periodate-Schiff stained gels were scanned at 560nm.

Stained or unstained radioactive gels were sliced into 1mm slices for counting, using a Mickle gel slicer (Mickle Laboratory Engineering Co., Gernshill, Surrey, England).

2.16 Liquid Scintillation Counting

Tritium was determined in a scintillation fluid, compatible with aqueous samples, which tolerated 1-2ml of aqueous sample per 10ml scintillator. The scintillation fluid was a 35% (v/v) solution of Triton X 114 (Koch-Light Laboratories Ltd., Colnbrook, Bucks., England) in toluene and contained 0.5% (w/v) 2,5-diphenyloxazole (also Koch-Light) and 0.05% (w/v) p-bis (4-methylstyryl) benzene (Eastman Kodak Co., Rochester, New York, U.S.A.).

Aqueous suspensions of erythrocyte membranes were added to scintillation fluid without additional treatment. Whole cells (5 μ l) were decolorised with 0.3ml 30% (w/v) hydrogen peroxide (British Drug Houses Chemicals Ltd., Poole, England). Water was added prior to scintillation fluid. Gel slices were treated with 0.3ml 30% (w/v) hydrogen peroxide and solubilised overnight at 60°C prior to the addition of water and scintillator. Counts were determined on a Nuclear Chicago Isocap 300. Efficiency of counting was approximately 45%.

2.17 Agglutination Studies

Human anti-B serum was obtained from Hyland Div. Travenol Laboratories Inc., Costa Mesa, California, U.S.A. Wheat-germ agglutinin was purified by the affinity method of Reitherman et al. (1974) and was used at a concentration of 0.4mg/ml. Carcinus lectin was partially purified from the haemolymph of Carcinus crabs by adsorption and elution from a column of N-acetylglycyl-AH-sepharose (A. Baxter, unpublished work). The concentration of agglutinin in this preparation was unknown.

Serial dilutions of the agglutinins, into PBS 7.4, were carried out in a plastic agglutination tile and to each well was added one half volume of 1.5% (v/v) erythrocytes. Erythrocytes were fractionated, trypsinised or neuraminidase treated, depending on the conditions being studied.

Trypsinised cells were obtained by incubating 0.2ml washed red cells for 1h at 37°C in 5.3ml of PBS 7.4 containing 5mg bovine pancreatic trypsin (EC 3.4.21.4), obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England. After 3 washes in PBS 7.4, the cells were resuspended to 1.5% (v/v).

Cells treated with neuraminidase (EC 3.4.21.14) were obtained by incubating 1.5ml packed red cells in 1.5ml 5mM sodium acetate, 0.15M sodium chloride buffered at pH 6.5, containing 0.2mg neuraminidase. Samples were incubated at 37°C for 15min or 1h and after washing, were resuspended at 1.5% (v/v) and used for agglutination.

2.18 Standardisation of Agglutination Procedure

In the previous section, the preparation of erythrocytes, the agglutinins used and the buffer have been defined. Agglutination was allowed to proceed for 30min with occasional shaking. After this time, the well in which 50% agglutination had taken place was noted. Assuming the initial concentration of the agglutinin in each case to be unity, each well in a row of serial dilutions can be assigned a number, corresponding to the agglutinin dilution factor. For example, well 1 would be designated 2, well 2 designated 4, well 3 designated 8, etc. In assessing the response of differently treated erythrocytes to equal concentrations of agglutinin, the dilution factor at which 50% agglutination of a particular type of cell occurred, was defined as that cell's agglutination index. In this way, the interaction of differently treated cells with the same agglutinin could be assessed semi-quantitatively.

2.19 Iodination of Wheat-Germ Agglutinin

The iodination reaction was a modification of the method of Marchalonis (1969). The reaction was carried out in a total volume of 0.4ml containing 0.15M sodium chloride, 0.05M sodium dihydrogen orthophosphate, 0.02mM potassium iodide, 0.1M N-acetylglucosamine (Sigma Chemical Co., St Louis, Missouri, U.S.A.), 20µg lactoperoxidase (EC 1.11.1.7) (Calbiochem, Los Angeles,

California, U.S.A.), 2mg wheat-germ agglutinin and 1.4 μ Ci sodium [¹²⁵I]-iodide. The reaction was initiated by the addition of hydrogen peroxide to give a final concentration of 0.176mM. After 30min at 20°C, the reaction was terminated by the addition of 1ml 20mM 2-mercaptoethanol (Koch-Light Laboratories Ltd., Colnbrook, Bucks., England). The agglutinin was dialysed against distilled water for 24h at 4°C, followed by two 24h dialyses against PBS 7.4. It was subsequently used at a concentration of 0.4mg/ml and its specific activity was determined as 0.34 μ Ci/mg.

2.20 Binding Studies Using [¹²⁵I]-Wheat-Germ Agglutinin

Binding studies were carried out in polypropylene Beckman Bio-vials which had been presoaked for 4h with 2ml of bovine albumin, which was 5mg/ml in PBS 7.4. The albumin was aspirated before use. For binding studies, 0.8 μ g-12 μ g of [¹²⁵I]-wheat-germ agglutinin and 10⁷ erythrocytes were incubated at 20°C in a total volume of 0.4ml PBS 7.4. For the purposes of studying saturation binding, 8 μ g, 12 μ g and 16 μ g agglutinin were added to 2 x 10⁶ erythrocytes. After 30min with occasional shaking, the cells were washed three times with 2ml PBS 7.4, and the amount of bound agglutinin was determined in a Beckman Biogamma T.M. counter (Beckman Instruments Inc., Palo Alto, California, U.S.A.). Corrections were made for non-specific binding to the polypropylene tubes, although this was minimised by the albumin presoaking step (Lesney *et al.*, 1972).

Inhibition of binding to erythrocytes by ovomucoid fraction II (Beeley, 1971) was carried out by incubating 12 μ g agglutinin and 10⁷ erythrocytes in 0.4ml PBS 7.4 containing 0.05 μ g-3mg ovomucoid fraction II.

2.21 Freeze-Fracture of Intact Erythrocytes

Freeze-fracture was carried out on both fixed and unfixed cells. Where fixed cells were used, washed, packed cells were suspended at 1% (v/v) in PBS 7.4 which was 0.5% (v/v) in glutaraldehyde (Taab Laboratories, Emmer Green, Reading, England). The cells were immediately pelleted by centrifugation at 800g for 5min, followed by washes in distilled water and 20% (v/v) aqueous glycerol (British Drug Houses, Chemicals Ltd., Poole, England). Samples of fixed, glycerinated or unfixed packed cells were applied to gold specimen support discs, using a fine capillary pipette, and were immediately rapidly frozen by immersion for 2-3s in Freon 22 at liquid nitrogen temperature. Samples were stored at this temperature until used. Freeze-fracturing was carried out in a Balzer's 360M Freeze-Etch Unit with a specimen temperature of -100°C during the fracturing process. Replication was started immediately after the last fracture stroke. After floating on to distilled water, the replicas were cleaned by overnight immersion in 70% (v/v) Chloros. The replicas were rinsed twice with distilled water, followed by transfer to a 70% (v/v) sulphuric acid solution for 3-4h and three 30min rinses in distilled water. The replicas were collected on grids carrying carbon-coated formvar films and examined in a Philip's EM300 electron microscope operating at 60kV.

2.22 Incorporation of [^{125}I]-Label into the External Surface Proteins of Erythrocytes

Washed erythrocytes were suspended to a 30% haematocrit in PBS 7.4 and incubated at 20°C with 0.2mg lactoperoxidase and 4 μCi sodium [^{125}I]-iodide. To this was added 20 μl 2.112mM

hydrogen peroxide at 2min intervals for 1h. Labelled erythrocytes were washed and membranes isolated as described.

2.23 Treatment of Erythrocytes with Proteolytic Enzymes

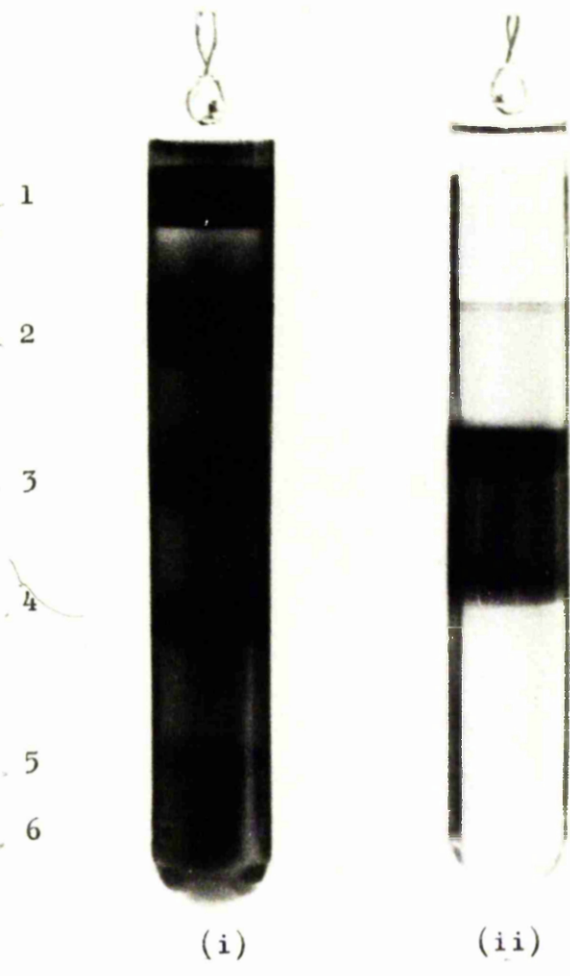
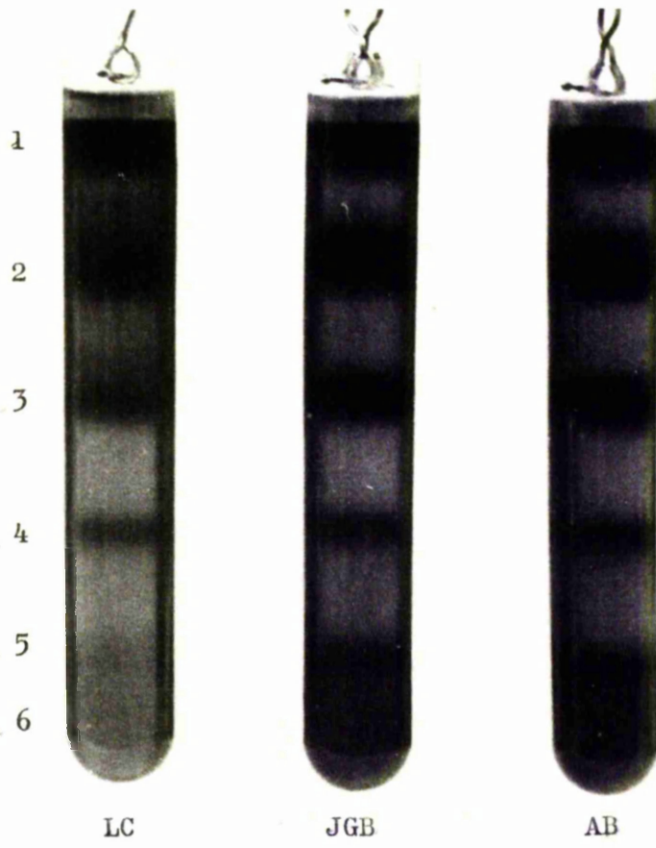
A grade α -chymotrypsin (EC 3.4.21.1) from bovine pancreas and B grade pronase (EC 3.4.24.4) were obtained from Calbiochem, Los Angeles, California, U.S.A. Subtilisin Type VII (EC 3.4.21.14) was obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. TPCK-trypsin was prepared from Sigma's bovine pancreatic trypsin by the method of Carpenter (1967).

Erythrocytes, at 33% haematocrit in PBS 7.4, were incubated at 37⁰C in the presence of 0.5mg enzyme per ml red cells. Duplicate samples were withdrawn at 3, 20 and 60 min, and immediately centrifuged in cold PBS 7.4 for a total of four washes. One sample was hydrolysed under standard conditions to determine its N-acetylneuraminic acid content. Membranes were prepared from the duplicate sample for electrophoresis.

RESULTS

Figure 2a Fractionation of Human Erythrocytes on Discontinuous Density Gradients of Iso-osmotic Bovine Albumin
Erythrocytes (1ml) from three donors, L.C., J.G.B. & A.B., were centrifuged on five-step discontinuous gradients, of density range 1.095-1.115g/ml by steps of 0.005, to yield six fractions of differently aged cells at the interfaces.

Figure 2b Recentrifugation of Erythrocyte Fraction 3
(i) Erythrocytes from A.B. were fractionated as described above. Fraction 3 was removed, washed free of albumin and reapplied to an identical gradient.
(ii) 78% of the erythrocytes returned to the expected position. The remainder banded at a position of increased specific gravity.



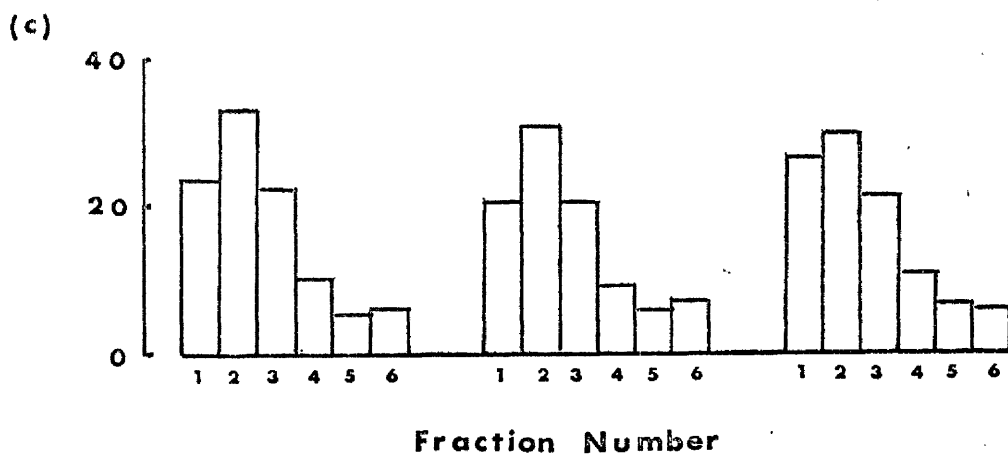
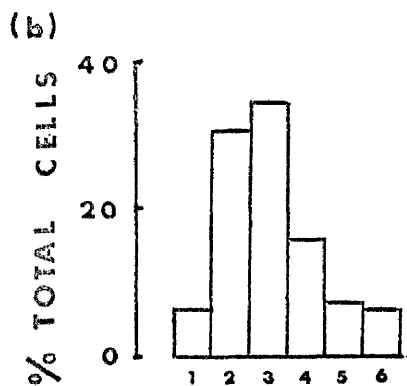
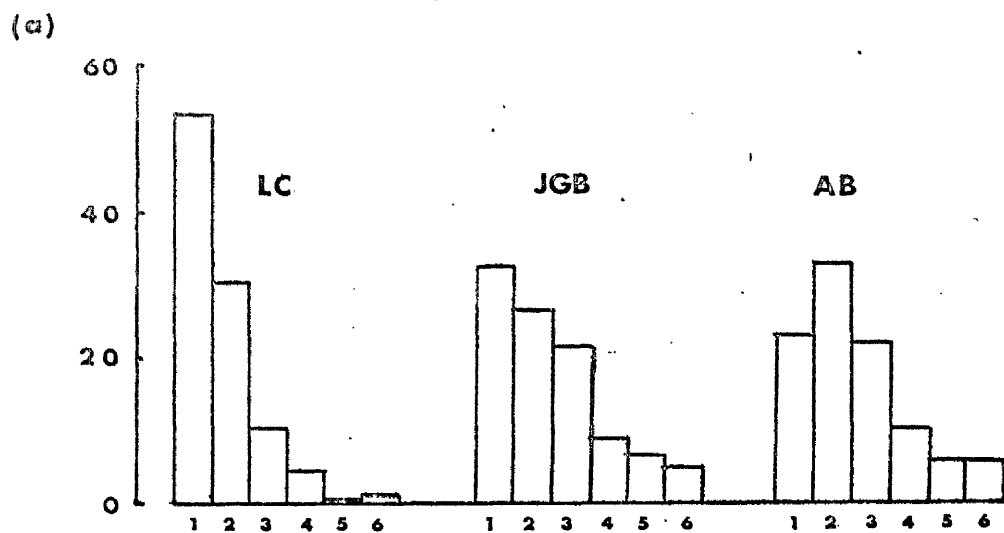


Figure 3 Distribution of Human Erythrocytes on Discontinuous
Density Gradients of Iso-osmotic Bovine Albumin

- (a) Erythrocytes from 3 individuals L.C., J.G.B. and A.B. were separated into six differently aged fractions on a five-step gradient of density range 1.095-1.115g/ml by steps of 0.005 to yield the distributions shown. These conditions were considered suitable for the cells of A.B.
- (b) Erythrocytes from J.G.B. were separated on a slightly modified gradient of density range 1.090-1.114g/ml by steps of 0.006.
- (c) The fractionation procedure yielded fairly reproducible distributions of the cells of A.B. in three separate experiments.

3.1.1 Fractionation of Human Erythrocytes

The separation of human erythrocytes on gradients of bovine albumin is dependent on the specific gravity distribution of an individual's red cells. Fig. 2a shows the distribution of cells of three individuals on a five-step gradient of density 1.095-1.115g/ml by steps of 0.005. Fig. 3a shows these distributions as percentages of the total cells applied to the gradients. Subject L.C. is a female donor whose erythrocytes were of significantly lower mean specific gravity than those of the other two male donors. In all subsequent studies, fresh erythrocytes were obtained from male subjects J.G.B. and A.B. Cells from J.G.B. were, however, fractionated on a five-step gradient of density 1.090-1.114g/ml by steps of 0.006 (Fig. 3b). These conditions were considered suitable for the investigation of changes occurring on aging, since they allowed the isolation of the densest 7-10% of the cells.

Fig. 3c demonstrates the reproducibility of the fractionation procedure for the erythrocytes of subject A.B. on the same gradient system in three separate experiments. Furthermore, if cells isolated from density fraction 5 are washed and re-applied to a fresh gradient, 78% of the cells reappear at the expected position (Fig. 2b). The remaining 22% show an increase in density, possibly due to additional manipulations or change in sample size.

3.1.2 Homogeneity of Cell Fractions

The preparation of a sample of erythrocytes for density gradient fractionation required removal of plasma and buffy coat of white cells and platelets by aspiration. To do this effectively entailed a loss of some 5-10% of the youngest erythrocytes but as

a result residual contamination by other blood cells was limited to the least dense fraction. Staining of the different density fractions with Giemsa stain indicated that minor contamination of the least dense fraction by white cells was of the order of 0.1%. Reticulocytes were present in the least dense fraction but were not routinely quantitated. Platelets were not observed under the microscope in any of the cell fractions. Furthermore, polyacrylamide gel electrophoresis in sodium dodecyl sulphate of ghosts prepared from cell fractions, consistently showed no contaminating non-erythrocyte polypeptides. Therefore all fractions, with the exception of the least dense, were considered to be homogeneous erythrocytes.

3.1.3 Haemoglobin Content of Fractionated Cells

Table 2 shows the cellular haemoglobin content of erythrocytes from different density fractions. The mean value obtained over the six fractions was 46.5pg haemoglobin per cell. The deviation from the mean is not considered to be significant, because of the large errors involved in visual cell counting, although these errors were minimised by counting at least 600 cells for each fraction. Leif & Vinograd (1964), using an electronic cell counting method, found that the haemoglobin content of human erythrocytes did not vary during in vivo aging.

Table 2 Haemoglobin Content of Fractionated Erythrocytes

Fraction Number	pg Haemoglobin/cell
1	50
2	48
3	45
4	42
5	43
6	46

These observations preclude the necessity of routine cell counting. Equal numbers of cells from different density fractions can be obtained by a simple haemoglobin assay.

3.1.4 Glucose-6-Phosphate Dehydrogenase Activity of Fractionated Erythrocytes

The relationship between age and specific gravity was established by studies on labelled human erythrocytes. Since similar labelling experiments with human erythrocytes were considered impractical in these studies, an independent parameter known to vary with erythrocyte age, was measured to test whether the fractionation on the basis of density was indeed yielding cells of different age. Table 3 shows the relative activity of glucose-6-phosphate dehydrogenase for different density fractions.

Table 3 Activity of Glucose-6-Phosphate Dehydrogenase of Fractionated Erythrocytes

Fraction Number	Distribution % of total cells	Glucose-6-phosphate dehydrogenase relative activity (%)
1	19.5	100
2	26.9	37.1
3	21.9	37.1
4	10.6	38.6
5	10.3	14.7
6	10.8	7.6

Piomelli *et al.* (1968) have shown that the activity of this enzyme decreases by about 75% during the human erythrocyte life-span, the mean value being 4.95 units per g haemoglobin. In the present study a mean of 4.19 units per g haemoglobin was found and the enzyme activity was found to decrease progressively, especially among the densest cells. These findings support the

suggestion that fractionation of human erythrocytes by ultracentrifugation on discontinuous density gradients, yields populations of cells of different in vivo age.

3.1.5 General Observations on Fractionated Erythrocytes

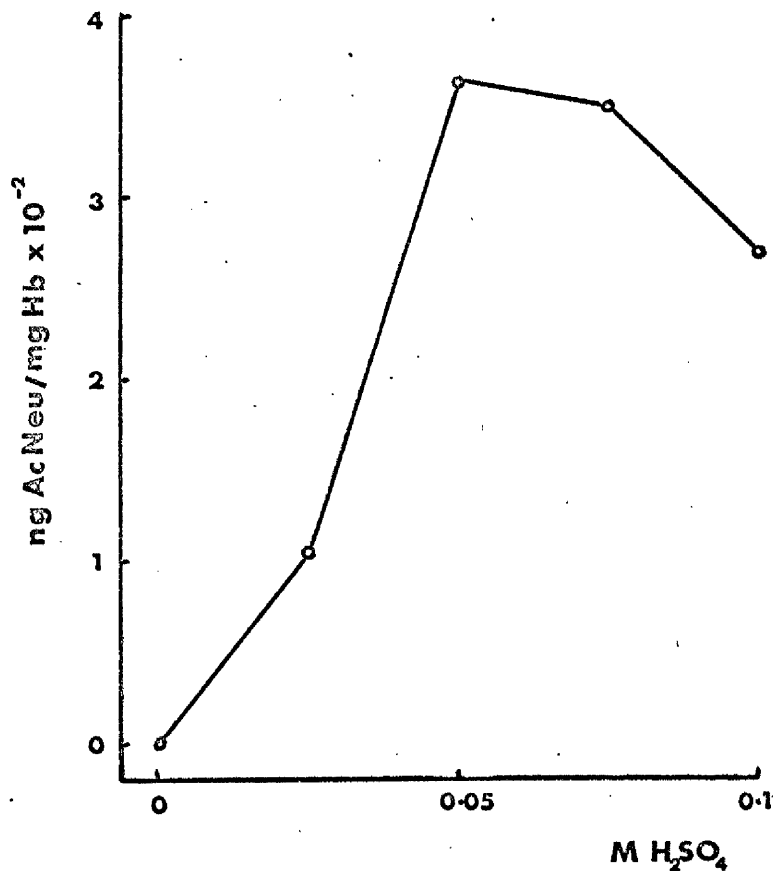
The densest fraction of cells obtained was markedly more susceptible to lysis on in vitro storage in isotonic buffer at 4°C. Moreover, the two densest cell fractions yielded pellets on centrifugation at 800g for 10min which were more difficult to resuspend than those of less dense fractions. Neuraminidase and trypsin treated cells were also more difficult to resuspend than control cells. Thirdly, it was observed that, in the densest fraction there were more cells per unit packed cell volume than in the lighter fractions.

3.2.1 The Estimation of N-Acetylneuraminic Acid

The most sensitive method available for the determination of N-acetylneuraminic acid is that of Aminoff (1961) which requires the presence of the free acid. This is oxidised by acidic sodium metaperiodate to β -formylpyruvic acid which reacts with thiobarbituric acid reagent to yield a pink chromogen of λ_{\max} 549nm (Warren, 1959).

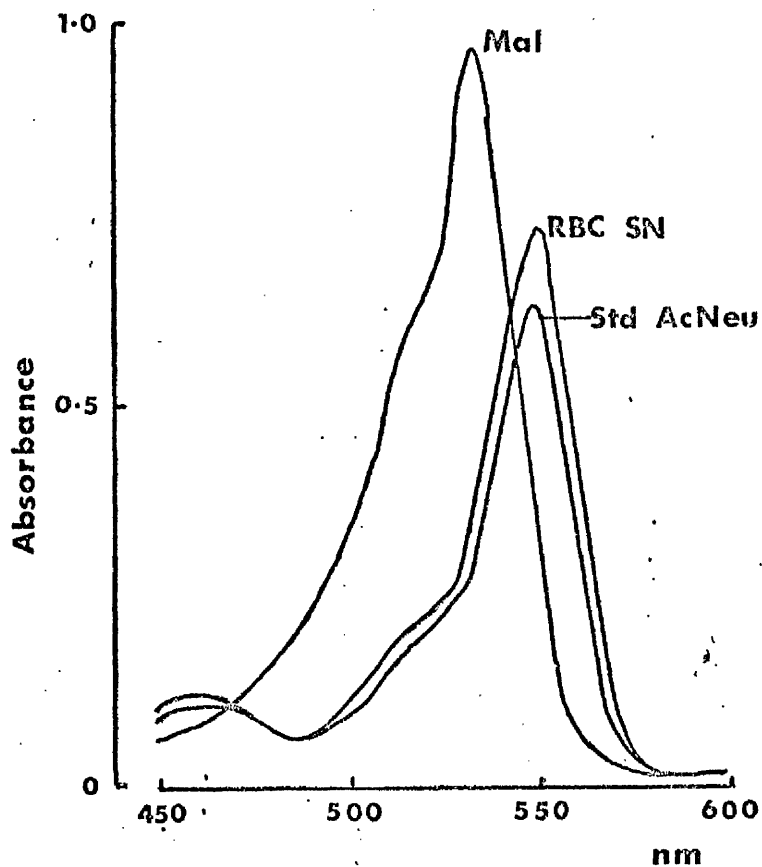
The release of N-acetylneuraminic acid by acid hydrolysis of cells was carried out under standardised conditions because of the buffering effect of large amounts of haemoglobin on the low concentration of acid required to liberate N-acetylneuraminic acid without its destruction. Fig. 4 shows the release of N-acetylneuraminic acid at different concentrations of sulphuric acid, from samples in which the concentration of haemoglobin was kept at 46.9mg/ml. Hydrolyses were performed at 80°C for 1h. Under such conditions, the optimum concentration of sulphuric

Figure 4 Effect of Sulphuric Acid Concentration on the Release of N-Acetylneuraminic Acid from Intact Erythrocytes



Erythrocytes, equivalent to 46.9mg haemoglobin, were hydrolysed at 80°C for 1h in a total volume of 1ml of varying concentrations of sulphuric acid. AcNeu was assayed by the method of Aminoff (1961). The optimal acid concentration was determined to be 0.05M.

Figure 5 Spectra of Chromogens Obtained in the Thiobarbituric Acid Assay



The thiobarbituric acid assay of N-acetylneuraminic acid was carried out according to the method of Aminoff (1961). Under the assay conditions, a hydrolysed red blood cell supernatant (RBC SN) and a standard solution of N-acetylneuraminic acid (Std AcNeu) gave identical pink chromogens of λ_{\max} 549nm. Malonaldehyde (Mal) gave rise to a pink chromogen of λ_{\max} 532nm.

acid was 0.05M and all subsequent hydrolyses were carried out under these standard conditions.

The colorimetric determination of N-acetylneuraminic acid is subject to interference by substances such as 2-deoxyribose, which on treatment with periodic acid gives rise to malonaldehyde (Waravdekar & Saslaw, 1959). The latter substance will react with thiobarbituric acid to yield a pink chromogen of λ_{\max} 532nm (Fig. 5). However, the supernatant from a standardised hydrolysis of red blood cells can be seen in fig. 5 to be identical to the N-acetylneuraminic acid standard under the assay conditions, and so cell hydrolysates were assayed, after precipitation of the protein, without additional treatment.

3.2.2 Lipid Peroxidation

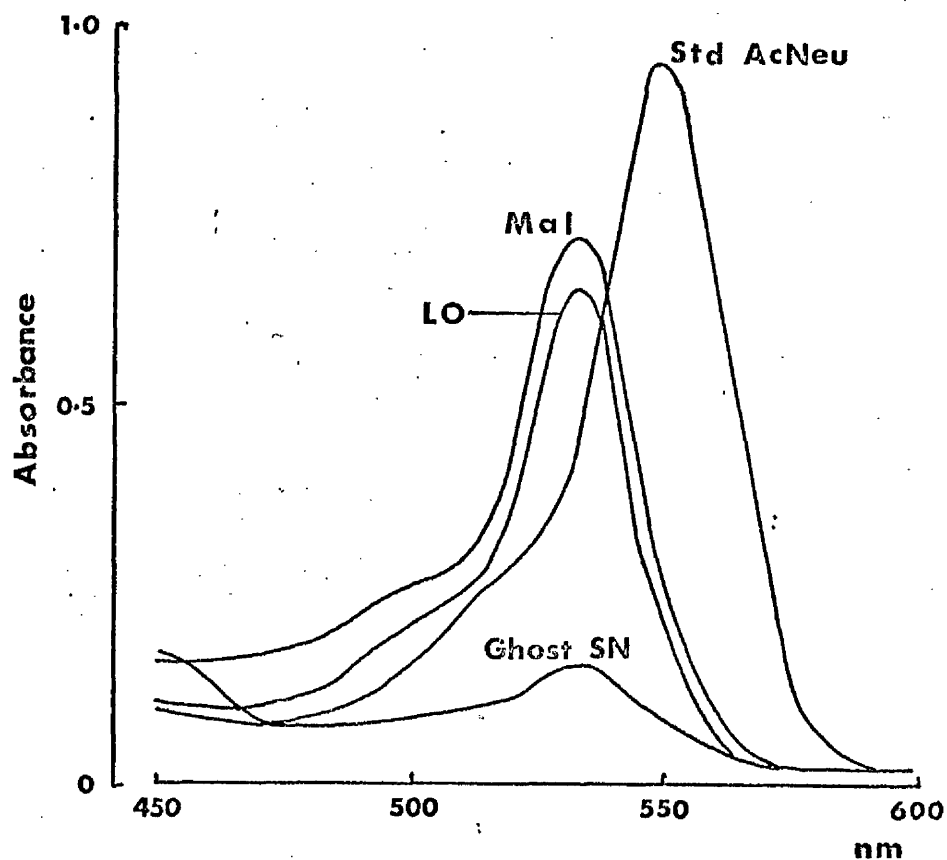
In the course of these studies it was necessary to carry out two experiments.

(i) to measure the N-acetylneuraminic acid content of erythrocyte membranes.

(ii) to treat erythrocytes with tritiated sodium borohydride in order to reduce aldehyde functional groups, generated by treatment of erythrocyte membrane carbohydrate with sodium metaperiodate or galactose oxidase.

It was observed with regard to (i) that a control sample of ghosts, which had not been hydrolysed to release N-acetylneuraminic acid, nor treated with periodate prior to the addition of thiobarbituric acid, gave rise to a pink chromogen of λ_{\max} 532nm (Fig. 6). Its spectrum was identical to that of malonaldehyde and to that of a substance present in oxidised linseed oil (Fig. 6), which has a high content of unsaturated fatty acids.

Figure 6 Spectra of Chromogens Produced by Substances which Interfere in the Thiobarbituric Acid Assay



The supernatant of a ghost suspension (Ghost SN), the supernatant of a linseed oil (LO) emulsion and malonaldehyde (Mal) give rise to pink chromogens of λ_{\max} 532nm under the conditions of the thiobarbituric acid assay of N-acetylneuraminic acid and so could interfere in the determination of AcNeu, λ_{\max} 549nm.

It was observed with regard to (ii) that control preparations of erythrocytes, which had not been oxidised by sodium metaperiodate or galactose oxidase, incorporated tritium label into the membrane. Gel electrophoresis showed that the non-specific label was predominantly in lipids. This non-specific labelling was greatly increased if ghosts were first isolated and then labelled (Table 4).

Table 4 Non-Specific Tritium Labelling of Erythrocyte Membranes

preparation labelled	d.p.m. per mg membrane protein
intact erythrocytes	8,200
erythrocyte ghosts	64,900

Since a thiobarbituric acid assay has been used to measure the products of the oxidation of lipids (Wilbur *et al.*, 1949), the above observations suggest that oxidation of the lipids of red cell membranes does take place especially in haemoglobin-free membranes. Since products of lipid oxidation interfere with the assay of N-acetylneuraminic acid, a column purification step was incorporated into the assay procedure, when N-acetylneuraminic acid of isolated membranes was being determined (2.8).

In labelling experiments, specific labelling of membrane components was identified by a separation of proteins and lipid on polyacrylamide gels.

3.2.3 N-Acetylneuraminic Acid of Fractionated Cells

Table 5 shows the N-acetylneuraminic acid content of blood group 0 erythrocytes for the density distribution shown. The data shows the average values of analyses of two separate experiments. In both cases assays were carried out after release of

N-acetylneuraminic acid by Clostridium perfringens neuraminidase in an acetate buffer at pH5, and by 0.05M sulphuric acid hydrolysis under standard conditions.

Table 5 N-Acetylneuraminic Acid Content of Fractionated Group O Erythrocytes

Fraction Number	Distribution % total cells	ng <u>N</u> -Acetylneuraminic Acid/ mg Haemoglobin	
		C.P. neuraminidase	0.05M H ₂ SO ₄
1	23.1	407	412
2	28.9	387	372
3	21.4	370	349
4	9.8	375	341
5	8.1	340	320
6	8.7	335	320

The accuracy of these values was estimated by analysis of the N-acetylneuraminic acid released by acid hydrolysis from replicate samples of erythrocytes. The value obtained was 354 ±5 (S.D., for eight determinations) ng of N-acetylneuraminic acid per mg of haemoglobin. These figures demonstrate that a decrease in N-acetylneuraminic acid of the order of 18-22% accompanies aging.

In order to establish that these results are not limited to one individual or blood group, the erythrocytes from a blood group B male donor were similarly hydrolysed under standard conditions. The results are shown in table 6. Since the trend of values in fractions 2-6 parallels that of the group O erythrocytes, it may be reasoned that the exceptionally high value in fraction 1 is due to contamination by cells other than mature erythrocytes, and is exaggerated by the small size of fraction 1. Thus if the

Table 6 N-Acetylneuraminic Acid Content of Fractionated GroupB Erythrocytes

Fraction Number	Distribution % total cells	ng N-Acetylneuraminic Acid/ mg Haemoglobin
1	5.4	539.4
2	23.9	375.3
3	36.2	365.4
4	18.6	359.3
5	8.8	337.7
6	7.1	324.3

values of fractions 1 and 2 are averaged, in which case the contamination becomes less important, the value of 405.5ng N-acetylneuraminic acid per mg haemoglobin is obtained. Comparing this value with 324.3 for fraction 6, it can be seen that there is again a decrease of the order of 20% in the cell content of N-acetylneuraminic acid accompanying aging.

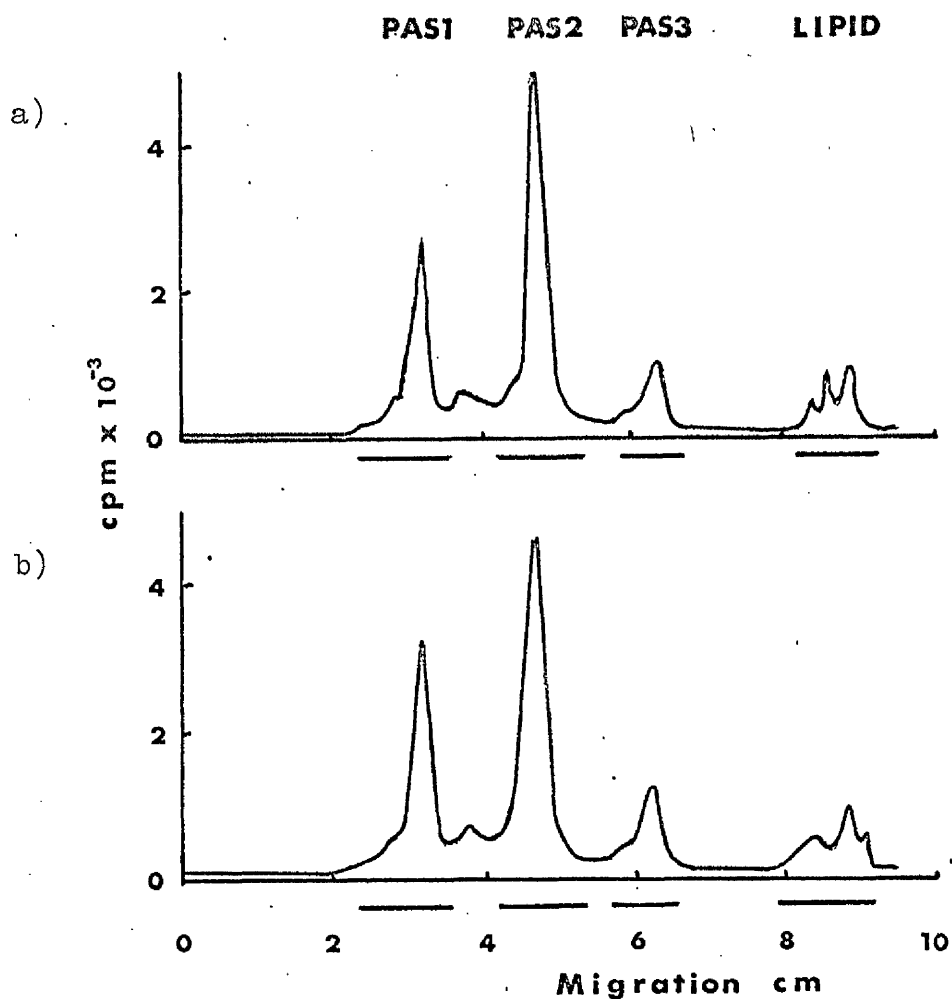
3.2.4 Characterisation of N-Acetylneuraminic Acid Losses

The N-acetylneuraminic acid of red cells can be labelled specifically using a mild sodium metaperiodate oxidation followed by tritiated sodium borohydride reduction (Blumenfeld *et al.*, 1972). Table 7 shows the composite results of two such experiments in which fractionated cells were labelled. These results

Table 7 Labelling of Red Cell Membrane N-Acetylneuraminic Acid

Fraction Number	c.p.m./mg haemoglobin		
	sodium metaperiodate sodium [³ H]-borohydride	sodium [³ H]-borohydride	AcNeu labelling
1	20715	6575	14140
2	19559	6770	12789
3	17387	5702	11685
4	17059	5717	11542
5	17941	5637	12304
6	15896	7011	8835

Figure 7 N-Acetylneuraminic Acid Specific Labelling of the Sialoglycoproteins of the Erythrocyte Membrane



A tritium label was incorporated into membrane sialoglycoproteins and lipids of young (a) and old (b) erythrocytes by sequential periodate oxidation/^[3H]-borohydride reduction (Blumenfeld *et al.*, 1972). Membranes were isolated, subjected to SDS/polyacrylamide gel electrophoresis and the gels were sliced and counted. The labelling patterns were indistinguishable.

show a 37% decrease in specific labelling, supporting the analytical data which showed a decrease in N-acetylneuraminic acid.

There are three major N-acetylneuraminic acid containing glycoprotein components of the red cell membrane, and these can be demonstrated on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (Fig. 1c). These are designated PAS 1, 2 & 3 by Fairbanks et al. (1971) and their labelling profile after sequential treatment with periodate and tritiated borohydride is shown in figure 7, for an old and young fraction of group O erythrocytes. It can be seen that there is no change in the general labelling profile of the glycoprotein components accompanying aging. Moreover, if the counts in each peak, indicated by the bars in figure 7, are summed up and expressed as a percentage of the total counts incorporated, it can be seen from table 8 that there is no significant change in the relative counts in each peak. For the glycoproteins, this indicates that there is no specific loss of

Table 8 Labelling of Individual Red Cell Membrane Components

Cell Fraction	PAS 1	PAS 2	PAS 3	LIPID
YOUNG	25.6%	47.4%	12.5%	14.5%
OLD	25.0%	45.6%	13.6%	15.9%

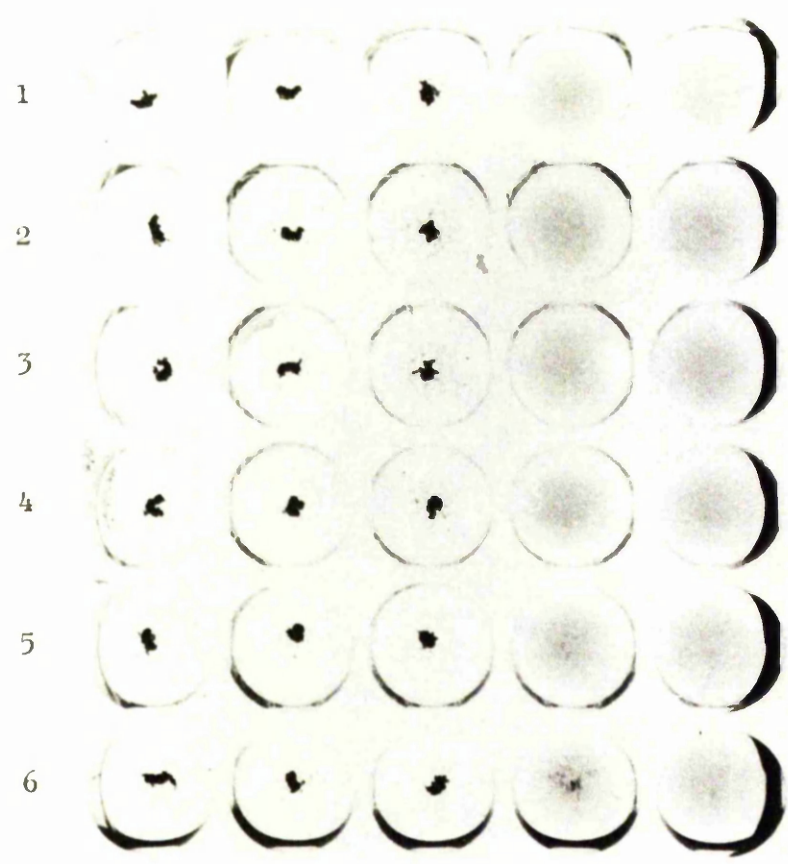
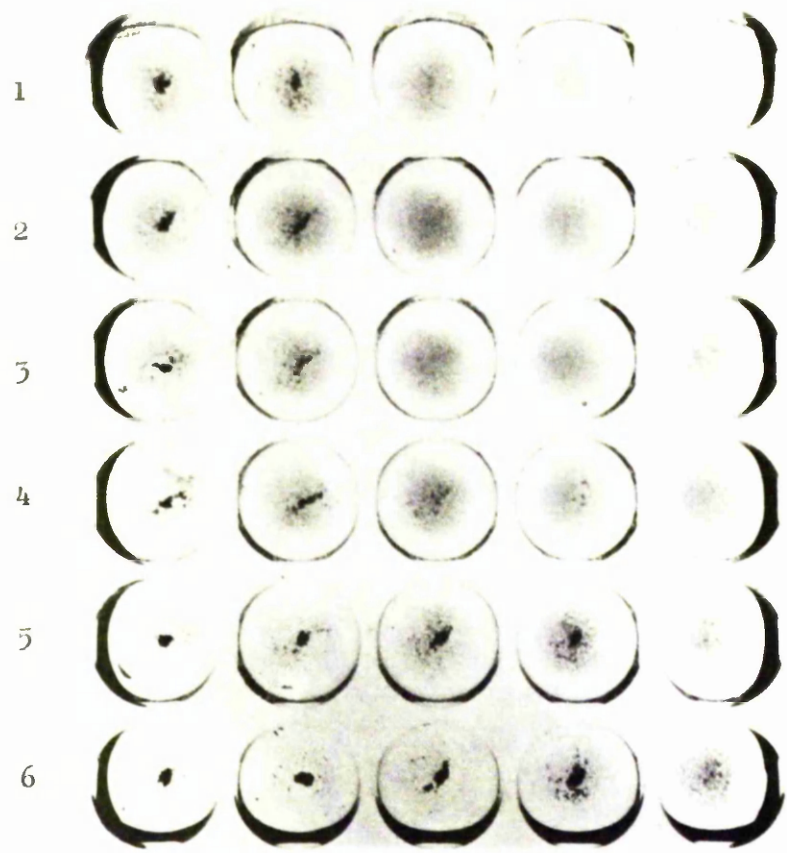
N-acetylneuraminic acid from any one particular component, but a generalised loss from all three. With regard to the lipid, these figures indicate that there is no marked increase in lipid oxidation within old cell membranes, despite their having been exposed to oxygen for a much greater period of time in the cells' lifespan.

Figure 8a Agglutination of Fractionated Blood Group B
Erythrocytes by Human Anti-B Antiserum

Equal numbers of blood group B erythrocytes of different age (1-6) were added to serial dilutions of human anti-B antiserum in PBS 7.4. Old cells (fractions 5 & 6) were more agglutinable than younger cells for any given concentration of agglutinin.

Figure 8b Agglutination of Fractionated Erythrocytes by
Lectins

This figure is representative of the agglutination of blood group B or O human erythrocytes by serial dilutions of the lectins wheat-germ agglutinin or Carcinus agglutinin. Identical results were obtained in all cases and showed that old cells (fraction 6) were more readily agglutinable at a 16-fold dilution of the lectins than younger cells.



3.3.1 The Agglutinability of Fractionated Cells

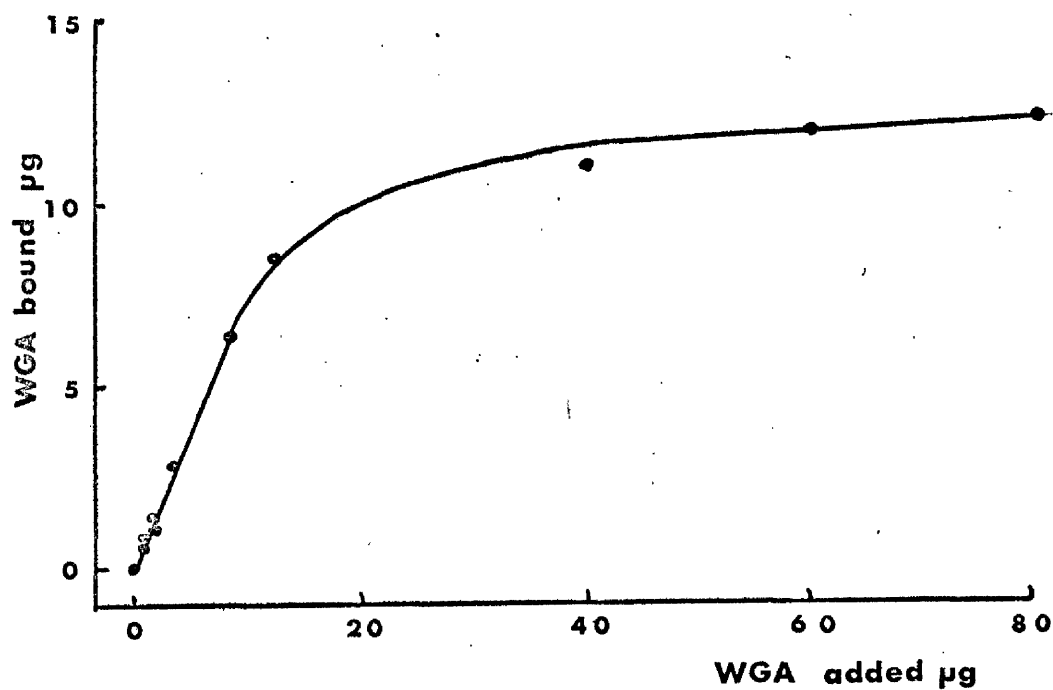
Since changes in cell surface carbohydrate take place as erythrocytes age in vivo, it is possible that these changes are recognised in the mechanism of removal of old cells from the circulation. As the nature of this removal mechanism has not been established, the interaction of fractionated erythrocytes with various agglutinins was studied as a model of the recognition process.

The agglutination of fractionated group B erythrocytes with human anti-B antiserum is shown in figure 8a. Using the agglutination index, defined in 2.18, it can be seen that young cells have a value of 6 and old cells have a value of 32, indicating a marked increase in the agglutinability of old red cells. Figure 8b shows a similar though less dramatic effect for Carcinus agglutinin, whose specificity is for N-acetyl sugars. Identical results were obtained with wheat-germ agglutinin (WGA), whose specificity is for N-acetylglucosamine. In both these cases, for fractionated cells of blood groups B and O, agglutination indices of 12 and 16 were obtained for young and old cells respectively. This again demonstrated the increased agglutinability of old cells. Concanavalin A and soya-bean agglutinin did not agglutinate untrypsinised erythrocytes. It appears, however, that some cell surface change, peculiar to old cells, can be "recognised" by certain agglutinins.

3.3.2 The binding of [¹²⁵I]-Wheat-Germ Agglutinin to Erythrocytes

In order to discover if the increased agglutinability of old cells was due to an increase in the number of exposed agglutinin binding sites, binding studies were carried out using wheat-germ

Figure 9 Binding of [¹²⁵I]-Wheat-Germ Agglutinin (WGA) to Human Erythrocytes



Erythrocytes were incubated with different amounts of [¹²⁵I]-WGA for 30min at 20°C in PBS 7.4. Cells were centrifuged and washed and the bound lectin determined by gamma-counting. The binding curve plateaus at about 13µg WGA bound per 9×10^6 erythrocytes.

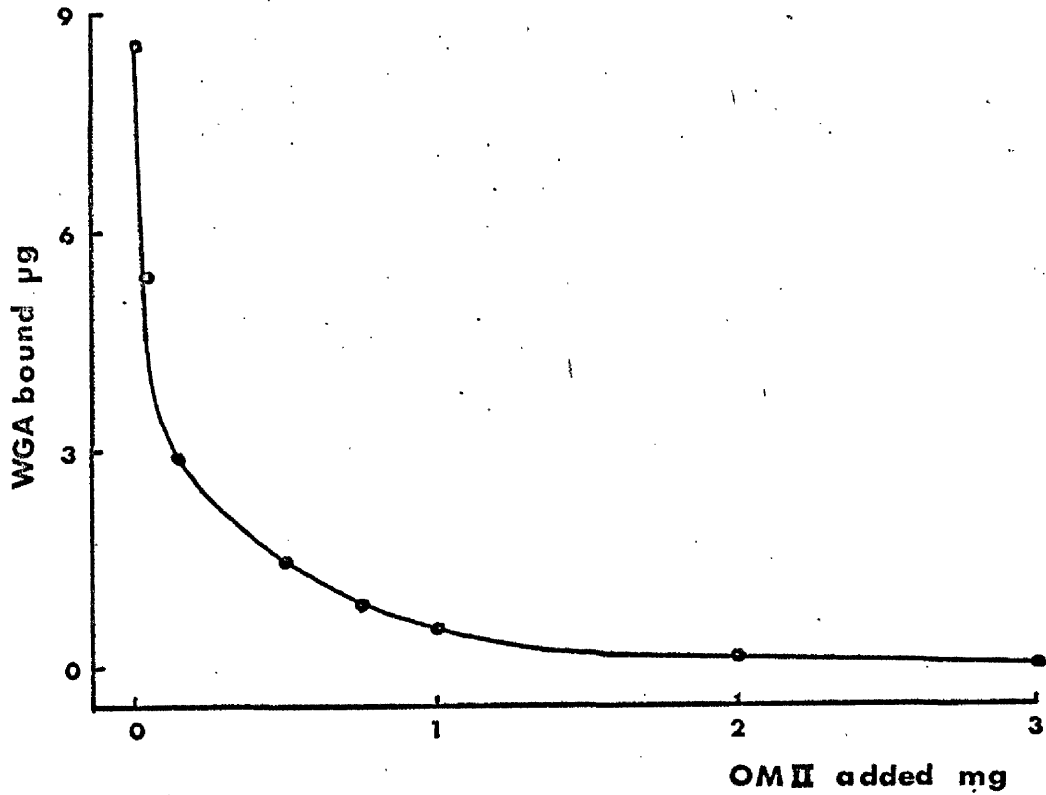
agglutinin which had been labelled with radioactive iodine. The binding was initially characterised on unfractionated group O erythrocytes. Figure 9 shows the binding curve which is initially fairly linear and tends toward a plateau at about 13 μ g wheat-germ agglutinin bound per 9×10^6 erythrocytes. This represents 1.7×10^7 binding sites per cell. From analyses of N-acetylglucosamine (3.4.3), it can be estimated that there are about 2.5×10^7 molecules of N-acetylglucosamine per cell. Moreover, Greenaway and Le Vine (1973) suggest that wheat-germ agglutinin also binds N-acetylneuraminic acid. It can be estimated that there are about 2.9×10^7 molecules of N-acetylneuraminic acid per cell (3.4.3). Some evidence that wheat-germ agglutinin does bind N-acetylneuraminic acid is shown in table 9.

Table 9 Binding of [¹²⁵I]-Wheat-Germ Agglutinin to Neuraminidase Treated Erythrocytes

Treatment	% AcNeu removed	molecules WGA bound/cell x 10 ⁻⁷
-	0	1.83
Neuraminidase 15min	67	1.30
Neuraminidase 60min	78	0.89

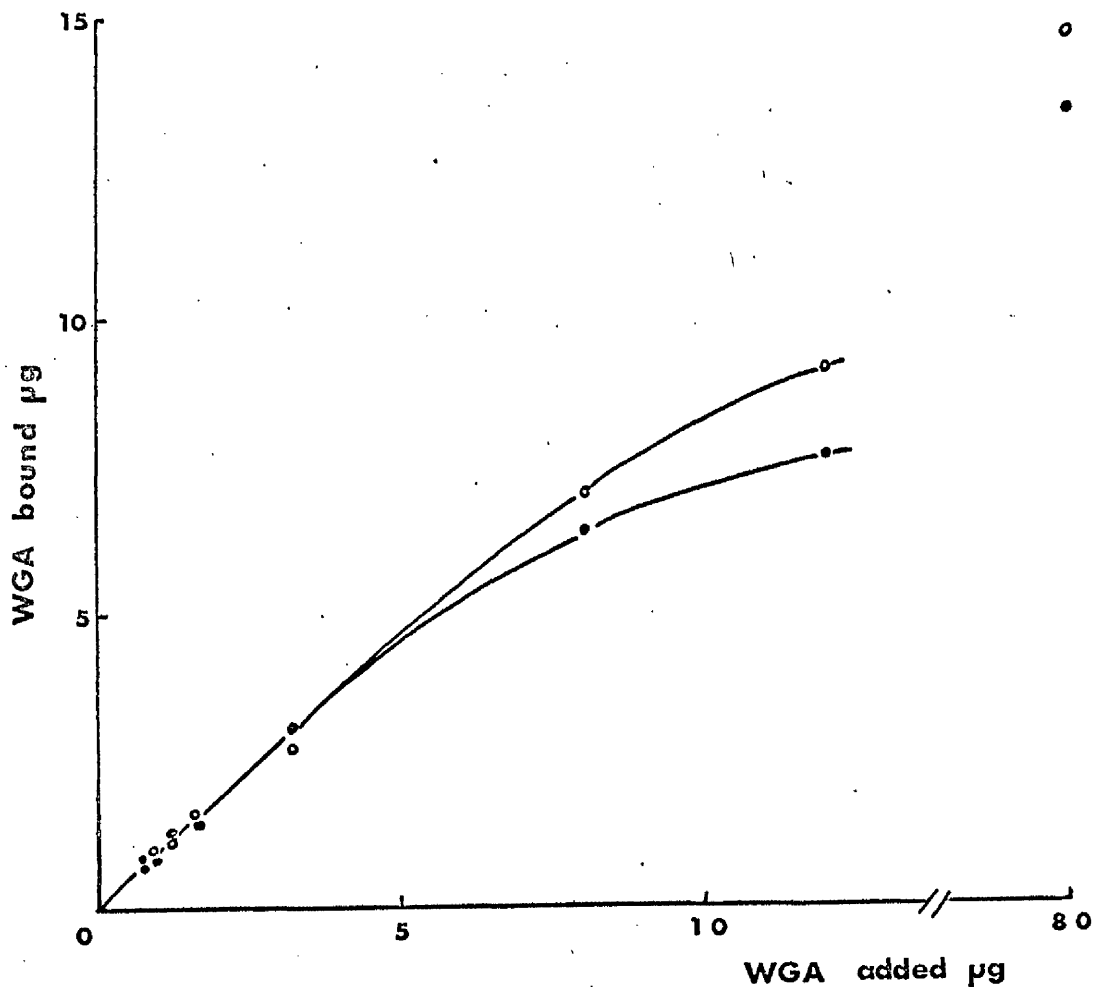
Erythrocytes were treated with neuraminidase for different periods of time and table 9 shows the decrease in the number of molecules of wheat-germ agglutinin bound, which accompanies the removal of N-acetylneuraminic acid. Furthermore, the data shown in figure 9 cannot be fitted to a double reciprocal plot by the method of Steck & Wallach (1965) to yield a straight line. This indicates that the binding is not simple and that there is more than one type of binding site. The complex binding is not due to non-specific binding since, in the presence of the glycoprotein

Figure 10 Inhibition of WGA Binding to Human Erythrocytes



[¹²⁵I]-WGA (12µg) and erythrocytes (10⁷) were incubated with various amounts of OMII at 20°C for 30min in PBS 7.4. After the cells were washed, the bound WGA was determined by gamma-counting. Binding could be completely inhibited by OMII.

Figure 11 Binding of [125 I]-WGA to Fractionated Erythrocytes



Young (o—o) and old (●—●) erythrocytes were incubated with [125 I]-WGA and the binding was determined by gamma-counting. The binding curves were divergent and values at saturation binding (extreme right) indicate that old cells have 10% fewer binding sites than young cells.

ovomucoid fraction II, all the binding is inhibited (Fig. 10).

3.3.3 The Binding of [¹²⁵I]-Wheat-Germ Agglutinin to

3.3.3.3 The Binding of [¹²⁵I]-Wheat-Germ Agglutinin to

Binding assays were carried out on the six density fractions of group 0 erythrocytes using [¹²⁵I]-wheat-germ agglutinin. The binding curves for fractions 2 and 6 are shown in figure 11. The values at saturation binding are also shown and it can be seen that there is no increase in binding sites for wheat-germ agglutinin on old cells. There is in fact a decrease of the order of 10%. However, the important conclusion from this experiment is that the increased agglutinability of old cells with wheat-germ agglutinin cannot be accounted for by an increase in the number of lectin binding sites.

3.3.4 The Intramembranous Particles of Fractionated Erythrocytes

Since intramembranous particles and lectin binding sites both involve glycoproteins (Pinto da Silva & Nicolson, 1974), the distribution of intramembranous particles of old and young erythrocytes was compared to determine if the distribution in old cells had changed in such a way as to predispose the old cells to agglutinate more readily in the presence of lectin. The freeze-fracture electron micrographs of young and old cells are shown in figures 12a and 12b respectively. It can be seen that there is no marked change in the distribution of intramembranous particles in the old cell membranes which might account for their increased agglutinability.

3.3.5 Interaction of Wheat-Germ Agglutinin with Enzyme Treated Cells

Table 10 shows one further observation concerning the

Figure 12a Intramembranous Particle Distribution of Young
Erythrocytes

Young glutaraldehyde fixed, glycerinated intact erythrocytes were subjected to freeze-fracture electron microscopy to expose the intramembranous particles.

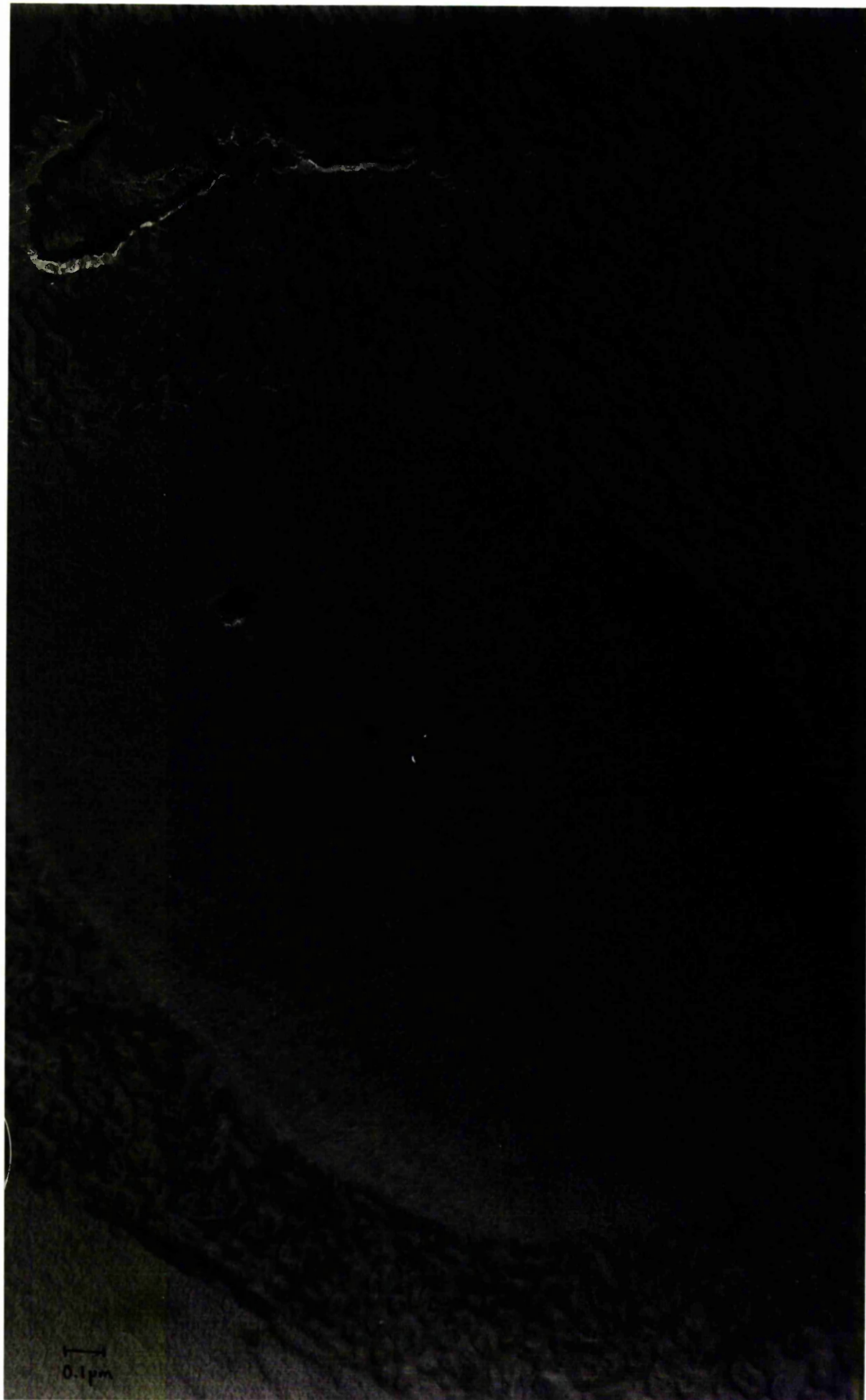
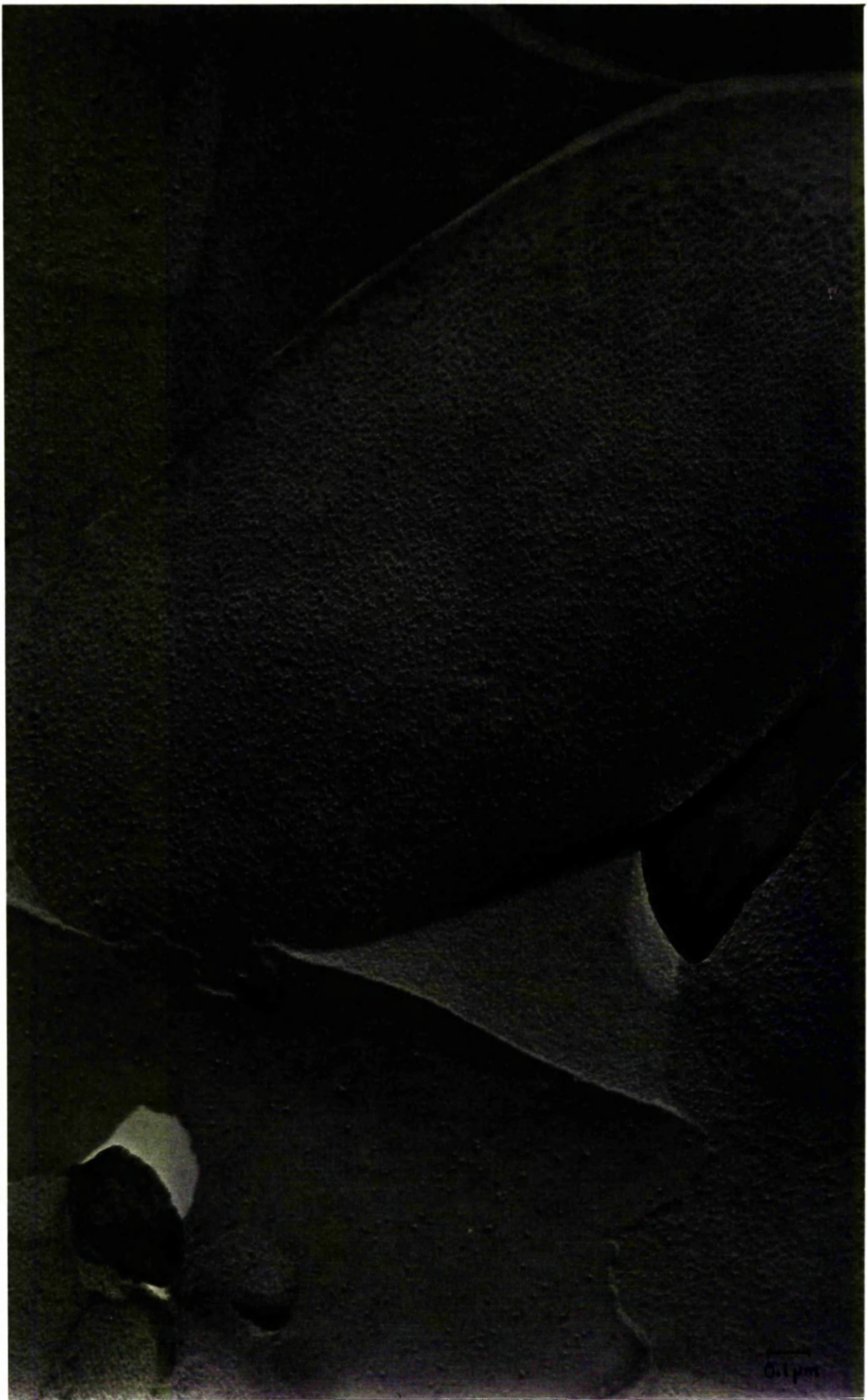


Figure 12b Intramembranous Particle Distribution of Old

Erythrocytes

Old glutaraldehyde fixed, glycerinated intact erythrocytes were subjected to freeze-fracture electron microscopy to expose the intramembranous particles.



interaction of erythrocytes with wheat-germ agglutinin. The

Table 10 Effects of Enzyme Treatment of Red Cells on Agglutination and Lectin Binding

Enzyme Treatment	% AcNeu released	Agglutination Index	Molecules WGA bound/cell x 10 ⁻⁷
--	0	32	1.87
trypsin	58	512	1.54
neuraminidase	67	128	1.30

characteristics of in vivo aged cells, namely, increased agglutinability, decreased binding sites and loss of N-acetylneuraminic acid can be mimicked not only by neuraminidase treated cells but also by trypsin treated cells.

3.4.1 Hydrolysis of Membrane Bound Amino Sugars

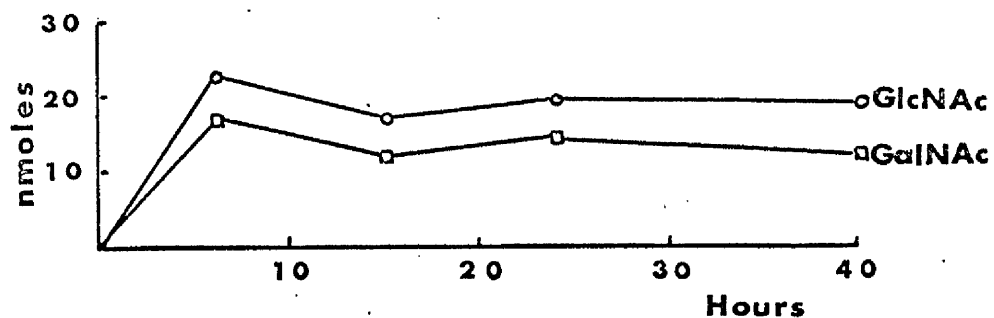
Figure 13a shows the time course of release of membrane bound amino sugars by 4M methanesulphonic acid hydrolysis at 100°C. High values at 6h are possibly due to interference by products of incomplete protein hydrolysis. Since the release of amino sugars appears to be maximal around 24h, and this results in fairly complete release of amino acids (Fig. 13b), a 24h hydrolysis was routinely employed.

3.4.2 Amino Acid Composition of Erythrocyte Membranes

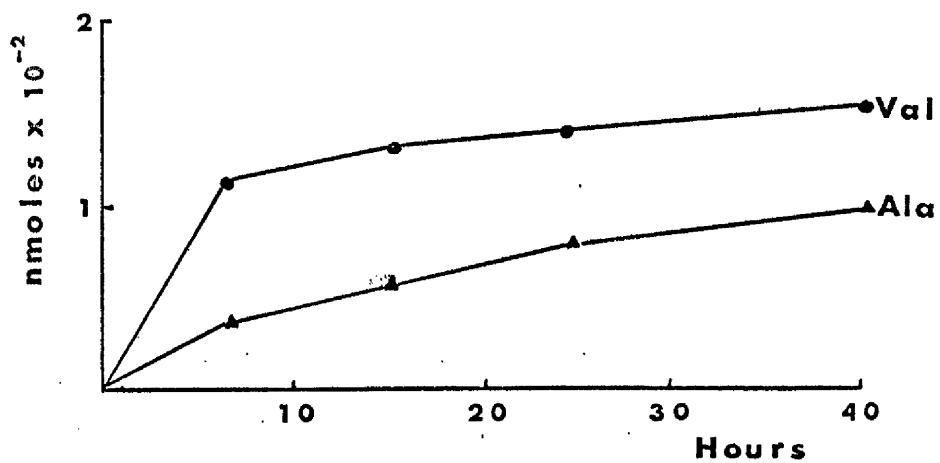
Table 11 shows the amino acid composition of the membranes isolated from three cell fractions of different age and, for comparison, the mean of some literature values (Rosenberg & Guidotti, 1969). There are no significant variations in amino acid composition among the different aged fractions, and all correspond

Figure 13 Time Course of Hydrolysis of Membrane Amino Acids
and Amino Sugars

(a)



(b)



Erythrocyte membranes were hydrolysed at 100⁰C in 4M methanesulphonic acid for different time intervals. Amino sugars (a) and amino acids (b) were determined on an amino acid analyser. Optimal time of hydrolysis for both, was 24h.

Table 11 Amino Acid Composition of Fractionated Erythrocyte Membranes

Amino Acid	Residues per 100 Residues			
	Fraction 1	Fraction 4	Fraction 6	Literature Value
Lys	4.90	5.15	5.14	4.96
His	3.24	2.79	2.92	2.38
Arg	5.36	5.72	5.22	4.62
Asp	9.37	8.86	9.05	8.69
Thr	5.41	5.58	5.52	5.76
Ser	9.37	8.72	8.21	7.20
Glu	13.12	13.08	12.27	12.39
Pro	6.19	7.01	6.60	4.28
Gly	7.21	7.01	6.82	6.80
Ala	8.65	8.36	8.51	7.84
Cys	0.22	0.29	0.30	1.14
Val	4.04	4.15	4.14	6.59
Met	2.53	2.50	2.38	1.85
Ile	2.95	3.07	2.76	4.92
Leu	11.03	11.29	11.04	12.06
Tyr	2.59	2.57	2.53	2.25
Phe	3.82	3.86	3.83	4.09

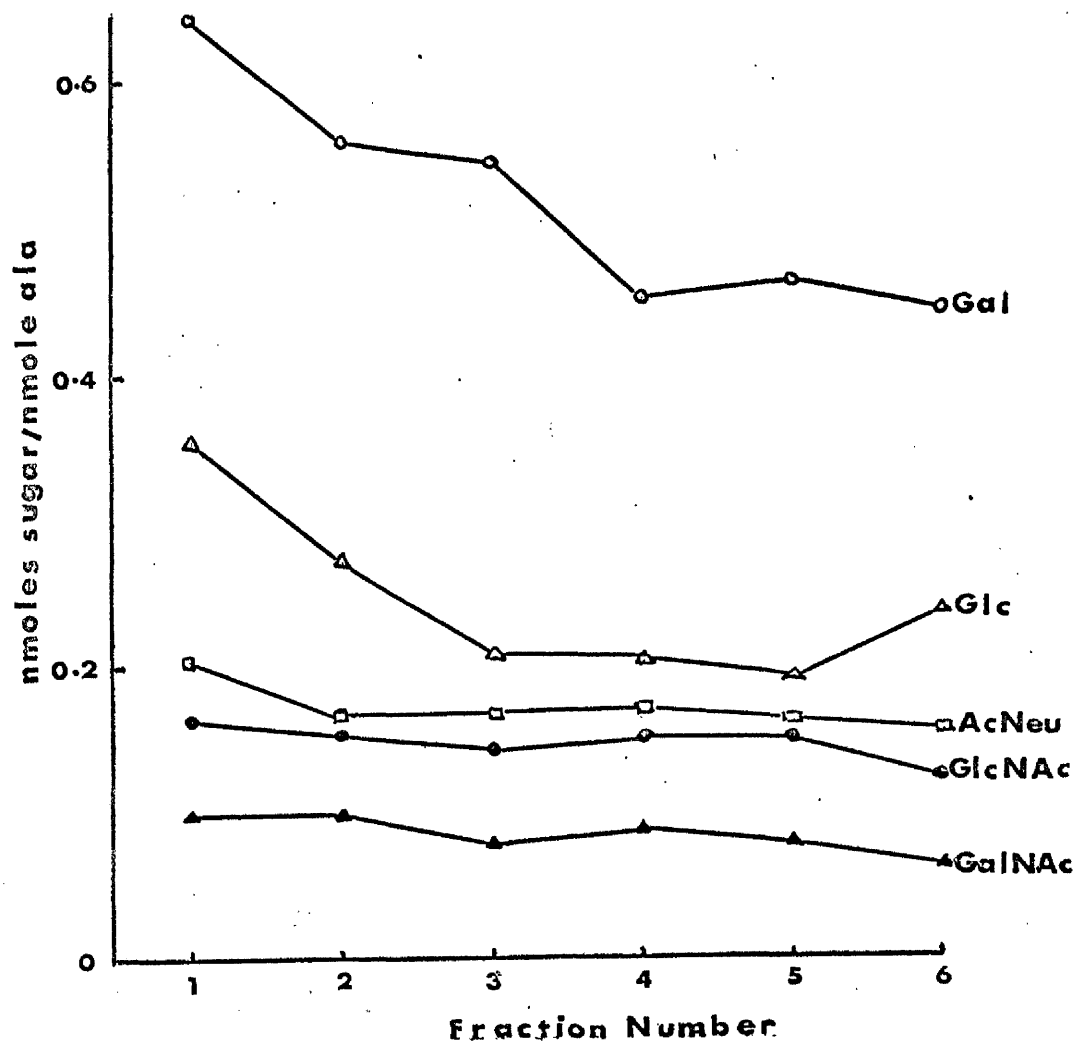
well with literature values. Moreover, table 12 shows the alanine

Table 12 Membrane Alanine Content of Fractionated Cells

Fraction Number	Nmoles alanine per mg haemoglobin
1	5.96
2	6.64
3	6.54
4	6.56
5	6.31
6	6.59

content of membranes isolated from equal numbers of fractionated cells. These values show that the protein content of red cells remains relatively constant during the erythrocyte lifespan - an

Figure 14 Hexose, Hexosamine and AcNeu Content of Red Cell Membranes



Membranes from fractionated erythrocytes were analysed for carbohydrate. Gal and Glc were determined by gas liquid chromatography by the method of Clamp et al. (1971). Amino sugars and alanine were determined on an amino acid analyser and AcNeu was assayed by the method of Aminoff (1961). All carbohydrates were found to decrease as the cell age increased.

observation supported by the protein profiles obtained by polyacrylamide gel electrophoresis of membranes (Figs. 16a & 16b). Consequently carbohydrate analyses of erythrocyte membranes are all related to alanine content.

3.4.3 Carbohydrate Content of Red Cell membranes

Figure 14 shows the carbohydrate composition of ghosts of fractionated group O erythrocytes. It can be seen that there are fairly complex changes occurring in the membrane carbohydrate as the cell age increases. The magnitude of the overall decreases in individual sugars is shown in table 13. The

Table 13 Changes in Membrane Carbohydrate of Fractionated Erythrocytes

Sugar	% decrease
<u>N</u> -acetylneuraminic acid	24
glucose	33
galactose	31
<u>N</u> -acetylglucosamine	24
<u>N</u> -acetylgalactosamine	37

decrease in N-acetylneuraminic acid of 24% confirms the findings for intact cells (3.2.3), where the decrease has been related to changes in glycoproteins.

3.4.4 Labelling of Galactose/Galactosamine Residues of Erythrocyte Membranes

The structures of glycopeptides, isolated from human erythrocytes, show that the sugars internal to N-acetylneuraminic acid in oligosaccharide sequences are galactose and N-acetylgalactosamine (Thomas & Winzler, 1969; Kornfeld & Kornfeld, 1970). Gahmberg & Hakomori (1973) have shown that a number of proteins and lipids of the erythrocyte membrane can be labelled using

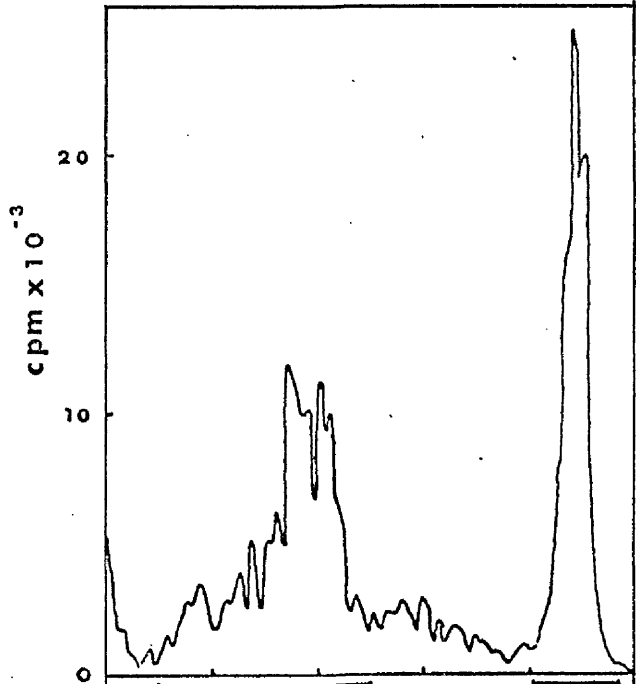
galactose oxidase and tritiated sodium borohydride. Moreover, pretreatment of the cells with neuraminidase causes one particular protein component to become highly labelled. It might be expected, therefore, that the removal of N-acetylneuraminic acid which accompanies aging, might lead to the exposure of galactose and galactosamine residues in old cells, which could be preferentially labelled by this technique. Figures 15a and 15b show the labelling profiles of young and old labelled fractions. Figure 15c shows the sialoglycoprotein profile of control membranes for comparison. The profiles in figures 15a and 15b are very similar, with possibly the suggestion of an increased band of labelling in old cells around 4cm. However, comparison with figure 15c shows that this does not correspond to a sialoglycoprotein component. Moreover, table 14 shows the c.p.m. per μ g protein applied to the gels, for the peaks indicated by the bars in figures 15a and 15b. The protein specific activity shows that there is no increased incorporation of label on aging, suggesting that galactose/N-acetylgalactosamine residues are also being lost from membrane glycoprotein as the cells age. The

Table 14 Distribution of Galactose/Galactosamine Label Between Protein and Lipid in Young and Old Erythrocyte Membranes

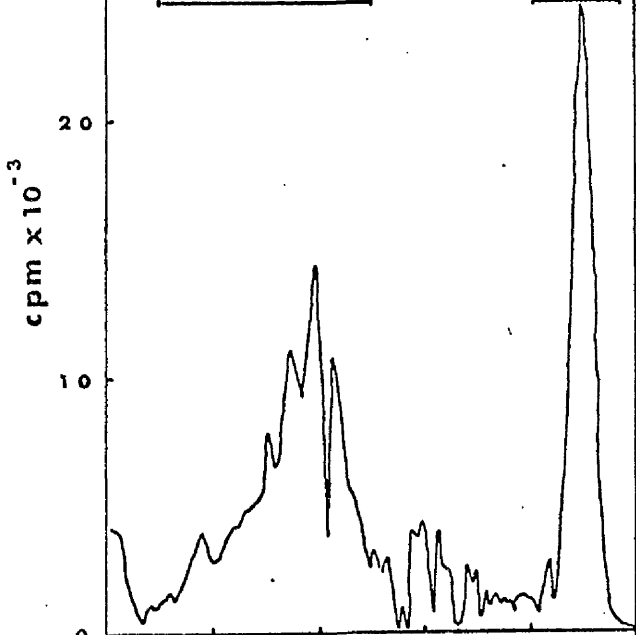
Cell Type	c.p.m./ μ g protein applied to gels	
	protein	lipid
Young	2085	1507
Old	2067	1187

lipid specific activity is seen to decrease as the red cell ages.

1a



1b



1c

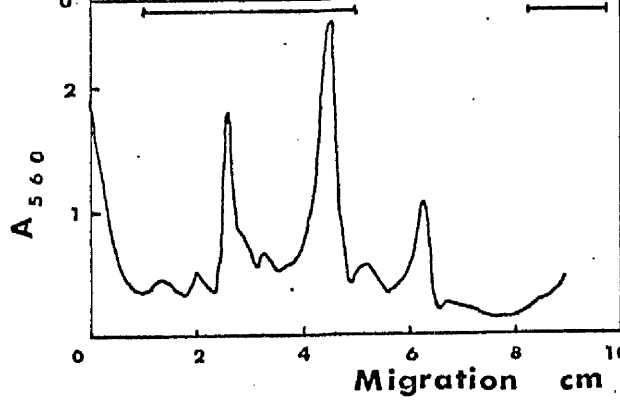


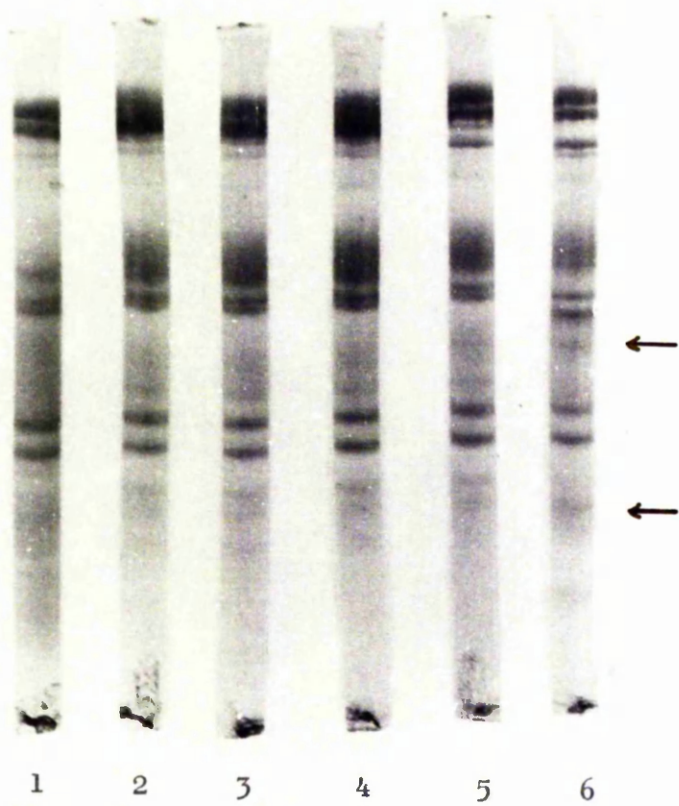
Figure 15 Specific Labelling of Galactose/Galactosamine Residues of Erythrocyte Membrane Glycoproteins & Glycolipids

A tritium label was incorporated into galactose/galactosamine residues of membrane glycoproteins and glycolipids of young (a) and old (b) erythrocytes by sequential galactose oxidase oxidation/ $[^3\text{H}]$ -borohydride reduction (Gahmberg & Hakomori, 1973). Membranes were isolated and subjected to electrophoresis on 7.6% polyacrylamide gels in the presence of SDS. Gels were sliced and counted. A control gel (c) was stained with periodic acid/Schiff reagent and scanned at 560nm to reveal the sialoglycoproteins.

Figure 16 Membrane Proteins of Fractionated Erythrocytes

Erythrocytes obtained from A.B. (a) and J.G.B. (b) were fractionated into populations of increasing age (1-6) and (3-6) respectively. Membranes were isolated and subjected to SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie blue to reveal two new components present in the oldest cells (arrowed).

a



b

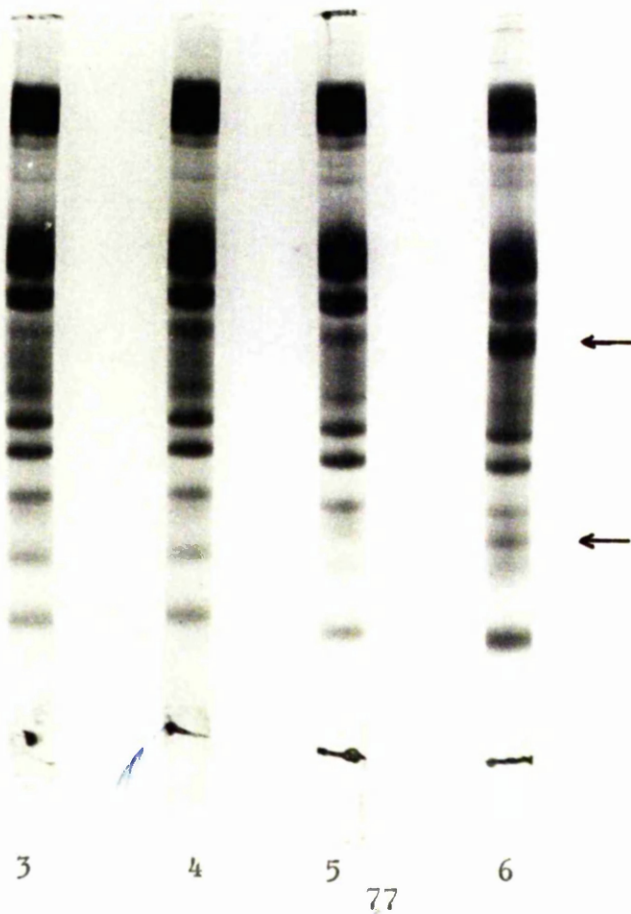
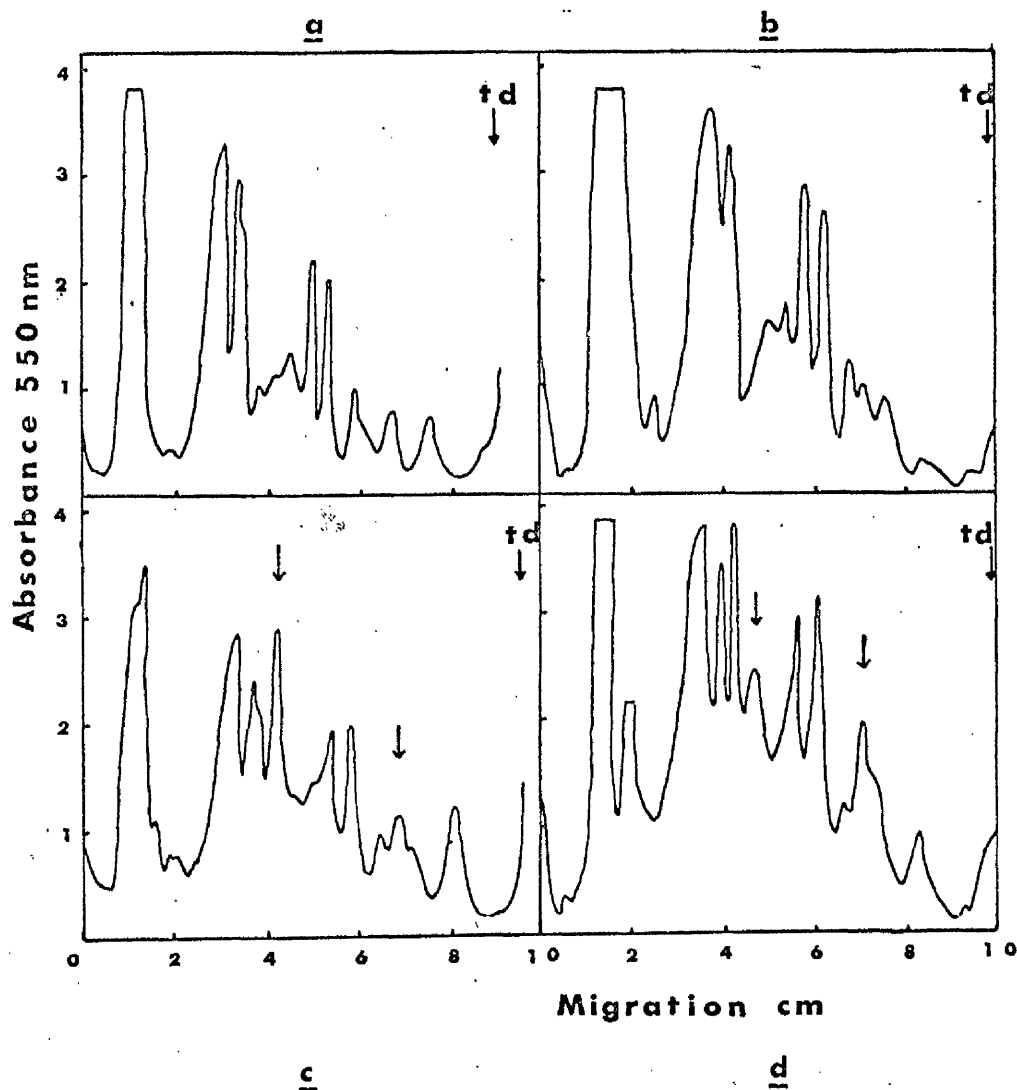
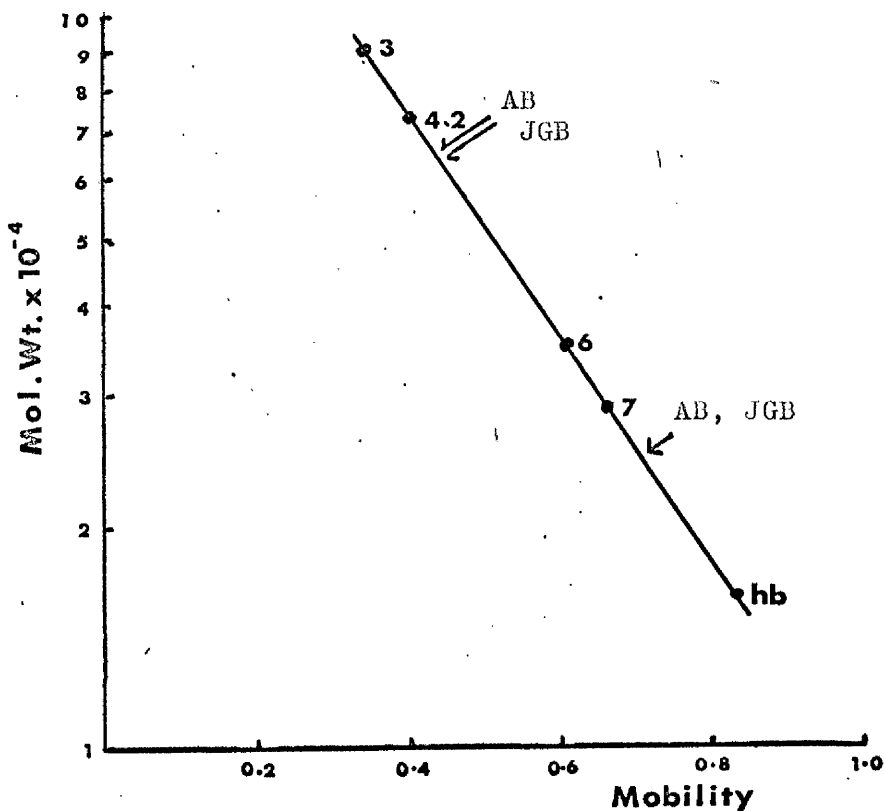


Figure 17 Appearance of Proteins in In Vivo Aged Erythrocyte Membranes



Cells obtained from two individuals were fractionated into populations of different age. Membranes were isolated and subjected to electrophoresis and gels were stained with Coomassie blue and scanned at 550nm. Comparing old and young cells (c with a and d with b), the components which appear during aging can be seen (arrowed). td represents the position of the tracking dye.

Figure 18 Molecular Weight Determination of Erythrocyte Membrane Polypeptides by SDS - Polyacrylamide Gel Electrophoresis



The electrophoretic mobilities of membrane proteins are shown as a linear function of the logarithm of their respective molecular weights. Molecular weights were taken from Steck (1974). Arrows indicate the mobilities of the components which appear during in vivo aging of the erythrocytes of two individuals A.B. and J.G.B. From these mobilities mol. wts. of 65,000 and 25,000 were estimated for A.B. and 61,000 and 25,000 for J.G.B.

3.5.1 Membrane Proteins of Erythrocytes Aged In Vivo

The proteins of the erythrocyte membrane may be separated by polyacrylamide gel electrophoresis in 1% sodium dodecyl sulphate and stained with Coomassie blue. Figures 16a and 16b show the changes in protein pattern which occur as the red cells of two individuals age in vivo. Figures 17a-d show comparative densitometric scans of the young and old fractions from these two sets of gels. In cells of J.G.B. (blood group B), two bands of molecular weights 25,000 and 61,000 appear (Figs. 16b, 17a & 17c). In cells of A.B. (blood group O), two bands of molecular weights 25,000 and 65,000 appear. The molecular weight values are obtained from the electrophoretic mobilities of the bands relative to the mobilities of membrane components (Fig. 18), whose molecular weights are known (Steck, 1974). It is also possible that component 3 is decreased in fraction 6 of figures 16a and 16b.

3.5.2 Proteolysis of the Erythrocyte Membrane Surface

The observations, made on in vivo aged cells, of additional protein components, decrease in component 3 and substantial loss of carbohydrate, suggested that aging may be accompanied by proteolysis of the extracellular membrane surface. Therefore unfractionated erythrocytes were treated with proteolytic enzymes for different time intervals and the effect on the membrane proteins was investigated by electrophoresis of the isolated membranes. The enzymes used were chymotrypsin, pronase, subtilisin and trypsin.

Table 15 shows the release of N-acetylneuraminic acid from the cells by these enzymes, over the time during which proteolysis took place. It can be seen that N-acetylneuraminic acid is

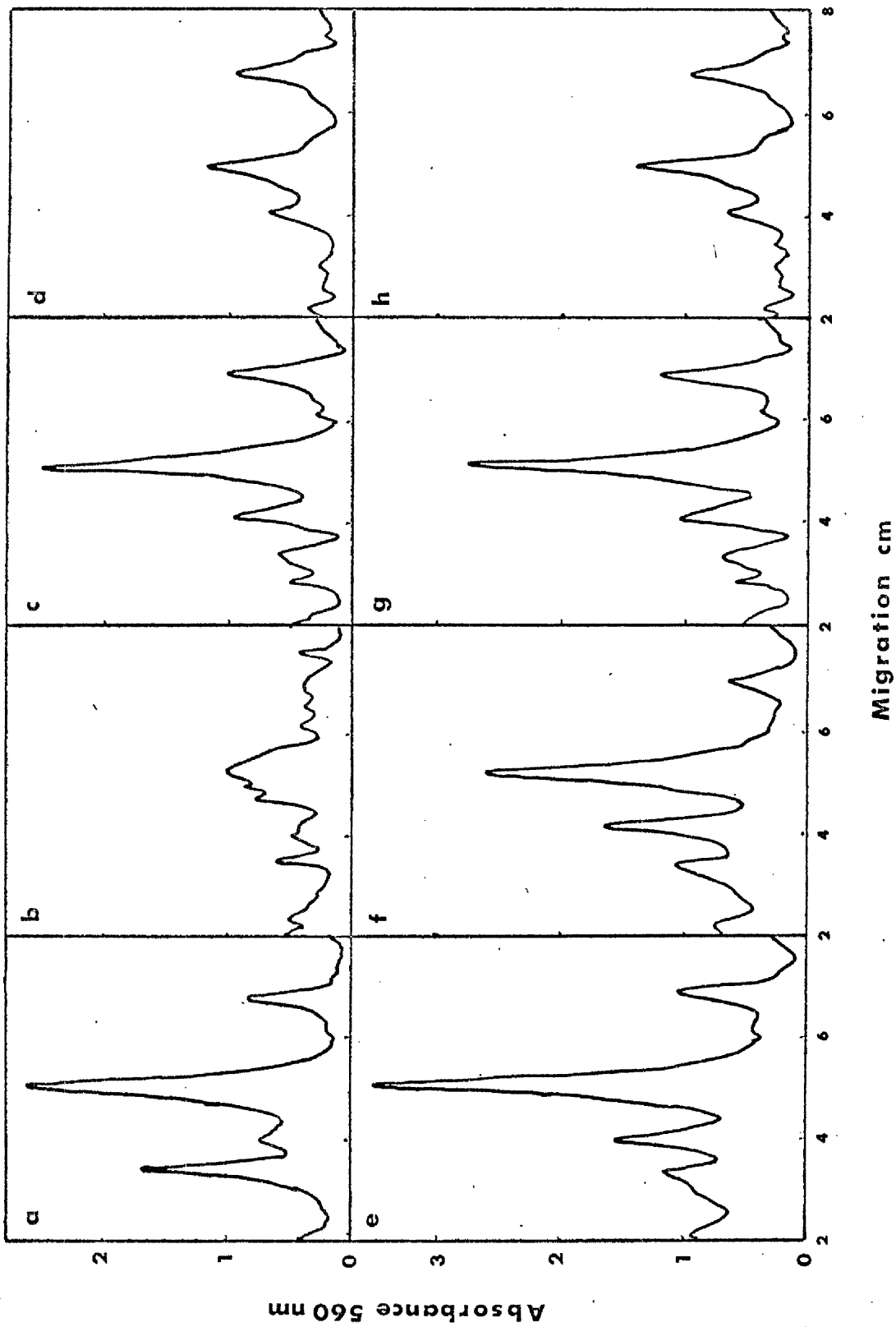


Figure 19 Effects of Proteolysis on the Sialoglycoproteins of Human Erythrocyte Membranes

Intact human red cells were incubated with proteolytic enzymes for different periods of time and the effect of proteolysis on the membrane sialoglycoproteins investigated by gel electrophoresis in SDS. Gels were stained with periodic acid/Schiff reagent and scanned at 560nm. (a) control PAS profile; (b) pronase 3min; (c), (d) subtilisin 3, 60min; (e), (f) chymotrypsin 3, 60min; (g), (h) trypsin 3, 20min.

N-Acetylneuraminic Acid

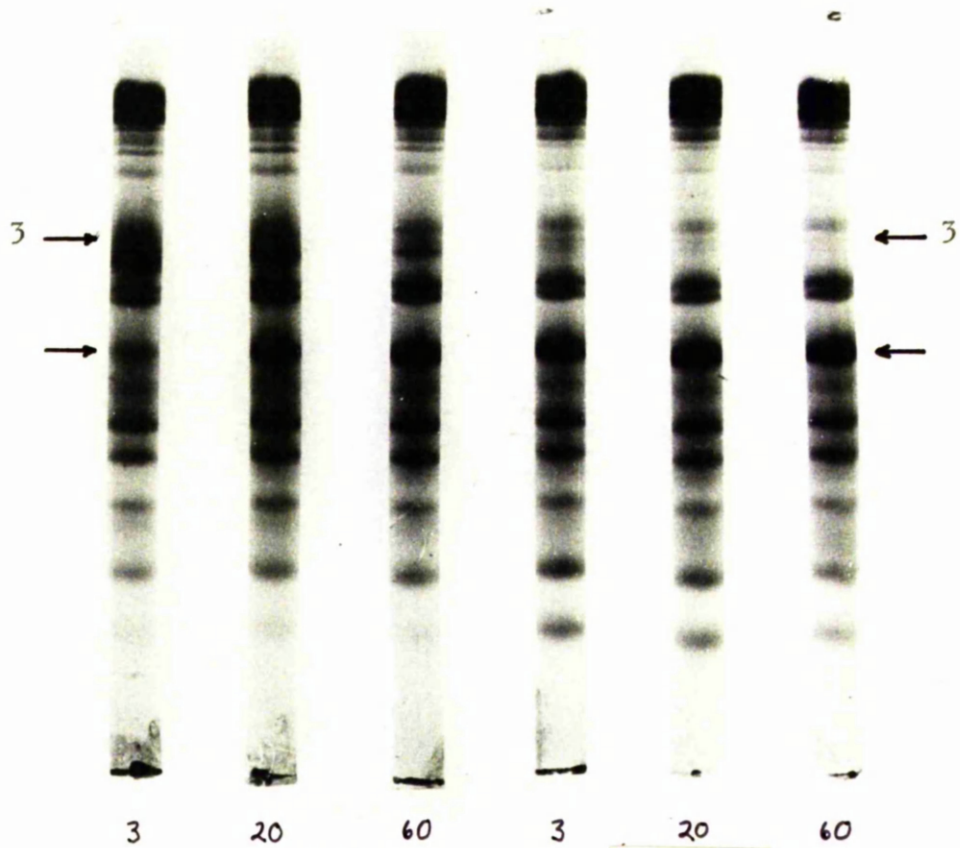
Enzyme	Time/min	% AcNeu released
chymotrypsin	3	11.5
chymotrypsin	20	22.1
chymotrypsin	60	25.0
pronase	3	55.8
pronase	20	63.5
pronase	60	66.3
subtilisin	3	17.3
subtilisin	20	32.7
subtilisin	60	48.1
trypsin	3	28.8
trypsin	20	45.2
trypsin	60	47.1

progressively released from the cells, due to proteolytic degradation of the sialoglycoproteins. This degradation can be seen in figure 19, where the progressive proteolysis of the PAS staining profile by the four enzymes is illustrated. Pronase has the most marked effect on the gel pattern (Fig. 19b), followed by trypsin, subtilisin and chymotrypsin.

Figure 20 shows the effect of proteolytic enzymes on the protein pattern of red cell membranes, as seen in Coomassie stained gels. Pronase and subtilisin cause extensive degradation of component 3, while with chymotrypsin, proteolysis of this component takes place to a lesser extent. Trypsin does not appear to affect component 3 at all. Also from figure 20 it can be seen that, with the exception of trypsin, these enzymes progressively effect the appearance of a new protein component similar to that seen in

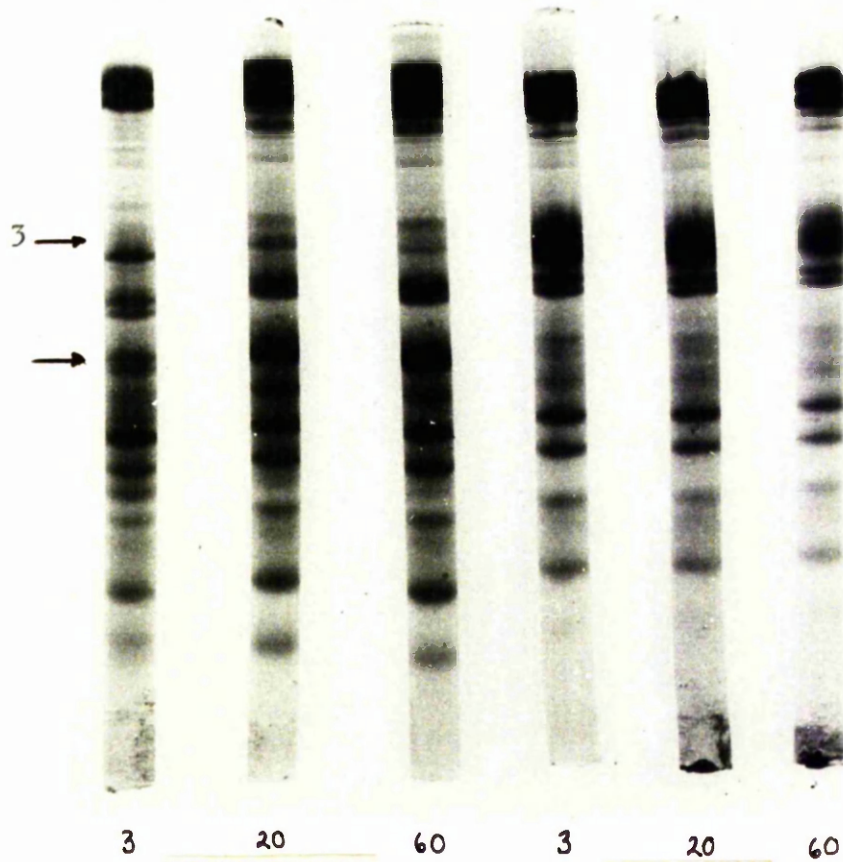
Figure 20 Effects of Proteolysis on the Proteins of Human Erythrocyte Membranes

Intact human red cells were incubated with proteolytic enzymes for 3, 20 or 60min and the effect of proteolysis on the major Coomassie blue staining components of the membrane was investigated by gel electrophoresis. On chymotrypsin, pronase or subtilisin treatment, component 3 was progressively digested and a new band appeared (arrows). Trypsin treated membranes appeared unaffected.



Chymotrypsin

Pronase



Subtilisin

Trypsin

in vivo aged cells. From gel densitometric measurements, the components produced by the three proteolytic enzymes had electrophoretic mobilities corresponding to an approximate molecular weight of 61-63,000. There is no indication of a 25,000 molecular weight product, similar to that seen in in vivo aged cells.

3.5.3 Iodination of Erythrocyte Membrane Proteins Exposed at the Extracellular Surface

The radioiodination of membrane proteins exposed at the extracellular surface of cells can be achieved specifically using lactoperoxidase, hydrogen peroxide and sodium [¹²⁵I]-iodide. Lactoperoxidase is assumed not to penetrate the cell membrane and so labelling is confined to the outside surface. Therefore labelling of fractionated erythrocytes was undertaken to determine if the proteins, which appear on aging, are exposed at the extracellular surface.

An old and young fraction of blood group B erythrocytes were iodinated and the membranes were isolated and subjected to electrophoresis. Gels stained with Coomassie blue (Fig. 21) indicate the presence of two new proteins in the old erythrocyte membrane. No peaks of radioactivity were seen to correspond to the extra bands.

b

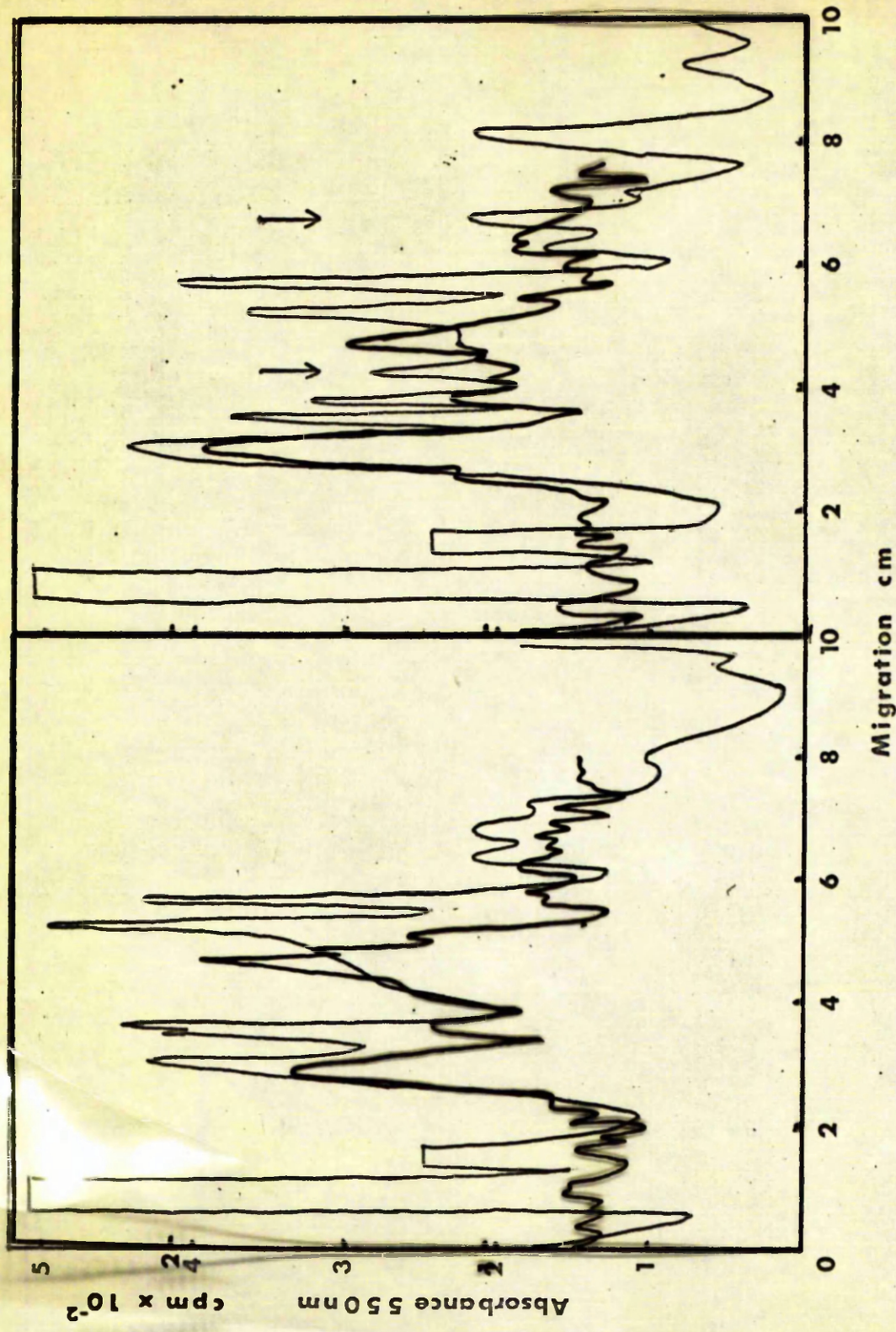


Figure 21 Radioiodination of Erythrocyte Membrane Proteins
Exposed at the Extracellular Surface

Young and old intact erythrocytes were labelled externally by lactoperoxidase catalysed iodination with [^{125}I]-iodide. Membranes were isolated and subjected to gel electrophoresis. Gels were stained with Coomassie blue to reveal the components which appear on aging. Components are arrowed in (b) old cells, relative to (a) young cells. Gels were then sliced and counted. Overlay shows labelling profile with no marked labelling of new components in old cells.

DISCUSSION

An aging process has been invoked to explain the non-random removal of human erythrocytes from the circulation, brought about by reticuloendothelial macrophages (Berlin, 1964). A number of properties of the erythrocyte change progressively as the cell ages in vivo (Table 1). It was reasoned therefore, that progressive changes on the red cell surface might accompany in vivo aging, and that such cell surface changes might culminate in an effete red cell being recognised and removed from the circulation.

A sample of blood, withdrawn from the circulation, has been shown to retain residual ability for protein synthesis, due to reticulocytes present, and this can be used to label a cohort of cells of similar age (reviewed by Berlin, 1964). If the cells are reinjected into an animal, the fate of the label over a period of time can be followed, the erythrocyte lifespan can be computed and, as the labelled cells will age in vivo, senescent cells can be identified and their properties can be determined. Using cohort-labelled cells, Borun et al. (1957) have shown that the specific gravity of human erythrocytes increases on aging. Simon & Topper (1957) have shown that the osmotic fragility of human erythrocytes increased on aging, although there also seemed to be a fragile young population. Similarly a separation method based on countercurrent distribution gave rise to contamination of old cells by a young population (Walter et al., 1964). The effectiveness of a centrifugal method for separating young and old erythrocytes has been improved by the use of density gradients of iso-osmotic albumin to enhance the small density differences (Bishop & Prentice, 1966; Pionelli et al., 1967).

Since a centrifugal separation produced uncontaminated old

cells, and since it gave rise to fractions of intact cells, density gradient ultracentrifugation was the method chosen to provide old and young erythrocytes.

The mean red cell specific gravity observed in the present studies was 1.100g/ml. A similar value was obtained by Danon & Marikovsky (1964), using density gradients of phthalate esters. These results conflict with the mean red cell specific gravity of 1.080-1.085g/ml obtained by Leif & Vinograd (1964). These authors have shown that the buoyant density at which a cell will settle during centrifugation is dependent on the tonicity of the solution. However, this author has found, using a number of different freezing-point depression osmometers, that osmometers calibrated with identical standards can give varying values for the osmolality of dense albumin solutions. However by standardising the gradient solution preparation, reproducibility was ensured (Fig. 3c). Moreover it is likely that the absolute tonicity of a gradient solution is unimportant, provided that, for a given series of experiments, the conditions are standardised, since Leif & Vinograd (1964) have shown that the entire distribution shifts with a change in tonicity.

Labelling studies have established that a fractionation of human erythrocytes on the basis of density gives rise to populations of cells of different age. Pionelli *et al.* (1968) showed that the activity of glucose-6-phosphate dehydrogenase in human erythrocytes was dependent on the age of the cell and so this criterion was used to confirm that the separation obtained in these studies did in fact give rise to cells of different age (Table 3). This conclusion was supported by other observations. The densest fraction of cells was most fragile, consistent with

the observation of Simon & Topper (1957) that old cells are more fragile than young. Moreover, the observation that there are more cells per unit volume in the densest fraction agrees with the findings of Leif & Vinograd (1964), who observed a decreased cell volume for older erythrocytes.

The preparation of a sample of essentially pure erythrocytes entailed the loss of some 5-10% of the total haemoglobin of a whole blood sample. Minor reticulocyte and white cell contamination was limited to the youngest fraction of erythrocytes. Therefore the absolute value of any parameter, measured for the youngest fraction, is subject to error in that this fraction cannot be said to consist of homogeneous young mature erythrocytes. The other five fractions were homogeneous.

The quantitation of any changes which take place during erythrocyte aging in vivo, requires reference to some parameter which remains constant with age. Leif and Vinograd (1964) found that the haemoglobin content of human erythrocytes remains constant during red cell aging. This was confirmed (3.1.3).

Two observations in the literature suggested that old erythrocytes would contain less N-acetylneuraminic acid than young cells. Danon et al. (1971) had shown that old erythrocytes had a reduced surface charge compared to young cells and Eylar et al. (1962) had shown that membrane bound sialic acids were largely responsible for the cell surface charge. Such a decrease has been quantitated (3.2.3; 3.4.3) and shown to be of the order of 20% for the erythrocytes of two individuals. Membranes, isolated from the fractionated cells of one of these donors, exhibit a similar decrease of 24%. Walter et al. (1965), using a counter-current distribution method of cell separation, were unable to

demonstrate an age related decrease in sialic acid. These authors, however, did not use a comparable method for the preparation of ghosts, nor did they purify the sialic acid from lipid contaminants. Greenwalt & Steane (1973a) centrifuged a column of red cells without resort to a gradient and showed a 9% decrease in red cell N-acetylneuraminic acid. However, this technique of separating cells probably only leads to a relative enrichment of the top and bottom fractions with young and old cells respectively. Moreover their oldest fraction consisted of about 14% of the total cells compared to about 9% in the studies presented here. Balduini et al. (1974) prepared cell fractions of different ages by differential lysis to yield three equally sized fractions. Although this does not correspond to very young or very old fractions, these authors found that, after pronase digestion to remove sialoglycopeptides, the decrease in N-acetylneuraminic acid was 20-30% in glycopeptides from old cells.

As described previously, separation of cells by ultracentrifugation on a density gradient is probably the most reliable method presently available for obtaining erythrocytes of different in vivo age. Therefore the data on the age related loss of N-acetylneuraminic acid, measured after release from intact cells by neuraminidase or acid hydrolysis, and confirmed by analysis of isolated membranes and on the intact cells of another individual, provides good quantitative evidence that red cell aging is accompanied by a decrease of 20-25% in cell surface N-acetylneuraminic acid. This estimate may be somewhat low because of the loss of some very young erythrocytes, while removing other contaminating cell types.

Eylar et al. (1962) suggested that the sialic acid of human erythrocytes is mostly, if not exclusively, N-acetylneuraminic acid, although they did not test for labile O-acetyl groups, shown by Schauer (1973) to be present in some sialic acids. They also suggested that all of the N-acetylneuraminic acid is on the extracellular surface, since it can be removed by neuraminidase, and that it is exclusively bound to glycoprotein. However, Wherret & Brown (1969) report the presence of human red cell gangliosides containing approximately 5% of the total N-acetylneuraminic acid. Despite this, it is obvious that a reduction of 20-25% in N-acetylneuraminic acid is greater than could be accounted for simply by loss of lipid bound sialic acids. These changes accompanying aging are therefore related to membrane glycoproteins.

The major Schiff staining glycoprotein components of the erythrocyte membrane (Figs. 1c & 1d) are also sialoglycoproteins as they can be labelled by the sialic acid specific labelling method of Blumenfeld et al. (1972) as is shown in figure 7. Sialic acid labelling of young and old red cells showed a difference of 37% in specific incorporation, again supporting the finding that this sugar is lost from the cell surface on aging. The difference in the values of 20-25% reduction in N-acetylneuraminic acid compared with 37% reduction in incorporation of tritium label, possibly indicates that some of the remaining 75-80% of the molecules of N-acetylneuraminic acid in old cells are not so reactive in the chemical labelling procedure. Blumenfeld et al. (1972) noted that the labelling of sialic acid residues was enhanced in ghosts, suggesting that the transition from intact cells to ghosts exposed some previously unreactive residues.

There are three major sialoglycoproteins in the human red cell membrane and, as discussed in 1.2.1 and 1.2.4, PAS 1 and PAS 2 may constitute different forms of the same molecule. Recent studies (Mueller & Morrison, 1974; Slutzky & Ji, 1974) have suggested that the components, behaving on acrylamide gels as PAS 1 and PAS 2, may possess different reactivity, possibly due to different conformations within the membrane. Therefore if PAS 1, PAS 2 and PAS 3 can be considered as separate entities, it was of interest to determine whether N-acetylneuraminic acid was lost specifically from any of these three components. It was shown in table 8, that there appears to be a general loss from all three components.

The simple removal of sialic acid from a glycoprotein would require the action of a neuraminidase. Warren & Spearing (1960) report the presence of a neuraminidase in a bovine plasma fraction. The enzyme was present at very low levels. However, it can be calculated that to release 20% of the red cell N-acetylneuraminic acid in 120 days requires only 0.001 units of enzyme.

Jancik & Schauer (1974) have demonstrated that 60% of [⁵¹Cr]-labelled rabbit erythrocytes, from which 65% of the cell surface sialic acids have been removed, are lost from the circulation within 24h. This implies that sialic acids are necessary for normal erythrocyte circulatory survival. These authors did not study the effects of removing less sialic acid and so the circulatory survival characteristics of erythrocytes from which 20-25% sialic acid has been removed are not known.

Lectins have been widely used in the detection of cell surface differences between normal and transformed cells.

Therefore, the interaction of agglutinins with the cell surfaces of old and young erythrocytes was compared. Old cells displayed an increased susceptibility to agglutination with the three agglutinins used. Greenwalt & Steane (1973b) have obtained similar results using other agglutinins. Binding studies using [^{125}I]-wheat-germ agglutinin showed that the increase in agglutinability was not due to an increase in the numbers of exposed lectin binding sites on old cells. The sialoglycoprotein and a number of tryptic sialoglycopeptides of the erythrocyte have been shown to inhibit the agglutination of red cells by wheat-germ agglutinin (Jackson *et al.*, 1973). This suggests that the red cell sialoglycoproteins possess receptors for wheat-germ agglutinin binding. The binding specificity of this lectin is generally accepted to be for N-acetylglucosamine (Najata & Burger, 1972). However Greenaway & Le Vine (1973) presented evidence that wheat-germ agglutinin also binds N-acetylneuraminic acid. This has been confirmed in the present studies (Table 9), where the treatment of red cells with neuraminidase has been shown to reduce the number of wheat-germ agglutinin binding sites.

The interaction of wheat-germ agglutinin with old erythrocytes could be mimicked by treating an unfractionated population of red cells with neuraminidase or trypsin. Both of these treatments produced a reduction in the numbers of wheat-germ agglutinin binding sites, but an increase in agglutinability (Table 10), implying that agglutination is not simply dependent on numbers of binding sites. Since enzyme treatment also released sialic acids which are responsible for the red cell surface charge, agglutinability of the cells may be dependent on

intercellular electrostatic repulsion. However, Luner et al. (1975) have shown that agglutination can take place with protease treated cells at a higher zeta-potential than that at which no agglutination takes place with neuraminidase treated cells. This implies that there is also a steric consideration in agglutination.

One other possibility was considered. It was pointed out in sections 1.2.2.2 and 1.2.2.3 that lectin binding sites and intramembranous particles are randomly distributed and possibly under the control of the spectrin complex. Since some workers claimed that the increased susceptibility to agglutination of transformed cells was due to a different topographical distribution of receptors on the cell surface, compared to that of normal cells (reviewed by Lis & Sharon, 1973), it was thought to be possible that such a redistribution might be responsible, in in vivo aged cells, for increased agglutinability. This presupposes that some change had taken place in the spectrin complex. Therefore, in old cells, decreasing the electrostatic repulsion between sialoglycoproteins, by loss of sialic acid, in conjunction with less control over their distribution by a less efficient spectrin complex might lead to a redistribution of these receptors, such that the agglutinability of old cells would be increased. A similar mechanism has been used to explain such findings in ghosts (Elgsaeter & Branton, 1974). However, examination of the freeze-fracture electron micrographs of old and young erythrocytes suggests that there is no marked redistribution.

It has already been pointed out that in vivo aged erythrocytes can pack closer together than young cells, and the

agglutination model studies show that old cells, through the action of an independent factor, can be brought together more readily. This might imply that the in vivo removal of senescent erythrocytes can be facilitated by their surface charge having been reduced, such that they can interact more readily with phagocytic cells of the reticuloendothelial system, either directly or after opsonisation. Some evidence for the latter type of mechanism comes from the work of Lee (1968), who showed that heterologous erythrocytes were more rapidly ingested by mouse peritoneal macrophages after neuraminidase treatment of the red cells.

The observation of Morell et al. (1971) that desialylated plasma glycoproteins are removed from the circulation by virtue of the exposure of an underlying galactose residue, suggested a possible role for sialic acid. Winterburn & Phelps (1972) postulated that the oligosaccharides attached to glycoproteins contain a code for the destruction of the glycoprotein, but that it is neutralised by the terminal sialic acid residues. Jancik & Schauer (1974) however, claim that, in contrast to the findings for plasma glycoproteins, the sequestration of desialylated rabbit cells cannot be prevented by modification of underlying galactose residues.

It is well established that sialic acid can be removed from the red cell surface, as part of a glycopeptide, by the action of proteases (Winzler, 1969). Therefore, the loss of cell surface N-acetylneuraminic acid from in vivo aged cells could have resulted from proteolytic digestion. This would be accompanied by the loss of other sugars. The analysis of some other major sugars of the erythrocyte membrane required the preparation of

ghosts from fractionated cells. Table 12 shows that the membrane alanine/haemoglobin ratio is constant for fractions 2-6 suggesting that the membrane protein remains fairly constant during aging. The loss of material in fraction 1 is probably associated with removal of the button. Since membrane alanine remains constant with age, carbohydrate analyses could be related to membrane alanine, for the different age fractions.

Carbohydrate analyses of ghosts showed that in vivo aging of erythrocytes is accompanied by fairly complex changes in the membrane bound sugars (Table 13). The value for N-acetylneuraminic acid confirms previous measurements made on intact cells. The decrease in glucose suggests glycolipid losses, since glucose is not normally a glycoprotein constituent. Moreover part of the decreases in N-acetylgalactosamine, galactose and glucose will be attributable to the loss of globoside and its analogues, shown by Sweeley & Dawson (1969) to accompany red cell aging.

To attempt to relate some of these carbohydrate changes to glycoproteins, it was reasoned that if N-acetylneuraminic acid alone was removed from the sialoglycoproteins of the old erythrocyte membrane, galactose residues would become exposed. Structures of the oligosaccharides of erythrocyte sialoglycoproteins have shown terminal N-acetylneuraminic acid linked to galactose (Winzler, 1969; Kornfeld & Kornfeld, 1970). Moreover Gahmberg & Hakomori (1973) showed that removal of N-acetylneuraminic acid from erythrocytes led to an increased incorporation of tritium label, after reaction with galactose oxidase and tritiated sodium borohydride, into a specific component. However, such a labelling experiment, comparing old and young cells

indicated no increase in galactose labelling of a similar component, or of any other sialoglycoprotein component (Fig. 15 & Table 14). This implies that galactose, as well as N-acetylneuraminic acid is lost from the sialoglycoproteins on aging. This finding has recently received support from the observation of Lotan (personal communication), that old red cells possess fewer binding sites for the lectin soya-bean agglutinin, with a specificity for galactose, than young cells.

Recently, Balduini et al. (1974) have isolated glycopeptides from old and young erythrocytes and have analysed the carbohydrate content of a major glycopeptide. They report that sialic acid and N-acetylgalactosamine are reduced by equimolar amounts. They interpret this observation to mean that a disaccharide, consisting of N-acetylneuraminic acid and N-acetylgalactosamine, is lost from the red cell on aging.

Table 14 shows that the incorporation of tritium label into the lipid fraction of old erythrocyte membranes is decreased by 20%. Previously it was shown (3.2.4) that there is little difference in the non-specific labelling of the lipids of old and young red cells, suggesting that erythrocyte aging does not appear to be accompanied by any major membrane lipid peroxidation, despite the fact that for 120 days this cell is constantly exposed to oxygen. Therefore, the decreased incorporation of label accompanying galactose oxidase/tritiated borohydride treatment, is probably associated specifically with glycolipids and is consistent with the observation of Sweeley & Dawson (1969) that galactose and galactosamine containing glycolipids are lost as the red cell ages.

The evidence presented so far indicates that specific carbohydrate losses from membrane glycoproteins accompany red cell

aging in vivo. The data does not, however, distinguish between sequential glycosidase action or proteolytic removal of glycopeptides.

Gel electrophoresis of the membranes of old cells obtained from two individuals, showed the presence of two components not observed in younger cells (Fig. 17). For one individual (A.B., blood group O), the components were of molecular weight 65,000 and 25,000, while for the other donor (J.G.B., blood group B), the components were of molecular weight 61,000 and 25,000 (Fig. 18).

Since figures 16a and 16b indicated the possible degradation of component 3 in old cells, and since the evidence presented so far is consistent with aging being accompanied by cell surface proteolysis, the effect of proteolytic enzymes on intact red cells was studied. Proteolysis of intact red cells by chymotrypsin, pronase and subtilisin resulted in the appearance of a polypeptide of molecular weight 61-63,000 (Fig. 20), similar to that present in aged red cells. No lower molecular weight components were detected. These three enzymes also digested the sialoglycoproteins (Table 15 & Fig. 19). However only one degradation product was evident, although several membrane components were being degraded. The behaviour of trypsin presents a similar anomaly in that, although the sialoglycoproteins were digested (Figs. 19g & 19h), no new low molecular weight component was detected. Moreover, trypsin appeared unreactive towards component 3 (Fig. 20). These findings are consistent with observations of Hubbard & Cohn (1972), Triplett & Carraway (1972) and Cabantchik & Rothstein (1974b), on the effects of proteases on the red cell. The most likely explanation of these observations

is that the 61-63,000 molecular weight component is derived from proteolysis of component 3. Proteolysis of the sialoglycoproteins must leave behind a residue too small to be detected on 5.6% polyacrylamide gels. From the analytical data of Segrest et al. (1973), the hydrophobic core and the carboxy terminal portion of the sialoglycoprotein, which would not be susceptible to proteolytic attack in intact cells, accounts for about 75 residues -- a peptide of approximate molecular weight 9,000.

Kadlubowski & Harris (1974) reported the presence, in old erythrocytes, of a protein which possibly corresponds to the high molecular weight component described above. Since these authors could not detect any apparent degradation of major membrane proteins, they suggested that the new component was adsorbed to the red cell membrane from the cytoplasm. There is no obvious labelling experiment which could distinguish an adsorbed protein at the cytoplasmic surface from a membrane protein which is exposed at the cytoplasmic surface. However, an alternative explanation for the new high molecular weight component might be that it was adsorbed from the plasma on to the extracellular membrane surface of old cells, since it has been shown that this surface is modified during in vivo aging. Lactoperoxidase catalysed radioiodination of intact red cells did not lead to labelling of the new components (Fig. 21). They are unlikely, therefore, to be adsorbed plasma proteins, although it is possible that they may be such proteins and possess no reactive tyrosine residues. A more attractive hypothesis, supported by the work with proteases, is that the high molecular weight component is a degradation product of an integral membrane glycoprotein, and has been digested such that no reactive tyrosine

residues are available to be labelled. Since labelling studies with radioactive iodine have shown that the sialoglycoproteins and component 3 can be iodinated, the observation that the components, resulting from in vivo aging, cannot be iodinated at the extracellular surface, suggests that proteolytic degradation has taken place, rather than sequential glycosidase action, since the latter should not affect markedly the availability of tyrosine residues.

There are a number of specialised proteolytic activities associated with plasma. These are mostly related to the clot formation mechanism. Movat et al. (1968) have shown that the protease activity of rabbit serum has an acidic pH optimum, and Bishop (1971b) points out that protease inhibitors are present in plasma. However, the time spent by the red cell in the plasma makes it likely that the changes observed during in vivo aging could be carried out by low levels of plasma protease activity.

Whether proteolysis is a further example of a deteriorative change for which the erythrocyte cannot compensate (compare 1.1), or whether it is a specific code for destruction, either by loss of a determinant necessary for survival, or exposure of some cryptic determinant leading to recognition and removal, is a matter for speculation. Maruta & Mizuno (1971) found that mouse red cells, treated with trypsin, while not immunogenic in mice, were nevertheless phagocytised by isologous macrophages. Although these authors did not test their system in the presence of serum, the results seem to suggest that phagocytosis of an effete red cell is not mediated by immunoglobulin antibodies. They found that ~~the~~metabolically depleted cells behaved similarly to trypsin treated cells in their system. Jancik et al. (1975), in

a study of the sequestration of desialylated erythrocytes, have shown, both in vitro and in vivo, that these cells will attach to liver and spleen and that this attachment is enhanced in the presence of serum. In contrast Lee (1968) showed that mouse peritoneal macrophages would neither attach to nor ingest desialylated isologous red cells. This experiment was performed with serum present. Phagocytosis of heterologous red cells did take place and was enhanced by desialylation. While there is some conflict between the results of Lee (1968) and Jancik et al. (1975), if they are compared with the findings of Maruta and Mizuno (1971), they suggest that desialylated and protease-treated erythrocytes require a different mechanism of macrophage recognition and uptake. Desialylated erythrocytes may not be good models of in vivo aged erythrocytes.

Rifkind (1966) has reviewed the mechanism by which the reticuloendothelial system destroys damaged red blood cells. Three factors are involved. Firstly injury to the red cell is required, and evidence has been presented that this occurs during in vivo aging. The second step is sequestration, that is, removal of the damaged cell by the organs of the reticuloendothelial system, namely the liver, spleen and bone marrow. Finally there is degradation, consisting of phagocytosis by and digestion within the reticuloendothelial macrophages. This has been briefly discussed in the preceding paragraph.

The possible relevance of the data presented in this thesis to the sequestration mechanism will now be discussed. The liver and spleen are generally accepted to be the main sites of red blood cell destruction. Rifkind (1966) reviews the evidence that the liver is principally involved in the clearance of cells which

have suffered major damage, whereas the spleen seems sensitive to more subtle injuries. It might be anticipated, therefore, that if red cell aging does not lead to antibody recognition and complement fixation, the site of sequestration of old red cells would be the spleen. The splenic blood flow can be resolved into two components. Where a cell is obliged to negotiate the slower splenic cordal pathway, it is exposed to reticuloendothelial cells lining its narrow vascular channels. Any factor which will prolong the erythrocyte's stay in this environment, such as a loss of repulsive surface charge, will increase its chances of being phagocytised.

In conclusion, the differences in membrane carbohydrate, in membrane protein components and in reactivity towards agglutinins, which are exhibited by in vivo aged human erythrocytes, relative to their younger counterparts, are consistent with and can in part be mimicked by proteolytic digestion of the red cell surface. This degradative change may be a primary factor in the recognition of effete cells and in their removal from the circulation by reticuloendothelial macrophages.

BIBLIOGRAPHY

- Allen, D.W. (1964) in *The Red Blood Cell* (Bishop, C. & Surgenor, D.M., eds.), pp. 309-337, Academic Press, London and New York
- Aminoff, D. (1961) *Biochem. J.* 81, 384-392
- Ashwell, G. & Morell, A.G. (1974) *Adv. Enzymol.* 41, 99-128
- Avissar, N., de Vries, A., Ben-Shaul, Y. & Cohen, I. (1975) *Biochim. Biophys. Acta* 375, 35-43
- Avruch, J. & Fairbanks, G. (1972) *Proc. Nat. Acad. Sci. U.S.* 69, 1216-1220
- Balduini, C., Balduini, C.L. & Ascari, E. (1974) *Biochem. J.* 140, 557-560
- Beeley, J.G. (1971) *Biochem. J.* 123, 399-405
- Berlin, N.I. (1964) in *The Red Blood Cell* (Bishop, C. & Surgenor, D.M., eds.), pp. 423-450, Academic Press, London and New York
- Bernacki, R.J. & Bosmann, H.B. (1973) *Eur. J. Biochem.* 34, 425-433
- Bishop, C. (1960) *J. Biol. Chem.* 235, 3228-3232
- Bishop, C. (1971) in *Blood*, p. 108, Blood Information Service, Buffalo, New York
- Bishop, C. (1971a) in *Blood*, pp. 97-100, Blood Information Service, Buffalo, New York
- Bishop, C. (1971b) in *Blood*, p. 49, Blood Information Service, Buffalo, New York
- Bishop, C. & Prentice, T.C. (1966) *J. Cell Physiol.* 67, 197-208
- Bishop, C. & Surgenor, D.M. (1964) *The Red Blood Cell*, pp. 162-175, Academic Press, London and New York
- Blumenfeld, O.O., Gallop, P.M. & Liao, T.H. (1972) *Biochem. Biophys. Res. Comm.* 48, 242-251

Borun, E.R., Figueroa, W.G. & Perry, S.M. (1957) *J. Clin. Invest.*

36, 676-679

Bosmann, H.B. (1971) *Biochem. Biophys. Res. Comm.* 43, 1118-1124

Bosmann, H.B. (1972) *Biochem. Biophys. Res. Comm.* 48, 523-529

Bretscher, M.S. (1971) *J. Mol. Biol.* 58, 775-781

Bukantz, S.C., Rein, C.R. & Kent, J.F. (1946) *J. Lab. Clin. Med.*

31, 394-399

Cabantchik, Z.I. & Rothstein, A. (1972) *J. Mem. Biol.* 10, 311-330

Cabantchik, Z.I. & Rothstein, A. (1974a) *J. Mem. Biol.* 15, 207-

226

Cabantchik, Z.I. & Rothstein, A. (1974b) *J. Mem. Biol.* 15, 227-

248

Carpenter, F.H. (1967) in *Methods in Enzymology* (Hirs, C.H.W.,

ed.), vol. 11, p.237, Academic Press, London and New York

Clamp, J.R., Bhatti, T. & Chambers, R.E. (1971) *Methods of*

Biochemical Analysis 19, 229-344

Cleve, H., Hamaguchi, H. & Hütteroth, T. (1972) *J. Exp. Med.* 136,

1140-1156

Danon, D. & Marikovsky, Y. (1964) *J. Lab. Clin. Med.* 64, 668-674

Danon, D., Marikovsky, Y. & Skutelsky, E. (1971) in *Red Cell*

Structure and Metabolism (Ramot, B., ed.) pp. 23-28,

Academic Press, London and New York

De Loecker, W.C.J. & Frankerd, T.A.J. (1961) *Clin. Chim. Acta* 6,

641-647

Dodge, J.T., Mitchell, C. & Hanahan, D.J. (1963) *Arch. Biochem.*

Biophys. 100, 110-130

Dunn, M.J. & Maddy, A.H. (1973) *FEBS Letters* 36, 79-82

Elgsaeter, A. & Branton, D. (1974) *J. Cell Biol.* 63, 1018-1030

Elgsaeter, A., Shotton, D. & Branton, D. (1973) *J. Cell Biol.* 59,

89a

- Evans, W.H. & Wisher, M.H. (1975) Abstr. 3rd Int. Symp. of
Glycoconjugates: Functions in Animals, pp. 20-21
- Eylar, E.H., Madoff, M.A., Brody, O.V. & Oncely, J.L. (1962) J.
Biol. Chem. 237, 1992-2000
- Fairbanks, G., Steck, T.L. & Wallach, D.F.H. (1971) Biochemistry
10, 2606-2617
- Farquhar, J.W. & Ahrens, E.H., Jr. (1963) J. Clin. Invest. 42,
675-685
- Fukuda, M. & Osawa, T. (1973) J. Biol. Chem. 248, 5100-5105
- Gahmberg, C.G. & Hakomori, S. (1973) J. Biol. Chem. 248, 4311-
4317
- Gardas, A. & Kościelak, J. (1971) Vox Sang. 20, 137-149
- Gesner, B.M. & Ginsburg, V. (1964) Proc. Nat. Acad. Sci. U.S. 52,
750-755
- Greenaway, P.J. & Le Vine, D. (1973) Nature New Biol. 241, 191-192
- Greenwalt, T.J. & Steane, E.A. (1973a) Brit. J. Haematol. 25,
207-215
- Greenwalt, T.J. & Steane, E.A. (1973b) Brit. J. Haematol. 25, 217-
226
- Grefrath, S.P. & Reynolds, J.A. (1974) Proc. Nat. Acad. Sci. U.S.
71, 3913-3916
- Hamaguchi, H. & Cleve, H. (1972) Biochim. Biophys. Acta 278, 271-
280
- Hickman, J., Ashwell, G., Morell, A.G., Van Den Hamer, C.J.A. &
Scheinberg, I.H. (1970) J. Biol. Chem. 245, 759-766
- Hoffman, J.F. (1962) J. Gen. Physiol. 45, 837-859
- Hubbard, A.L. & Cohn, Z.A. (1972) J. Cell Biol. 55, 390-405
- Hudgin, R.L. & Ashwell, G. (1974) J. Biol. Chem. 249, 7369-7372
- Hudgin, R.L., Pricer, W.E. & Ashwell, G. (1974) J. Biol. Chem.
249, 5536-5543

- Jackson, R.L., Segrest, J.P., Kahane, I. & Marchesi, V.T. (1973)
Biochemistry 12, 3131-3138
- Jaffé, E.H. (1964) in The Red Blood Cell (Bishop, C. &
Surgenor, D.M., eds.) pp. 397-422, Academic Press, London
and New York
- Jamieson, G.A., Urban, C.L. & Barber, A.J. (1971) Nature New
Biol. 234, 5-7
- Jancik, J. & Schauer, R. (1974) Hoppe-Seyler's Z. Physiol. Chem.
355, 395-400
- Jancik, J., Schauer, R., von Düring, M. & Andres, K.H. (1975)
Abstr. 3rd Int. Symp. of Glycoconjugates: Functions in
Animals, p. 21
- Ji, T.H. (1974) Proc. Nat. Acad. Sci. U.S. 71, 93-95
- Juliano, R.L. (1973) Biochim. Biophys. Acta 300, 341-378
- Kadlubowski, M. & Harris, J.R. (1974) FEBS Letters 47, 252-254
- Kant, J.A. & Steck, T.L. (1973) J. Biol. Chem. 248, 8457-8464
- Kathan, R.H. & Winzler, R.J. (1963) J. Biol. Chem. 238, 21-25
- Kathan, R.H., Winzler, R.J. & Johnson, C.A. (1961) J. Exp. Med.
113, 37-45
- Koch, P.A., Gardner, F.H. & Carter, J.R. (1973) Biochem. Biophys.
Res. Comm. 54, 1296-1299
- Konsek, J. & Bishop, C. (1962) Proc. Soc. Exp. Biol. Med. 110,
813-817
- Kornfeld, R. & Kornfeld, S. (1970) J. Biol. Chem. 245, 2556-2545
- La Celle, P.L. & Arkin, B. (1970) Blood 36, 837
- Lea, E.J.A., Rich, G.T. & Segrest, J.P. (1975) Biochim. Biophys.
Acta 382, 41-50
- Lee, A. (1968) Proc. Soc. Exp. Biol. Med. 128, 891-894
- Leif, R.C. & Vinograd, J. (1964) Proc. Nat. Acad. Sci. U.S. 51,
520-528

- Lesency, A.M., Bourrillon, R. & Kornfeld, S. (1972) Arch. Biochem. Biophys. 153, 831-836
- Lis, H. & Sharon, N. (1973) Annu. Rev. Biochem. 42, 541-574
- Lodish, H.F. (1973) Proc. Nat. Acad. Sci. U.S. 70, 1526-1530
- Lodish, H.F. & Deslaur, O. (1973) J. Biol. Chem. 248, 3520-3527
- London, I.M. & Schwarz, H. (1953) J. Clin. Invest. 32, 1248-1252
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
- Lowy, B.A., Williams, M.K. & London, I.M. (1962) J. Biol. Chem. 237, 1622-1625
- Luner, S.J., Sturgeon, P., Szklarek, D. & McQuiston, D.T. (1975) Vox Sang. 28, 184-199
- Marchalonis, J.J. (1969) Biochem. J. 113, 299-305
- Marchesi, V.T. & Palade, G.E. (1967) J. Cell Biol. 35, 385-404
- Marchesi, V.T. & Steers, E. (1968) Science 159, 203-204
- Marchesi, V.T., Steers, E., Tillack, T.W. & Marchesi, S.L. (1969) in Red Cell Membrane Structure and Function (Jamieson, G.A. and Greenwalt, T.J., eds.) pp. 117-130, J.B. Lippincott Co., Philadelphia
- Marchesi, V.T., Tillack, T.W., Jackson, R.L., Segrest, J.P. & Scott, R.E. (1972) Proc. Nat. Acad. Sci. U.S. 69, 1445-1449
- Marks, P.A., Gellhorn, A. & Kidson, C. (1960) J. Biol. Chem. 235, 2579-2583
- Marton, L.S.G. & Garvin, J.E. (1973) Biochem. Biophys. Res. Comm. 52, 1457-1462
- Maruta, H. & Mizuno, D. (1971) Nature New Biol. 234, 246-248
- Mills, G.C. (1959) J. Biol. Chem. 234, 502-506

- Moore, G.L., Kocholaty, W.F., Cooper, D.A., Gray, J.L. &
Robinson, S.L. (1970) *Biochim. Biophys. Acta* 212, 126-133
- Morawiecki, A. (1964) *Biochim. Biophys. Acta* 33, 339-347
- Morell, A.G., Van Den Hamer, C.J.A., Scheinberg, I.H. & Ashwell, G.
(1966) *J. Biol. Chem.* 241, 3745-3749
- Morell, A.G., Irvine, R.A., Sternlieb, I., Scheinberg, I.H. &
Ashwell, G. (1968) *J. Biol. Chem.* 243, 155-159
- Morell, A.G., Gregoriadis, G., Scheinberg, I.H., Hickman, J. &
Ashwell, G. (1971) *J. Biol. Chem.* 246, 1461-1467
- Morrison, W.L. & Neurath, H. (1953) *J. Biol. Chem.* 200, 39-51
- Morrison, M., Mueller, T.J. & Huber, C.T. (1974) *J. Biol. Chem.*
249, 2658-2660
- Movat, H.Z., Uriuhara, T., Taichman, N.S., Rowse, H.C. &
Mustard, J.F. (1968) *Immunology* 14, 637-648
- Mueller, T.J. & Morrison, M. (1974) *J. Biol. Chem.* 249, 7568-
7573
- Mulder, E. & Van Deenen, L.L.M. (1965) *Biochim. Biophys. Acta*
106, 106-117
- Murphy, J.R. (1962) *J. Lab. Clin. Med.* 60, 571-578
- Najata, Y. & Burger, M.M. (1972) *J. Biol. Chem.* 247, 2248-2250
- Nicolson, G.L. (1973) *J. Cell Biol.* 57, 373-387
- Nicolson, G.L. & Painter, R.G. (1973) *J. Cell Biol.* 59, 395-406
- Nicolson, G.L. & Singer, S.J. (1971) *Proc. Nat. Acad. Sci. U.S.*
68, 942-945
- Oliveira, M.M. & Vaughan, M. (1964) *J. Lip. Res.* 5, 156-162
- Palek, J., Curby, W.A. & Lionetti, F.J. (1971) *Am. J. Physiol.*
220, 1028-1032
- Pennell, R.B. (1964) in *The Red Blood Cell* (Bishop, C. &
Surgenor, D.M., eds.) p. 53, Academic Press, London and New
York

- Phillips, D.R. & Morrison, M. (1971) *Biochemistry* 10, 1766-1771
- Pinto da Silva, P. & Branton, D. (1970) *J. Cell Biol.* 45, 598-605
- Pinto da Silva, P., Douglas, S.D. & Branton, D. (1971) *Nature*
London 232, 194-196
- Pinto da Silva, P. & Nicolson, G.L. (1974) *Biochim. Biophys. Acta*
363, 311-319
- Piomelli, S., Lurinsky, G. & Wasserman, L.R. (1967) *J. Lab. Clin.*
Med. 69, 659-674
- Piomelli, S., Carash, L.M., Davenport, D.D., Miraglia, J. &
Amorosi, E.L. (1968) *J. Clin. Invest.* 47, 940-948
- Pollack, W. (1965) *Ann. N.Y. Acad. Sci.* 127, 892-900
- Frankerd, T.A.J. (1958) *J. Physiol. (London)* 143, 325-331
- Rall, T.W. & Lehninger, A.L. (1952) *J. Biol. Chem.* 194, 119-130
- Reed, C.F. (1959) *J. Clin. Invest.* 38, 1032-1033
- Reichstein, E. & Blostein, R. (1973) *Biochem. Biophys. Res.*
Comm. 54, 494-500
- Reitherman, R.W., Rosen, S.D. & Barondes, S.H. (1974) *Nature* 248,
599-600
- Rifkind, R.A. (1966) *Am. J. Med.* 41, 711-723
- Roseman, S. (1970) *Chem. Phys. Lipids* 5, 270-297
- Rosenberg, S.A. & Guidotti, G. (1968) *J. Biol. Chem.* 243, 1985-
1992
- Rosenberg, S.A. & Guidotti, G. (1969) in *Red Cell Membrane*
Structure and Function (Jamieson, G.A. & Greenwalt, T.J.,
eds.) p. 94, J.B. Lippincott Co., Philadelphia
- Roth, S., McGuire, E.J. & Roseman, S. (1971) *J. Cell Biol.* 51,
536-547
- Roth, S. & White, D. (1972) *Proc. Nat. Acad. Sci. U.S.* 69, 485-
489

- Schatzmann, H.J. & Vincenzi, F.F. (1969) *J. Physiol.* 201, 369-395
- Schauer, R. (1973) *Angew. Chem. Int. Edn.* 12, 127-138
- Schengrund, G., Jenzen, D.S. & Rosenberg, A. (1972) *J. Biol. Chem.* 247, 2742-2746
- Scott, E.M. & McGraw, J.C. (1962) *J. Biol. Chem.* 237, 249-252
- Segrest, J.P., Jackson, R.L., Andrews, E.P. & Marchesi, V.T. (1971) *Biochem. Biophys. Res. Comm.* 44, 390-395
- Segrest, J.P., Jackson, R.L., Marchesi, V.T., Guyer, R.B. & Terry, W. (1972) *Biochem. Biophys. Res. Comm.* 49, 964-969
- Segrest, J.P., Kahane, I., Jackson, R.L. & Marchesi, V.T. (1973) *Arch. Biochem. Biophys.* 155, 167-183
- Segrest, J.P., Gulik-Krzywicki, T. & Sardet, C. (1974) *Proc. Nat. Acad. Sci. U.S.* 71, 3294-3298
- Simon, E.R. & Topper, Y.J. (1957) *Nature London* 180, 1211-1212
- Sloviter, H.A. & Bose, R.K. (1966) *Biochim. Biophys. Acta* 116, 156-158
- Slutzky, G.M. & Ji, T.H. (1974) *Biochim. Biophys. Acta* 373, 337-346
- Steck, T.L. (1972) *J. Mol. Biol.* 66, 295-305
- Steck, T.L. (1974) *J. Cell Biol.* 62, 1-19
- Steck, T.L. & Wallach, D.F.H. (1965) *Biochim. Biophys. Acta* 97, 510-522
- Steck, T.L. & Yu, J. (1973) *J. Supramol. Struct.* 1, 220-232
- Steck, T.L., Weinstein, R.S., Strauss, J.H. & Wallach, D.F.H. (1970) *Science* 168, 255-257
- Steck, T.L., Fairbanks, G. & Wallach, D.F.H. (1971) *Biochemistry* 10, 2617-2624
- Sweeley, C.C. & Dawson, G. (1969) in *Red Cell Membrane Structure and Function* (Jamieson, G.A. & Greenwalt, T.J., eds.) pp. 172-227, J.B. Lippincott Co., Philadelphia

- Tanner, M.J.A. & Boxer, D.H. (1972) *Biochem. J.* 129, 335-347
- Taverna, R.D. & Langdon, R.G. (1973) *Biochem. Biophys. Res. Comm.* 54, 593-599
- Thomas, D.B. & Winzler, H.J. (1969) *J. Biol. Chem.* 244, 5943-5946
- Tillack, T.W., Scott, R.E. & Marchesi, V.T. (1972) *J. Exp. Med.* 135, 1209-1227
- Triplett, R.B. & Carraway, K.L. (1972) *Biochemistry* 11, 2897-2903
- Tuech, J.K. & Morrison, M. (1974) *Biochem. Biophys. Res. Comm.* 59, 352-360
- Van Deenen, L.L.M. & de Gier, J. (1964) in *The Red Blood Cell* (Bishop, C. & Surgenor, D.M. eds.) pp. 243-307, Academic Press, London and New York
- Van Den Hamer, C.J.A., Morell, A.G., Scheinberg, I.H., Hickman, J. & Ashwell, G. (1970) *J. Biol. Chem.* 245, 4397-4402
- Van Kampen, E.J. & Zijlstra, W.G. (1961) *Clin. Chim. Acta* 6, 538-544
- Walter, H., Selby, F.W. & Brake, J.M. (1964) *Biochem. Biophys. Res. Comm.* 15, 497-501
- Walter, W., Winge, R. & Selby, F.W. (1965) *Biochim. Biophys. Acta* 109, 293-301
- Wang, K. & Richards, F.M. (1974) *J. Biol. Chem.* 249, 8005-8018
- Waravdekar, V.S. & Saslaw, L.D. (1959) *J. Biol. Chem.* 234, 1945-1950
- Warren, L. (1959) *J. Biol. Chem.* 234, 1971-1975
- Warren, L. & Spearing, C.W. (1960) *Biochem. Biophys. Res. Comm.* 3, 489-492
- Weed, R.I. & La Celle, P.L. (1969) in *Red Cell Membrane Structure and Function* (Jamieson, G.A. & Greenwalt, T.J., eds.) pp. 318-338, J.B. Lippincott Co., Philadelphia

- Westerman, M.P., Pierce, L.E. & Jensen, W.N. (1963) J. Lab. Clin. Med. 62, 394-400
- Wherret, J.R. & Brown, B.L. (1969) Neurology 19, 489-493
- White, A., Handler, P. & Smith, E.L. (1973) Principles of Biochemistry (fifth edition) pp. 629-655, McGraw Hill
- Whittam, R. & Ager, M.E. (1965) Biochem. J. 97, 214-227
- Wilbur, K.M., Bernheim, F. & Shapiro, O.W. (1949) Arch. Biochem. 24, 305-313
- Winterburn, P.J. & Phelps, C.F. (1972) Nature London 236, 147-151
- Winzler, R.J. (1969) in Red Cell Membrane Structure and Function (Jamieson, G.A. & Greenwalt, T.J., eds.) pp. 157-171, J.B. Lippincott Co., Philadelphia
- Woodruff, J.J. & Gesner, B.M. (1968) Science 161, 176-178
- Woodruff, J.J. & Gesner, B.M. (1969) J. Exp. Med. 129, 551-567
- Yu, J., Fischman, D.A. & Steck, T.L. (1973) J. Supramol. Struct. 1, 233-248