THE PROTEINS OF THE HUMAN ERYTHROCYTE PLASMA MEMBRANE WITH RESPECT TO IN VIVO AGEING

Michael Kadlubowski

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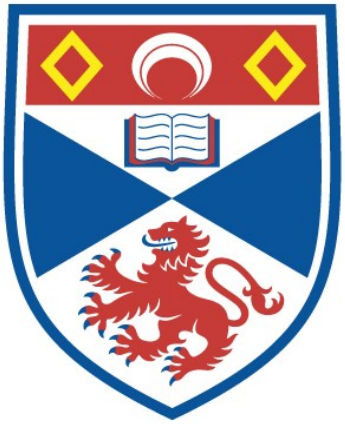
at the
University of St Andrews



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ABSTRACT

Fresh human red blood cells were fractionated according to their *in vivo* age by simple centrifugation in order that changes in the membrane proteins could be investigated. Accepted criteria were used to assess the fractionation obtained. A packed column of the youngest cells was found to contain much more extra-cellular space when compared to the oldest cells and the evidence pointed to this having arisen from a difference in surface charge repulsion rather than geometry. 25% of both the youngest and oldest cells appeared to be sphered.

Analysis of the membrane proteins by SDS PAGE revealed an increase in proteins 2.5 and 4.1 and a decrease in protein 7 with age. A tentative classification of the proteins revealed by SDS PAGE was put forward based on their age-dependent behaviour and *in situ* loci, special attention being paid to membrane-cytoplasm interactions. *In vitro* ageing was found to produce quantitatively very different changes in the membrane proteins.

The large increase in protein 4.1 with age and the temperature dependence of its binding to the membrane made its isolation seem worthwhile. A protein which was almost certainly 4.1 was purified from the cytoplasm of outdated blood.

A method was developed for the total solubilisation of the membrane proteins, permitting polyacrylamide gel electrophoresis whilst maintaining a very high degree of functional integrity. This was achieved using the four nonionic surfactants NP40, B35, T20 and T40. LDH, PNP, AChEase, NADH-MR and GAPD were visualised with this system and of these PNP, AChEase and NADH-MR exhibited an age-dependent decrease in band intensity. LDH was resolved into four discrete isozyme bands and PNP

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appeared to contain many unresolved bands.

Nine membrane-associated enzymes were assayed by standard techniques. No change was found for Na, K-ATPase and GAPD but the specific activities of AChEase, PGK, PNP, AKase, Mg-ATPase, NADH-MR and alkaline phosphatase were all found to decrease with age by varying amounts. Reticulocyte contamination could only have accounted for the small AChEase decrease. Of the rest, all the decreases were considered to be genuinely age-dependent, although only the decreases in NADH-MR and alkaline phosphatase could unequivocally be said to have given a good indication of the *in situ* situation. The other decreases could easily have been caused by a decrease in membrane integrity with age leading to a greater loss of enzyme protein from the older membranes during ghost preparation.

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A Thesis

Submitted to the University of St. Andrews for the Degree of Doctor of Philosophy.

by

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February 1976.

DECLARATION

I hereby declare that the research reported in this thesis was carried out by me and that the thesis is my own composition. No part of this work has been previously submitted for a higher degree.

The research was conducted in the Department of Physiology, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Dr. J.R. Harris.

ACADEMIC RECORD

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CERTIFICATE

I hereby certify that M. Kadlubowski has spent nine terms engaged in research work under my direction, and that he has fulfilled the conditions of General Ordinance No. 12 (Resolution of the University Court No. 1 1967), and that he is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

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It is my pleasant duty to acknowledge very gratefully the assistance of the following: Professor J.F. Lamb and Dr. J.R. Harris for providing the facilities and resources, the many donors who made it all possible, Dr. C.G. Ingram and Jean Polson for withdrawing the blood, the occupants of the departmental coffee room for many useful discussions, the technical staff, Andria Sylvester for preparing the figures, proof-reading and moral support, and Paul Agutter for everything.

ABBREVIATIONS

AAT: Aspartate Aminotransferase (EC.2.6.1.10).

AChEase: Acetylcholinesterase (EC.3.1.1.7).

ADP: Adenosine-5'-Diphosphate

Adenosine Deaminase: (EC.3.5.4.4).

AKase: Adenylate Kinase (EC.2.7.4.3).

Aldolase: (EC.4.2.1.7).

Alkaline Phosphatase: (EC.3.1.3.1).

AMP: Adenosine-5'-Monophosphate

Aminotripeptidase: (EC.3.4.1.3).

ATP: Adenosine-5'-Triphosphate

B35: Brij 35

Bis: NN'-Methylenebisacrylamide

Ca-ATPase: Ca-stimulated Mg-dependent Adenosine-5'-Triphosphatase (EC.3.6.1.3).

Catalase: (EC.1.11.1.16).

CMC: Critical Micellar Concentration

DCIP: 2,6-Dichloroindophenol

2,3-DPG: D-2,3-Diphosphoglyceric Acid

DPG Phosphatase: Diphosphoglyceric Acid Phosphatase (EC.3.1.3.13).

DTNB: 5,5'-Dithio-bis-(2-nitrobenzoate)

ECS: Extracellular Space

EDTA: Ethylenediaminetetra-acetic Acid

FDC: Frequency Distribution Curve

FDP: D-Fructose-1,6-Diphosphate

Fumarase: (EC.4.2.1.2).

GAPD: Glyceraldehyde-3-Phosphate Dehydrogenase (EC.1.2.1.12).

GDH: Glutamate Dehydrogenase (EC.1.4.1.2).

Glycylglycine Dipeptidase: (EC.3.4.3.1).

Glyoxalase: (EC.4.4.1.5).

GOT: Glutamate Oxalacetate Transaminase (EC.2.6.1.1).

G6P: D-Glucose-6-Phosphate

G6Pase: Glucose-6-Phosphatase (EC.3.1.3.9).

G6PD: Glucose-6-Phosphate Dehydrogenase (EC.1.1.1.49).

GPT: Glutamate Pyruvate Transaminase (EC.2.6.1.2).

GSH: Reduced Glutathione

GSSG: Oxidised Glutathione

Hb: Haemoglobin

HK: Hexokinase (EC.2.7.1.1).

HLB: Hydrophilic-Lipophilic Balance

Ht: Haematocrit

ICD: Isocitrate Dehydrogenase (EC.1.1.1.41).

ISD: Integrated Stain Density

ITPase: Inosine-5'-Triphosphatase (EC.3.6.1.4).

LDH: Lactate Dehydrogenase (EC.1.1.1.27).

Leucine Aminopeptidase: (EC.3.4.1.1).

Lysophospholipase: (EC.3.1.1.5).

MCH: Mean Cell Haemoglobin

MCHC: Mean Cell Haemoglobin Concentration

MCV: Mean Cell Volume

2-ME: 2-Mercaptoethanol

Mg-ATPase: Non-specific Mg-dependent Adenosine-5' Triphosphatase

MTT: 3-(4,5-Dimethyl Thiazolyl-2)-2,5-Diphenyl Tetrazolium Bromide

NAD(H): Nicotinamide Adenine Dinucleotide (reduced)

NADH-MR: NADH-dependent Methaemoglobin Reductase (EC.1.6.2.1).

NAD Nucleosidase: Nicotinamide Adenine Dinucleotide Nucleosidase (EC.3.2.2.5).

NADP(H): Nicotinamide Adenine Dinucleotide Phosphate (Reduced)

NADPH-MR: NADP-dependent Methaemoglobin Reductase (EC.1.6.2.3).

Na,K-ATPase:	Na + K-stimulated Mg-dependent Adenosine-5'-Triphosphatase (EC.3.6.1.4).
NIS:	Nonionic Surfactant
NP40:	Nonidet P40
ODC:	Orotidine-5'-Monophosphate Decarboxylase (EC. 4.1.1.23).
OPRT:	Orotidylate Phosphoriboeyltransferase (EC.2.4.2.10
PAGE:	Polyacrylamide Gel Electrophoresis
PEP:	Phosphoenolpyruvate
PFK:	Phosphofructokinase (EC.2.7.1.11).
PGA:	D(-)-3-Phosphoglyceric Acid
6PGD:	6-Phosphogluconate Dehydrogenase (EC.1.1.1.44).
PGK:	D-1,3-Diphosphoglycerate Kinase (EC.2.7.2.3).
PHI:	Phosphohexoisomerase (EC.5.3.1.9).
Phosphoglucomutase:	(EC.2.7.5.1).
Pi:	Inorganic Phosphate
PK:	Pyruvate Kinase (EC.2.7.1.40).
PMS:	Phenazine Methosulphate
PNP:	Purine Nucleoside Phosphorylase (EC.2.4.2.1).
PNPP:	p-Nitrophenyl Phosphate
PPP:	Pentose Phosphate Pathway
PVP	Polyvinylpyrrolidone
SDH:	Succinate Dehydrogenase (EC.1.3.99.1).
SDS:	Sodium Dodecyl Sulphate
T20:	Tween 20
T40:	Tween 40
TA:	Transaldolase (EC.2.2.1.2).
TEA:	Triethanolamine
TEMED:	NNN'N'-Tetramethylethylenediamine
TK:	Transketolase (EC.2.2.1.1).
Tris:	Tris (Hydroxymethyl) Aminomethane
TPI:	Triose Phosphate Isomerase (EC.5.3.1.1).
Xanthine Oxidase:	(EC.1.2.3.2).

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INTRODUCTION

Several reviews have appeared in the literature on the subjects of the kinetics of red cell ageing, the techniques used to achieve an age-dependent separation of the red cells and the subsequent characterisation of these age-fractionated cells (1,2,3,4,5) and the help of these authors in compiling the following review is gratefully acknowledged. These authors, however, have not been wholly selective in their choice of material, occasionally including studies from more than one species and also from outdated blood bank blood. It has thus been essential, for the purposes of this review, to consult all the original publications and it is pertinent to stress at this point that all the observations referred to here were concerned with the *in vivo* ageing of the *human* erythrocyte, unless otherwise stated.

RED BLOOD CELL SURVIVAL STUDIES

W. Ashby (6) showed as early as 1919 that the life span of the human red blood cell was approximately 120 days but this result was by no means universally accepted. Her work was first confirmed by D. Shemin *et al.* (7) in 1946 and since then by many others. The two problems that were foremost in workers' minds were the time that the erythrocyte spends in the circulation and the nature of its removal. That is to say, are the erythrocytes subject to an ageing process and therefore have a finite life span or are they removed randomly from the circulation? These problems were resolved using the techniques listed in Table 1, the five most important of these being discussed in detail below.

Differential Agglutination.

This was first described by C. Todd (8) but was developed for use in the human by W. Ashby (6). It remains today the only reliable non-radioactive method but being technically very difficult to perform it has fallen into disuse. The technique involves the transfusion of serologically compatible but antigenically distinguishable cells into a recipient, and the counting of these cells at intervals by agglutinating the recipient's cells in a sample and counting those remaining. As it is necessary to have strongly agglutinating anti-sera the technique is limited to the transfusion of group O into group A or B, and group MN into group MM. Also, in order that there be enough cells to count accurately, a transfusion of at least one litre is required, and therefore the recipient has to be relieved of an equivalent amount prior to the transfusion to maintain a steady state throughout the experiment. Thus, there is a great risk of contracting jaundice. The main criticism of this method is that the cells are not in their natural environment.

A slight modification of this technique is differential haemolysis as described by T.H. Hurley et al. (9). As the name suggests, this technique uses haemolysing instead of agglutinating anti-sera and therefore cannot be used for M and N blood groups. It is more difficult to perform and is subject to the same drawbacks.

Chromium-51

Developed by F.G. Ebaugh et al. (10), this is one of the simplest techniques and hence is widely used clinically even though it is very difficult to arrive at an absolute value for the life span. This is because the decrement in label with

Method	Mechanism
Differential Agglutination	Antigenically distinguishable blood transfused into recipient.
Differential Haemolysis	" "
Chromium-51	Taken up *in vitro* as chromate ion, bound covalently to globin.
DFP-32	Bound to serine-OH of AChEase and many other proteins.
Iodine-125	Bound covalently to membrane protein by lactoperoxidase method.
C-14 or N-15 Glycine	Given orally and incorporated into protoporphyrin.
Fe-59	Given orally and incorporated into haem.
C-14 Cyanate	Irreversibly bound to amino-terminal valine of haemoglobin.
C-14 Glucose	Incorporated into membrane glyco-sphingolipids.
N-15 Faecal Stercobilin	Results from breakdown of N-15 labelled haem.

Table 1. Techniques used for the measurement of red blood cell life span.

time is a function of ageing loss, elution from the cell (approximately 1%/day) and decay of the label. Consequently, it is the half-life of the label in the circulation which is used. A small sample of blood is withdrawn, washed and added to a sodium chromate-51 solution and after a short time interval is reinfused. Chromium enters as the chromate ion, changes valency and is firmly bound to the haemoglobin. Any eluted label is therefore in the reduced state and so is not reutilised (5). Chromium-51 being a strong gamma emitter permits surface counting and thus enables the sites of red cell sequestration to be charted (11).

Phosphorus-32

This is in the form of Diisopropylfluoro-phosphate (DFP) and was first used by J.A. Cohen et al. (12) who showed that the label is bound irreversibly by the cell. J.R. Bove et al. (13) showed that DFP was a potent inhibitor of acetylcholinesterase and for this reason it was initially thought to be a specific membrane label. However, D.A. Sears et al. (14) showed clearly that only approximately 10% of the label was bound to the membrane. Nevertheless, as the label is firmly bound after 10 days (13) (as with most labelling techniques there is initial loss of presumably unbound material) a linear decay curve is obtained, as in the Ashby technique, for normal subjects. DFP-32 is a beta emitter and so surface counting is not possible, the technique is difficult to perform and therefore is rarely used.

Carbon-14 and Nitrogen-15 Glycine

Historically this is important as the first radioactive experiment which confirmed Ashby's work (7). The label is given orally and is incorporated by the bone marrow, the alpha

carbon atom of glycine being a precursor for 8 of the 34 carbon atoms of haem. Thus, a specific age group of red blood cells is labelled and their sojourn in the circulation can be followed. Unfortunately, there is considerable reutilisation of the label.

Iodine-125

This is a very recent technique and is described in full by M. Weintraub et al. (15). It is, however, the most interesting as it is the only one which specifically labels the proteins of the membrane and so should help to decide whether the cytoplasmic components and those of the membrane behave differently or not with respect to age. It should also help to elucidate the ultimate fate of the membrane proteins after red cell sequestration, this being unknown at present.

All these methods when used in the human have shown that the normal healthy subject has negligible random destruction of red cells, but that the latter all have a similar life span and that this life span is approximately 120 days. This is illustrated in Fig. 1 (a + b), taken from J.W. Harris and R.W. Kellermeyer (5), which also shows the results from a situation in which there is complete random destruction taking place for comparison (c + d). A.C. Dornhorst (16) showed that even if there is an ageing process taking place, a linear survival curve will only be obtained if all the cells have a very similar life span, i.e. if there is a low coefficient of variation. Referring to Fig. 1 he showed that as the coefficient of variation increased a. will approximate to c. and b. will approximate to d. Consequently, it is very difficult to distinguish between an ageing process with a high coefficient of variation and a random process. This observation has recently

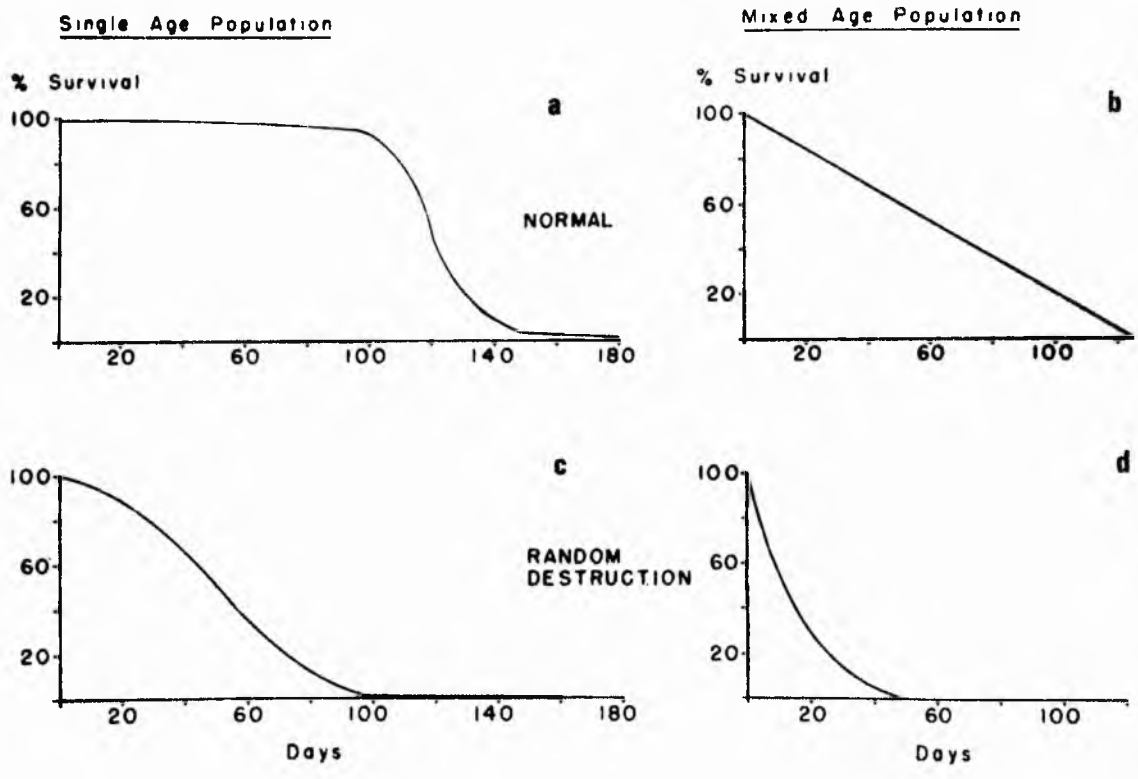

Fig.1. The survival of red cells in a normal ageing situation (a + b) and the effect of random destruction (c + d).

been used by G.N. Smith (17) to show that this was actually the case in the rabbit, and that there was negligible random destruction, contrary to what had previously been shown (18, 19). A similar explanation might be offered for the observation of S.T. Callender et al. (20) that human females have a small random component which is too large to be accounted for by menstruation. It is therefore fortuitous from the point of a mechanistic understanding of red cell destruction that in the human male at least there is a very low coefficient of variation.

As clear cut as this situation seems, it must be pointed out that there are some conflicting reports. R.L. Evans (21) measuring nitrogen-15 faecal steroobilin claimed that one eighth of the red blood cells were very short lived (0-10 days). Whilst this report itself has not been confirmed, there are several lines of evidence (22, 23, 24) to suggest the existence of two populations of red blood cells.

RED BLOOD CELL SEPARATION STUDIES

Having shown that an ageing process is involved in the destruction of the circulating erythrocytes it is natural to enquire as to its nature. Obviously, the characterisation of erythrocytes of different ages has as its prerequisite the age-dependent fractionation of the total population. Table 2 lists the techniques available.

In Vitro	In Vivo
Centrifugation	Differential Agglutination
Serial Osmotic Lysis	Induced Reticulocytosis
Counter-current - Distribution	Suppressed Reticulocytosis
	Hypertransfusion

Table 2. Techniques available for separation of red blood cells according to their chronological age and for producing age-enriched populations.

Centrifugation

Using Ferrous-59 labelling E.R. Borun et al. (25) confirmed that there was a correlation between the chronological age and the density of the red blood cell, although as C. Bishop et al. (26) pointed out much later, this correlation is not as exact as one would like it to be. Since then centrifugation has become the most popular and most diverse technique for separation, but a major preoccupation of many workers has been the cause of the observed density change. This was initially thought to be due to a change in the lipid content (27) but it is now the consensus of opinion that during the life cycle of the cell, water is lost with little if any loss of haemoglobin (26), causing an increase in density. Thus it can be said that the older the red cell, the greater is its sedimentation rate in a gravitational field likely to be.

Centrifugation can be of the simple kind performed in an anticoagulant treated plasma or some other isotonic medium or it can be performed on a density gradient. The former is simplest and can handle larger volumes of cells but it is not thought to give as good a separation as the cells are unable to reach their buoyant density equilibrium (2). D.A. Rigas et al.

(29) evaluated the effect of centrifugal speed and showed that the greater the speed, the better was the stratifaction of the erythrocyte population according to age. Most of these studies have been performed at below room temperature but J.R. Murphy (30) has recently shown that the efficiency of separation is greatly increased if the temperature is maintained at 30°C. This is because an increase in temperature is accompanied by a decrease in viscosity and an increase in deformability of the red blood cells, thus allowing a greater freedom of movement in the gravitational field.

A number of Media have been used in density gradient centrifugation and many of these were investigated by C. Bishop et al. (31). They found Ficoll, sucrose, PVP and dextran to be unsatisfactory as they caused clumping or were not dense enough, but found BSA to be highly satisfactory. The latter is usually used in the form of a discontinuous gradient (32), so that bands of increasing mean age are trapped at each successive interphase.

As a modification, D. Danon et al. (33) introduced the use of water-immiscible phthallete esters. Two esters are mixed to give a separating fluid of the required density and a small amount is spun with a column of blood. The older denser cells are trapped below the ester and the younger lighter cells above. This technique will be discussed in more detail later on.

Serial Osmotic Lysis

D. Chalfin (34) showed that older rabbit erythrocytes were more susceptible to hypotonic lysis than were the younger cells, whereas W.B. Stewart et al. (35) showed that the opposite was true for dog erythrocytes. E.R. Simon et al. (36) using ferrous-59 labelling confirmed the correlation between age and

osmotic fragility in the human erythrocyte and in this case it is the oldest erythrocytes which lyse first as the osmotic strength of the suspending medium is lowered. P.A. Marks et al. (37) using a similar technique claimed that the separation was superior to that obtained by simple centrifugation but this technique has now fallen into almost complete disuse due to the difficulty of accurately controlling the tonicity of the lysing medium and also because one obviously cannot obtain whole cells for further study. The increase in osmotic fragility with age is thought to be due to a decrease in the surface area : volume ratio so that the older cells are unable to accommodate an equivalent increase in volume, but one cannot rule out changes in the structure of the membrane itself.

Counter-Current Distribution

This technique was developed for use with red blood cells by H. Walter et al. (23) who based it on one of the two-phase aqueous systems of P.A. Albertsson (38). The technique is extremely complex to perform and it is not clear what is the deciding property for the observed separation. They found a marked correlation between separation and electrophoretic mobility (39) but no correlation with sialic acid content and were forced to conclude that there must be a change in the effective surface charge due to membrane alteration with age. They remain the only workers to use this technique.

Differential Agglutination

This technique has been described at length previously and due to the obvious technical difficulties has been seldom used (40, 41, 42).

Induced Reticulocytosis

This involves either the administration of phenylhydrazine which destroys mature red cells (43, 44), or repeated bleeding (34). Both of these procedures result in a massive reticulocytosis to counteract the loss of erythrocytes. Consequently, these studies have been concerned more with the reticulocyte to erythrocyte transformation than the senescence of the mature erythrocyte. These kind of experiments, however, are of undoubted importance for the interpretation of senescence studies which do not usually distinguish these two phenomena. There is considerable evidence that the 'stress' reticulocytes or macroreticulocytes produced in this way differ from their normal physiological counterparts especially as regards longevity (45, 46, 47), although a more recent report by S.O. Shattil (48) concluded that the macroreticulocyte had a normal survival time.

Suppressed Reticulocytosis

The preferred drug for this kind of experiment is Actinomycin D which suppresses the formation of new cells and therefore the mean age of the red cell population gradually rises during the course of treatment. This drug was used by C. Van Gastel et al. (49) on mice which together with rats are claimed to be much more resistant to this treatment than are humans, dogs and rabbits. As the erythrocytes do not possess the apparatus for protein synthesis (5) they are presumably unaffected by the drug but one cannot rule out the possibility that Actinomycin D has effects other than the inhibition of RNA Polymerase.

Hypertransfusion

This technique has been used twice (50, 51). Essentially, a strain of highly inbred rats were used and the cell mass from a given number was transfused into ever smaller numbers of animals. Thus, the formation of new cells was suppressed by maintaining the haematocrit above 50% which clearly avoids the sort of criticisms that can be levelled against the administration of a protein synthesis inhibitor. In this way a given population of cells could be followed for up to 48 days.

The use of phenylhydrazine, Actinomycin D and hypertransfusion is obviously out of the question in human studies. Serial osmotic lysis, counter-current distribution, differential agglutination and repeated bleeding have proved to be of very limited usefulness and so by far the majority of the ageing characteristics that are listed in Tables 3 to 8 are from studies that have used some form of centrifugation as the separating technique.

	Decrease	No Change
HK	42,52	53
PHI	54,55	-
PFK	42	-
Aldolase	52,54	-
GAPD	41,42	53,56
PK	57,58	-
G6PD	55,59	-
6PGD	55,60	59
NADH-MR	29	-
NADPH-MR	29	-
Catalase	29,40	22
TK	61	-
TA	61	-
PNP	62	59
GOT	30,63	-
AAT	55,64	-
ICD	59	-
OPRT	65	-
ODC	65	-
Glyoxalase	40	66

Table 3. References showing changes occurring in the cytoplasmic enzymes during *in vivo* ageing of the human erythrocyte.

	Decrease	Increase
Glycolysis	54	-
ATP Utilisation	67	-
PPP : Glycolysis	-	54*
Inosine → 2,3-DPG	68	-

Table 4. References showing changes occurring in the metabolism of the human erythrocyte during *in vivo* ageing. *Both the PPP and glycolysis decrease with age but the former has a greater proportional decrease.

	Decrease	No Change	Increase
ATP	54,69	27	-
ADP	4	-	-
AMP	4	-	-
2,3-DPG	54,68	-	-
GSH	29	27,61	-
GSSG	-	-	70
NAD	5	-	-
NADP	4	-	-
NADPH	70	-	-
Pyridoxal Phosphate	64	-	-
Na^+	69	-	54,27
K^+	27,69	-	-
Ca^{++}	54	-	-
Cl^-	28,69	-	-
Organic Anion	28	-	-
Water	28,69	-	-
Non-haemoglobin Protein	71	-	-

Table 5. References showing changes occurring in the non-haemoglobin cytoplasmic components of the human erythrocyte during *in vivo* ageing.

	Decrease	No Change	Increase
Methaemoglobin	-	72,73	74,75
Oxygen Affinity	-	-	76,77
Haemoglobin Cooperativity	73	-	-

Table 6. References showing changes occurring in haemoglobin during *in vivo* ageing of the human erythrocyte.

	Decrease	No Change	Increase
AChEase	40	-	-
Phosphatidylcholine Synthesis	-	-	78
Phospholipid	79,80	-	-
Cholesterol	79,81	-	-
Anionic Sites	82,83	-	-
Sialic acid	84,85	39	-
Antigen Density	-	-	86
Stromalytic Forms	87	-	-
Granularity	88	-	-

Table 7. References showing changes occurring in the composition, structure and metabolism of the plasma membrane of the human erythrocyte during *in vivo* ageing.

	Decrease	Increase
Volume	63,81	-
Surface Area	81	-
Density	-	25,28
MCHC	-	30,69
Osmotic Fragility	34,37	-
Deformability	89,90	-
Agglutinability	-	63,89
Electrophoretic Mobility	91,92	-

Table 8. References showing biophysical changes of the human erythrocyte occurring during *in vivo* ageing.

POSSIBLE MECHANISMS OF RED CELL SENESCENCE

Tables 3 to 8 illustrate that a vast amount of information has been collected with regard to changes occurring in the ageing erythrocyte, but this does not seem to have brought an explanation of the mechanism of senescence much nearer. Before we examine the possibilities it would be as well to comment on the large number of discrepancies in the literature.

Although it has been stated that most studies use some form of centrifugal separation, very few authors use exactly the same technique. Differences in the degree of separation or age-enrichment are therefore to be expected. As some of the changes are quite small it is possible that these would be observed only if a technique achieving a high degree of separation were used. Another explanation that has already

been mentioned is the failure of many authors to distinguish between reticulocyte maturation and erythrocyte senescence. Thus, for example, B.M. Turner et al. (62) originally claimed that there was an age-dependent decrease of the enzyme PNP, but in a later paper (59) they showed that this decrease could be attributed entirely to the reticulocyte to erythrocyte transformation. They proposed a similar mechanism for the decrease in 6PGD which had previously been observed (55, 60).

The situation concerning the red cell metabolites (Table 5) is even more confusing. This was dealt with in great depth by J. Astrup (93) who was concerned with the method in which results were expressed. He showed that in the case of sodium ions, if the latter were expressed /Litre of red cells or /Litre of red cell water than an increase was observed with age, but if they were expressed /Kg of red cell solids then there was a lack of any change. With potassium ions, expressing the content /Litre of red cell water abolished the decrease observed /Litre of red cells and /Kg of red cell solids. H.G. Keital et al. (69) suggested that as the red cell loses water with age it would be preferable to express ionic content /Kg of red cell solids, but as there is some information regarding the loss of non-haemoglobin solids with age (71) even this might lead to some error. As it is agreed that there is little if any loss of haemoglobin with age (28) it seems that R.E. Bernstein's (54) method of expressing ionic content (uMoles/gm Haemoglobin) is the best that one can hope for. The discrepancy in sialic acid content (39, 84, 85) can be explained in a similar fashion and this will be discussed in the next chapter.

Metabolic Changes

As the erythrocyte derives at least 90% of its energy from the glycolytic pathway (54) it is natural to look here for the loss of a critical enzyme function and especially at the four phosphohydrolase reactions. Hexokinase has been shown to decrease with age (42, 52) but all are not agreed (53). Although phosphofructokinase exhibited a large decrease in specific activity (42) the latter was always greater than that of hexokinase which remained rate-limiting. No change was found in phosphoglycerate kinase (41) but pyruvate kinase was shown to decrease (57, 58).

The situation is, however, complicated by the fact that enzymes are normally measured under standard assay conditions, utilising optimal substrate and cofactor concentrations. Thus, while it is apparently a simple matter to show some change in the maximum turnover of an enzyme, how this relates to the *in situ* condition is almost impossible to deduce. This was shown by D.E. Paglia *et al.* (94) who observed a change in Km, V max. and pH optimum of pyruvate kinase with age towards impaired catalytic efficiency. Therefore the actual decrease in the specific activity of this enzyme could be greater than is apparent from present studies. The decrease in glycolytic rate is presumably responsible for the fall in ATP (again all are not agreed), and it is this fall which is considered by some (52) to be of prime importance. It is argued that this decrease would inhibit the rate-limiting hexokinase causing a decrease in the glycolytic rate with all its attendant metabolic consequences. Apart from this being a somewhat circular argument, hexokinase is normally inhibited by 2,3-DPG (95) and the latter has been shown to decrease with

age (68), so one should not be too eager to give credence to such facile explanations. Moreover, it has been shown that ageing is associated with a decreased ability to utilise ATP (67), and so the latter, even if it does decrease, is not necessarily likely to be rate-limiting.

The weight of evidence (41, 42, 53, 56) suggests that there is no decrease in glyceraldehyde-3-phosphate dehydrogenase and it is accepted that there is no change in lactate dehydrogenase (22). Thus there would appear to be no decrease in the capacity to produce NADH from NAD. However, if there is some overall decrease in the glycolytic rate, this function must be impaired to some extent. This cofactor is required for the enzyme NADH methaemoglobin reductase which is the main protective mechanism against oxidative denaturation of haemoglobin (4). Opinion is divided as to whether there is an increase in methaemoglobin content with age (72, 73, 74, 75), so any decrease in the activity of this enzyme or reduced cofactor is obviously not great enough to prevent protection of haemoglobin to any significant degree.

There does seem to be a real decrease in glucose-6-phosphate dehydrogenase (55, 59) with a presumably associated decrease in NADPH (70). This reduced cofactor is vital for the functioning of the glutathione reductase-glutathione peroxidase system which is responsible for maintaining a reducing environment (GSH), and for breaking down hydrogen peroxide (4). Once again, as the latter causes the formation of methaemoglobin, there would not appear to be a critical decrease in the level of this cofactor or the activity of the two enzymes. This is supported by the lack of any clear-cut change in reduced glutathione (29, 27, 61).

Several other enzymes have been shown to decrease with age (Table 3) but as their importance in the metabolic functioning of the cell is unclear it is impossible to say whether these changes are significant or not. There is also a general loss of nucleotides and cofactors from the cell on ageing (Table 5) but as it is the ratios of these substances which are all-important, no conclusions can as yet be drawn.

Impaired Haemoglobin Function

Whilst the methaemoglobin situation is unclear, it is known (76,77) that haemoglobin from older cells has an increased oxygen affinity and this probably arises from intrinsic changes in the haemoglobin itself (77, 96), the decrease in 2,3-DPG (68), a decrease in haemoglobin cooperativity (75) and the formation of mixed disulphides as a result of the increase in GSSG (70). The latter was shown to inhibit some enzymes and may also be responsible for the apparent decrease in non-haemoglobin proteins that has been observed (71). That is, some of the non-haemoglobin proteins may become sequestered by haemoglobin in the form of mixed disulphides. The increase in oxygen affinity of haemoglobin undoubtedly leads to an impairment of the prime function of the red cell but a causal relationship between this function and red cell viability has not been shown.

Recognition

Removal of sialic acid from the surface of the red cell using neuraminidase, followed by reinfusion results in the rapid clearance of these treated cells from the circulation in the case of the rabbit (97, 98, 99) and the rat (97). These observations and similar experiments with glycoproteins (100, 101) suggest that sialic acid has an antirecognition or protective function. In these studies, however, a very large

proportion of the sialic acid residues were removed and as it is unclear in the human whether or not there is a decrease in sialic acid content with age (39, 84, 85), the importance of this substance as a determinant of red cell life span must be held in great doubt. This doubt is reinforced by the studies of S. Chien et al. (102) and J. Darnborough et al. et al. (103), in which patients were found with red cells possessing considerably decreased sialic acid levels but normal life spans. The supposed loss of sialic acid is suggested to be the cause of the increase in antigenic sites (86) with a resulting increase in agglutinability. Whatever the cause of this increase in agglutinability, it is an attractive mechanism for senescence but it remains pure speculation.

Biophysical Changes

Bolus flow through the capillaries and passage through the spleen necessitate that the erythrocyte be a highly deformable structure. The change in the surface area : volume ratio with age is thought to be responsible for the decrease in deformability (90, 104) and this is likely to be aggravated by any fall in the ATP level which is known to have a marked effect on the shape and mechanical properties of the membrane (105). It has been suggested by P.L. LaCelle et al. (104) that the fall in the ATP level with age is enhanced by increased binding to haemoglobin, the latter being facilitated by the fall in the 2,3-DPG level. This then makes more calcium ions available for interaction with the plasma membrane causing the observed increase in rigidity. In an over-zealous attempt to prove their point they add that the oldest cells probably contain no ATP at all. The mechanical properties of the membrane are also likely to be affected by the lipid changes (81),

especially as cholesterol has been implicated as being responsible for the stabilisation of membrane structures (106). Once again it is still necessary to show that this parameter falls below a critical level.

FINAL REMARKS

The outcome of senescence must be either the spontaneous haemolysis of the red cell or its sequestration by the reticuloendothelial system following recognition of some kind. Thus, whatever the primary cause of senescence and the subsequent chain of events, the final result of this process must be a membrane event. As yet only the properties of the membrane have been studied as regards *in vivo* ageing in any detail and so an investigation of the membrane components seemed long overdue. The aim of this study is not to solve the problem of the mechanism of red cell senescence but to characterise the proteins of the membrane and to observe any changes in them in the hope that some light may be shed on this very complex problem.

A study of this nature is also necessary, because as D.J. Hanahan (107) pointed out, too many workers consider erythrocytes to be an homogeneous population and consequently treat the plasma membrane as a static entity. It is therefore the somewhat more attainable goal of this study to quantify as fully as possible the variability that one would expect to find in an heterogeneous population.

Lastly, several studies have been undertaken to characterise the red cell membrane proteins from patients with various congenital haemolytic anaemias (108, 109, 110), some of which are thought to be due to premature ageing. As these studies

have been undertaken before the normal ageing situation has been understood it is hoped that this gap will have been narrowed thus enabling more accurate conclusions to be drawn.

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CHAPTER 1

CELL SEPARATION AND WHOLE CELL STUDIES

Summary

1. Simple centrifugation was found to be a convenient technique for producing an age-dependent stratification of human red blood cells within a centrifugal field.
2. Assessment of the fractionation using the specific activity of GOT, the MCHC and the reticulocyte count showed it to be comparable to that found in the literature, but far from complete.
3. The decrease in MCV measured by the Coulter Counter was approximately half that found using standard haematological techniques. This was caused by the presence of a greater extracellular space in the packed column of younger cells as compared to the older cells and was probably caused by a greater surface charge repulsion of the former. Consequently, just over half the MCHC increase that was observed had been artifactually produced.
4. Both the youngest and oldest fractions contained 75% biconcave discs and 25% spheres. As both of these populations decreased in volume with age it was concluded that a constant proportion of all age groups are sphered, and not that the spheres represented the youngest cells.

Introduction

Before a study of the human erythrocyte membrane proteins could be begun a suitable technique for separating the cells according to their *in vivo* age had to be found. Most of the previous work in this field (see Introduction) has been of a biochemical, biophysical or ultrastructural nature which requires very small amounts of material. A study of the membrane proteins, however, requires somewhat more material and this is especially so for a functional study due to the generally lower catalytic rates of these proteins as compared to those found in the cytoplasm.

Two techniques were therefore evaluated from the point of view of ease of performance and the degree of separation achieved. The latter was assessed using standard criteria and these themselves were subjected to critical evaluation. This inevitably involved the repetition of some previous work for the sake of comparison, and more importantly where it was felt that sometimes tenuous or even erroneous conclusions had been drawn. As a result the sialic acid content of the membrane and the volume of the whole cell were investigated at great length.

Materials and Methods

Centrifugation

The age-dependent fractionation of whole blood was carried out using a Beckman L2 65B ultracentrifuge with a SW 27 rotor. When required, ghosts were made by a modification of the method of J. T. Dodge *et al*. (1) using a Beckman J21 preparative centrifuge. This involved a final Tris wash after the three phosphate washes to remove as much haemoglobin as possible, as described by J.R. Harris (2). In the procedure shown in detail below, all steps were performed at 4°C unless otherwise stated.

Obtain fresh blood
↓
Spin 2X at 5 Krpm for 10 min. at 30°C to remove buffy coat and increase Ht. to approx. 90%
↓
Spin at 15 Krpm for 1 hr. at 30°C
↓
Separate RBC column into 8 fractions.
↓
Resuspend in PBS at 30°C, spin at 5 Krpm for 5 min. at 4°C → Take sample of each fraction for whole cell studies
↓
Resuspend in ice cold PBS, spin at 5 Krpm for 5 min. at 4°C
↓
Haemolyse in 10 mM Na phosphate pH 7.4, spin at 20 Krpm for 15 min. → Take sample of supernatant for GOT assay

Repeat twice with
pellet
↓
Resuspend 3rd. phosphate
pellet in 10 mM Tris/Cl
pH 7.2, spin at 20 Krpm
for 20 min.

Unless otherwise stated group A+ or O+ blood was collected from healthy males between the ages of 18 and 30, from the brachial vein into a 50 ml. plastic or glass syringe containing 2.0 ml. heparin (5000 Units/ml.). To ensure that the donor was in an haematologically steady state, blood was never withdrawn more often than once every four months.

As stated above, the red cell population was separated into eight fractions. These fractions were numbered from the top of the centrifuge tube so that the youngest lightest cells constituted fraction 1, fraction 2 was just below this and so on. This nomenclature is adhered to throughout this work.

Centrifugation of whole blood was also performed using the phthallate ester technique of D. Danon et al. (3). Owing to an interaction between the phthallate esters and the plastic tubes normally used with the Beckman L2 65B, glass tubes were used. 6.0 ml. of blood was placed in a 7.0 ml. graduated glass centrifuge tube and on top of this red cell column was placed 0.5 ml. of the appropriate phthallate ester. The whole was spun at 4.5 Krpm for 45 minutes at room temperature.

Haematological Constants

The haematocrit (Ht.) was measured by spinning the red cell suspension for 5 minutes in a Hawksley Micro-Haematocrit Centrifuge. The haemoglobin concentration was obtained by the cyanmethaemoglobin method of J.V. Dacie (4) as follows. 20 uL of cells were added to 4.0 ml. of a solution containing 200 mg. K ferricyanide, 50 mg. KCN, 40 mg. KH_2 Phosphate and 1.0 ml. Nonidet P40 per litre. The resulting colour was read at 540 nm using a Beckman DB GT spectrophotometer and compared to a standard of known concentration prepared by BDH. Red cell counts and volumes were measured using a Model Zf Coulter Counter with a Channelizer output. The cells were diluted 50,000 fold with the Coulter diluent, Isoton. The constants were calculated as follows.

$$\text{Mean Cell Volume (MCV)} = \frac{\text{Ht (\%) x 10}}{\text{Count } (/mm^3 \text{ x } 10^6)}$$

$$\text{Mean Cell Haemoglobin (MCH)} = \frac{\text{Hb. (gm.\%)}}{\text{Count } (/mm^3 \text{ x } 10^6)}$$

$$\text{Mean Cell Haemoglobin (MCHC) Concentration} = \frac{\text{Hb. (gm.\%) x 100}}{\text{Ht. (\%)}}$$

Reticulocytes were stained with 0.6% New Methylene Blue 1.4% potassium oxalate (5). 2 drops of packed cells were mixed with 2 drops of plasma and 3 drops of stain and incubated for 30 minutes at 37°C. After spinning for 30 seconds in a Beckman/ Spinco 152 Microfuge the supernatant was discarded and a smear made of the pellet. At least 1000 cells from each fraction were counted under oil immersion.

Glutamate-Oxalacetate Transaminase (GOT)

This was assayed with a BDH kit as follows. 0.2 ml. of haemolysate was added to 1.0 ml. of 2 mM 2-oxoglutarate/200 mM DL-aspartate/ 0.1 M phosphate pH 7.4 and incubated for 1 hour at 37°C. 1.0 ml. of 2, 4-Dinitrophenylhydrazine was then added to this mixture which was incubated for 20 minutes at 37°C. After the addition of 4.0 ml. of 0.4 N sodium hydroxide the tubes were left to stand for 10 minutes and read at 505 nm. against a water blank. A standard curve was constructed using pyruvic acid. The activity was expressed per mg. of haemoglobin as described below.

Sialic Acid

The sialic acid content of the ghosts was measured by the method of D. Aminoff (6). The sialic acid in 0.4 ml. of ghosts was hydrolysed by the addition of 0.1 ml. of 0.5 N sulphuric acid followed by heating at 80°C for 30 minutes. 0.25 ml. of 25 mM periodic acid/ 0.125 N sulphuric acid was then added and the mixture incubated at 37°C for 30 minutes to oxidise the exposed residues. The unreacted periodic acid was removed by the addition of 0.2 ml. of 2% sodium arsenite/ 0.5 N hydrochloric acid which was left for 1-2 minutes. The chromophore was produced by the addition of 2.0 ml. of 0.1 M thiobarbituric acid and heating for 7.5 minutes at 100°C. This was cooled on ice and then the pink chromophore was extracted into 5.0 ml. of acid butanol (10% v/v with hydrochloric acid). The colour was read at 549 nm against a membrane blank assuming a molar extinction coefficient of 70.7×10^3 (6).

A modification of this method was required for the measurement of whole cell sialic acid owing to the enormous amounts of haemoglobin present. 3.0 ml. of water was added to 1.0 ml. of packed cells and the mixture was shaken vigorously. Two 0.1 ml. samples were removed, diluted 100 fold with water and read at 540 nm so that the sialic acid content could be expressed per mg. of haemoglobin as will be explained below. 3.2 ml. of the rest was added to 0.8 ml. of 0.5 N sulphuric acid and hydrolysed at 80°C for 30 minutes. This was then mixed with 1.0 ml. of 10% phosphotunstic acid (7) and spun at 5K for 15 minutes in a Mistral 6L centrifuge to remove all the contaminating haemolgobin which would interfere with the optical measurements. To 0.5 ml. of the supernatant was added 0.25 ml. 25 mM periodic acid/ 0.125 N sulphuric acid and from this point onwards the procedure was identical to that of the membrane experiment.

Protein

This was measured by the method of O.H. Lowry et al. (8) using bovine serum albumin as the standard. The sample (0.1 ml. in the case of ghosts) was made up to 2.0 ml. with water. To this was added 2.0 ml. of a mixture containing 50 parts 2% sodium carbonate/ 0.1 N sodium hydroxide + 0.5 parts 1% cuprous sulphate + 0.5 parts 2% sodium tartrate. This was mixed and incubated for 30 minutes at 60°C to clear the suspension. 0.2 ml. of 50% (v/v) Folin and Ciocalteu's phenol reagent was then added and after 30 minutes at room temperature was read at 750 nm against a reagent blank.

Haemoglobin Estimation

The 540 nm reading of 100 fold diluted haemolysate or cytoplasm was converted into mgs. of haemoglobin by performing a Lowry estimation of the undiluted sample and assuming that 97% of the cell contents were haemoglobin (9). Thus 1 O.D. Unit at 540 nm is equivalent to 81.1 mg. haemoglobin per ml.

All reagents were purchased from British Drug Houses.

Results and Discussion

The first attempt to effect an age-dependent fractionation of whole blood was made using the method of D. Danon *et al.* (3). This was carried out using blood from both males and females and the results are shown in Fig. 1.1. This illustrates the characteristic sigmoidal density distribution curve (DDC) described by these authors. This kind of curve suggests that the majority of cells have a similar density and that it is only the very youngest and oldest cells which exhibit any marked deviation from this norm. Whatever the explanation, this method would seem suited to collecting the lightest and densest few percent of the erythrocyte population. Fig. 1.1 also shows that the DDC obtained using female blood has a significant shift to the right indicative of a lower mean cell density, and that the points on this curve all have a much greater error. These two features are probably the result of the varying degrees of mild anaemia that post-adolescent females are prone to. These observations taken with the results of S.T. Callender *et al.* (10) which have been described in the Introduction, made it desirable to restrict this study to male blood.

As to the phthallate ester technique itself, this was abandoned for a number of reasons.

1. As each spin took 45 minutes and as the density of the erythrocytes changes on standing (3), it is not desirable to have more than one spin on any one sample of blood. Thus only the lightest and densest fractions could be collected. The results for Fig. 1.1 were obtained simply by reading the calibrations on the centrifuge tubes.
2. Collecting the fractionated samples proved very difficult. It was a relatively simple matter to aspirate cells above the

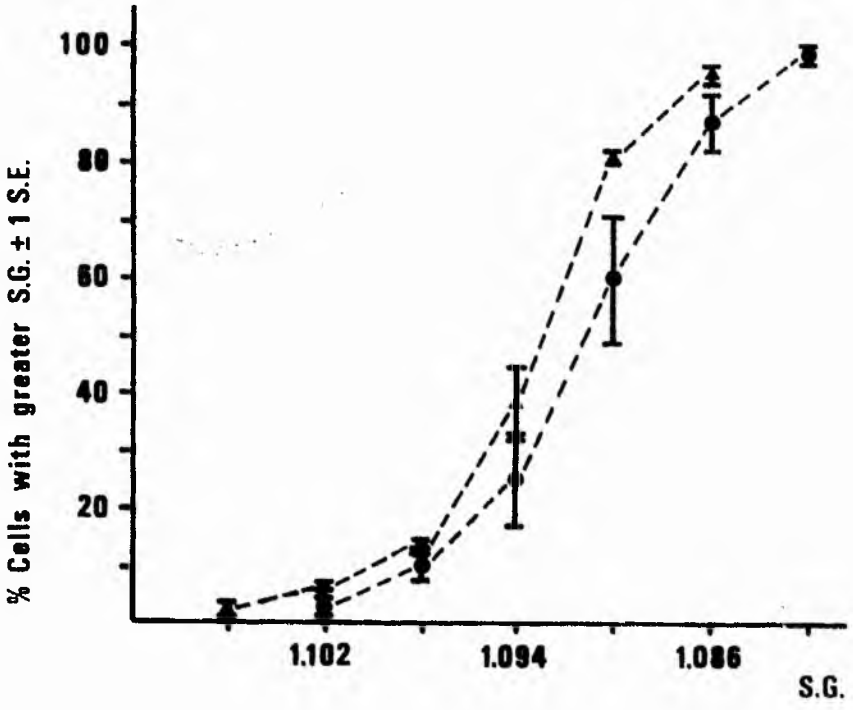

Fig.1.1. The Density Distribution Curves of unfractionated male (▲) and female (●) blood using the phthallate ester technique.

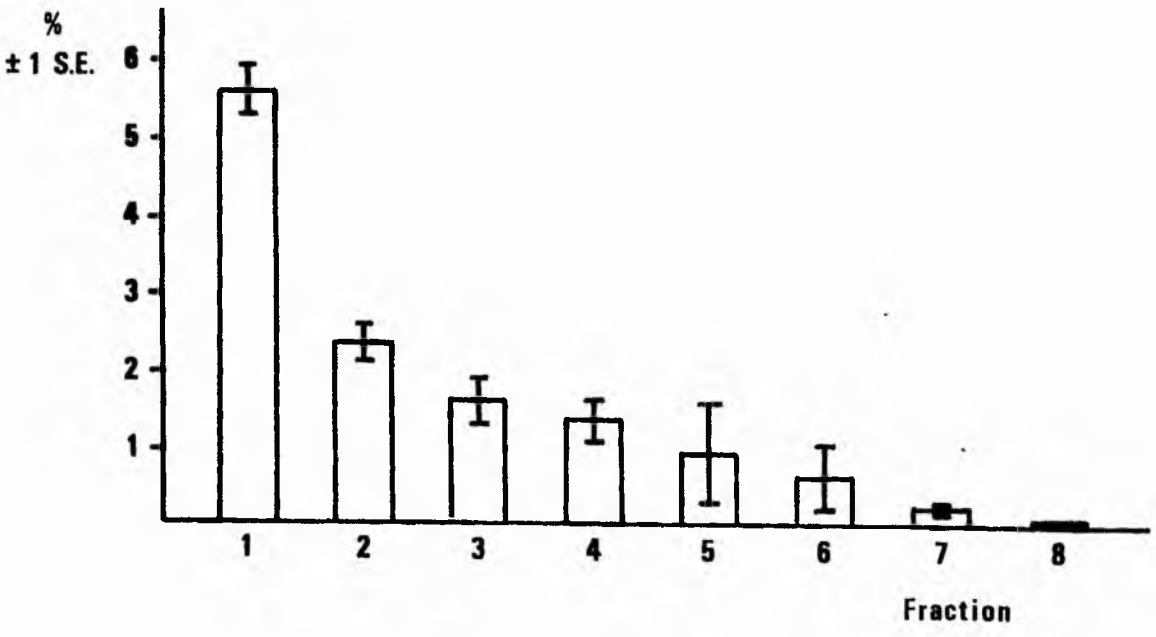

Fig.1.2. The Reticulocyte content of the age-dependent fractions.

ester layer but to obtain the denser cells required first removing this layer. Due to the oily nature of the esters there was some inevitable contamination and it was impossible to remove the emulsified droplets.

3. A more ominous problem was the effect of these esters on the erythrocytes themselves. D. Danon et al. had shown that these substances had no effect on the cytoplasmic enzymes that they investigated, but T.J. Greenwalt et al. (11) had reported a loss of agglutinability following the appropriate manipulations due presumably to adsorption onto the membrane surface. On one occasion in this study a dense and light fraction of erythrocytes was prepared and ghosts were made. These membranes which are normally very loose packing formed a very hard, dense pellet and when washed were almost completely resistant to solubilisation in the nonionic surfactant mixture which is the subject of Chapter 4. The marked increase in density and the resistance to solubilisation of the membranes were both indicative of adsorption of the esters to the membrane surface. This has very serious consequences when one considers the volume of work published by D. Danon, Y. Marikovsky and E. Skutelsky (see Introduction) on the biophysical aspects of the ageing erythrocyte using this technique.

Simple centrifugation offered the only alternative that would provide enough material for a study of the membrane proteins. As suggested by J.R. Murphy (12) this was performed at 30°C as the resulting lower viscosity and greater deformability aids the density stratification of the red cells in the centrifugal field. He also suggested that a fixed angle rotor would provide an even better separation by facilitating an internal circulation of cells. What is meant by this is that the denser cells will move

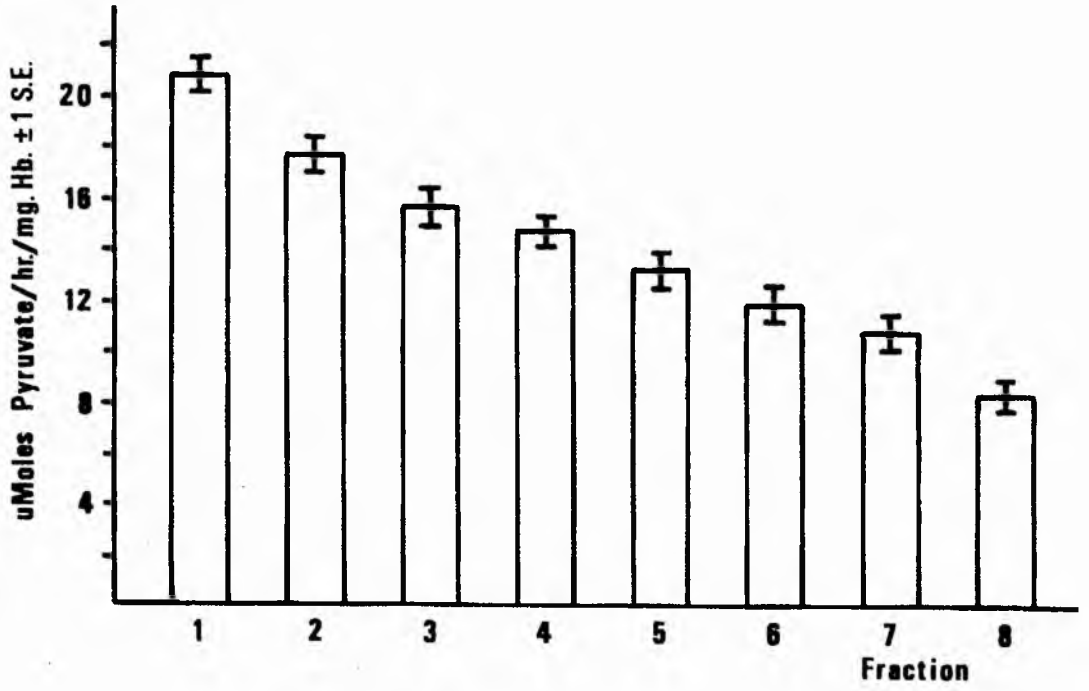

Fig.1.3. The effect of in vivo age on cytoplasmic GOT.

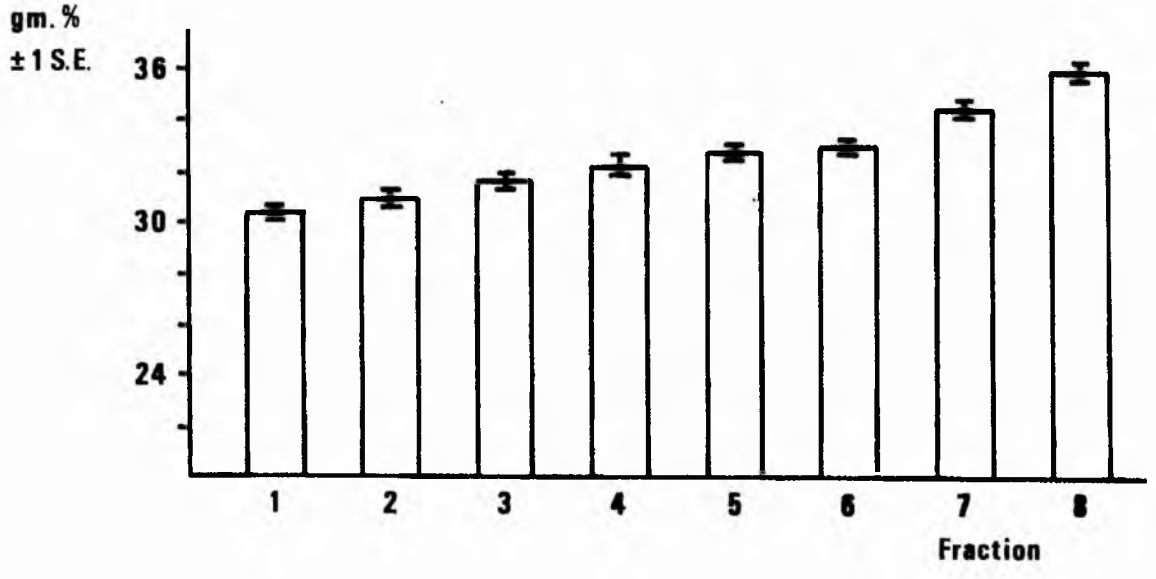

Fig.1.4. The effect of in vivo age on the MCHC.

towards the apex of the tube along the lower surface and the lighter cells will move towards the top of the tube along the upper surface. A suitable rotor was not available for this hypothesis to be tested and thus a swing-out rotor had to be used.

Preliminary experiments using the enzyme GOT as a marker showed that a spin of 60 minutes at 15 Krpm. at 30°C gave the best separation that could be obtained using this technique. Having settled on these conditions, it was necessary to show that a satisfactory separation had been achieved. Three criteria were chosen. The first, reticulocyte count, is shown in Fig. 1.2, where it can be seen that there is a considerable degree of reticulocyte enrichment in the top layers, but that the separation is by no means complete. The second, GOT, had been shown (11, 13, 14) to be a good indicator of mean cell age, and the decrease in specific activity shown in Fig. 1.3 agrees very well with these authors. The third, MCHC, was used by J.R. Murphy himself and the increase of approximately 18% shown in Fig. 1.4 is marginally less than he recorded, but as he compared the youngest 5% of the population with the oldest 5% this is not surprising. Thus, using criteria that have become accepted in the literature as meaningful, it seems that the technique of simple centrifugation adopted here does afford a reasonable degree of age-dependent fractionation. No attempt has been made here to calculate the mean age of each fraction. This would have involved radioactive labelling of the red cells and it is certain that the volunteers would not have been forthcoming. Figs. 1.3 and 1.4 show that the separation is at least as good as that obtained by other authors in this field, and Fig. 1.2 shows that there is still room for improvement.

There are, however, serious objections that can be levelled against the criteria which have been used to verify the fractionation.

Reticulocyte Contamination

The reticulocyte must have a surface area no less than that of the erythrocyte and it is known to be spherical (15). Therefore, it must have a significantly greater volume than the erythrocyte. As a result, it could be argued that the larger reticulocyte must have a lower MCHC and that the increase in MCHC shown in Fig. 1.4 is a consequence of decreasing reticulocyte contamination. This can be countered by subjecting this argument to mathematical verification. In the formula derived below it is assumed that the reticulocytes and erythrocytes have a similar and constant MCH and that the volume of the erythrocyte is constant with age. The purpose of the formula, therefore, is to calculate what the volume of the reticulocyte would have to be, using the observed values for MCHC, % reticulocytes and % erythrocytes, to produce the observed increase in MCHC.

V_e = volume of the erythrocyte

V_r = volume of the reticulocyte

N_e = % erythrocytes

N_r = % reticulocytes

Assume constant MCH with age

" " V_e " "

$$\text{MCHC } (\%) = \frac{N_e}{V_e} + \frac{N_r}{V_r} \qquad (\text{If } N_e + N_r = 100)$$

$$\frac{\text{MCHC} \times V_e}{N_r} = \frac{N_e}{N_r} + \frac{V_e}{V_r}$$

$$\frac{V_e}{Vr} = \frac{MCHC \times V_e}{N_r} - \frac{N_e}{N_r}$$

$$\frac{V_e}{V_r} = \frac{MCHC \times V_e - N_e}{N_r}$$

$$\frac{V_r}{V_e} = \frac{1}{\frac{MCHC \times V_e - N_e}{N_r}}$$

Let $V_e = 1$

$$V_r = \frac{1}{\frac{MCHC - N_e}{N_r}}$$

Fraction	% MCHC	Ratios.	% RBC	V_r
8	100	0	100	-
7	95.83	0.23	99.77	- 0.059
6	91.67	0.63	99.37	- 0.082
5	91.11	0.93	99.07	- 0.117
4	89.72	1.36	98.64	- 0.153
3	88.06	1.60	98.40	- 0.155
2	86.11	2.37	97.63	- 0.206
1	84.44	5.63	94.37	- 0.565

Table 1.1. The calculation of V_r from the above formula and the information contained in Figs. 1.2, and 1.4.

It is clear from Table 1.1 that we have set the reticulocyte an impossible task as the formula consistently generates negative values. In fact S.J. Shattil et al. (16) who used the technique of induced reticulocytosis on rats (see Introduction) calculated that the reticulocyte to erythrocyte transformation was accompanied by a loss of 25% of the cell haemoglobin. Thus if a similar situation were to exist in the

human, the reticulocyte MCHC would be nowhere near as low as the simple volume ratio would suggest. It seems, therefore, that we can disregard the suggestion of reticulocyte contamination for the moment.

Age-Dependent Packing

The weight of evidence suggests that the sialic acid content of the erythrocyte decreases with age and as this substance is thought to be the major if not only determinant of surface charge (17), the older cells might be expected to pack more tightly when the haematocrit is measured due to a decrease in repulsion. It is thus feasible that there could be no decrease in volume with age but an increase in packing density giving an artifactual increase in MCHC. As the GOT measurements are independent of cell packing, the degree to which the latter is involved can in no way invalidate the conclusion that an age-dependent separation has taken place, but it could have a great effect on the volume and MCHC changes which are thought to occur with age.

A brief look at the literature regarding the sialic acid content of the ageing erythrocyte revealed that the conflict (Table 7 - Introduction) was almost undoubtedly due to the manner in which the results had been expressed. When the sialic acid content was expressed per cell, a decrease of 10-15% was found (7, 18), but when it was expressed per mg. of membrane protein, no change was found (19). Another study (20) involving organic solvent extraction of the membrane glycoproteins was undertaken, but as no attempt was made to assess % recovery, the results need not concern us. In order to resolve this situation sialic acid was measured per mg. of membrane protein (Fig. 1.5) and

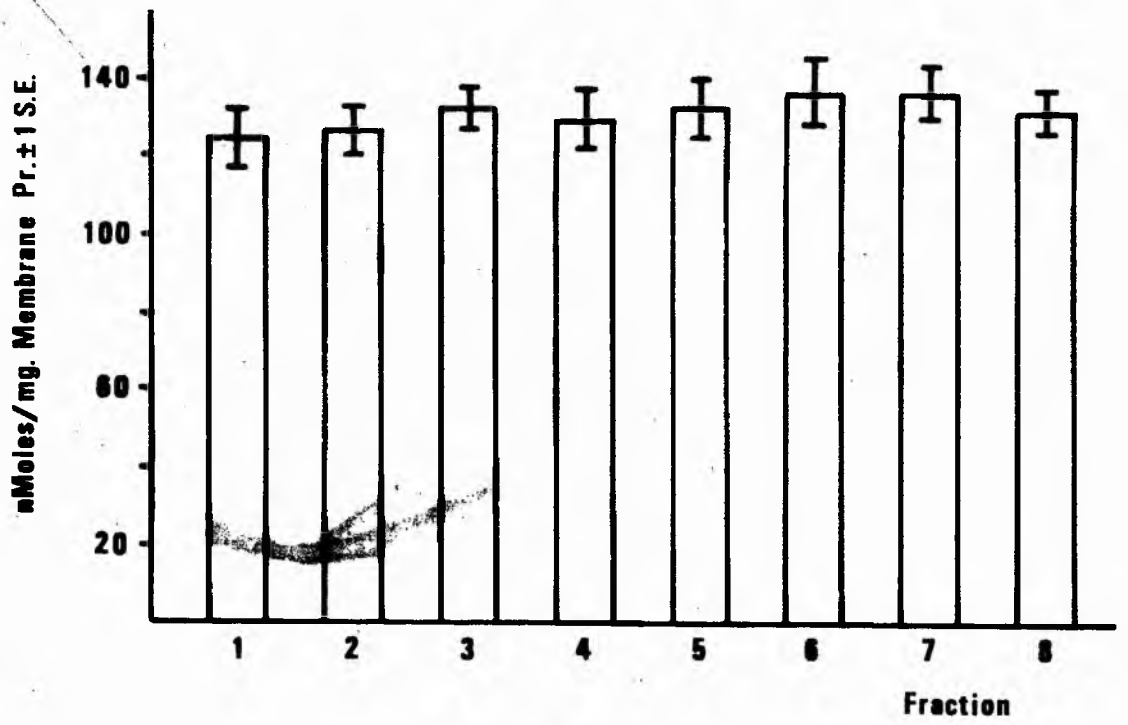

Fig.1.5. The effect of <u>in vivo</u> age on the Sialic acid content of the isolated membrane.

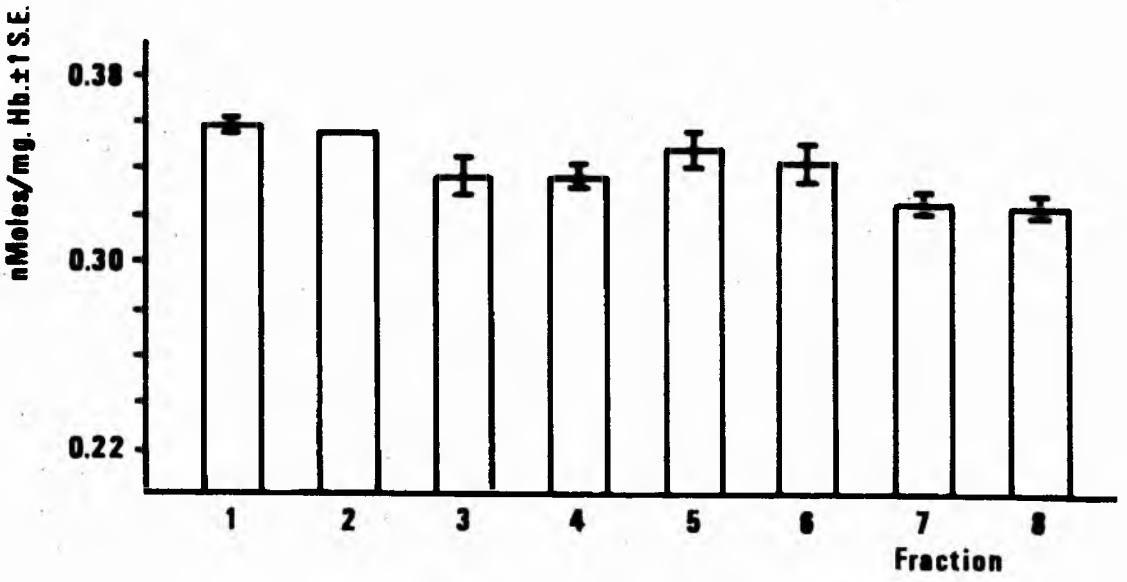

Fig.1.6. The effect of <u>in vivo</u> age on the Sialic acid content of the whole cell. $p < 0.01$ by Analysis of Variance.

per mg. haemoglobin (Fig. 1.6). Haemoglobin was chosen since it can be measured more accurately and easily than cell number and is thought to be constant with age (21). As expected there was no change per mg. of membrane protein but a significant decrease per mg. haemoglobin. Thus, the previous reports have been confirmed and the likely explanation of this apparent conflict is that there is no selective elution of sialic acid from the membrane with age, but that there is a loss of membrane fragments leading to the previously reported decrease in surface area (22). The decrease in surface area of 11% reported by C. Van Gastel et al. (22) agrees well with the 10% decrease in sialic acid content of the whole cell found here.

We now have to ask whether a loss of surface area, associated with a constant charge density (assuming that the sialic acid does not somehow become masked or unmasked with age) can produce an increase in packing density. This problem is compounded by the suggestion that the loss of surface area with ageing is associated with a sphering or thickening of the cell (22, 23). Not only would the change in shape effect the packing by simple geometry but alterations in the radius of curvature would also effect the charge density.

Before proceeding any further, the MCV, MCH and MCHC of fractions 1 and 8 were measured by standard haematological techniques to put this enquiry onto a firm footing. The results shown in Fig. 1.7 confirm that the haemoglobin content of the erythrocyte is constant with age and so if there is any loss at the time of reticulocyte maturation it is not great enough to be detected here. This parameter is calculated by dividing the haemoglobin content of a given volume of blood by the number of cells present and so is not affected by cell packing. The

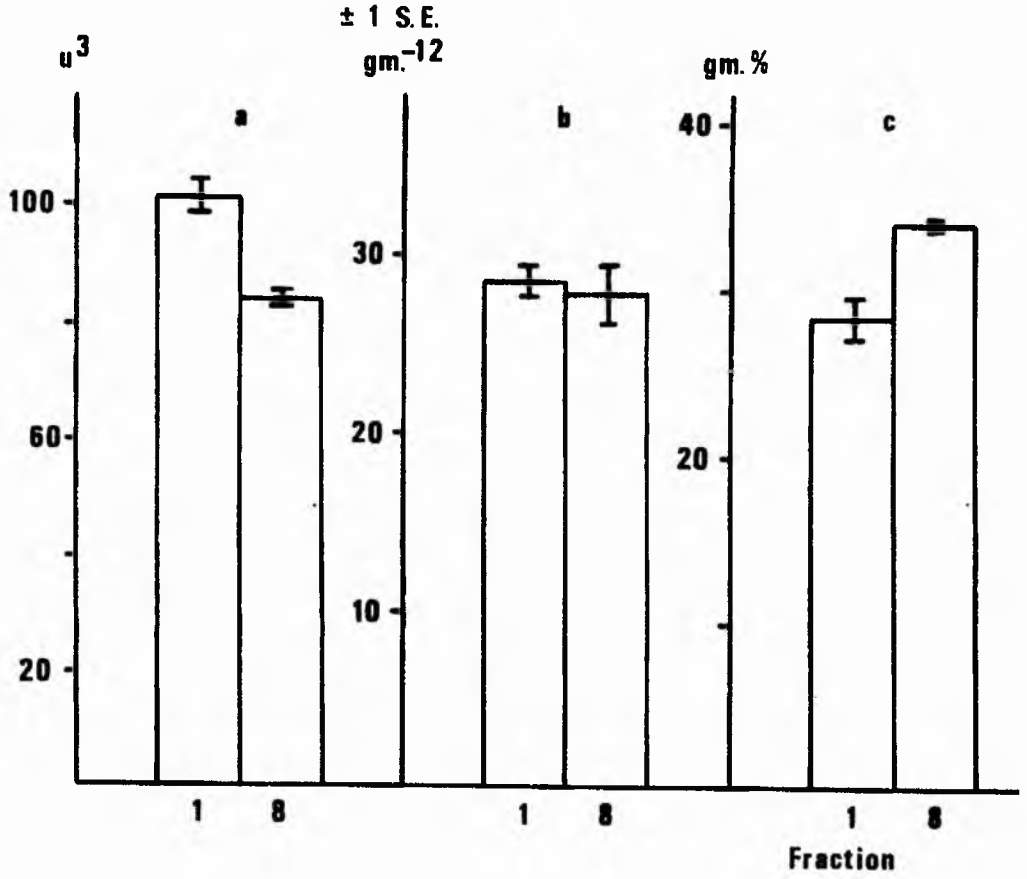

Fig.1.7. The effect of <u>in vivo</u> age on the MCV (a), MCH (b) and MCHC (c).

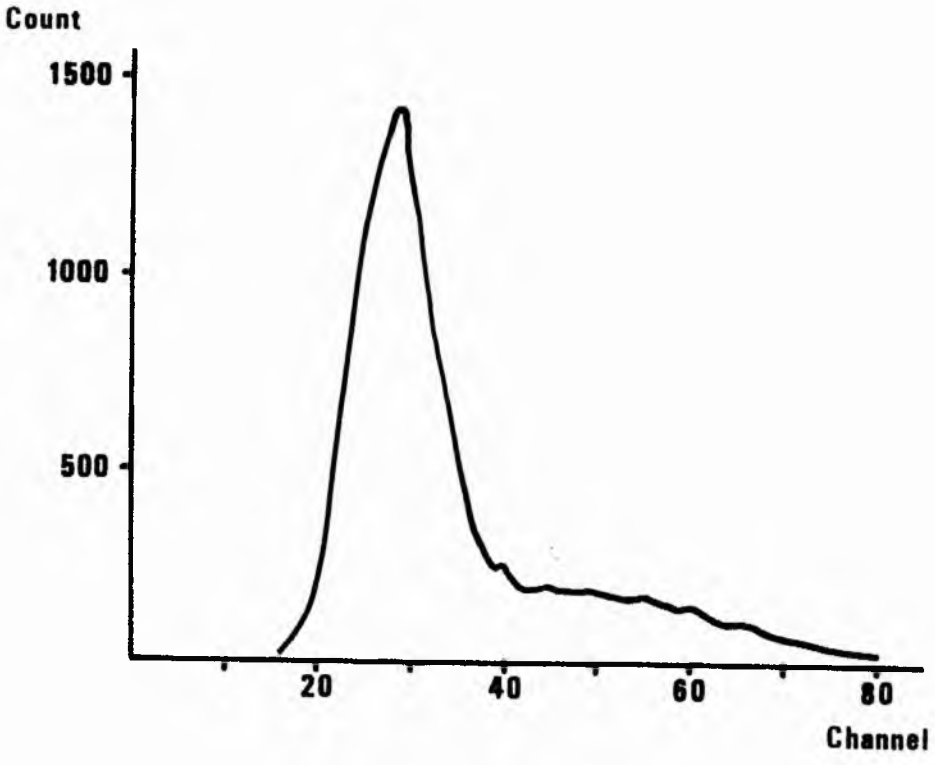

Fig.1.8. The typical FDC of unfractionated red blood cells given by the Coulter technique.

constancy of the MCH adds weight to the sialic acid measurement and confirms that Fig. 1.6 gives a true account of the change in sialic acid content per cell. A 17% decrease in MCV with an equivalent increase in MCHC was also found.

No further progress being possible using these indirect techniques, it became necessary to measure the cell volume directly. The Coulter Counter offered the best possibility for doing this but there is unfortunately considerable disagreement as to the interpretation of the frequency distribution curve (FDC) that is obtained. Fig. 1.8 demonstrates that neither a normal nor a log. normal distribution is obtained but instead there is a considerable skew on the right-hand, descending slope. As will be seen below the arguments centre on whether this skewness of the FDC is indicative of a real population of large cells or whether it is the result of an artifact of the Coulter method. The various suggestions can be discussed in the light of the relevant publications and experiments conducted in this study.

Coincidence

Coincidence is the likelihood of more than one cell passing simultaneously through the counting orifice and being counted as a single cell of a much larger volume. G. Brecher *et al.* (24) reported that increasing the number of cells per unit volume caused an increase in the skew, which suggested that the latter was the product of coincidence. Conversely, A. Ur *et al.* (25) found that such a procedure had no effect on the curve until a certain critical point was reached. Beyond this point there was a slight shift of the curve to the left which can in no way be explained by coincidence. They suggested that increasing the

pulse frequency of the counting device may eventually lead to a sudden increase in impedance. H. Wintor et al. (26) calculated that coincidence could not possibly account for the skew and Fig. 1.9 supports this view by showing that over a concentration range of fifty fold there was no change in the appearance of the skew.

Deformability

B. Buckhold Shank et al. (27) attributed the skew to the deformability of the red cell during its passage through the counting orifice. In support of this they found that treatment with glutaraldehyde and also counting at 4°C eliminated the skew. In this study, counting the cells at 4°C (Fig. 1.10) had a negligible affect on the FDC whereas 1% glutaraldehyde did produce the predicted affect (Fig. 1. 11). However, pretreatment of the cells with 0.025% trypsin (Fig. 1. 12) produced almost the same effect as did the glutaraldehyde. As trypsin might be expected to have the opposite effect on membrane deformability it seems unlikely that this property of the erythrocyte is responsible for the skew. A more likely explanation is that one is observing a direct effect of these agents on the size of the erythrocytes.

Orientation

J. Gutmann et al. (28) argued that the two peaks were caused by the two orientations which the red cell can adopt in the current field across the counting orifice, that is, parallel or vertical to it. Using plastic models in an electrolytic trough they concluded that a red cell passing parallel to the current flow would cause negligible distortion of the field and give a

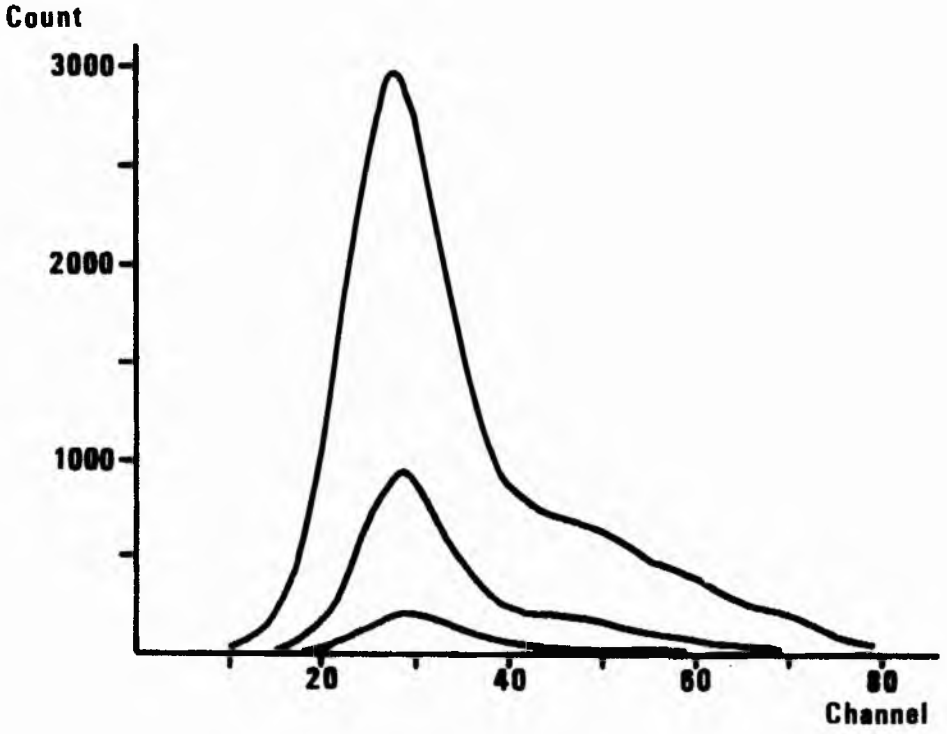

Fig.1.9. The effect of cell number per unit volume on the FDC of unfractionated red blood cells.

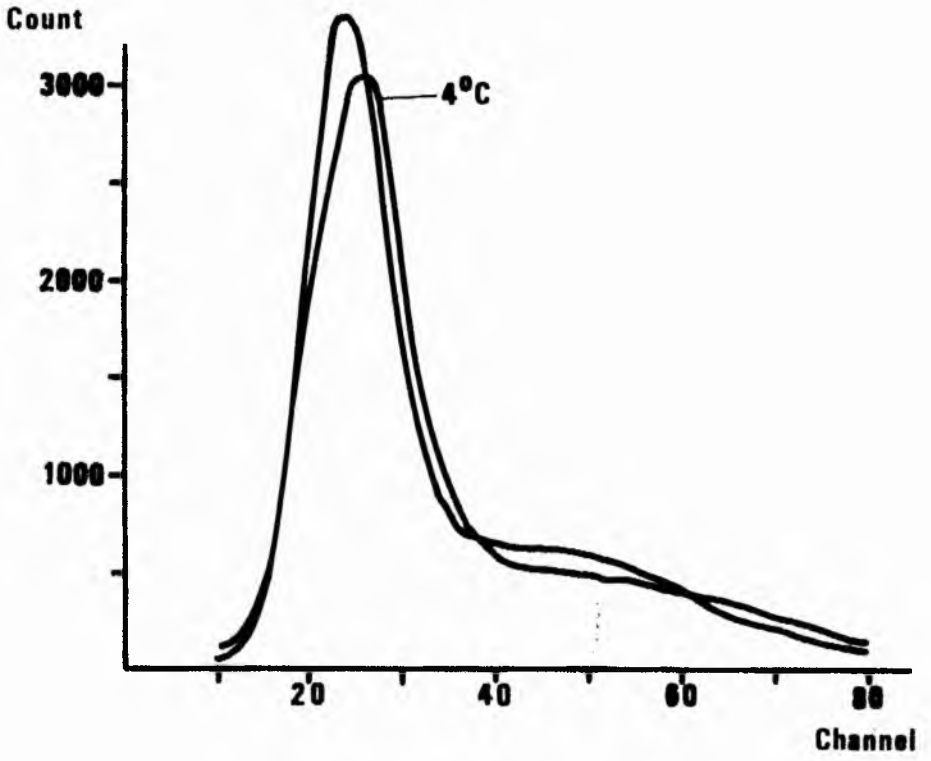

Fig.1.10. The effect of cooling to $4^{o}C$ on the FDC of unfractionated red blood cells.

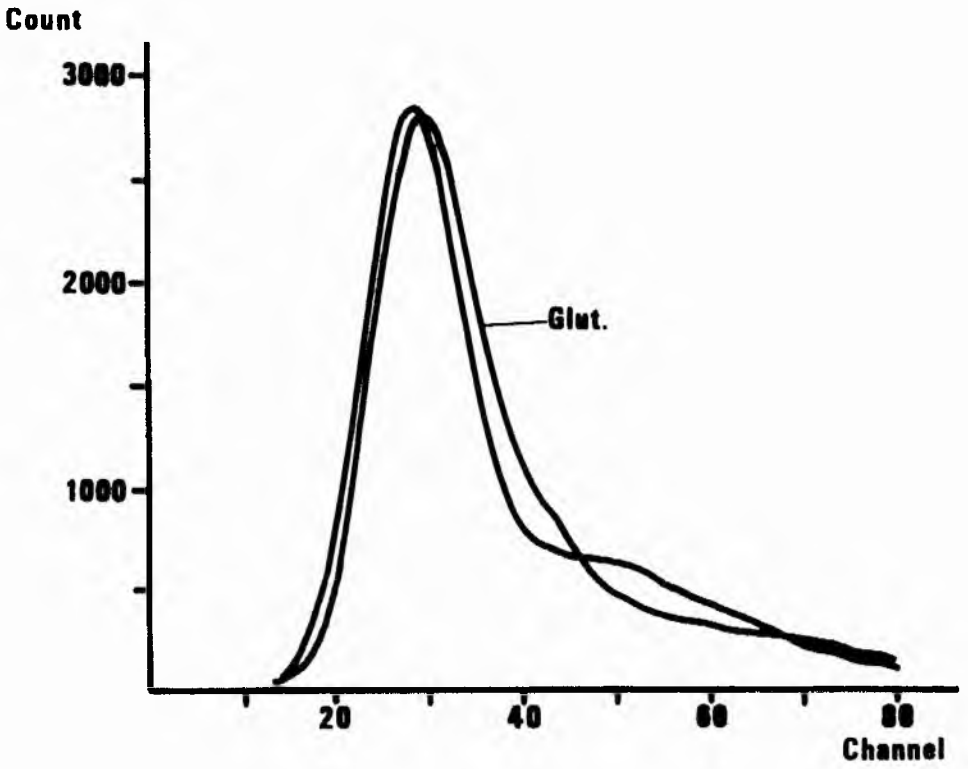

Fig.1.11. The effect of 1% Glutaraldehyde on the FDC of unfractionated red blood cells.

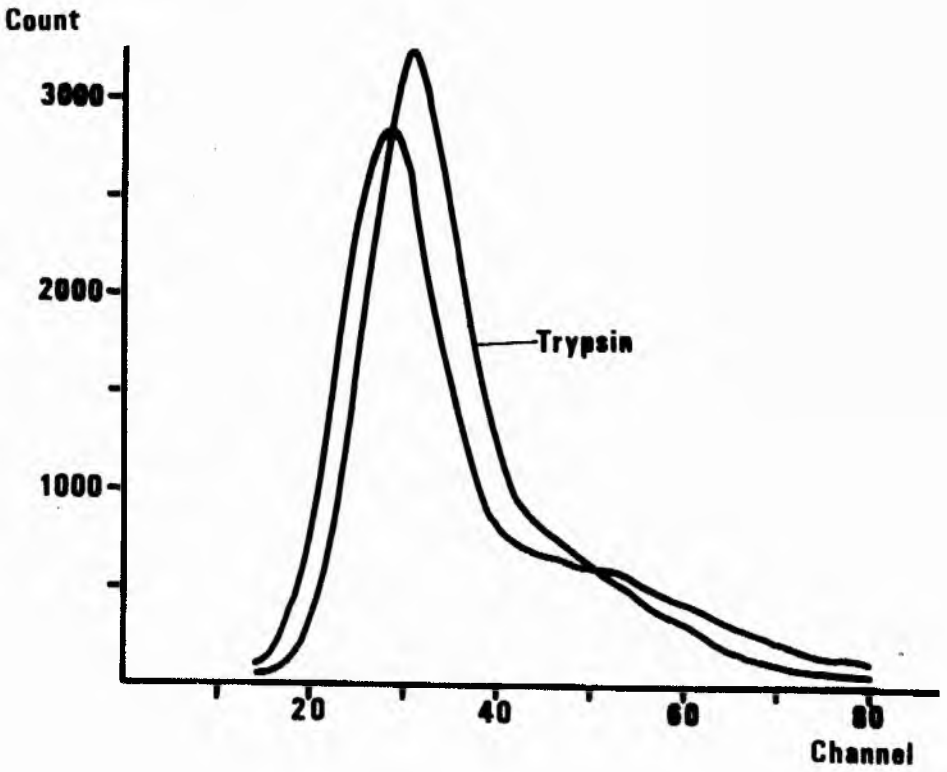

Fig.1.12. The effect of 0.025% Trypsin on the FDC of unfractionated red blood cells.

true volume reading, whereas those passing vertically would give rise to an electrical shadow causing a 40% increase in the volume reading. A. Ur *et al.* (25), however, felt sure that at the flow rates used, all the cells would be passing parallel to the current axis and showed that decreasing the flow rate (which should cause an increase in the incidence of cells passing vertically) had no effect on the skew. They also pointed out that this mechanism could account for the observed skew only if among all the possible orientations there were but two that predominated. In addition R.I. Weed *et al.* (29) found that yeast cells, which are considerably more sphered than erythrocytes, gave a skewed curve, and that ghosts which are flatter than erythrocytes gave a normal distribution. Thus, the explanation is unlikely to be due to a simple shape effect.

Haemoglobin Effect

R.I. Weed *et al.* (29) looked at the problem from the other side of the curve and suggested that the distribution pattern differs from the Gaussian because of the displacement of the small cells towards the population mean, rather than of normal cells away from the mean towards a larger apparent volume. The proposed mechanism for this is that haemoglobin at high concentrations has an anomalously high osmotic pressure. Thus the very smallest cells (assumed to be the oldest) having the greatest MCHC swell in the isotonic medium to give an aberrantly high volume.

Miscellaneous Effects.

H. Winter et al. (26) discussed the possibility that the skew may arise from the conversion of a linear scale of diameters to the cubic scale of volumes. This was dismissed mathematically and in addition by pointing out that the heights and weights of a homogeneous population of adult males are both normally distributed. A. Ur et al. (25) found that a continuous exposure of aperture current of at least 25 minutes was necessary to produce volume changes in the erythrocyte. The changes could be observed microscopically, adjacent to the cathode and were thought to have been caused by alterations in the pH of the suspending medium. As the red cell spends 32 - 35 microseconds in the counting orifice a direct effect of the electrical field on the red cell itself is most improbable. This shortness of transit time also precludes such suggestions as variations in electrophoretic mobility and local heating.

Volume

The simplest explanation of the skewed or bimodal FDC is that there are two populations of cells with overlapping FDC's present. This view has been put forward by C.C. Lushbaugh et al. (30) and A. Ur et al. (25) in the human and H. Winter et al. (26) in sheep. All of these groups have suggested that the larger population of cells, which constitutes the major part of the skewed region, represents the younger cells. H. Winter et al. postulated that all the cells have a similar diameter giving rise to the familiar, normally distributed Price-Jones curve (31) and that the larger cells are merely thicker. This they concluded,

could explain the inability of C.C. Lushbaugh *et al.* to demonstrate microscopically the existence of two populations.

It can be seen in the methods section that the MCH and MCV are calculated using the red cell count as the denominator. The counts used to obtain the values in Fig. 1.7 were arrived at by integrating the total area under the FDC and as these agree very well with those obtained by the standard haemocytometer method, common sense argues that coincidence cannot be occurring to any significant extent. Thus even if the shape of the FDC is an artifact (and it will be demonstrated that this is unlikely) we can at least have faith in the red cell count given by this procedure. We can take this common sense argument one step further when considering the direct MCV given by the Coulter Counter. If the skewed region represents the presence of real cells which have somehow been detected as having an aberrantly large volume, then one is forced to conclude that the cells present in the main peak have somehow been registered as having a volume just low enough to counteract the effect of the skewed region, so yielding a similar MCV to that given by standard techniques. This is what B. Buckhold Shank *et al.* (27) are asking us to believe. This conclusion also argues against the suggestion of J. Gutmann *et al.* (28) who have stated that the main peak represents the true volume. As will be seen below, this yields much too low a value for the MCV.

Spheres

The one accepted observation is that osmotic swelling of the erythrocytes produces a unimodal FDC as seen in Fig. 1.13. This phenomenon has been interpreted by B. Buckhold Shank *et al.*, J. Gutmann *et al.* and R.I. Wood *et al.* as supporting their

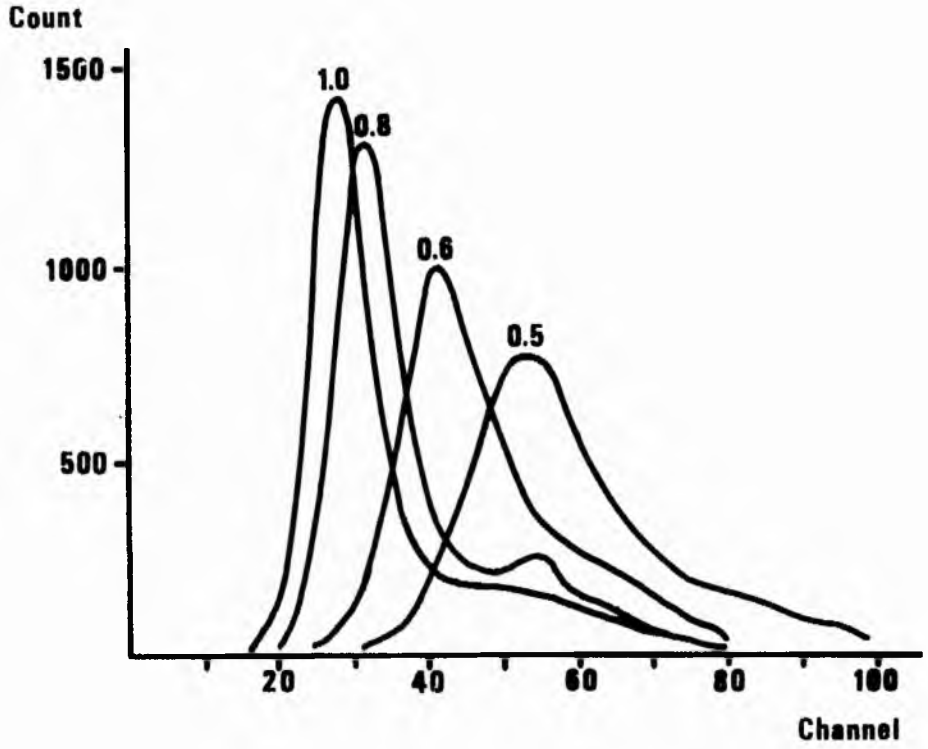

Fig.1.13. The effect of hypotonicity on the FDC of unfractionated red blood cells. (1.0 = isotonicity).

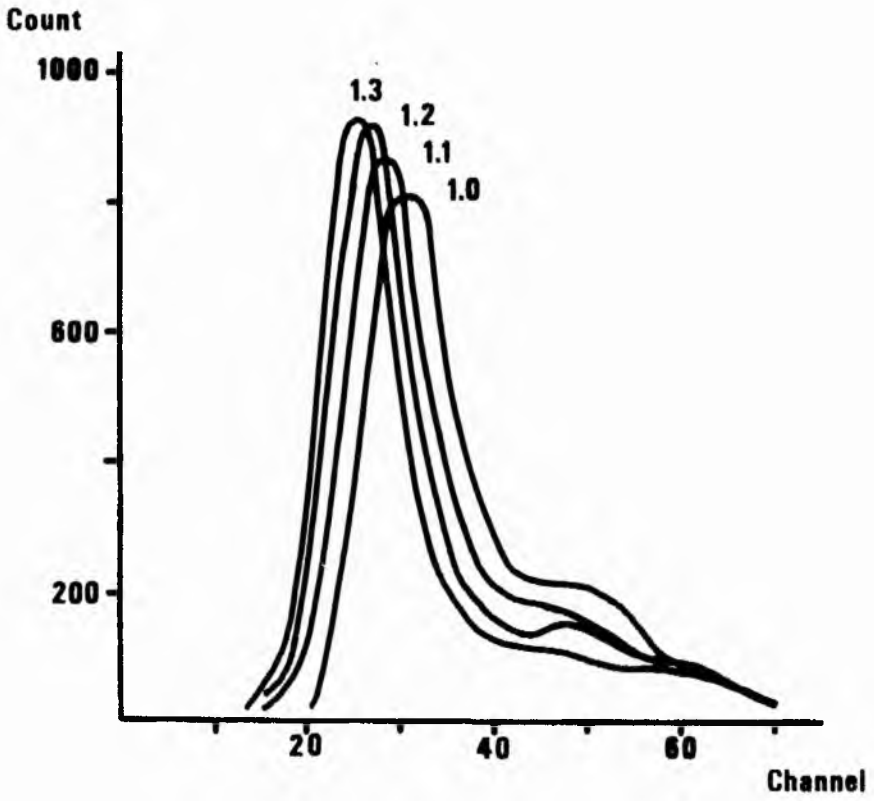

Fig.1.14. The effect of hypertonicity on the FDC of unfractionated red blood cells. (1.0 = isotonicity).

particular hypotheses, i.e. spheres are less deformable than biconcave discs; spheres have only one possible orientation; dilution of the cell contents abolishes the anomalous osmotic effect of haemoglobin. What has not been mentioned or perhaps not noticed by these authors, but which is very clear from this figure, is that this population of spheres is positioned in the region of the skew. The lowest concentration used in this experiment was 50% Isoton (below which haemolysis is certain to begin) and so the MCV in this case must approximate to the maximum pre-haemolytic volume. The MCV for this population was calculated from several experiments to be 153 ± 3.1 u^3 which is very similar to the calculated volume of a sphere possessing the same surface area as the initial biconcave disc (32, 150 u^3) and so the presence of spheres is assured.

This finding, that what are known to be spheres are found in the same region as the skewed portion of the normal FDC, has led to the postulation here that the skew is produced by the presence of a certain proportion of sphered or nearly sphered cells as well as biconcave discs. F. Doljanski et al. (33) have observed this skew when sizing the cells in 100% plasma and thus if this hypothesis is correct the sphere may not necessarily be the pathological conditions that one usually accepts it as being. Of course, even if this hypothesis is correct it is possible that the spheres are being produced as a result of the stress of passing through the counting orifice. This would fit with the observations of J. Gutmann et al. and F. Doljanski et al. that the characteristics of the skew are very dependent on the dimensions of the orifice. It could, however, be just as easily argued, as A. Ur et al. have done, that altering the dimensions of the counting orifice can affect

the ability of the machine to discriminate the two different populations.

In order to test the hypothesis further, red cells were subjected to a mild hypertonic stress in phosphate buffered saline (it being more convenient to prepare this medium than to concentrate Isoton). This caused a shift of the main peak to the left and what at first appeared to be a diminution in the proportion of cells in the skewed region (Fig. 1. 14). However, when the percentage of cells contained in the skewed region was calculated (this was achieved by assuming that the point of inflection of the curve represented the perpendicular boundary between the two populations) it was found to be approximately 25% for all four tonicities. That is to say, increasing the osmotic strength of the diluting medium produced a very noticeable decrease in the volume of the main peak but had very little effect on the skewed population.

Can the suggestions of deformability and orientation explain this observation? Both are dependent on the biconcave shape for their *modus operandi* but as R.P. Rand *et al.* (23) have shown that at a concentration of 1.3 times tonicity the red cell population consists almost entirely of crenated spheres, they are obviously rendered impotent. A crenated sphere is neither highly deformable, nor does it have two orientations. In the light of the observation of R.P. Rand *et al.* both of these hypotheses predict that hypertonicity would cause the FDC to shift towards the left and become more unimodal. The haemoglobin effect is also unable to explain the observation as the hypertonicity would be expected to produce a greater number of cells with an anomalously high osmotic pressure giving rise to an increase in the skewed population.

Once again it is clear that there are two populations of cells present with very different volumes. As the population of cells having the greater MCV and what are known to be spheres have very similar volumes, there can be no viable alternative to the conclusion that the cells present in the skewed region are spheres and they will be referred to as such from this point onwards. The only other explanation, within the bounds of a true volume difference, is that the cells of the skewed region are biconcave discs with a much increased diameter. However, the appearance of the already mentioned Gaussian Price-Jones curve, which depicts the distribution of red cell diameters, refutes this. In the case of Fig. 1.14, the working hypothesis of this study predicts that two populations of crenated spheres have been formed by the hypertonic treatment.

Spheres have been shown in the present study to occur in both Isoton and phosphate buffered saline and have been reported in the presence of 100% plasma (33). As the concept of a healthy but sphered erythrocyte is anathema to the haematologist, considerable thought was given to how this situation might have arisen following removal of the cells from their natural environment. Stress within the counting orifice has already been mentioned and this will be discussed later. The only other possibility was temperature, the experiments being conducted at room temperature which is a full 15°C below normal body temperature. Fig. 1.15 shows that counting the cells in Isoton prewarmed to 37°C had no qualitative effect on the FDC. Thus, while it cannot be concluded that sphered erythrocytes are normally present in the circulation, they are almost certainly present <u>in vitro</u>.

<u>Direct Measurement of Red Cell Volume</u>

From the point of view of the aim of this particular

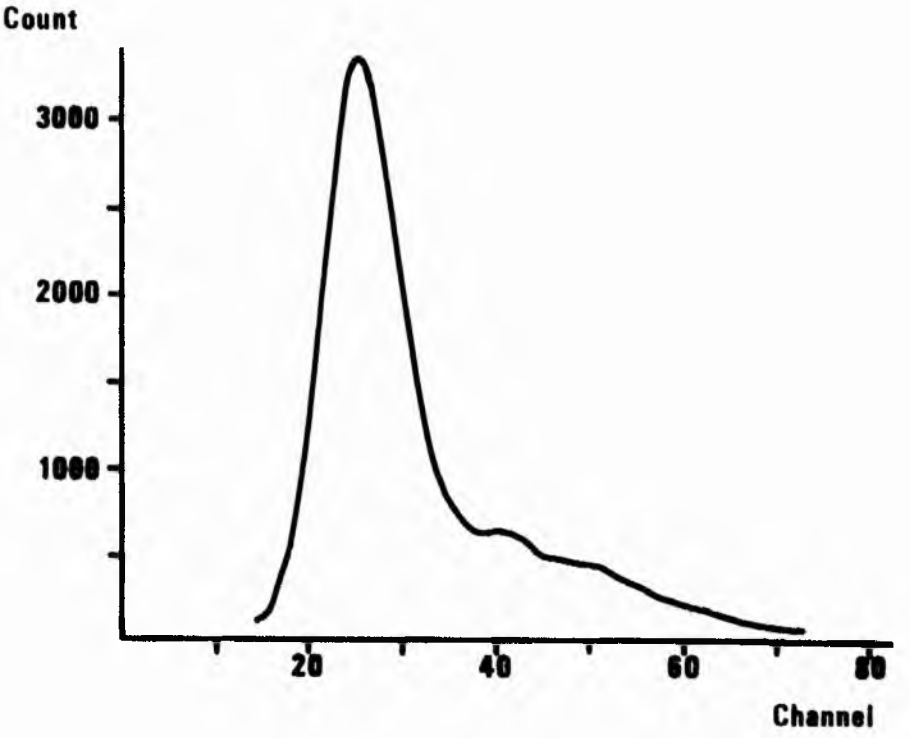

Fig.1.15. The effect of warming to 37°C on the FDC of unfractionated red blood cells.

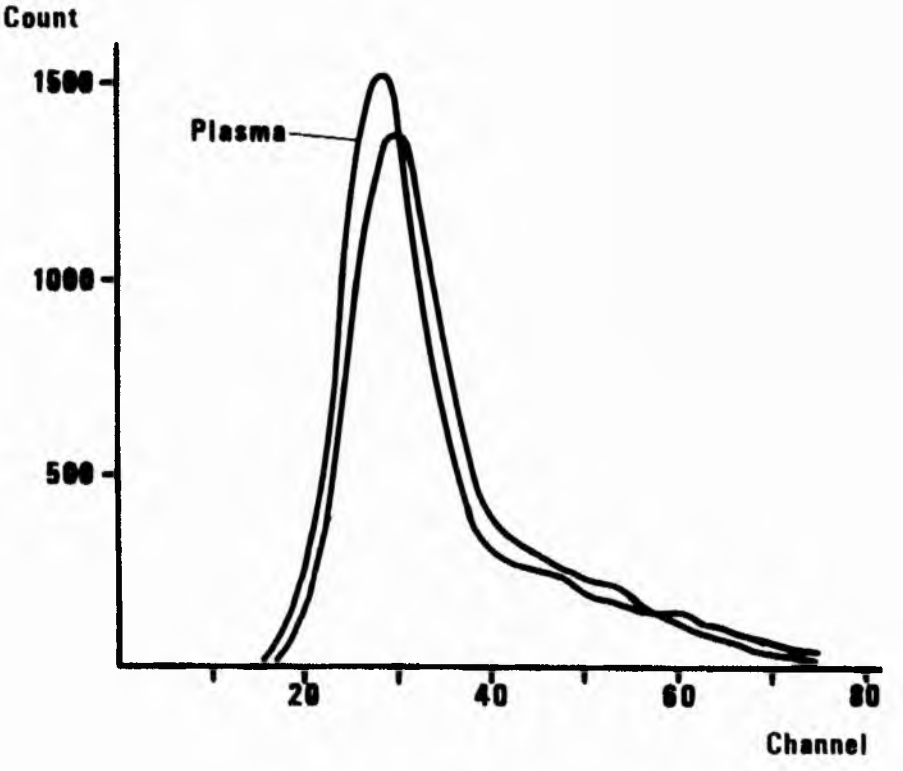

Fig.1.16. The effect of 10% plasma in the counting fluid on the FDC of unfractionated red blood cells.

experiment (to ascertain the true volume behaviour with ageing) it has been shown that the whole FDC must be considered as giving a true representation of the volume of the red cell population. It would naturally have been preferable to measure the MCV in the donor's own plasma but the volume of fluid needed to aspirate and dilute the cells prevented the final plasma concentration from exceeding 10% (v/v). It can be seen from Fig. 1. 16 that the presence of 10% plasma had a negligible qualitative effect but caused a slight decrease in the MCV. As there was no effect on the distribution of latex spheres (results not shown) this was not caused by a change in the conductivity of the counting medium. This decrease is similar to the observation of A.W.L. Jay (34) on the effect of bovine and human serum albumin on red cell volume, and it is probably more correct to conclude that the removal of serum proteins causes a slight increase in the MCV.

Although Coulter claim that the red cell volume remains constant for 30 minutes in Isoton, it was felt necessary to put this to the test in the presence and absence of 10% plasma (Fig. 1.17). The graph does not go beyond 15 minutes as the cells were always sized within this time following separation, and again 10% plasma had no qualitative effect. Both curves were essentially static over this period of time and so the ageing experiment was performed in the presence of 10% plasma, this being as near physiological as could be achieved.

This experiment was conducted on the youngest (fraction 1) and the oldest (fraction 8) erythrocytes and a typical result is given in Fig. 1. 18 in which there are two points of interest. Firstly, the main peak is shifted to the left and secondly, the skew is considerably reduced in the oldest cells. It was found

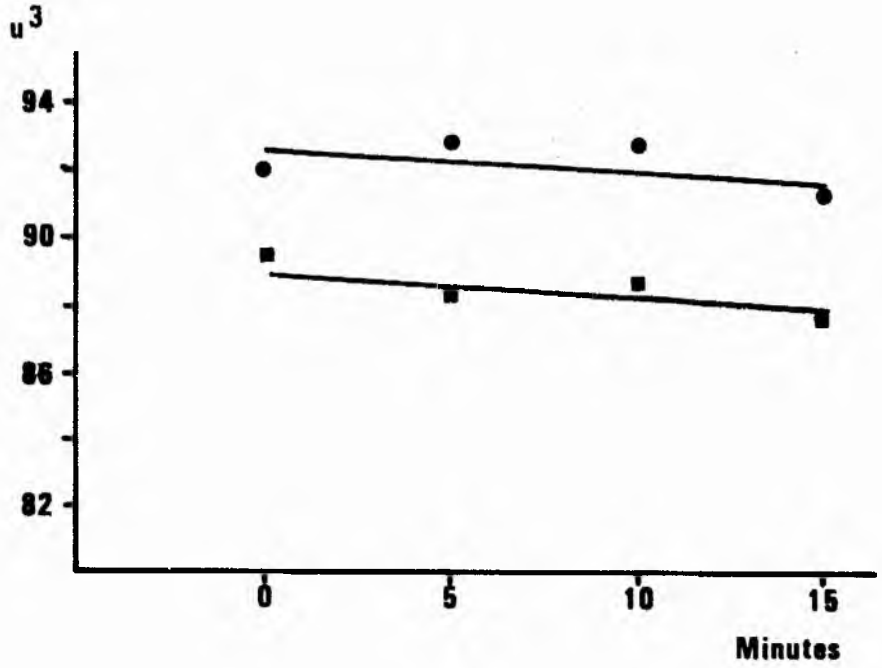

Fig.1.17. The effect of time on the MCV calculated from the FDC in the presence (■) and absence (●) of 10% plasma.

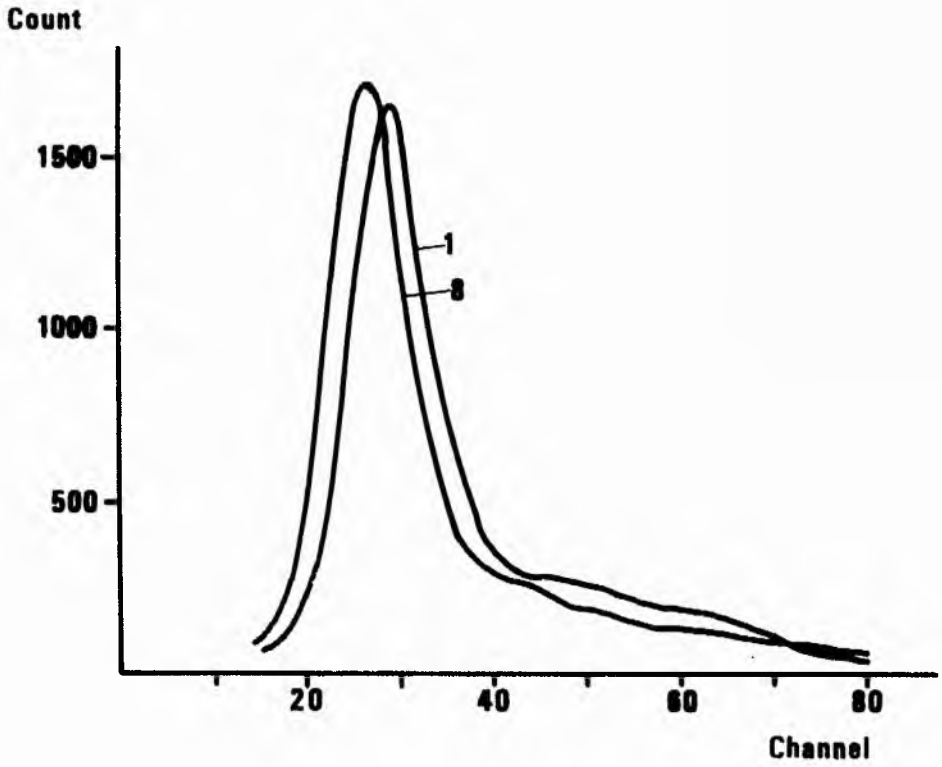

Fig.1.18. The effect of <u>in vivo</u> age on the FDC.

that subtracting the counts in the skewed region of fraction 8 from that of fraction 1 gave a population shown in Fig. 1. 19. The FDC of fraction 1 from Fig. 1.18 is included for comparison. This curve contained 4.6 ± 0.6% of the total cells present and this is close to the percentage of reticulocytes found in fraction 1 (5.6% - see Fig. 1.2). It was therefore concluded that this age-dependent depression of the skew was caused by the absence of reticulocytes having an MCV of 141 ± 3.3 u^3. This was taken as yet further evidence for the skew representing a true population of larger cells, for if the presence of spherical reticulocytes is acknowledged then postulating the existence of spherical erythrocytes does not require a great logical jump.

In order to analyse the erythrocyte's FDC further it was decided to assess as accurately as possible the relative proportions of cells contained in the two portions of the curve. This was achieved by making two assumptions:

1. The cells present in the main peak follow a log normal distribution.
2. The left-hand, ascending slope of the main peak is not contaminated by the sphered cells of the second peak.

The procedure adopted was then as follows:

1. Sum the successive channel counts and make a note of the final total.
2. Reduce this final total by various percentages to give trial totals.
3. Express the summated values of the ascending slope of the main peak as percentages of the trial totals.
4. Plot these percentages on log-probability paper and extrapolate to generate the values of the descending slope.

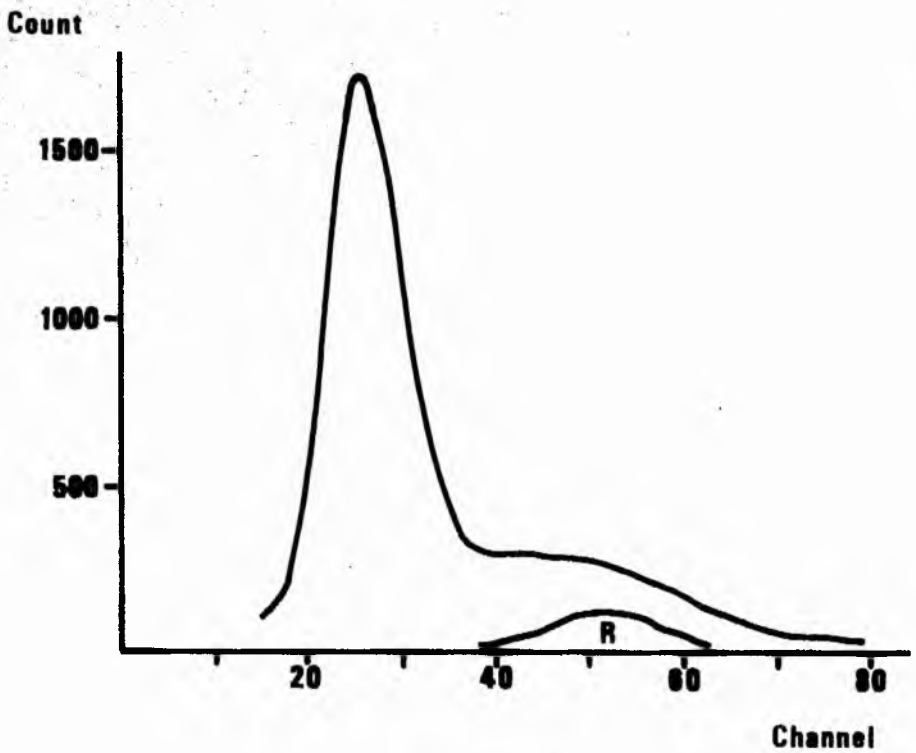

Fig.1.19. The FDC of the reticulocyte population (R) contained in the skewed region of fraction 1.

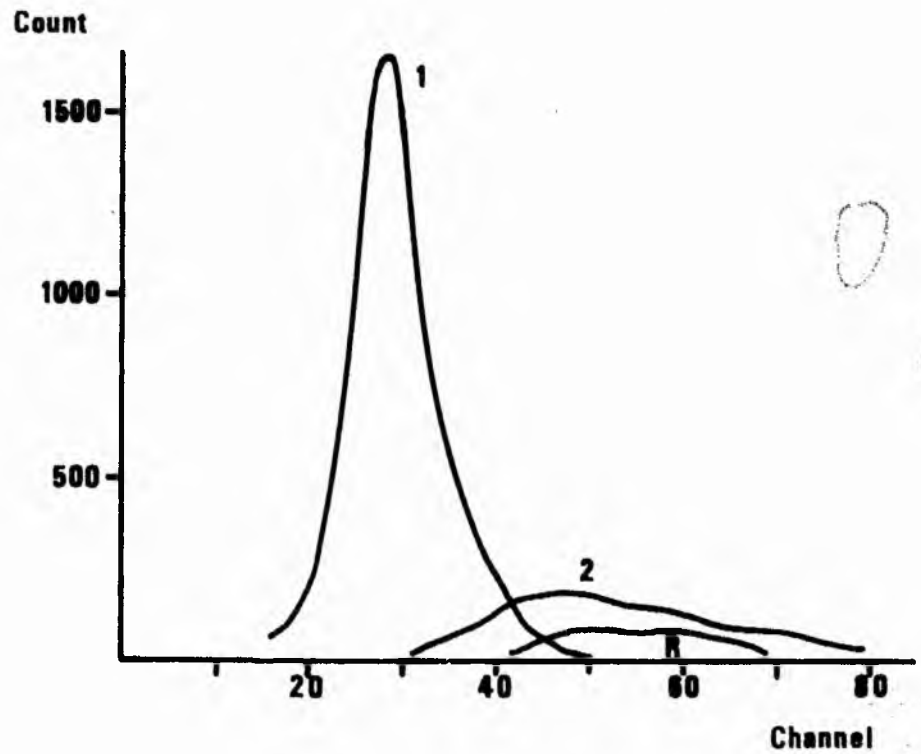

Fig.1.20. The projected breakdown of the total FDC for fraction 1 showing Peak 1 (biconcave discs), Peak 2 (spheres) and Reticulocytes (R).

This was carried out using several amended totals and it was found that the best fit was obtained when the main peak contained 75% of the reticulocyte-free count. The extrapolated values were then subtracted from the initial counts to give the values for the second sphered peak. This information, together with the results from the experiment as a whole, is presented in Table 1.2, Fig. 1.20 revealing the projected breakdown of the total FDC for fraction 1. Apart from the difference in MCV's, fraction 8 merely lacks the reticulocyte peak.

	Volume u^3			
Fraction	Total MCV	MCV -ve Retics.	Peak 1	Peak 2
1	90.4 ± 2.5	87.8 ± 1.9	73.0 ± 1.8	135 ± 3.4
8	83.3 ± 1.6	83.3 ± 1.6	67.8 ± 1.0	131 ± 2.3
% decrease	7.9	5.1	7.1	3.0

Table 1.2. The effect of in vivo ageing on the volume of the human erythrocyte as revealed by the Coulter Counter.

Fraction	MCV	Ht / Count	% ECS (Total FDC)	% ECS (Peak 1 only)
1	102 u^3		12.8	39.7
8	84.7 u^3		1.6	24.9

Table 1.3. Estimations of the % ECS present in the haematocrit tube using the data of Fig. 1.7 and Table 1.2.

The first column of Table 1.3 gives the MCV's shown graphically in Fig. 1.7. These have been compared with the values from Table 1.2 to calculate the % extracellular space (ECS) that must have been present in the haematocrit tubes. Clearly, the percentages obtained taking the whole FDC at face

value are much more credible than those assuming the skewed population to have an artifactually large volume. Finally, Table 1.4 breaks down the overall 17.0% decrease in MCV found in Fig. 1.7 into its components. After eliminating the effect of differential packing and the presence of reticulocytes, we are left with a real decrease of 5.1% in the MCV of the erythrocytes upon ageing.

	% Decrease in MCV
Haematocrit/Count	17.0
Total FDC	7.9
Retic.-free FDC	5.1
Presence of Retics.	2.8
Effect of ECS	9.1

Table 1.4. Factors contributing to the overall decrease in MCV as measured by standard haematological techniques.

Discussion

The aim of this chapter has been to evaluate the efficacy of simple centrifugation in effecting an age-dependent fractionation of a whole red cell population. That the fractionation compared favourably with the literature was shown by a 60% decrease in the specific activity of GOT and an 18% increase in the MCHC. The former being expressed per mg. of haemoglobin, which was shown to remain constant with age, can only be effectively explained in terms of an age-dependent fractionation. Not only does the rate of the GOT decrease bear no resemblance to the decrease in reticulocyte contamination, but as will be explained in Chapter 5, the reticulocyte specific activity would have to be much too great.

Having accepted that some degree of separation had taken place, criticism was focussed on the validity of some measured biophysical parameters. This was initiated by the realisation that the increase in observed MCHC is dependent on at least a constant extracellular space throughout the eight fractions. As a non-selective loss of membrane sialic acid was found (i.e. no change per mg. of membrane protein) it seemed very possible that the oldest cells might be packing more closely, giving a relatively lower volume than the younger cells. The Coulter Counter confirmed this and it was found that just over half of the decrease in MCV measured by the standard haematological techniques could be accounted for by an increase in packing density with age. In fact, the MCV by both techniques yielded similar results in the case of the oldest fraction indicating that it was the youngest cells which were giving an erroneously high volume. The small change in total cell volume could not possibly have brought about this large change in packing and so

sialic acid would seem by default to offer the only explanation. As the technique used here measures total membrane sialic acid, it is possible that there are more subtle changes, in perhaps the effective surface charge, taking place physiologically.

A considerable part of this work was concerned with the Coulter FDC and there appeared no alternative to the conclusion that approximately 25% of the erythrocytes are present in a spherical or nearly spherical form. The volume of the pre-haemolytic spheres was found to be 153 u^3, which is slightly above that calculated for the MCV of the skewed population, but as R.P. Rand et al. (23) found that haemolysis was preceded by an increase in surface area this is not surprising. Although these spheres are present under physiological conditions (100% plasma, 37°C) it was nevertheless considered wise not to exclude the possibility that these had been formed as a result of stress within the counting orifice. How then are we to account for the similarity of the MCV for fraction 8 measured under the two conditions? Could the completely different kind of stress encountered in the haematocrit tube have given rise to exactly the same percentage of spherings? This seems most improbable. The slightly greater MCV for fraction 8 obtained using standard techniques is likely to have arisen from the near absence of plasma during this procedure and so there may be virtually no extracellular space in the oldest fraction. The effect of 10% plasma on the MCV (Figs. 1.16 and 1.17) was investigated on whole blood and it is not known at present whether or not this phenomenon is also age-dependent. An age-dependent effect, however, superimposed on what is itself a minor component is hardly likely to affect significantly the conclusions drawn here.

There is therefore at present no experimental data for considering that spheres, or more likely very thick cells, are not normally present.

However strong the evidence is for the presence of spheres, it must be admitted that it is all circumstantial. Microscopy was considered but we already have the failure of H. Winter et al. (26) to detect any such cells. The lability of the red cell shape is well known and this has led to the use of glutaraldehyde as a fixative, but the demonstration here (Fig. 1.11) of a volume change produced by this agent precluded its use. These two factors did not make microscopy seem worthwhile.

The larger group of cells (referred to as spheres in this study) have been postulated as being the younger cells (25, 26, 30) and as far as the reticulocytes are concerned this has been confirmed here. However, this contradicts the normally held view that ageing is associated with an increase in sphering or thickness (22, 23). This is in fact one of the main assumptions of P.L. LaCelle et al. (35) who have postulated that this increase in sphering leads to a mechanical disadvantage and eventual haemolysis in the splenic sinusoids. Mention is not made of the ability of the larger and more spherical reticulocyte to overcome this anatomical hazard. The results of this study do not land themselves to either school of thought.

First of all, it is evident that both the youngest and oldest fractions contain the same proportion of sphered cells. To suggest that these are the youngest cells in the population is not compatible with the conclusion that a reasonable degree of age-dependent fractionation had been achieved. The

reticulocytes, for example, while not being confined to the lightest fraction do at least show a decrease in incidence towards the bottom of the centrifuge tube.

Secondly, the results make it possible to draw some conclusions about the shape of the erythrocyte with respect to age. No-one would disagree that the main peak consists of biconcave discs, whatever their orientation, and their volume was calculated to decrease by 7.1%. By virtue of its shape, any increase in the thickness of this biconcave disc would result in a very large increase in cell volume. Consequently there would have to be a massive loss of surface area just to maintain a constant volume and yet we can see that the volume actually decreases. There was also a small decrease in the volume of the spheres but obviously these cannot become thicker or more spherical. As the sialic acid measurement indicates a 10% loss of surface area there can clearly be very little change in the shape of the biconcave discs associated with ageing. Thus, the suggestion of P.L. LaCelle *et al.* is not supported by empirical observation. It is unfortunately not possible to decide how the surface area and volume changes are related for as we have seen we would have to contend with reticulocytes, spheres and biconcave discs.

To conclude this discussion on spheres, there are four possible explanations.

1. Their presence *in vitro* is not a true indication of events *in situ*. The finding, however, that this spherical population must be included to give a reasonable MCV argues strongly against this.

2. The spherical erythrocytes expel the remains of their cytoplasmic organelles to become mature erythrocytes, but this

transformation is not accompanied by any sudden change in shape. These young mature erythrocytes remain in this spherical or near-spherical state for approximately one quarter of the total erythrocyte life span, gradually assuming the familiar biconcave shape. The similar proportion of spheres in both fraction 1 and 8 makes this unlikely.

3. Reticulocyte maturation proceeds as described above but being a complex process has a high failure rate. The successfully transformed cells have the characteristically 'normal' biconcave shape whereas the rest are spherical. The latter, being spherical, are not able to withstand the rigours of the circulation and consequently have a shortened life span. This then is a possible explanation for the observation of R.L. Evans (36) concerning a population of short-lived cells (see Introduction). This could also explain why M.D. Sass et al. (37) found that there were apparently two populations of young cells-one most resistant to and the other least resistant to osmotic lysis. The explanation that would be offered here is that the latter constitute the spherical 'abnormal' cells. H. Walter et al. (38) also detected the presence of two populations of young cells by counter-current distribution. Doubt has, however, already been cast on the hypothesis that sphering leads per se to a shortened life span, and there is a discrepancy in that R.L. Evans calculated these short-lived cells to have a survival time of 0-10 days, whereas 30 days is indicated here. As well as this, there is the observation here that there appears to be a slight decrease in the MCV of the spheres with ageing.

4. The decrease in the MCV of the main peak and the probable decrease in that of the spheres make it most likely that a

constant propeortion of all age groups are sphered. If the spheres represented the youngest cells it is doubtful that any great decrease in the specific activity of GOT would have been observed. This suggestion appears best able to satisfy all the observations and so, whilst agreeing with others (25, 26, 30) that there is present a real population of very large cells, the latter are unlikely to be the youngest cells.

This discussion would be incomplete if no mention were made of the fact that the notion of a constant proportion of sphered cells is irreconcilable with the widely held view that the increase in red cell density is caused by an increase in haemoglobin concentration. Unless there are two populations of reticulocytes, one giving rise to biconcave discs and the other to spheres possessing almost double the normal haemoglobin content, the spheres must have a greatly reduced MCHC and presumably density as well. The relationship between density and position in the centrifugal field appears to have held for the reticulocytes, which were found largely at the top of the centrifuge tube, but not for the spheres which were distributed equally throughout the centrifugal field. This anomaly argues very strongly for suggestion (1) above being accepted as the best explanation of the recorded events.

In defence of the position taken in this discussion only R.O. Leif et al. (39) can be called upon. These authors calculated the expected distribution of red cell volumes which would result from the known density distribution, assuming constant cell composition. They found that the resulting FDC was much narrower than is normally observed and concluded that it could not have arisen simply from a variable water content.

The observed distribution of spheres suggests that some credence be given to this finding, but it is obviously not satisfactory to rely solely on this for the sake of an explanation. The slight decrease in the MCV of the spheres implies that even these are behaving as predicted and so the problem must be left unresolved.

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CHAPTER 2

THE MEMBRANE PROTEINS AS REVEALED BY SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS PAGE).

Summary

1. In vivo ageing of the human erythrocyte was found to be associated with three significant alterations in the membrane proteins. The increase in protein 4.1 content was attributed to a high affinity binding of this species from the cytoplasm, while the loss of protein 7 was probably due to an age-dependent decrease in membrane integrity. The heterogeneity of band 2.5 and its proximity to the doublet made interpretation difficult in the case of the increase of this component.

2. In contrast, in vitro ageing of the human erythrocyte, by blood bank storage, leads to qualitatively different alterations in the membrane proteins. This, and observations on the age-dependent resistance of membranes to SDS solubilisation, forces the conclusion that in vivo and in vitro ageing are not identical processes.

3. Proteins 4.1 and 4.2 were shown not to be subunits of a protein 4. Although they are both initially completely cytoplasmic, each exhibits a highly characteristic behaviour, 4.2 showing itself to be present in the membrane only as a cytoplasmic contaminant.

4. The binding of protein 4.1 to the membrane was found to be critically dependent on the temperature, but owing to the variability of this temperature effect a likely mechanism could not be advanced.

5. The term 'membrane protein' was found to be inadequate when it was realised that the proteins as revealed by SDS PAGE were by no means identical in their *in situ* origins and behaviour. A tentative classification has been proposed.

Introduction

Electrophoresis using acrylamide as the supporting medium has been in use since 1959 (1), but it was not until 1964 that L. Ornstein (2) placed this technique on a firm footing. He illustrated its superiority as compared to starch gels (3) for resolving complex mixtures such as plasma proteins and in general the greater resolution that could be obtained.

This technique, as were its predecessors, was obviously limited to the visualisation of soluble proteins, and very soon after L. Ornstein's publication several workers began to use sodium dodecyl sulphate to render various membrane-bound proteins soluble (4, 5, 6, 7). The demonstration by A.L. Shapiro et al. (8) of a relationship between the molecular weight of the SDS-protein complex and its migration rate has made SDS PAGE by far the most widely used of all the electrophoretic techniques. They showed, using a variety of proteins with a wide range of isoelectric points, that the logarithm of the molecular weight of the SDS-protein complex was proportional to the migration distance over the molecular weight range 15,500 to 165,000.

It is the SDS monomer which binds to proteins and at low monomer concentrations these bind with a high affinity to specific sites on the protein surface, the number of these being a characteristic of the protein (9). Above 0.5 mM monomer concentration there is a sudden non-specific, low affinity increase in binding by a cooperative process which results in the unfolding and denaturation of the protein. Between 0.5 and 0.8 mM SDS monomer, approximately 0.4 gm. of SDS is bound per gm. of protein, and above 0.8 mM SDS monomer this jumps to

1.4 gm. (10). This probably represents two ways in which the SDS monomer can associate with the protein, the first case consisting of one amphiphile molecule per 7 amino acid residues and the second, one amphiphile per 2 amino acid residues (9). This uniformity of SDS binding by diverse protein species in the presence of saturating SDS monomer concentrations, which is dependent on the reduction of any disulphide bridges that may be present (11), suggested a uniformity in the structure of the SDS-protein complexes. J.A. Reynolds et al. (10) postulated a complete unfolding of the polypeptide chain to give rod-like particles with a constant amount of SDS bound per unit length, and offered this (12) as an explanation for the observation of A.L. Shapiro et al. (8). That is, a loss of native conformation and masking of native charge leading to a migration rate dependent solely on the length of the unfolded polypeptide.

The binding of SDS to proteins was shown to be independent of ionic strength and pH (12) and was concluded to be largely a hydrophobic interaction between the hydrocarbon tail of the amphiphile and hydrophobic regions on the protein. R. Pitt-Rivers et al. (11) had previously concluded that it was the polypeptide moiety of the protein which was the binding locus, but had also shown that if a high degree of charge were present, such as in the highly basic polylysylglutamic acid, anomalous binding will occur, due to electrostatic interactions. Taking this one step further, D.K. Igou et al. (13) recently found very little interaction between SDS and synthetic un-ionised polypeptides, suggesting an hitherto dismissed importance for the cationic sites on the polypeptide chain. This is supported by the work of J. Oakes (14) who found that binding of SDS was

accompanied by a greater immobilisation of the polar head group than the hydrocarbon tail. These observations do not dispute the empirical observation of L. Shapiro (8) but they make it likely that the mechanism of SDS binding may involve coulombic as well as hydrophobic interactions. Lastly, it has been demonstrated that some membrane proteins (15), highly charged proteins (16) and glycoproteins (17) do not exhibit this logarithmic relationship between molecular weight and mobility and so care must be exercised in the interpretation of unknown systems.

Fig. 2ihas been reproduced from G. Fairbanks et al. (18) and shows the typical Coomassie Blue and Periodic Acid Schiff staining patterns that are obtained when human red cell ghosts are subjected to SDS PAGE. The nomenclature is that of G. Fairbanks et al. except that in the case of the proteins, Arabic numerals have replaced the original Roman ones. Seven major protein bands as well as haemoglobin can be seen and these are numbered in order of decreasing apparent molecular weight. The PAS technique gives three glycoprotein bands known as PAS 1, PAS 2 and PAS 3 as well as a glycolipid region at the solvent front. Although this is the most commonly used technique for visualising the erythrocyte membrane proteins and glycoproteins, the loss of functional integrity following SDS solubilisation means that very little is known about their in vivo function.

As bands 1 and 2 are very rarely found separately they are invariably dealt with together and have acquired such names as 'spectrin' (19) and 'tektin' (20). G.L. Nicolson et al. (21) showed that these two bands together with band 5 can be eluted from the membrane using a low ionic strength

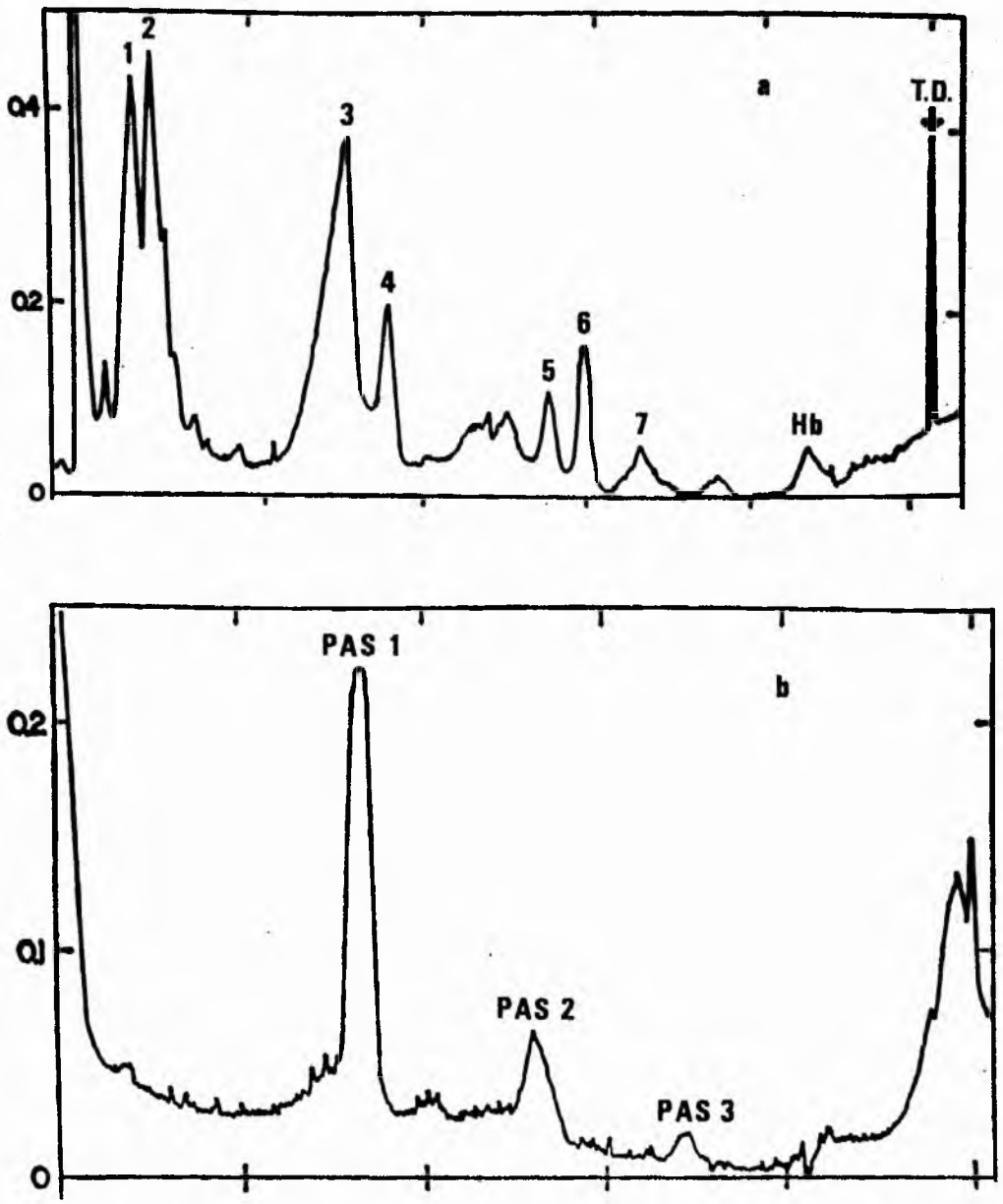

Fig.2.1. The proteins (a) and glycoproteins (b) of the plasma membrane as revealed by SDS PAGE (18).

procedure. They were able to purify spectrin from this extract, found it to be free of lipid and carbohydrate and calculated its molecular weight to be 140,000. By raising rabbit antibodies they were able to localise this component to the inner surface of the membrane. A.S. Rosenthal et al. (22) noticed that the addition of divalent cation to the low ionic strength extract caused the formation of fibrils, similar to those seen on the inner surface of the membrane. These fibrils were no longer apparent after the low ionic strength extraction procedure. The inevitable comparison between this and the muscle actinomyosin system was made and it was suggested that this explains the mechanism of the metabolic dependence of red cell deformability observed by R.I. Weed et al. (23). R.O. Williams (24) and J. Avruch et al. (25) have both shown that ATP will phophorylate band 2 in the presence of magnesium ions and they postulated that this mechanism might be involved in shape maintainance. However E. Weidekamm et al. (26) have located the Ca, Mg-ATPase activity here and found it to be kinetically very similar to the calcium transport system described by H. J. Schatzman et al. (27). They point out that these two functions are both activated by calcium and magnesium ions whereas myosin-ATPase is inhibited by magnesium ions.

The molecular weight of spectrin is now considered to be approximately 220,000 to 240,000 (28), but more importantly, G.M. Fuller et al. (28) and H. Knufermann et al. (29) showed that this component contained at least five N-terminal amino acids, although F.W. Hulla (30) was adamant that spectrin was not composed of subunits. The heterogeneity of spectrin was, however, confirmed by M.J. Dunn et al. (31) who also provided evidence for considering band 5 to be a subunit of spectrin. It would seem therefore, that spectrin behaves anomalously in

an SDS system as has already been observed for highly charged proteins (16) and glycoproteins (17), and that great care must be exercised in the interpretation of these results.

Band 3 is the broadest SDS band and this is likely to result from there being many components situated here (32, 33). Regardless of this admitted heterogeneity, properties and conformations continue to be attributed to this diffuse area. M.J.A. Tanner et al. (34) showed that it contained approximately 8% carbohydrate and M.S. Bretscher (35) claimed that it spanned the membrane. The latter phenomenon has been the subject of widespread debate and would certainly fit in with the anion transport (36, 37), sugar transport (38) and water transport (39) properties and the Na, K-ATPase activity (40, 41) which has been localised here. Studies such as those of N.M. Whiteley et al. (33) and M.S. Bretscher (35) rely on there being no structural rearrangement of the membrane proteins following lysis and resealing and assume that any protein which is accessible to the label on the inner surface is normally in contact with the cytoplasmic environment in situ. Recently J.V. Staros et al. (42) showed that this assumption is no longer valid. They found that after lysis and resealing under controlled conditions, protein 4 becomes accessible to the label from the outer surface and that this is certainly not the case in the intact cell. They also found that the greater the volume in which the lysis was performed relative to the cell volume, the greater was the degree of structural reorganisation of the membrane proteins. At a more basic level, the heterogeneity itself makes it unreasonable to conclude from external and internal labelling studies that one component is acting as the binding locus. Apart from those functions already mentioned, M.B. Bellhorn et al. (43) have located the reduced acetylchol-

inesterase monomer here. Thus, although there is nothing intrinsically inconceivable about the idea of a transmembrane protein, it remains to be conclusively shown that such a protein does, in fact, exist.

The major glycoprotein (PAS 1), often called glycophorin (44), has also been shown by M.S. Bretscher (17) and V.T. Marchesi et al. (44) to span the membrane but the same criticisms as outlined above apply. This component has an apparent molecular weight of approximately 90,000 but M.S. Bretscher (17) showed that its behaviour in the SDS system was anomalous. Numerous studies have thus been undertaken to calculate the molecular weight and a short sample of the results gives 29,000 (45), 34,000 (17), 50,000 (44) and 58,000 (46). It is also uncertain whether the three PAS staining bands represent individual components or are subunits of one glycoprotein. The latter is thought to be the most likely (47, 48), G.M. Slutzky et al. (49) having postulated that glycophorin is composed of two subunits which can be associated by two different means in situ. The first is SDS resistant and gives rise to PAS 1 and the second is readily dissociated by SDS to give PAS 2. These workers concluded that PAS 3 arose from a separate component. It is fairly well established, however, that these components contain the antigenic sites on the membrane surface (44), H. Hamaguchi et al. (46) suggesting that PAS 1 was responsible for M and N activity, whilst PAS 3 was responsible for A, I and S activity. B.C. Shin et al. (50) were also in favour of glycophorin spanning the membrane and suggested that on its inner surface it was associated with spectrin. In this way, it was postulated that information could be transmitted across the membrane and around its inner surface.

M.J.A. Tanner et al. (51) showed that band 6 could be

selectively eluted from the membranes by high ionic strength treatment, and following amino acid analysis this protein was found to be glyceraldehyde-3-phosphate dehydrogenase. C.F. McDaniel *et al*. (52) and J.A. Kant *et al*. (53) showed that the binding of GAPD was selective in that it occurred only on the inner membrane surface, and that it had a very high affinity. They both suggested that this location might have some physiological significance such as the control of ion movements.

M.S. Bretscher (17, 35) and B.C. Shin *et al*. (50) have shown that band 3 and PAS 1 are definitely exposed at the outer membrane surface. In addition J.V. Staros *et al*. (54), using a smaller and more penetrating probe, have claimed that many of the minor components between bands 2 and 3 are present on the outer surface. By elimination, therefore, the other components are likely to be buried within the lipid core of the membrane and/or exposed at the inner surface. The latter has already been demonstrated for spectrin (21) and band 6 (52, 53).

No work has yet been published on changes in the proteins of the human erythrocyte membrane upon *in vivo* ageing, but it is worth mentioning the few related studies which have been carried out. Two groups have investigated changes associated with reticulocyte maturation in the rabbit. In the first by P.A. Koch *et al*. (55), maturation was found to be associated with the loss of a single SDS band and the latter was shown not to have originated from ribosomal contamination of the reticulocyte membrane. In the second study, D. Wreschner *et al*. (56) claimed that at least ten changes could be observed and that the majority of these were losses of low molecular weight bands. As P.A. Koch *et al*. had shown that most of the ribosomal proteins migrated in this area, it is interesting to

speculate that these second authors were observing, in the main, a loss of ribosomal material.

M.J. Conrad *et al.* (57) investigated the effect of blood bank storage and found that after three weeks band 3 became 'denatured' with the result that it was highly resistant to solubilisation by 0.1% SDS. This was reversed by raising the SDS concentration to 1.0% but not by the addition of thiol reagents. It seemed, therefore, that this 'denaturation' was not the result of disulphide bridge or other SDS resistant bond formation. These authors proceeded to speculate that this process might be involved in the *in vivo* ageing process. As a result, *in vitro* as well as *in vivo* ageing was investigated in the present study to find out whether or not the two processes are in any way comparable.

Materials and Methods

Ghosts were prepared as previously described unless otherwise stated.

Polyacrylamide Gel Electrophoresis (PAGE)

All electrophoretic reagents were purchased from BDH. Acrylamide was purified by the method of U.E. Loening (58) as follows. 70 gm. of acrylamide was added to 1 litre of chloroform at 50°C, stirred in until completely dissolved, cooled overnight at -20°C and the resulting supernatant discarded. The acrylamide crystals were rinsed on Whatman No. 1 filter paper with 500 ml. chloroform at -20°C and dried *in vacuo* with a Wright's rotary evaporator.

Electrophoresis was performed with the following solutions.

1. 5% acrylamide, 0.05 Bis., 0.2% SDS, 50 mM Tris/Cl pH 7.4
2. 10% TEMED in 99% ethanol
3. 10% ammonium persulphate
4. 5% SDS, 20% glycerol, 20 mM Tris/Cl pH 7.4
5. 0.2% SDS, 50 mM Tris/Cl pH 7.4
6. 10% acetic acid, 20% methanol, 70% water
7. 0.025% Coomassie Brilliant Blue R250 in solution 6

Gels were made by adding 0.1 parts solution 2 and 0.05 parts solution 3 to 10 parts solution 1, pipetting 1.1 ml. of the mixture into each glass holder, overlaying with a few drops of solution 5 and allowing to polymerise for at least 30 minutes. The overlay was then replaced and the gels were used after 12 hours. Quickfit apparatus holding eight of the cylindrical gels was employed for the electrophoresis. All gels were pre-run

with solution 5 for 30 minutes at 6 mA/tube, after which this solution was replaced. Each electrode compartment contained approximately 200 ml. of solution 5. The samples were prepared by adding 1 part material + 1 part solution 4 + 3 parts water, mixing and incubating for 15 minutes at 37°C. Up to 0.03 ml. of this mixture was layered onto the top of each gel with a 1.0 ml. graduated plastic syringe and 0.01 ml. of 0.01% Bromophenol Blue tracker dye was added separately to one of the gels at random. The gels were run for 5 minutes at 2 mA/tube to allow the polypeptides to enter and then at 6 mA/tube until the tracker dye was almost at the bottom of the gel. This took approximately 70 minutes. After the first 30 minutes had elapsed the two electrode solutions were mixed to prevent buffer exhaustion.

The gels were removed from their holders using a water filled syringe with a very long flexible needle, the latter being forced between the glass and gel surfaces. They were fixed in 20% TCA for approximately 1 hour, removed and placed in 75 ml. of solution 6, shaken vigorously for at least one hour to remove the TCA and SDS and left overnight in 5 ml. of solution 7 to stain. Destaining was achieved by repeated washing in solution 6. The gels were photographed by transmitted light using Kodak FP4 film and a Practika camera. Scanning densitometry was performed with a Joyce Loebl Chromoscan 201 at 560 nm with a 0.5 mm. slit width. The integrated stain densities were calculated by cutting the peaks out of the scans and weighing them.

In one series of experiments electrophoresis was performed with 1.0% SDS and in the presence and absence of 1 mM 2-ME and 1 mM EDTA. The latter two were added only to the sample mixture as follows; 1 part material + 1 part solution

4 + 1 part 5 mM 2-ME + 1 part 5 mM EDTA + 1 part water. 1.0% SDS electrophoresis was carried out by increasing the SDS concentration in solution 5 to this value and pre-running the gels for 30 minutes at 6 mA/tube as above. Non-surfactant electrophoresis was performed by omitting SDS from all the solutions and increasing the pH of the buffers to 9.0.

Exclusion Chromatography

This was carried out with Biogel P 200 purchased from Biorad Laboratories. The eluting buffers will be described in the text. The eluate from the columns was monitored by passage through an LKB Uvicord II with chart printout and collected in an LKB Ultrorac fraction collector.

High Salt Membrane Extract

One volume of ghosts was added to four volumes of 0.2 M NaCl/ 10 mM Tris/Cl pH 7.4 and incubated overnight at 4°C. The residue was removed by centrifugation at 10 Krpm for 30 minutes.

Results and Discussion

Proteins were visualised in this study using the acid wool dye Coomassie Brilliant Blue R250 because of its great sensitivity (59). There was also the added advantage that the protein-dye complex was found to be extremely stable. Unlike Amido Black 128 stained gels, which began to fade visibly within a matter of days, these gels could be stored for months without any apparent decrease in staining intensity. The binding of Amido Black is known to be closely correlated with the basic amino acid content of the protein (60) and this is likely to be the case for Coomassie Blue. Therefore a quantitative comparison between different protein species is not possible. It was nevertheless felt that some degree of quantification was worthwhile and so first of all it was necessary to ascertain whether there was any correlation in the system used here between the amount of protein added to a gel and its resulting integrated stain density (ISD).

S. Fazekas de St. Groth *et al.* (59) using cellulose acetate strips and six pure proteins found a linear relationship between these two parameters that help up to 50 ug. protein/cm. W.N. Fishbein (61) observed a similar relationship using polyacrylamide as the supporting medium, although he noticed a deviation from linearity in those protein bands which were very compact. He suggested that this might have arisen from an inability of this large dye molecule to penetrate the very compact protein bands.

Bovine serum albumin was used here and Fig. 22 shows increasing amounts of this protein which had been run under identical conditions to the membrane proteins. It should be

Fig.2.2. Increasing amounts of BSA run under standard conditions (see Methods).

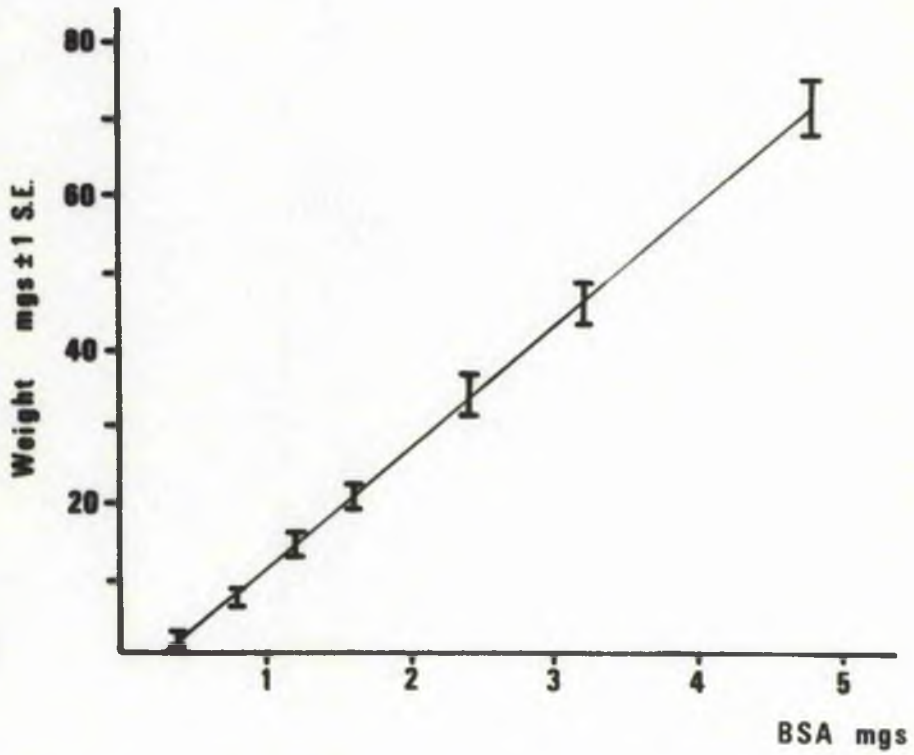

Fig.2.3. The correlation between the amount of BSA applied to a gel and the resulting ISD. The line was drawn by the method of least squares.

noted that even with this soluble protein, SDS treatment does not always apparently result in complete monomerisation. It can be seen from Fig. 23 that there is a very good correlation between the amount of protein and its ISD and it was thus considered valid to use this method of expressing the results in the following study.

Fig. 24 illustrates a typical scan obtained from whole plasma membranes and it can be seen how the scans were divided between the various protein species present. As the beam width was 0.5 mm., only between bands separated by a distance greater than or equal to this value would the trace return to the baseline. This is so only for the regions separating bands 6, 7 and haemoglobin which therefore caused no problem. All the other bands were separated by troughs of varying magnitude and in addition bands 4.1, 5, and 4.2 when present, were superimposed on a diffuse, unresolved region known as 4.5. Except for the 4.5 region, perpendiculars were drawn from the bottom of the separating troughs to the baseline as is shown for bands 1, 2, 3 and 6, the first two being treated as one species. That this system could not be adopted for band 4.1 is illustrated by fraction 1 in Fig. 2.6 in which it can be seen that in its virtual absence a large shoulder is still present due to the 4.5 region. That is, band 4.1 is superimposed entirely on 4.5. Band 5 was considered to be present at the leading edge of the 4.5 region and so a diagonal line was adopted for its trailing edge. Confirmation for the adopted procedure was obtained by the similarity between the % ISD of 1 + 2 + 5 found in this study (35-38%) and the estimate of the percentage protein released from the red cell ghosts by dilute EDTA given by J.R. Green et al. (62), (40%). The technique used was not sensitive enough to discriminate between

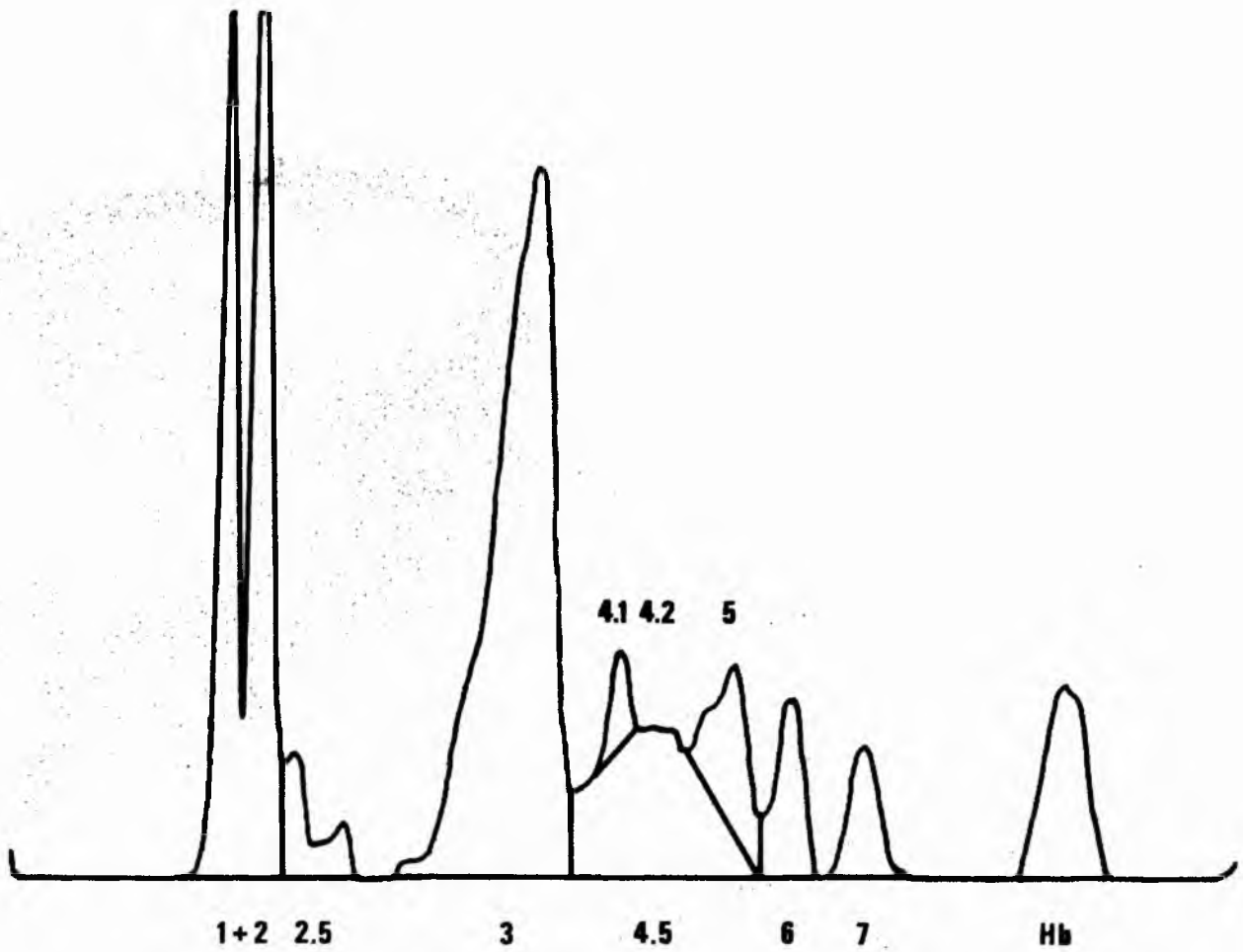

Fig.2.4. A scan of the proteins from unfractionated membranes showing the nomenclature used in this study and the procedure adopted for dividing it amongst the constituent species. No protein 4.2 can be seen but its position when present is indicated.

the various minor components between bands 2 and 3 and so these are collectively referred to as 2.5.

In Vivo Ageing

Fig. 2.5a shows the membrane protein patterns from the eight age-dependent fractions. It can be seen that protein 4.1 is hardly present in the youngest fraction and gradually increase to become a major species. This observation is the subject of a previous paper (63). The only other visible change is that of residual haemoglobin; this was usually a decrease. To confirm that the latter could not in any way have brought about the 4.1 change, Figs. 2.5b and 2.5c show an increasing 4.1 gradient in the presence of no haemoglobin change and an increase in haemoglobin binding respectively. The determining factor in haemoglobin binding is not known. Scans of the gels in Fig. 2.5a are given in Fig. 2.6 and the heterogeneity of band 3 is quite clear, there being two obvious shoulders on its ascending limb.

In order to quantify the change in 4.1 and perhaps to detect any other more subtle age-dependent changes the % ISD's were calculated. Table 2.1 reveals that there are three significant changes with age. That is, an increase in bands 2.5 and 4.1 and a decrease in band 7, these being illustrated in Fig. 2.7.

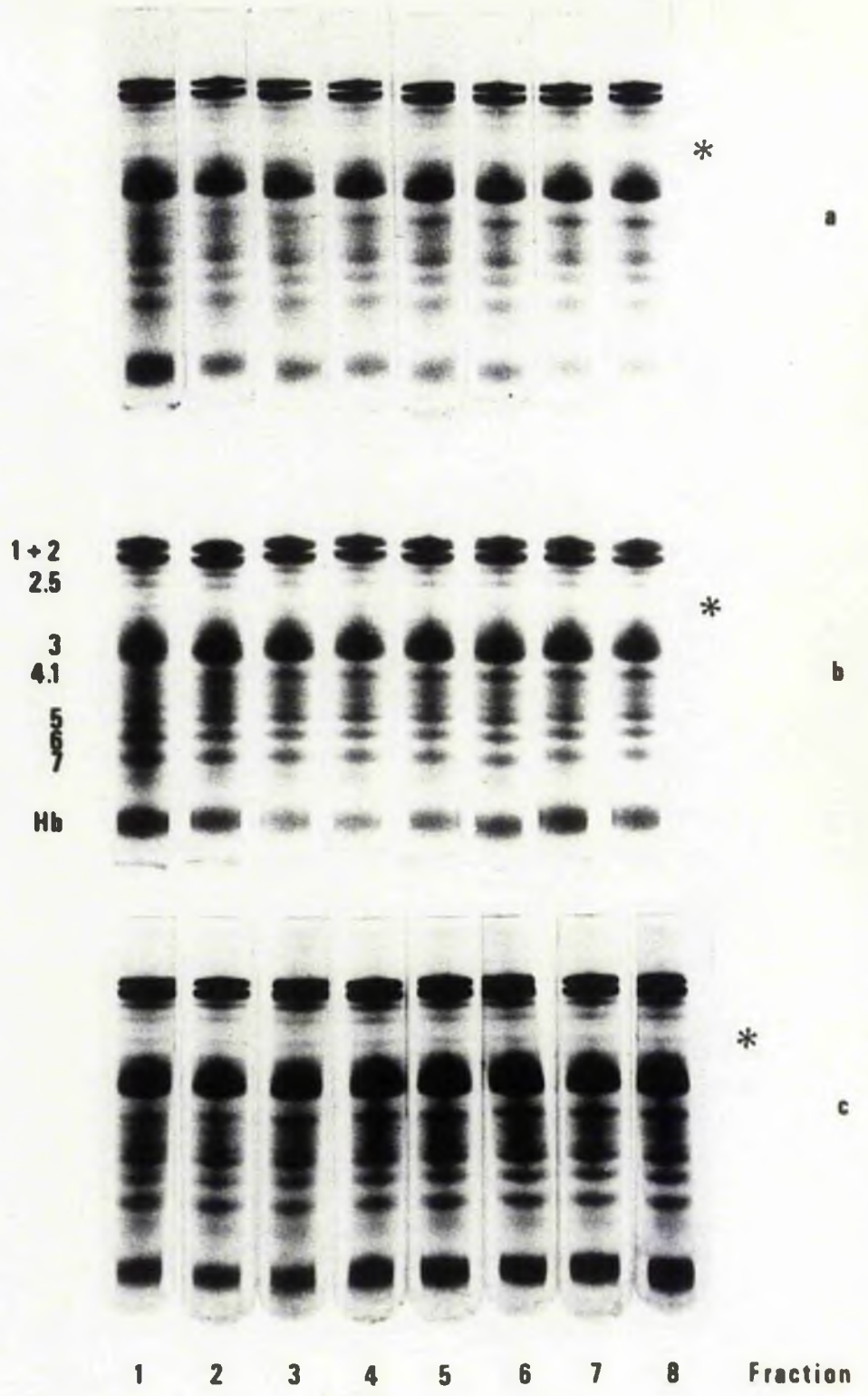

Fig.2.5. The effect of <u>in vivo</u> age on the membrane proteins in the presence of (a) a decrease in Hb. content, (b) no change in Hb. comtent and (c) an increase in Hb. content.

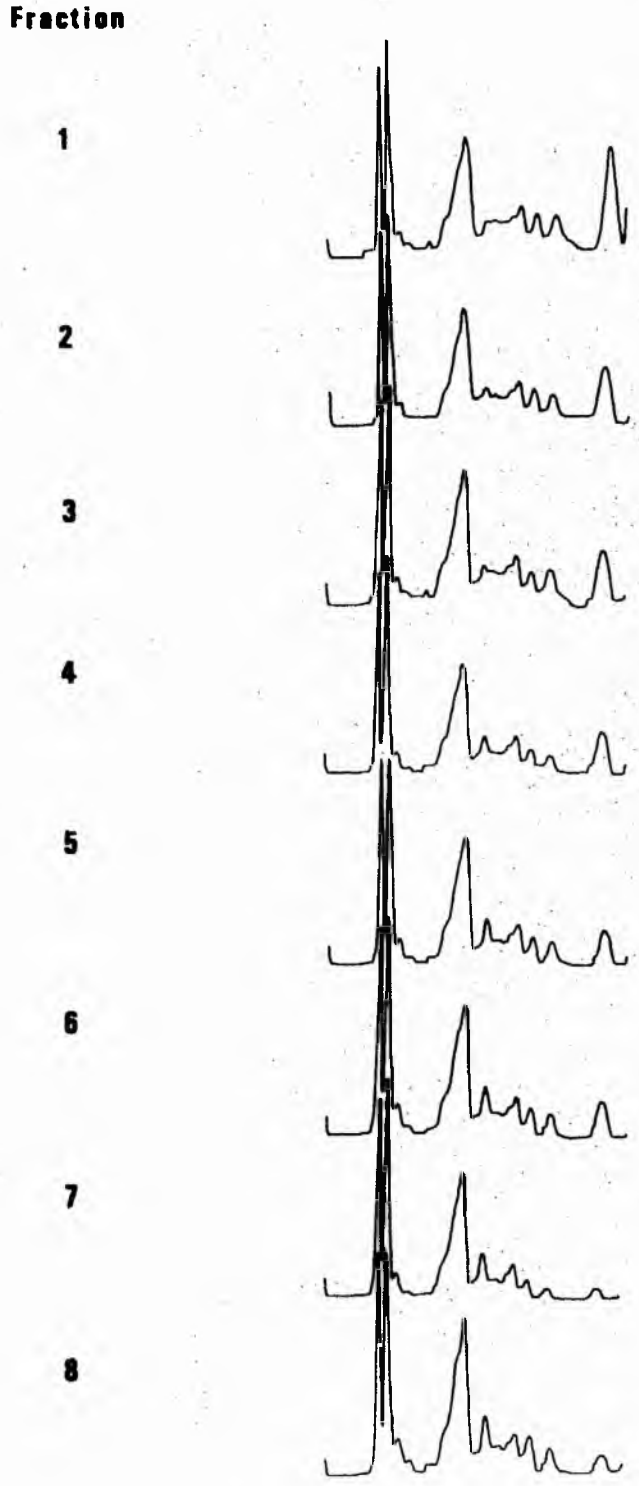

Fig.2.6. Scans of the gels shown in Fig.2.5.a.

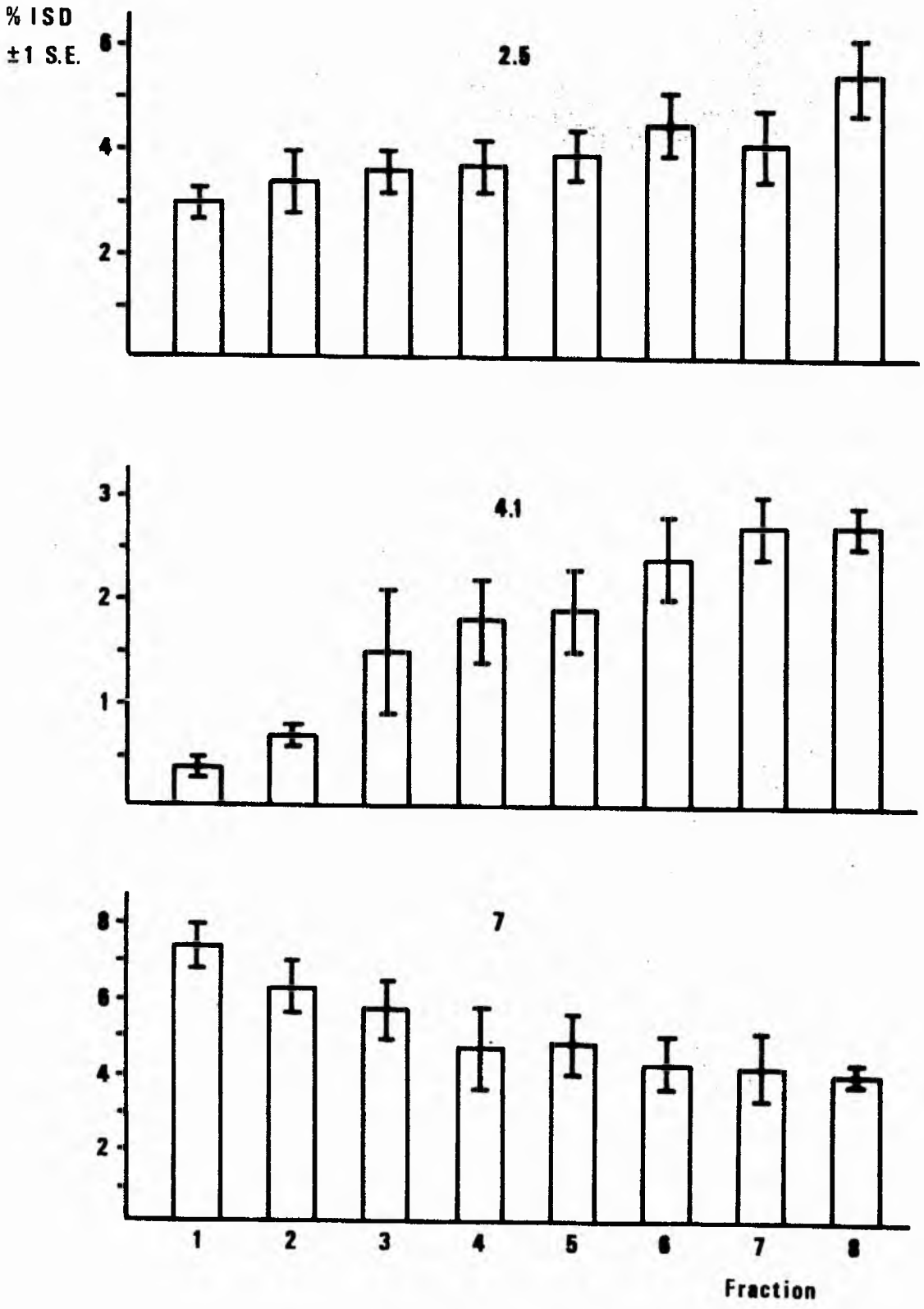

Fig.2.7. The effect of <u>in vivo</u> age on the % ISD's of proteins 2.5, 4.1 and 7 in the membrane.

Fraction	Protein; % ISD ± S.E. 1 + 2	2.5	3	4.1
1	28.0 ± 0.4	3.0 ± 0.3	34.3 ± 0.5	0.4 ± 0.1
2	30.7 ± 1.3	3.4 ± 0.6	34.5 ± 0.3	0.7 ± 0.1
3	30.9 ± 2.1	3.6 ± 0.4	34.9 ± 0.4	1.5 ± 0.6
4	31.3 ± 1.6	3.7 ± 0.5	35.2 ± 0.7	1.8 ± 0.4
5	30.7 ± 1.5	3.9 ± 0.5	35.2 ± 0.7	1.9 ± 0.4
6	30.7 ± 0.9	4.5 ± 0.6	35.8 ± 0.5	2.4 ± 0.4
7	30.1 ± 1.9	4.1 ± 0.7	35.5 ± 0.8	2.7 ± 0.3
8	29.5 ± 1.0	5.4 ± 0.7	34.8 ± 0.5	2.7 ± 0.2
p	<.5	<.001*	<.5	<.001*
	4.5	5	6	7
1	15.8 ± 0.4	5.8 ± 0.4	4.7 ± 0.5	7.4 ± 0.6
2	15.0 ± 0.3	5.4 ± 0.2	3.9 ± 0.7	6.3 ± 0.7
3	14.8 ± 1.3	5.1 ± 0.3	3.8 ± 0.7	5.7 ± 0.8
4	14.5 ± 1.1	5.0 ± 0.1	3.9 ± 0.2	4.7 ± 1.1
5	14.6 ± 0.9	4.9 ± 0.3	3.8 ± 0.5	4.8 ± 0.8
6	14.1 ± 0.7	4.9 ± 0.3	3.3 ± 0.7	4.3 ± 0.7
7	14.9 ± 1.5	4.9 ± 0.2	3.6 ± 0.5	4.2 ± 0.9
8	14.1 ± 0.9	4.8 ± 0.4	4.0 ± 0.6	4.0 ± 0.3
p	<.75	>.1	>.1	<.005*

Table 2.1. The effect of *in vivo* ageing on the proteins of the human erythrocyte membrane as revealed by SDS PAGE. The results were subjected to analysis of variance. * denotes significance.

In Vitro Ageing

This was investigated by preparing ghosts from fresh unfractionated and three week outdated blood under identical conditions. Table 2.2 shows that *in vitro* ageing is associated with slight but significant increases in proteins 3, 4.1 and 6. It is clear, therefore, that not only do changes occur in the protein components of the membrane upon storage but that these changes are by no means similar to those occurring during the process of *in vivo* ageing. The exception is protein 4.1 which seems to increase under both conditions. These results suggest very strongly that workers such as M.J. Conrad *et al.* (57) should avoid extrapolating from *in vitro* experiments to the *in vivo* system.

Protein	% I.S.D. ± S.E. Fresh	Outdated	p
1 + 2	34.0 ± 1.3	31.5 ± 1.5	.2
2.5	2.9 ± 0.4	3.6 ± 0.2	<.9
3	35.3 ± 0.4	36.4 ± 0.2	<.05*
4.1	1.0 ± 0.1	1.9 ± 0.1	<.001*
4.5	14.0 ± 0.7	14.6 ± 0.7	.5
5	4.6 ± 0.4	4.3 ± 0.3	.5
6	3.2 ± 0.4	4.3 ± 0.2	.02*
7	4.8 ± 0.4	4.1 ± 0.7	<.4

Table 2.2. The effect of *in vitro* ageing on the proteins of the human erythrocyte membrane as revealed by SDS PAGE. The results were subjected to analysis of variance. * denotes significance.

Temperature Studies

One of the simplest explanations of the *in vivo* age-dependent protein changes observed was an age-dependent resistance or susceptibility to the haemolysing media. Thus, in the case of protein 4.1, it could be that this protein is more firmly bound in the older membranes and so is preferentially lost from the younger membranes during the washing procedure. This could be resolved to a great extent by an examination of the membrane pellets after each centrifugation step. Unfortunately, preliminary attempts to carry this out resulted in a complete loss of the 4.1 gradient.

Fig. 2.8 shows two experiments carried out on the same donor. In the initial experiment (Fig. 2.8a) an increase in 4.1 and a decrease in haemoglobin was observed. Fig. 2.8b demonstrates the effect on the final ghosts of removing a sample of the pellet after each stage in the washing procedure. It can be seen that the 4.1 gradient has been completely lost and a similar amount of this protein is present in all the fractions. The haemoglobin gradient has also been reversed but as indicated previously (Fig. 2.5), this is probably not significant. It was thought possible that in this second experiment, for some reason an age-dependent separation had not been achieved in the initial centrifugation, but it can be seen in Fig. 2.9 that in both cases a very similar decrease in the specific activity of the enzyme GOT was found.

An examination of the likely causes of this effect revealed temperature as the most promising. The ambient temperature in the centrifuge room during the preparation of the ghosts was 30 - 33°C, and as the pellet volume after each stage was approximately 1 ml. one might expect a significant

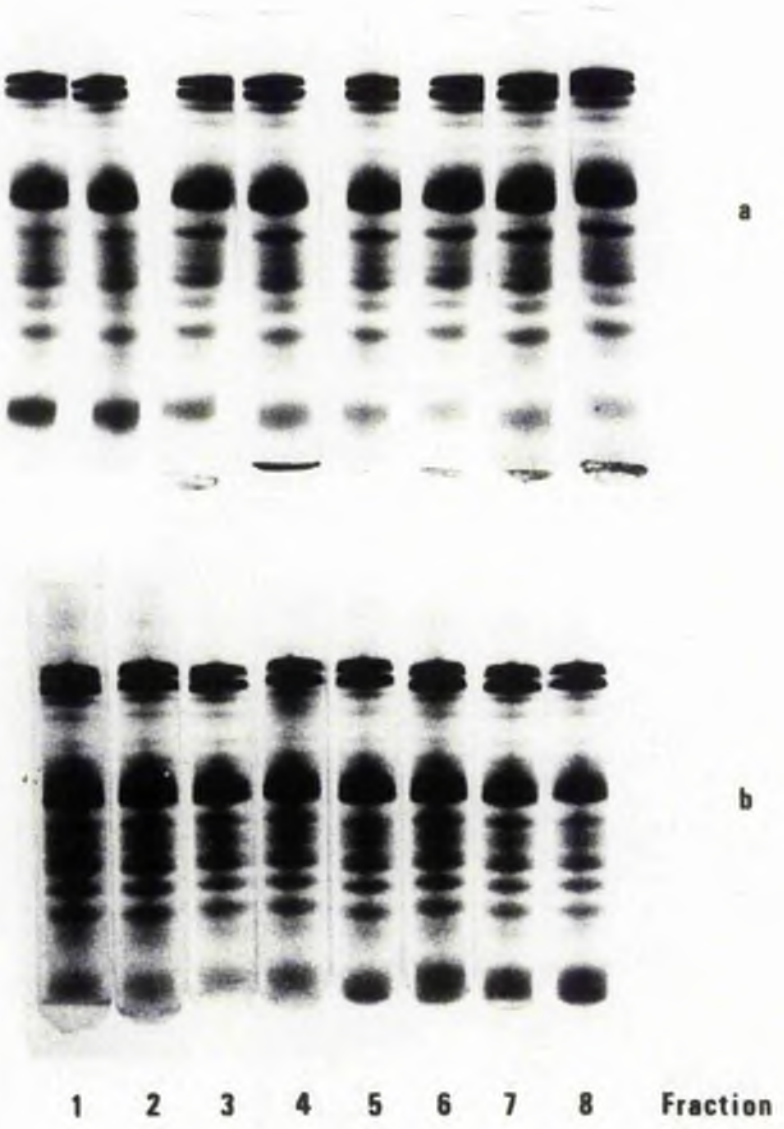

Fig.2.8. Two experiments performed on the same donor on the effect of <u>in vivo</u> age on the membrane proteins showing (a) the normal increase in protein 4.1 and (b) no change in the protein 4.1 content.

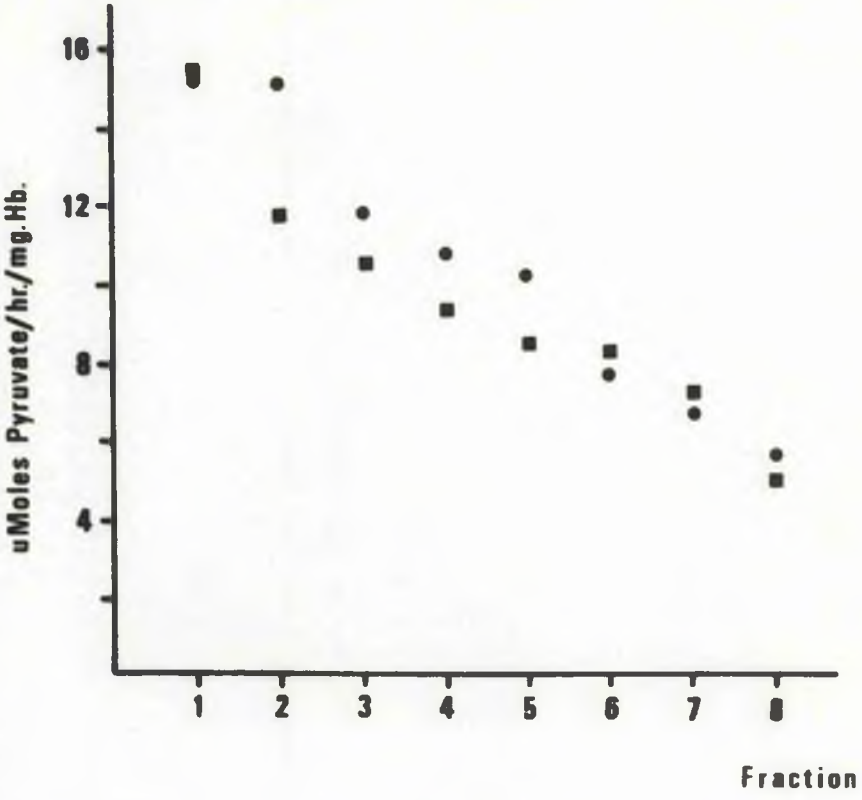

Fig.2.9. Cytoplasmic GOT from the experiments shown in Figs.2.8.a (■) and 2.8.b (●).

Fig.2.10. The effect of an uncontrolled increase in temperature during preparation on the final protein 4.1 content of unfractionated membranes. (a) normal membranes and (b) membranes subjected to the temperature increase.

rise in the temperature of this pellet while a sample was being withdrawn from each of the eight fractions. This hypothesis was examined by conducting the following experiment. Ghosts were made from two identical samples of unfractionated blood. After the first sodium phosphate spin one sample had its supernatant removed, was topped up with fresh cold buffer and placed on ice. The other, after removal of its supernatant was left standing on the bench for five minutes before fresh cold buffer was added. The rest of the procedure was as described in the methods. The results in Fig. 2.10 show that in this case, incubating the first membrane pellet caused a visible increase in 4.1 content and a decrease in haemoglobin. This suggests that not only is the final amount of 4.1 present in the ghosts dependent on the temperature during the centrifugation procedure, but it also seems likely that protein 4.1 is present in the cytoplasm of the red cells unless one postulates that the increase in temperature prevented it from being washed away. This phenomenon of temperature dependence seemed worthy of further investigation.

It was decided to investigate the effect of more controlled temperature changes and to see if EDTA might display a protective effect in the hope of throwing some light on the mechanism of this effect. EDTA was added at a concentration of 0.1 mM to the haemolysing sodium phosphate buffer, all subsequent solutions being EDTA-free. Fig. 2.11 illustrates what happened when the first haemolysate was incubated at increasing temperatures. In this case, the increase in temperature caused a loss of 4.1 which was partially counteracted by the presence of EDTA. As the previous experiment, in which the membrane pellet had been subjected to

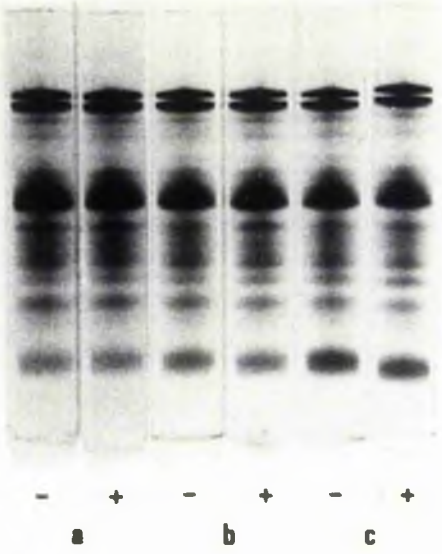

Fig.2.11. The effect of incubating the first haemolysate for 5 min. at (a) 4°C, (b) room temperature and (c) 37°C on the final protein 4.1 content of unfractionated membranes (± 0.1 mM EDTA in the haemolysing medium).

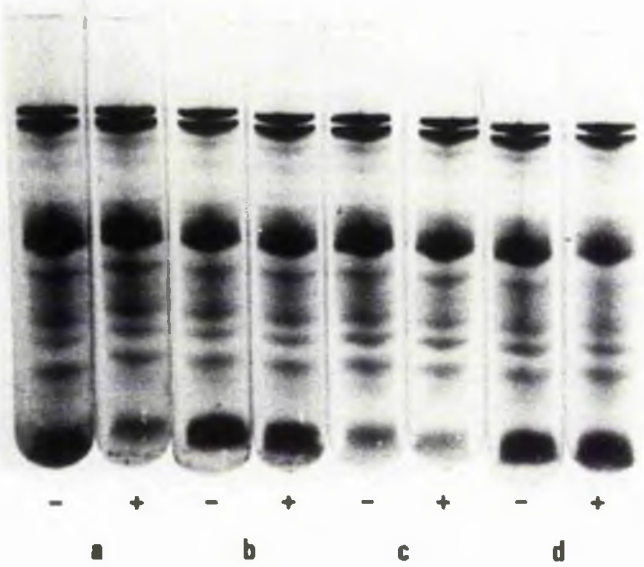

Fig.2.12. The effect of incubating for 5 min. at room temperature (b) the first haemolysate, (c) the first pellet and (d) both, on the final protein 4.1 content of unfractionated membranes (± 0.1 mM EDTA in the haemolysing medium). The control is shown in (a).

the temperature increase, had shown an increase in 4.1, it seemed possible that the membrane concentration might be having some effect. Thus, Fig. 2.12 shows the effect of incubating at room temperature, the first haemolysate, the first pellet and both. Unfortunately, this failed to confirm the previous observations and in this case EDTA seemed to enhance the loss of 4.1. Further attempts to elucidate this problem produced random occurrences of all possible 4.1 effects. Inspection of Fig. 2.12, however, shows a rather regular haemoglobin effect. That is, increasing the temperature of the membrane pellet caused significant binding of haemoglobin but an increase in the temperature of the first haemolysate caused no such increase. Further experiments confirmed this although it must be admitted that the experiment illustrated in Fig. 2.10 contradicts it. The conclusions that can be drawn from this series of experiments are that an increase in the temperature during the preparation of the ghosts is almost certain to have some unpredictable effect on the final 4.1 content, and in the case of haemoglobin is likely to result in an increase of this component, especially if it is the pellet which suffers the increase in temperature.

Membrane-Cytoplasm Interactions

Bearing in mind the conclusions of the previous section, it was decided to place the centrifuge tubes on ice while the samples from each stage were being withdrawn. To minimise the operating time only the youngest and oldest membranes were investigated. In this way, the results shown in Fig. 2.13 were obtained. The scans of these gels are given in Figs. 2.14

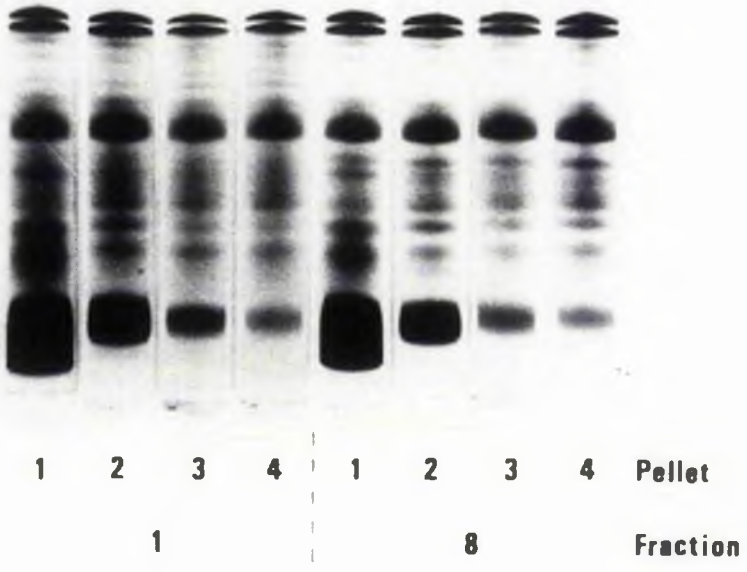

Fig.2.13. The effect of in vivo age on the proteins present in the membrane pellet after each of the four washes.

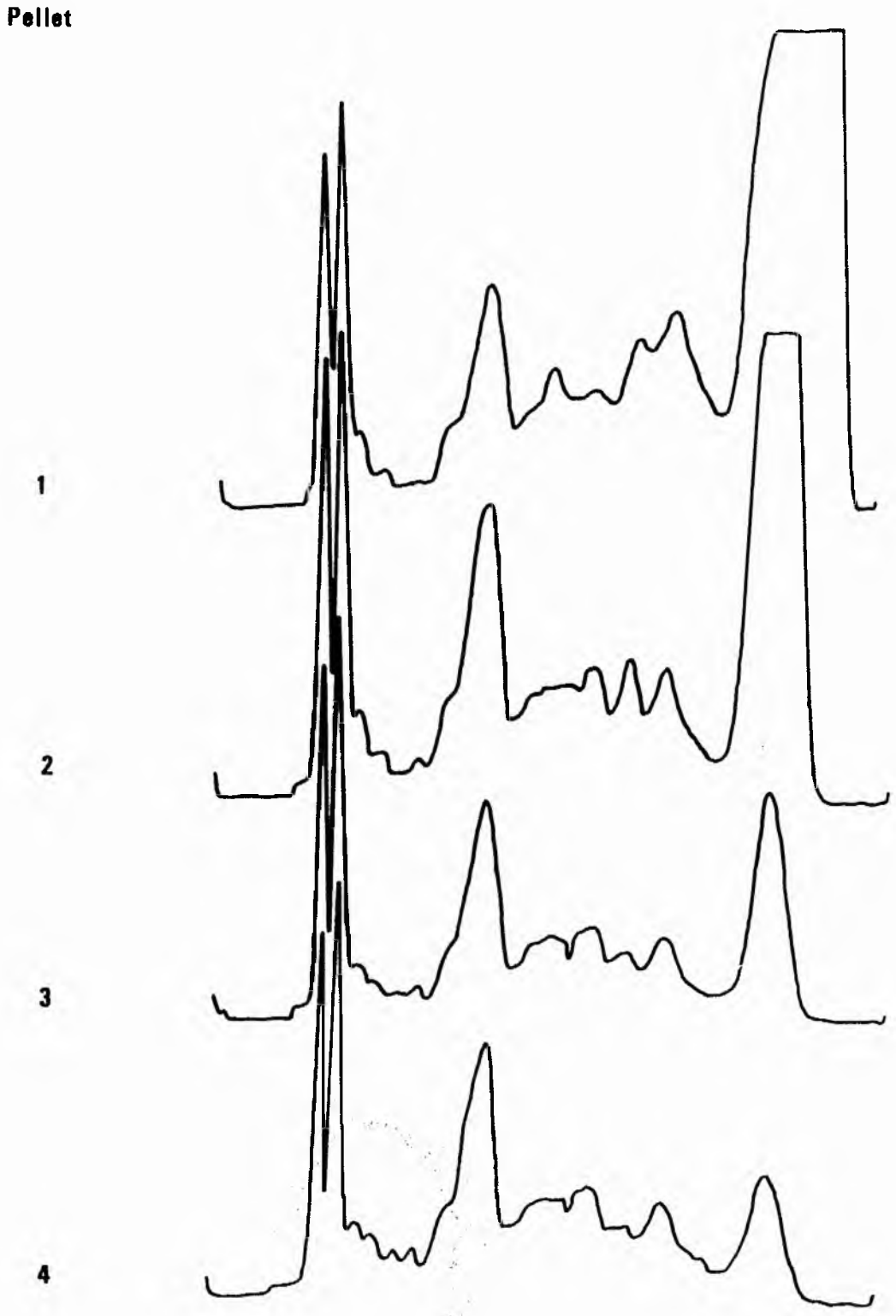

Fig.2.14. Scans of the fraction 1 gels shown in Fig.2.13.

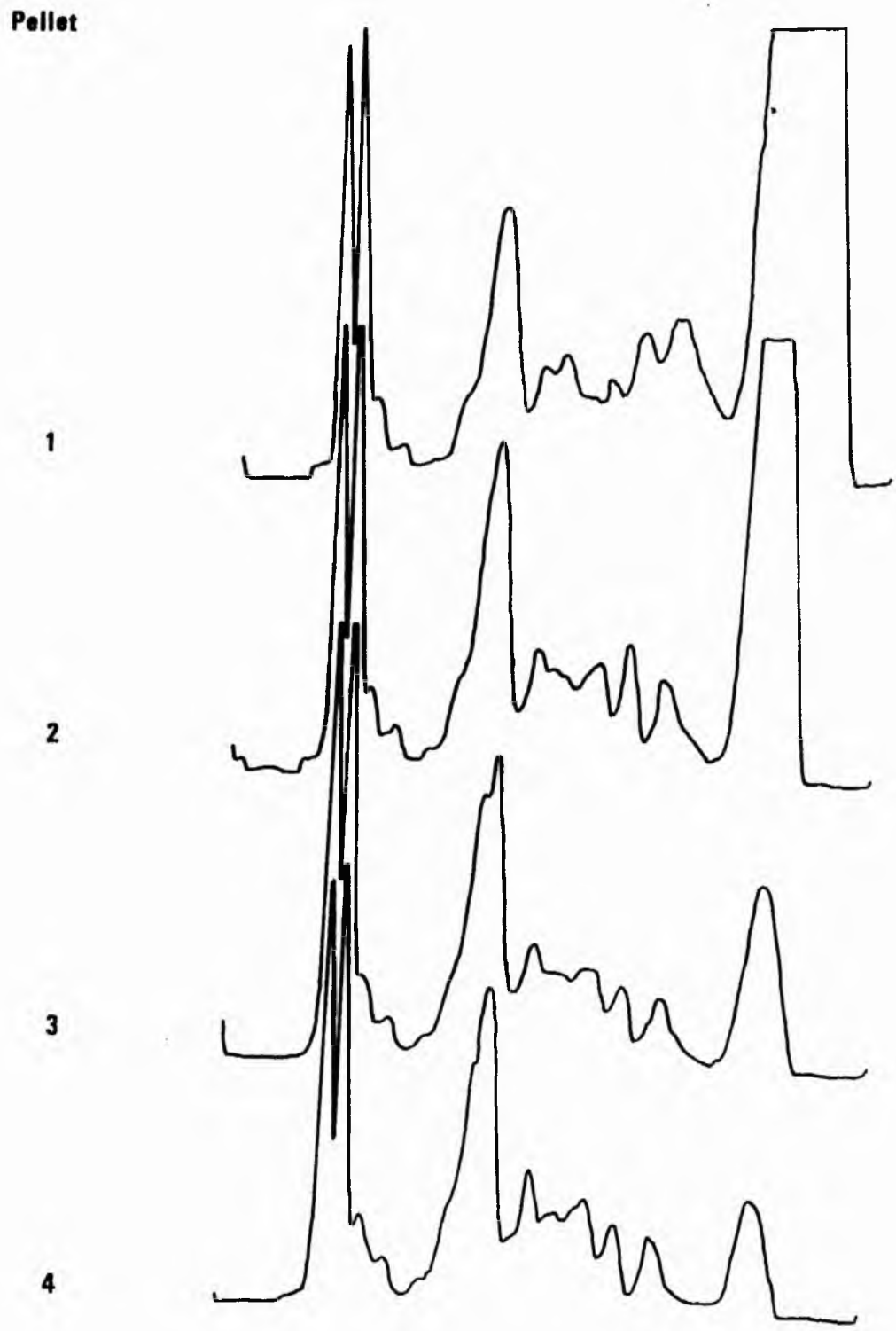

Fig.2.15. Scans of the fraction 8 gels shown in Fig.2.13.

and 2.15. It can be seen that at no stage in the washing procedure is there any 4.1 in the youngest membranes whilst it is present in all stages of the oldest membranes. Thus, whatever the cause of this protein effect it is not selective elution, although one cannot rule out protein loss at the instant of haemolysis. Due to the complex yet minor nature of the 2.5 region and the large amounts of a protein in the same position a 7 in the cytoplasm, it is not possible to make any such pronouncements in these cases.

There are, however, two very interesting observations to be made from this experiment. Firstly, there are two protein species present in large quantities in the cytoplasm, whose mobilities correspond to proteins 6 and 7 in the plasma membrane. These will be discussed in full below. Secondly, it can be seen that the first pellet in both the youngest and oldest fractions contained a prominent band 4.2 which is rapidly washed out from the membrane pellet. This assumes importance when it is recalled that G. Fairbanks et al. (18) have claimed that PAGE performed in the presence of 1.0% SDS gives a single band called 4, whereas in the presence of 0.1 to 0.2% SDS, this splits into two bands later called 4.1 and 4.2. Not only was no picture of this interesting situation given by these authors but it seems parodoxical that decreasing the surfactant concentration should result in the splitting of a protein. More important is the fact that the cleavage of a protein into two bands of roughly equal intensity would be expected to impart to these two products a much greater mobility than that of the parent protein. It seems highly unlikely that the two products of this cleavage with presumably much lower molecular weights would position themselves on either side of the parent protein.

Finally, Y. Tashima (41) and R. Schmidt-Ullrich *et al.* (64) both used high SDS concentrations and obtained proteins 4.1 and 4.2 on their gels. These considerations together with the observations from Figs. 2.13-15 suggest that these two proteins are in no way related and in fact display very dissimilar patterns of behaviour under the same conditions of ghost preparation.

This situation was further investigated by performing SDS PAGE of the four membrane pellets from unfractionated blood under four conditions. The first two of these conditions were 0.2% and 1.0% SDS and as can be seen in Figs. 2.16a and 2.16b the observation of G. Fairbanks *et al.* was not confirmed. Increasing the concentration of surfactant, apart from a slight effect on overall relative band positions, had no effect on the presence of these two proteins. In both cases, both are present in the first pellet and protein 4.2 is washed out in the successive washes. The curvature of the doublet bands in the presence of 1.0% SDS was also reported by these authors. Figs. 2.16c and 2.16d show the same two SDS concentrations but 1 mM 2-ME and 1 mM EDTA had been added to their solubilising mixtures as it is believed by some (15) that those two compounds are necessary for complete solubilisation. It can be seen that these also had no effect on the disappearance of protein 4.2, although the final wash in 2.16d does seem to have a slight trace of this component. It is not possible to be sure whether the latter is actually 4.2 or whether the high SDS combined with the 2ME and EDTA had caused some modification in the many minor bands that comprise the 4.5 region. It seems, therefore, that the contentions of G. Fairbanks *et al.* do not stand up to investigation and moreover it would appear that protein 4.2 is

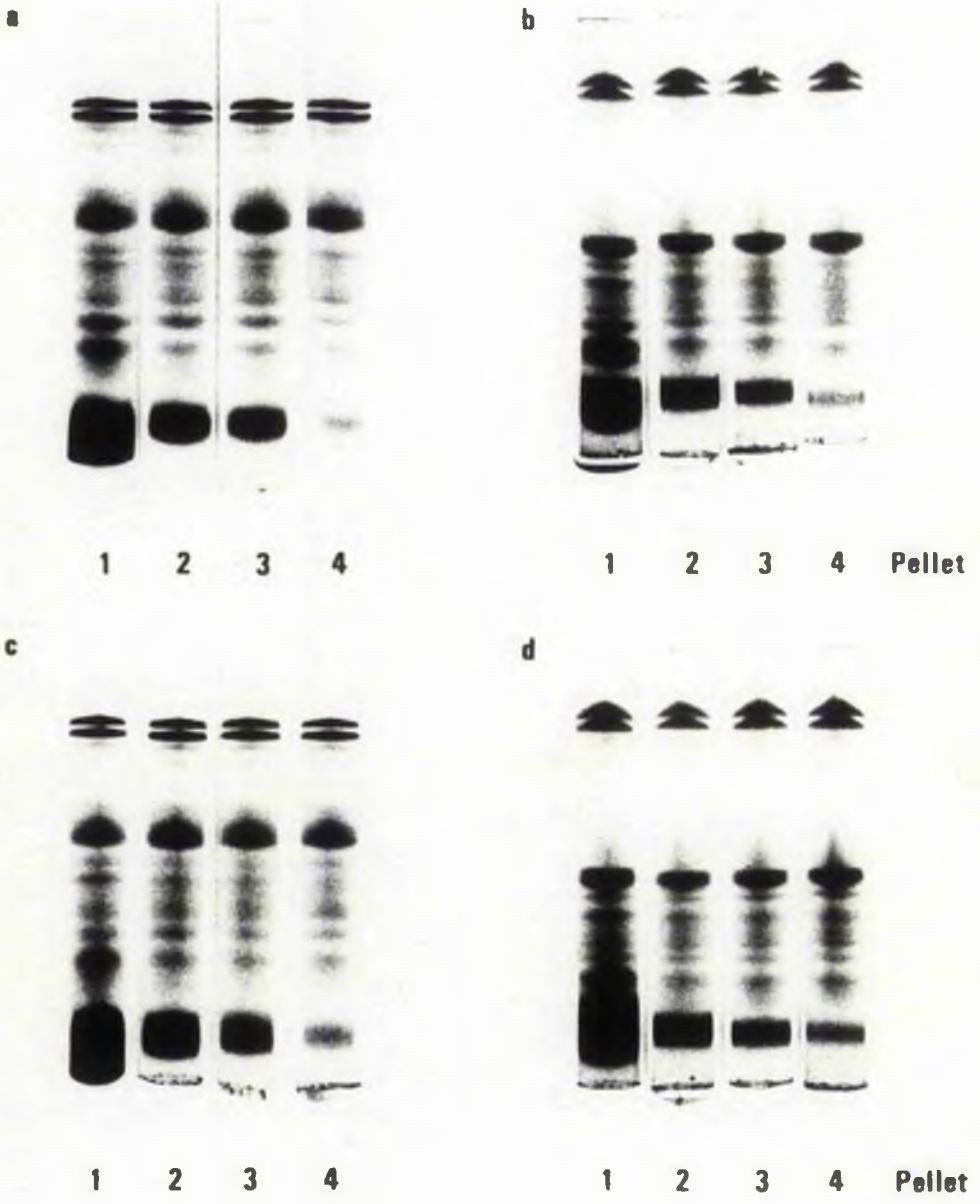

Fig.2.16. The four membrane pellets of unfractionated membranes as revealed by PAGE with (a) 0.2% SDS, (b) 1.0% SDS, (c) 0.2% SDS + 1 mM EDTA + 1 mM 2-ME and (d) 1.0% SDS + 1 mM EDTA + 1 mM 2-ME.

either a very loosely bound membrane protein or that it is present only as a cytoplasmic contaminant. The scans of the gels in Fig. 2.16a are presented in Fig. 2.17 and the % ISD's of the various components are given in Table 2.3. For the purposes of this Table, those proteins in the cytoplasm migrating in similar positions to plasma membrane proteins were given the same numbers as their coincident bands.

It is possible using these data to hypothesise about the in situ origin of the protein species. Successive washing of the membrane pellets by definition removes at least haemoglobin and other cytoplasmic components and so those proteins which constitute the membrane would be expected to become more dominant. In this category we can place proteins 1, 2, 2.5, 3, 4.1, 4.5 and 5. It can be seen that protein 4.2 is present only in membranes containing a large degree of cytoplasmic contamination and this makes it very likely that it is not associated with the membrane in situ. Proteins 6 and 7 appear to be present in significant quantities in both the membrane

	% I.S.D.			
Protein	1st. Pellet	2nd Pellet	3rd. Pellet	4th Pelle
1 + 2	20.7	25.3	23.7	28.4
2.5	2.9	3.7	3.6	3.7
3	27.6	33.6	35.7	37.2
4.1	1.5	1.7	1.7	1.8
4.2	1.4	0.7	0.2	0.0
4.5	12.5	14.9	15.8	14.3
5	4.6	4.2	4.9	5.3
6	10.2	8.2	6.9	4.9
7	18.4	7.4	7.3	4.3

Table 2.3. Alterations in protein content during the preparation of human erythrocyte ghosts from unfractionated blood

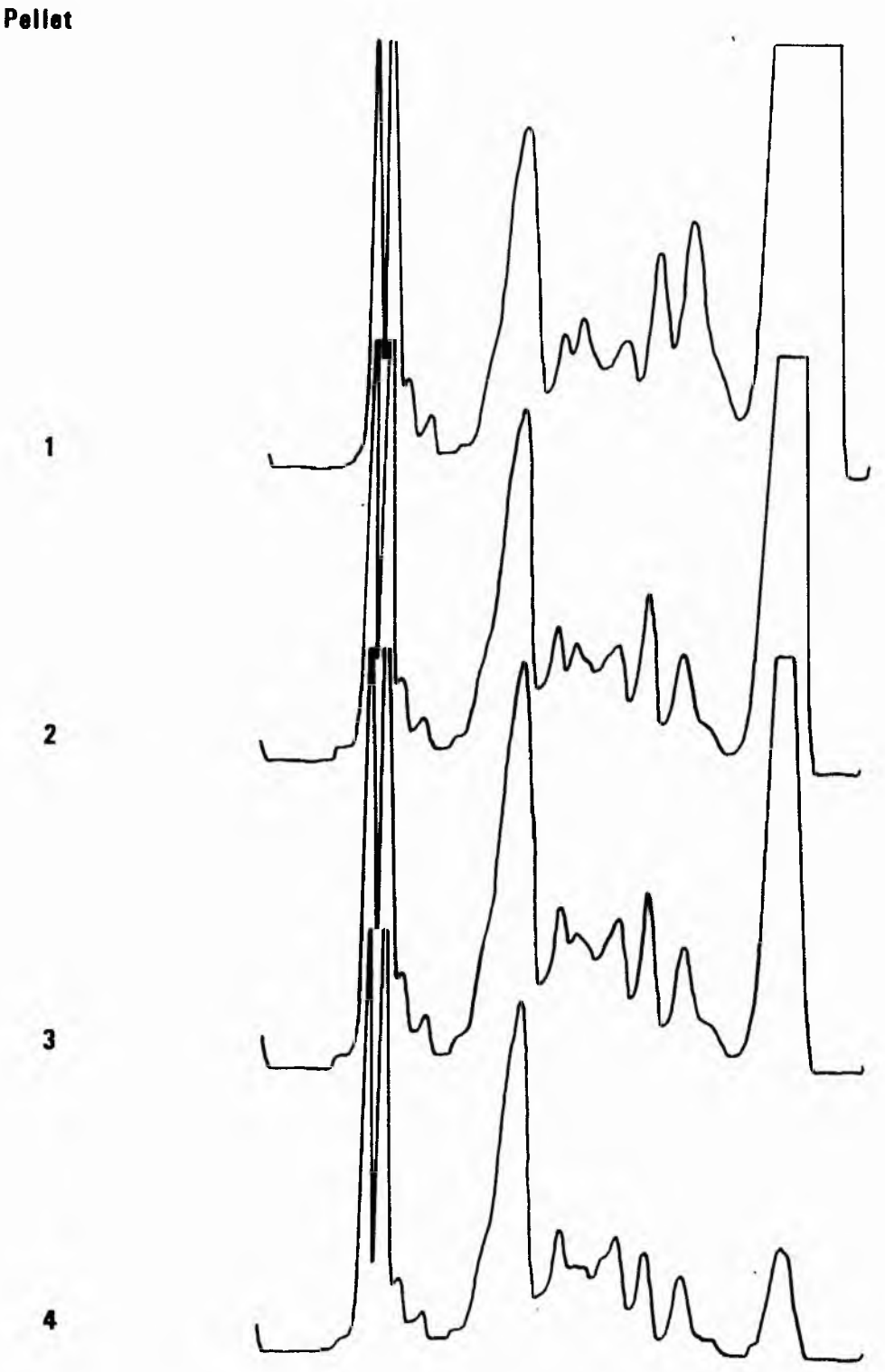

Fig.2.17. Scans of the gels shown in Fig.2.16.a.

and the cytoplasm but obviously coincident mobility is no proof of similar identity. Let us therefore, for the time being, refer to these two species in the cytoplasm as 6' and 7'.

Because of the apparent dual location of two of the SDS PAGE visualised proteins, several studies were initiated in order to clarify this issue. First of all, a brief study was made of the first haemolysates to determine whether or not any age-dependent protein changes could be observed in the cytoplasm. The absence of any qualitative change is clear in Figs. 2.18a and 2.18b. There is naturally a very prominent haemoglobin band and also clearly present are bands 6' and 7'. Unexpectedly, another protein was found which exhibited a very strange behaviour. In Fig. 2.18a it is situated between the doublet and band 3, whereas in 2.18b it is in the 4.2 position. This was repeated several times and the position of this band was quite random. On one occasion seen in Fig. 2.19 bands appeared in both of these positions. The band 4.2 position corresponds to a molecular weight of approximately 75,000 (18) and the other position of this species could easily have been approximately 150,000, and therefore dimerisation seemed very likely.

In order to attempt to confirm this, haemolysate was taken after SDS PAGE had confirmed the presence of the faster variety and passed down a Biogel P200 column using 10 mM Tris/Cl pH 7.4 as the eluant. This was done in the hope that the faster species might be converted into the slower species in a similar manner to the conversion of protein 5 to doublet as shown by M.J. Dunn et al. (31). Both the included and excluded peaks were pooled and concentrated, and a sample of this compared with the untreated haemolysate (Fig. 2.20).

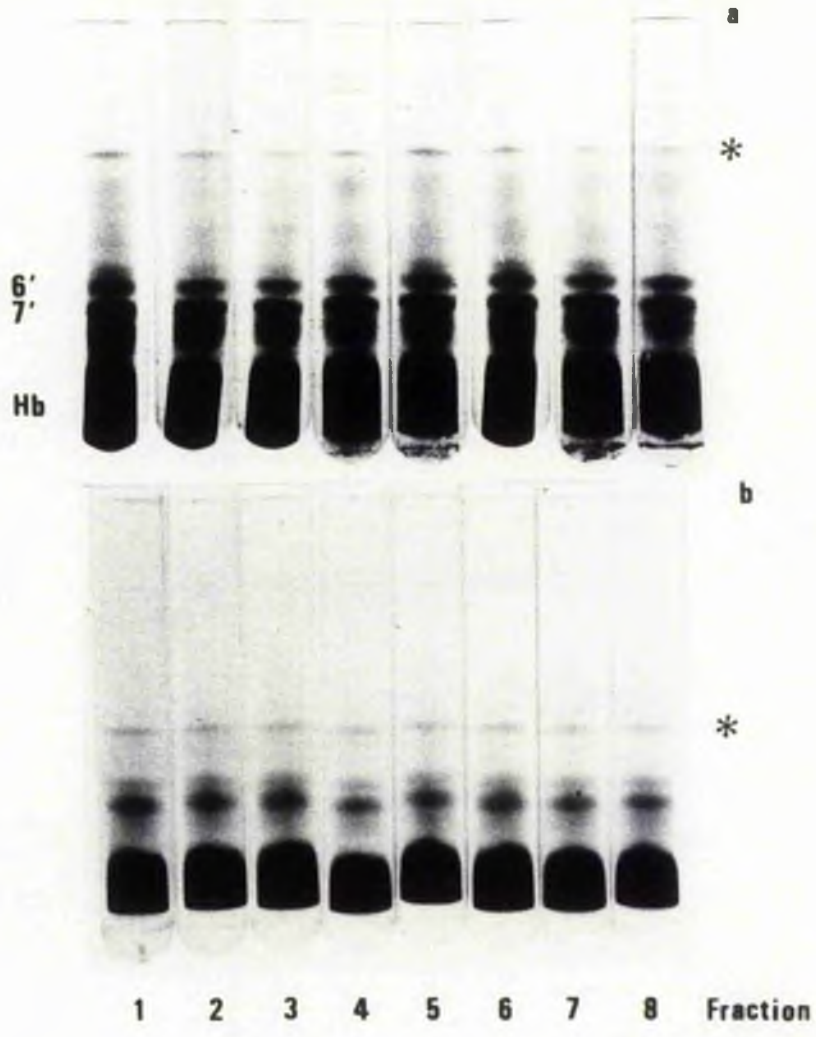

Fig.2.18. The effect of <u>in vivo</u> age on the cytoplasmic proteins showing a protein in (a) the 150,000 M.W. position and (b) the 75,000 M.W. position.

Fig.2.19. The cytoplasmic proteins of unfractionated blood showing proteins in both the 150,000 and 75,000 M.W. positions.

As can be seen this had no effect on the protein in the 4.2 position but did completely remove band 6' which was presumably adsorbed onto the column.

At this point it did not seem worthwhile pursuing this particular problem any further and so the nature of the apparent dimerisation remains a mystery. Figs. 2.13 to 2.17 show that membranes in the presence of high cytoplasmic contamination (1st. Pellet) contain a prominent protein 4.2, and Figs. 2.18 and 2.19 show that the cytoplasm contains a prominent protein which can be in the 4.2 position. It is therefore likely that this mysterious protein and 4.2 are one and the same. If so, this is further evidence for protein 4.2 being present in the membrane only as a cytoplasmic contaminant.

The observation in Fig. 2.20 that passage of haemolysate selectively removed band 6', although quite accidental, proved fortuitous in that it provided a means of deciding the identity of this species. Protein 6 is known to be selectively eluted from the membrane by high salt treatment (51) and so if the adsorbed protein 6' could be eluted from the Biogel column by a similar means, comparative tests could be carried out. Needless to say, the mere successful elution would have been very strong circumstantial evidence for the similar identity of 6 and 6'.

A large amount of haemolysate was therefore added to a Biogel P200 column in order to saturate the binding sites and maximise the yield, and eluted as before. After the complete passage of pigmented material the column was eluted with 10 mM Tris/Cl pH 7.4 containing 1 M NaCl. As can be seen in Fig. 2.21 this high salt treatment succeeded in eluting a

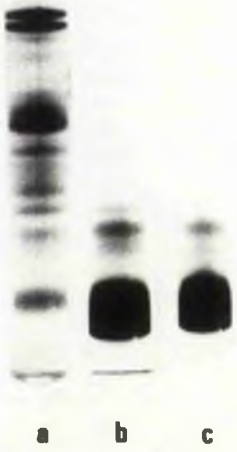

Fig.2.20. The effect of exclusion chromatography with Biogel P200 on the cytoplasmic proteins of unfractionated blood. (a) whole membranes for reference, (b) whole cytoplasm and (c) pooled included + excluded proteins.

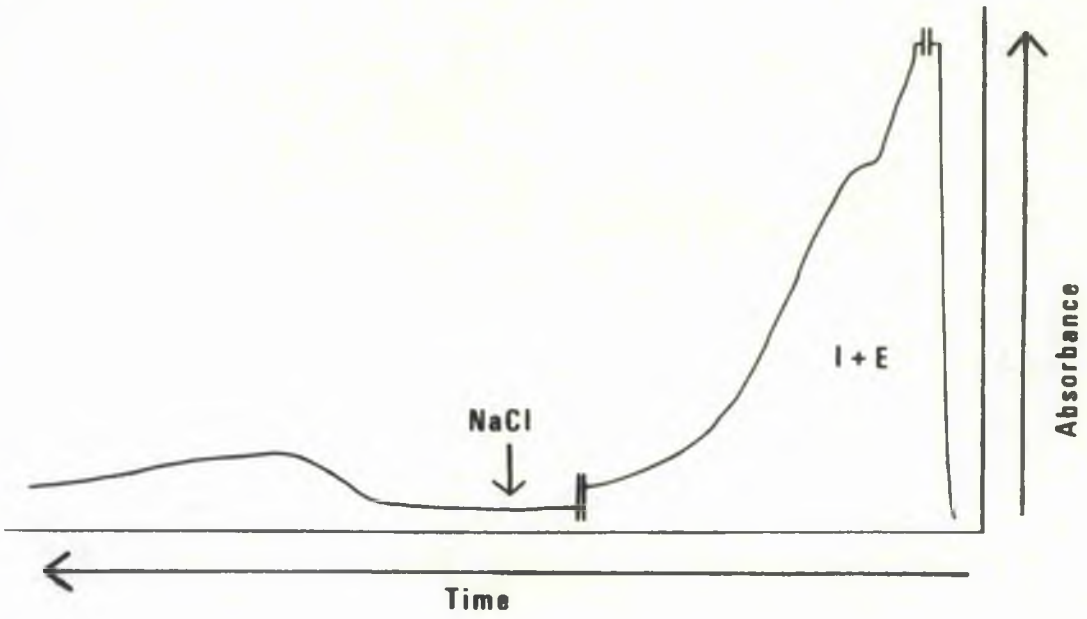

Fig.2.21. The elution of adsorbed cytoplasmic protein from Biogel P200 with 1 M NaCl. (I and E refer to included and excluded material respectively.)

protein peak. This high salt eluted material was then compared with a high salt membrane extract (see Methods for details) by SDS PAGE as shown on Fig. 2.22, which also includes a sample of whole ghosts and the high salt residues for comparison. The sharp band that can be seen between the origin and the doublet is often present in ghosts prepared from outdated blood but its nature is unclear. The high salt treatment of the membranes, as well as liberating virtually all of protein 6, removed most of the residual haemoglobin and a minor protein which is positioned just above band 3. The Biogel extract consisted of protein 6' and a trace of haemoglobin. The two high salt extracts were then subjected to non-surfactant PAGE (Fig. 2.23). The membrane extract gave a broad haemoglobin band and two others labelled (a) and (b). Band(a) was much more prominent than band (b) and when compared with SDS PAGE was therefore designated as being protein 6. Its mobility was coincident with that of protein 6'. The absence of trace haemoglobin in the Biogel extract was probably due to this band being much more diffuse in the non-surfactant system. The coincident mobilities of proteins 6 and 6' in these two very different electrophoretic systems was considered sufficient evidence for concluding that they were the same protein. This conclusion is strengthened by the findings of S.L. Schrier et al. (65) that glyceraldehyde-3-phosphate dehydrogenase (which is known to migrate in the band 6 position-ref. 51) prepared from both the membrane and the cytoplasm could not be distinguished by gel filtration chromatography, kinetic parameters and antibody reaction.

No simple method was found for preparing relatively enriched fractions of proteins 7 and 7' but there was sufficient

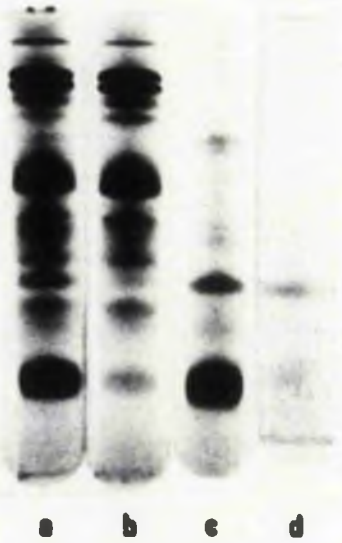

Fig.2.22. A comparison of high salt membrane and cytoplasmic extracts. (a) whole membrane, (b) high salt membrane residue, (c) high salt membrane extract and (d) high salt Biogel P200 extract.

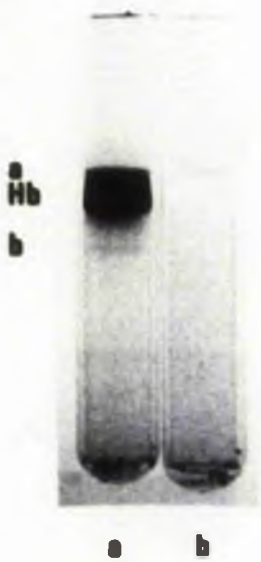

Fig.2.23. A comparison of (a) high salt membrane extract and (b) high salt Biogel P200 extract by non-surfactant PAGE.

circumstantial evidence to indicate the similarity of their identities. Fig. 3.10 in the following chapter shows the separation of a cytoplasmic acid extract by sucrose density gradient centrifugation, the point of interest being Peak III. The material in this peak when visualised by SDS PAGE (Fig. 3.10) gave a prominent species in the band 7 position plus a minor species just above the band 3 position. As stated in the text the hollow cylinder protein (66), which is extracted from the plasma membrane, is known to migrate in the same position on the sucrose density gradient. This membrane located protein also gives a major band in the 7 position and a minor band just above the 3 position on SDS PAGE. Lastly, with reference to Fig. 2.5 in this chapter, it can be seen that there is a minor species just above band 3 (*) which visibly decreases with age in two out of the three cases shown. The importance of this becomes clear when one remembers that protein 7 was found to exhibit a significant decrease in % ISD with age. The following conclusions can therefore be drawn.

1. Proteins 7 and 7' are the same.
2. These two proteins represent the subunits of the very high molecular weight Hollow cylinder protein observed by J.R. Harris (66).
3. A small proportion of this protein is not completely broken down into constituent subunits by SDS treatment.

Before proceeding, it must be pointed out that the minor species released by high salt treatment, and the protein that is likely to be a polymer of protein 7 would also appear to be one and the same. The discussion on the identity of band 7 is dependent on the latter being found invariably in the

presence of this minor species and so some doubt must exist with regard to the conclusion. However, it is possible (67) that the four subunits of the hollow cylinder protein (known as single torus proteins) are not identical and so the high salt treatment may specifically release that subunit which is not monomerised by SDS.

Having shown that in the cases of protein 6 and 7 a coincident mobility is almost certainly a result of similar identity, it is now possible to conclude the discussion on the finding listed in Table 2.3. Proteins 6 and 7 are by no means exclusively located in the plasma membrane as proteins 1, 2, 2.5, 3, 4.1, 4.5 and 5 would appear to be, and so we shall have to define carefully what we mean by the term 'membrane protein'. The failure of the washing procedure to remove a significant proportion of proteins 6 and 7 suggests that these two species are normally resident in the plasma membrane as well as the cytoplasm. A similar situation is likely to be the case for protein 4.1, for although it showed a high affinity for the plasma membrane and was not noticeably removed by the washing procedure, the temperature experiments provided evidence that it might also be found in the cytoplasm. Finally, protein 4.2 showed no affinity for the plasma membrane and so is most likely to reside exclusively in the cytoplasm.

SDS Anomaly

On several occasions while performing SDS PAGE of membrane samples, one or more of the gels ran very badly and had to be discarded. At first this was explained as random failure as one cannot expect every experiment to be successful, no matter how much care is taken. Two such occurrences are

revealed in Fig. 2.24. It can be seen though from Fig. 2.25 that this is not a random effect but is restricted to the younger fractions, especially fraction 2. This could not possibly have resulted from the experimental procedure. The lack of any flocculated material at the origin of the gels was puzzling. Instead the bands were rendered highly diffuse so that they merged into each other and in some cases (e.g. Fig. 2.24a) the high molecular weight bands were also virtually absent.

By chance, an experiment was being carried out under the conditions used in Fig. 2.16 when this effect happened to the membrane proteins of fraction 1. It can be seen in Fig. 2.26 that increasing the concentration of SDS to 1.0% reversed this effect but 1 mM 2-ME and 1 mM EDTA were inactive. By the very nature of the occurrence of this effect it was not possible to repeat this experiment or take it any further.

In the instances when the effect was characterised merely by the loss of band definition it is easy to postulate that an age-dependent resistance to SDS solubilisation was in operation rather than the presence of SDS resistant bonds. However, the disappearance of the high molecular weight bands cannot be explained. There is some similarity between this effect and the observation of M.J. Conrad et al. (57) that the membrane proteins of outdated blood were more resistant to SDS solubilisation. In this study, however, it is the younger membrane proteins which were more resistant, thus illustrating yet another difference between in vivo and in vitro ageing.

Fig.2.24. Two experiments on the effect of <u>in vivo</u> age on the membrane proteins in which one of the gels in each ran anomalously.

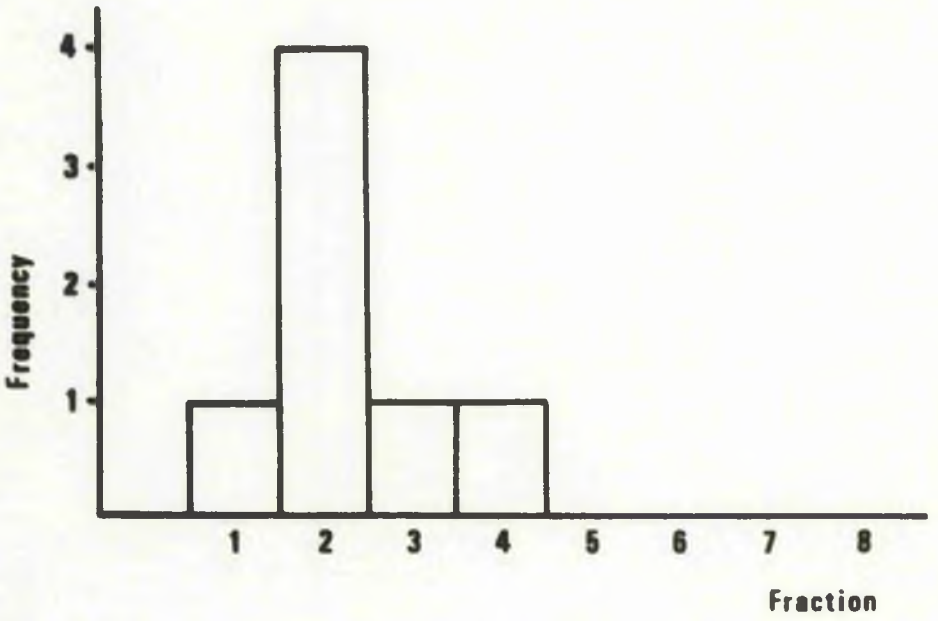

Fig.2.25. The effect of <u>in vivo</u> age on the occurence of anomalous gels.

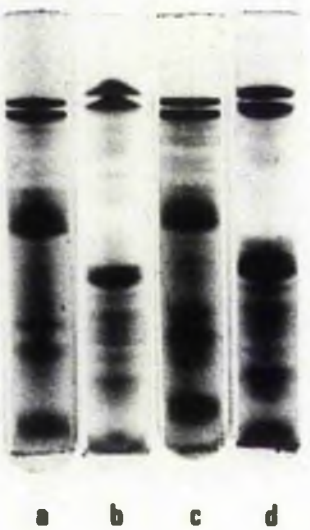

Fig.2.26. The effect of (a) 0.2% SDS, (b) 1.0% SDS, (c) 0.2% SDS + 1 mM EDTA + 1 mM 2-ME and (d) 1.0% SDS + 1 mM EDTA + 1 mM 2-ME, on the appearance of the membrane proteins from fraction 1.

Discussion

It has been shown that *in vivo* ageing is associated with three significant alterations in the membrane proteins so let us consider these individually.

Protein 2.5

The protein referred to as 2.5 in this study is in fact all the minor components between proteins 2 and 3. This region is usually dominated by two bands but there are often more, J.V. Staros *et al.* (42) claiming to have labelled six components. In fact, although the region as a whole was found to increase with age, one very minor component positioned immediately above band 3 was observed to decrease. Also, the proximity of the doublet means that some of these components are on the lower portion of the descending limb of the doublet scan and thus the resulting % ISD is undoubtedly high. Therefore, conclusions about this band's increase are prevented not only because of the heterogeneity, but also because any change in the doublet scan could have some effect on the % ISD. An example of the latter is a broadening of the doublet band with age which would force the 2.5 trace further up the descending limb causing an artifactual increase due to the manner of calculating the % ISD's. The lack of any change in the doublet renders the latter possibility somewhat academic but it is still a point worth bearing in mind.

Protein 4.1

Having shown that under the conditions used in this study this is a single, though not necessarily homogeneous, component, its increase is available for hypothesis. It has already been shown that it does not result from selective elution during the ghosts' preparation, and the red cell being enucleate, there should be no objection to ruling out protein synthesis. This leaves us with two possible explanations.

1. Aggregation of low molecular weight proteins or breakdown of high molecular weight proteins.
2. Adsorption of a cytoplasmic protein.

Considering the first possibility, we must look at all the other proteins as revealed by SDS PAGE and decide whether or not the observed increase in protein 4.1 could have arisen from a decrease in one or more of these. We can immediately eliminate protein 2.5 as this also showed an increase with age but the decrease in protein 7 was more than sufficient to accommodate the 4.1 increase. That protein 4.1 is not a polymer of protein 7 is evident from the following considerations.

1. The isolation of protein 7 from both the plasma and the cytoplasm was discussed and protein 4.1 was never found to be associated with it.
2. If protein 4.1 were a polymer of protein 7 then from the results of Table 2.1, 5% of this protein in the youngest membranes would have been in the polymerised form rising to 40% in the oldest membranes. Protein 7 has been demonstrated to be a predominant species in the cytoplasm and so if between 5 and 40% of it were in the polymerised form this would have

been clearly visible. There is, however, a conspicuous lack of any protein in the 4.1 position in the cytoplasm.

3. Table 3.3 reveals that washing of the membrane pellets was associated with a drastic loss of protein 7 but a slight increase in the relative proportion of protein 4.1.

4. R.G. Langdon (68) found proteins 4.1 and 7 to have highly dissimilar N-terminal amino acids.

None of the other protein species showed a significant decrease in % ISD but due to the relatively minor nature of protein 4.1 it is quite possible that a decrease may have occurred, but as a result of the errors involved in the measurements this was not noticed. In this category must be placed proteins 1, 2 and 4.5. Protein 3 although being the most prominent of all the protein bands exhibited such a low standard error (see Tables 2.1 and 2.2) that it is safe to conclude that a loss of material would certainly have been detected. On the other hand, proteins 5 and 6 cannot by any means be considered as subunits of protein 4.1. Reluctantly, protein 4.5, due to its diffuse and undoubtedly heterogeneous nature, must be left out of the discussion and so we are left with the doublet.

It is well known that low ionic strength treatment of the ghost selectively elutes proteins 1, 2 and 5 (21, 31) and that protein 4.1 is never found in this extract. Thus, although there is evidence for considering protein 5 as being a subunit of the doublet (31), it is unlikely that protein 4.1 can be so classified. This is again supported by R.G. Langdon (68) who showed that doublet and protein 4.1 do not share the same N-terminal amino acids.

This leaves us with the adsorption of a cytoplasmic protein, which could possibly be onto the outer surface of the membrane following haemolysis. Why then should the older membranes bind more of this protein? One explanation would be the suggested decrease in the sialic acid content of the membrane with age (69, 70) which would result in a decreased repulsion of a negatively charged protein. The demonstration in the previous chapter of a constant sialic acid content when expressed per mg. of membrane protein makes this most unlikely. However, it was suggested that there might be a more subtle change in the surface charge with age than the sialic acid measurements would have us believe.

Protein 4.1 is not eluted from the membrane by low ionic strength (21, 31), high ionic strength (51, 52) and 0.5% Triton X-100 (47). Thus its association with the membrane can be said to be one of high affinity as compared to protein 4.2, for example, which shows no affinity at all for the membrane. Labelling of resealed ghosts reveals that, after washing to remove cytoplasmic contamination, protein 4.1, is not present on the outer surface (54, 71), although it is accessible from the outer surface under certain conditions (42). This makes the location of protein 4.1 on the inner surface of the membrane the most likely one. The resistance of protein 4.1 to ionic manipulations suggests that the increase in the membrane complement of this protein with age is not as artifact, but is likely to have arisen from the high affinity binding of a protein which is initially, in the young mature erythrocyte, purely cytoplasmic. It is clearly not possible to make any statement about the nature of this binding except to restate that it does seem to be unusually

susceptible to temperature. Weight is added to this hypothesis by the observation of I. Fischer et al. (72) that the older erythrocytes contain less non-haemoglobin protein in their cytoplasm compared to the younger ones, although their decrease of 20% is vastly in excess of the situation here.

Protein 7

As Protein 7 has the lowest apparent molecular weight in the SDS system, we need only consider whether this has polymerised to form another of the bands. Proteins 2.5 and 4.1, being the only areas where a significant increase had been recorded, must be the most probable locations of such a polymer if it had been formed. Protein 4.1 has already been ruled out as a likely candidate and some of the same arguments can be applied to protein 2.5. That is, if the latter were a polymer of protein 7, then 29 to 52% would have to exist in this form making it easily visible in the cytoplasm, and washing the ghosts leads to a decrease in 7 and an increase in 2.5. The situation is not quite as straightforward as for 4.1 though, due to the observation that one of the bands constituting the 2.5 region did seem to be a polymer of protein 7; this showed a decrease with age as did 7 in contrast to the rest of the region. This obviously strengthens the case for not considering the increase in 2.5 to have arisen at the expense of 7. The same arguments also apply as regards proteins 1, 2, 3, 4.5, 5 and 6.

It is therefore most likely that this is simply a 'loosely' bound protein which is lost to the cytoplasm as a consequence of the ageing process. It is a matter for conjecture

whether this decrease of protein 7 represents an *in situ* loss of the membrane bound protein into the cytoplasm or an age-dependent inability to withstand the rigours of haemolysis and successive hypotonic washing.

Protein Classification

This discussion on the effect of *in vivo* ageing and the results from Figs. 2.13 to 2.19 and Table 2.3 enable us to classify the proteins revealed by SDS PAGE into four groups.

1. Proteins present solely in the membrane; 1, 2, 2.5, 3, 4.5 and 5.
2. Proteins always present in both the membrane and the cytoplasm; 6 and 7.
3. Cytoplasmic proteins present in the membrane only as contaminants; 4.2.
4. Cytoplasmic proteins which become firmly bound to the membrane during *in vivo* ageing; 4.1.

The classification is based on the results contained in this chapter concerning the location and behaviour of the so-called membrane proteins and is intended as a guide to a fuller understanding of them. It is not unlikely that all the membrane proteins are represented in the cytoplasm, but that those in group 1 being present in very small amounts, are masked by the enormous amounts of haemoglobin. Thus, protein 4.1 has not been empirically observed in the cytoplasm, but its presence there has been inferred from its behaviour in the membrane with respect to red cell ageing.

In Vitro Ageing

Significant, if slight, increases in proteins 3, 4.1 and 6 were noted as a consequence of *in vitro* ageing. The significance of the protein 3 result is probably due to its constancy. That is, because of the very low error involved in its measurement, a very slight general decrease in the relative proportions of the other proteins would result in an increase being recorded.

It is tempting to conclude that the increase in protein 4.1 noted for *in vivo* ageing continues *in vitro* and therefore represents some intrinsic property of the membrane that continues to change regardless of the red cell environment. As the % ISD for the fresh membranes was somewhat lower than that recorded in Tables 2.1 and 2.3 it might, however, be unwise to draw such a hasty conclusion.

The increase in protein 6 was strange in that one might expect a loss of membrane integrity with *in vitro* ageing with a subsequent loss of the less firmly bound group 2 proteins. It may be that the losses of proteins 1, 2 and 7, which are not themselves significant, result in more area being made available on the inner surface of the membrane for this major cytoplasmic species to become adsorbed. Perhaps this process does not require the loss of other proteins but results simply from adsorption onto the inner membrane surface due to the conditions of storage.

It is obvious that *in vivo* and *in vitro* ageing are not identical with reference to changes in the membrane proteins. Further evidence for a distinction between these two processes was afforded by the observation that it was the younger *in vivo* aged membranes which seemed to be more resistant to SDS

solubilisation, whereas N.J. Conrad *et al.* (57) had shown that in the case of *in vitro* ageing it was the older membranes which were more resistant. It is to be hoped that authors in the future will be more careful in the conclusions that they draw from *in vitro* experiments and that reviewers will be more diligent in differentiating the two processes. G. Fornaini (73) for example, in his review on the biochemical aspects of red cell ageing collated results from both kinds of experiments, this giving a very misleading picture.

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CHAPTER 3

PURIFICATION OF PROTEIN 4.1

Summary

1. Protein 4.1 could not be purified from the membrane.

2. A protein which was thought to be protein 4.1 was purified from the cytoplasm using non-denaturing techniques.

Introduction

The previous chapter revealed several changes in the membrane proteins of the human erythrocyte with respect to *in vivo* ageing. One of these, the apparent binding of protein 4.1 from the cytoplasm, seemed worthy of further study, not only as a means for a deeper understanding of the ageing process, but also to enable the elucidation of the criteria that determine whether a protein be found in the cytoplasm or bound to the membrane. Protein 4.1 would appear to start its existence in the newly formed mature erythrocyte in a completely soluble form and gradually a fraction becomes firmly bound to the membrane, as judged by its resistance to various solubilising procedures (see Chapter 2). The binding of 4.1 to the membrane was also effected by temperature and the chelating agent EDTA, although these effects were highly variable. Thus, protein 4.1 seems to be an eminently suitable subject for the study of membrane-cytoplasm interactions, the results of which could throw more light on the ageing process itself.

As was revealed in Chapter 2, it was not possible to perform any reproducible binding studies in the presence of the whole cytoplasm, due no doubt at least partially to the complexity of the system, and so it was decided to attempt to attempt to devise a purification scheme for this protein. M.J.A. Tanner et al. (1) purified a very small amount of a protein F which seemed to correspond to protein 4.1 in the classification used in this study, but this involved repeated pyridine extraction, repeated acetone extraction and exclusion chromatography in the presence of very high concentrations of sodium dodecyl sulphate. While this may have been satisfactory for the elucidation of the primary structure of this protein, denaturing agents of this calibre certainly cannot be used if any degree of physiological integrity is intended to be retained. It was the prime directive of the work carried out in this chapter that protein 4.1 be isolated by non-denaturing means.

Methods and Materials

Ghosts were prepared from outdated blood as previously described. All reagents were purchased from BDH except Brij 35 and Tween 20 which were obtained from Sigma Ltd.

Membrane Extracts

The initial low ionic strength extract was prepared by adding 1 volume of ghosts to 1 volume of 5 mM EDTA/ 5mM 2-ME/ 5 mM Tris/Glycine pH 9.5, incubating the mixture overnight at 4°C and centrifuging for 30 minutes at 20 Krpm. The residue was then added 1:1 to a 1% Tween 20 solution in 10 mM Glycine/ NaOH pH 9.8, incubated similarly and centrifuged for 30 minutes at 60 Krpm. Brij 35 and Nonidet P40 were also used at a concentration of 1% in the same buffer as Tween 20. The combined solubilising mixture consisted of 1% Tween 20/ 5 mM EDTA/ 5 mM 2-ME/ 5 mM Tris/Glycine pH 9.5.

Acid Precipitation

1 M citric acid was used in all cases. The details will be given in the text.

Ammonium Sulphate Precipitation

1 volume of resolubilised acid precipitate at pH 8.0 was added to 1 volume of ammonium sulphate in 0.1 M Tris/Cl pH 8.0, stirred for 5 minutes and then centrifuged for 5 minutes at 5 Krpm.

Sucrose Density Gradient Centrifugation

This was performed using the Beckman L2 65B ultracentrifuge with an SW 27 rotor. Sucrose was dissolved in 10 mM Tris/Cl pH 8.0 4.0 ml. of 2.0 M sucrose was placed at the bottom of 40 ml. centrifuge tubes and the 35 ml. linear gradients were formed on top. A standard two-chamber gradient mixer was used to prepare the 0.25 to 1.0 M and 0.4 to 1.0 M gradients which were pre-cooled to 4^{o}C prior to the addition of the acid extract. The gradients were spun at 27 Krpm for 24 hours after which analysis was performed by piercing the apex of the tube and running the gradient through an LKB fraction collector as described previously.

Analytical PAGE

Both SDS and non-surfactant PAGE were carried out as described previously.

Preparative PAGE

Quickfit Instrumentation apparatus was used in which the proteins were eluted at the anodic surface of the polyacrylamide gel and collected in the LKB fraction collector. A 15 ml. gel was used and 2.0 ml. of the SDG extract containing a very small amount of bromophenol blue was added. A current of 50 mA was used for the first 10 minutes, increasing to 100 mA for the second 10 minutes and 150 mA for the rest of the run which lasted approximately 2 hours. The gel and buffer were cooled with constantly running tap water.

Ficoll

Very dilute protein solutions were concentrated against a Ficoll solution at 4°C. The latter was prepared by dissolving as much of the powder as possible in a volume of distilled water, dialysing exhaustively for several days against distilled water and concentrating in a Wright's rotary film evaporator until crystals began to appear. After the Ficoll had been used three times this procedure was repeated.

Immunodiffusion.

The Ouchterlony double diffusion technique (2) was used. Antibody was prepared by injecting a rabbit with 1 mg. of ghosts plus 2.5 ml. of Freund's adjuvant followed by 9 weekly injections of 1 mg. of ghosts. A week after the final injection 50 ml. of blood was withdrawn from the ear, allowed to coagulate and centrifuged to yield the antibody-containing supernatant. 1% Agar dissolved in 1% sodium azide and phosphate buffered saline (PBS) was cast in 5 cm plastic petri dishes to a depth of 1 mm. The central well and six surrounding walls were cut out with a cork borer attached to a vacuum pump. One drop of antibody was added to the central well and the various protein extracts added to the surrounding wells. The precipitin lines were left to develop for 48 hours at 4°C after which the gels were repeatedly washed in PBS for another 48 hours. Finally, the gels were stained with Coomassie Blue as described in the previous chapter.

Photography was as described previously.

Results and Discussion

Protein 4.1 is present in the membrane and its existence in the cytoplasm has been strongly inferred, providing a choice of two possible starting materials. The membrane has the obvious advantage of definition. That is, protein 4.1 is by definition that protein which migrates in a characteristic position when whole erythrocyte ghosts are subjected to SDS PAGE. Consequently, following the fate of this band through whatever procedures are undertaken would avoid the possible ambiguity of coincidental mobility. The cytoplasm is at a disadvantage because there is no band visible in the 4.1 position, but as the protein is presumably in a soluble form it should be easier to deal with.

Membrane Extracts

The initial attempt at purification was made using the membrane not only for the reasons stated above, but because of a report by L. Liljas *et al.* (3) on the selective elution of protein 4.1 using the nonionic surfactant Tween 20. Although it was intended to avoid the use of surfactants, the nonionic class of these compounds is considered to be exceptionally mild (see Introduction to Chapter 5). Essentially, the technique consisted of an initial low ionic strength EDTA treatment of the membrane to remove most of proteins 1, 2 and 5 (4), followed by disruption of the residue with low ionic strength, glycine buffered Tween 20 as described in the methods. According to L. Liljas *et al.* this latter solution should have solubilised all the protein 4.1 and the remaining doublet.

Unfortunately, despite their conditions being rigidly adhered to, this apparently simple extraction procedure could not be repeated. It seems from gels c and d in Fig. 3.1 that if anything, protein 4.1 had been selectively left behind in the residue. For the sake of comparison the low ionic strength residue was also treated with two other nonionic surfactants, Brij 35 (gels e and f) and Nonidet P40 (gels g and h) under the same conditions as Tween 20. From Fig. 3.1 it seemed that Tween 20 and Brij 35 were having very similar effects except that protein 4.1 was more equally distributed between residue and supernatant in the latter, and so as a last resort an attempt was made to combine these two surfactants. This attempt, which also failed, is illustrated in Fig. 3.2. Gels a and b show the whole membrane and low ionic strength extract as in Fig. 3.1, gel c is the Tween 20 supernatant and gels d and e are the residue and supernatant of the Brij 35 extraction of the Tween 20 residue. Gel f is the supernatant from a combined low ionic strength EDTA/ Tween 20 extraction of the whole membrane, the residue being treated with Brij 35 to give residue (gel g) and supernatant (gel h). There was obviously no point in continuing this approach. A.H. Maddy (5) has listed the solvents in common use for the partial and total solubilisation of the erythrocyte membrane proteins, but these were all rejected as being possibly deleterious to functional integrity.

Cytoplasmic Extract

There was thus no choice but to consider the cytoplasm as a possible source for protein 4.1. Before all else, a

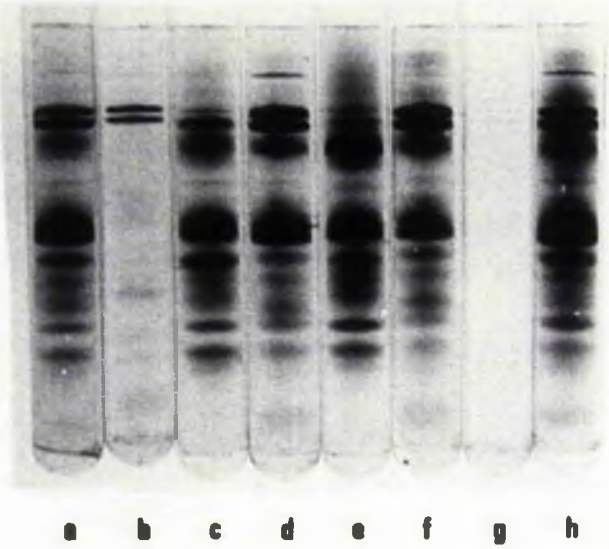

Fig.3.1. The effect of nonionic surfactants on the membrane proteins. (a) whole membranes, (b) low ionic strength extract, (c) T20 residue, (d) T20 supernatant, (e) B35 residue, (f) B35 supernatant, (g) NP40 residue and (h) NP40 supernatant.

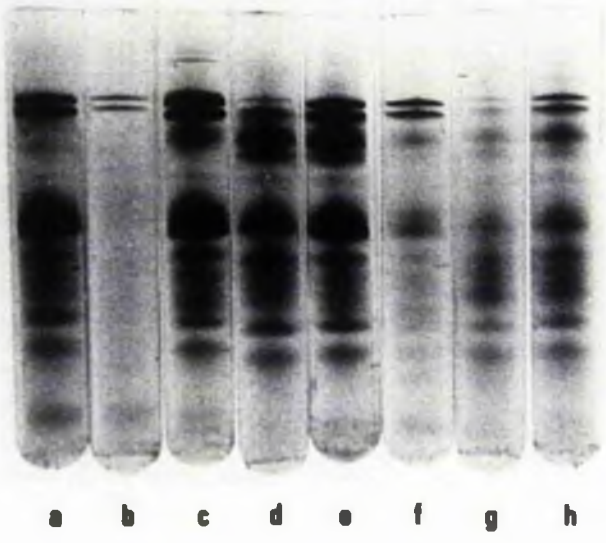

Fig.3.2. A sequential nonionic surfactant solubilisation of the membrane proteins. (a) whole membranes, (b) low ionic strength extract, (c) T20 supernatant, (d) B35 residue of T20 residue, (e) B35 supernatant of T20 residue, (f) low ionic strength/T20 supernatant, (g) B35 residue of low ionic strength/T20 residue, (h) B35 supernatant of low ionic strength/T20 residue.

convenient method had to be found for removing the vast excess of haemoglobin to permit the visualisation of the remaining proteins. Ammonium sulphate was tried but was found to be non-selective, precipitating haemoglobin and non-haemoglobin proteins alike. Acid precipitation offered more hope as the membrane proteins are known to have an isoelectric point of approximately pH 5.0, (6) whereas the isoelectric point of haemoglobin is 7.0 (7). A range of pH values was tried, the cytoplasm being adjusted to the selected pH by titration with 1 M citric acid and centrifuged. The resulting pellet was resuspended at pH 7.5 by the addition of 1 M Tris, centrifuged and the process repeated on the supernatant. It can be seen in Fig. 3.3 that this was successful in selectively removing the majority of haemoglobin and consequently revealing a considerable number of hitherto hidden protein species. The presence of doublet is likely to be due to contamination by membrane fragments which are also brought down by the acid treatment, but as this component is located on the inner surface of the membrane (4) it is possible that a small fraction is normally resident in the cytoplasm (see discussion on group 1 proteins-Chapter 2). Lowering the pH to 5.5 brought down very little protein, but decreasing it further to 5.0 and 4.5 was very effective, also producing one or two likely candidates for protein 4.1. As there was very little to choose between pH 4.5 and 5.0 it was decided to use the latter for further study so as to minimise any harmful effects which the acidification might be having on the proteins.

The acid extract was resuspended at pH 8.0 and treated with increasing amounts of ammonium sulphate to yield the

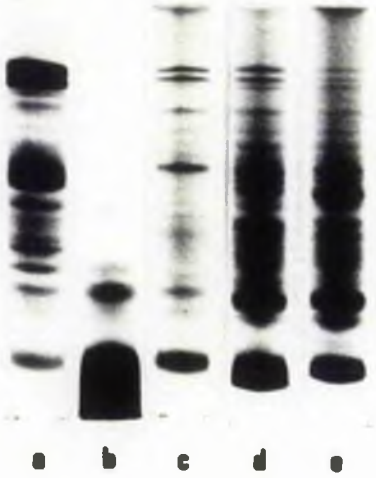

Fig.3.3. The precipitation of cytoplasmic proteins with Citric acid. (a) whole membranes, (b) whole cytoplasm, (c) pH 5.5 ppt., (d) pH 5.0 ppt., (e) pH 4.5 ppt.

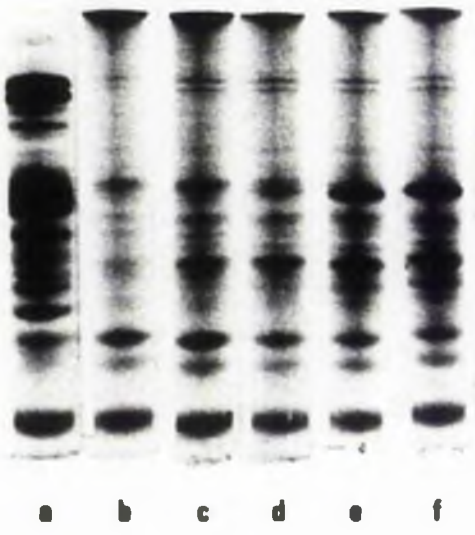

Fig.3.4. The precipitation of resuspended pH 5.0-precipitated cytoplasmic proteins with $(NH_4)_2SO_4$. (a) whole membranes; final $(NH_4)_2SO_4$ concentration of (b) 2%, (c) 4%, (d) 6%, (e) 8%, (f) 10%.

precipitates shown in Fig. 3.4. Whilst there was obviously a protein in the 4.1 position, it was neither being selectively retained in solution nor precipitated by the ammonium sulphate. Out of curiosity, the supernatant remaining from the pH 5.0 precipitation was titrated to pH 4.5 by the addition of more 1 M citric acid and centrifuged. This extract contained a very prominent species with a migration rate only fractionally slower than that of protein 4.1 (Fig. 3.5). Addition of this extract to a whole membrane sample (Fig. 3.6) caused the migration rate of this interesting protein to increase slightly so that it coincided with the position of protein 4.1.

Thus, by the only criterion available, that of mobility in SDS PAGE in the presence of all the membrane proteins, this was protein 4.1. It is accepted that this is a tenuous conclusion as it is quite likely that more than one protein could have the same migration rate, but this study was begun with the full realisation that such a situation must arise. It was accepted that the purification of a protein from the cytoplasm must be accompanied by a degree of uncertainty, but that once the protein had been purified it would be possible to ascertain whether or not it was in fact protein 4.1 by immunological means.

For some unknown reason this sequential acid extraction could not be repeated but it had been shown that a protein behaving like protein 4.1 as far as SDS PAGE was concerned, was present in the pH 4.5 precipitate, so let us refer to this protein for the time being as 4.1'. The procedure for preparing the acid precipitate was therefore modified accordingly and lengthened somewhat to remove even more

Fig.3.5. The proteins precipitated by decreasing the pH of the pH 5.0 cytoplasmic supernatant to pH 4.5 with Citric acid (b). The whole membrane is shown in (a).

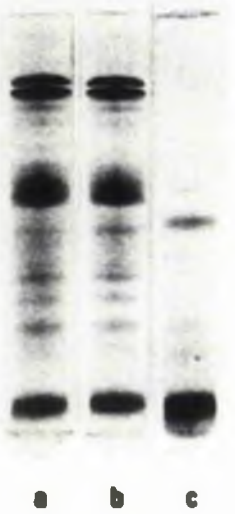

Fig.3.6. The addition of the pH 5.0 - pH 4.5 cytoplasmic extract to whole membranes. (a) whole membranes, (b) whole membranes + cytoplasmic extract, (c) cytoplasmic extract.

haemoglobin. It is shown in full in Fig. 3.7, the numbers in parenthesis referring to the gels in Fig. 3.8 which demonstrates the proteins present at each stage. The selective removal of haemoglobin with the resulting enrichment of the other proteins can be clearly followed.

Sucrose Density Gradient (SDG) Centrifugation

The resuspended acid extract was subjected to SDG centrifugation employing a 0.25 to 1.0 M gradient (Fig. 3.9a) and a 0.4 to 1.0 M gradient (Fig. 3.9b). The 4.0 ml. pad of 2.0 M sucrose on which these gradients were formed was to prevent any high density fragments from reaching the bottom of the centrifuge tube and clogging the fraction analyser after the tube had been pierced. Although a sucrose gradient was used, the separation achieved after 24 hours was likely to have been velocity-dependent rather than isopycnic (8). 2.0 ml. of acid extract was used per gradient and this unfortunately caused a slight overloading but it can still be seen that the 0.25 to 1.0 M sucrose gradient gave a better separation of the components as judged by the width of the shoulder on the main peak (*) and the depth of the trough between the two peaks (**).

A detailed characterisation of the 0.25 to 1.0 M gradient was therefore undertaken using 1.0 ml. of the acid extract per gradient with a much more satisfactory result (Fig. 3.10). Below the Uvicord trace are SDS PAGE gels which reveal the proteins present throughout the gradient. The material comprising the jagged peak at the bottom of the gradient failed to enter or only just entered the gels and so

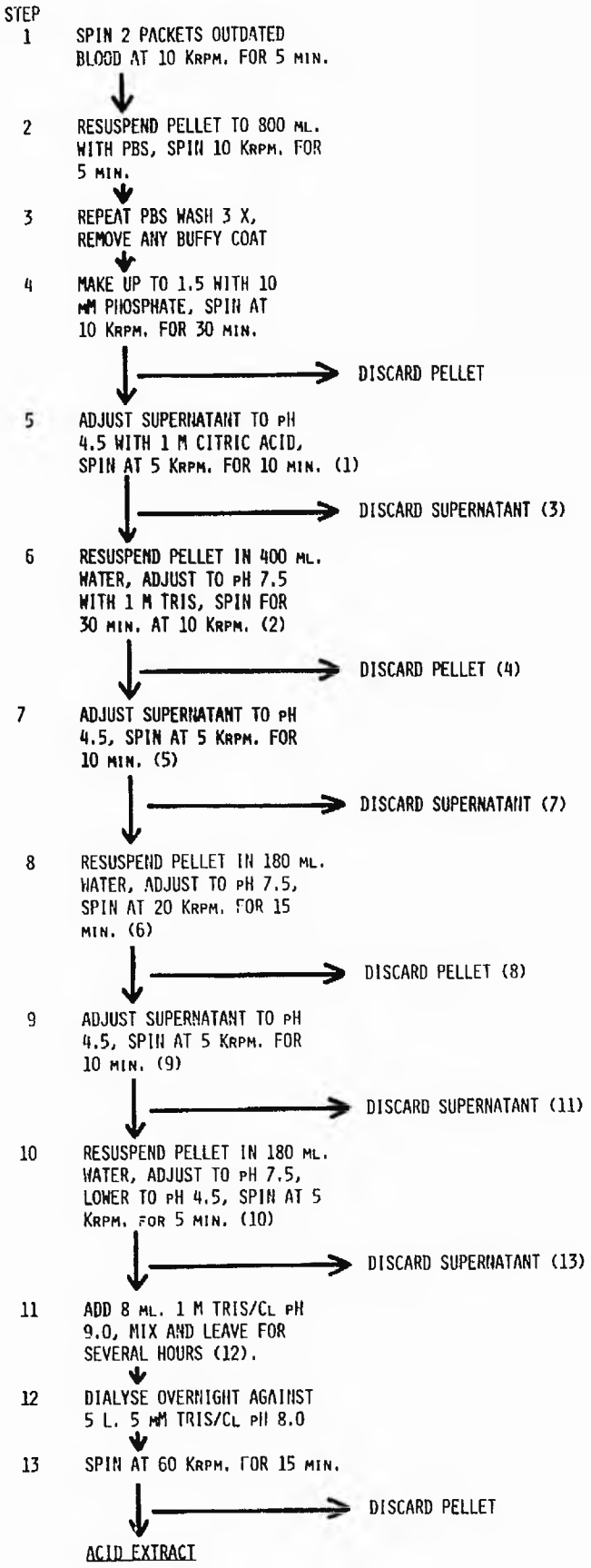

Fig.3.7. The procedure adopted for preparing the cytoplasmic acid extract. Steps 1 - 7 were performed in a Beckman J 21 centrifuge using a J 10 rotor, steps 8 - 10 used a J 20 rotor and step 13 used a Beckman L2 65B centrifuge with a SW 65 rotor. All steps were performed at $4^{o}C$.

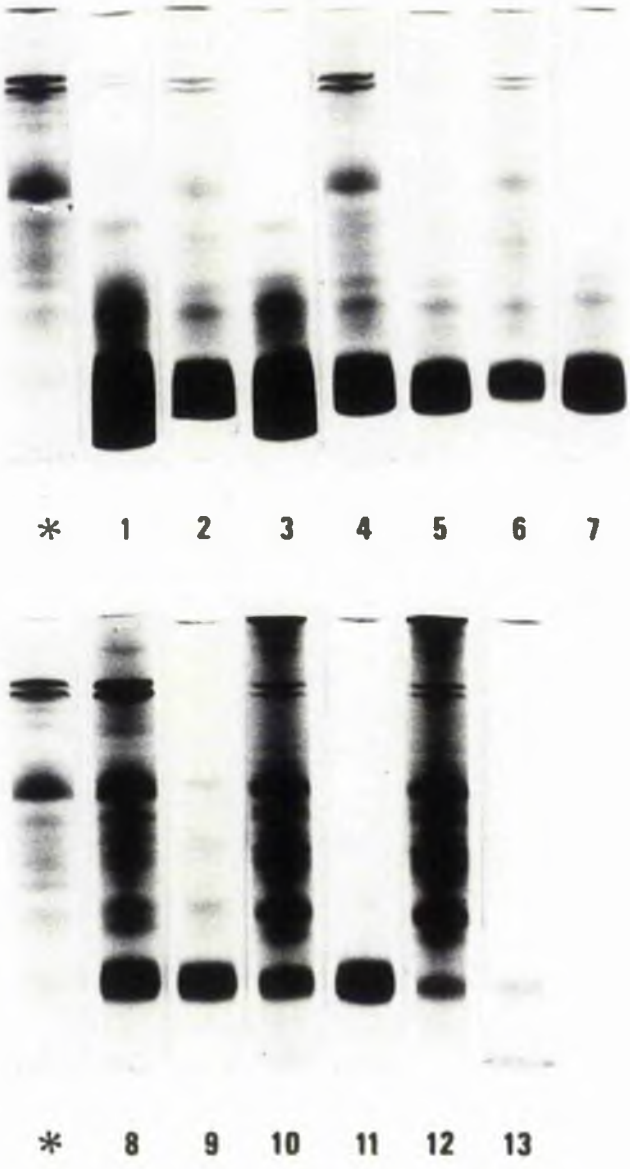

Fig.3.8. The proteins present at wach stage of the acid extraction procedure. The numbers refer to the numbers in parenthesis in Fig.3.7. Whole membranes (*) are included for comparison.

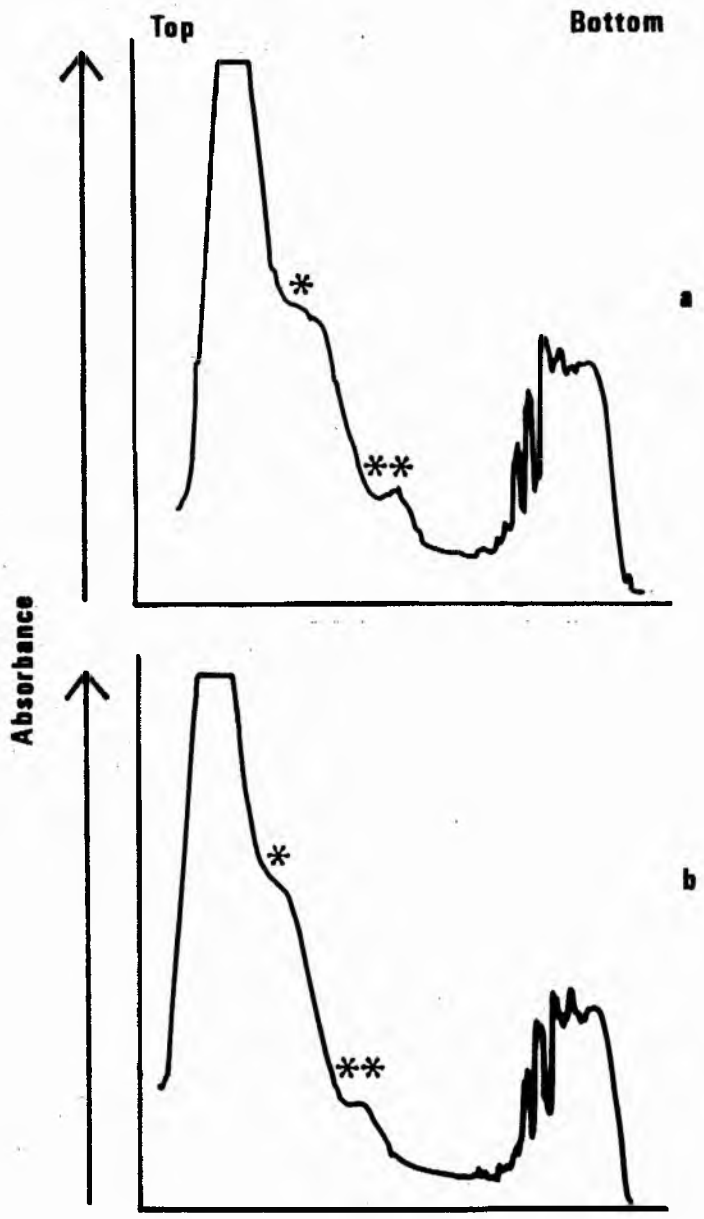

Fig.3.9. U.V. analysis of the acid extract after SDG centrifugation employing (a) a 0.25 to 1.0 M gradient and (b) a 0.4 to 1.0 M gradient.

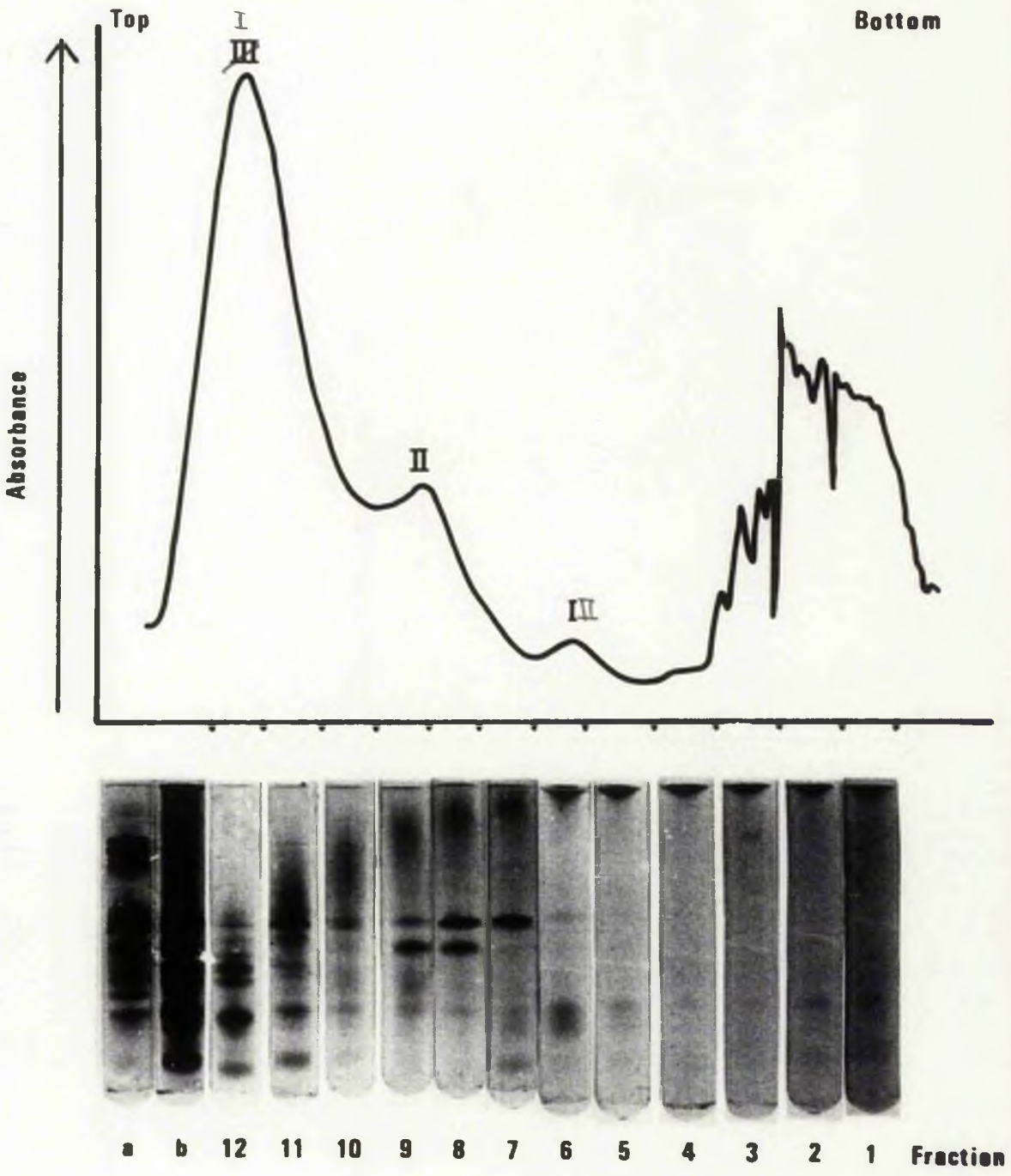

Fig.3.10. The proteins present throughout the 0.25 to 1.0 M sucrose density gradient. (a) whole membranes and (b) acid extract are included for comparison.

was likely to have consisted of proteins which had been denatured or aggregated by the acid precipitation and/or the SDG centrifugation. The final high speed spin (Fig. 3.7, step 13) in the acid extraction procedure should effectively have removed all the aggregates present at that stage, so these aggregates are likely to have formed during the SDG centrifugation.

The proteins remaining in solution had arranged themselves in three peaks which are labelled I, II and III in Fig. 3.10. Peak III contained a small amount of protein which is known to consist largely of the hollow cylinder protein (9). The major Peak I contained the majority of the acid extracted proteins whilst Peak II consisted predominantly of two proteins with very little contamination. One of these was protein 4.1' and the other, more of which was usually present, had a slightly greater apparent molecular weight. The second stage of the purification scheme consequently became the isolation of this Peak II with the aid of an LKB Uvicord and fraction collector.

Preparative PAGE

The simplicity of the SDG extract and the not too similar mobilities of the two major constituents would have made preparative SDS PAGE a highly attractive proposition, had it not been decided at the outset that denaturing techniques were to be avoided. Preparative PAGE in the absence of surfactant would, however, be acceptable if these proteins still had sufficiently dissimilar migration rates and also if they could be identified in this system. The samples used for

gels 7, 8 and 9 in Fig. 3.10 were therefore run on an analytical non-surfactant system giving the protein banding shown in Fig. 3.11b. These three samples run in the SDS system are reproduced in Fig. 3.11a for comparison. It was apparent that not only did these two species still migrate as two, but that the protein of interest had a considerably faster migration rate. Conveniently, all the minor contaminating species appeared to be left behind very near the origin. This made preparative non-surfactant electrophoresis by elution from the bottom of a short gel worth trying.

2.0 ml. of the SDG extract was added direct to the preparative gel and the Uvicord trace given by the eluate is shown in Fig. 3.12. It was clear from this trace that even allowing for dilution, a large proportion of the sample had been lost, this probably being due to adsorption onto the membrane separating the anodic buffer from the elution chamber. The eluate was divided into four as seen on the trace, the fractions in each pooled, concentrated against Ficoll and SDS PAGE performed. Fig. 3.13 confirms that the first peak contained protein 4.1' whereas the second extremely flat peak contained the other major protein, as would have been expected from Fig. 3.11.

The first peak also contained three higher molecular weight bands which were not present in the original SDG extract. On the assumption that these three bands were the dimer, trimer and tetramer of protein 4.1' the $\log_{10}$ of their relative molecular weights were plotted against their migration distances. This exercise gave a very good straight line (Fig. 3.14) consistent with this hypothesis. Thus preparative PAGE and/or concentrating against Ficoll seemed to cause a degree of SDS-resistant polymerisation of protein 4.1'.

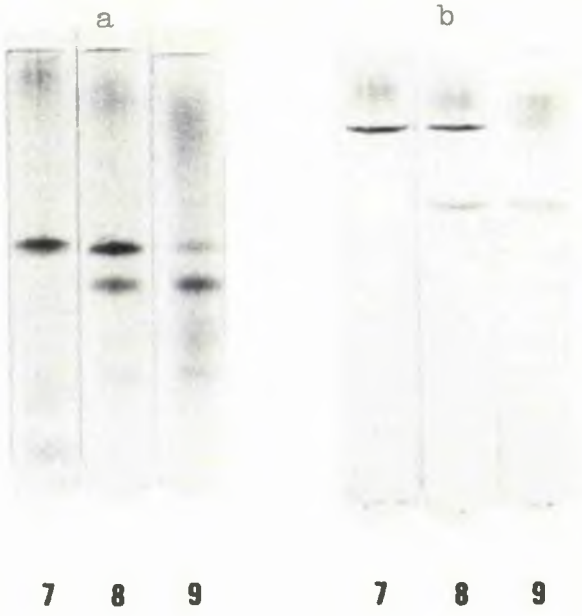

Fig.3.11. The proteins present in fractions 7, 8 and 9 in Fig.3.10 examined by (a) SDS PAGE and (b) non-surfactant PAGE.

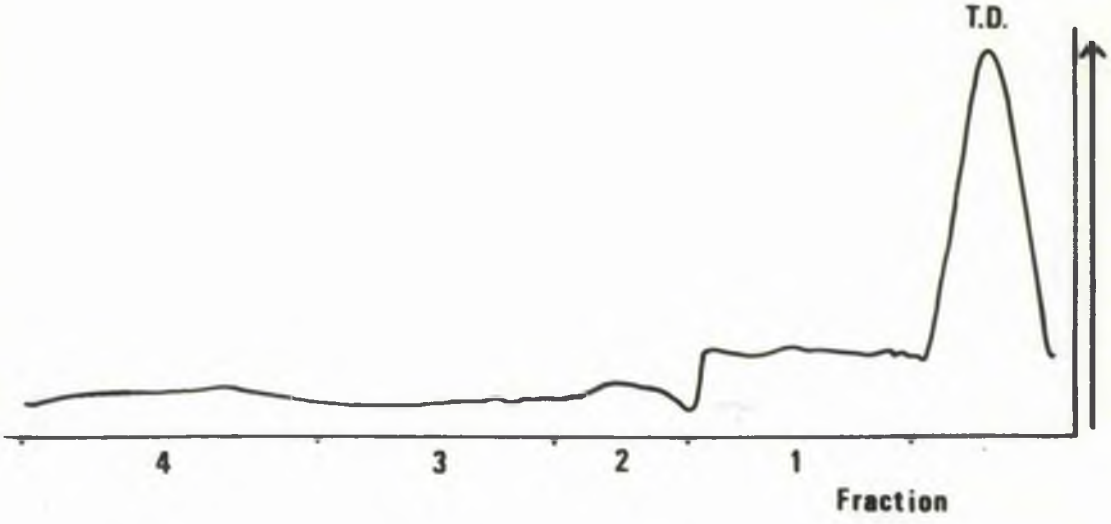

Fig.3.12. Fractionation of the SDG extract by preparative non-surfactant PAGE. (T.D. = tracker dye).

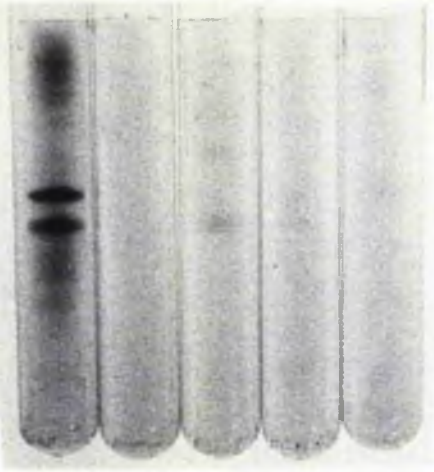

Fig.3.13. Analysis of the four fractions isolated by preparative non-surfactant PAGE shown in Fig.3.12. (S = SDG extract).

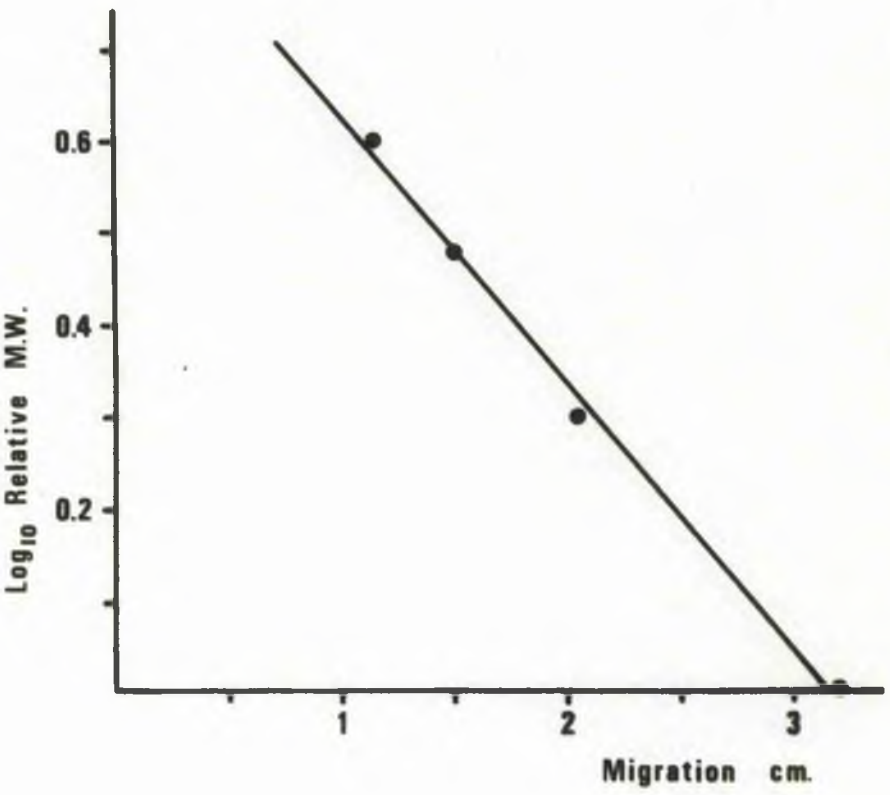

Fig.3.14. Log_{10} of the relative molecular weights of the four bands seen in fraction 2 of Fig.3.13 plotted against their migration distances.

Immunodiffusion.

Fig. 3.13 shows that protein 4.1' had been purified from the cytoplasm but there was insufficient material for the double diffusion experiment which would have decided whether or not it was the same as protein 4.1 from the membrane. Thus, this technique was limited to the acid and SDG extracts. Fig. 3.15 illustrates that the former gave many precipitin lines as one would have expected due to the presence of haemoglobin, proteins 6 and 7 (see Chapter 2) and other contaminants. The SDG extract gave a single line and so it can be concluded that one of the two proteins in this extract has a counterpart in the membrane.

In the absence of empirical observation, which of the two SDG proteins is most likely to have given rise to the precipitin line? The protein species which migrated in the band 3 position could quite easily have been one of the components of band 3. However, the exceptionally low standard error found in the measurement of band 3 (see Chapter 2) despite its heretogoneity, makes its presence in the cytoplasm doubtful whereas the behaviour of protein 4.1 in the membrane with respect to *in vivo* ageing could only be satisfactorily explained if it was postulated as being present also in the cytoplasm. It is hoped to be able to decide this issue in the not too distant future.

Fig.3.15. An investigation of the proteins in the acid extract (A) and the SDG extract (S) by the double diffusion technique. The central well contained rabbit antibodies to whole membranes.

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CHAPTER 4

THE VISUALISATION OF A FEW ENZYMES OF THE HUMAN ERYTHROCYTE MEMBRANE BY NONIONIC SURFACTANT POLYACRYLAMIDE GEL ELECTROPHORESIS (NIS PAGE)

Summary

1. A method has been developed for the electrophoretic separation of the human erythrocyte plasma membrane proteins using nonionic surfactants which cause a minimal loss of functional integrity.

2. Five enzyme activities were visualised in this system. LDH was resolved into four discrete isozyme bands, none of which showed any age-dependent activity. GAPD, AChEase and NADH-MR were all present as single bands and the latter two decreased with age. PNP gave a broad band which was concluded as representing a number of overlapping isozymes. Ageing was associated with a decrease in staining intensity and an increase in the proportion of the faster varieties.

Introduction

Chapter 2 was concerned with the human erythrocyte membrane proteins as revealed by SDS PAGE and as was explained, the dissolution of the membrane proteins using this surfactant leads to denaturation and a loss of functional integrity. It was thus felt that visualisation of these proteins whilst maintaining functional integrity should be attempted, attention being naturally focussed on the nonionic surfactants which are known to be much milder than the ionic surfactants and rarely cause any functional denaturation (1). These nonionic surfactants have found wide use in the liberation of membrane-bound enzyme activities from such systems as erythrocytes (2,3,4), liver cells (5), Ehrlich ascites cells (6), mitochondria (7) and viruses (8). There is also some evidence to suggest that they may activate some enzymes (5, 9) but it is very difficult to differentiate this from simple solubilisation and also a protective effect.

What is the reason for this major difference in the biological effects of nonionic and ionic surfactants? It has already been explained in Chapter 2 that it is the surfactant monomer which binds to the protein and that if the concentration of this ligand exceeds 0.5 mM then cooperative binding leading to denaturation takes place. The critical micellar concentration (CMC) is below 0.5 mM for most nonionic surfactants (10) and so cooperative binding is unable to occur. The CMC for sodium dodecyl sulphate, on the other hand, is well above this value (10). There is also the fact that alkyl sulphates have flexible unbranched tails which can almost literally wriggle into the protein interior to expose new binding sites, whereas nonionics tend to have very large, rigid

lipophilic groups. Lastly, the latter by definition have uncharged headgroups and so there can be no mutual electrostatic repulsion of the bound amphiphiles which might aid the unfolding of the polypeptide chain (11), such as is the case with ionic surfactants.

Another difference between these two classes of amphiphile was shown by A. Helenius et al. (1) when they found that Triton X-100 was bound only by hydrophobic proteins. They concluded that solubilisation was simply the replacement of in situ lipid by the detergent lipid which was bound at the same sites, thus presumably preserving the protein conformation. Consequently, the binding of nonionic surfactant tends to be at specific high-affinity sites by a hydrophobic interaction (11). Therefore it is the ability to displace membrane lipid that determines the effectiveness of a nonionic surfactant, the latter being thought to aggregate around the hydrophobic region of the protein in a micellar fashion such that a completely hydrophilic surface is presented to the aqueous environment (1).

J.N. Umbreit et al. (12) tested the ability of a great number of nonionics to solubilise bacterial membrane enzymes and found that the ability to do so could not be correlated with surface tension, wetting, foaming, interfacial tension, C.M.C. or the chemical nature of the hydrophobic region. It was, however, extremely well correlated with the Hydrophilic-Lipophilic Balance (H.L.B.) which as its name suggests is a value describing the relative contributions of the polar and apolar moieties to the whole molecule. The most hydrophobic surfactants have an H.L.B. number that approaches 0 whilst the most hydrophilic have an H.L.B. number of approximately 20. They found that the best results were achieved with nonionic surfactants whose H.L.B. numbers lay between 12.4 and 13.5.

Presumably the properties of these particular nonionics most closely approximate to those of the native lipid.

As with most hypotheses there is usually one dissenting voice and this time it belongs to W.W. Sukow et al. (13) who have found evidence for the cooperative binding of Triton A-100 to bovine serum albumin.

The purpose of developing an electrophoretic system capable of visualising functional proteins was not simply to observe changes in activity associated with in vivo ageing, as this can be achieved by routine assay techniques, but to observe any possible isozyme changes. Such changes have already been observed for purine nucleoside phophorylase (14), aldolase (15), glutamate-oxalacetate transaminase (16), lactate dehydrogenase (17) and hexokinase (18) in the red cell cytoplasm, although all but the first two were limited to the reticulocyte to erythrocyte transformation.

Methods and Materials

Tissue Preparation

Human red cell ghosts were prepared as described previously. Rats were killed by a sharp blow to the back of the head followed by dislocation of the spinal column. Blood was withdrawn by cardiac puncture and the heart, devoid of all connective tissue and as much blood as possible, was placed in 20 ml. of 10 mM Tris/Cl pH 7.4 containing 2 mM $MgCl_2$. The latter was to keep the loss of chromatin from the nuclei to a minimum. A similar amount of skeletal muscle was also placed in 20 ml. of this solution and both were homogenised for 5 minutes using an Ultra-Turrax Rotary-Blade Homogeniser at setting 4.0. The resulting suspensions were cleared by centrifugation at 20 x Krpm. for 15 minutes and a sample of each supernatant was dialysed as described below. Rat red cell haemolysate was obtained by a method identical to that for the human material.

Electrophoresis

All solutions were made up in de-ionised water.

1. 4% acrylamide/ 0.05% Bis./ 0.2% NP40/ 0.1% T20/ 0.1% T40/ 0.1% B35/ 5 mM Tris/Cl pH 8.4.
2. Same as 1. but 3.5% acrylamide.
3. 5% NP40/ 2.5% T20/ 2.5% T40/ 2.5% B35/ 20% glycerol/ 0.1 mM 2-ME/ 0.1 mM EDTA/ 5 mM Tris, pH approximately 7.9.
4. 0.5% NP40/ 0.25% T20/ 0.25% T40/ 0.25% B35/ 0.1 mM 2-ME/ 0.1 mM EDTA/ 5 mM Tris/Cl pH 8.4.

5. 0.2% NP40/ 0.1% T20/ 0.1% T40/ 0.1% B35/ 5 mM Tris/Cl pH 8.4.
6. 0.1 mM 2-ME/ 0.1 mM EDTA/ 5 mM Tris/Cl pH 8.4
7. 10% TEMED in 99% ethanol.
8. 10% ammonium persulphate.

The same Quickfit apparatus as described previously was used. The gels were cast in stages as follows. A mixture of 10 parts 1 + 0.1 parts 7 + 0.05 parts 8 was made, 1.0 ml. being placed in each tube and overlaid with several drops of solution 5. At least 30 minutes were allowed for polymerisation, whereupon the overlay was discarded and 0.1 ml. of a mixture consisting of 10 parts 2 + 0.1 parts 7 + 0.05 parts 8 was added. This was also overlaid with several drops of solution 5 and left for at least 30 minutes. After replacing the overlay the gels were left overnight at room temperature and used within 24 hours of polymerisation as the surface began to deteriorate with time.

Electrophoresis was performed with 200 ml. of solution 4 in each electrode compartment. The gels were given two preruns, each using fresh buffer and lasting for 15 minutes at 1.5 mA/ tube in order to remove as much persulphate as possible. All samples for electrophoresis were dialysed against at least 1000 times their own volume of solution 6. Dialysis tubing was prepared by boiling for approximately 30 minutes in 2% sodium carbonate/ 0.1% EDTA followed by repeated washing with distilled water to remove any reagent. 1 part dialysed sample was then added to 1 part solution 3 and 3 parts solution 6 and incubated for 30 minutes at 37°C. Up to 0.05 ml. of this solubilised material was applied to each gel with a graduated 1.0 ml. plastic syringe. The temperature during the run was

kept as near 37°C as possible by pre-heating the buffers and passing heated water through a coil in the lower anodic chamber. Current strength was 0.5 mA/ tube for the first 10 minutes followed by 1.5 mA/ tube for 60 minutes, the two electrode buffers being mixed every 15 minutes to prevent exhaustion. Bromophenol blue tracker dye was not used in this system as its migration rate was slower than some of the protein species. Protein staining of the gels was by the same procedure described for SDS PAGE.

Enzyme Histochemistry

Staining was performed by placing the gels into one of the following solutions and incubating at room temperature until the band(s) had developed.

1. AChEase

3.46 mM Acetylthiocholine Iodide.
40 mM Glycine
8.0 mM $CuSO_4$
120 mM NaCl
30 mM $MgSO_4$
20 mM Tris Maleate/ NaOH pH 6.0

2. LDH

5 parts 0.1 M Tris/Cl pH 7.5
1 part 0.27% MTT
1 part 0.1% PMS
1 part 8 mM NAD
1 part 0.54 M sodium lactate
1 part water

3. NADH-MR

5 parts 6.0 mM NADH
2.5 parts 6.0 mM DCIP
5 parts 6.5 mM MTT
6.5 parts 1 M Tris/Cl pH 8.4
6.0 parts water

4. GAPD

5 parts 60 mM Nicotinamide/ 0.2 M Tris/Cl pH 7.5
1 part 60 mM FDP
1 part 0.27% MTT
1 part 0.1% PMS
1 part 8.0 mM NAD
1 part 10 mM sodium phosphate pH 7.5
0.1 part aldolase, 120 U/ ml.

5. PNP

5 parts 50 mM sodium phosphate pH 7.5
1 part 0.27% MTT
1 part 0.2% PMS
1 part 0.2% Inosine
2 parts water

Standard Enzyme Assays

The routine assays used here are given in the Methods section to Chapter 5.

All biochemicals and Brij 35, Tween 20 and Tween 40 were purchased from Sigma Ltd. All other reagents were purchased from BDH.

General

Purification of acrylamide, measurement of protein and photography were as previously described. The optical density of surfactant treated ghosts was measured with a Beckman DB GT double beam spectrophotometer.

Results and Discussion

Development of Nonionic Surfactant Polyacrylamide Gel Electrophoresis

Nonionic surfactants are polyoxyethylene condensates covalently linked to a variety of apolar groups which determine the generic name. There are countless numbers of those reagents and so those used are simply the ones most likely to be found in a laboratory. A technique was required which would totally solubilise the erythrocyte membrane proteins enabling electrophoresis to be performed with the minimum of functional denaturation, and the approach used was of a highly empirical nature. Preliminary investigations showed that the four surfactants listed in Table 4.1 offered the best chance of achieving these goals and so a number of experiments were carried out to characterise this system.

Commercial Name	Chemical Name	HLB Number
Nonidet P40 (NP 40)	Polyoxyethylene p-t-octyl phenol	13.1
Tween 20 (T 20)	Polyoxyethylene-sorbitol monolaurate	16.7
Tween 40 (T 40)	Polyoxyethylene-sorbitol monopalmitate	15.6
Brij 35 (B35)	Polyoxyethylene lauryl alcohol	16.9

Table 4.1. Some characteristics of the nonionic surfactants used in this study (from 10 and 12).

Two experimental systems were used. Firstly, the surfactants were added to ghost suspensions and the degree of solubilisation was assessed by light scattering at 530 nm., and then the most potent mixtures were examined electrophoretically. The results of these electrophoretic trials are not included as they were very numerous and, until the correct mixture was found, consisted usually of a flocculated layer of protein at the gel-electrolyte interface with little if any protein in the gels.

Fig. 4.1 shows the effect of increasing concentrations of these four surfactants on the optical density of the ghost suspensions and it is clear that using this criterion NP40 was by far the most active membrane solubilising agent with T40 apparently totally ineffective. The experiment illustrated in Fig. 4.2 consisted of incubating membrane samples in the presence of 1% surfactant for up to 1 hour at 37°C. Once again NP40 appeared the most promising, displaying a 50% decrease in optical density over this period of incubation. This finding adds weight to the conclusion of J.N. Umbreit et al. (12) that nonionic surfactants with an HLB number between 12.4 and 13.5 (see Table 4.1) were the most useful. The other three surfactants were apparently resistant to any temperature effect. These two experiments made it clear that 1% NP40 should be the basis of any solubilising mixture and that the presence of the others would be merely supportive. That the latter were useful can be seen from Fig. 4.3 in which B35, T20 and T40 can all be seen to have enhanced the effect of 1% NP40 and that this reached a maximum at approximately 0.5% surfactant concentration. Furthermore, it was decided to include an half hour 37°C incubation period to aid the membrane dissolution.

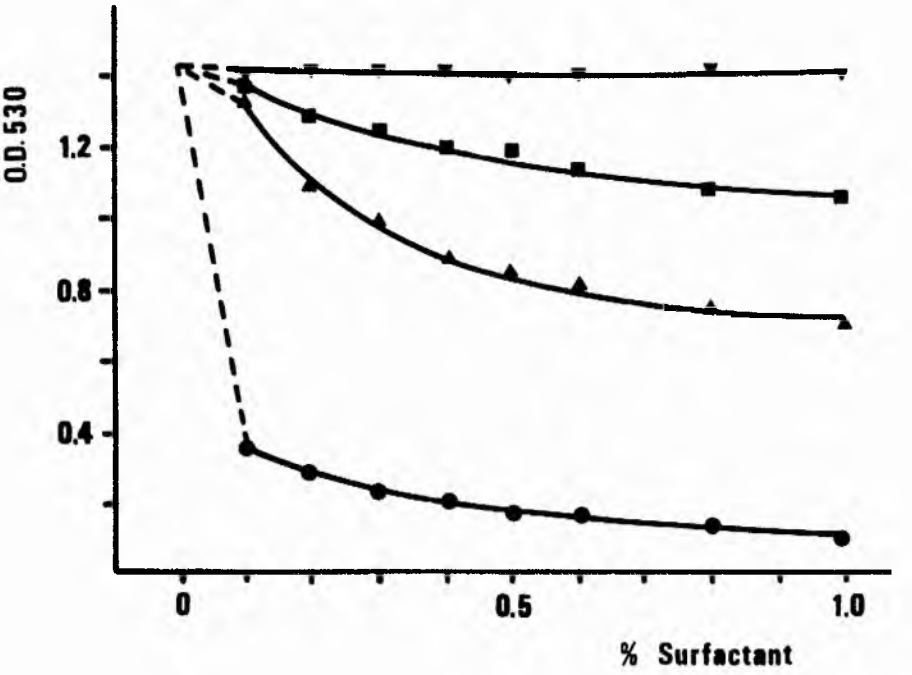


Fig.4.1. The effect of NP40 (●), T20 (▲), T40 (▼) and B35 (■) on the optical density of an erythrocyte membrane suspension.

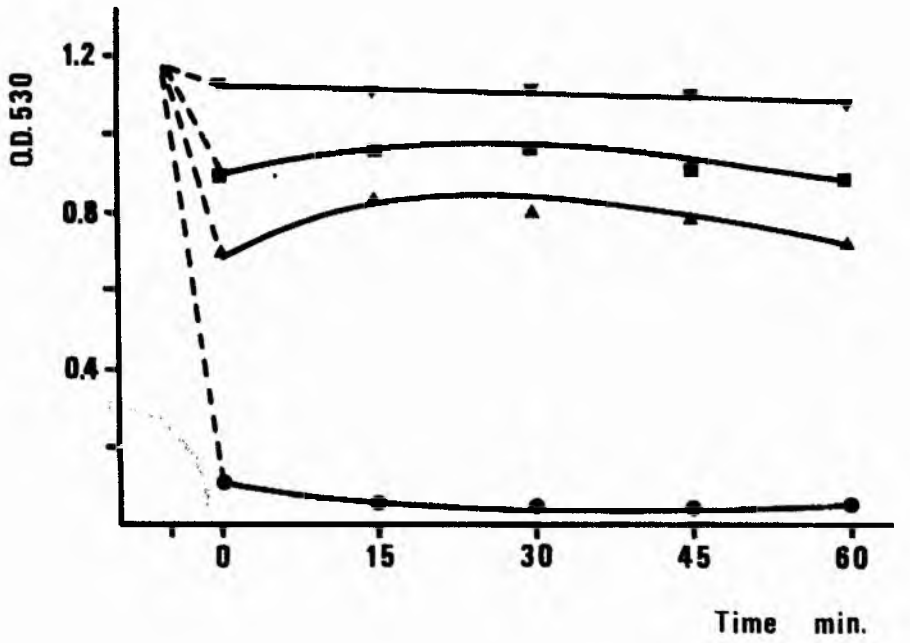


Fig.4.2. The effect of incubation at 37°C on the optical density of an erythrocyte membrane suspension in the presence of 1% NP40 (●), 1% T20 (▲), 1% T40 (▼) and 1% B35 (■).

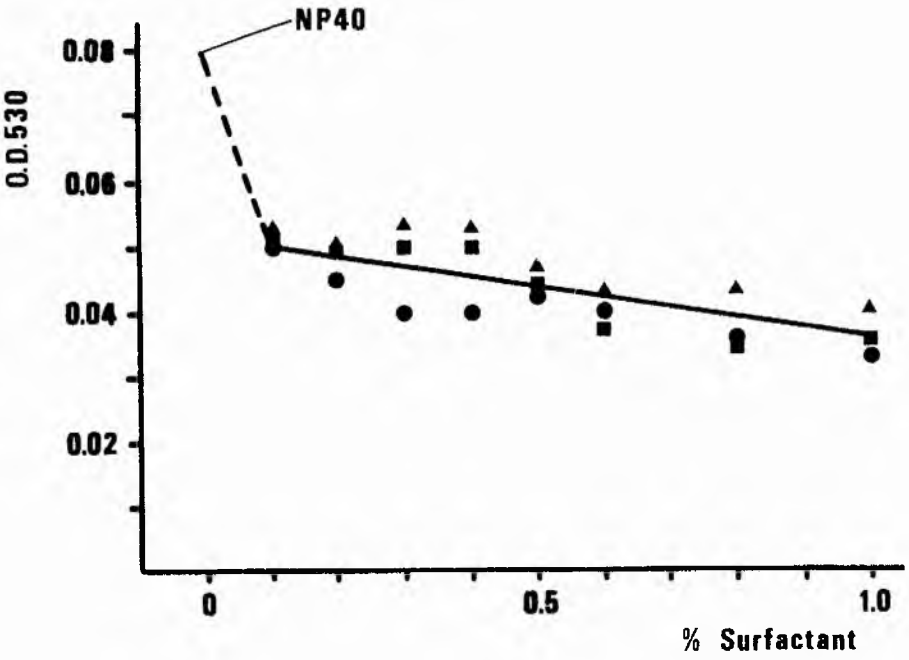


Fig.4.3. The effect of T20 (▲), T40 (■) and B35 (●) on the optical density of an erythrocyte membrane suspension in the presence of 1% NP40.

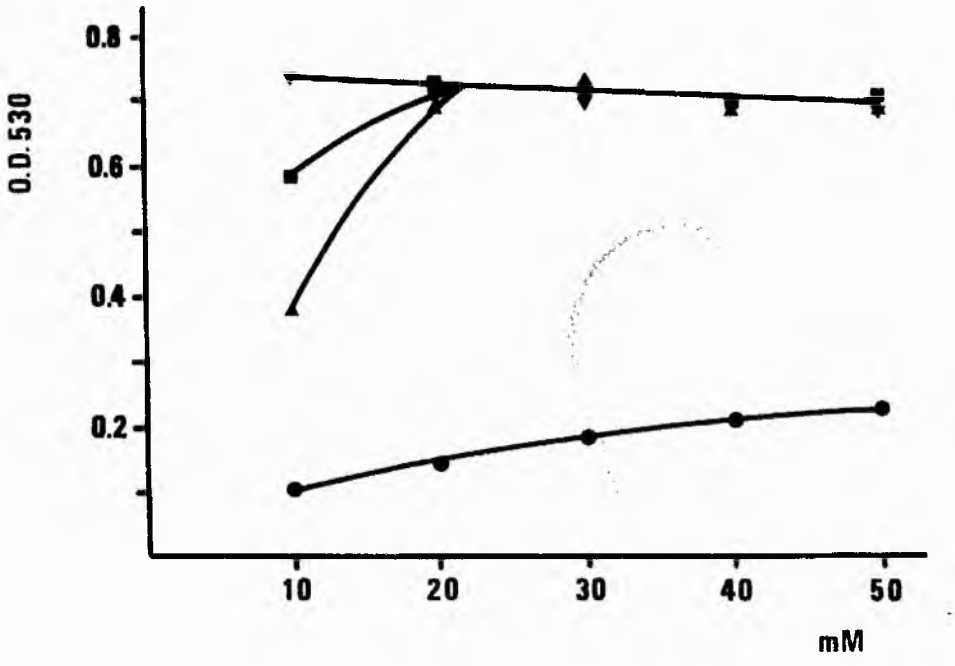


Fig.4.4. The effect of ionic strength (Tris/Cl pH 7.4) on the optical density of an erythrocyte membrane suspension containing 1% NP40 (●), 1% T20 (▲), 1% T40 (▼) and 1% B35 (■).

The experiments described so far were performed in the presence of 10 mM buffer, but electrophoresis being carried out concurrently and with 50 mM buffer was being hampered by flocculation of the protein at the gel-electrolyte interface. D.M. Miller (19) had shown that the ability of Triton X-100 to solubilise erythrocyte ghosts was ionic strength-dependent and the results in Fig. 4.4 confirmed that this was the case with the four surfactants being used here. The efficacy of these surfactants is dependent on a minimal ionic strength and there was no reason to suggest that disaggregation would not be further augmented by a decrease below 10 mM.

As decreasing the ionic strength of the haemolysing medium would undoubtedly have resulted in the loss of membrane protein, this decrease in ionic strength could only be achieved by dialysis of the ghosts. Alkaline pH is known to cause a certain amount of membrane dissolution (20) and so it was decided to investigate the combination of a decrease in the ionic strength with an increase in the pH of the dialysing medium. It can be seen from Fig. 4.5 that decreasing the ionic strength had no visible effect but that increasing the pH to 8.4 was very effective. Nevertheless electrophoresis, being somewhat more sensitive to particle size than light scattering measurements, dictated that 5 mM ionic strength be used and so a 5 mM Tris/Cl pH 8.4 dialysis step was incorporated.

The necessity of performing electrophoresis at 5 mM ionic strength (any increase caused significant flocculation) was the major disadvantage of this system. Zone sharpening is dependent on an ionic gradient between the electrolyte and the applied sample (21) and such low ionic strengths also make buffer exhaustion a constant risk. The former drawback had to be accepted and the latter was overcome by the frequent mixing of

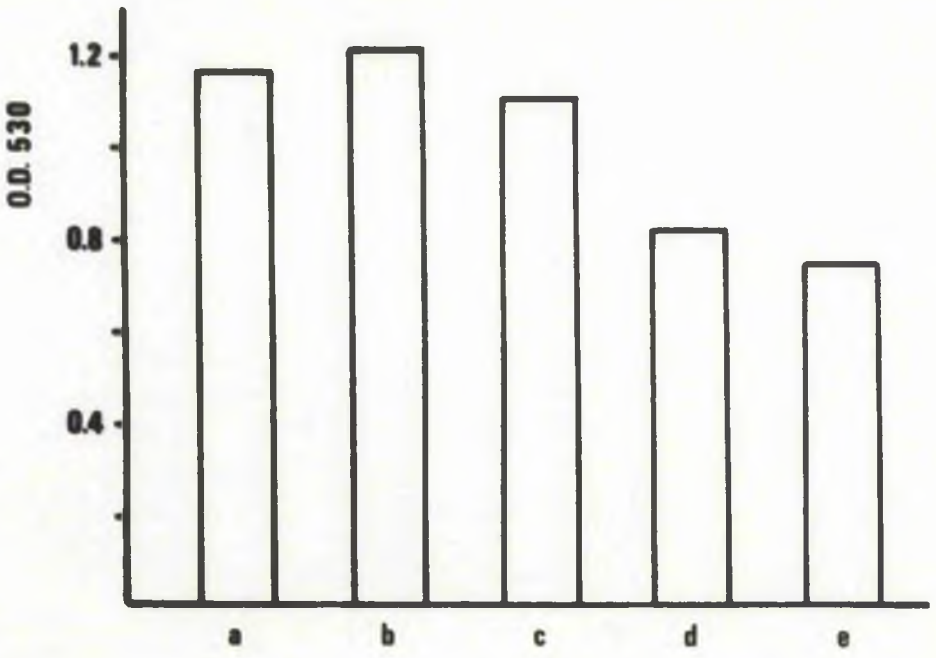


Fig.4.5. The effect of decreasing the ionic strength and increasing the pH on the optical density of an erythrocyte membrane suspension by dialysis overnight against (a) control-10 mM Tris/Cl pH 7.4, (b) 5 mM Tris/Cl pH 7.4, (c) 5 mM Tris/Cl pH 7.9, (d) 5 mM Tris/Cl pH 8.4 and (e) 5 mM Tris/Cl pH 8.9.

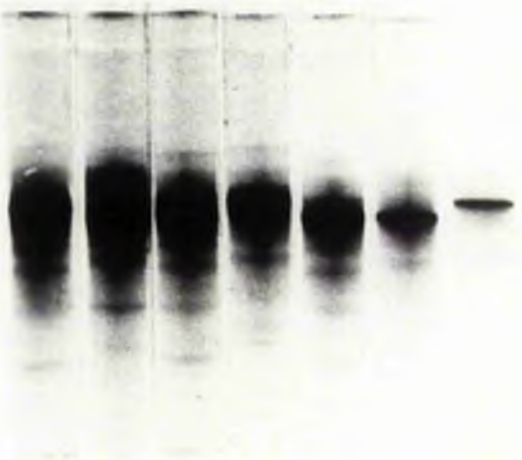


Fig.4.6. The electrophoresis of increasing amounts of unfractionated membranes using 1/10 surfactant concentration in the gels and buffers.

the two electrolytes. Electrophoresis performed in conjunction with the optical studies confirmed that NP40 was the prime component necessary for the entry of all the membrane proteins into the gels, but the other three surfactants were found to be necessary at a concentration of 0.5% each largely to resolve these proteins into as many discrete bands as possible.

All the evidence therefore decided that the optimum conditions for solubilisation and electrophoresis were 1% NP40/ 0.5 B35/ 0.5% T20/ 0.5% T40/ 5 mM Tris/Cl pH 8.4. It can be seen from the methods that the surfactant concentrations of the solubilising mixture (solution 3) were actually 5 times these values and this also contained 0.1 mM EDTA and 0.1 mM 2-ME to protect the enzymes and 20% glycerol to facilitate layering of the samples onto the gels. All these components are acidic and the 5 mM Tris base on its own could not raise the pH of this mixture above 7.9. However, as the membrane sample and the diluent were both at pH 8.4 the final pH of the solubilised membrane sample was unlikely to have been significantly below this value.

The acrylamide concentration had to be decreased from the 5% used for SDS PAGE to 4% to allow all the proteins to enter but even so there was one species which only just entered and tended to interfere with the migration of the other proteins. This was resolved by placing a small layer of 3.5% acrylamide on top of the 4% gel. This layer was rather precariously attached and was often lost during the fixing and staining procedures as will be seen from some of the following figures.

The last major parameter that had to be settled was the surfactant concentration to be used in the gels and electrolyte buffers. If the surfactant concentration used to solubilise the membranes (solution 3 diluted 5 times) is unity then Fig.

4.6 shows the effect of electrophoresing various amounts of unfractionated ghosts when the gels and buffer both contained 1/10 this concentration. It is obvious that individual band mobility was highly dependent on the total protein applied suggesting that there was insufficient surfactant present to maintain complete solubilisation. Figs. 4.7 and 4.8 show the same membrane samples added to gels containing 1/5 surfactant concentration, as did the electrolyte in Fig. 4.7 whereas the electrolyte in Fig. 4.8 contained 1/2. Both are significantly better than Fig. 4.6 in that band mobility was almost completely independent of the amount of protein added, but the band definition was best in Fig. 4.8. It was not possible to increase the surfactant concentration in the gels any more as the gels easily fell out of the glass tubes and so the conditions used in Fig. 4.8 were adopted.

Figs. 4.7 and 4.8 contained an extra gel to which no protein had been added and it can be seen that these and all the other gels possessed a layer of protein staining material as the gel-electrolyte interface. As this was present in the absence of applied protein it could not have been due to denaturation of some of the sample and so this phenomenon was not investigated further. Two possibilities are surfactant precipitation and dust in the electrolyte.

Some of the protein species visualised in this system migrated faster than bromophenol blue and therefore the run length also had to be determined empirically. As a result of the experiment in Fig. 4.9, 60 minutes was chosen and to prevent buffer exhaustion the two electrolytes were mixed every 15 minutes. 0.1 mM EDTA and 0.1 mM 2-ME were used in all the solutions that the proteins came into contact with in an attempt to minimise loss of functional integrity. To this end the gels

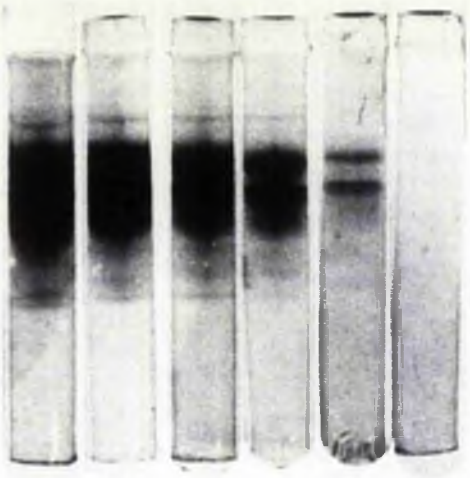


Fig.4.7. The electrophoresis of increasing amounts of unfractionated membranes using 1/5 surfactant concentration in the gels and buffers.

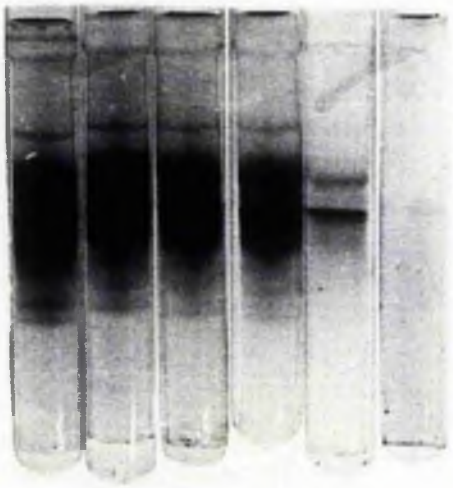


Fig.4.8. The electrophoresis of increasing amounts of unfractionated membranes using 1/5 surfactant concentration in the gels and 1/2 in the buffers.

Fig.4.9. The electrophoresis of unfractionated membranes for increasing periods of time.

were given a thorough pre-run in order to elute or reduce as much of the persulphate catalyst as possible. It would naturally have been preferable to use riboflavin as the catalyst but this was found to be ineffective in the presence of the nonionic surfactants.

Comparison with SDS PAGE

Fig. 4.10 gives the results of three experiments in which fractionated ghosts were run in this nonionic surfactant system followed by Coomassie Blue staining. It is clear that NIS PAGE was by no means as reproducible as the SDS system and that there was a great deal of experimental and/or individual variation. The overall patterns were, however, very similar and there were two points of interest. There was one band (*) which was virtually absent from the youngest membranes and gradually became more pronounced with age. It was very tempting to make the comparison between this protein and protein 4.1 seen in Chapter 2 but this was not followed up. Another band (**) which had a rather variable mobility decreased drastically with age. It is clear from Fig. 4.11 that there was no correlation between mobility and molecular weight in this system and so no discussion on these two changes is possible.

Enzyme Histochemistry

The major difference between protein staining and histochemical staining of the gels is that the former is preceded by immediate fixation to avoid any diffusion which

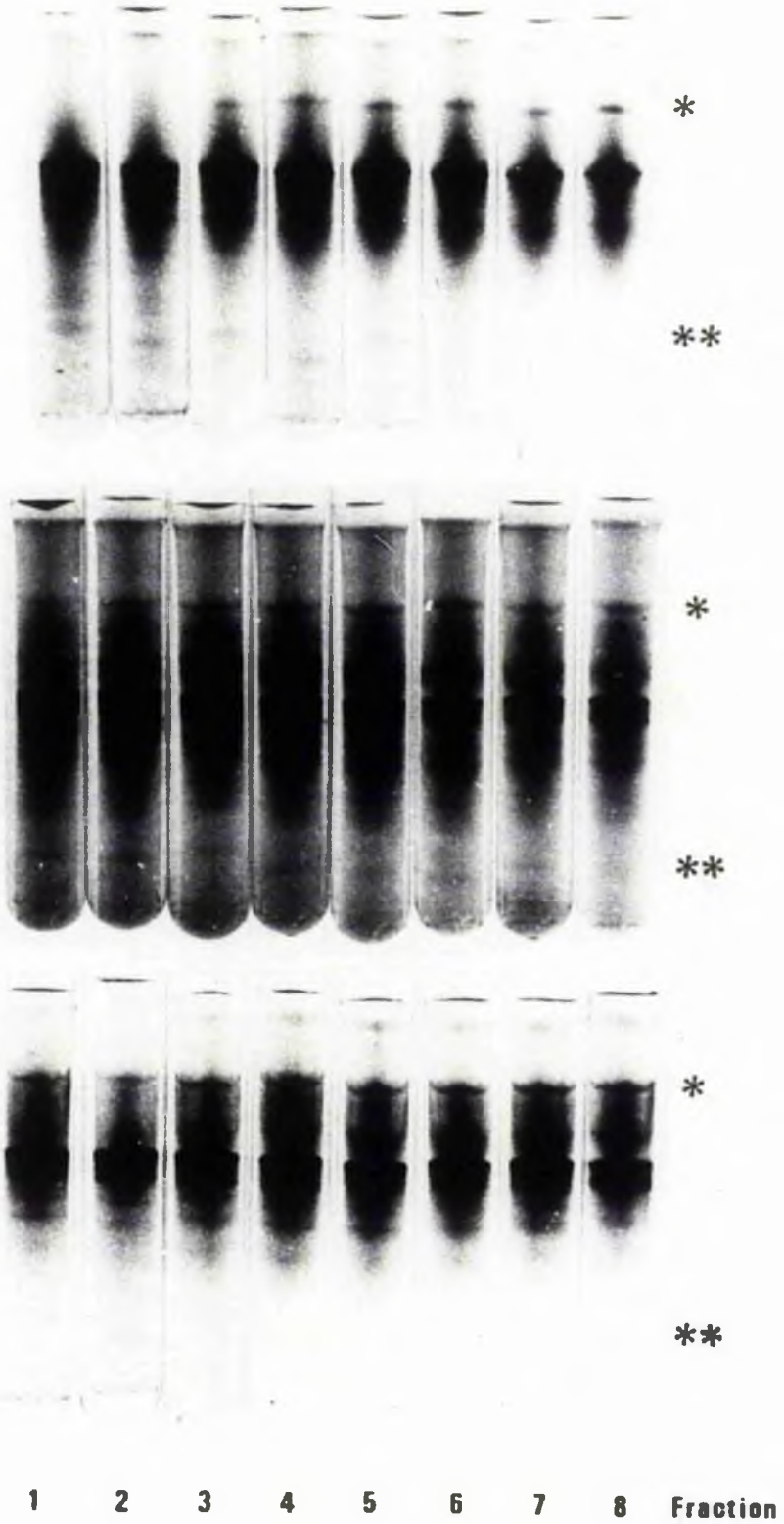


Fig.4.10. Three experiments on the effect of in vivo age on the membrane proteins as revealed by NIS PAGE.

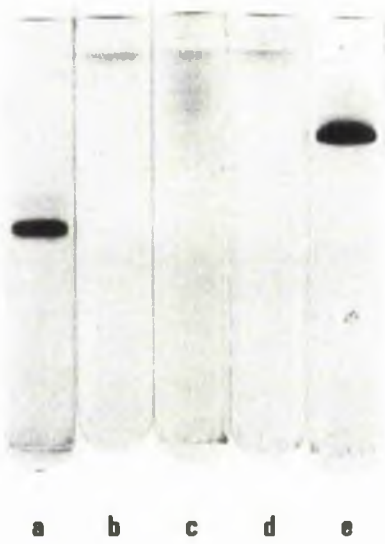


Fig.4.11. Electrophoresis of (a) BSA, (b) cytochrome c, (c) β globulin, (d) γ globulin and (e) Hb.

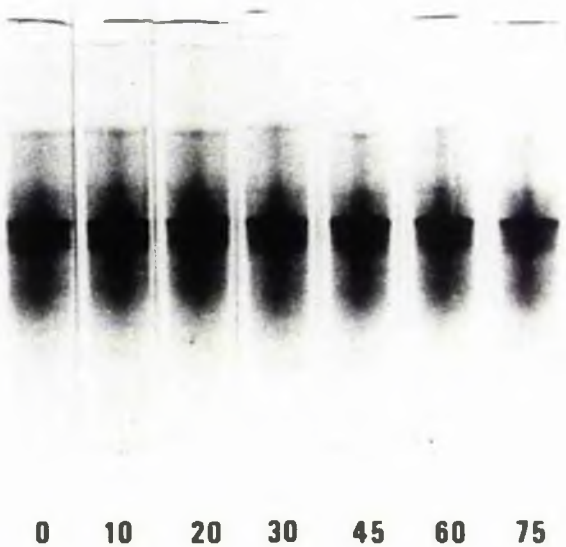


Fig.4.12. The effect of increasing the time prior to fixation on the band pattern of unfractionated membranes.

would result in the loss of protein and a decrease in resolution. Enzyme histochemistry often took up to one hour before there was sufficient band intensity to permit photography and it seemed likely that some degeneration of the band pattern was likely to have occurred in this time. Fig. 4.12 confirmed this suspicion but at the same time showed that even after one hour the band pattern was still very similar to that fixed immediately. Therefore the prolonged incubations are unlikely to have seriously affected the localisation of the enzyme proteins.

The choice of enzymes for this investigation was limited by the availability of staining techniques and the very low activity of most membrane enzymes. Thus, the five enzymes included were chosen largely by a process of elimination although it was hoped to maintain as much overlap as possible with the contents of Chapter 5. Before embarking on the histochemical staining it was first necessary to demonstrate that these five activities could survive the low ionic strength dialysis followed by incubation in the presence of very high concentrations of nonionic surfactants. That this was the case is shown in Table 4.2.

The specific activities of all but AChEase increased during the preparative procedures and this is likely to have been caused by a cryptic effect as is described in the next chapter. Briefly, dissolution of the membrane probably exposed more active sites to the medium or allowed freer access of substrate molecules. Dialysis caused a significant decrease in the specific activity of AChEase but this was returned to the initial value by surfactant solubilisation.

Enzyme	Ghosts	% Specific Activity ± 1 S.E. Dialysed ghosts	Solubilised ghosts
AChEase	100	60.1 ± 7.2	105 ± 4.9
LDH	100	387 ± 45	348 ± 11
NADH-MR	100	105 ± 7.9	140 ± 28
GAPD	100	310 ± 114	547 ± 117
PNP	100	114 ± 24	159 ± 24

Table 4.2. The effect of low ionic strength dialysis followed by nonionic surfactant solubilisation on the membrane enzymes of the human erythrocyte.

A possible explanation is that the low ionic strength dialysis caused the formation of a large number of sealed inside-out vesicles and as this enzyme is thought to be present solely on the external surface (22) the result was an apparent decrease in specific activity. The addition of surfactant would naturally break up these vesicles giving at least the initial specific activity. The lack of any increase in specific activity after total solubilisation of the ghosts strongly supports the contention that AChEase is located exclusively at the membrane surface and as the activity can be recorded in the intact cell (22) this is likely to be the external surface.

Acetylcholinesterase (AChEase)

It can be seen from Fig. 4.13 that in the absence of substrate no band was visible. Fig. 4.14 shows that there was a single AChEase staining band and that its intensity decreased

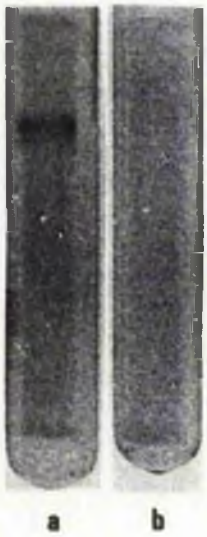


Fig.4.13. AChEase of unfractionated membranes in (a) the presence and (b) the absence of acetylthiocholine iodide.

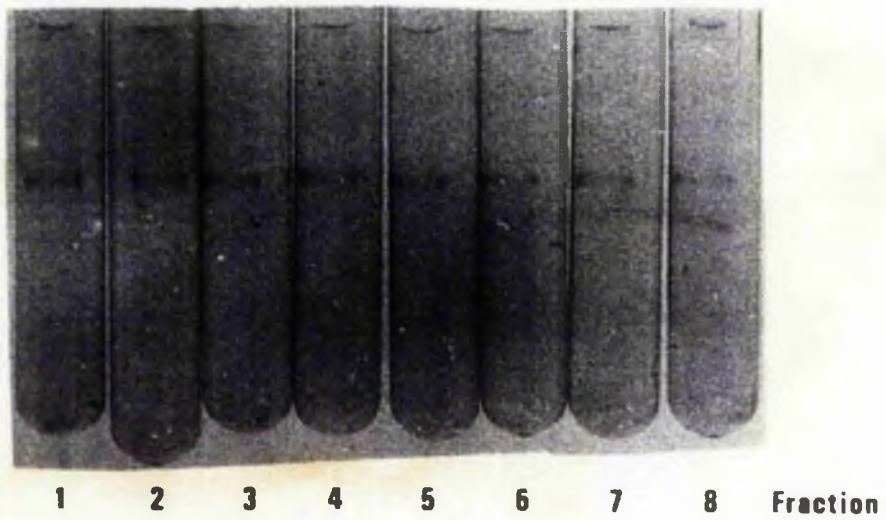


Fig.4.14. The effect of <u>in vivo</u> age on membrane-associated AChEase.

visibly with ageing. Stain was also taken up at the top of the gels but as this was seen for all the enzymes this was probably a non-specific adsorption of the stain by the denatured layer mentioned earlier. It was found (Fig. 4.15) that the non-specific butyryl cholinesterase present in the plasma had a significantly faster migration rate, making it likely that this technique could be used to separate these two activities. The specific activity of the plasma enzyme was much lower than that in the red cell membrane and therefore the incubation time was much longer, causing the broader more diffuse bands than those seen in Fig. 4.14. No activity could be found in the cytoplasm, confirming the membrane as the locus for red cell activity.

Lactate Dehydrogenase (LDH)

Fig. 4.16 confirms that the stain was specific for LDH as the omission of either NAD or sodium lactate eliminated any band formation. There were four bands present in the membrane preparation, one of them being extremely faint. Figs. 4.17a and 4.17b give the results of two experiments, one of which recorded a decrease in all four bands and the other an increase with age. It seems safe to conclude from this that LDH is probably loosely attached to the membrane in a manner similar to that of haemoglobin, and as was seen in Chapter 2 the latter showed all possible permutations of binding affinities with age.

The presence of the four bands was considered to be indicative of four LDH isozymes, but as NIS PAGE is an uncharacterised system it was just as likely that these four

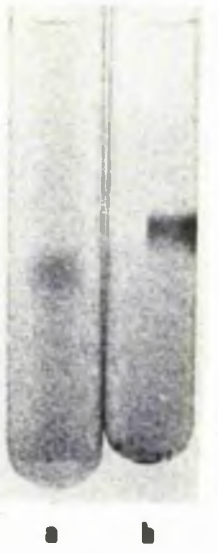


Fig.4.15. A comparison of (a) plasma and (b) membrane-associated cholinesterases.

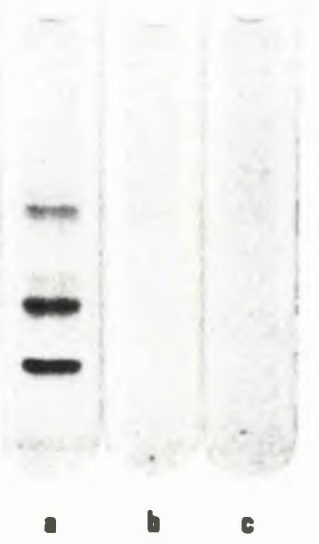


Fig.4.16. LDH of unfractionated membranes. (a) complete medium, (b) -ve NAD and (c) -ve Lactate.

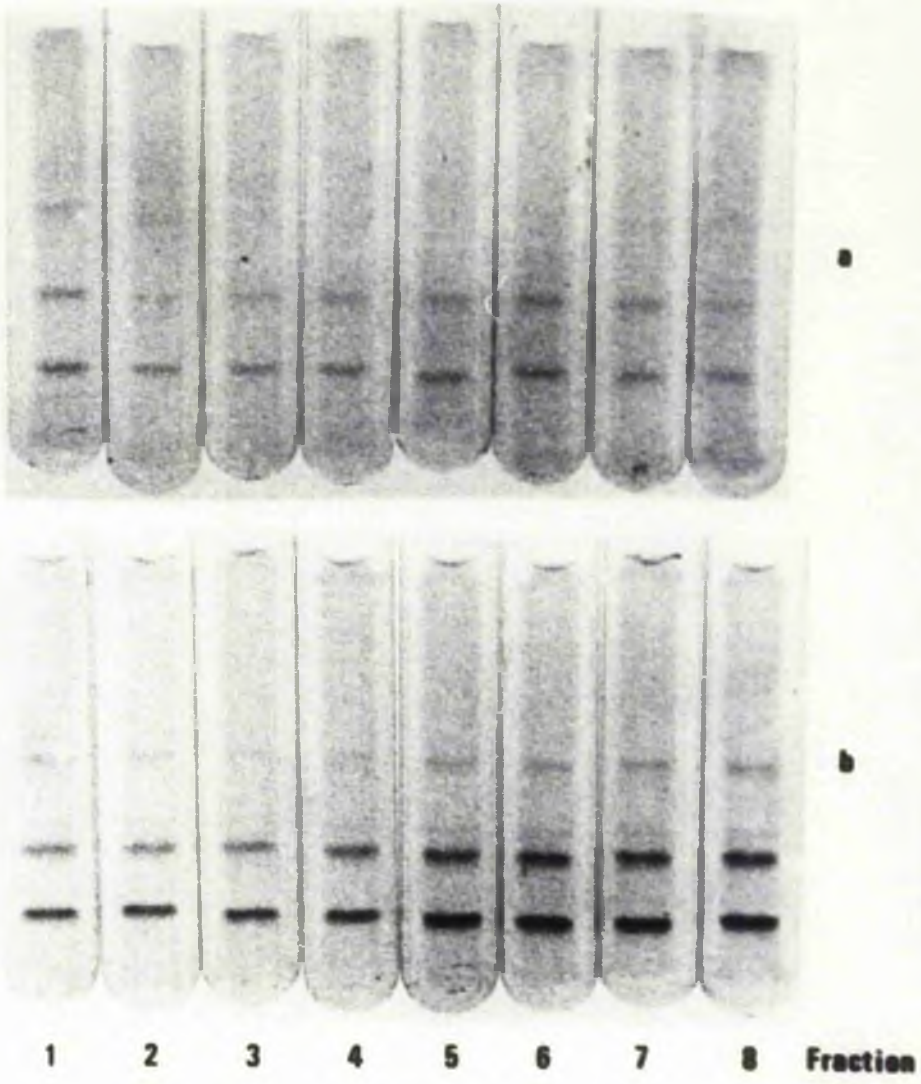


Fig.4.17. The effect of in vivo age on membrane-associated LDH showing (a) a decrease and (b) an increase in total activity.

bands represented various aggregation states of one enzyme. Further information was therefore obtained by comparison with the enzyme patterns derived from an already soluble activity. The band patterns obtained from the plasma membrane and the cytoplasm were identical (the extra band in the cytoplasm is haemoglobin) whereas the plasma lacked one of the major bands and probably the minor one as well (Fig. 4.18). The presence of multiple bands in the soluble as well as membrane samples strongly favoured the suggestion of isozymes but naturally the final proof would have been to compare heart and skeletal muscle as these are known to possess very different isozyme compliments (23).

Not surprisingly, this was not possible in the human and so an experiment was performed using rat tissue. Rat red cell cytoplasm was prepared in the same manner as the human equivalent and the heart and skeletal muscle extracts were made as described in the methods. Unfortunately, of these three, only the heart extract gave discrete bands free from any blurring (Fig. 4.19), illustrating the specificity of nonionic surfactant mixtures which must be tailor-made for each tissue. The red cell cytoplasm was heavily blurred and the skeletal muscle extract always had a very dense staining layer at the top of the gel. Nevertheless, altogether five bands were visible (numbered from the top) and it can be seen that the heart extract possessed bands 2, 3, 4 and 5, whilst the other two tissues possessed the missing 1 as well as the others. Consequently, there would appear to be little doubt that these bands do indeed represent the isozymes of LDH which can therefore be said to have widely differing mobilities in the nonionic system. This illustrates once again the complete lack of any correlation between molecular

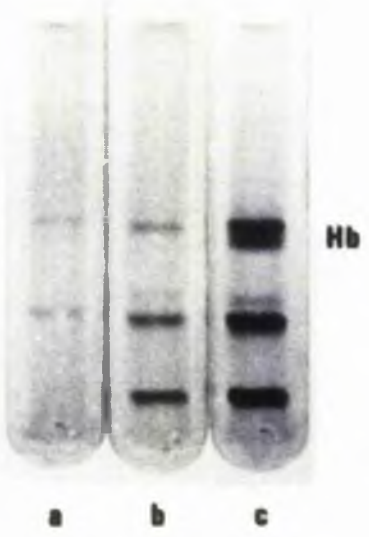


Fig.4.18. A comparison of (a) plasma, (b) membrane-associated and (c) cytoplasmic LDH.

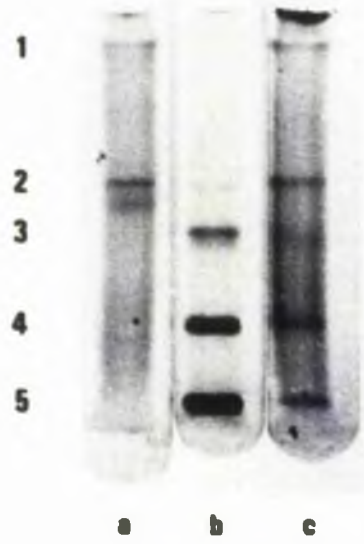


Fig.4.19. LDH of rat tissues showing (a) red cell cytoplasm, (b) heart muscle extract and (c) skeletal muscle extract.

weight and mobility.

NADH-Methaemoglobin Reductase (NADH-MR)

The stain for this enzyme used DCIP as the electron acceptor rather than the physiological substrate. Fig. 4.20 reveals that the absence of DCIP eliminated all activity but that there was a slight activity in the absence of NADH. As with AChEase there was a single band which also decreased in intensity upon ageing (Fig. 4.21). Fig. 4.22 shows that unlike the previous two enzymes, no activity of NADH-MR could be detected in the cytoplasm or the plasma.

Glyceraldehyde-3-Phosphate Dehydrogenase (GAPD)

This activity gave a broad band which was not present in the absence of NAD, FDP or aldolase (Fig. 4.23) and showed no change in intensity or mobility with age (Fig. 4.24). The only point of interest concerning GAPD came from the experiment in Fig. 4.25 which revealed a faint staining band in the cytoplasm with a much greater mobility than that seen for the membrane activity. This could have been due to a difference in the physical properties of the soluble and membrane-bound enzyme but as explained in Chapter 2 this is unlikely. A more plausible explanation is retardation of the membrane enzyme as a result of incomplete dissociation from the other membrane components which could also have caused the diffuseness of the membrane band.

Fig.4.20. NADH-MR of unfractionated membranes. (a) complete medium, (b) -ve NADH and (c) -ve DCIP.

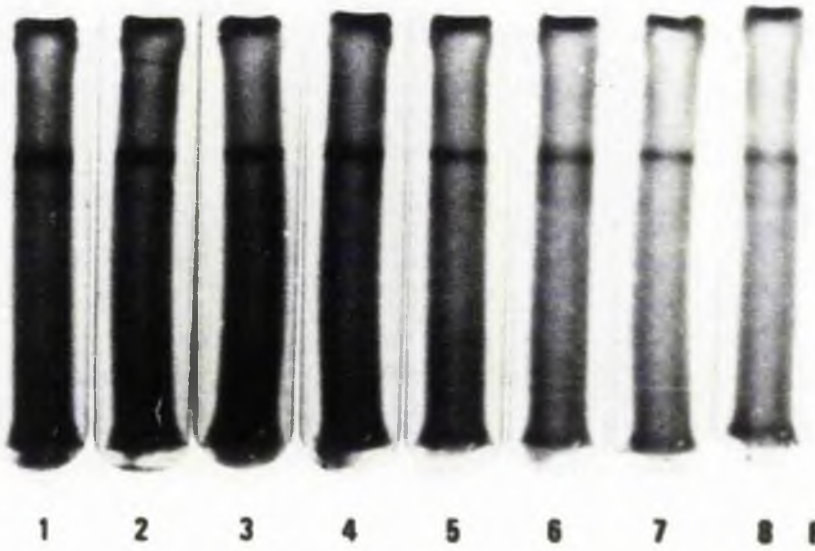


Fig.4.21. The effect of <u>in vivo</u> age on membrane-associated NADH-MR.

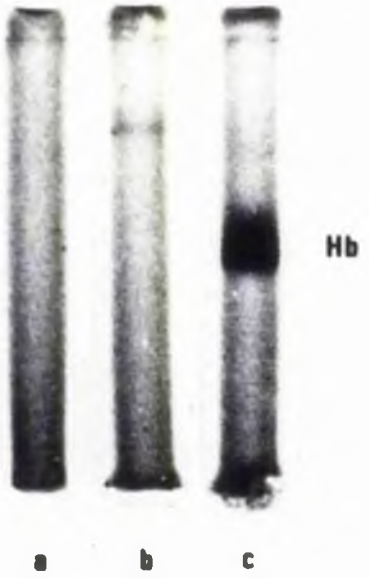


Fig.4.22. A comparison of (a) plasma, (b) membrane-associated and (c) cytoplasmic NADH-MR.

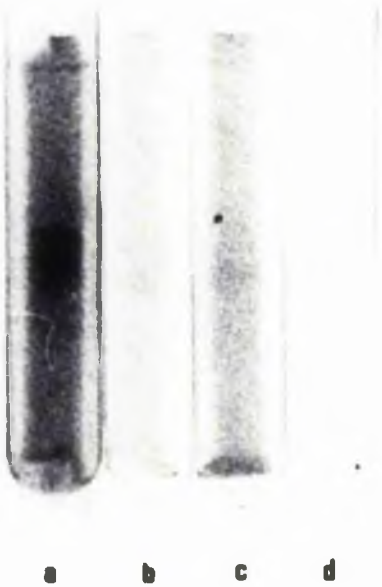


Fig.4.23. GAPD of unfractionated membranes. (a) complete medium, (b) -ve FDP, (c) -ve NAD and (d) -ve Aldolase.

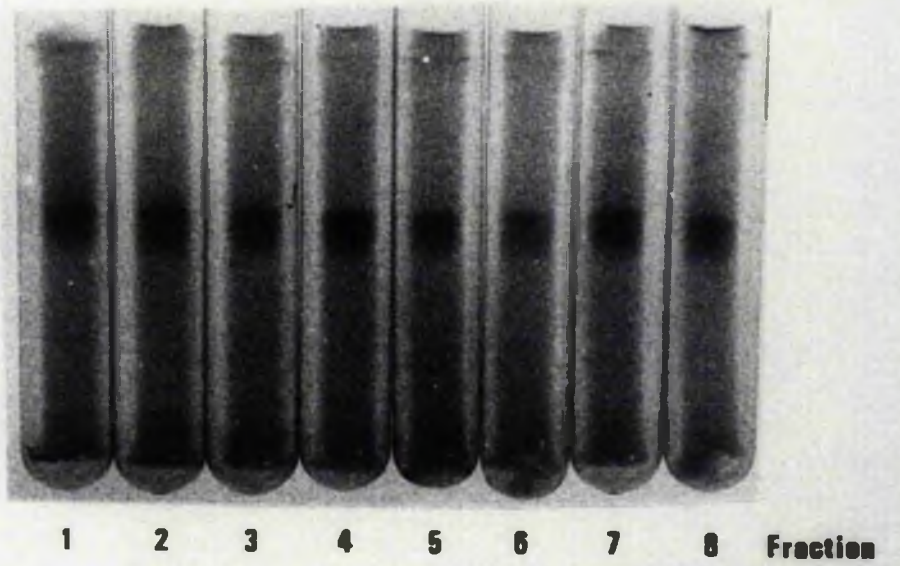


Fig.4.24. The effect of <u>in vivo</u> age on membrane-associated GAPD.

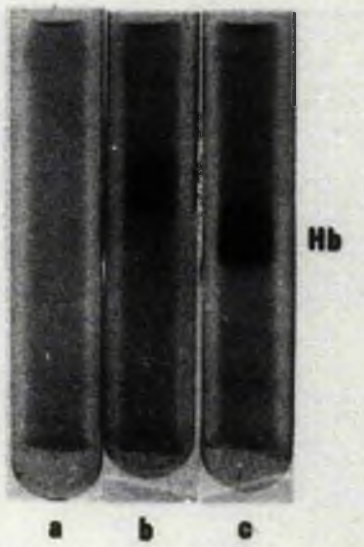


Fig.4.25. A comparison of (a) plasma, (b) membrane-associated and (c) cytoplasmic GAPD.

Purine Nucleoside Phosphorylase (PNP)

This too failed to give a staining band in the absence of substrate (Fig. 4.26) and at first glance there seemed to be only a decrease in stain intensity with age (Fig. 4.27). Closer examination, however, revealed a narrowing of band width which had come about by an advancement of the trailing edge with the leading edge remaining fixed. That this decrease in width was not caused simply by a decrease in enzyme protein is demonstrated by Fig. 4.28 in which two concentrations of the youngest membranes were run and stained. It can be seen that the difference in enzyme protein concentration was reflected in the band intensity and not the band width. Lastly, the presence of PNP was detected in both the plasma and cytoplasm (Fig. 4.29). The plasma enzyme had a slightly slower mobility with a narrower band width whilst the cytoplasmic enzyme was much broader with perhaps a slightly faster mobility. The merging of the trailing edge with the haemoglobin makes judgement difficult.

Localisation of Enzyme Proteins

In order to localise the enzyme proteins gels, after being histochemically stained and photographed, were fixed and stained for protein. This was carried out for all five enzymes and from these results the composite diagram in Fig. 4.30 was produced.

Fig.4.26. PNP of unfractionated membranes. (a) complete medium and (b) -ve inosine.

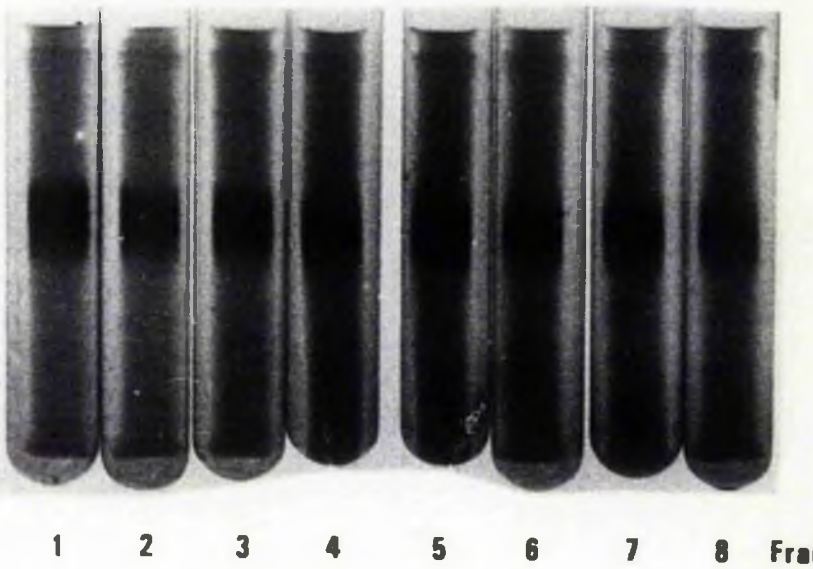
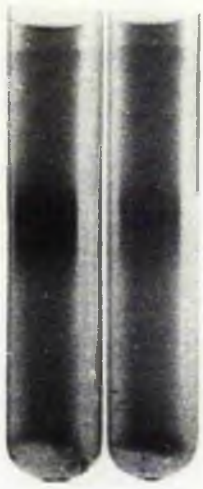
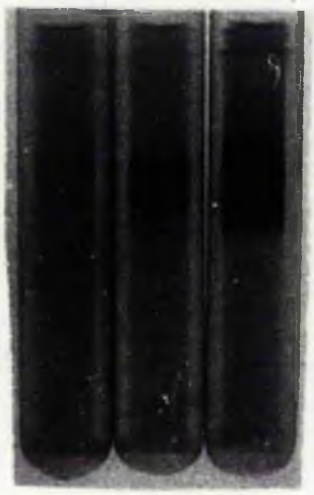


Fig.4.27. The effect of <u>in vivo</u> age on membrane-associated PNP.



a b

Fig.4.28. The effect of halving the amount of fraction 1 membrane protein applied to the gel on the resulting PNP band width.



a b c

Fig.4.29. A comparison of (a) plasma, (b) membrane-associated and (c) cytoplasmic PNP.

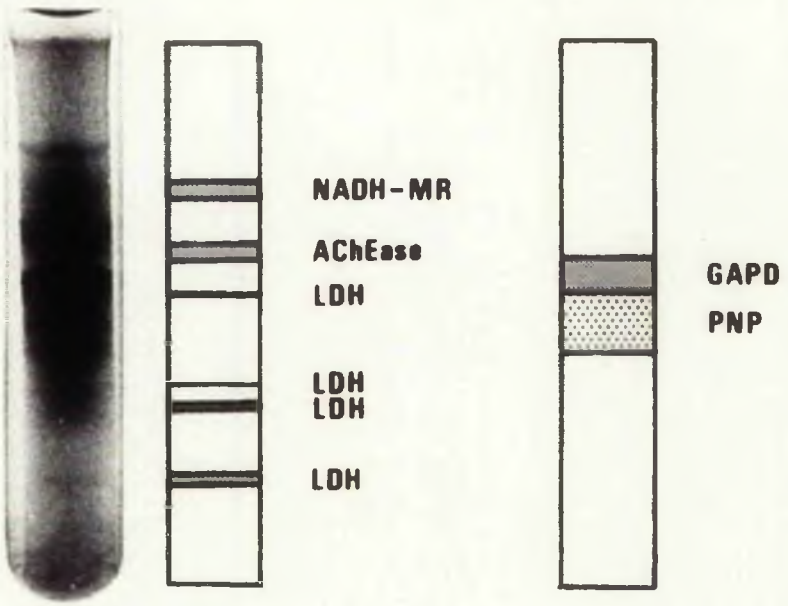


Fig.4.30. The localisation of the five membrane-associated enzyme activities investigated in this study.

Discussion

Five membrane enzymes have been histochemically investigated with respect to *in vivo* ageing. Of these, LDH was the only one which was resolved into more than one discrete band, and the four observed were shown to represent individual isozymes. The observation of both an increase and a decrease in activity with age strongly suggested that this was a very loosely bound enzyme and no change was noted in the relative staining intensities of the individual bands. GAPD, AChEase and NADH-MR were each present as single bands, the former showing no age-dependent change whilst the other two displayed visible decreases in band intensity. As well as these changes or lack of changes in band intensities, none of the bands associated with these four enzyme activities showed any change in band mobility whatsoever.

PNP showed a visible decrease in band intensity and was also the only enzyme to display any mobility change. The staining band was very broad but there was a decrease in its width with age, this coming about solely by an advancement of the trailing edge. It was conclusively demonstrated that this decrease in band width could not be explained by a decrease in enzyme protein but that it was a property of the ageing red cell. B.M. Turner *et al.* (14) performing starch-gel electrophoresis on the cytoplasmic enzyme found 5-7 isozymes present in the red cells but more importantly they noticed a progressive increase in the number and intensity of the faster bands as the mean age of the red cells increased. It is therefore proposed that the very broad staining band obtained with PNP in this study is due to the proximity of a number of isozymes and that there is a loss of the slower moving varieties with age as suggested by

B.M. Turner *et al.*

B.M. Turner *et al.* also looked at PNP in cultured fibroblasts where they found only one isozyme, corresponding to the slowest band in the red cell cytoplasm. Storage at $4^{\circ}C$ induced the formation of several faster bands in this tissue and this was not prevented by 2-ME. The interpretation offered was that the slowest variety is the species synthesised by the cell and in a situation of rapid protein turnover, such as in the cultured fibroblast, this is the only species seen. However, if this species is not removed it suffers a number of molecular alterations, some of which result in an altered electrophoretic mobility, as seen in the red cell. This hypothesis is tentatively supported by Fig. 4.29 in which it can be seen that the plasma enzyme stain is narrower and slower than that from the red cell.

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CHAPTER 5

THE ACTIVITY OF SOME MEMBRANE-ASSOCIATED ENZYMES.

Summary

1. Nine enzyme activities of the human erythrocyte plasma membrane have been measured with respect to *in vivo* ageing and all but Na, K-ATPase and GAPD exhibited significant decreases.

2. AChEase displayed the smallest decrease, this being the only one that could have been caused by reticulocyte ghost contamination.

3. The proportions of the whole cell compliments of PGK, PNP and AKase in the membrane were so small that their decreases could easily have resulted from an age-dependent loss of enzyme protein during ghost preparation or *in situ*.

4. The large decreases in Mg-ATPase, NADH-MR and alkaline phosphatase were concluded to have been caused by unambiguous losses of membrane enzyme activity although the percentage recovery in the case of Mg-ATPase was not assessed.

Introduction

The work described so far has been concerned largely with structural aspects of the membrane proteins and so it was felt that a look at some functional aspects was called for. This is especially important as this is undoubtedly the most neglected area of research in the field of red cell senescence. Well over 140 enzymes have so far been found in various mammalian erythrocytes (1) and many of these are situated, at least in part, in the membrane. Clearly, some form of selection was necessary and this was achieved largely on the basis of apparent overall metabolic importance. As the main aim of this study was to characterise the erythrocyte membrane proteins, exclusive location of an enzyme in the membrane was also considered a sufficient justification for its inclusion.

Naturally, a study of the membrane enzymes would be incomplete without some mention of ATPase. That the erythrocyte membrane could liberate phosphate from ATP had been noticed by several workers in the early and middle 1950's (2, 3, 4), but it was not until 1957 when J.C. Skou (5) published his famous work on the crab nerve fibre that the 'ATPase' was clearly defined. This enzyme had an absolute requirement for Mg^{++} and was stimulated by Na^{+} and K^{+} and so became known as the Na, K-stimulated Mg-ATPase. If present in the erythrocyte membrane what could its function be? A year earlier I.M. Glynn (6) had shown that the major fractions of red blood cell potassium influx and sodium efflux were stoichiometrically linked and required energy that was derived from glycolysis. It was but a few years before R.L. Post *et al.* (7) and E.T. Dunham *et al.* (8) showed that this 'sodium pump' and the Na, K-Mg, ATPase were identical

as regards kinetics, substrate requirements and ouabain inhibition. Thus, if these two processes are not expressions of one molecular species they are at least intimately connected.

In addition, E.T. Dunham et al. (8) found a ouabain insensitive ATPase which also required magnesium for its activity but was stimulated by low concentrations of calcium ions. A.S. Rosenthal et al. (9) suggested that this Ca-ATPase activity was associated with the proteins released after low ionic strength treatment of the ghosts and offered this association as a mechanistic explanation for the metabolic dependence of red cell deformability observed by R.I. Weed et al. (10). This enzyme has also been implicated by H.J. Schatzmann et al. (11) in the active extrusion of calcium ions from the red cell, i.e. the calcium pump. Recently, L.K. Drickamer (12) presented evidence from studies using γ-^{32}P-ATP labelling of the membrane followed by SDS PAGE that the Mg-ATPase, Na, K-ATPase and Ca-ATPase were functional expressions of three separate molecular species.

At about the same time as the ATPase studies were beginning, D.R.H. Gourley (13) and T.A.J. Prankerd (14) found that ghosts could adenylate inorganic phosphate to produce ATP. This was followed up by G.R. Bartlett (15) who claimed that this was in fact the mechanism of phosphate influx. That is, phosphate traverses the erythrocyte membrane in the adenylated form before being released at the inner surface in its initial state. He suggested that this was accomplished by the location of the enzymes GAPD and PGK within the membrane as follows. The first of these converted intracellular GAP and NADH plus the extracellular P_i into 1, 3-DPG, the phosphate then being transferred to ADP to give ATP by the PGK. To maintain a

constant intracellular P_i and ATP he was forced to include a membrane ATPase which preferentially used this ATP, somehow liberating the ADP on the inner surface and the P_i on the outer surface. This study came just before the demonstration of the coupling of the Na, K-ATPase with the sodium pump and so he postulated that the liberated energy might be used to 'impart vigorous motion' to the membrane.

Unfortunately V.T. Marchesi et al. (16) have since shown that the Na, K-ATPase liberates its phosphate exclusively on the inner surface of the membrane. This in no way negates the concept of a functional coupling of some of the more important membrane enzymes and S.L. Schrier et al. have published extensively on this issue (17, 18, 19). Their scheme is very similar to that of G.R. Bartlett's in that they imagine GAPD, PGK and the Na, K-ATPase to be tightly coupled, but the energy derived from the first two is used to drive the sodium pump via a specific membrane pool of ATP.

A slightly different idea was initiated by the observation of R. Whittam et al. (20) that external potassium and internal sodium stimulated lactate production. The latter and the production of P_i were both inhibited by ouabain which forced the conclusion that energy production and cation transport were linked. J.C. Parker et al. (21) found that it was only the steps before PGK in the glycolytic sequence which were inhibited by ouabain and that this inhibition did not occur in membrane-free haemolysates. In addition, the rate of the GAPD-PGK sequence was found to be a function of the ADP concentration and that it was only the forward reaction that was ouabain sensitive. They therefore concluded that the Na/K transport system exerted an effect on the membrane PGK via a

compartmentalised form of ADP within the membrane, for this to be so, a large fraction of the total cell PGK would have to be resident in or on the membrane and the estimates of 1% and 2% from S.L. Schrier et al. (22) and C.F. McDaniel et al. (23) would not seem to bear this out, although D.E. Green et al. (24) put this value at 11.4%. It is of course quite possible that in situ this enzyme is closely associated with the membrane and that it is lost during the ghost preparation.

There is considerable dispute concerning the amount of membrane-bound GAPD and the nature of its association with the membrane. The percentage of the total cell activity in the membrane has been found to be 60 (22), 79 (24), 60-80 (25) and 87 (23) but this is complicated by the observation of C.D. Mitchell et al. (26) that GAPD retention in the membrane is highly dependent on the pH and ionic strength. This property has been used by M.J.A. Tanner et al. (27), C.F. McDaniel et al. (23) and J.A. Kant et al. (28), who have shown that increasing the ionic strength in the presence of ghosts results in an almost complete elution of the enzyme. Consequently, D. Maretzki et al. (29) concluded that the finding of GAPD in the membrane was an artifact caused by hypotonic haemolysis being the preferred method of ghost preparation. As confirmation for this they showed that membranes prepared by sonication under isotonic conditions contained no GAPD whereas if the sonication was performed under standard hypotonic conditions they did not contain GAPD.

However, simple isotonicity in no way completely mimics the intracellular environment. Thus J.A. Kant et al. (28) and G. Letko et al. (30) have shown that a variety of glycolytic intermediates effect GAPD binding. Furthermore J.A. Kant et al. (28) showed that at isotonicity, if the pH was above 7.0 there

was negligible binding whereas between 6.0 and 7.0 binding became considerable. If as D.E. Green *et al.* (24) have stated, the entire glycolytic pathway is located within the plasma membrane, it would be quite possible for the inner surface to be at a significantly lower pH than the rest of the interior and so *in situ* binding might be feasible. Also C.F. McDaniel *et al.* (23) and J.A. Kant *et al.* (28) have shown that the binding of GAPD to the membrane occurs at specific high affinity sites located on the inner membrane surface. Therefore binding, when it does occur, is by no means as a result of non-specific adsorption and it is conceivable that *in situ* this binding is responsive to local variations in pH, ionic strength and metabolite concentrations.

In addition to this, the argument for the complete intracellular location of GAPD is challenged by G. Ronquist (31, 32). He showed that intact red cells could synthesise ATP from P_i if all the substrates and cofactors for GAPD and PGK were present in the external medium. Altogether he found GAPD, PGK and AKase activities on the red cell external surface and calculated that these activities represented 3.1%, 4.5% and 2.0% respectively of the total membrane activity. He did however, acknowledge that membrane damage may have caused these findings.

Of prime importance to any investigation of membrane enzyme activity is the position of the enzyme within the membrane. For example, an enzyme present on the surface of the membrane with its active site orientated towards the soluble phase would be expected to reveal its total activity in an assay situation. If, however, the enzyme is buried within the membrane or if its active site is orientated

towards the membrane, then a portion of its activity could be hidden due to steric hundrance of substrate and product diffusion.

This aspect was first fully investigated by G. Duchon et al. (33) who described the phenomenon as 'crypticity'. They looked at many of the glycolytic enzymes after various membrane disrupting procedures such as very low ionic strength, increasing pH and nonionic surfactant solubilisation and classified the enzymes as loosely bound or firmly bound on their ability to withstand these treatments and the degree of crypticity. They concluded that GAPD and aldolase were firmly bound, that PGK was intermediate and that PK, LDH and TPI were loosely bound. This concept had been used earlier by S.L. Schrier et al. (18) when they had been formulating their ideas on enzyme coupling. They had measured enzyme activity after sonication in the presence and absence of magnesium ions (which are thought to stabilise the membrane structure) and after treatment with lipid active agents. They concluded that GAPD was located on the inner surface and that PGK was more deeply buried. Thus substrates could be drawn from the intracellular environment and the products passed to the central lipid core of the membrane where they assumed the ATPase to reside. The latter was decided on the 'commonsense' argument that as this enzyme has access to both membrane surfaces, it is likely to be situated in the middle of the membrane and the fact that it requires lipid for full activity.

In contrast to this situation, the total activity of AChEase can be detected in the intact cell (34) and so not only is it exclusively located in the membrane but it is presumably present on the external surface with its active site completely

available to the external environment. A decrease in the activity of this enzyme was reported in one of the earliest *in vivo* ageing studies (35) but this has not yet been confirmed. An investigation of this activity is also justified because it has been found to be responsive to cell volume (36) and various pathological states (37). However, as F. Metz *et al.* (38) found that the *in vivo* inhibition of erythrocyte AChEase had no effect on the cell's life span it may be that changes in its activity are a consequence of rather than a cause of membrane alterations. This situation may become clear when the functional significance of this enzyme is resolved.

T.L. Steck (34) has claimed that NADH-MR is located on the inner surface of the membrane and considering its vital function of protecting haemoglobin (39) this would certainly be a convenient location, but I. Zamudio *et al.* (40) using hypotonicity and sonication found a degree of crypticity. S.L. Schrier *et al.* (17) have shown that ghosts can produce glycolytic intermediates from ribose-5-P via the non-oxidative limb of the pentose phosphate pathway. Due to the presence of PNP this ribose-5-P could come from purines, were it not for the reported absence of phosphoribomutase. Nevertheless, it was felt that this may be an important pathway *in situ*, especially as S.L. Schrier *et al.* could not detect the presence of hexokinase in the membrane. Lastly, it was thought worthwhile to investigate further the nucleotide metabolism of the membrane. Specific membrane pools of ATP (19) and ADP (21) have been mentioned and if these exist then the presence of adenylate kinase (32, 41) might be critical to the functioning of the membrane.

Materials and Methods

General

Ghosts, prepared as described previously were used for all but the phosphohydrolase assays. For the latter, the ghosts were given an extra wash in 10 mM Tris/Cl pH 7.4 to reduce the phosphate background to an acceptable level. Protein was measured as described previously. All biochemicals were purchased from Sigma Ltd., and the other reagents from B.D.H.

Enzyme Assays

The non-phosphohydrolase enzymes were assayed at 37°C by continuous spectrophotometry using a Beckman DB GT double beam spectrophotometer as follows.

GAPD + PGK (17), 2.0 ml. 7.5 mM $MgCl_2$/ 0.2% 2-ME/ 50 mM Tris/Cl pH 7.5

0.2 ml. 20 mM ATP

0.2 ml. 3.0 mM NADH

0.2 ml. water

0.1 ml. 10 Units/ml. GAPD (for PGK assay)

0.1 ml. 20 Units/ml. PGK (for GAPD assay)

0.1 ml. sample

Preincubate until all substrate independent reaction ceases

Add 0.2 ml. 50mM PGA

Read at 340 nm. against water blank

AChEase (42), 2.7 ml. 0.1 M Na Phosphate pH 8.0

0.1 ml. 160 mg% DTNB/ 60 mg% Na Bicarbonate.

0.1 ml. sample

Preincubate for 5 min.

Add 0.1 ml. 75 mM Acetylthiocholine Iodide

Read at 412 nm. against water blank

NADH-MR (40), 2.4 ml. 0.3 M Tris/Cl pH 8.5

0.1 ml. 10 mM K Ferricyanide

0.3 ml. water

0.1 ml. sample

Preincubate for 5 minutes

Add 0.1 ml. 6 mM NADH

Read at 340 nm. against water blank

PNP (17), 2.5 ml. 0.1 M Na Phosphate pH 7.4

0.2 ml. 0.06 Units/ml. Xanthine oxidase

0.1 ml. sample

Preincubate until all substrate independent reaction ceases

Add 0.2 ml. 30 mM Inosine

Read at 290 nm. against water blank

AKase (41), 2.0 ml. 7.5 mM $MgCl_2$/ 0.2 M NaCl/ 21 mM KCl/ 75 mM Tris/Cl pH 7.5

0.2 ml. 3 mM NADH

0.2 ml. 15 mM PEP

0.1 ml. 100 Units/ml. LDH

0.1 ml. 80 Units/ml. PK

0.2 ml. 30 mM ATP

0.1 ml. 3 mM Ouabain

0.1 ml. sample

Preincubate until reaction has been linear for 5 min.

Add 0.1 ml. 6 mM AMP

Read at 340 nm. against water blank.

SDH (43), 2.4 ml. 0.4 mM EDTA/ 25 mM Tris/Cl pH 7.4

0.2 ml. 50 mM Na Succinate pH 7.4

0.2 ml. sample

Preincubate for 5 min.

Add 0.2 ml. 50 mM K Ferricyanide

Read at 420 nm. against water blank

Alkaline (44) Phosphatase. Due to the low level of activity this was not assayed by continuous spectrophotometry.

a. membranes 2.4 ml. 50 mM Na Borate pH 9.2

0.4 ml. 45 mM PNPP

0.2 ml. sample

Incubate at 35°C for 2 hours

Stop reaction by placing on ice

Add 0.5 ml. 0.5 M Na Carbonate/ 10 mM EDTA

Read at 410 nm. against water blank

b. whole cells 2.4 ml. 60 mM Na Borate pH 9.2

0.4 ml. 45 mM PNPP

0.4 ml. red cells

Incubate at 35°C for 2 hours

Stop reaction by cooling on ice

Add 0.5 ml. PATSA (10% v/v perchloric acid/ 8% tungstosilicilic acid) and mix

Spin at 5 Krpm for 5 min.

Add 0.5 ml. 1M NaOH to 2.5 ml. supernatant

Read at 410 nm. against water blank

The assay mixtures for the phosphohydrolase enzymes were as follows.

Mg-ATPase (43), 1.0 ml. 40 mM TEA/Cl pH 7.6

0.4 ml. 4 mM MgATP

0.2 ml. sample

Na/K + (43) 1.0 ml. 20 mM KCl/ 0.2 M NaCl/ 40 mM TEA/Cl

Mg-ATPase pH 7.6

0.4 ml. 4 mM MgATP

0.2 ml. sample

G6Pase (43), 1.0 ml. 80 mM Succinate/Cl pH 6.5

0.2 ml. 80 mM G6P

0.2 ml. water

0.2 ml. sample

All three were incubated at 35°C for 1-2 hours, the reaction being stopped by placing on ice. The procedure was then as follows.

0.5 ml. PATSA

1.5 ml. 1.45% Na Molybdate/ 13.1% NaCl

4.0 ml. Ethyl acetate

Mix vigorously for 30 sec. and allow phases to separate

Remove top ethyl acetate layer and read at 310 nm. against ethyl acetate.

For those enzymes which were measured by continuous spectrophotometry a constant rate of reaction had to be observed for at least 5 minutes for the result to be used. The one exception was NADH-MR, the rate of which was never linear. This was likely to have been caused by product inhibition and

in this case the initial rate of reaction was used. The rates of reaction for all the other enzymes had been found to be linear over the incubation period used, prior to the actual experiments.

Assays, carried out in the presence of nonionic surfactant had a final concentration of 0.1% P40/ 0.05% T20/ 0.05% T40/ 0.05% B35.

Results and Discussion

The red cell has been shown to lose surface area during the ageing process (see Chapter 1) and an associated decrease in total membrane enzymes activity is to be expected. Therefore to avoid any confusion the enzyme activities have been expressed per mg. of membrane protein to detect any selective loss of activity. The question of reticulocyte contamination was considered in some depth in Chapter 1 and in this Chapter account had to be taken of the presence of reticulocyte ghosts and also the possible presence of mitochondria and endoplasmic reticulum. S.J. Shattil *et al.* (44) and S.M. Rapoport *et al.* (45) examining reticulocyte ghosts under the electron microscope claimed that these were usually contaminated with mitochondria. A.K. Percy *et al.* (46) were not able to confirm this but they did accept the possibility of organelle contamination. The degree of contamination in this study was assessed with specific enzyme markers, SDH and G6Pase being used to detect the presence of mitochondria and endoplasmic reticulum respectively. Neither of these enzymes could be detected and so the reticulocyte ghosts were considered to be free from organelle contamination.

Adenosine Triphosphatases

The various ATPases have undoubtedly been the subject of more publications than any other enzyme (see 47 for recent review), and yet they have so far escaped scrutiny as regards red cell ageing. Considering, for example, the vital role

played by the Na, K-ATPase in the maintainance of essential ionic gradients and the fact that ageing research is devoted to detecting the loss of an essential component, this omission is strange indeed. A.A. Yunis *et al.*(48) found that rat and rabbit reticulocyte ghosts had a higher specific activity for this enzyme compared to their respective erythrocyte ghosts but were unable to decide whether this was due to reticular contamination or not.

Fraction	% Na, K-ATPase / Total ATPase
1	16
2	27
3	26
4	25
5	34
6	28
7	39
8	42

Table 5.1. The change in Na, K-ATPase activity as a percentage of the total membrane ATPase with respect to *in vivo* ageing.

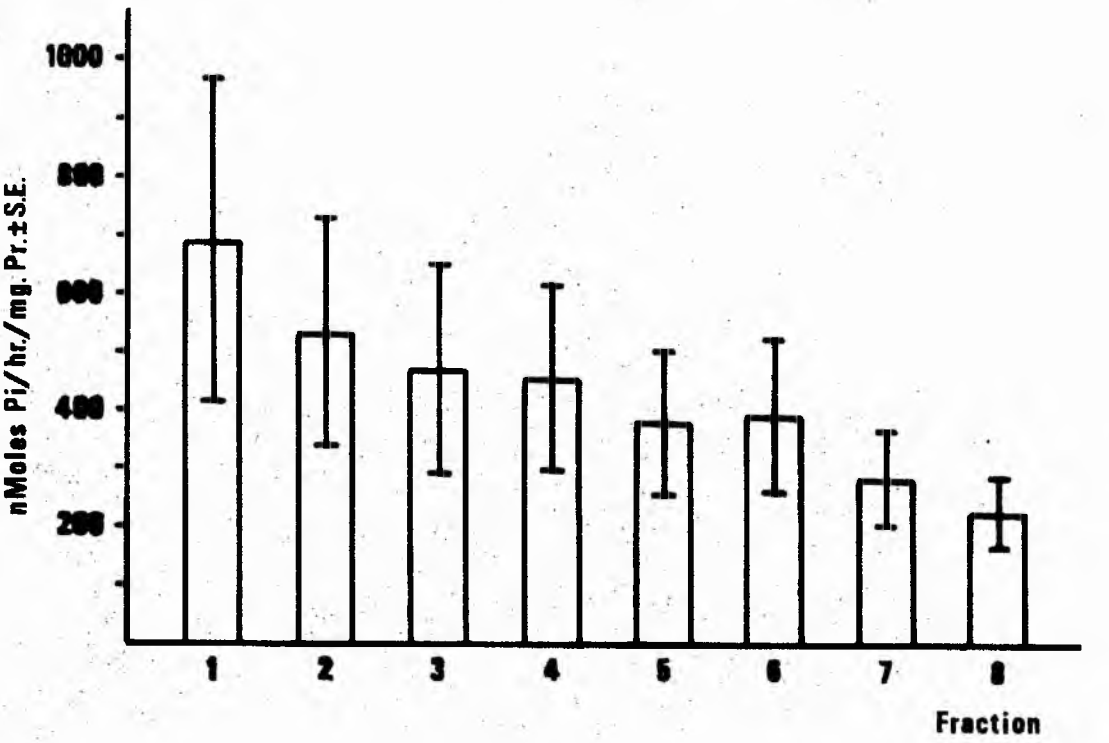

Fig.5.1. The effect of <u>in vivo</u> age on membrane-associated Mg-ATPase. $p = 0.025$ by Analysis of Variance.

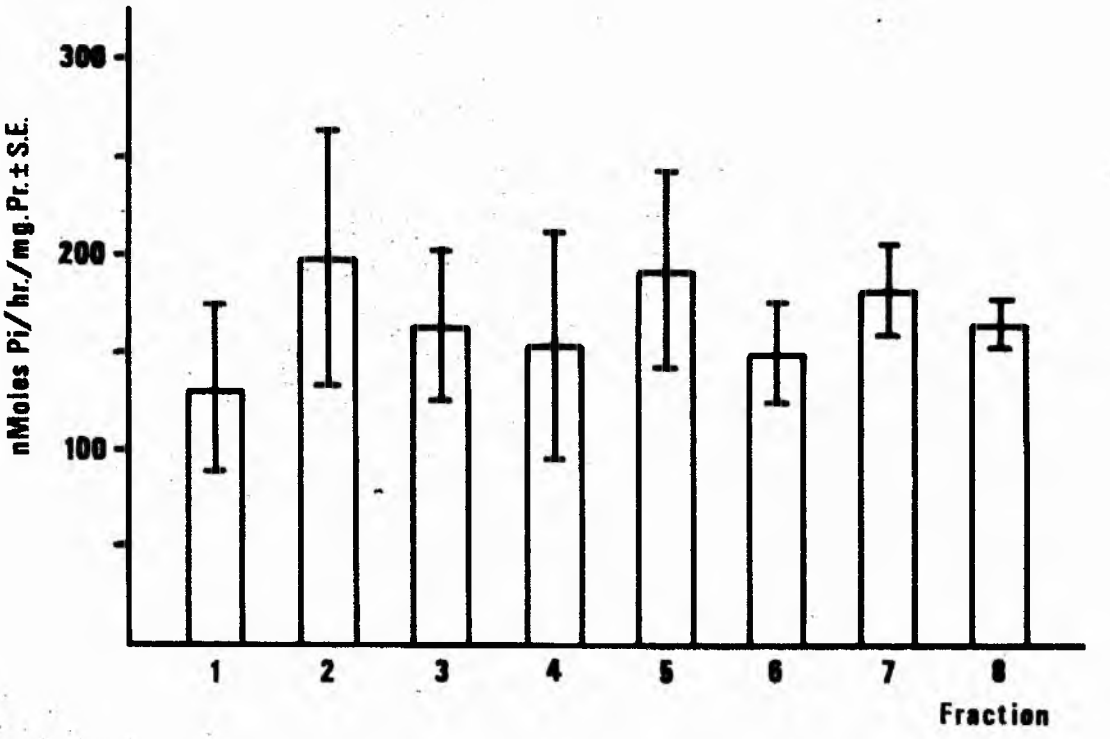

Fig.5.2. The effect of <u>in vivo</u> age on membrane-associated Na,K-ATPase. $p > 0.75$ by Analysis of Variance.

Fraction	% S.E. Mean Mg-ATPase	Na, K-ATPase
1	40	32
2	36	33
3	38	24
4	35	38
5	32	26
6	34	17
7	29	13
8	27	8

Table 5.2. The variability of ATPase activities as a function of *in vivo* ageing.

Figs. 5.1 and 5.2 illustrate the effect of age on the Mg-ATPase and the Na, K-ATPase respectively. The former was subject to a large significant decrease whilst the latter showed no change. This results in a drastic alteration in the ratio of these two activities as is revealed in Table 5.1. What is more interesting is the decrease in the variability of the specific activities with age. Table 5.2 shows a slight decrease in this parameter (the standard error expressed as a percentage of the mean) for the Mg-ATPase and a large decrease for the Na, K-ATPase. This could be the result of very slight but undetected organelle contamination.

Glyceraldehyde Phosphate Dehydrogenase + Phosphoglycerate Kinase

These two enzymes have been placed together partly

because they were assayed in the same system but also because of the evidence already cited suggesting a functional coupling in the membrane. It was found that there was no significant decrease in the specific activity of GAPD (Fig. 5.3) but PGK exhibited a marked decrease (Fig. 5.4) which was confined largely to the first four fractions. The GAPD result ruled out the need for further experimentation but the PGK result needed to be followed up to investigate the possible involvement of crypticity. That is, the possibility that the result was a consequence of the orientation and/or location of the enzyme within the membrane. Thus, two possible explanations for Fig. 5.4 other than a real age-dependent decrease in specific activity are, a. diffusion of substrate and/or product to and from the active site became impeded with age due to some change in the membrane structure or b. there was an age-dependent tendency towards resealing and the enzyme was located on the inner surface. The addition of nonionic surfactant to the assay medium as described in the Methods should have abolished these cryptic effects if they were occurring. It can be seen from Fig. 5.5 that this procedure simply caused a slight increase in the specific activities of all the fractions, indicating that the effect was not due to crypticity or resealing. It does, however, show that there is a small fraction of activity which is hidden under the conditions of the assay, unless of course the surfactant itself has somehow activated the enzyme.

Acetylcholinesterase

Unlike the previous report (35) which claimed quite a

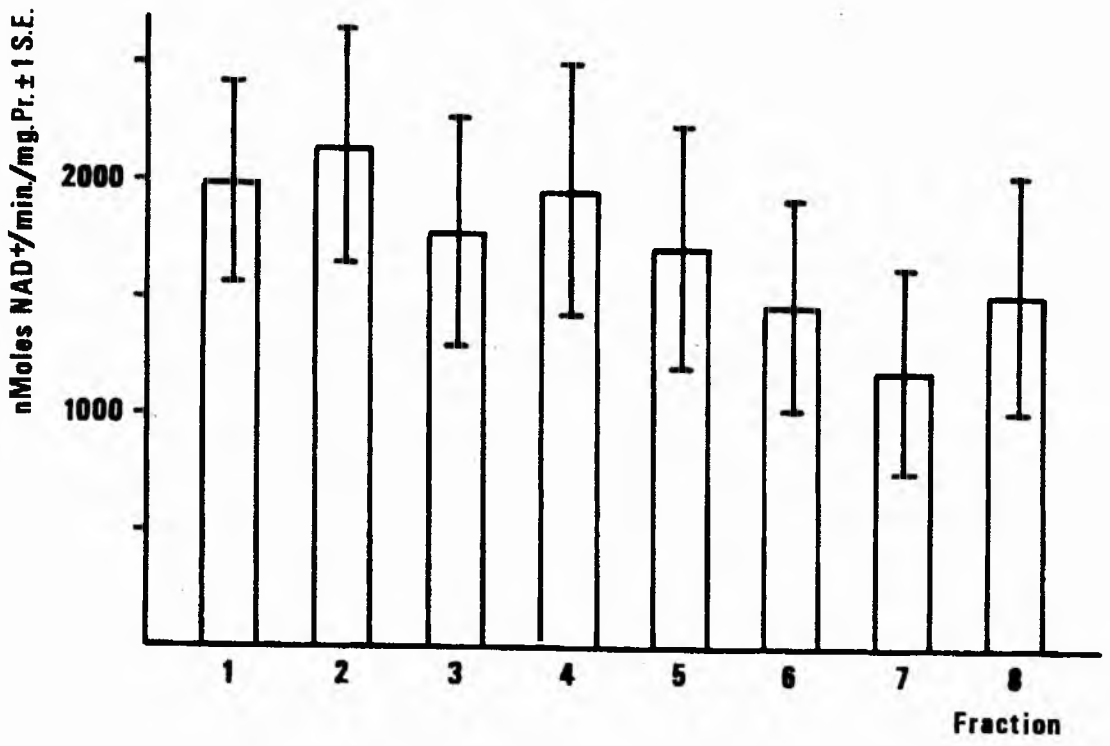

Fig.5.3. The effect of <u>in vivo</u> age on membrane-associated GAPD. $p < 0.1$ by Analysis of Variance.

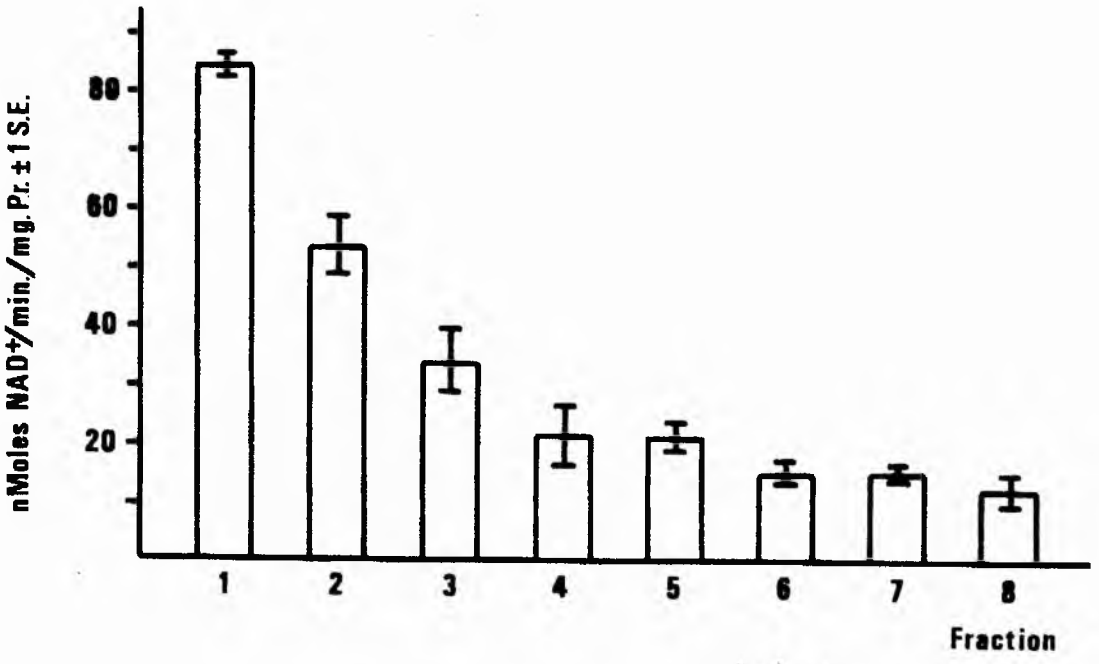

Fig.5.4. The effect of <u>in vivo</u> age on membrane-associated PGK. $p < 0.001$ by Analysis of Variance.

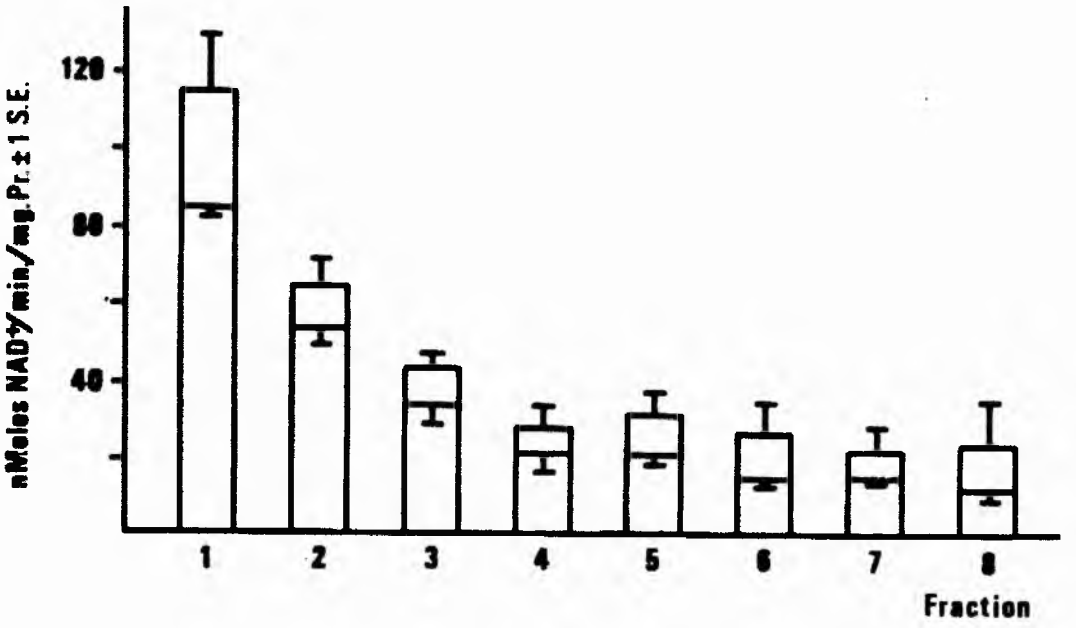

Fig.5.5. The effect of <u>in vivo</u> age on membrane-associated PGK in the presence (T) and absence (⊥) of nonionic surfactant (see Methods).

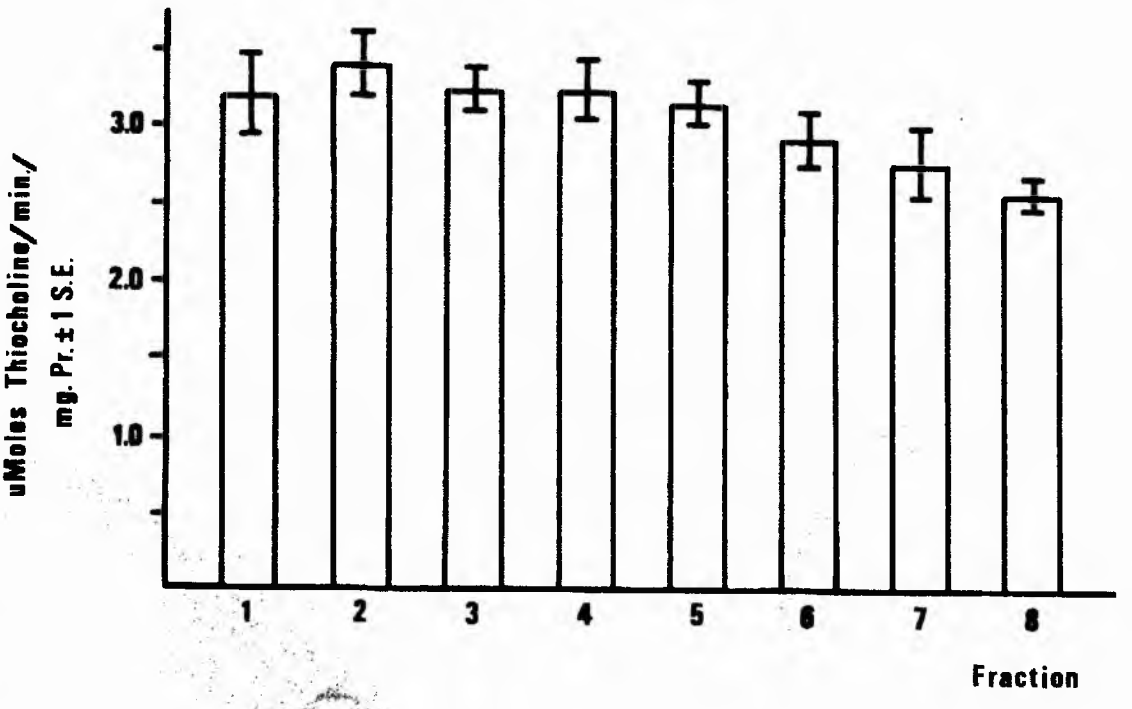

Fig.5.6. The effect of <u>in vivo</u> age on membrane-associated AChEase. p = 0.001 by Analysis of Variance.

large decrease in the specific activity of this enzyme, only a very slight but nevertheless significant decrease was found in this study (Fig. 5.6). The slight initial increase in specific activity was found in the majority of cases and was thought to have been due to the larger amounts of haemoglobin bound by the youngest membranes which would have given a higher protein estimation with a consequent decrease in specific activity. As AChEase is situated on the external surface of the membrane, crypticity is unlikely to have been involved but one could have been looking at a resealing effect, i.e. an age-dependent tendency to form inside-out vesicles. Once again surfactant was used (Fig. 5.7) and this caused a constant and significant decrease in specific activity irrespective of age. It has been reported (49) that surfactants cause a decrease in the optical density of the DTNB complex and this is the most likely cause of the observed decrease in specific activity. This finding is, however, at odds with the demonstration in the previous chapter that total solubilisation of the membrane proteins with nonionic surfactants had no apparent effect on the activity of this enzyme. The discrepancy has probably arisen because in the previous chapter the final surfactant concentration in the assay mixture was $\frac{1}{5}$ that used in this chapter and so may have been insufficient to cause the decrease in optical density.

As was pointed out in the Introduction, it is not too wise to place too much faith in the measurement of enzyme activities under optimal conditions and so it was decided to briefly investigate the kinetics of the acetylcholinesterase reaction using the youngest and oldest fractions. An Eadie plot (50) revealed (Fig. 5.8) a slight increase in Km and a 7%

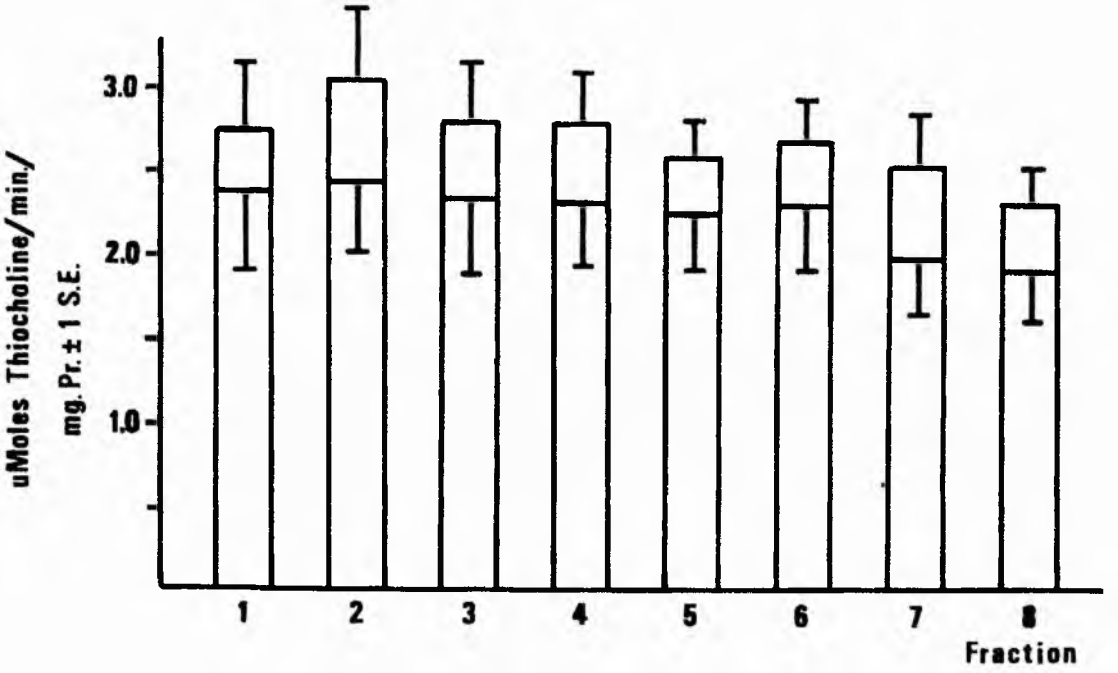

Fig.5.7. The effect of in vivo age on membrane-associated AChEase in the presence (⊥) and absence (T) of nonionic surfactant (see Methods).

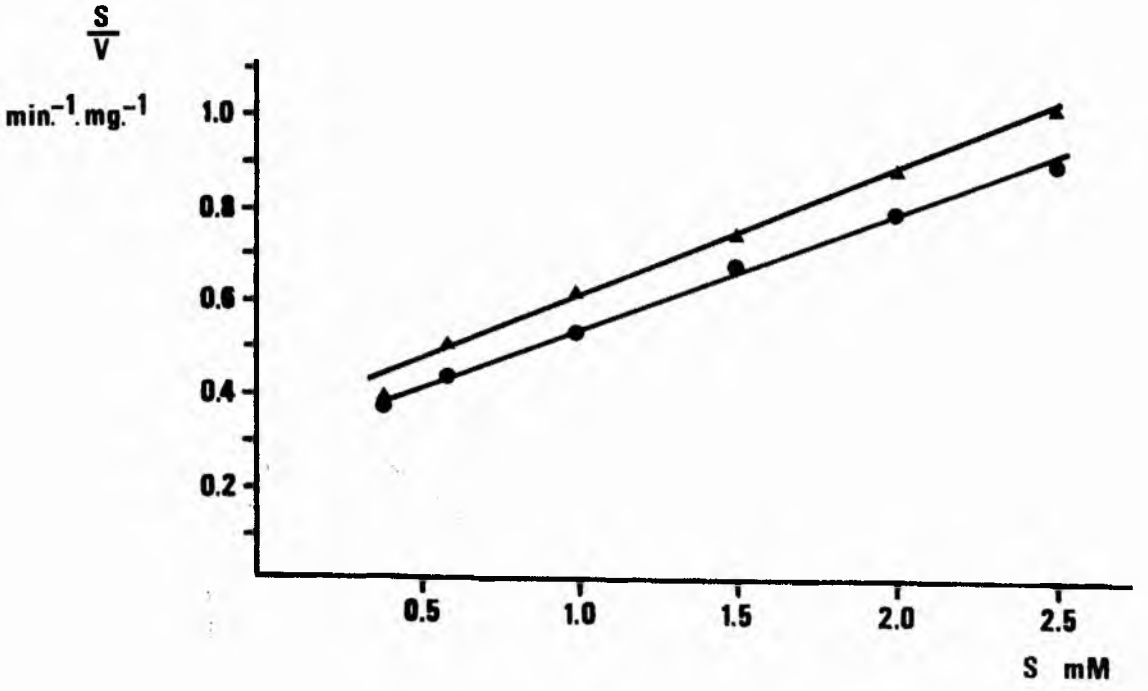

Fig.5.8. An Eadie plot of membrane-associated AChEase; fraction 1 (●) and fraction 8 (▲).

decrease in Vmax. with age. This also showed that the experiments in Figs. 5.6 and 5.7 had not been performed at supra-optimal substrate conditions but as the % of Vmax. recorded at 2.5 mM substrate concentration for fractions 1 and 8 were 82 and 77 respectively, this will not have affected the results to any significant extent assuming of course Michaelis-Menton kinetics. An Arrhenius plot (51) also failed to detect any difference between the two fractions apart from the already observed difference in specific activities at 2.5 mM substrate concentration (Fig. 5.9). Both fractions seemed to undergo a similar change in activation energy at approximately 33°C as judged by eye; the four lines shown then being drawn by the method of least squares. It is not possible to say whether the change in activation energy is due to the presence of two or more sub-reactions each with different temperature coefficients or to a temperature-dependent phase change in the membrane.

NADH-Methaemoglobin Reductase

This enzyme is involved in the vital function of protecting haemoglobin from oxidative denaturation (39) and consequently its physiological substrate is methaemoglobin. It is, however, usually measured using ferricyanide as the electron acceptor as described by I. Zemudio et al. (40) and so there was the accepted risk that another enzyme was being investigated. A slight but insignificant increase in the specific activity was found (Fig. 5.10) which was odd because in the previous chapter a clear decrease in the histochemical staining intensity of this enzyme had been shown (Fig. 4.21).

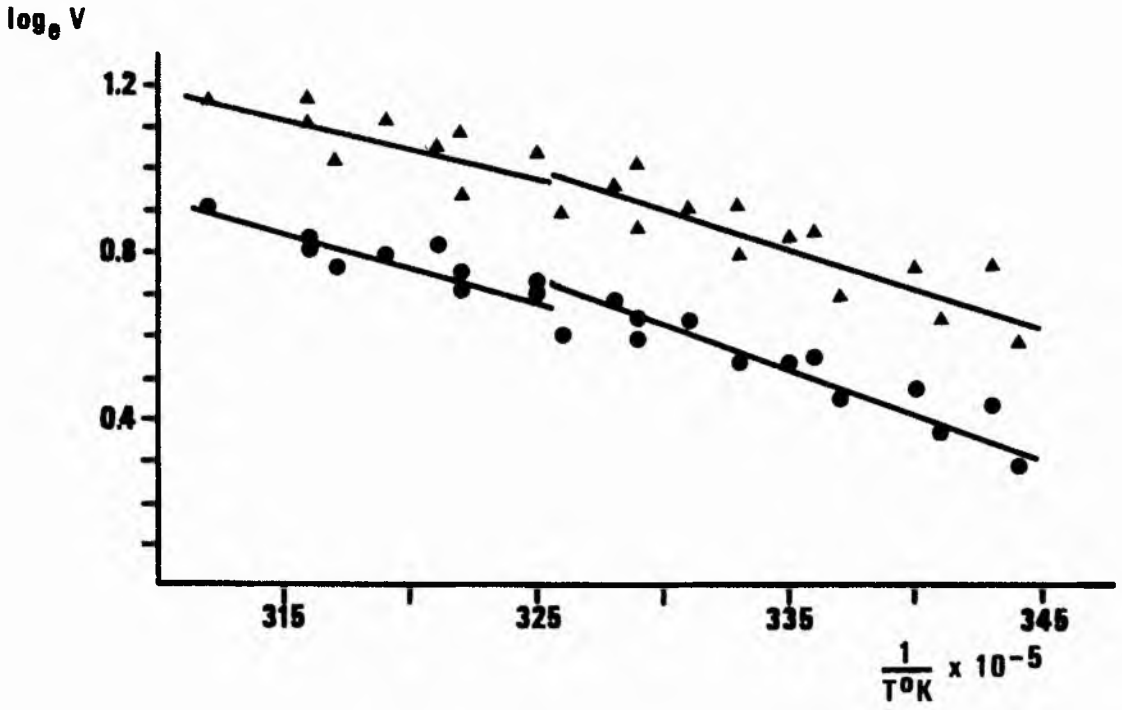

Fig.5.9. An Arrhenius plot of membrane-associated AChEase; fraction 1 (▲) and fraction 8 (●).

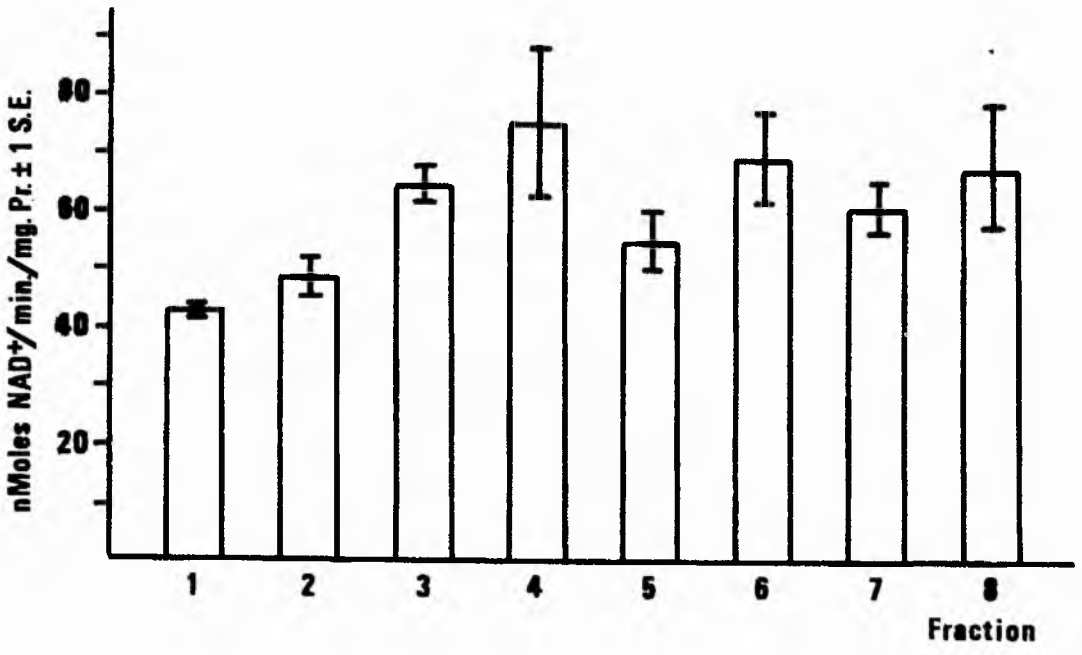

Fig.5.10. The effect of <u>in vivo</u> age on membrane-associated NADH-MR. $p < 0.1$ by Analysis of Variance.

A likely explanation for this discrepancy was that of crypticity as already discussed. This hypothesis was consistent with the results shown in Fig. 5.11 in which the assay was performed in the presence of surfactant. The results from Fig. 5.10 are included for comparison. The surfactant caused a large increase in specific activity in all the age groups, but as this increase was proportionately much greater in the younger membranes the slight insignificant increase initially observed was transformed into a significant decrease. This illustrates all too clearly one of the major hazards involved in measuring the activities of membrane enzymes and confirms the findings of I. Zamudio et al. that this enzyme is subject to a considerable cryptic effect.

The degree of surfactant stimulation observed here is once again in conflict with the results of the previous chapter (Table 4.2) which recorded a much smaller increase. As stated above the results of Table4.2 were obtained using $\frac{1}{3}$ the present surfactant concentration but as the membranes had been subjected to complete solubilisation prior to this, we cannot look to crypticity for an answer this time. Perhaps the low ionic strength dialysis or the very high surfactant concentrations had a small detrimental effect on the enzyme.

If we assume that the activity in the presence of surfactant shown here represents the maximum activity, then expressing the activity in the absence of surfactant as a percentage of this should give some idea as to the amount of activity being revealed. It can be seen in Table 5.3 that in the youngest membranes only 8.4% of the activity is revealed in the intact ghost and that this rises to 25% in the oldest ghosts. If this interpretation is correct then it is good evidence for

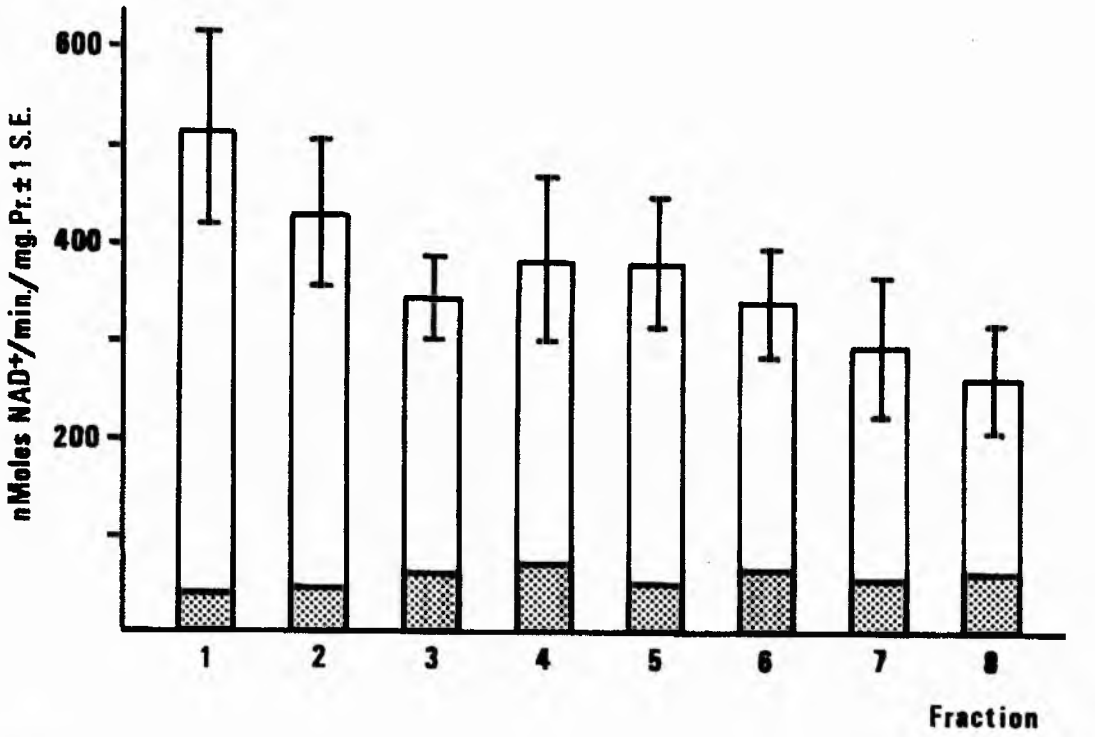

Fig.5.11. The effect of in vivo age on membrane-associated NADH-MR in the presence (unshaded) and absence (shaded) of nonionic surfactant (see Methods).

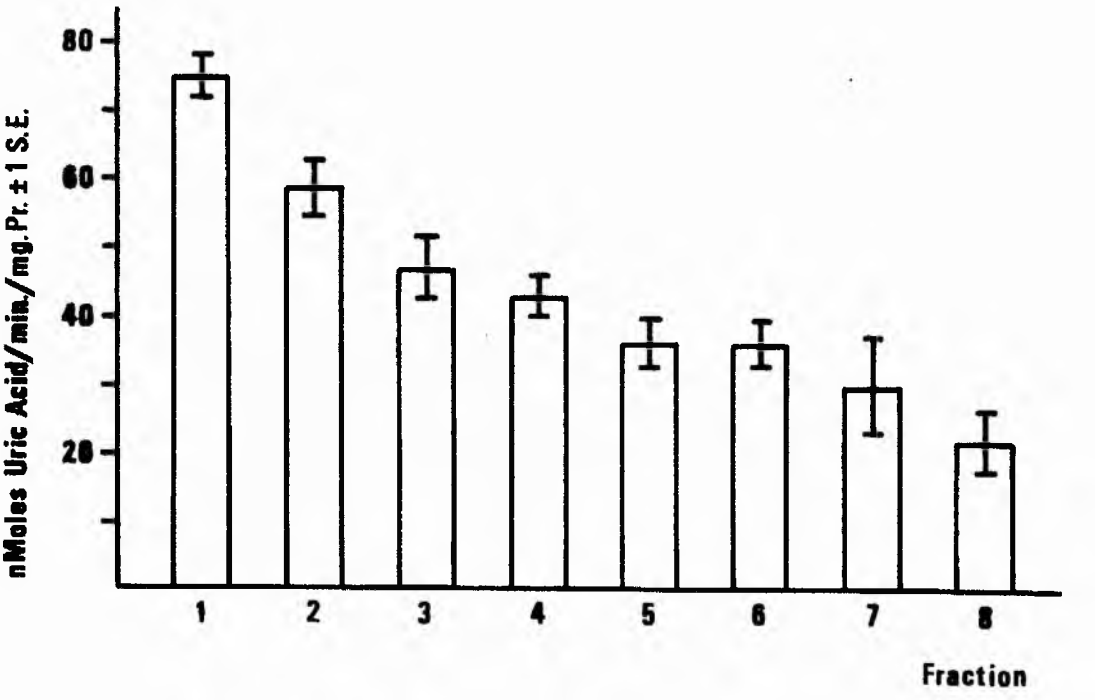

Fig.5.12. The effect of in vivo age on membrane-associated PNP. $p < 0.001$ by Analysis of Variance.

believing that ageing is associated with a decrease in membrane integrity. An alternative explanation would be that the younger ghosts preferentially reseal but as this has already been shown not to occur in the case of AChEase and PGK, this is unlikely.

Fraction	% $\frac{\text{- Surfactant}}{\text{+ Surfactant}}$
1	8.4
2	11
3	19
4	20
5	14
6	20
7	20
8	25

Table 5.3. The effect of *in vivo* ageing on the apparent crypticity of membrane NADH-MR.

Purine Nucleoside Phosphorylase

A highly significant decrease in the specific activity of this enzyme was found (Fig. 5.12). The activity fell fairly rapidly for the first four fractions to about half the maximum activity, levelled off somewhat and then showed another slight decrease in the oldest fractions.

Adenylate Kinase

A very dramatic fall of approximately 80% was found in the first five fractions after which there was no change (Fig.

5.13).

Alkaline Phosphatase

E. Cacciari et al. (52) measured the total cell activity and also the membrane activity and found the latter to be just under 40% of the total. However, as they made no attempt to assess recovery it seemed likely that the membrane might be a much more important site for this enzyme than their results would suggest. This was confirmed in the present study in which no activity could be found in the cytoplasm and so it would appear that virtually all of the red cell alkaline phosphatase is membrane bound. Fig. 5.14 shows that there was a significant decrease in specific activity which was qualitatively similar to that seen for PNP but much more pronounced. That is, the activity fell immediately to approximately 50%, followed by a long plateau and then another rapid decrease in activity in the oldest fractions. The overall decrease in specific activity was approximately 90% and was the largest recorded for all the enzymes studied.

Cytoplasmic Enzyme Studies

Membrane PGK, NADH-MR, PNP and AKase have all been shown to suffer a large decrease in specific activity with *in vivo* age but as they are also present in the cytoplasm these changes might not be a true reflection of the situation in the whole cell. Therefore, the specific activities of all four of these enzymes was measured in the cytoplasm and these results are given in Fig. 5.15. It is apparent that no decrease could

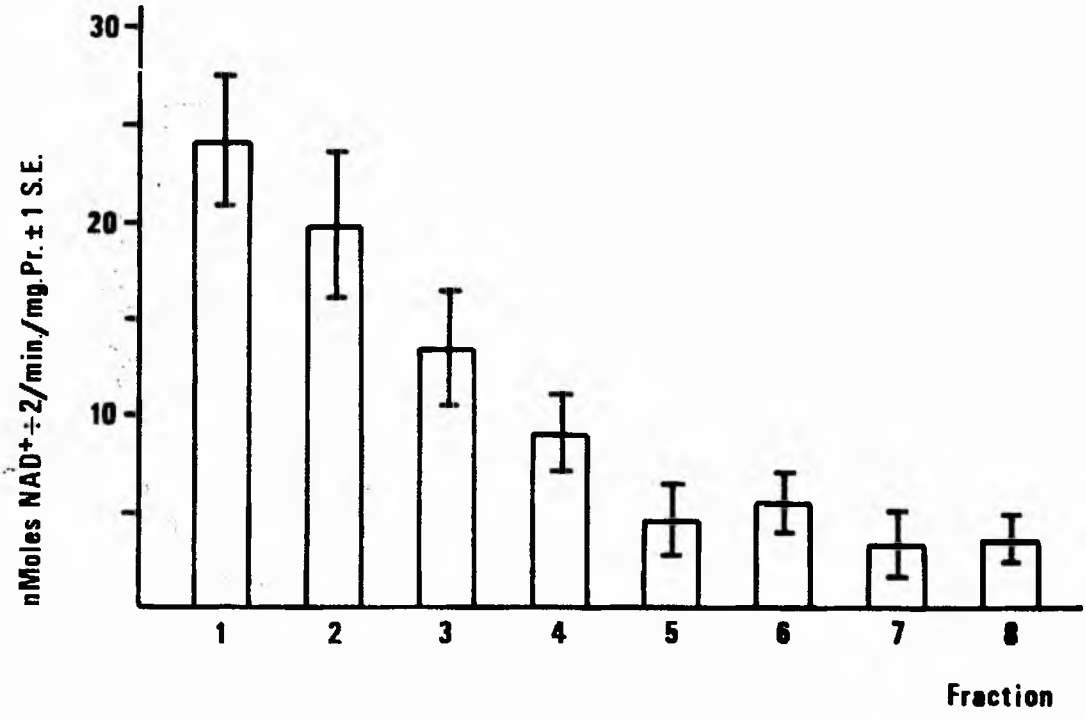

Fig.5.13. The effect of <u>in vivo</u> age on membrane-associated AKase.

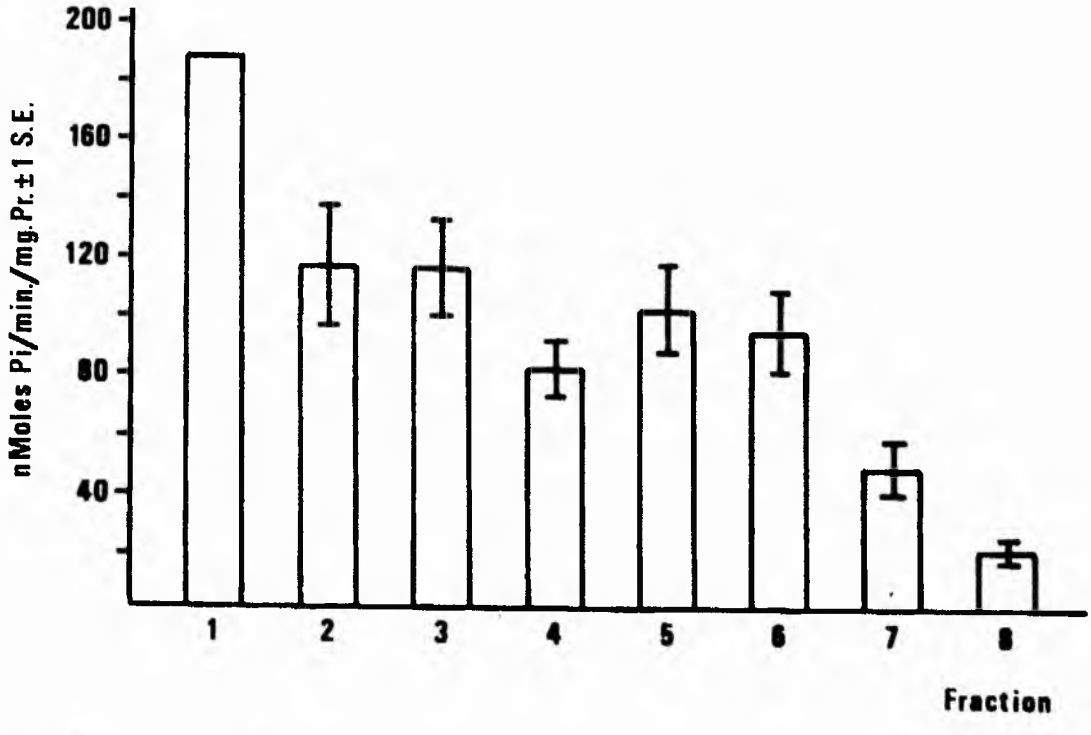

Fig.5.14. The effect of <u>in vivo</u> age on membrane-associated Alkaline Phosphatase.

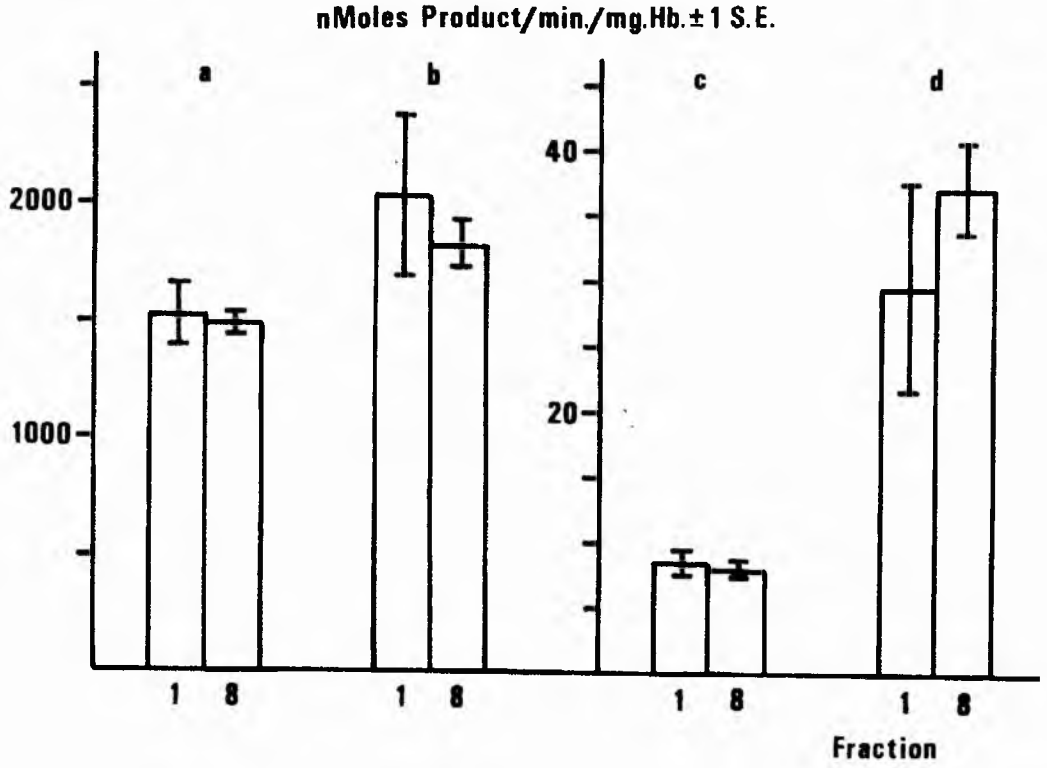

Fig.5.15. The effect of in vivo age on cytoplasmic (a) PGK, (b) PNP, (c) NADH-MR and (d) AKase.

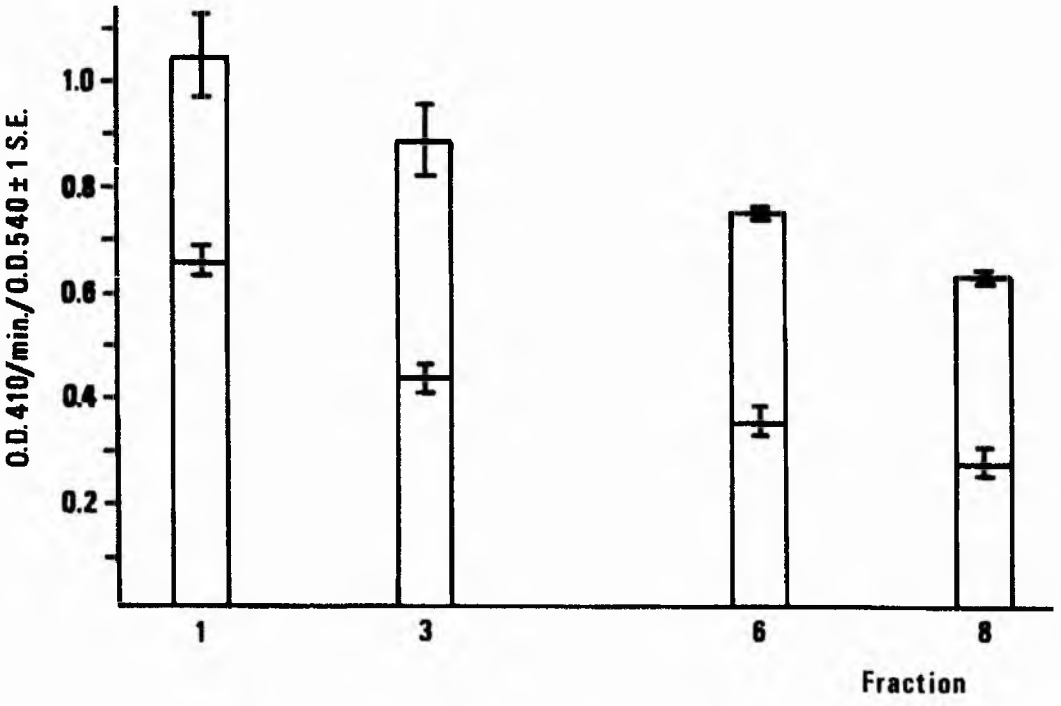

Fig.5.16. The effect of in vivo age on whole cell Alkaline Phosphatase after partial haemolysis (low values) and complete haemolysis (high values).

be detected in the soluble phase and so we are left with two explanations. Either the membrane complements of these enzymes are subject to an ageing loss of activity, whilst their soluble complements are not, or there is an age-dependent loss of these enzymes from the membranes during the washing procedure or even *in situ*. If the latter were the case one would expect an increase in the specific activity of the cytoplasm, which there obviously was not, unless there is a highly disproportionate partition between the two phases. To ascertain whether or not this was the case the % of the total red cell activity of each enzyme present in the membrane was calculated from the information in Figs. 5.5, 5.11, 5.12, 5.13 and 5.15. This is given in Table 5.4 in which it was assumed that the red cell contains 100 times as much haemoglobin protein as stromal protein (53).

	% of Total Activity in Membrane	
Enzyme	Fraction 1	Fraction 8
PGK	.07	.02
NADH-MR	37.5	25.0
PNP	.04	.01
AKase	.8	.1

Table 5.4. The percentage of the total red cell enzyme activity present in the youngest and oldest membranes.

The figures for PGK and Akase are considerably lower than those given in the literature (22, 23, 24, 32) but information on the other two could not be found. It is apparent

that in the case of PGK, PNP and Akase a significant fraction of the membrane enzyme activity could be lost to the cytoplasm during in vivo ageing or at the moment of haemolysis and this would not be detected. Therefore no conclusions can be drawn on the mechanism of the age-dependent membrane loss.

The very high activity of NADH-MR makes it highly unlikely that it has been lost to the cytoplasm and so it is possible to conclude that either the enzyme is preferentially lost from the older membranes during the washing procedure or that this represents a true loss of functional integrity with age. The apparent high degree of crypticity associated with this enzyme suggests a firmness of binding which favours the latter hypothesis.

Whole Cell Study

As alkaline phosphatase could not be detected in the cytoplasm, whole cells had to be used in order to obtain the true picture of the age-dependent behaviour of this enzyme. Only fractions 1, 3, 6 and 8 were used for this study but due to an oversight two sets of values were obtained (Fig. 5.16). Initially, the cells were added to the incubation medium suspended in PBS, it being assumed that lysis would follow. The observation of red cell sedimentation after one experiment indicated otherwise and a calculation revealed the osmotic strength of the assay mixture to be approximately 150 mOsm. This condition gave rise to the lower set of values. As a result of this observation, prior to the incubation, the red cells were added to approximately two volumes of 10 mM Tris/Cl pH 7.4 to ensure lysis. The higher set of values was thus obtained. Both experiments indicated that there was a 50%

decrease in the activity of this enzyme per mg. haemoglobin.

Chapter 1 had shown that mg. of haemoglobin is proportional to cell number and also that there was a 10% loss of membrane surface area with ageing. Alkaline phosphatase having been found to be located exclusively in the membrane, it follows that a 10% decrease in activity per mg. haemoglobin would be expected in the absence of any specific decrease in its activity. Therefore, the real decrease in specific membrane activity is 44%* and as the whole cell was used this must have arisen from a loss of functional integrity of the enzyme molecule itself. The almost twenty fold dilution of the cell contents for the assay rules out the possibility of the build-up of a toxic metabolite.

The results of Fig. 5.14 reveal a 90% decrease in the specific activity expressed per mg. of membrane protein, i.e. the ghosts show a 45% greater decrease in specific activity than the whole cells. The simplest conclusion that can be drawn from this is that the oldest, as a result of a lower structural integrity, are losing enzyme protein to a greater extent than the youngest ghosts in the washing procedure. If we make the very tenuous assumption that no protein is lost from the youngest membranes then ghost preparation causes a 45% loss of enzyme protein from the oldest membranes. It is clear from the two curves in Fig. 5.16 that at least half of the enzyme activity is not available to the exterior surface but unfortunately the somewhat uncontrolled nature of the experiment makes it impossible to be more specific.

* $\frac{50 - 10}{100 - 10}$

Reticulocyte Ghosts

It was stated at the beginning of this section that mitochondrial and reticular contamination were not detectable. We must now consider the reticulocyte ghost itself and decide as in Chapter 1 whether the results obtained could have been due to the decreasing number of reticulocytes present in the erythrocyte fractions of increasing age. Let us imagine that all the erythrocyte ghosts have a constant specific activity and that the reticulocyte ghosts also have a constant but greater specific activity. Then if,

S.A.e = specific activity of erythrocyte ghosts

S.A.r = " " " reticulocyte ghosts

%e = % erythrocytes in each fraction

%r = % reticulocytes in each fraction

% Total specific activity =

$$(\%e \times S.A.e) + (\%r \times S.A.r)$$

Using this equation and the values of %e and %r from Chapter 1 (Table 1.1) one can generate theoretical specific activity curves as shown in Fig. 5.17 in which the numbers in parenthesis represent the S.A.r : S.A.e ratios. In the absence of further information these curves can only be used for a qualitative comparison. For example, it can be seen that in order for fraction 1 to have a specific activity double that of fraction 8 the reticulocyte ghost must have a specific activity 20 times that of the erythrocyte ghost. In order to assess what is a reasonable ratio Table 5.5 was collated from H. Friedmann *et al.* (1) and B.M. Turner *et al.* (54).

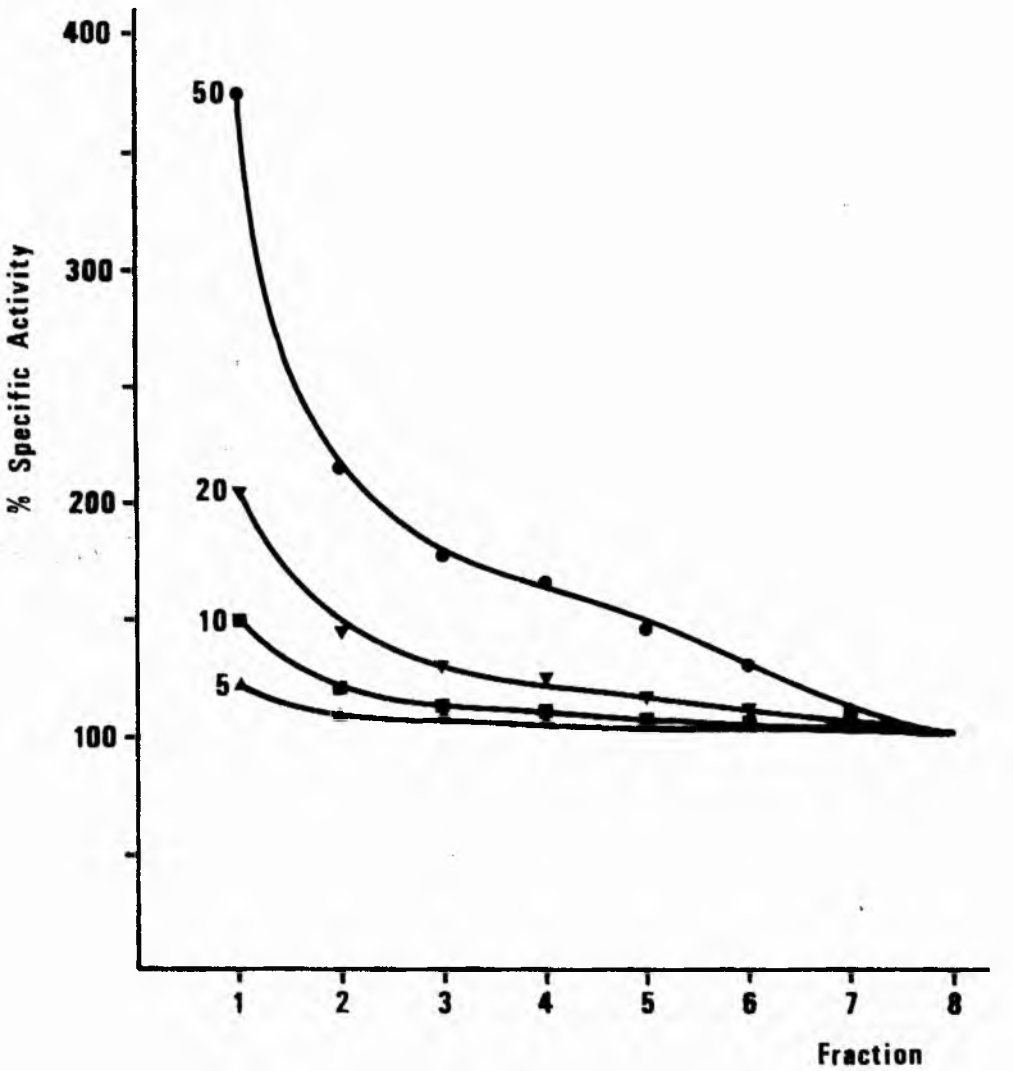

Fig.5.17. Theoretical specific activity curves assuming constant erythrocyte activity (100%). Numbers in parenthesis refer to the S.A.r : S.A.e ratio.

Enzyme	S.A.r : S.A.e	Species
Aldolase	3	Rabbit
AChEase	2	Neonatal Human
Adenosine Deaminase	2	Rabbit
Aminotripeptidase	1	Man
DPG Phosphatase	1.6	Rabbit
Fumarase	1.8	"
G6PD	2.5	Man
GDH	1.5	Rabbit
GOT	5.6	"
GPT	1.4	Rat
Glycylglycine Dipeptidase	3.6	Rabbit
ITPase	6.1	"
ICD	1.4,4.0	Man
Leucine Aminopeptidase	1.2	Rabbit
Lysophospholipase	1.6	Man
NAD Nucleosidase	1.0,2.2	Rabbit
6PGD	7.1	Man
Phosphoglucomutase	1.4	Rabbit
PNP	6.8	Man

Table 5.5. The activity of some reticulocyte enzymes as a function of their corresponding erythrocyte activity (1, 54).

The average ratio calculated from this data is 2.8 although the highest is 7.1 and so for the purposes of the discussion it seems that an upper limit of 10 would be acceptable If this assumption is correct then reticulocyte ghost contamination cannot account for more than a fall of approximately 33% in the specific activity.

Discussion

The specific activities of nine enzymes have been measured in the human erythrocyte plasma membrane with respect to *in vivo* ageing and all but Na, K-ATPase and GAPD exhibited a significant decrease. To conclude that this is an accurate representation of *in vivo* ageing would, as in previous chapters, be premature. The alternative explanations are;

1. Reticulocyte organelle contamination
2. Reticulocyte membrane contamination
3. Cryptic effect
4. Age-dependent loss of enzyme protein from membrane during ghost preparation or *in situ*

Contamination

The inability to detect any SDH and G6Pase activity effectively rules out contamination by reticulocyte organelles thus leaving the reticulocyte plasma membrane. The possibility that the effect observed in this chapter could have been due to the reticulocyte ghosts having a higher specific activity than the erythrocyte ghosts has been examined in some depth (Table 5.5, Fig. 5.16) and the evidence makes this unlikely. Using what data we have available it was calculated that the presence of reticulocyte ghosts could not be expected to account for a decrease of more than 33% in the specific activity of any enzyme. Of the seven activities that were found to decrease only AChEase comes within this limit although a decrease of 50% in NADH-MR activity in the presence of surfactant might be considered just possible. With regard

to NADH-MR, Fig. 5.15 shows absolutely no change in the specific activity of the cytoplasmic compliment of this enzyme. Thus, this argument can be upheld only if one makes the somewhat improbable assertion that the specific activities of the reticulocyte and erythrocyte soluble phases are identical whilst there is a 10-20 fold difference in the specific activities of their respective membranes. As AChEase is not found in the cytoplasm it is quite possible, in the context of the assumptions made here, that the observed decrease in its specific activity was due to reticulocyte plasma membrane contamination.

The presence of reticulocyte ghosts may have been the cause of the decrease in variability associated with the Mg-ATPase and Na, K-ATPase (Table 5.2) but as the reticulocyte is metabolically more active than the erythrocyte (1, 45, 54) one would expect this decrease in variability to be accompanied by a decrease in the mean specific activity. This is certainly the case for the Mg-ATPase but is not so for the Na, K-ATPase. This can be resolved only by proposing that the reticulocyte plasma membrane contains a highly active Mg-ATPase and the specific activity of its Na, K-ATPase is similar to that of the erythrocyte plasma membrane. The variability of the latter would therefore be an inherent property of the younger erythrocyte ghosts. Ignoring the large decrease in the specific activity of the Mg-ATPase which as explained above is too great to be accounted for by reticulocyte contamination, this hypothesis is mere conjecture and cannot be confirmed or otherwise by the available data.

Cryptic Effect

Two forms of cryptic effect have been considered. The first results from the active site of the enzyme being buried within the membrane matrix as suggested by S.L. Schrier et al. (18) and G. Duchon et al. (33) and the second is caused by resealing of the ghosts. Some of the enzymes were investigated in the presence of surfactant which should have drastically reduced the first effect and totally abolished the second. A slight cryptic effect was found in the case of PGK which was not age-dependent but the surfactant unfortunately interfered with the AChEase assay. Once again though, the cryptic effect if present must have been very slight. This procedure cannot differentiate between the two kinds of crypticity but it can be concluded that for these two activities neither was significant.

NADH-MR was much more interesting because a decrease in specific activity was observed only in the presence of surfactant. Thus, not only was there a very large cryptic effect (Table 5.3) but it was also age-dependent. Although the assay conditions were dissimilar, as resealing had been shown to be insignificant for PGK and AChEase the most likely explanation seemed to be that the enzyme is buried within the membrane and that there is an age-dependent loss of membrane integrity. The resulting 'porosity' of the older membranes gives them a spuriously high specific activity with respect to the younger membranes in the absence of membrane disruption.

Protein Loss

It seems, therefore that with the exception of AchEase, unambiguous age-dependent decreases in specific activities have

been recorded and so it remains to be decided whether or not they are a result of an age-dependent ability to withstand the applied *in vitro* manipulations. The activities of PGK, NADH-MR, PNP, AKase and alkaline phosphatase were measured in the cytoplasm of the youngest and oldest fractions, no difference being found for the first four whilst alkaline phosphatase could not be detected. It was then calculated (Table 5.4) that the amounts of PGK, PNP and Akase present in the membrane, expressed as percentages of the total cell content, were small enough to render it impossible to detect massive losses into the cytoplasm and so no conclusions could be drawn. NADH-MR on the other hand, was considered to be present in the membrane in a sufficient quantity to permit such a detection. It is therefore unlikely that this enzyme was lost to the cytoplasm during haemolysis or *in vivo* ageing and this is further corroborated by the conclusion that its active site is deep within the membrane.

Conclusions

Mg-ATPase and AChEase are exclusively membrane enzymes *in situ* and so the decreases in specific activity of these two are probably genuine, although in neither case was percentage recovery investigated. PGK, PNP and AKase exhibited marked decreases but because of the relatively small percentages present in the membrane it has not been possible to decide between an *in vitro* and an *in vivo* mechanism. The evidence as regards NADH-MR is fairly conclusive that the observed decrease is genuine and probably results from an age-dependent denaturation of the enzyme. It is strange, however, that such a large proportion of the cell activity should be located deep within

the membrane when it has an exclusively intracellular function. It is of course possible that as the physiological substrate has not been used, we have been looking at another activity.

Alkaline phosphatase suffered a 90% decrease in specific activity with age, this being the largest observed and it could not be detected in the soluble phase. Measurements on the whole cells revealed that the true decrease in specific activity was half this, the discrepancy arising probably from an age-induced loss of enzyme protein. This is still a large decrease but its importance in terms of a determinant of red cell life span awaits a decision on the role of this enzyme activity.

Only AChEase was examined in any depth and this proved disappointing. There was very little difference in Km or Vmax, and the Arrhenius plots gave very similar changes in activation energy at approximately 33°C. As G. Zimmer et al. (55) showed that extracted erythrocyte membrane lipids undergo a change in their physical state (measured by viscosity) at 18-19°C, the observed change is probably due to the presence of two or more sub-reactions differing in the temperature coefficients of their rate constants.

Until a few months ago no study had been published on the changes in enzyme activity of the red cell membrane with respect to in vivo ageing. W. Tillmann et al. (56) as part of an investigation into membrane enzyme organisation separated red cells by a method similar to that used here and measured the specific activities of 16 enzymes concerned largely with carbohydrate metabolism. Only two enzymes are common to both studies but as their activities were expressed by W. Tillmann et al. as I.U./10^{11} ghosts, absolute comparison is impossible.

It seems pertinent to question the wisdom of counting ghosts by the Coulter method when one might expect some fragmentation. It was claimed that at the lysing osmolarity no particles or fragments could be seen by phase microscopy in unfractionated ghosts, but it is quite probable that the older ghosts were more prone to breaking up giving a falsely low count. This would obviously counteract any measured specific activity change. Also, as mentioned previously this method of expressing the results cannot differentiate between losses of activity by non-selective loss of membrane surface area and specific age-dependent losses of activity. Nevertheless, they found that the activity of GAPD decreased by approximately 30% and that of PGK by approximately 20%. Neither of these values is in agreement with the present study but as no comparison is possible the reason for this is unclear.

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Discussion

All aspects of the work presented in this study have already been adequately discussed and summarised and so it is hoped to avoid unnecessary repetition. Rather, the purpose of this section is to focus attention on those areas which proved most interesting and those which remained unresolved, with a view to outlining possible courses of future action.

Red Cell Separation

The red cells were separated into age groups by high speed centrifugation and were then divided into eight fractions. Therefore, even if a complete age-dependent stratification had been effected the comparison would have been between the youngest 12% and the oldest 12% of the population. However, the distribution of the reticulocytes within the eight fractions confirmed that the stratification had been far from complete. Consequently, it is reasonable to believe that the age-dependent changes found in the haematological parameters of the whole red cell and the protein content and enzyme activities of the membrane do not give a true indication of the magnitude of the changes occurring during the ageing process.

How could this situation be improved? Within the confines of the experimental procedure used, the first suggestion would naturally be to take smaller fractions, e.g. 0.5 ml. instead of 2.0 ml. This would be reasonable for a microscopic study or the measurement of most cytoplasmic enzymes but as explained, 2.0 ml. of packed cells barely

yielded sufficient ghost material for some of the investigations undertaken. Obviously, this modification would be possible if more than one centrifuge tube were used with a small amount being taken from each. This was not done, primarily because the experiments performed here were of an exploratory nature and the amount of material needed would not have made this a worthwhile exercise.

Having localised several areas that would benefit from further research (see below) it now seems eminently more sensible to think in terms of a better separation technique rather than a scaling-up of one that has been shown to be not completely satisfactory. This is most likely to be one that involves isopyonic rather than velocity-dependent separation. That is, the cells must be layered on a suitable gradient and be allowed to reach their true buoyant densities. As explained in the Introduction this is a widely used technique but has two major drawbacks.

1. A good separation is dependent on a large sample volume : gradient volume ratio and so only very small volumes of blood can be dealt with.

2. The cells are by necessity placed in a non-physiological environment and therefore great care has to be exercised in manipulating the pH, tonicity and osmolarity of the medium as well as its specific gravity. Slight changes in these parameters can easily lead to morphological alterations which invariably affect the specific gravity of the red cells. Many media also produce significant agglutination.

Zonal centrifugation offers the best solution to the first problem. These rotors usually hold 1 to 1.5 litres and therefore should easily cope with at least 50 ml. of packed red cells, which is approximately three times the volume used in

this study. As to the gradient material, this will always be a problem but improvements must be possible. Bovine serum albumin cannot be considered because of the cost and most of the other commonly used materials have been found wanting in one respect or another. L.M. Corash et al. (1) recently described the use of the polysaccharide Stractan II which, apart from being relatively inexpensive, was found to be biologically inert, caused minimal agglutination which was abolished by a small amount of bovine serum albumin and had a low viscosity. It is hoped that zonal centrifugation using Stractan II as the gradient medium can be investigated as soon as possible.

Spherical Red Cells

With the evidence available there was no alternative to the conclusion that approximately 25% of the normal red cell population had a volume almost twice that of the rest and that the most likely shape for these was a sphere. This was unfortunately not supported by direct observation and so every effort must be made to test this unpalatable conclusion. This could possibly be done using Nomarski interference optics or scanning electron microscopy but there is the very great problem that glutaraldehyde, the most common fixative, appears to alter the shape and volume of the erythrocytes.

Membrane Proteins

Several changes were noted in the protein composition of the membrane and an attempt was made to follow up one of

these by purifying protein 4.1. A very small amount of what is likely to have been this protein was obtained but its identity was not unequivocally confirmed. A method must be found for preparing adequate quantities of this protein in order to investigate the nature of its binding to the membrane. The binding of this protein from the whole cytoplasm has already been shown to be responsive to temperature and this effect could be investigated more easily with the purified material as well as the possible involvement of ions and metabolic intermediates. This should not only help to further elucidate the ageing process but also throw some light upon the factors that control the locus of a protein within the cell.

Nonionic Surfactant Electrophoresis

Total solubilisation of the membrane proteins is somewhat incompatible with the preservation of functional integrity and yet a great deal of success was achieved in the development of this system. One of the main drawbacks was that a compromise had to be arrived at between the surfactant concentration within the electrophoretic apparatus and the maintainance of the vertically mounted gels in their glass holders. It seemed very likely that if this surfactant concentration could have been increased to that of the solubilising mixture greater resolution would have been obtained. Therefore a possible modification is thin-layer horizontal-gel electrophoresis. In this way the surfactant concentration could be increased without much fear of destroying the glass-gel adhesion. An alternative would be to extend the study to other nonionic surfactants.

Enzymology

This was basically a broad characterisation of some functional aspects of the membrane and many decreases in enzyme activity were observed. More importantly, it was shown in the case of one enzyme and is probably true for others that the preparation of ghosts results in the loss of enzyme protein and that this loss is age-dependent. This study could easily be extended to cover more of the membrane enzymes but it might prove more interesting to look at the kinetics of some of those already characterised.

Conclusion

Have the results obtained in this study in any way succeeded in fulfilling the broad aims that were outlined in the Introduction? Considerable amounts of data have been accumulated with respect to the protein changes with age, both from a structural and functional point of view, but as expected no obvious possibilities for the cause of red cell senescence have become apparent. For the sake of conjecture, one contributing factor may be the binding of cytoplasmic protein to the membrane with a resulting increase in rigidity. Further speculation is needless to say pointless.

As expected, more success was met with in the characterisation of the heterogeneous red cell population. The greatest variation was found in enzyme activity with up to a ten fold difference in the specific activity of the youngest membranes compared to the oldest. It has therefore been adequately demonstrated that not only should allowance be made for this age-dependent variability but that there is also

in some cases an age-dependent susceptibility to the experimental manipulations.

No comparison is possible with studies on the membrane proteins from patients with congenital haemolytic anamias due to the lack of experimental overlap. The closest work is that of E.D. Gomperts et al. (2) who have shown protein differences as a result of hereditary spherocytosis and antibody induced haemolytic anaemia but unfortunately they performed urea-starch gel and polyacrylamide gel electrophoresis of an acetic acid extract. However, their failure to detect any difference when n-butanol was used as the solubilising agent suggests that they may have been observing a differing susceptibility to acetic acid extraction rather than a difference in protein composition. Another possibility is n-butanol induced aggregation which may have masked the protein differences.

References

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2. E.D. Gomperts, J. Metz, S.S. Zail, Brit. J. Haemat. 23: 363-370, 1972.