TO MY PARENTS

CALCIUM TRANSPORT AND PROTEIN PHOSPHORYLATION OF HUMAN RED CELL MEMBRANE WITH REFERENCE TO CONGENITAL HAEMOLYTIC ANAEMIA

Thesis submitted by

SAWSAN ABDUL-HAMID ALI

in candidature for the degree of

Doctor of Philosophy

in the

University of London

1978

ACKNOWLEDGMENTS

I wish to thank Professor Sir J.V. Dacie for allowing me to carry out this study in the Department of Haematology, Royal Postgraduate Medical School, Hammersmith Hospital.

I am deeply endebted to Dr. H.S. Selhi for his supervision and full support throughout this study.

I wish to thank the following:-
Professor J.M. White for his initial joint supervision (1974 - 1975).

Dr. E.C. Gordon-Smith also for his joint supervision (1975 - 1978). I am most grateful for his collaboration in providing samples from his patients in the studies concerning hereditary spherocytosis.

Mr. P. Lister and Mr. S. Sidhu for their continued efforts in teaching me the alectron microscopy techniques.

Mr. A. Cooper for allowing me to use the analytical centrifuge at MSE Crawley.

Mrs. J. Black for typing the first draft.

Miss S. Masterson for her unfailing efforts for finishing the final proof on time.

SYNOPSIS

Ca^{2+} ATPase activity was determined in membranes isolated from human red blood cells. Several types of Ca^{2+} ATPase were found to exist. Due to the lack of availability of a known specific inhibitor of (Ca^{2+} + Mg^{2+}) - ATPase activity (which is part of the Ca^{2+} transport system), the transport of Ca^{2+} into the IO prep was investigated. Initially it was found that the vesicles were unable to retain Ca^{2+} in excess of 2.2 nmoles per mg protein. A possible cause of this instability may be the formation of non-specific channels in the membrane vesicles. Ca^{2+} uptake in IO prep of HS membrane was not investigated due to this instability. However, later studies showed that by use of EGTA buffer systems and the Ca^{2+} ionophore A23187 fairly accurate measurement of Ca^{2+} uptake could be achieved in the IO prep, although this was still not possible using intact cells.

Studies on phosphorylation of normal and H.S. membrane showed lowered level of phosphate bound to the latter, although the results were not significant at $P = 0.05$ level. The Km for ATP for the normal membrane was found to be 24.8 uM and that for H.S. membrane 39.0 uM. No significant difference in Km value for extracted protein kinase of the membrane was found. Heterogeneity of phosphorylation kinetics in H.S. was also found.

Using SDS polyacrylamide gel electrophoresis the H.S. membrane band (I & II), which represents spectrin, showed lowered level

of phosphorylation than normal. In both membrane samples, a distindtiwe cycle of phosphorylation, dephosphorylation and rephosphorylation of band (I & II) was observed.

Membranes derived from circulating RBC of different age showed that the old cells were capable of a higher level of endogenous phosphorylation than the younger cells. Exogenous protein kinase had little effect on the young cell membrane but markedly inhibited the phosphorylation of the old cell membrane.

Iodination studies on isolated spectrin showed that the protein could undergo conformational changes. The ratios of moles of spectrin bound to a mole of phosphate and to a mole of iodine was used to construct a model of superstructure of spectrin in the red blood cell membrane on phosphorylation.

ABBREVIATIONS

All abbreviations of common chemicals are as quoted in instructions to authors in the British Journal of Haematology. Other abbreviations used are as follows:-

BSA	Bovine serum albumin
CPD	Citrate phosphate dextrose anticoagulant
Conc	Concentration
DDSA	Dodecenylsuccinin anhydride
DMP	2,4,6-tri(dimethylaminomethyl) phenol
O.D.	Optical density
P.K.	Protein kinase
S.A.	Specific activity
S.D.	Standard deviation
SDS	Sodium dodecyl sulphate
Std	Standard

MATERIALS

All common laboratory chemicals were from BDH, Poole, England and were of analytical grade.

All fine chemicals and all enzymes used were obtained from Sigma Company, England.

The following radioactive compounds were obtained from the Radiochemical Centre, Amersham, England.

$[^{14}C]$ - Inulin (Hydroxy Methyl) S.A. = 5 to 15 mCi/mmole

$[^{45}Ca]$ - $CaCl_2$ 5 A approx. 10 to 40 mCi/mg Ca

$[^{125}I]$ - KI S.A. = 100 mCi/ml

$[^{22}Na]$ - NaCl S.A. approx. 100 mCi/mg Na

$[^{32}P]$ - Carrier free inorganic phosphate in dilute HCl

S.A. = Approximately 33 Ci/mg

$[\gamma - ^{32}P]$ - ATP S.A. approx. 2 to 3 Ci/mmole

Dioxane cocktail: 40gm butyl-PBO, 400gm naphalene added to 1L ethanol and made upto 5L with dioxane. This was filtered and kept in dark glass bottles at 4 °C.

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GENERAL CONCLUSION AND DISCUSSION

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SECTION 1

BIOLOGICAL MEMBRANES: GENERAL PROPERTIES

1.1 BIOLOGICAL MEMBRANE COMPONENTS

Membranes are composed mainly of lipids and proteins. It is the interaction of these major components with each other which gives the membrane its functional characteristics. The conformation of the proteins in the lipid bilayer also determine the shape of the membrane.

1.1.1 The lipids

Several kinds of membrane lipids are found. The phospholipids are predominantly present with small proportions of sterols (e.g. cholesterol) in mammalian cell membranes. The ratios of different types of phospholipids as well as cholesterol differ from one cell type to the other. They also determine the functional properties of the cell membrane. The phospholipids are rodlike molecules, with bimodal or amphipathic characteristics, i.e. they contain hydrophilic groups and hydrophobic fatty acid side chains. In an aqueous environment they behave in such a manner as to reach a stable configuration. Thermodynamically this could be achieved by placing the charged hydrophilic groups facing the aqueous environment so that interaction of these molecules with water leads to minimum free energy.

If the hydrophobic tail groups of the lipids were in contact with the aqueous environment the water molecules will try to arrange themselves in a way to minimize the interaction with these groups. This will lead to decreased entropy.

Further studies suggested that the only possible organization of the lipid molecules in an aqueous environment which satisfies these energetic requirements is the bilayer organization (Gorter and Grendle, 1925) - that is the lipid polar heads face the aqueous environment on both sides of the bilayer while the hydrophobic non-polar tails will be oriented to the interior of the bilayer to minimize their interaction with water. Cholesterol was found to modify the fluidity of the phospholipid side chain and restrict their mobility (Chapman and Penkett, 1966). The effect of cholesterol on the fluidity of the lipid bilayer is important in determining the lipid-protein interaction and certain enzymic and transport activities associated with the membranes.

1.1.2 Proteins

Initially, knowledge about the structure and arrangement of proteins in the lipid bilayer were not available due mainly to the difficulty in techniques concerning the isolation of these proteins. Recently some membrane proteins have been successfully isolated without any severe or irreversible denaturation during the isolation procedures and some direct information about the conformation of the protein molecule has been established. The association of the proteins with the lipids in the membranes is achieved by the hydrophobic and hydrophilic interactions. The hydrophilic groups of the proteins should be exposed to an ionic or aqueous environment (e.g. the ionic groups of the proteins and glycolipids). The hydrophobic groups (e.g. the non polar amino acids) are internalized or directed away from the aqueous exterior towards the interior of the lipid bilayer where association with the lipids is through hydrophobic interactions.

The proteins in the membranes fall into two categories (Singer & Nicolson, 1972). The first represents those proteins which are bimodal, i.e. are associated with the lipid bilayer through hydrophobic and hydrophylic interaction; these proteins are called intrinsic or integral proteins which penetrate the bilayer and may span it. This type of protein is tightly bound to the membrane by hydrophobic interactions.

The other category is the peripheral protein; these proteins are located on either surfaces of the lipid bilayer. They are not bimodal and they do not diffuse into the lipid bilayer. These proteins could be released from the membrane by high salt concentration or metal chelating agents; they are water soluble and usually free from lipids when isolated. Further details concerning the association of the integral, and peripheral proteins are discussed in the preceding section.

1.2 MEMBRANE MODELS

The specific interaction of membrane components and their structural arrangements are essential factors in understanding the functional properties of the membranes.

The different proportions of lipids and proteins in membranes from cells of different types raises the question of whether a general model would explain the distinctive features of all cell membranes. The answer lies in the fact that there is some similarity in the relative orientation of lipids and proteins in the membranes, although a single model cannot explain the properties of all membranes. The following membrane models examine the common features of the cell membrane.

1.2.1 Gorter and Grendel (1925)

based their model on the fact that the lipids are an important feature of membrane structure. They extracted the lipids of the red cell membrane by acetone and reported that it formed a monolayer in air-water interface.

The calculated surface area occupied by this film was found to be twice that of the original membrane. Therefore they concluded that the lipids in cell membrane are arranged as a bilayer with the hydrophobic chains oriented towards the interior. Their conclusions were further scrutinised by Bar et al (1966) who found that there were some technical errors in the approach to the measurements of the surface area of the film. When acetone was used to extract the lipids, nearly 70 - 80% of the lipids were solubilised from the membrane. This was compensated by their underestimation of the surface area.

1.2.2 Davson and Danielli (1935) proposed another model for the cell membrane. The arrangement of protein in the lipid layer was discussed for the first time. It was proposed that proteins in their globular conformation are attached to the polar head groups of the lipids on each side of the bilayer, a conclusion which has since been shown to be a considerable oversimplification.

Meanwhile, advances in the electonmicroscopy concerning suitable conditions of staining and fixation techniques revealed further information on the ultrastructural features of the membranes. This led Robertson in 1959 to propose a model known as the unit membrane hypothesis and was a modification of the Davson & Danielli model.

1.2.3 Davson-Danielli-Robertson model

This model was further supported by the x-ray diffraction studies. Although most of Robertson's observations were on the plasma membranes of the myelin sheaths of the Schwann cell (which contain very high proportions of membrane lipids compared to other cells), the unit membrane hypothesis was considered as a general basis for the membrane structure. Robertson observed

that the membrane is a three-layered structure consisting of 2 electronedense layers of 20 Å width each and a third 35 Å thick light zone between the two layers - making a unit of 75Å in thickness (see Fig. 1.1). These measurements were theoretically sufficient to accommodate the lipid bilayer. However, according to this model the lipid bilayer is in continuous arrangement in the plane of the membrane. Extensive studies on the membrane structure has shown a discontinuous arrangement of the lipids in membranes with higher proportions of proteins than the myeline, (Bar et al, 1966; Bangham & Horne, 1964). The amount of lipids in membranes of these cells (such as the red cell) was found to be insufficient to cover the whole cell in the bilayer configuration, assuming that the lipids are in compact structure. Further studies by Bangham & Horne (1964) and Lucy & Glauret (1964) have developed the possibility of globular arrangements of phospholipids in the membrane. Based on x-ray diffraction studies, the unit membrane concept would indicate that there is not much room for the proteins to be present in the globular configuration as in Davson-Danielli model. This suggested that the proteins are extended in beta pleated sheet configurations, with ionic and polar side chains interacting with polar heads of the lipids from one side, and the aqueous environment from the other side. However, this will not allow the interaction of the polar heads of the lipids with the aqueous environment.

Furthermore, the protein lipid interactions will take place in a non-polar environment, excluding completely hydrophobic interaction of proteins with lipide. It would also expose the non-polar residues of proteins to the aqueous environment resulting in a very high free energy of the system and would be thermodynamically unfavourable. Studies by Maddy and Malcolm (1965;1966)

Lenard & Singer (1966) on isolated protein from RBC ghosts have shown that the protein appeared to be globular or in a helical configuration.

1.2.4 Singer & Lenard (1966) Model

This model is based on the lipid as a bilayer as in the model of Daveon-Danielli, but the arrangement of proteins in this model is different. As a result of the optical rotary dispersion and circular dichroism measurement of the red cell membranes, Singer & Lenard have shown that the proteins are in globular structure, part of which exhibit alpha helix configuration. It was found that this type of protein is related to the structural proteins described by Green and co-workers (Richardson et al, 1963) as a predominant protein in most types of cell membranes and exhibiting the same conformation. Therefore this model proposed that the structural protein have a unique structure in that the ionic and polar side chains are exposed to the external surface of the membrane which may interact with polar heads of the lipids or the aqueous environment through Van der Waals interactions, while the non polar side chains are located in the interior of the membrane where they interact hydrophobically with the non polar hydrocarbon chain (tails) of the lipids. This model was further extended by Singer and Nicolson in 1972.

1.2.5 The Benson Model

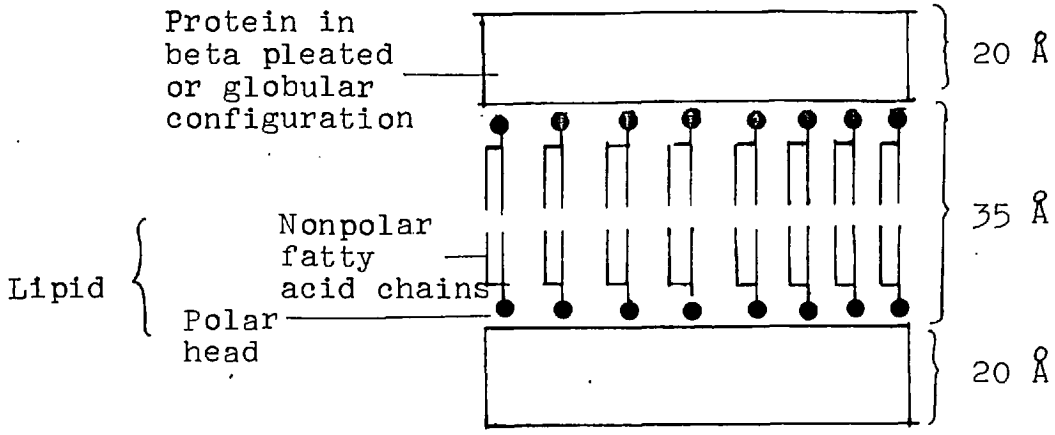
This model was described by Benson & Singer (1965) and later by Benson (1966). According to this model (see Fig. 1.2) the lipids are not arranged in a bilayer type structure but are involved with the non polar side chains of the globular proteins in hydrophobic interactions, while the polar heads are exposed to the aqueous environment at the exterior surface. This model is found to be thermodynamically satisfactory compared to the Davson-Danielli-Robertson model because it allows the hydrophobic

interactions between the lipids and the proteins which the previous model did not. In this model the membrane appeared to be composed of complexes of lipoprotein represented as subunits held together by hydrophobic interactions in the plane of the membrane. However, the interaction of lipid with protein does not leave any opportunity for protein interchain hydrogen bonding in the membrane interior, which leads to the stability of the protein molecule by providing the lowest free energy possible.

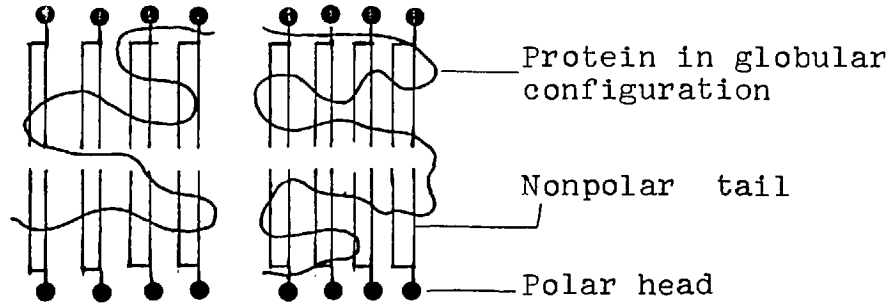
1.2.6 The Fluid Mosaic Model

In 1972 Singer and Nicolson proposed the fluid mosaic concept of the membrane emphasising the role of peripheral proteins and their interaction with the lipids. In this model the lipids are arranged in a bilayer structure with the polar head groups exposed to the exterior aqueous environment and the non polar hydrophobic chains (tails) orientated towards the interior (see Fig. 1.3).

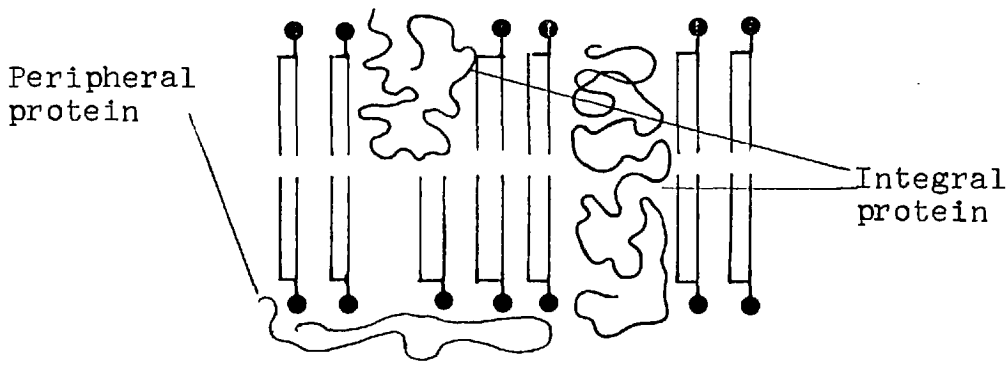
The integral proteins exhibit hydrophobic and hydrophilic characteristics. Studies on these proteins suggested that a considerable part of these globular proteins are present in alpha helix configurations (Lenard & Singer, 1966 and Wallach & Zelhar, 1966). The polar or ionic residues of such proteins are exposed to the exterior of the membrane in contact with aqueous environment, while the non polar parts are hidden in the interior of the membrane and interact with the non polar hydrocarbon chains of the lipids through hydrophobic interaction. The hydrophobic and the hydrophilic interaction of the bimodal molecule of the integral protein with the molecular environment provide a minimum free energy state and therefore it is thermodynamically favourable. These globular molecules or aggregates of one protein subunit of the integral protein may be present in a size that they may



The Davson-Danielli-Robertson model



The Benson model



The fluid-mosaic model.

Fig 1.1 Diagramatic representation of membrane models

traverse the entire thickness of the lipid bilayer. In this case the polar and ionic residues will be exposed to the aqueous environment from both sides of the membrane. However, some of the integral protein molecules do not have an adequate size to span the entire membrane. The ionic residues will therefore be exposed to only one side of the membrane. Some protein-protein interaction could also occur either with groups of peripheral protein with exterior groups of the integral protein, or two or more molecules of the same integral protein may be associated to form a subunit. The existence of integral proteins was supported by freeze fracture electron microscopy, a technique described by Steere (1957). In this technique a fresh sample is frozen under vacuum and a microknife is passed through the hydrophobic region of the membrane producing a fractured face; this is then metal shadowed, replicated and examined under the electron microscope. The water could be removed (sublimed or etched) from the fractured surface before shadowing.

This technique was widely used to reveal several types of membrane particles (Pinto da Silva and Branton, 1970). Tillack & Marchesi (1970) observed some tiny particles on the fractured membrane face which appeared to be the same size as was suggested for the integral proteins. On the other hand, such observation on the myline plasma membrane did not reveal these particles and the fractured surface was almost smooth. This may be explained by the fact that nearly 80% of the myline plasma membrane is composed of lipid and very little protein is present.

2 MOLECULAR ORGANIZATION OF THE MEMBRANE

2.1 Lipids

As mentioned, the bilayer arrangement of the lipids in the membrane was early described (Gortor & Grendel, 1925) and formed the basis for construction of other models that followed (Benson, 1966; Green & Pardue, 1966). However, these models did not take into consideration the lipid-lipid as an important stabilizing force holding the membrane components together. This is due to the fact that in construction of these models evidences concerning the lipids bilayer as a major organizational configuration in all membrane is well established. These evidences were based on the thermodynamic studies on microorganisms (Stein et al, 1969) and were confirmed by x-ray differaction measurements of the membranes compared with that of the synthesised lipid bilayers (Levine & Wilkins, 1971; Wilkins et al, 1971). Spin label probes of the membrane lipids were used to investigate the orientation and motion of the lipids molecules compared with the orientation of the same label in the synthetic bilayer (Hubbell & McConnell, 1969). Such studies on the synthetic bilayers revealed many facts about the fluidity of the bilayer configuration of the lipids in the membrane. The spin label studies (Hubbell McConnell, 1971; Jost et al, 1971), confirmed by NMR studies (Lee et al, 1972; Godici & Landsberger, 1974), showed difference in the fluidity of the lipids in different regions of the bilayer. The fluidity decreases towards the glycerol moiety. The aitroxide groups of the label was found to be in a highly rigid environment when it was attached to a carbon atom on the fatty acid chain close to the glycerol group and in much more fluid environment when attached to a carbon atom in the fatty acid chain further away

from the glycerol group. This means that the lipid bilayer is characterised by rigid regions in the exterior with an increase in the fluidity towards the middle of the bilayer. The fluidity of the bilayer depends on the degree of the saturation of the fatty acids forming the tail of the phospholipid molecule in the interior of the bilayers (Rottem et al, 1970); Levine et al, 1972; Godici & Landesberger, 1974). The presence of cholesterol and of different temperatures (whether it is above or below the gel to liquid-crystalline transition of the lipid) has also great effect on the fluidity of the bilayer (Chapman, 1973).

The cholesterol affects the fluidity of the bilayer by abolishing the gel-liquid crystalline thermal transition (Chapman, 1973). Above the transition temperature, the cholesterol increases the rigidity of the bilayer especially in the middle of the fatty acid chain (Godici & Landsberger, 1975; McConnell & McFarland, 1972), while below the transition temperature the cholesterol increases the fluidity of the bilayer (Oldfield & Chapman, 1972). The mechanism of this effect is not fully understood. It is suggested that cholesterol forms complex with the phospholipid molecule in a phospholipid: cholesterol ratio of 1 : 1 (Chapman, 1973) or 2 : 1 (Darke et al, 1972; Godici & Landesberger, 1975). However, a second motion above the glycerol moiety was found in the choline residue of the phosphotidyl choline and this motion increase in carbon atoms further away from the glycerol moiety as demonstrated by 13C - NMR analysis (Sheetz & Chan, 1972; Levin et al, 1972). The motion of the phospholipid molecule above and below the glycerol moiety suggested that the glycerol acts as a rigid structure providing stability for the molecule. It is not known whether the rigidity of the

glycerol arises from interaction of the glycerol groups or the effect of the hydrophobic interaction above and below the glycerol. Another type of movement of the phospholipid molecule was described by Kornberg & McConnell (1971a)as a lateral movement parallel to the plane of the membrane. This was indicated by the broadening of the spin label probe to the bilayer. This movement was so rapid that it occurred within a fraction of a minute (Scandella et al, 1972). It has been demonstrated that there is a wide range of temperature over which two lipid phases, a solid-like and liquid-like, coexist (Shimshick & McConnell, 1973). The physiological temperature for growth of microorganism fall within this region (Linden et al, 1973) suggesting that this coexistence occurs in the biological membrane as well. This will increase the chances of lateral diffusion of the lipid molecule which allow insertion of newly synthesised material. However, the lateral and intermolecular motion of the phospholipid in the membrane are correlated and form the molecular basis for fluidity of certain membranes (Devaux & McConnell, 1972).

2.2 Protein

The protein comprises about half to two thirds of the total weight of biological membrane. Most of these membranes are insoluble in aqueous environment making their extraction from the membrane rather impossible. These difficulties hamper most investigators. However, the development of suitable techniques such as gel electrophoresis employing anionic reagent SDS to disperse these proteins from their membranes provided a major advance in the knowledge of these proteins. These proteins were classified according to their association with the membrane (Singer, 1971) into two categories. These two types are discussed below.

2.2.1 Integral Proteins

Most of the membrane proteins are integral in that they are associated with the lipids in the membrane. Some membrane enzymes require lipids to express their full activity (Coleman, 1973). Certain antigens, e.g. the Rh antigens of the red cell also require lipids (Green, 1968). The interaction of the integral protein with the lipids in the membrane may provide the structural matrix of the membrane. The integral protsins were proposed to exhibit a globular configuration with amphipathic characteristics - that is one end of the protsin is hydrophobic bearing the ionic and the highly polar residues of the protein, whereas the other end is hydrophobic, embedded in the lipid interior (Wallach & Zather, 1966). The integral protein may possibly span the width of the bilayer with two hydrophilic ends emerging on both sides of the membrane and a hydrophylic region embedded in the lipid interior (Lenard & Singer, 1966). The possibility that the protein could be completely embedded in the lipid bilayer is not favoured since such proteins should contain very few ionic residues and the free energy required to embed them may be several hundreds kilocalories (Singer, 1971).

2.2.2 Peripheral Proteins

These proteins are considered to be loosely attached to the membrane; in fact they are easily extracted from the membrane merely by lowering the ionic strength. Examples of these proteins are cytochrome C located in the outer side of the inner surface of the mitochondrial membrane (Ernster & Kuylenstierna, 1970) and the red cell membrane protein, spectrin, which is localised also in the inner surface of the membrane (Marchesi & Steers, 1968). The peripheral proteins are not generally associated with lipids when extracted. They are soluble in aqueous solutions and are

considered to be in globular configuration when attached in the membrane in order to maximize their interactions (Gulik-Krzywicki et al, 1969).

2.3 Carbohydrates

The carbohydrates are present in the membranes in association with lipids or protein forming the glycolipids or glycoproteins respectively. The glycoprotein have shown to be exclusively localised at the outer surface of the membrane (Marchesi et al, 1972; Nicolson & Singer, 1971), suggesting a highly asymmetric distribution of proteins and lipids in the cell membrane.

2.4 The lipid-protein interaction

In the bilayer configuration of amphipathic lipid molecules, the hydrophilic interaction of the polar groups are maximized by sequestering the hydrophobic groups away from the aqueous environment. This provides decreased free energy and increased entropy of the system (Tanford, 1972; Singer, 1971). The hydrophobic and hydrophilic interactions are the stabilizing forces for the protein in the membranes.

The peripheral proteins which are attached to the surface only, require hydrophilic interaction for their stability. Therefore, they should assume a configuration in which most of their ionic residues are on the outside while the non polar residues are sequestered away from the aqueous environment.

In the integral protein, the exposure of the residues in certain parts of the molecule depends upon the region of the membrane that particular part is in; i.e. the polar or ionic residues will be located in the parts of the molecule located on either side of the membrane, whilst the nonionic or non polar groups are located in the part of the molecule which is carried in the lipid interior.

The physical and thermal status of the lipid bilayer were shown to be important in determining the functional activities of certain membrane enzymes as it was found that the transition temperature of the enzyme is altered with the change in the lipid composition, (Machtiger & Fox, 1973).

The proteins can also alter the properties of the lipids as it was shown that the vesicles prepared from lipids extracted from the membranes are more fluid and have lower transition temperature (Stein, 1972; Seelig & Hasselbach, 1971).

2.5 The Red Cell Membrane

The membranes obtained from a red cell has a mass of $11 - 12 \times 10^{-13}$gm (Dodge et al, 1963) and a density of 1.15 gm/ml (Zalher & Weiber, 1970). The surface area of $140\mu m^2$ (Weinstein, 1974) and calculated thickness of $75^{\circ}A$ agree with the value obtained from electron microscopy data (Robertson, 1972). The membranes are prepared by osmotic haemolysis of the cell in a mildly alkaline pH buffer. The most widely used procedure is that of hypotonic haemolysis due to its rapidity in releasing the haemoglobin (Seeman, 1967). The membrane obtained by this procedure recovered the original activities of most enzymes (Dodge et al, 1963). Haemolysis in isotonic buffer can be achieved although the validity of the preparation depends on the haemolysing buffer used (Hanahan et al, 1973).

2.5.1 Analysis of the ghost proteins

One of the most suitable techniques for analysing membrane proteins is gel electrophoresis in the presence of SDS developed by Shapiro et al,(1967). This detergent, when used with reducing agent, attacks most of the hydrophobic bonds in the protein leading to unfolding of the polypeptide chain (Reynolds & Tanford, 1970) with gross negative surface charge due to detergent binding. The classification and nomenclature of the proteins in the red cell

membrane have been described by Fairbanks et al (1971). According to this classification eight major polypeptides are stained by coomasie blue and the PAS reagents, although some minor polypeptides which contribute to important membrane function (such as the $Na^{+}+K^{+}$ ATPase) are also present.

The PAS staining glycoprotein can be distinguished as four bands: PAS I, II, III & IV. These are the sialoglycoprotein carrying approximately 90 - 95% sialic acid content of the membrane. More glycopeptides have been detected by enzymic labelling techniques, which could not be revealed by the above procedure (Steck & Dawson, 1974).

However, the proteins of the erythrocyte membrane can be classified as intrinsic or integral and extrinsic or peripheral proteins according to the classification of Singer & Nicolson (1972). Another way of classifying these proteins is according to their spatial arrangements and location in the lipid bilayer. This was carried out by using radioactive labelling techniques of the intact red cell with probes or enzyme molecules of a size large enough not to penetrate the cell membrane (Berg, 1969; Bretscher, 1971). According to this procedure only the two glycoproteins were found to be attached to the outer surface of the membrane. However, only two major polypeptides will be discussed in detail (since these are fairly well established) with brief discussion of the rest.

2.5.1.1 Band III

This band has been isolated and characterized by several workers (Tanner & Boxer, 1972; Findlay, 1974; Jenkins & Tanner, 1975; Furthmayr et al, 1976). It contains carbohydrate (5 - 8% of its dry weight) in the form of mannose, galactose and N-acetylglucosamine. The apparent molecular weight of this band is 90,000 dalton on SDS gel electrophoresis. Enzymic labelling

techniques have shown that part of this band is labelled in the intact cells indicating its exposure to the outer surface of the membrane (Bretscher, 1971a). Other parts of this band were shown to be labelled in sealed inside-out vesicles (Mueller & Morrison, 1974; Shine & Caraway, 1974) indicating that this part is exposed to the interior surface. Therefore, this molecule seems to span the entire width of the membrane protruding from both sides. Treatment of this protein by pronase showed that it can form two fragments - only the smaller fragments contain all the carbohydrates (Cabantchik & Rothestein, 1974). However, this band was shown to bind concavaline A and its conjugates, providing convenient tool for localisation of the binding sites of this protein on the external surface (Pinto da Silva and Nicolson, 1974).

Evidence has accumulated concerning the participation of this band in membrane transport activities, such as sugar and anion transport (Lin & Spudick, 1974).

2.5.1.2 Sialoglycoproteins

As mentioned above, the PAS stained protein is separated into four polypeptides. These are present in oligomeric form in the cell membrane and are thought to have molecular weight of 83,000, 45,000 and 25,000 dalton for bands 1,2 and 3 respectively (Fairbanks et al, 1971). Separation and purification of the sialoglycopeptides using different solubilizing agents have been developed (Kalhan et al, 1961; Hamaguchi and Cleve, 1972). Glycophorin A, the basic subunit of the PAS-I dimer constitutes nearly 75% of the total sialoglycopeptide of the red cell membrane, and the oligosaccharide comprises nearly 60% of its weight (Furthmayr et al, 1975). All the oligosaccharide residues are bound to the N-terminal part of the polypeptide and all of these (except one) are bound to serine or threonin

residues through O-glycosidic bond (Thomas & Winzler, 1969; Springer & Desai, 1974). The MN-antigenic activity was found to be located at the oligosaccharide (Springer & Desai, 1974). The C-terminal end of the polypeptide contains most of the acidic amino acids, making this end very polar and suitable for hydrophilic interaction and cation binding (Tomita & Marchesi, 1976). The middle part of the polypeptide between the N-terminal and the C-terminal ends have been shown earlier to be hydrophobic containing the non-polar amino acids and therefore are insoluble in the aqueous solutions (Winzler, 1969).

The arrangement of the glycophorin A as an integral protein spanning the lipid bilayer with the N-terminal end at the exterior, and the C-terminal end at the interior, has been shown by the enzymic labelling technique (Bretscher, 1971b).

Further studies suggested the association of the glycophorin A with band 3 to form globular structures in the interior of the lipid bilayer. These structures appear as particles when observed by freeze-etch electron microscopy on the etched surface, (Pinto da Silva & Branton 1970). Also the ferritin - conjugated lectins were found to bind to sites on the external surface corresponding to positions of the intra-membraneous particles. This data suggested that the glycophorin A binds the lectin conjugated at other external surface while extending to the internal surface in association with other integral protein to form the intra-membraneous particles (Pinto da Silva and Nicolson, 1974; Tillack et al, 1972). The C-terminal end of the glycophorin A was shown to be in close association with spectrin at the internal surface of the membrane (Nicolson & Painter, 1973). This will be discussed further in the following section.

Other minor glycopeptides, PAS 3, has also been isolated and characterized. They have carbohydrates composition similar to glycophorin A, although the C-terminal end was observed at the outer surface, (Furthmayr et al, 1975). This suggests that the glycoprotein is extending to the outer surface and penetrated partially into the lipid bilayer but not spanning it. Some blood groups activities other than the MN activity, e.g. the AB groups, were found on this glycopeptide (Anstee and Tanner, 1974) although evidence is presented that this is an artifract due to contamination (Greensed & Goldstein, 1974).

The other glycopeptide PAS IV has not been identified as yet and it appears in the region between the PAS 1 and II in SDS polyacrylamide gel electrophoresis (Fairbanks et al, 1971). Other minor unidentified glycopeptides which could not be detected on PAS staining for their low content of carbohydrates has been described (Weiss et al, 1971).

Band VII is among the glycoproteins which could not have been characterized fully due to its poor resolution by gel electrophoresis. Enzymic labelling techniques have shown that this band is integrated fully in the lipid interior although evidence is not yet available.

The enzyme acetylcholine esterase is thought to be located in the outer surface while the $(Na^{+}+K^{+})$-ATPase is on the inner surface as suggested by the proteolytic digestion studies of the intact red cells (Martin, 1970). Other studies however, have suggested that part of the ATPase should be exposed to the external surface in order to react with its inhibitor ouabain (Haley & Hoffman, 1974).

The peripheral or extrinsic proteins of the erythrocyte membrane are located on the inside surface as suggested by the proteolytic digestion studies (Bender et al, 1971) and enzymatic labelling techniques using impermeable probes (Phillips and Morrison, 1970) to the intact red cell. It was concluded from these studies that the lipid bilayer protects these proteins from the action of such enzymes, although it has been argued recently that these proteins could not be labelled due to their negative reactivities with the labels used and not because of the protective effect of the lipid bilayer (Wallach et al, 1974) This was supported by the finding that some of these impermeable reagents can penetrate the membrane due to their ability to alter the conformational state of the membrane during their reaction (Cuberntchik et al, 1975). The extrinsic proteins form 40% of the membrane protein and comprises bands I, II, IV and VI, (Fairbanks et al, 1971). They have been studied extensively due to the ease of release by low ionic strength and elevated pH. (Furthmayr & Timple, 1970).

2.5.1.3 Spectrin

This protein comprises two large molecular weight polypeptides (over 200,000 daltons) designated as bands I & II in SDS gel electrophoresis, (Fairbanks et al, 1971). It is an extrinsic protein, located on the inner surface of the membrane, as has been shown by ferritin - conjugated antibodies binding studies (Nicolson & Marchesi, 1971). The observation that an actin-like filament produced after tryptic digestion of the red cell membrane (Marchesi & Palad , 1967) has led these authors to believe that it is a contractile protein. A procedure for its extractions by chelating agents in the presence of ATP and

reducing agents similar to those used for extraction of muscle proteins have been developed (Marchesi & Steers, 1968).

However, the fibrillar appearance of this protein was thought to be due to actin or actin-like protein which comprises band V on SDS gel electrophoresis (Marchesi & Palade, 1967). The great susceptibility of spectrin to proteolytic digestion by contaminating leucocytes or by the presence of endogenous pronase in the red cell (Berank & Boemann, 1972), hampered most investigators from further studies. Another argument has been suggested - the possibility that spectrin exists as an aggregate of smaller polypeptides which could aggregate to higher molecular weight polypeptides even in the presence of SDS (Dunn & Maddy, 1973). This was further confirmed by the finding that dilute hydrochloric acid can dissociate the spectrin polypeptides into smaller subunits (Schiechl, 1973) although these conditions are considered to be optimal for proteolytic digestion for susceptible protein like spectrin (Halla, 1974). Analysis of this protein has revealed the existence of multiple N-terminal amino acids (Dunn et al, 1975; Fuller et al, 1974; Knuferman et al, 1973). This suggested that either the spectrin is composed of multiple polypeptide chains with similar molecular size or it is an aggregate of smaller polypeptides. However, it is argued that the presence of multiple N-terminal amino acids is an artifact due to the presence of other polypeptides which migrate in the same region.

The tendency of the spectrin polypeptide to aggregate in aqueous solutions, particularly in the presence of cation, is one of the major difficulties facing the workers in this field to assess its structure. Immunoelectrophoretic studies revealed five separate bands (Bhakdi et al, 1974). These bands can be cross-linked immunologically with each other suggesting the

idea that they belong to the eame polypeptide chaine but are present in different stages of aggregation (Green et al, 1974; Bhakdi et al, 1975).

The aesociation betwaen spectrin and the integral proteins has been demonstrated (Ji, 1973). When the anti-spectrin antibody was bound to spectrin inside the membrane agglutination of the siglo-glycoprotein sites on the outer surface occurred (Nicolson & Painter, 1973) as observed electronmicroecopically. This suggests that a dynamic relationship concerning the movement of the glycoprotein sites at the outer surface and epectrin may exiet. Also the agglutination of the galactoeylc reeidues on the outer surface by Ricinus communie agglutin led to rearrangement of the epectrin moleculee (Ji & Nicolson, 1975). These observatione suggeet that epectrin playe an eeeential role in controlling the mevement of membrane eitee. Another obeervation reported the agglutination of the intra-membraneoue particle at pH 5.5, which ie the ieoelectric point of spectrin, and that the movement of the integral peotein ie at its highest level when spectrin is extracted from the membrane (Elgeaeter & Branton, 1974). Theee observations euggeet that epectrin preventa the movement of theee proteins probably by their involvement in cytoplaemic connectione on the epeotrin network.

Lipids

Theee are discueeed in eection 2.1. For further detail eee Van Deenen and De Gier, (1974).

SECTION 3

TRANSPORT ACROSS BIOLOGICAL MEMBRANES

3.1 Transport Mechanisms

All living cells are bound by a membrane which separates the inside of the cell from its exterior allowing the development of a spacial internal environment, the characteristics of which depend upon the functional needs of the cell. This membrane should allow essential metabolites and products to enter and leave the cell at controlled rates. The membrane serves as a regulator of the intracellular levels of certain ions and metabolites by its selective permeability to such substances in appropriate directions. The processes through which metabolites, ions and waste products traverse the cell membrane is as follows:

3.1.1 Simple diffusion

The process of diffusion in the living cell is of great importance in maintaining its normal physiological state. It is a simple way by which the cell gains the substances necessary for its energy requirement for its metabolic activity and extrudes the waste products of these activities. In 1850, Graham investigated the diffusion phenomenon in free solutions and showed that the rate of diffusion depends on the type and size of diffusion particles. Considering the general mechanism of simple diffusion in a solution, this process is defined as the migration of solute molecules through a unit area from one solution to the other due to random motion of the molecules. The rate of diffusion is directly proportional to the difference in concentration of the two solutions and the unit area through which the solute molecules pass and inversely proportional to the size of the molecule and the distance between the two solutions.

This may also be represented mathematically by an equation derived from Fick's law which is applicable to free diffusion of the solutes:

$$\frac{ds}{dt} = DA \frac{dc}{dx}$$

Where ds/dt represents the rate of diffusion, i.e. the amount of the solute, diffused in unit time, t. A is the unit area through which the diffusion occurs, c is the difference in concentrations of the two solutions, x is the distance the solute migrate from one solution to the other and D is a constant known as diffusion coefficient and is the diffusional characteristic of a particular substance. It represents the amount of solute that diffuses through a unit area when the concentration gradient is unity.

Owing to random motion of molecules in solution, diffusion takes place continuously in all directions. In homogenous solution the average flux rate over a unit area in opposite directions to any point in the solution is equal. A net change in concentration does not, therefore, take place. Intermolecular collision does occur but this is more important in gaseous phase where the process is more rapid and frequent, due primarily to a smaller intermolecular attraction than in solutions. When a region of unequal concentration of solute molecules is achieved then a net diffusional movement is present from the high concentration region to the low. This is simple diffusion and does not require any other energy than that of the kinetic energy of motion of the solute molecule itself.

3.1.2 Activated diffusion

From the thermodynamic point of view, the diffusion process in the solution is different from that of the gas. In the gas the molecules are more random and they are only subjected to small intermolecular processes. Therefore the kinetic energy of the diffusion is not related to the chemical nature of the diffusing molecule. In solution, the movement of the solvent molecule, as well as the solute, should be taken into consideration, for the movement of the solute could be due to the movement of the solvent molecules surrounding it. In order for the solute molecule to move a certain distance it should acquire energy to break itself from the solvent molecule. The diffusion of a molecule therefore occure in a series of "jumps" as it acquired the activation energy necessary for its movement. The diffusion coefficient will be related to the viscosity of the solution and the friction between the solute and the solvent molecule. The rate of diffusion could be rapid in certain cases where the forces of attraction are mainly weak (Van der Waals). The concept of activated diffusion was put forward by Danielli (1947), who derived the relationship shown below:

$$DM^{\frac{1}{2}} = Q_{10} \frac{T + 10}{10}$$

Where Q_{10} is the temperature coefficient of diffusion (D) and it varies for different solutions according to the activation energy (M) the solute molecules require for the "jumps" to take place. Therefore, the higher the activation energy (i.e. the higher the forces to be overcome) the higher the value of Q_{10} should be. Applying this concept to biological membranes, these

"jumps" of the molecule allow them to penetrate the lipid bilayer of the membrane pushing their way through by making a temporary pore for its passage in and out of the cell.

3.1.3 Facilitated diffusion

Although the diffusion across the biological membranes is a very slow process due to the barrier of the membranes, it was noticed that certain substances diffuse through these membranes at a much faster rate than that expected when their mass is taken into consideration. This suggests that the diffusion of these molecules must be facilitated in some way. The facilitated diffusion could be defined as a diffusion of substances across the membrane at a faster rate than expected from the nature of the diffusing molecule and the diffusion medium. The rate of facilitated diffusion is proportional to the concentration gradient (as with simple diffusion) and no additional energy input is required. The transfer of the permeating molecule across the membrane is due to its interaction with some specific membrane component, most probably a protein, which is directional in its action.

The term membrane carrier (see Wyssbrod et al, 1971) is used to describe a component of the membrane that exhibits the capability to associate transiently or temporarily with the permeating molecule (the passenger) in a way that allows this passenger to cross the membrane at a rate which is significantly greater than that of the passenger in simple diffusion. The carrier may allow or enhance the solubilisation of the passenger in the membrane (Useing 1952) and therefore facilitate the component to cross the barrier. Many models of membrane transport proposed that the substance is transported across the membrane

by specific binding to a carrier in the membrane followed by translocation of the "carrier-substrate complex" through the membrane and release of the substrate from the carrier. The transport of the carrier-substrate complex is either by simple diffusion or activated diffusion down a concentration gradient.

Facilitated diffusion occurs through specific sites on the membrane and these sites show a characteristic ability to be saturated when there is sufficient amount of substrate. This is different from the free diffusion in that the latter reaches a steady state equilibrium rather than saturation.

The association of the substance with the carrier is a reversible process resembling the mechanism of enzyme-substrate interaction, i.e.

$$C + S \rightleftharpoons CS$$

where C is the carrier in the membrane and S is the permeating substance. D-glucose transport across the red cell membrane is a good example of the carrier transport concept.

Studies on facilitated transport have been carried out on membranes by isolation, characterisation and identification of the carrier from the membrane. Penrose et al, (1968) have isolated the leucine binding proteins from the E coli K12 cells by osmotic shock and characterised the protein by gel electrophoresis, ultracentrifugation and immunodiffusion. This protein is highly specific for L-isomer of leucine. The authors showed that the rate of diffusion of this amino acid in E coli was greatly decreased after extraction of the binding protein. Similar work was carried out by Araku (1968a) who isolated the binding protein from E. coli K12 and showed that the diffusion process by this carrier exhibited saturation kinetics (Araku, 1968b).

3.1.4 Active transport

For many years investigators attempted to differentiate and define the transport phenomenon observed in the biological system. Most of these phenomena were discussed according to the forces involved in the system. Advances in research in transport phenomena across the biological membranes led to a deeper understanding of the active transport process. Investigators explained the term active transport as "a process that required energy for the transport of the species which is accompanied by coupling of metabolic reaction". Thus they believed that the driving force and the energy supply should be internal rather than external to the biological system. However, Rosenberg (1948) defined the active transport as a process which leads to transfer of species across the membrane against their electrochemical potential gradient. This approach was useful in applications to experimental observation since the measurements of the electrochemical potential difference was relatively simple. However, Rosenberg excluded the possibility that the active transport may be coupled to external metabolic reaction and may take place in the direction of external electrochemical potential, as was observed in the active transport of the Na^+ ions from outside to inside of the frog skin. Attempts were made by several investigators to define the active transport more precisely and involved non-equilibrium thermodynamic concepts for interpreting the process (Kedem, 1961). Similar concepts were used by other investigators(Moszynisky *et al*, 1963; Jardutzky & Snell, 1960) but no experimentally useful definition to this process was evolved. However, the coupling of metabolic energy to the active transport is a complicated process and its understanding requires knowledge of the source of energy,

the way it is conserved or used in the biological system and interaction of the membrane components with the transported substances.

The energy source for the active transport differs among different types of cells and organisms. In mammalian cells the energy is derived mainly from the cleavage of energy rich phosphate bond of the terminal phosphate group of ATP. On the other hand the mitochondria and certain types of bacteria use energy derived from the membrane linked oxidation processes as a result of the activity of membrane bound oxidative enzymes. This type of energy source is used by nerve, muscle and nucleated red cells of birds (Shanes, 1951; Maizels, 1954; Hodgkins & Keynes, 1955). The conservation of the energy in the mammalian cell membranes may be achieved through the formation of covalent bonding in the membrane protein and the utilisation of this energy could be observed in conformational changes of the membrane components involved in the transport.

The remainder of this section will be concerned about the active transport mechanisms of cations with particular reference to the red cell membrane.

3.2 $(Na^+ + K^+)$ - ATPase

This is one of the most extensively studied active transport system. It utilizes ATP as a source of energy which is derived from the cleavage of its terminal phosphate bond.

The studies of the cation gradient across the cell membrane were established first by Gohn & Cohn (1939) when they observed a rapid uptake of ^{22}Na by the blood cell of the dog which suggested the existence of cation gradient due to the permeability of the cell membrane to sodium ions. This gradient was abolished by

incubation of the cell at low temperature (Harrie, 1941) but was re-established after incubation at physiological temperature. These observations led to the "pump-leak" concept. According to this concept both Na^+ and K^+ ions could leak passively into and out of the red cell in the direction of their concentration gradient, while an active pump extrudes Na^+ out of and draws K^+ into the cell also against a chemical gradient. The pump activity was found to be abolished when glucose was absent from the medium or when inhibitors of the glycolytic pathway, e.g. fluoride, N-ethylacetamide, were present (Harris, 1941; Dawski, 1949). The cation pump is not associated with the glycolytic pathway but the ATP which is used is supplied by this pathway and is essential for the pump activity. Further experiments provided evidence that the cation pump activity was restored when ATP was regenerated in energy depleted cells (Hoffman et al, 1960). However, the possibility of existence of an enzyme required to utilize the energy available from ATP for the cation pump was discussed by Skou (1957) who described an activity of the enzyme ATPase from crab nerve which is stimulated by Mg^{2+}. This activity was increased on addition of Na^+ and K^+ ions. The enzyme was strongly inhibited by the cardial glycoside ouabain (Schatzmann, 1953), which was found to be specific for this enzyme (Skou, 1960). Skou also suggested that the ouabain-sensitive $(Na^+ + K^+)$ - ATPase might be a part of the Na^+ transport system.

3.2.1. General properties of the $(Na^+ + K^+)$ - ATPase

The properties of this enzyme are more or less similar in most tissues. Therefore they are discussed here in general with reference to the enzyme activity in the red cell.

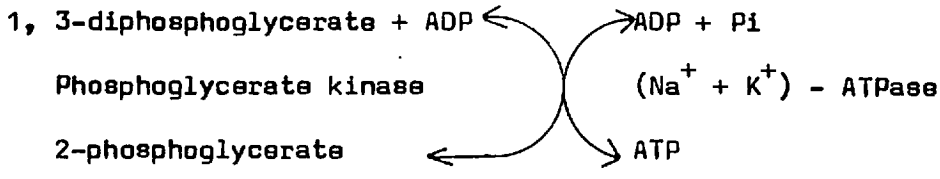
The pH optimum of the $(Na^+ + K^+)$ - ATPase in several tissues are in the range of 7.2 in the nerve (Skou, 1957) to 7.5 - 8

in the intestine of the guinea pig (Taylor, 1962). The stimulation of the enzyme by Mg^{2+} in rat liver is optimum when the ratio of ATP : Mg^{2+} is 1 : 1 (Bakkeren & Bonting, 1968). It was suggested that the true substrate for the enzyme is Mg - ATP complex rather than ATP only or that the ATP binds to the enzyme via Mg^{2+} (Atkinson et al, 1968). The half maximal activation (Km) of the enzyme by Na^{+} was investigated in many tissues and found to vary from one tissue to the other (Skou, 1957; Hafkenscheid & Bonting, 1969). At high Na^{+} concentration (above 125 mM) the enzyme activity was found to decrease. This suggests that Na^{+} ions competes with K^{+} ions and remove them from their binding sites on the enzyme (Priestland & Whittam, 1968). Similarly, K^{+} activation of the enzyme was studied (Bonting et al, 1964) and it was found that maximal activation is reached at K^{+} concentration of 5 mM followed by decrease in the activity when the K^{+} concentration was greater than 30 mM. Other cations, e.g. thalium, rubidium, were shown to replace Na^{+} or K^{+}, although the activity of the enzyme was much lower for these cations.
As mentioned previously, cardiac glycosides were shown to inhibit the enzyme specifically, the best known being ouabain. This is used experimentally because it is fairly soluble in water.
The mechanism of action of ouabain on the enzyme was shown to be biphasic, i.e. it stimulates the activity at very low concentration (10^{-8} - 10^{-9}M) and inhibits it at higher concentration 10^{-4}M (Bonting, 1966). K^{+} ions antagonise the inhibition by ouabain in erythrocyte membrane (Dunham and Glynn, 1961). The association of the enzyme with the membrane lipid was studied by the action of detergents and phospholipases. It was shown that the removal

of certain lipids from the membrane led to loss of the enzyme activity which was partially restored after addition of lipids, particularly phosphotadylcholine (Tanaka & Strickland, 1965). This suggested that the $(Na^+ + K^+)$ - ATPase is an intrinsic protein associated with the membrane by lipid-protein interactions. Molecular weight of the enzyme was estimated by several authors to be 670,000 (Vesugi et al, 1969) and 250,000 (Keprrer& McCay, 1968).

The $(Na^+ + K^+)$ - ATPase was first demonstrated in the red cell by Dunham & Glynn (1960, 1961) and Post et al (1960). These authors suggested that the cation pump and the $(Na^+ + K^+)$ - ATPase are virtually the same because they exhibit the same characteristics concerning their location in the membrane, utilisation of ATP, requirement of Na^+ and K^+ ions and their inhibition by ouabain. Investigation concerning the ionic strength effect on the enzyme showed that K^+ activates the system outside the RBC whereas Na^+ activates in internally (Glynn, 1962; Whittam, 1962). As mentioned, the ATP should be on the interior surface of the cell to serve as a substrate (Hoffman, 1962). The phosphate is released outside the cell and passively diffused in (Schatzmann, 1964). These facts suggested that the active site of the enzyme is located at the internal surface of the membrane and activated by Na^+ ions whereas the dephosphorylation site is in the outer surface, activated by K^+ and inhibited by ouabain. The stoichiometric relation between the cations and ATP is 3 for Na^+ and between 2 to 2.5 for K^+ i.e. 3 Na^+ ions and 2 to 2.5 K^+ ions are transported for one molecule of ATP hydrolysed (Sen & Post, 1964; Gordose, 1964). Correlation

between the pump and the glycolytic pathway was established in the erythrocyte. The reaction step in the pathway which lead to the generation of ATP is influenced by the pump activity, as shown:



This influence is represented in supplying the ADP (resulting from the hydrolysis of ATP by the pump) to this step leading to increased production of new molecules of ATP which will be utilised by the pump, the reaction being cyclic (Parker & Hoffman, 1967).

3.2.2 The mechanism of the $(Na^+ + K^+)$ - ATPase

The first step in the reaction mechanism is the formation of the phosphorylated intermediates after hydrolysis of the ATP. This complex formation is activated by Na^+ and Mg^{2+} ions (Fahn et al, 1966). This reaction is represented as follows:

$$ATP + E \xrightarrow{Mg^{2+},\ Na^+} E - Pi + ADP$$

K^+ ions enhance the dephosphorylation of the high energy complex, E - Pi. This step is inhibited by Ouabain (Post et al, 1965; Gibbs et al, 1965). The possibility of labelling the phospholipide with ^{32}P as a phosphorylated intermediate was eliminated because it was found that 95% of the label is incorporated into the phosphoprotein fraction (Ahmed and Judah, 1965) and the isolation of various phospholipids from the membrane after the labelling showed no significant incorporation of ^{32}P in the lipid fractions (Glynn et al, 1965). The second step is the dephosphorylation of this phosphorylated intermediate. This dephosphorylation is stimulated by K^+ and inhibited by ouabain as shown below (Rega et al, 1968):

$$E - Pi \underset{\text{ouabain inhibitable}}{\overset{K^+}{\rightleftharpoons}} E + Pi$$

This enzyme was further stimulated upon the addition of ATP and Na^+.

This suggested a close relationship between the phosphatase and the ($Na^+ + K^+$) - ATPase system (Yoshida et al, 1969). However, more detailed reaction mechanism of the ($Na^+ + K^+$)-ATPase system was proposed (Seigel & Albers, 1967) which includes two types of phosphorylated intermediates. The sequence is presented as follows:

$$E_1 + ATP \xrightleftharpoons{Na^+,\ Mg^{2+}} E_1 - Pi + ADP$$

$$E_1 - Pi \rightleftharpoons E_2 - P$$

$$E_2 - Pi + H2O \xrightarrow{K^+} E_2 + Pi$$

$$E_2 \rightleftharpoons E_1$$

The E_1 and E_1— Pi are oriented to the interior of the cell membrane with high affinity to Na^+ ions and low affinity to K^+ ions. The E_2 and E_2 — P are located at the exterior of the cell membrane with high affinity to K^+ and low affinity to Na^+ ions (Albers et al, 1968). This model is presented in fig. 3.1.

In earlier studies a different model was proposed (Opit & Charnock, 1965) based on the Davson-Danielli protein-lipid bilayer. This model suggested the location of an ionic site of the protein at the interior of the membrane. To these sites cations, e.g. Na^+, K^+ were said to be bound. Further, in the presence of excess Na^+, the ATP will react with the phosphorylation sites of the enzyme leading to elongation of the polypeptide chain at the inner surface thus causing the chain to rotate about the centre of the lipid exposing the anionic sites to the exterior. The phosphorylation sites will remain at the interior surface due to their resistance to rotation. The anionic sites at the exterior loose their affinity for Na^+ and exchange

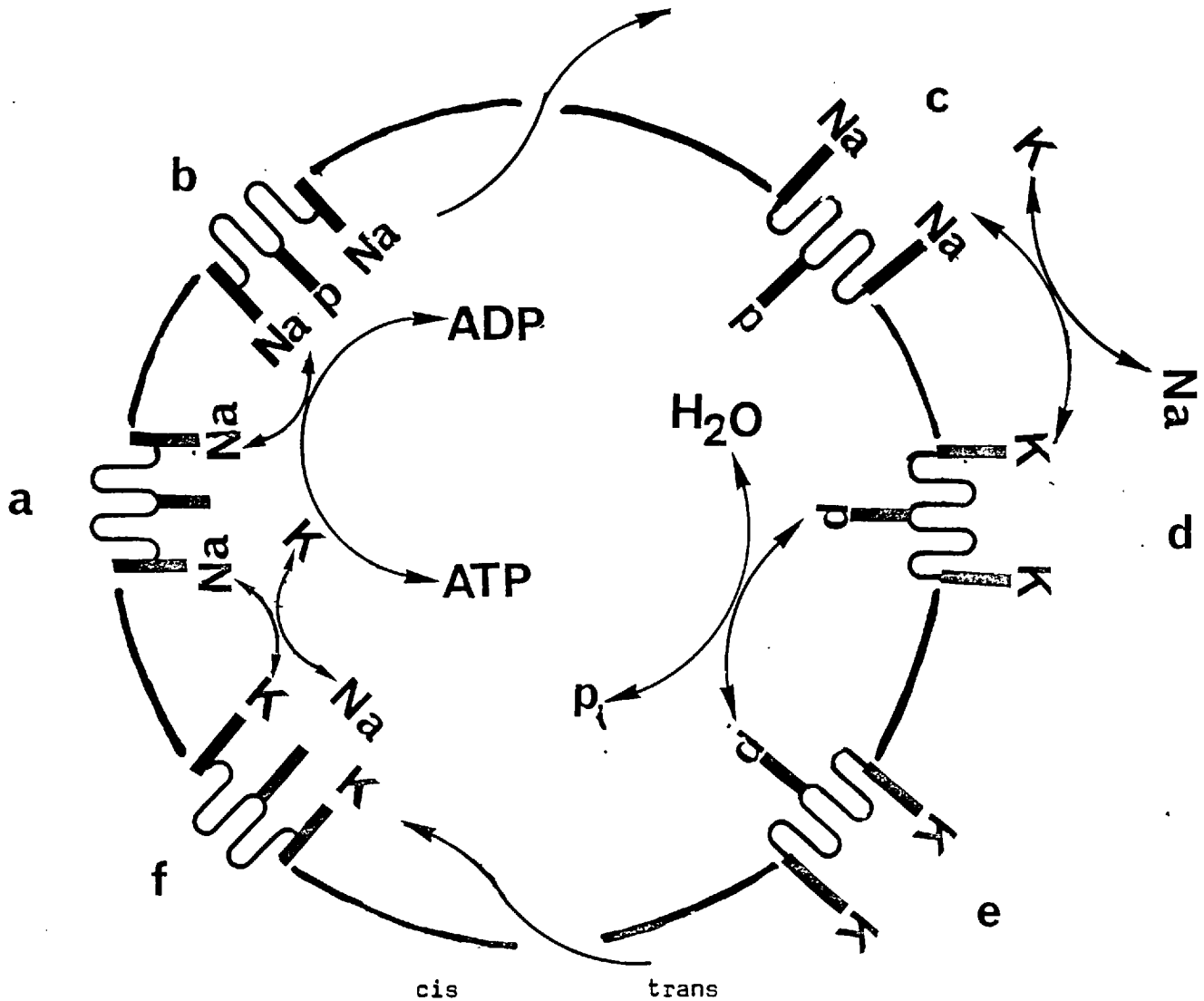


Fig 3.1 Model of ($Na^+ + K^+$) - ATPase according to Albers et al (1968). The circle represents cell membrane. Anionic sites are represented by solid bars. (a) Na^+ dependent ATP-ADP exchange. (b): Change of cis to trans-form or phosphorylation. (c): Na^+, K^+ exchange; presences of K^+ causes dephosphorylation and the enzyme converts to cis-form when exchange of K^+ for Na^+ takes place.

it for K^+. K^+ binding will stimulate the dephosphorylation of the phosphorylated intermediate. However, this model has not been substantiated.

3.2.3 The Mechanism of Inhibition by Ouabain

It was observed that ouabain combines with the phosphorylated enzyme and inhibits the dephosphorylation step (Matusi & Schwartz, 1968). This reaction is antagonised by K^+ ions and is reversed at low concentration of ouabain (Matusi & Schwartz, 1966). The Ki depends on the Na^+ : K^+ ratio. The binding requires Mg^{2+} and ATP, is stimulated by Na^+ and depressed by K^+. This suggests that the binding of ouabain is to the phosphorylated enzyme. However, the binding of ouabain was shown to take place even when the phosphorylated intermediates were not formed (Schwartz et al, 1968). This suggested that the binding results from the conformation of the enzyme brought about by the phosphorylation or other process. This was further supported by the evidence that the enzyme could bind inorganic phosphate in the presence of ouabain whereas this did not occur in the absence of ouabain. This can be explained by assuming that ATP and Na^+ binds to the enzyme (rather than its conversion to E — Pi form) which will induce the conformational changes in a way which is favourable to K^+ stimulated hydrolysis and that ouabain binds to the enzyme in this form.

3.3 ACTIVE Ca^{2+} TRANSPORT ACROSS THE RED CELL MEMBRANE

The permeability of the red cell membrane to Ca^{2+} was shown to be low (Passow, 1961 ; Rummel et al, 1962) as is the Ca^{2+} concentration inside the cell. These observations were further confirmed by Schatzmann and Vincenzi (1969). They observed very slow diffusion of Ca^{2+} into the cell after

incubation of the intact red cells at 4 C in a medium containing radioactive $^{45}Ca^{2+}$ for several weeks. Exchange between Ca^{2+} ions inside and outside the red cell was also demonstrated (Porzig, 1970). However, many techniques were used in an attempt to determine the precise concentration of free Ca^{2+} in the cell. The values were variable due to over-estimation of the real value by calculating for the Ca^{2+} bound to the membrane on the interior surface of the cell membrane. Eventually, the concentration of the Ca^{2+} inside the cell was accepted as 10^{-6}M (Lichman and Weed, 1973; Vincinzi and Schatzmann, 1967).

The red cell membrane binds considerable amount of Ca^{2+} and at least three binding sites of different affinities for Ca^{2+} have been shown to be located on the inner surface of the membrane (Long & Monat, 1971). Several binding sites were also found to be located on the spectrin-like protein (La Celle et al, 1972).

The existence of $(Ca^{2+} + Mg^{2+})$ - ATPase in the red cell was known for some time (Dunham & Glynn, 1961) before the demonstration of the active Ca^{2+} extrusion pump in the red cell membrane (which was not inhibited by ouabain) by Schatzmann (1966). Schatzmann's studies were based on loading the cell with Ca^{2+} by the method of reversal haemolysis described by Whittam (1962). According to this method the cells were exposed to hypotonic shock which render the membrane leaky to cations. The impermeability of the membrane to certain cations is restored as soon as the cells are transferred to isotonic medium. In this medium the membrane eventually reseals, enclosing the cations which have leaked into the cell during the hypotonic shock.

Although 80% of the membranes are resealed by this method they are still leaky to Na^+ and K^+ ions and the sealing for these ions take place only after prolonged incubation of the cells at 37 C. (Bodemann & Passow, 1972). These cells do, however, reseal to Ca^{2+}, Mg^{2+} and ATP almost immediately after the restoration of tonicity.

The level of Ca^{2+} inside the cell can be controlled by EGTA (Schatzmann, 1973). The cells were incubated at 37 C under sterile conditions for 15 hours in glucose free medium to deplete the cells from energy and were loaded with Ca^{2+} (in concentrations less than that of the medium), Mg^{2+} and ATP. In the presence of ATP and Mg^{2+} the rapid movement of Ca^{2+} across the cell membrane to the outside was noticed. This was accompanied by the presence of inorganic phosphate (Pi) inside the cell (Schatzmann & Vincenzi, 1969). In the absence of ATP no net movement of Ca^{2+} ions was observed. These observations suggest that there is an active pump which drives the Ca^{2+} ions from inside the cell against a chemical gradient (Jay & Burton, 1969). The results were confirmed by other workers (Olson & Gazort, 1969; Lee & Shin , 1969; Porzig, 1970).

3.3.1 <u>The energy source for the Ca^{2+} pump and cation requirements</u>

Since the energy deprived cells did not transport Ca^{2+} in the absence of ATP it was concluded that ATP provided the pump with energy by its hydrolysis. It was argued that ATP or its hydrolysis compound ADP and Pi act as a carrier for Ca^{2+} ions across the cell membrane as they move by simple diffusion to the external medium but when the net diffusion of these compounds was abolished by increasing their concentrations outside the cell, the overall movement of Ca^{2+} was not affected (Olson & Gazort, 1974).

The ATP utilized in Ca^{2+} transport should be present inside the cells. Experiments showed that ATP in the external medium failed to serve as a substrate for the enzyme (Palek et al, 1971). The enzyme also requires Mg^{2+} ions inside the cell (Lee & Shin , 1969). The optimal concentration for Mg^{2+} is 5 mM or equal to the concentration of ATP. Any change in the concentration of Mg^{2+} in the presence of ATP affects the Ca^{2+} concentration. This was thought to be due to competition of Mg^{2+} and Ca^{2+} for complexing with ATP to form the (Mg ATP) and (Ca ATP) complexes respectivsly (Schatzmann, 1969). The true substrate for the enzyme is thought to be (Mg ATP) complex rather than ATP alone, (Epstein & Whittam, 1966). Mg^{2+} is not transported and its level inside the cell is maintained throughout the reaction (Schatzmann, 1969).

The enzyme (Ca^{2+} + Mg^{2+}) - ATPase shows a relative specificity for other metal ions: Sr^{2+} can replace Ca^{2+} and was shown to be transported at the same rate (Schatzmann & Vincenzi, 1969; Olson & Gazzort, 1969) and compete with Ca^{2+} at the active site of the enzyme. The relative affinity of the enzyme for both ions is virtually the same; Mn^{2+} on the other hand activates the enzyme partially and is not transported, nor does it compete with Ca^{2+}.

The enzyme shows high specificity for ATP although different nucleoside triphosphates, e.g. UTP and CTP (Olson & Gazzort, 1969) are less effective in serving as a substrate (Lee & Shin, 1969).

3.3.2 Inhibitors

As mentioned previously ouabain did not inhibit the Ca^{2+} transport or the (Ca^{2+} + Mg^{2+}) - ATPase (Schatzmann, 1966). Other inhibitors such as oligomycine, caffeine (Schatzmann & Vincenzi, 1969) were also not effective. Sulphydryl reagents

which are potent inhibitors for $(Na^+ + K^+)$ - ATPase, are effective in inhibition of Ca^{2+} transport and $(Ca^{2+} + Mg^{2+})$ - ATPase, and also for Mg^{2+} - ATPase (Vincenzi, 1968; Schatzmann & Vincenzi, 1969; and Palek et al, 1971). Ruthenium-red, a hexavalent cation, which was shown to be a strong inhibitor for Ca-transport in mitochondria, could inhibit the $(Ca^{2+} + Mg^{2+})$ - ATPase of red cells (Watson et al, 1971). Mg^{2+} - ATPase and $(Na^+ + K^+)$ - ATPase were also found to be inhibited by this cation but to a lesser extent. However, the Ca^{2+} transport was not inhibited by this cation (Schatzmann, 1975). Ruthenium-red is available commercially contaminated with other reagents and its purification is necessary (Luft, 1971). The impurities in the ruthenium-red led to the suggestion that its inhibitory action is an artifact due to the presence of these impurities (Schatzmann, 1975). The $(Ca^{2+} + Mg^{2+})$ - ATPase activity as well as the Ca^{2+} transport were shown to be blocked completely by the trivalent lanthanides, holium (Ho^{3+}) and praseodymium (Pr^{3+}). At 1 mM concentration of Ho^{3+}, full inhibition occurs, suggesting the possibility of complex formation with ATP. It may also compete with Ca^{2+} ions at the active site of the enzyme (Schatzmann & Techabold, 1971).

It may be mentioned here that the activation of Ca^{2+} pump can be achieved by the hormone calcitonine (which induces Ca^{2+} absorption in the bone tissues). Release of Ca^{2+} from the red cell was observed after introduction of this hormone (Parkinson & Rudd, 1969). A protein activator from the haemolysate of membranes prepared in the presence of Tris-EDTA buffer was shown to stimulate the activity of $(Ca^{2+} + Mg^{2+})$ - ATPase without any effect on the activities of Mg^{2+} - ATPase and $(Na^+ + K^+)$ - ATPase (Bond & Clough, 1973).

3.3.4 Different types of Ca^{2+}-activated ATPases

In the red cell membrane, apart from the $(Na^{+} + K^{+})$ - ATPase, there exist other types of ATPases that may be stimulated by Ca^{2+} but may depend on other cations for their full activity.

The Mg^{2+} activated ATPase operates in the presence of Mg^{2+} and ATP inside the cell or membrane prep and its activity is detectable when Ca^{2+} and other cations are absent. It is not involved in transport of any of the cations and its functional significance is thought to be in its influence on the red cell permeability (Romero, 1974). The Ca^{2+}-activated ATPase operates presumably when high level of Ca^{2+} inside the cell is reached. The Km of this enzyme for Ca^{2+} was estimated to be 1 - 2 mM (Rosenthal et al, 1970). This enzyme is thought to be associated with the fibrillar protein spectrin because it could be solubilized from the membrane under the same conditions used for solubilisation of this protein, e.g. the presence of low ionic strength, elevated pH and chelator (Marchesi & Steere, 1968). It was suggested by these authors that spectrin is involved in the regulation of the deformability of the cell membrane and plays an important role in many physiological functions of the red cell.

The high level of Ca^{2+} inside the cell is very unlikely to be reached unless, under certain pathological conditions associated with alteration in the physical status and permeability of the membrane. This in turn will lead to stimulation of this enzyme. Mg^{2+} ions were shown to inhibit this activation when present inside the cell with Ca^{2+} (Rosenthal et al, 1970). It may also act as a regulator of the permeability of the cell

membrane (Romero, 1974).

$(Ca^{2+} + Mg^{2+})$ - ATPase is an important enzyme because, besides the $(Na^{+} + K^{+})$, it is thought to be associated with Ca^{2+} transport (Schatzmann, 1975). Kinetic studies showed that the true substrate for this enzyme is (Mg ATP) complex (Wolf, 1972). This enzyme is activated at very low Ca^{2+} concentration inside the cell compared to the Ca^{2+}-ATPase discussed above. This is one of the reasons to believe its association with Ca^{2+} transport pump (Schatzmann, 1975). The Km value for this enzyme was estimated by several workers and found to be in the range of 10^{-5}M (Vincenzi & Schatzmann, 1967; Wolf, 1972). 2×10^{-5}M (Bond, 1972) and 6×10^{-6}M (Davis & Vincenzi, 1971). This enzyme may also stimulate binding of Ca^{2+} to the membrane in the presence of Mg^{2+} and ATP (Duffy & Schwarz, 1973).

The $(Ca^{2+} + Mg^{2+} + Na^{+})$ and $(Ca^{2+} + Mg^{2+} + K^{+})$ - ATPases are probably a conversion of the $(Ca^{2+} + Mg^{2+})$ - ATPase which operates in the presence of one of the alkali cations K^{+} or Na^{+} which may induce further stimulation of the $(Ca^{2+} + Mg^{2+})$ATPase (Schatzmann & Rossi, 1971; Bond & Green, 1971). The expression of the activity of these enzymes is greatly dependent on the mode of preparation of the membrane and the assay mixture (Shcarff, 1972). It is also suggested to be an artifact of preparation (Schatzmann, 1973). However, the same author also suggested that the two monovalent cation activities may result from decoupling of $(Na^{+} + K^{+})$ - ATPase.

The $(Ca^{2+} + Mg^{2+})$ - ATPase was found to exhibit different affinities for Ca^{2+}, depending on the range of Ca^{2+} concentration used in the experiments (Schatzmann & Rossi, 1971; Wolf, 1973). When a wide range of Ca^{2+} concentration was employed (0.1×10^{-4}

to 5×10^{-4}M) the (Ca^{2+} + Mg^{2+}) - ATPase showed a complex kinetic behaviour indicating the presence of more than one binding site for Ca^{2+} with different affinities (Schatzmann & Rossi, 1971). Two sites of differing affinities for (Ca^{2+} + Mg^{2+}) - ATPase were also suggested by Wolf (see review by Schatzmann, 1975).

The Km value for the high affinity (Ca^{2+} + Mg^{2+}) - ATPase was reported to be 4 M by Schatzmann & Rossi (1971) and 2.3 M by Wolf (1972). For the low affinity, the Km value was found to be 0.1 mM (Shcatzmann & Rossi, 1971). The derivation of Km value for the low affinity site is made difficult due to contaminating Ca^{2+} ions in the preparation of the membrane. EGTA buffers have been used, but in most cases involve uncertainties in the dissociation constant of Ca.EGTA complex under the experimental conditions used (Schatzmann & Rossi, 1971).

However, Schatzmann (1973) has concluded that the low affinity site may be due to an artifact. He reported the Km value of Ca^{2+} transport out of the red cell to be 4 M, agreeing closely with that of high affinity (Ca^{2+} + Mg^{2+}) - ATPase. On this evidence it is generally believed that the high affinity ATPase is responsible for Ca^{2+} transport. The function of the low affinity (Ca^{2+} + Mg^{2+}) - ATPase has not yet been established.

3.4 The phosphoproteins of (Ca^{2+} + Mg^{2+}) - and (Na^{+} + K^{+}) - ATPases

SDS gel electrophoresis analysis of the ghost membranes showed at least two phosphoproteins, one in the region of the gel corresponding to molecular weight of 150,000, which appeared when phosphorylation of the membrane was carried out in the presence of 0.5 mM Ca^{2+} (Knauf *et al*, 1974). The other phosphoprotein appeared in the region of 103,000 molecular weight and

was considered as the Mg^{2+} and Na^{+} phosphoproteins (Knauf et al, 1974). The formation of two separate phosphoproteins suggested that the $(Na^{+} + K^{+})$ - ATPase and the $(Ca^{2+} + Mg^{2+})$ - ATPase are two separate enzymes operating independently of each other. The pronase digestion studies indicated the localization of the enzyme phosphorylation site on the internal surface (Knauf et al, 1974). A close relationship between the $(Ca^{2+} + Mg^{2+})$ - dependent ATPase and the p-nitrophenyl phosphatase was found to exist (Rega et al, 1973). This is based on the observations of a common optimal Ca^{2+} concentration required for activation, the requirement for ATP and the activation of both enzymes at the inner surface of the membrane. These observations suggested that both enzymes may be part of one system and may share the same active site for ATP and Ca^{2+}. Moreover, these authors have shown that the phosphatase substrate (p-nitrophenyl phosphate) combines with the enzyme system at the active site inhibiting the $(Ca^{2+} + Mg^{2+})$ - ATPase activity and the active Ca^{2+} extrusion.

3.5 CALCIUM TRANSPORT IN THE SARCOPLASMIC RETICULUM

Examination of studies carried out on sarcoplasmic reticulum with regard to Ca^{2+} transport and Ca^{2+}-ATPase shows that a very similar process also takes place in the red blood cell. Although it is clear that the pumps involved are different, the general principles involved are very similar in that a contractile type of system is involved in both in which Ca^{2+} appears to be of central importance.

3.5.1 Regulation of Ca^{2+} concentration in the sarcoplasm by membrane of the sarcoplasmic reticulum

In the presence of ATP and Mg^{2+} the sarcoplasmic fragments actively remove Ca^{2+} from the medium (Ebashi, 1960). This was described later (Hasselbach, 1964) to be due to active Ca^{2+} transport which derives its energy from the hydrolysis of ATP and actively accumulates Ca^{2+} in the sarcoplasmic reticulum. The mechanisms of regulation of the contractile system by Ca^{2+} is not fully understood but the dependence of muscular tension on free Ca^{2+} concentration in the sarcoplasm is accepted. The free Ca^{2+} concentration in the resting muscle was found to be less than 10^{-6}M (Portzehl *et al*, 1964). The level of intracellular free Ca in the red cell was also found to be in the micromolar range (Vincinzi & Schatzmann, 1967). The accumulation of Ca^{2+} by the sarcoplasmic reticulum will lead to lowering of the free Ca concentration below 10^{-7}M which is below the threshold concentration and will cause muscle relaxation (Weber *et al*, 1963). On the other hand, the release of Ca^{2+} ions from the sarcoplasmic reticulum upon excitation (Ashley & Ridgway, 1970) will increase the free Ca^{2+} concentration in the sarcoplasm leading to muscle contraction. In the presence of 5mM Mg^{2+} and 1mM ATP at free Ca

concentration of 10^{-6} to 10^{-5}M, hydrolysis of ATP, acetylphosphate (de Meis, 1969) and p-nitrophenylphosphate (Inesi, 1971) occur simultaneously suggesting the existence of a common enzyme for the above substrates.

3.5.2 Phosphoprotein intermediate

In the microsoms an exchange between the tri and di phosphates at the nucleoside occur: ATP + ADP = ADP + ATP (Ullbrecht, 1962; Makinose, 1969). This is dependent on free Ca^{2+} concentration in the medium which is similar to that of ATPase and Ca^{2+} transport system. The relation between ATP - ADP exchange, ATPase activity and Ca^{2+} transport was demonstrated as formation of phosphoprotein intermediate (Yamamoto & Tonomura, 1967; Makinose, 1969), after incubation of the microsome with ^{32}P labelled substrates (i.e. the ATP or acetylphosphate). The steady state optimum is reached at 5mM Mg^{2+} concentration which was shown to accelerate the formation of this intermediate (Martonosi et al, 1971). However, the possible mechanism of the hydrolysis of ATP by Ca^{2+} transport system could be:

$$E + ATP \rightleftharpoons E - ATP \quad (1)$$

$$E + ATP + 2Ca^{2+} \rightleftharpoons E - ATP - 2Ca^{2+} \quad (2)$$

$$E - ATP - 2Ca^{2+} \overset{Mg^{2+}}{\rightleftharpoons} E - P - 2Ca^{2+} + ADP \quad (3)$$

$$E - P - 2Ca^{2+} \rightleftharpoons E_1 - P - 2Ca^{2+} \quad (4)$$

$$E_1 - P - 2Ca^{2+} \rightleftharpoons E_1 + Pi + 2Ca^{2+} \quad (5)$$

$$E_1 \longrightarrow E \quad (6)$$

Step (1) does not require divalent cation. The phosphoprotein intermediate will result from interaction of Ca^{2+} with the E-ATP complex. Two Ca^{2+} ions are required for 1 molecule of ATP

to interact with enzyme (Hasselbach & Makinose, 1963). The formation of thie intermediate ie dependent on the free Ca^{2+} concentration and ATP - ADP exchange (Yamamoto & Tonomura, 1967) which will occur after the formation of ADP to the ATPase activity, will provide the energy for formation of ATP from ADP and inorganic phoephate (Makinose, 1971; Makinose & Haeselbach, 1971). The phoephate being accumulated outeide due to its diffusion through the highly anion permeable membrane (Duggan & Martonosi, 1970). The molecular organieation of the enzyme was further investigated by labelling the membrane with $AT^{32}P$ and separating the polypeptides by high voltage electrophoreeis after the extraction of membrane lipide and digestion with pepein (Martonosi, 1969; Martonosi & Halpin, 1971). Only one polypeptide wae labelled, although other weakly labelled polypeptidee were obeerved. A phoephoprotein in the red cell membrane wae aleo ehown to be formed in the preeence of 0.5 mM Ca^{2+}. The molecular weight of this protein was found to be 150,000 daltone (Knauf et al, 1974).

3.5.3 The releaee of Ca^{2+} from the earcoplaemic reticulum

The fragmente of the earcoplaemic reticulum previouely loaded, were ehown to release Ca^{2+} rapidly upon electrical etimulation of the frog muecle (Jobeie & O'Connor, 1966). The eetimated ionic flux in different typee of mueclee (Bianchi, 1966; Winegrad, 1961) and different epeciee (Tasaki & Singer, 1966) were found to be eimilar. Thie euggeete that the mechaniame involved in Ca^{2+} releaee and regulation of ite permeability ie eimilar (Katz, 1966).

However, two mechaniems are involved in Ca^{2+} efflux: carrier mediated efflux of Ca^{2+} and paeeive diffueion of Ca^{2+} and anione. Such mechaniems have not been found in the red cell yet, although

these may exist.

1. Carrier mediated efflux of Ca^{2+}

The exchange between $^{45}Ca^{2+}$ loaded in the sarcoplasmic reticulum and $^{40}Ca^{2+}$ added to the incubation medium was found to be rapid with no effect on the steady state concentration of Ca^{2+} (Martonosi & Feretos, 1963; Weber et al, 1966). This suggests that the steady state is maintained by balance of the Ca^{2+} influx and efflux (Weber, 1971a; Weber et al, 1966). The steady state efflux of the Ca ions is inhibited in the presence of ATP, Mg, by increasing the intravesicular Ca^{2+} concentration or decreasing the ion concentration in the external medium (Weber, 1971a.) The retention of Ca^{2+} inside the vesicles in the presence of Mg^{2+} and after suspension of the vesicles in Ca^{2+} free medium required ATP. The ATP is necessary for maintaining a relative impermeability of the microsomes to Ca^{2+} (Weber, 1971b). When ATP or Mg^{2+} are removed the Ca efflux increases via passive diffusion, due to changes in the permeability of the microsomal membrane after the removal of ATP. In the presence of Mg^{2+}, ADP and inorganic phosphate in Ca^{2+} free medium, the rate of efflux of Ca in Ca^{2+} loaded vesicles increases. This is accompanied by the synthesis of one mole of ATP for each two moles of Ca^{2+} ions released. The rate of synthesis of ATP is only 20% of its hydrolysis rate during active transport (Barlogie et al, 1971; Makinose & Haeselbach, 1971). The carrier mediated efflux of Ca^{2+} represents the reversal of the active Ca^{2+} uptake, evidenced by the finding that the Ca^{2+} efflux is induced by ADP, Mg and Pi, and is inhibited by increasing free Ca^{2+} concentration in the medium (Barlogie et al, 1971). The significance of the carrier mediated Ca^{2+} efflux is the regulation of the sarcoplasmic Ca^{2+} concentration.

The Ca^{2+} release during stimulation is regenerative process (Ford & Podolsky, 1970; Endo et al, 1970) because further increase in the sarcoplasmic Ca^{2+} concentration enhance further release of Ca^{2+} to the external medium.

The penetration of Ca^{2+} through a mobile carrier has been studied in the artificial lipid membranes. The rate of Ca^{2+} efflux was estimated to be 8×10^{-8} mole/cm^2/second (Kornberg & McConnell, 1971b) when the initial Ca^{2+} concentration of the phosphotidylecholine artificial bilayer was 1 to 3×10^{-2} mole of Ca^{2+}/mole of phospholipid. The movement of Ca^{2+} was thought to be due to movement of the phospholipid molecule from the outer to the inner surface of the bilayer, a mechanism known as flip-flop transition. The rate of flip-flop was estimated by Kornberg & McConnell (1971b) to be 1.7×10^{-14} mole/cm^2/second by the spin labelling techniques. The penetration of Ca^{2+} through the hydrophylic channels created across the artificial or natural bilayer by the use of macrocytic antibodies has also been studied (Mueller & Rodin, 1968). The Ca^{2+} permeability was enhanced by passage through these channels.

2. <u>The passive diffusion of Ca and anions across the sarcoplasmic reticulum</u>

The isolated sarcoplasmic reticulum membrane exhibits very low permeability for Ca^{2+} which may be similar to its state <u>in vivo</u>. This was observed due to the slow release of Ca^{2+} from the loaded vesicles even after electrical stimulation (Van der Kloot, 1966; Lee, 1967). The rate of Ca^{2+} release from the fragments in Ca^{2+} free medium was 50 to 100 times slower than initial active uptake and 10 to 20 times slower than the carrier mediated efflux (Duggen & Martonosi, 1970). Although this rate is slightly enhanced by elevated temperature, alkaline pH and the presence of

SH-group reagent, the rate is still much below the expected rate of the efflux during excitation. On the other hand, the sarcoplasmic reticulum fraction appear to be quite permeable to anions such as oxalate, pyrophosphate and orthophosphate, as evidenced by the complexation of oxalate with Ca^{2+} ion in the microsomal particles (Martonosi & Feretosa, 1964). It is not certain whether this permeability is due to partial damage of the membrane or to its natural state *in vivo*. If this is true, then anions may be incorporated in the synthesis of important metabolic intermediates necessary for energy producing activities of the sarcoplasmic reticulum (De Duve *et al*, 1962).

Fragments of the sarcoplasmic reticulum are impermeable to macromolecules, e.g. inuline (molecular weight 5000) or dextran (molecular weight 15,000 - 90,000) at pH 7.9. But, in the presence of ATP, a decline in the level of Ca^{2+} inside the vesicles was observed accompanied by penetration of ^{14}C-inulin or ^{14}C dextran. Similarly, treatment of the fragments with EDTA at pH8 produced the same effect (Duggan & Martonosi, 1970). Treatments with EDTA caused the release of two proteins of molecular weight 51,000 and 63,000, as estimated by gel electrophoresis (Martonosi & Halpine, 1971) which were reattached upon addition of 1mM $CaCl_2$.

SECTION 4

4.1 PHOSPHORYLATION - PROTEIN KINASE

The phosphorylation process is important in the regulation of several cellular activities carried out by components of the living cell. Protein phosphorylation generally involves the transfer of the gamma terminal phosphate group of ATP to a receptor protein. This process is catalysed by protein kinase.

Protein kinase was first extracted from liver tissue and was thought to be of mitochondrial origin (Burnett & Kennedy, 1954). Earlier attempts to isolate the enzyme were not successful (Friedkin & Lehninger, 1949). A similar enzyme was described in the rabbit mammary gland which could phosphorylate casein (Sunderajan et al, 1958). The existence of this enzyme in a variety of tissues, e.g. brain and yeast (Rabinowitz & Lipmann, 1960), was also demonstrated. Protein kinases involved in the regulation of a variety of cellular processes were described shortly after the discovery of these enzymes in the liver. Amongst these enzymes was the phosphorylase kinase which catalysed the phosphorylation and activation of the enzyme glycogen phosphorylase, (Krebs & Fischer, 1956). This enzyme was not able to phosphorylate phosvitine significantly (Rebinowitz & Lipmann, 1960).

Originally, the protein kinases were described and named according to their substrate, e.g. protamine kinase (Jergil & Dixon, 1970); histone kinase (Langan & Smith, 1967). However, this was altered since the protein kinases were found to have a broader specificity than first thought. Therefore the enzyme was named according to its dependence on cAMP or other cyclic nucleotides. The interest in the cAMP-dependent protein kinase

has been widely growing ever since these enzymes were described as a class of regulatory enzymes and since it was demonstrated that the protein kinase from the liver is regulated by cAMP (Langan, 1968).

The cAMP-dependent protein kinases were found to be widely distributed in a variety of tissues among different species (see Kuo & Greengard, 1969; 1970). These authors have put forward the hypothesis that all the physiological effects of cAMP are mediated by the protein kinases. Since cAMP is an important regulator of many cell processes, their hypothesis made this enzyme equally important in these regulations. This was further supported by the finding that the activity of the kinases and the protein phosphorylation are affected by hormones which are also known to control the level of cAMP in the cell, (Langan, 1969). A simplified diagram shows an example of action of cAMP in the regulation of cell function:

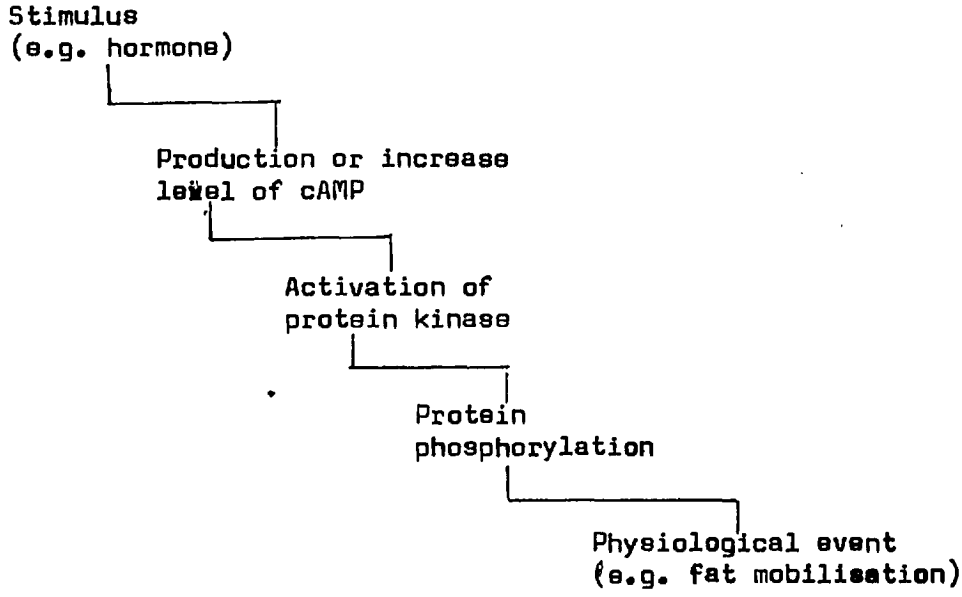


Fig 4.1 Sequence of reaction upon hormonal stimulation of a receptor.

4.1.1 Properties of Protein Kinases

Protein kinases have been characterised in most human and animal tissues, rabbit skeletal muscle protein kinase being the most extensively studied. This enzyme was found to be composed of two components when isolated by DEAE cellulose column chromatography. These two components (Peak I & II) were both cAMP-dependent (Reimann et al, 1971). Peak I was further separated into two subunits by sucrose density gradient centrifugation with sedimentation coefficients of 6.85s and 5.25s (Reimann et al, 1971; Corbin et al, 1972a). The 5.25s component was further separated on sucrose density gradient into two components of 5.45s and 5.05s (Corbin et al, 1972a).

Each of these components was found to be stimulated by cAMP and dissociated into catalytic subunits and regulatory subunit. The estimated molecular weight of Peak I component (6.85s) was 123,000 and 76,000 for Peak II (Corbin et al, 1972a). The pH optima for Peak I varied depending upon the substrate. A pH of 6.0 was found to be optimum when casein was used and of 6.5 when histone was used. The enzyme activity was increased 5 - 20 fold upon addition of cAMP (Reimann et al, 1971). Mg^{2+} was suggested to play an important role in the activation of the enzyme (though the mechanism of this is not fully understood) as well as complexing with ATP in (Mg.ATP) form as a substrate for the enzyme (Huijing & Larner, 1966). Protein kinase contains two subunits (Gill & Garren, 1970), catalytic (C) and regulatory (R) or binding subunits. The regulation by cAMP is represented by its binding to the R subunits forming the complex cAMP - R with concomitant release of the free active C subunits. The reaction could be represented as:

P.T.O to p 56

Contd. from p56

$$RC + I \longrightarrow RCI$$

Where R is the regulatory subunit. This complex dissociates to:

$$RCI \longrightarrow R + CI$$

When cAMP is present then the inhibitor (I) will react with the free catalytic subunit:

$$RC + cAMP \rightleftharpoons R.cAMP + C$$

$$C + I \longrightarrow CI$$

However, the requirement of cAMP for the activation of the enzyme is not always necessary. Certain cAMP dependent protein kinases can be dissociated and act independently under certain conditions. For example, the cAMP-dependent protein kinase prepared from bovine brain can dissociate independently in the presence of an exogenous substrate such as histone (Miyamoto et al, 1971). Similar behaviour was noticed for the enzyme derived from the Rabbit reticulocytes (Tao, 1972) and adipose cells (Corbin et al, 1972b). The multiplicity of the activation mechanism of the protein kinase is further complicated by the possible existence of more than one regulatory subunit for each catalytic subunit. One kind of subunit may be suppressive for the activity of the catalytic subunit while the other may be activating (Veda, 1971). Furthermore the different affinities for the regulatory subunits for cAMP makes the determination of definite level of cAMP which is required in the cell for activation difficult (Yamamura et al, 1971; Veda, 1971).

However, it was suggested that a modulator is present (Donnelly et al, 1973, a,b) that may be inhibitory in some cases and activating in others depending on the concentration of the substrate, cyclic nucleotide, metal ions and nature and source

P.T.O to p57

Contd. from p 54

$$R - C + cAMP \rightleftharpoons R - cAMP + C$$

The reaction is reversible and the equilibrium position may be influenced by factors in the cell. Mg^{++}ATP complex was reported to either bind tightly to the enzyme lowering its affinity for cAMP (Haddox et al, 1972) or it may facilitate the reverse reaction (Brostrom et al, 1971).

4.1.2 Modulation of the cAMP Dependent Protein Kinase

The study of the mechanism of modulation of protein kinase activity led to gain in knowledge of the factors which influence ite activity. The enzyme is sensitive to pH change and ionic strength. However, a heat stable protein was described to be a modulator of the protein kinase activity during the initial studies on the phosphorylase kinase activation (Gonzales, 1962). This was purified 750 fold from the rabbit skeletal muscles. This activity is destroyed by trypsin and it was found to be stable to treatment by DNase, RNase and phospholipases. (Walsh et al, 1971). Therefore it was considered to be a protein and also an inhibitor of the enzyme.

4.1.2.1 Mechanism of action of the inhibitor

The inhibitor protein is able to interact with the cAMP-dependent protein kinase with either the holoenzyme or the dissociated catalytic subunit (c) depending on the presence of cAMP (Asby & Walsh, 1972, 1973). When cAMP is absent from the incubation mixture, the inhibitor (I) will react with the holoenzyme (RC) to produce a complex (RCI) according to the following equation:

P.T.O to p 55

Contd. from p 55

of the enzyme or the modulator itself. The binding of cAMP to the regulatory subunits was found to occur in a definite fashion (Veda, 1971). A rapid, high-affinity binding reaction was noted first followed by a slow, low affinity, reaction. Storage of the enzyme has effects on the subunit composition without any effect on the dependence with regard to cAMP (Rubin et al, 1972); while in some cases ageing of the enzyme resulted in the dissociation of the enzyms subunits with concomitant loss of the cAMP-dependent stimulation (Pierre & Leob, 1971).

The formation of the catalytic inhibitor complex and the holoenzyme-inhibitor complex were detected by sucrose density gradient ultracentrifugation (Walsh & Ashby, 1973). According to these observations the interaction of the inhibitor with the holoenzyme in the absence of cAMP did not occur, i.e. RCI was not formed and the dissociation of this complex did not, therefore, take place. This was further evidenced by the observation that the inhibition of the enzyme occurred upon the addition of different concentrations of the free catalygic subunit to the incubation mixture containing the holoenzyme and the inhibitor without cAMP. This indicates that the free catalytic subunit activity was inhibited by the free I and not by forming the RCI complex (Walsh & Ashby, 1973).

Protein kinase of erythrocyte membrane

The membranes isolated from normal human red cell exhibited the ability of endogenous phosphorylation (Guthrow et al, 1972). The protein's bands II, III and IVc were found to be labelled with ^{32}P as a result of this endogenous phosphorylation (Guthrow et al, 1972); Rubin & Rosen, 1973; Rosen & Appel, 1973a). The

localisation of the enzyme protein kinase was investigated and it was found to be located at the internal surface of the membrane. These findings were supported by studies which compared the accessibility of this enzyme with other membrane enzyme markers in intact red cell as well as impermeable inside-out and right side-out vesicles prepared from erythrocyte membrane.

It was found that both the binding and catalytic subunits are located at the inner surface of the membrane (Rubin et al, 1973). Most of the catalytic subunit was extracted from the membrane using high salt concentration while the cAMP binding subunit remained bound to the membrane (Rubin et al, 1972). This suggested that the protein may be firmly integrated in the membrane.

The protein IVc has been claimed to be the cAMP subunit (Guthrow et al. 1973).

Abnormality of protein phosphorylation in erythrocyte membrane was shown to exist in certain diseases. Rosse & Appel (1973b) found that the rate of endogenous membrane phosphorylation was decreased 50% of normal in myotonic muscular dystrophy. Also in congenital haemolytic anaemia, a defect in membrane protein phosphorylation was reported (Greenquist & Shohet, 1974).

4.2 FORMATION OF cAMP

Since cAMP is an important regulator of many cell functions and since it is required for the control mechanisms of certain protein kinase activities, formation of this nucleotide and the enzymes involved in the regulation of its metabolism is briefly described. 3', 5' - cyclic adinosine monophosphate is formed

from ATP as shown in the following equation:

$$ATP \xrightarrow[Mg^{2+}]{\text{Adenyl cyclase}} cAMP + PPi$$

4.2.1 Metabolism of Adenyl Cyclase

Two enzymes are involved in the metabolism of cAMP.

1. Adenyl cyclase

This enzyme catalyses the formation of cAMP and requires Mg^{2+} or Ma^{2+} for full activity (Rall & Sutherland, 1962). This enzyme was found to be lacking or immeasurably low in mammalian red cells (Sutherland et al, 1962), although the avian nucleated erythrocytes do contain measurable amounts of this enzyme (Rosen & Rosen, 1969). Studies concerning the localization of this enzyme in the nucleated avian erythrocytes suggested that it is in the cell membrane (Rosen & Rosen, 1969; Davoren & Sutherland, 1963). The active site of this enzyme was also shown to reside at the inner surface of the membrane (Øye & Sutherland, 1966). Adenyl cyclase is stimulated by high concentrations of fluoride 5 - 10mM (Rall & Sutherland, 1958). This stimulation could not be observed at early stages of development and the fluoride was thought to be inhibitory at these stages (Schmidt et al, 1970). The reason for the reverse action of this ion is not known.

2. Phosphodiesterase

This enzyme was first observed in heart, brain and liver tissues and had the ability to destroy the activity of cAMP by hydrolysis (Sutherland & Rall, 1958).

$$cAMP \xrightarrow[Mg^{2+}]{\text{phosphodiesterase}} 5'\text{- AMP}$$

Mg^{2+} was found to be important for full activity of the enzyme, while caffein and theophylline strongly inhibited the activity (Butcher & Sutherland, 1962). The inhibition by theophylline was found to be competitive (Butcher & Sutherland, 1962) while that for caffeine was non-competitive (Nair, 1966). The Km values of phosphodiesterase differs from one type of tissue to the other and it was found to be 10^{-4} cAMP in the heart (Butcher & Sutherland, 1962) and slightly higher in the brain (Cheung, 1967).

4.3 HORMONE RECEPTORS AND cAMP AS A SECOND MESSENGER

Certain hormones are either closely related to or part of the adenyl cyclase system. The hormone-receptor interaction may be stimulatory or inhibitory to the enzyme adenyl cyclase. This will reflect the change of level of cAMP. The cell responds to this change and a physiological event takes place. Glycogenolysis in muscle is a good example for this sequence which is represented in fig 4.2.

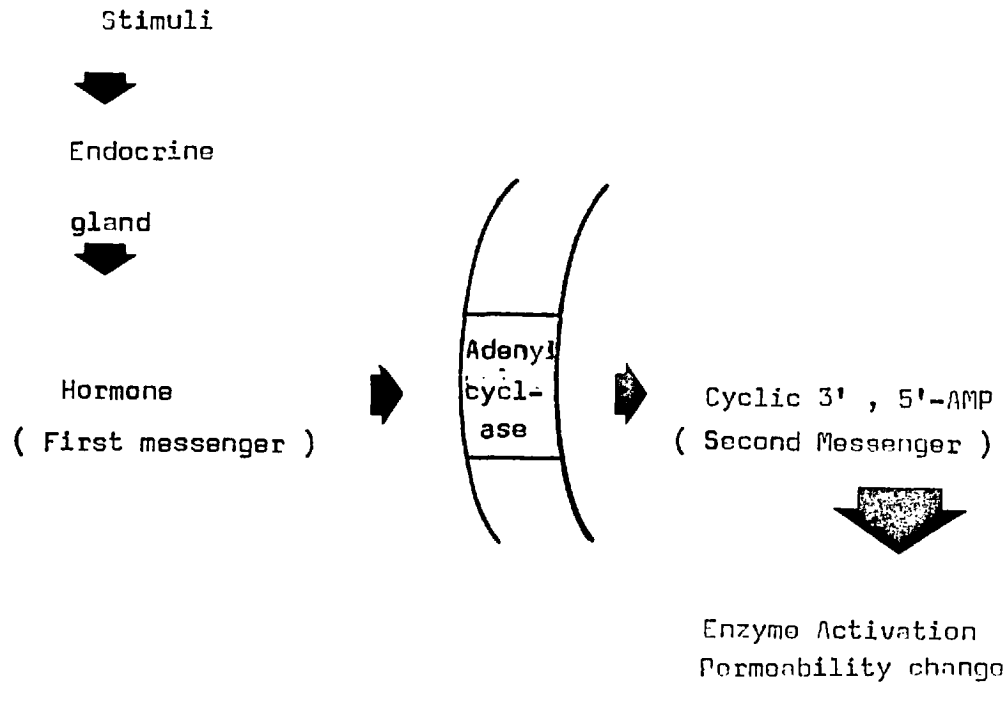


Fig 4.2 Diagramatic representation of second messenger concept.

Therefore the hormones carry the information to the cell thus acting as a first messenger leading to changed level of cAMP which acts as a second meesenger in carrying these informations to parts of the cell concerned with a specialised function.

SECTION 5

5.1 AGEING OF THE RED CELL

The average life span of the normal red cell in the circulation is 100 - 120 days. After this period the cells are squestered by the spleen due to changes in their viability, structure and metabolic activity. The reasen for these changes is not known yet. In order to fully understand and characterize the biochemical and biophysical features associated with these changes, many attempts were carried out to separate the old red cells from the heterogenous population of cells in the circulating blodd. The separation was achieved firstly by high speed or differential centrifugation based on the fact that the densities of the old cells are greater than the young cells, (Garby & Hjelm, 1963). Therefore the layer at the bottom of the centrifuged tubes are the old cells and the top represent the young celle (Garby & Hjelm, 1963; Valeri & MacCollum, 1965).

The use of density gradient centrifugation has revealed better separation of the cells into distinguishable layers according to their age, (Piomellis et al, 1967). As mentioned above, the cells undergo several alterations as they proceed in age in the circulation. These changes could be summarized as follows:

5.1.1 Physical alteration

The physical and mechanical properties of the red cells and their membranes change as the cells age. These properties are represented by changes in the surface area to volume ratio, the flexibility and elasticity of their membrane which allow the cell to conform to variable shapes during their passage through the capillaries of the micto circulation whose diameter is less than the diameter of the individual cell. The capability of the

cell to pass through these capillaries is entirely confined to its deformability. The spherocyte, for example, is not capable of passing through a narrow aperture due to the rigidity of its membrane. This rigidity is due to decreased surface area/volume ratio compared to that of the normal discocyte which allow the expansion and thinning of the cell during its passage through the aperture. The decreased surface area/volume ratio is due to loss of part of the membrane, through microfragmentation, without the loss of cell content during the life span of the cell. The *in vitro* controlled microfragmentation of the cell membrane without the loss of haemoglobin was developed by La Celle (1972). The deformability of the cell after each fragmentation process was measured and defined as the pressure required to cause the cells to pass through micropipette of certain diameter. The smaller the pressure required the more deformable the cell is and easier passage is achieved. The surface area and volume were measured also after each fragmentation process. The cells acquired the spherocytic shape accompanied by greater pressure required to cause them to pass through the aperture.

In vivo these spherocytes are incapable of passing through the capillaries and soon they will rupture and be equestred by the spleen. The decrease of the surface area/volume ratio of the old cell was demonstrated and referred to fragmentation as well as thickening of the cell membrane as examined by electronmicroscopy. (Bretcher & Bessis, 1972).

The osmotic fragility of the cells is increased with increasing age and cells become more vulnerable to haemolysis (Marks & Johnson, 1958; Danon, 1967). This increase of osmotic fragility of the old cell was shown to be due to decreased intracellular level of ATP (Weed, 1970).

5.2 Lipid contents during ageing

One of the biochemical alterations that occure during ageing of the cell is the change in the lipid content of the cell. The total lipid, phospholipid and cholesterol concentration was decreased in old cells compared to young cells, (Westerman et al, 1963; Van Gaster et al, 1966). Although these authors have shown that there is no significant alterations in the lipid and phospholipids content in the old and young cells when measurements were taken regarding the surface area and volume of the cells. The decrease was more significant on single cell level. However, the cellular cholesterol level decreased in the old cells regardless of the measurements taken. The level of the cellular cholesterol depends largely on its level in the plasma and many studies using labelling technique have shown that a rather rapid exchange of free cholesterol between the plasma and the cell occurs (Bell et al, 1972, Bell & Schwartz, 1971). This exchange process is non-enzymic and non-energy dependent although the precise molecular explanation for it is not available. The cholesterol content in the red cell membrane and the degree of saturation of the constituent fatty acids are vital factors in regulating the cell permeability to various substances, (Demel et al, 1972). Therefore a decrease in the cholesterol and other lipids may be responsible for the removal of the cells from the circulation since they contribute to the membrane structure and alteration in their concentration may lead to alteration in the membrane integrity and specific activities of the membrane bound enzymes, some of which have been shown to be associated with lipids.

The rate of uptake of linoleate and palmitate and lysophospholipids from the plasma into phospholipids in the cells or their membrane has been shown to be variable in different age groups,

(Witerbourn C. & Batt R.D., 1970). The mechanism involved in the uptake of the free fatty acids and lysophospholipids from the plasma could be represented by this equation:

$$\text{Fatty acid} + \text{ATP} + \text{COA} \xrightarrow{\text{Thiokinase}} \text{Acyl - CoA} + \text{AMP}$$

$$\text{Acyl CoA} + \text{lysophospholipids} \xrightarrow{\text{acyetreneferqsee}} \text{phospholipid}$$

The variation in the uptake of plasma fatty acid and lysophospholipids is probably due to change in the specific activity of the lysophospholipid-specific-acyltransferase with age of the cell, (Kohlschutter et al, 1968).

5.3 METABOLIC ALTERATION DURING AGEING

5.3.1 The glycolytic pathway

The glycolytic pathway provides the major source of energy vital for the survival of the mature erythrocyte in the circulation. Activities of certain enzymes of this pathway appeared to be decreased with a decrease of the overall rate of glycolysis in the old cell compared to young cells, (Bennett et al, 1966). Of particular importance are the enzymes which are normally present in low concentration in the cell such as phosphofructokinase (PFK) and hexokinase (HK) and are rate limiting in the control of glycolysis. The HK is a non-specific enzyme which catalyses the phosphorylation of glucose or hexosesto glucose -6- phosphate or hexose-6- phosphate; it is inhibited by its product and activated by ATP, or rather Mg-ATP (Rapoport, 1968). Four-fold decrease in the activity of this enzyme was estimated with increase in age, (Chapmann & Schaumburg, 1969). This decrease may lead to a decrease in the rate of glycolysis and hence a decrease in the

viability of the cell which leads to removal of the cell from the circulation.

5.3.2 The pentose phosphate pathway (PPP)

The activity of glucose-phosphate dehydrogenase (GGPD)

This enzyme is rate limiting in PPP. It catalyses the first step in this pathway by conversion of glucose -6- phosphate (G-6-P) to 6-phosphogluconate (6-PG) and ultimately reduces $NADP^+$ to NADPH which is subsequently used to reduce GSSG to GSH - by glutathione reductase. It also acts as a coenzyme for meth. haemoglobin reductase (diaphorase II) which reduces the oxidised haemoglobin, methaemoglobin. Therefore, G-6-PD protects the cell from oxidation. Decrease, or deficiency, of this enzyme render the cells to haemolysis in the presence of oxidants (e.g. H_2O_2 which accompanies phagocytosis by leukocytes), due to oxidation of cell contents (e.g. haemoglobin, glutathion etc.) and peroxidation of the membrane lipids. The thyroid hormone thyroxine and the peroxide radicals were suggested to be involved indirectly in the ageing process of the red cell due to the increased sensitivity of these cells to toxification by oxidants (Walle et al, 1976).

5.4 THE RED CELL DESTRUCTION

The biochemical and metabolic alteration accompanying the red cell ageing process leads eventually to the senescence and destruction of the cell. The reticuloendothelial system of the spleen is the major site for the destruction of the ecenescenced cell. The spleen mainly carries out the destruction although under certain conditions (such as that of increased rate of destruction of the cells) the liver may participate in performing

this function also. The exact mechaniem of the destructionof the red cell is not fully underetood.

The pathway of destruction of the red cell include osmotic lysis and perforation of the red cell membrane leading to loss of haemoglobin and other macromolecules. Also, fragmentation of the cell and phagocytosis of erythrocytes (by phagocytic cells of the reticuloendothelial system) play an important part.

5.4.1 Osmotic lysis

The increased permeability of the red cell membrane associated with the ageing process results in dieruption of the cation gradient. Water will enter the cell leading to its swelling and hence expanding the pores in the cell membrane. This will allow the haemoglobin and other macromolecules to leak out of the cell.

5.4.2 Perforation of the cell

This could be due to a defect in the membrane allowing the leakage of haemoglobin and cell contents without disruption of the cell's ionic gradient,

5.4.3 Fragmentation

This occurs due to loss of small pieces of the membrane from the cell surface. These pieces may or may not contain haemoglobin and the haemoglobin loss may not occur.

When fragments are small (Rand, 1964), extensive fragmentation will lead to decreased area/volume ratio resulting in spherocytosis. The fragmentation process is also accompanied by decreased deformability of the red cell membrane which interferes with the ability of the cell to pass through the microcirculation and hence sequestration occurs.

5.4.4 Erythrophagocytosis

This may occur intravascularly, i.e. the red cell may be removed from the circulation directly by phagocylosis of the monocytes in the circulation, or it may take place extra-vascularly, e.g. antibody coated erythrocytes (Lo Bulgio et al, 1967). It may also occur in drug-induced haemolytic anaemia (Croft et al, 1968).

SECTION 6

6.1 THE HAEMOLYTIC ANAEMIA

Haemolytic disorders are aeeociated with abnormally ehort survival of the red cell in the circulation and -- early deetruction by the epleen. This abnormality could, in certain conditions, be compenaated for by hyperactivity and increaeed rate of erythropoieeis. When this compeneation can not be achieved, due to increaeed rate of destruction compared to the rate of erythropoieeis, the level of haemoglobin in the circulating blood will be decreaeed and an anaemia develop. The haemolytic anaemiae are claeeified according to the type of abnormality, whether it ie intrineic to the cell, i.e. due to abnormal haemoglobin eynthesis or abnormal membranee, or it is extrineic arieing from abnormality in the eurrounding environment, e.g. plaema or in tieeue external to the red cell, ae in certain drug induced abnormalitiee. The former type of abnormality repreeente the hereditary haemolytic anaemia, while the latter repreeente the acquired haemolytic anaemia. In the following an intrineic haemolytic anaemia, known ae hereditary epherocytoeie, ie discuseed.

6.2 HEREDITARY SPHEROCYTOSIS (H.S.)

This is an inheritable dieeaee aeeociated with epherocytic erythrocytes with increased osmotic fragility. The abnormality ie thought to be inherited ae a eingle dominant gene (Race, 1942).

The celle are microspherocytic under the light and electron microecope compated to the biconcave shape of the normal red cell. The celle appear 'thicker' and take more intenee etain than normal cells. The surface area of the RBC is smaller compared to the

normal cells, although the volume is not very different from normal. The excessive Na^+ gain compared to K^+ loss and consequent gain of water leading to swelling are characteristic of the spherocytic cells (Selwyn & Dacie,1954). This lose was observed when normal cells were stored in a sucrose free medium either at 37 or 4 C (Harris, 1941). These findings led to the suggestion that a defect in the cation pump of the red cells membrane in patients with hereditary spherocytosis exists (Jacob, 1964). Also, an abnormality concerning glycolysis was sought, since ATP is the energy source of the cation pump. A defective phosphorylation of the glycolytic intermediates by ATP was suggested (Tabechian et al, 1956) although this has not been confirmed. Furthermore, the level of the glycolytic intermediates and ATP was reported to be normal (Shafer, 1964). The ATP level decreased more rapidly than in normal cells in the absence of glucose (Mohler, 1965). Several enzymes of the glycolytic pathway have been assayed and also found to be normal (Tanaka et al, 1972), eliminating the possibility that the defect is due to defficiency of a glycolytic enzyme.

The attention was drawn to the possible defect in the membrane components of the epherocytes since no defect was detected in the cell "cytoplasm". The lipid contents of the cell membrane were studied in cells from patients with hereditary spherocytosis and significant defficiency from the normal was found (Reed& Swisher,1966) Fragmentation of the membrane was suggested (Selwyn & Dacie, 1954) when the cells swell after 24 hrs of incubation under sterile conditions. This was followed by a decrease in size with considerable

haemolysis during the second 24 hrs of incubation. The loss of lipids in the spherocytes was more rapid compared to the normal cells incubated under the same conditions (Prankerd, 1960a). This was further confirmed by Jacob (1967) and Reed & Swisher (1966). These authors, however, did not explain the reason for the rapid lipid loss in the spherocytes. The lipids were lost in quantities proportional to their concentration in the cell membrane (Reed & Swisher, 1966) suggesting fragmentation of the cell membrane leading to smaller surface area/volume ratio. The fragmentation process was shown to occur by microscopic techniques (Weed & Weiss, 1966; Weed, 1968).

The increased osmotic fragility may be explained by the loss of the membrane fragments as there is less membrane into which the cells can swell before a critical rupture occurs. However, the gross lipid loss from membranes of the red cells from patients with hereditary spherocytosis does not explain the basic genetic abnormality. Therefore, an abnormality in one or more proteins in the cell membrane was suggested (Jacob & Kaynovsky, 1967). The protein spectrin was amongst those which were studied in the H.S. This protein formed microfillamentous network at the inner surface of the red cell membrane after the addition of Mg^{2+} ions and ATP, (Marchesi & Steers, 1968). The aggregation and the consequent formation of these microfillaments could be detected by ultracentrifugation techniques. This aggregation is detected by the increase in the sedimentation rate of the aggregated protein. The spectrin, extracted from normal and H.S. red cell membrane were examined by this technique. Jacobs and coworkers (1971) observed that when cations were added no change in the

sedimentation rate of the protein extracted from H.S. membrane was observed as it was observed in normal protein (Jacob et al, 1971). Further, analysis by the same author showed that this pattern is changed in some patients and that spectrin from H.S. membrane sediment in two peaks, one of which is similar to that of the normal, which represented the aggregated part of the protein, while the other peak represents the non-aggregated part, (Jacob, 1972).

These observations were further supported by the use of other techniques such as gel electrophoresis (Gomperts et al, 1972) which suggested a genetic defect in this protein in patients with hereditary spherocytosis. However, the results did not appear to be consistent. The effect of certain drugs on this protein was also studied. Antimitotic drugs e.g. vinblastine colchicine and strychnine, are known to reversibly denature and precipitate the microfillamentous proteins from which the mitotic spindle is formed (Marantz et al, 1969; Wilson et al, 1970). A microfillamentous protein spectrin is also precipitated by these drugs. The extent of the precipitation was examined on extracted protein from membranes of normal and H.S. cells (Jacob et al, 1971). Most of this protein of the normal cell membrane was precipitated while significantly less precipitated from the protein from H.S. membranes. The characteristic features of the antimitotic drugs is their ability to transform the normal red cells into characteristic shape of H.S. cell (Jacob, 1972). Short exposure of normal cells to these drugs leads to increased activity of the ($Na^+ + K^+$) pump with concomitant increase in the glycolytic rate. The cell

rigidity seemed to be altered as examined by the filtrability of the cell according to Teital (1964) technique. Prolonged incubation of these cells led to formation of microspherocytic cells (Jacob, 1972). Ultrastructural studies of the drug-treated normal cells showed that invagination of the membrane occurred following the fusion of the opposing edges of the invagination and with consequent release of the invaginated fragment inside the cell (Jacob et al, 1972). These observations were confirmed quantatively by radiolabelling of the extracellular fluid (Ben-Bassat et al, 1972).

E X P E R I M E N T A L

SECTION 7

7.1 THE (Ca^{2+} + Mg^{2+}) - ATPase OF THE RED CELL MEMBRANE

The active transport of Ca^{2+} ions from the red cell and the existence of a (Ca^{2+} + Mg^{2+}) - ATPase enzyme activity has been demonstrated by several workers (Coffrey et al, 1956; Herbert, 1956; Dunham & Glynn, 1961). Ca^{2+} was found to be stimulatory at low level and inhibitory at high levels (Wolf, 1970). Monovalent cations were also shown to be stimulatory at certain levels (Wolf, 1970); Shcatzmann & Rossi, 1971). These authors have shown that this enzyme may represent a part of an energy-dependent system which actively extrudes Ca^{2+} ions from the cell to maintain low level of this cation inside the cell.

The enzyme (Ca^{2+} + Mg^{2+}) - ATPase exhibits both high and low affinities for the substrate ATP (Wolf, 1972). It is not clear which part of this enzyme is involved in the Ca^{2+} transport.

Association of this enzyme activity with the fibrillar proteins of the red cell membrane was demonstrated (Ohnishi, 1962; Wins & Schoffenick, 1966; Rosenthal et al, 1970). The Ca^{2+} - ATPases in general are discussed further in section 8. In this section the activity of (Ca^{2+} + Mg^{2+}) - ATPase of the red cell membrane is investigated. Since the radioactively labelled substrate AT ^{32}P was not provided, a method for the synthesis of this substrate from ^{32}P is described.

7.2 METHODS

7.2.1 Synthesis of [γ - ^{32}P] - ATP

A carrier free ^{32}P inorganic phosphate in dilute HCl was used: the AT^{32}P was synthesised by the modification of the method of Post and Sen (1967). The specific activity was 33 Ci/mg.

The reaction was carried out in the fume-cupboard in the same vial in which the ^{32}P was supplied. Fig 7.1 shows a simplified diagram for the experimental set up. A long Yale needle (21G2) was inserted carefully into the rubber seal of the vial and pushed until it was in contact with the solution in the vial. Another short needle (20G2) was inserted which did not reach the level of the solution in the vial and this acted as an air vent. The concentration of H^+ ions was calculated from the value of pH stated on the vial and for each mole of HCl, 1.5 mole of tris was required for neutralisation. A solution of tris was prepared so that each 50µl contained exactly that amount of tris required to neutralize the vial. Using 1ml syringe 50ul was added to the vial via the long needle and the vial was swirled gently. After neutralization of the solution, 0.5ml of a co-factor solution was injected into the vial. The co-factor solution contained (in final concentrations) 4mM Na_2 EDTA, 5mM Na_2ATP, 1mM Na_2 ADP, 5.0mM 3-phosphoglycerate and 0.2mM NAD. This was followed by addition of 0.5ml of tris/HCl (pH 8.1) and 2µl of β-mercaptoethanol was added followed by 10µg of phosphglyceric kinase and 30µg of phosphoglyceraldehyde dehydrogenase. Finally 50µl of 0.1M $MgCl_2$ was added and the vial was swirled several times to ensure complete mixing of the reagents added. The vial was incubated at 37°C for 10 min and swirled again in order that the solution touched the tip of the long needle. The needle was then withdrawn gently and the tip was shaked in 5ml of 0.1mM KH_2PO4 in 5% TCA and then discarded. 10µl aliquot of this solution was taken for counting in 10mls of scintillation fluid and to the rest of the solution 0.8gm of charcoal was added. The tube was left at room temperature for 20 min with occasional shaking. This was then centrifuged for 5 min in MSE Mistral 4L centrifuge and the supernate filtered

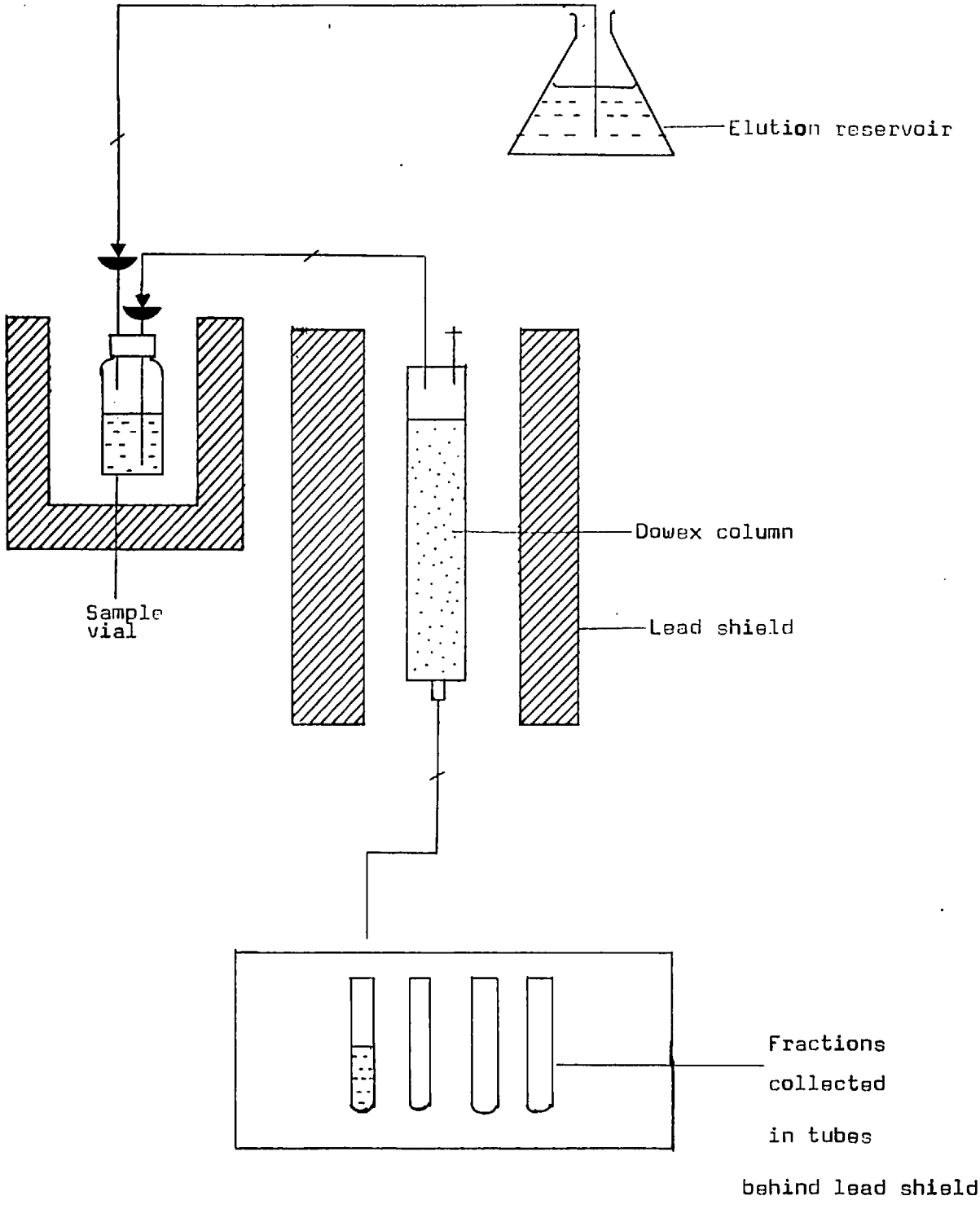


Fig 7.1 Diagramatic representation of set-up used for AT^{32}P synthesis.

through Whatman No. 1 filter paper. Aliquots were taken from the filtered supernatant and counted as before and the ratio of the counts after and before the addition of the charcoal was calculated. If this ratio was less than 0.5 then the reaction was terminated at 30 - 35 min of incubation by suspending the reaction bottle in boiling water bath for 3 min and allowing to cool at room temperature; otherwise the reaction was left to proceed until a ratio of less than 0.5 was obtained.

7.2.2 Column chromatography for $AT^{32}P$

Since the column chromatography method was used both in the purification of the $AT^{32}P$ and for assaying for ^{32}Pi liberated in the $(Ca^{2+} + Mg^{2+})$ - ATPase experiment it is important to measure the efficiency of the column in the recovery of the ^{32}P hydrolysed by the enzyme $(Ca^{2+} + Mg^{2+})$ - ATPase of the red cell membrane.

Different types of Dowex columns were used but it was found that the type AG-X~~1~~-8 (quartenary ammonium) was the most suitable. The resin (5gm approximately) was washed with 100 mls of 1M HCl for two minutes and then washed several times with distilled H_2O until the pH was in the range of 3 - 4, A piece of glass wool was added to the bottom of a small column (5ml plastic syringe was convenient) and the column was fixed in a vertical position. The resin slurry was then poured in carefully and (for trial experiments) 0.2ml of $AT^{32}P$ (2×10^6cpm) was added to the top of the column. The column was then eluted with 25ml each of 20, 200 and 500mM HCl. 5ml fractions were collected. Aliquots were taken from each fraction for O.D determination at 259 nm as well as for the radioactive estimation of the eluted free ^{32}P and $AT^{32}P$. External quench correction was applied by

spiking each vial with 10^3 dpm of AT^{32}P after each determination and counting again. Fig 7.2 shows the peaks of elution of ^{32}P as well as AT^{32}P as estimated by the O.D readings of the fractions at 259nm and the radioactive estimation. It is noticed that at HCl concentration of 20mM HCl, the ^{32}P was eluted first followed by the elution of AT^{32}P at concentration of 500mM HCl. The recovery of ATP was also calculated as percentage of the cpm added to the top of the column and that recovered in the fractions. As shown in Table 7.1 88% of the radioactivity was recovered. The phosphate peak was also detected by the colormetric method of Buginski et al (1967) for determination of inorganic phosphate. A small amount of AT^{32}P recovered from the fractions with absorption at 259nm was hydrolysed at 100°C in 1M HCl for 7 min and the samples reapplied to fresh dowex columns. The purity of AT^{32}P was further established by paper chromatography on Whatman No.1 paper in isobutyric acid; water; ammonia (0.88); EDTA (37 g/l) (500; 280; 21; 8). Cold ATP was added as a tracer. The spots were viewed under U.V. light. Two spots were generally obtained of ATP and ADP. The ATP fraction contained between 92 - 95% of the radioactivity.

7.2.3 Recovery of ^{32}P by the charcoal/celite mixture.

A mixture of 4gm each of activated charcoal/celite were suspended in 100ml of 0.1N HCl made in 5mM phosphate solution. The mixture was stirred constantly on ice. A stock solution of ^{32}P was prepared by the addition of 2μCi of ^{32}P (S.A.=5Ci/mM) to 10ml of 50μM ATP (in tris salt) pH 6.8. Aliquots of 100 - 500 μl of this stock were added to 1.25ml of 60mM histidine/imidazole buffer (pH 8.0) and the volume was completed to 2.5ml with water.

1ml of charcoal/celite mixture was added to each tube and shaken immediately. All tubes were kept in ice bath for 30 min with occasional stirring and then centrifuged at 2000 rpm for 10 min using MSE Mistral 4L refrigerated centrifuge. 100µl aliquots from the supernatant were taken in 10ml of scintillation fluid and counted in Intertechnique SL30 scintillation counter.

In some experiments ^{32}P was replaced by $AT^{32}P$ which was slightly hydrolysed. 1µCi of $AT^{32}P$ (specific activity; 2.1 mCi/mM) was added to 10ml of 50µM ATP pH 6.8 and aliquots of that were taken in histidine/imidazole buffer as described above.

7.2.4 Preparation of the red cell membrane

The procedure of membrane preparation was carried out at 4°C. Human blood taken in CPD as anticoagulant was used. The blood was suspended in twice its volume of isotonic saline (0.15M) and centrifuged at 1500 rpm (2000g approx.) in MSE Mistral 4L centrifuge for 5 min. The supernatant was aspirated and the white buffy coat removed with as little red cells as possible. This centrifugation procedure was repeated three times and the packed cells were haemolysed in 10 times its volume of hypotonic phosphate buffer (5mM, pH8). The haemolysate was centrifuged at 15000 rpm (16$\frac{1}{2}$000 × g) using Sorvall centrifuge and rotor.

The supernatant was aspirated as was the small packed sediment in the bottom of the tube underneath the ghosts which consist mainly of WBC. The ghosts were suspended in the same haemolysing buffer, mixed gently and recentrifuged as above. This procedure was repeated three times, or until a pale pink or cream colour of the ghosts was obtained. The ghosts were suspended in 5mM phosphate buffer (pH 8) to give protein concentration of 3 - 4 mg/ml and stored in small aliquots at -20°C until required.

7.2.5 Measurement of (Ca^{2+} + Mg^{2+}) - ATPase activity

The activity of the (Ca^{2+} + Mg^{2+}) - ATPase was expressed as the amount of the inorganic phosphate released due to the hydrolysis of the terminal phosphate of the substrate ATP by the membrane bound enzyme. The radioactivity labelled (γ - ^{32}P) ATP was used as substrate and the inorganic phosphate ^{32}P was determined by scintillation counting.

Accurate determination of the concentration of the free ionised calcium inside the cells could not be achieved, particularly at low levels due to the presence of contaminating Ca^{2+} ions. Therefore at these levels EGTA was used to maintain constant concentration of calcium ions during the experiment.

7.2.6 Preparation of the Ca-EGTA buffer

Histidine/imidazole buffer (pH 8.0) containing 0.1 mg/ml ouabain and 0.5mM EGTA was used. The concentration of EGTA and the total calcium concentration was adjusted to give the required free Ca^{2+} concentration. The concentrations were calculated according to Portzel et al, (1964). The following equation was used:

$$DK = \frac{[EGTA - Ca]}{[EGTA] \times [Ca^{2+}]\ free}$$

Where DK is the dissociation constant of the [Ca - EGTA] complex and [EGTA] is the concentration of free EGTA, [Ca^{2+}] free is that of free ionised calcium. Since DK, [EGTA] and total [Ca^{2+}] is known, the value of [Ca^{2+}] free can be obtained roughly. This value is reused in the corrected equation and another value is obtained. This procedure is repeated until a

constant value for $[Ca^{2+}]$ free is obtained.

7.2.7 Aeeay for the activity of the $(Ca^{2+} + Mg^{2+})$ - ATPase was carried out at different time intervals for up to 60 min. The final volume of the reaction mixture was 2.5ml. The final incubation mixture contained the following:-

30mM histidine/imidazole buffer pH 8.0

0.1mg/ml ouabain

0.5mM EGTA

0.2mM ATP (Tris salt)

5mM $MgCl_2$

0.2mM $CaCl_2$ (total concentration)

0.1M choline chloride

The membrane samplee were thawed, euspended in 0.1M choline chloride and centrifuged at 15,000 rpm. The pellet wae reeuspended in 0.1M choline chloride to original protein concentration. The tubes were preincubated for 5 min at 37°C and the reaction started by the addition of 0.5 - 0.2 mg of membrane protein. The tubee were incubated for the time period required and the reaction etopped by transferring the tubes to ice-bath and immediately adding 1ml of 4% charcoal/celite in 0.1N HCl from a beaker conetantly etirring the slurry. The tubes were ehaken and kept on ice for 30 minutes with occasional ehaking. The tubes were centrifuged for 5 min at 1500 rpm in MSE Mistral 4L centrifuge. Aliquote of 50 µl from the supernatant were taken for counting (for the amount of ^{32}P incorporated in the membrane), in 10 ml of ecintillation fluid (dioxane cocktail) ueing Intertechnique SL30 ecintillation counter. Tris-ATP was prepared by cation-exchange chromatography of Na_2-ATP.

7.2.8 The pH profile

The optimum pH value at which highest activity of the $(Ca^{2+} + Mg^{2+})$ - ATPase is obtained, was investigated. In this experiment the pH of the buffer was adjusted at incubation temperature (37°C) with the imidazole to values of 6.2, 6.6, 7.2, 7.6 and 8.0. The activity was measured for 10 min as described in section 7.2.7. Ca^{2+} in concentrations of 0.05, 0.1, 1.0 and 2.0mM were used.

7.2.9 Different concentrations of ATP

In these experiments varying concentrations of ATP were used and the Km of the enzyme was determined. The incubation conditions being the same as above.

7.2.10 Influence of monovalent cations

The possibility of the existence of different Ca^{2+} - ATPase according to the presence of monovalent cations was tested. To the incubation medium 0.1M of either NaCl or KCl was added and the activity was measured as described previously for up to 30 minutes of incubation. Equivalent amounts of choline chloride were omitted in order to maintain constant ionic strength.

7.2.11 Use of inhibitors

The effect of the inhibitors, ruthenium red and lanthanum chloride was tested. The assay for the $(Ca^{2+} + Mg^{2+})$ - ATPase was carried out as described above in the presence of 0.2mM CaCl and different concentration of the inhibitor. The buffer contained no ouabain or EGTA. The effect of the inhibitor on the $(Ca^{2+} + Mg^{2+})$ - ATPase at different Ca^{2+} concentrations was also tested. The ruthenum red solution was filtered before use.

7.3 RESULTS AND DISCUSSION

7.3.1 The syntheeis of $AT^{32}P$

The γ- ^{32}P-labelled ATP wae synthesised ae deecribed previouely and purified from contaminating unincorporated ^{32}Pi ae well ae ^{32}P labelled reaction intermediatee by chromatography. The efficiency of the Dowex AG1-x-8 used wae teeted. Thia wae done by meaeuring the O.D at 259nm of the fractione eluted using pure ATP eamplee. Aleo known amount of $AT^{32}P$ (1×10^6 cpm) wae heated in IM HCl in boiling water bath for 7 mine. The hydrolyeed $AT^{32}P$ was then rechromatographed using eimilar column. The column wae eluted with different concentration of HCl ae previouely deecribed and fractione were collected. Aliquote of each fraction were counted and ae it is shown in fig 7.2 all the radioactivity appeered in the ^{32}P fractions. Thie method showe that the $AT^{32}P$ is completely hydrolyeed by HCl and it containe ^{32}Pi only ae a contaminant which eluteo before the $AT^{32}P$ iteelf. However, the amount of ^{32}P incorporated in ATP when thie method wae employed varied between 39% to 84%. Other Dowex resins were used euch ae the typee AG 3-X-4 polyamine and the AG1-8-X polyamine. The type A-G 1-X-8 quatenary ammonium was found to be more eatiefactory and provided a good eeparation of the ^{32}P and the $AT^{32}P$ peake, (eee Fig 7.2).

7.3.2 Eetimation of ^{32}Pi by charcoal/celite mixture

Uaing miniature Dowex columns for a large number of experimental eamplee wae found to be troubleeome and time conauming. Therefore the charcoal/celite mixture wae used inetead. The activated charcoal epecifically binde $AT^{32}P$ only, the ^{32}Pi being meaured in the eupernatant. The charcoal/celite mixture wae made in phoephate eolution in order to eaturate the

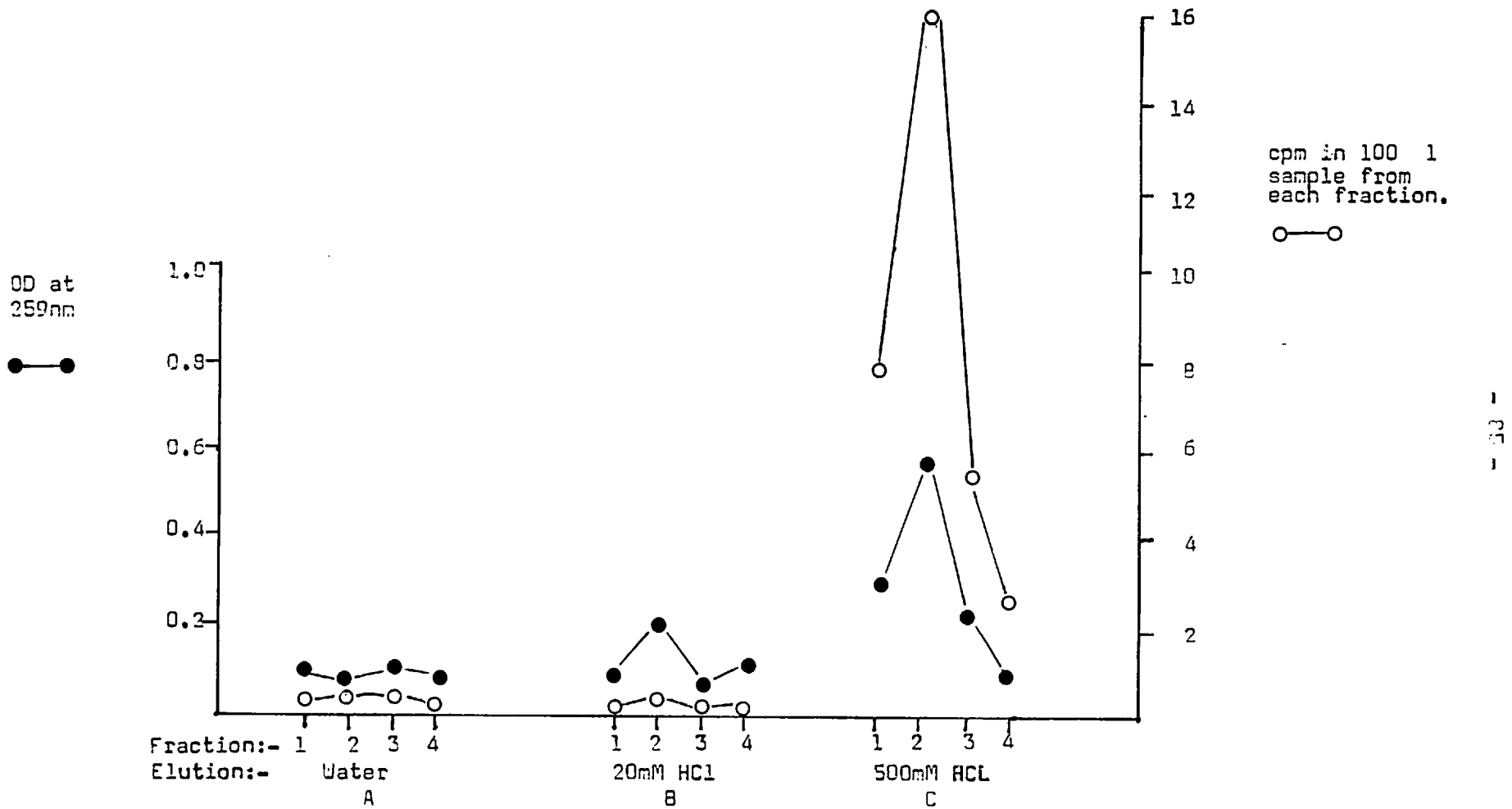


Fig 7.2 Elution of a freshly synthesised AT^{32}P sample on 5ml of Dowex AG-1-x-8 quarternary ammonium) column. Four fractions of 5ml each collected. The column was loaded normally with 2 x 10^6 dpm AT^{32}P. In this instance the loading was with 1.842 x 10^6 dpm AT^{32}P.. A: elution with water: no radioactivity or 259nm absorbing material eluted. B: elution with 20mM HCl: very low levels of radioactivity in fraction 2. Elution of ADP and phosphate detected. C: elution with 500mM HCl: approximately 88% of the activity loaded on the column found in this fraction. Detection of ATP without ADP in fraction 2. Note: to avoid superposition of activity and OD, a value of 0.05 added to each OD reading.

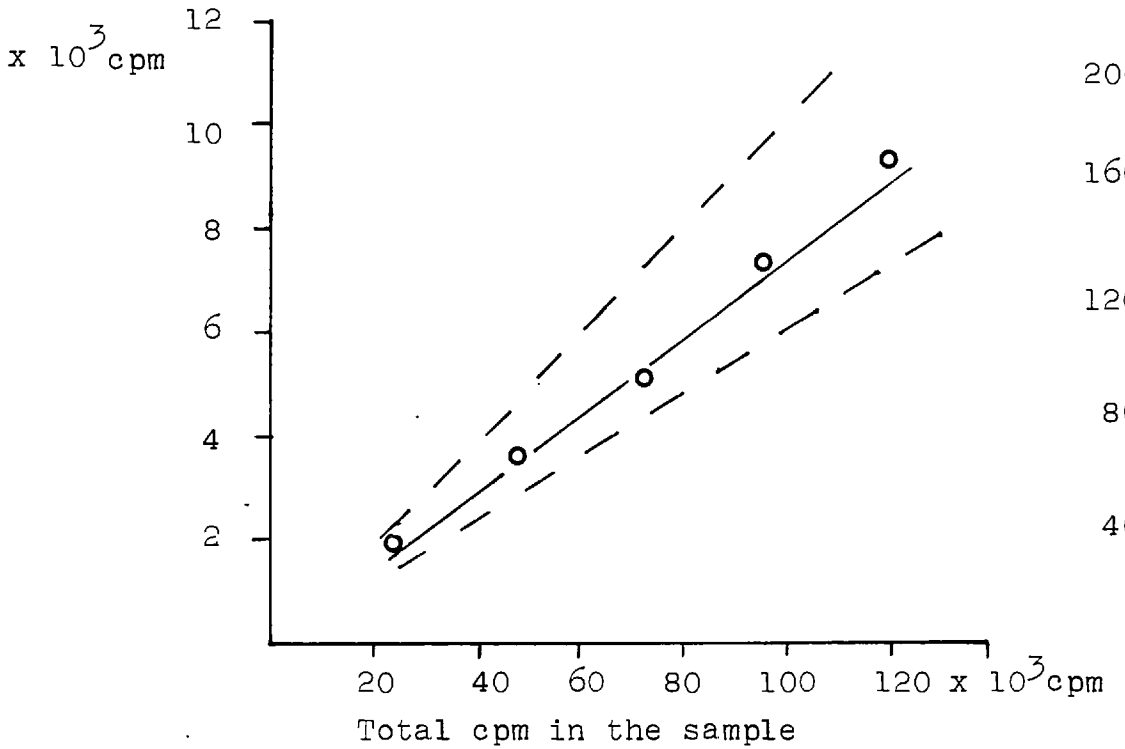


Fig 7.4 Estimation of ^{32}Pi in a sample of $AT^{32}P$ using charcoal/celite method of assay. The $AT^{32}P$ sample was purchased from the Radiochemical Centre, Amersham. All samples were measured at constant quench. Dotted lines show the limits suggested by the supplier. (See table 7.2)

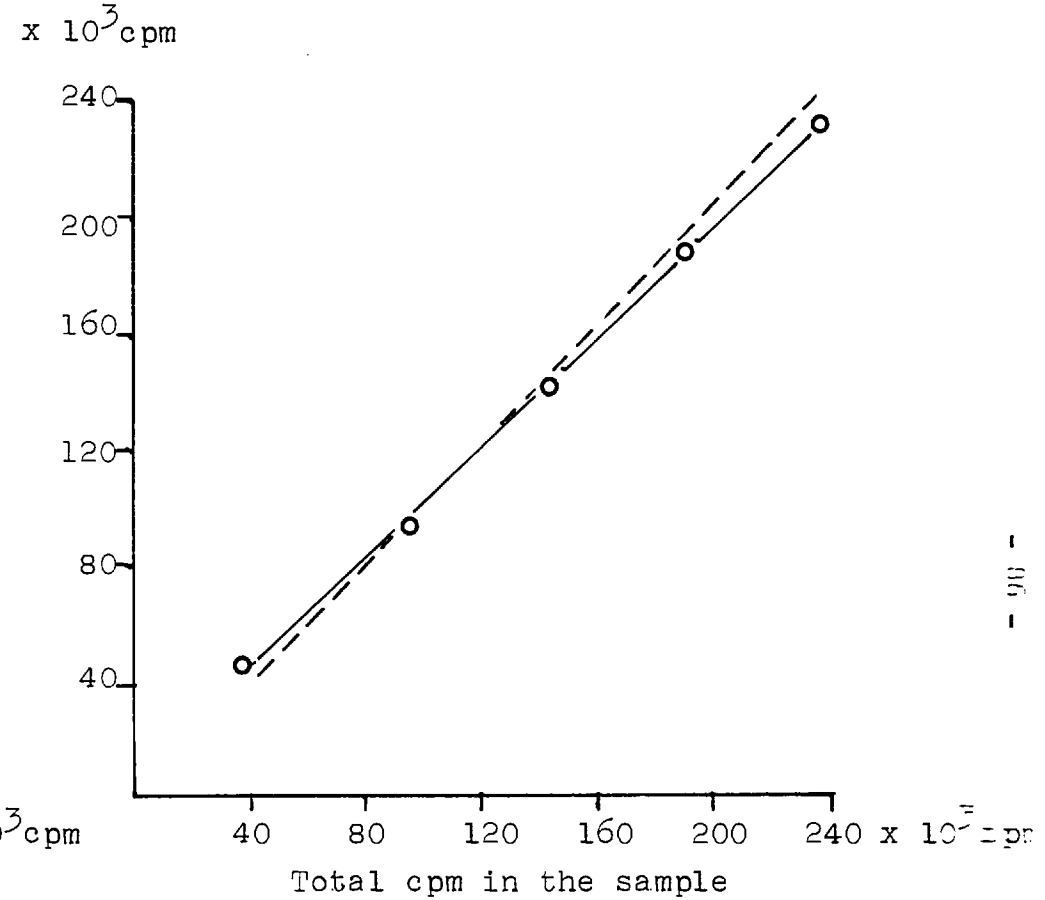


Fig 7.3 Recovery of ^{32}Pi using the charcoal/celite method of assay. Dotted line indicates a 100% recovery. Both Fig 7.3 and 7.4 show mean of three different duplicate determinations. (See table 7.1)

Table 7.1 Recovery of ^{32}Pi in the supernate of 4% charcoal/celite (2mM Na_2HPO_4) mixture for test of linearity of recovery of Pi. Correction was made for the volume of charcoal/celite in the slurry. Known counts of ^{32}Pi were added to each sample. The experiment was repeated three times in duplicate. Mean of each duplicate value is shown.

Sample No.	Total cpm in the sample	Total cpm recovered in charcoal/celite supernate			% Recovery of cpm	% Recovery of cpm (Mean)
1	48,560	46,925	45,969	46,957	96.6;94.7;96.7	96.0
2	97,120	94,151	93,006	92,893	96.9:95.8:95.6	96.1
3	145,680	138,893	139,985	140,177	95.3;96.1:96.2	95.8
4	194,240	186,501	185,930	187,219	96.0;95.7;96.4	96.0
5	242,800	228,205	231,724	229,186	94.0;95.4;94.4	94.6
					Mean of all Readings ± 1SD	= 95.70 ± 0.90

Table 7.2 Recovery of ^{32}Pi from ($AT^{32}P$ + ^{32}Pi) using charcoal/celite. The labelled ATP contained approximately 5 to 10% of free ^{32}P according to the manufacturer's estimate. The experiment was carried out three times in duplicate. The mean of duplicate value is shown.

Sample No.	Total cpm in the sample	Total cpm recovered in charcoal/celite supernate			% Recovery of cpm	% Recovery of cpm (Mean)
1	24,307	1,909	1,914	1,931	7.9;7.9;7.9	7.9
2	48,614	3,481	3,513	3,590	7.2;7.2;7.4	7.3
3	72,921	4,891	5,269	5,312	6.7;7.2;7.3	7.7
4	97,228	7,211	7,401	7,411	7.4;7.6;7.6	7.6
5	121,535	10,003	9,211	9,101	8.2;7.6;7.5	7.5

Mean ± ISD = 7.5 ± 0.01

non-specific binding sites on the charcoal which may bind ^{32}Pi. The celite helps a faster sedimentation of the charcoal and hence only low speed centrifugation is required. When ^{32}Pi was added and different aliquots were counted, a linear recovery was obtained over a six fold range and the recovery was 95.7% ($\pm$ 0.9) as shown in fig 7.3. When $AT^{32}P$ + ^{32}P was added the same linear response over six fold range in concentration was obtained as shown in fig 7.4. Also percentage recovery of ^{32}P was 7.5 ($\pm$ 0.01) as shown in Table 7.1 and 7.2. This agrees with the data quoted by the suppliers.

7.3.3 Preparation of the Ca-EGTA buffer system

Following the equation described in the method, the concentration of free Ca^{2+} was calculated in the presence of 0.5mM EGTA. Fig 7.5 represents the calibration curve of free Ca^{2+} concentration against different concentrations of total Ca^{2+}.

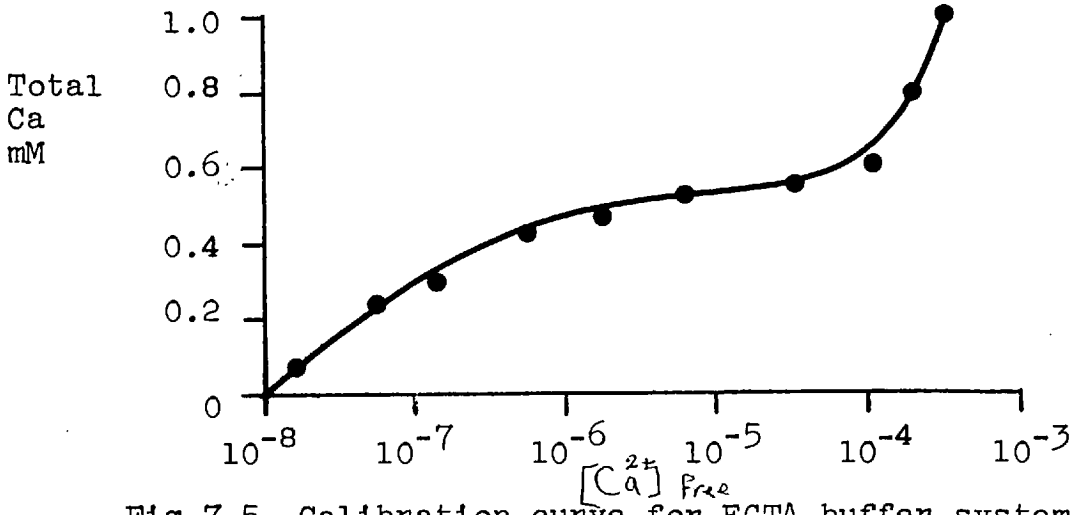


Fig 7.5 Calibration curve for EGTA buffer system

From this graph the corresponding total Ca^{2+} concentration was obtained for the desired free Ca^{2+} concentration. However, the EGTA buffer system was not generally used for free Ca^{2+} concentration above 0.2mM.

7.3.4 Time course studies

Time course experiments were carried out by incubation of the red cell ghosts for selected time periods under the conditions described in the method. The activity of the enzyme was expressed as the amount of Pi hydrolysed per mg ghost protein. As shown in fig 7.6 the reaction was linear for up to 30 min followed by reduced rate of hydrolysis reaching constant level in the second half hour of the incubation periods. The incubation time under these experimental conditions was chosen to be between 10 - 30 minutes for the preceding experiments. The hydrolysis rate is higher than that obtained by Schatzmann & Rossi (1971). One reason for the low values is probably due to measurements of the activity after 90 minutes of incubation (i.e. after the plateau is reached) in their studies.

7.3.5 The pH profile

Experiments were carried out to determine the pH value at which the maximum activity of the enzyme is obtained. Different Ca^{2+} concentrations were used in the assay as shown in fig 7.7. The pH value for maximum activity at all Ca^{2+} concentrations was 8.0. Values beyond this were not included in the experiment since assays carried out for the alkaline phosphatase showed that this enzyme does exhibit some activity, when histidine/imidazole buffer was used at pH 8.2. This activity was increased further when glycine buffer at pH 9.2 was used. Rega et al, (1973) also

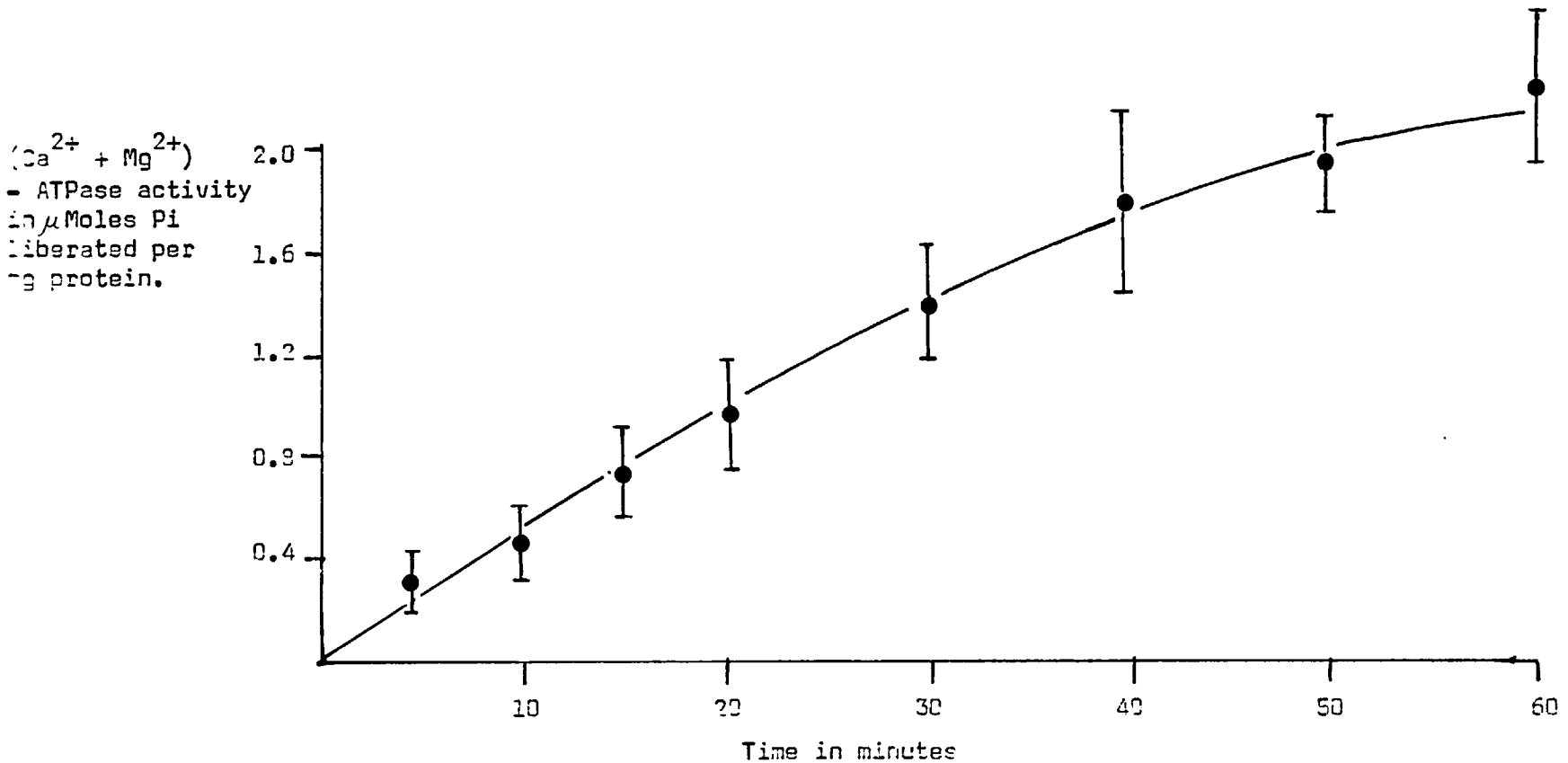


Fig 7.6 Time course for (Ca^{2+} + Mg^{2+}) - ATPase activity of normal red cell membranes. Ca^{2+} was present at a concentration of 0.2mM in absence of EGTA and ouabain. The activity was found to be non-linear beyond 30 minutes.

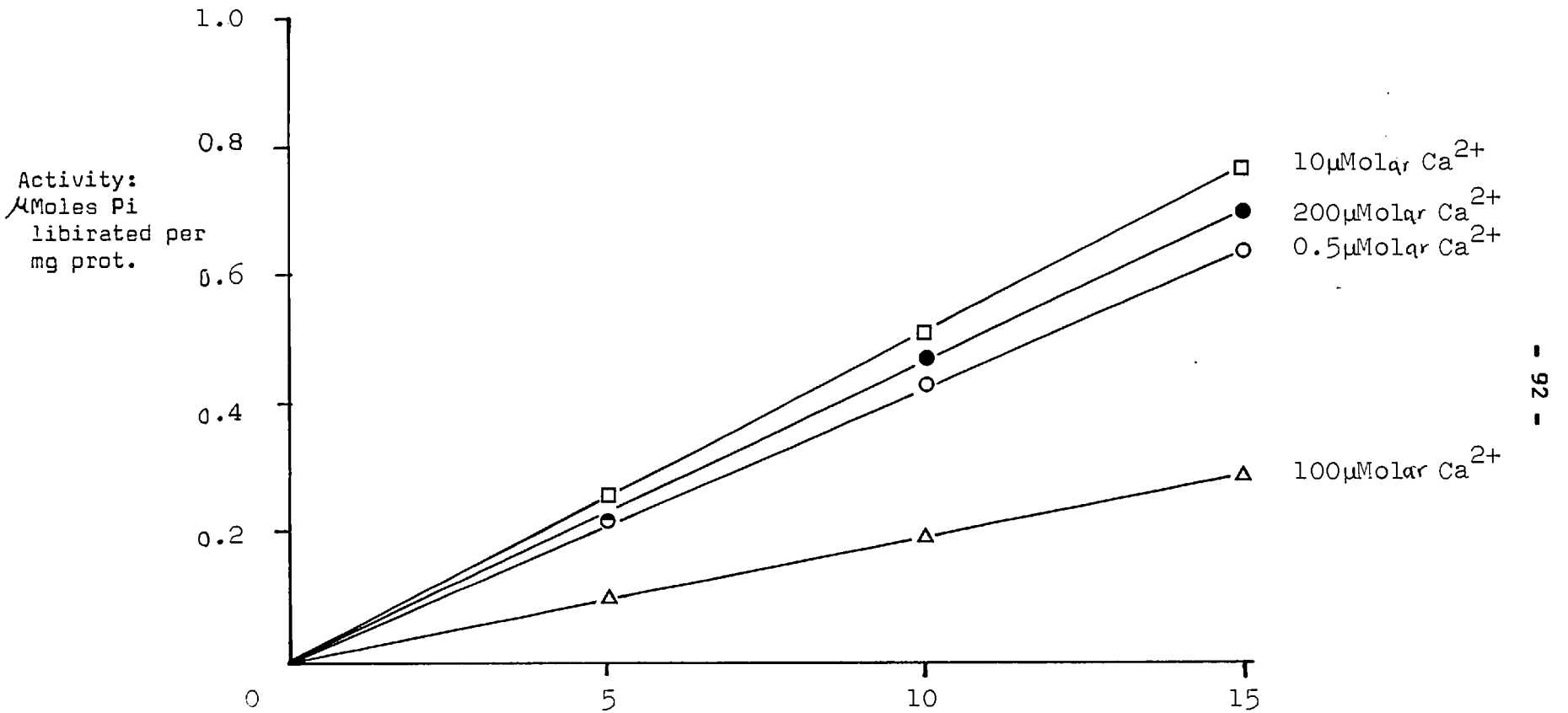


Fig 7.6a $(Ca^{2+} + Mg^{2+})$ - ATPase activity at pH 8.0 using various free Ca^{2+} concentrations.

$(Ca^{++} + Mg^{++})$ - ATPase
µMole Pi per minuts per mg
membrane protein.

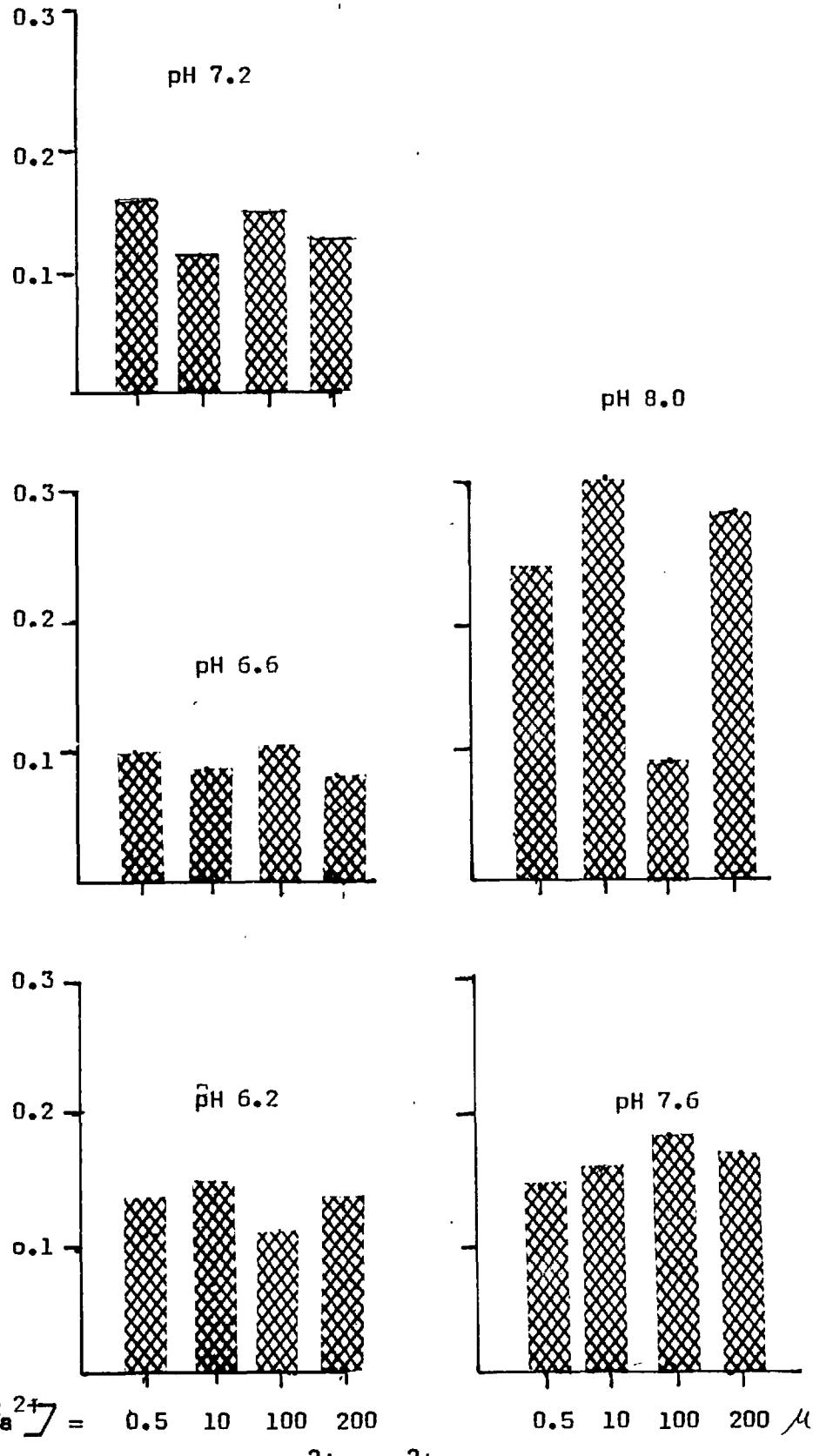


Fig 7.7 Activity of $(Ca^{2+} + Mg^{2+})$ - ATPase at various pHs in presence of EGTA buffer system.

Table 7.3

pH profile for (Ca^{2+} + Mg^{2+}) - ATPase using different concentrations of Ca^{2+}. Each determination is a mean of six values using three different normal membrane samples. The mean zero $\lceil Ca^{2+} \rfloor$ value for each pH was subtracted as background activity from each value of the determination made at a particular Ca^{2+} concentration. 1 S.D. is shown of the resulting values. *Choline chloride wash was ommited.*

Ca^{2+}	Activity: µmole Pi hydrolysed per mg protein per hr				
µMole	pH 6.2	pH 6.6	pH 7.2	pH 7.6	pH 8.0
0.0	1.74 ±(0.43)	2.09 ±(0.41)	2.64 ±(0.50)	3.03 ±(0.63)	3.62 ±(0.72)
0.5	1.06 ±(0.35)	1.14 ±(0.29)	1.61 ±(0.78)	1.42 ±(0.72)	2.55 ±(0.86)
10.0	1.55 ±(0.28)	0.84 ±(0.39)	1.34 ±(0.67)	1.57 ±(0.85)	3.08 ±(0.84)
100.0	1.03 ±(0.42)	0.78 ±(0.32)	1.51 ±(0.81)	1.85 ±(0.61)	1.15 ±(0.63)
200.0	1.29 ±(0.31)	0.70 ±(0.44)	1.32 ±(0.65)	1.69 ±(0.69)	2.79 ±(0.73)

have shown that under these conditions the alkaline phosphatase is activated. However, under the experimental conditions used in this study, the alkaline phosphatase activity does not significantly affect the $(Ca^{2+} + Mg^{2+})$ - ATPase assay.

7.3.6 Different Ca-ATPases

Apart from the $(Ca^{2+} + Mg^{2+})$ - ATPase which is activated at a wide range of Ca^{2+} concentrations, there appears to be additional activities of this enzyme when Na^{+} or K^{+} ions are present in the incubation medium. Fig 7.8 shows that during the time course of the experiment the $(Ca^{2+} + Mg^{2+})$ - ATPase is further stimulated by addition of the alkali monovalent cations Na^{+} and K^{+}. The stimulation of the enzyme by Na^{+} is greater than by K^{+}. This is not in agreement with the results obtained by Schatzmann & Rossi (1971).

These authors have excluded the possibility that these ATPases can participate in the transport of Ca^{2+} across the cell membrane because this stimulation occurs at fairly high concentration of Ca^{2+}. Therefore the low affinity part of the Ca^{2+}-ATPase only exhibits this stimulation by monovalent cations. Moreover, Schatzmann & Rossi (1971) stated that the rate of the outward movements of Ca^{2+} were not altered by the addition of K^{+} or Na^{+}. The possibility that these ATPases play a role in the $(Na^{+} + K^{+})$ - ATPase or probably are manifestations of this enzyme is excluded since ouabain (a potent inhibitor of the $(Na^{+} + K^{+})$ - ATPase was present in the incubation medium at a final concentration of 0.1 mg/ml. Ouabain was shown to have no effect on the activity of $(Ca^{2+} + Mg^{2+} + K^{+})$ - and $(Ca^{2+} + Mg^{2+} + Na^{+})$ - ATPases

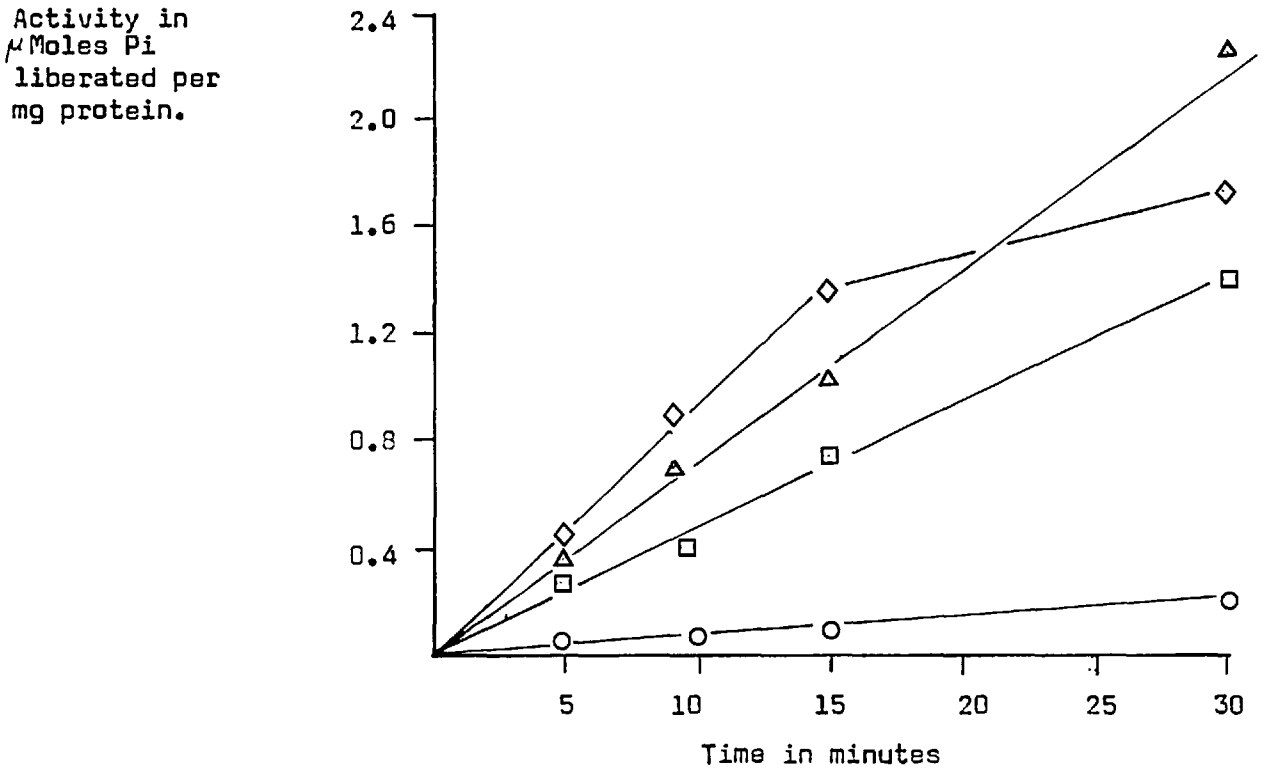


Fig 7.8 Different ATPase activity in presence of 0.2mM Ca^{2+} and 0.1mg/ml ouabain. When either Na^{+} or K^{+} were used (at 100mM) an equivalent amount of choline was removed from the incubation medium. Mg^{2+}ATPase:(O - O) Activity in absence of Ca^{2+}. (Ca^{2+} + Mg^{2+}) - ATPase (□ - □) (Ca^{2+} + Mg^{2+} + K^{+}) - ATPase (△ - △). (Ca^{2+} + Mg^{2+} + Na^{+}) - ATPase (◇ - ◇).

Table 7.4

Activity of different ATPases in the presence of the monovalent cations. Each point is a mean of 6 determinations ± 1 S.D. $\lceil Ca^{2+} \rfloor$ = 1μM; $\lceil Mg^{2+} \rfloor$ = 5mM; $\lceil K^{+} \rfloor$ = 100mM; $\lceil Na^{+} \rfloor$ = 100mM. EGTA buffer system was used.

Time (mins)	Activity: μmole Pi hydrolysed per mg protein			
	Mg^{2+}	Ca^{2+}+Mg^{2+}	Ca^{2+}+ Mg^{2+}+ K^{+}	Ca^{2+}+ Mg^{2+}+ Na^{+}
5	0.0422 ±(0.002)	0.328 ±(0.089)	0.358 ±(0.132)	0.361 ±(0.120)
10	0.062 ±(0.009)	0.401 ±(0.099)	0.710 ±(0.150)	0.880 ±(0.182)
15	0.0789 ±(0.013)	0.776 ±(0.130)	1.053 ±(0.261)	1.299 ±(0.300)
20	1.220 ±(0.010)	0.920 ±(0.351)	1.420 ±(0.285)	1.465 ±(0.332)
30	0.224 ±(0.018)	1.389 ±(0.280)	2.258 ±(0.305)	1.740 ±(0.271)

(Schatzmann & Rossi, 1971). These authors have also suggested that (Ca^{2+} + Mg^{2+} + Na^{+}) and (Ca^{2+} + Mg^{2+} + K^{+}) - ATPases may be a result of uncoupling action of Ca^{2+} on the (Na^{+} + K^{+}) - ATPase. If that is true then data presented by many authors on the (Ca^{2+} + Mg^{2+}) - ATPase activity measured in the presence of monovalent cation must be corrected for this excess uncoupled activity.

7.3.7 Determination of the Km value of MgATP for the (Ca^{2+} + Mg^{2+}) - ATPase

The activity of the enzyme was measured at different concentrations of the substrate ATP in the presence of 0.2mM Ca^{2+}. From Linweaver-Burk plots as shown in fig 7.9, the Km values were estimated to be 70μM and 310μM. These values are in agreement with that of Wolf (1972) which were estimated to be 40 to 50μM and 440μM respectively. The two values indicate the existence of more than one active site with different affinities for ATP. However, the concentration of ATP used in the incubation medium for this study for (Ca^{2+}+ Mg^{2+}) - ATPase ranges from 1 - 2.0mM, therefore the accessibility of the enzyme to its substrate is ensured. Argument arose that the true substrate for the enzyme is the complex (Mg.ATP) rather than ATP alone since Mg^{2+} is essential for the full activity of the enzyme (Wolf, 1972). Therefore an equal concentration of Mg^{2+} or more was used in the assay medium to allow sufficient Mg^{2+} for stimulation of the enzyme. No EGTA or ouabain was added to the buffer in these experiments.

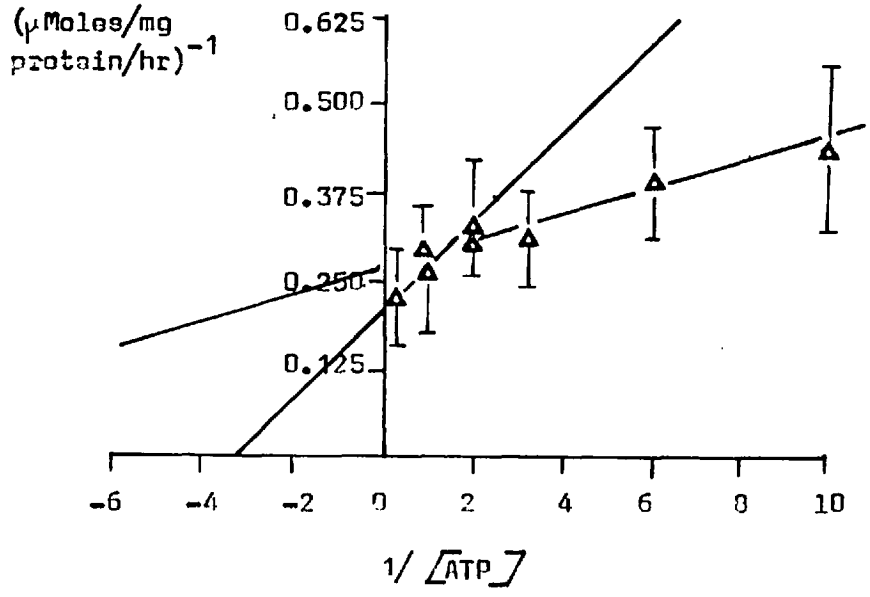


Fig 7.9 Km values for ATP for (Ca^{2+} + Mg^{2+}) - ATPase. The Km values were found to be 45 μM and 440 μM for ATP. Mean of three different membrane samples.

7.3.8 Use of inhibitors

Ruthenium red and lanthanum chloride were used as inhibitors for the (Ca^{2+} + Mg^{2+}) - ATPase in the red cell ghosts. The effect of ruthenium red was tested by varying concentration in the presence of 0.2mM Ca^{2+} as shown in fig 7.10. The K_i for ruthenium red was 0.4mM. Watson et al, (1971) have shown that 90% of the (Ca^{2+} + Mg^{2+}) activity is inhibited by 6 x 10^{-5}M ruthenium. The effect of lanthanum at concentration of 0.1mM on the high and low affinity (Ca^{2+} + Mg^{2+}) - ATPase was also investigated. Table 7.5 shows the percentage of inhibition of the enzyme at Ca^{2+} concentrations of 0.05mM and 0.2mM.

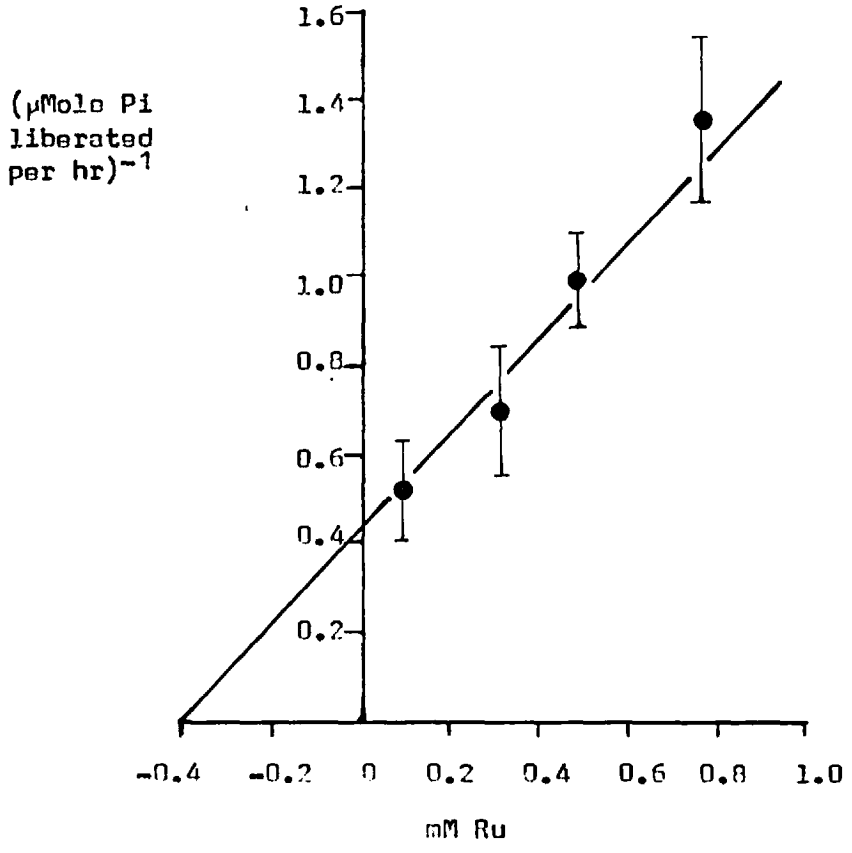


Fig 7.10 Inhibition of (Ca^{2+} + Mg^{2+}) - ATPase activity by Ru red. Mean of three determinations (± lSD shown).

Ruthenium red was, however, highly impure (20% impurity estimated by the manufacturers, see Luft,(1971). It was filtered on Whatman paper No. 1 before use. The mechanism of inhibition of lanthanum and ruthenium is not fully understood and both of these inhibitors fail to block the part of ATPase involved in Ca^{2+} - transport. Several other inhibitors have also been described (Wolf, 1973), but the effects of these on Ca^{2+} transport is unknown.

Table 7.5 Inhibition of (Ca^{2+} + Mg^{2+}) - ATPase activity of red cell membrane in presences of 2mM lanthanum. Each value is a mean of four determinations. lSD is shown.

$[Ca^{2+}]$ mM	No La^{3+}	+ 2mM La^{3+}	% inhibition
0.05	2.18 ± 0.54	0.83 ± 0.23	62%
0.20	2.63 ± 0.62	0.51 ± 0.18	81%

SECTION 8

8.1 Ca^{2+} TRANSPORT IN INTACT RED CELLS AND MEMBRANE VESICLES

The molecular constitution of the biological membranes differ from one side of the cell surface to the other, thus accounting for its asymmetrical organisation. An approach to the study of localisation and organisation of membrane component is the preparation of intact membrane vesicles, with a particular orientation, and to treat this with macromolecular (impermeable) probes.

Red cell membrane vesicles could be obtained either with the normal orientation known as the right-side-out (RO) vesicles or with the internal surface exposed to the outside hence called the inside-out vesicles (IO). Steck et al (1970a) described a procedure for the preparations of the RO and IO vesicles. The vesiculisation was induced by suspension in low ionic strength buffer. Gentle homogenisation disrupted these vesicles. Dextran density gradient centrifugation revealed two bands in different density zones. The difference in the densities of the vesicles was thought to be due to the difference in charge density on either sides of the membrane, (Steck et al, 1970b).

The RO have higher density than the IO due to high density of sialic acid anions on their surface which was found to be accessible to sialidase, (Steck et al, 1970a). However, when $MgSO_4$ was added to the homogenate, a different pattern was observed. An additional diffused band appeared containing a mixture of IO and RO vesicles. This was thought to be due to the effect of the cation which caused a tight sealing of the vesicles (Kant & Steck, 1972).

The mechanism of vesiculisation is not fully understood but it is believed that suspension of the membrane in low ionic strength causes budding of the membrane either towards the outside or towards the inside (endocytosis) giving rise to the RO and IO respectively (Steck & Wallach, 1970).

In this section the transport of Ca^{2+} was investigated using the IO vesicle preparation. Since the active Ca^{2+} transport site is located at the inner surface of the membrane, these vesicles were found to be ideal for providing full accessibility of the enzyme active site to its substrate. Ca^{2+} transport in the intact red cells was also investigated using the ionophore A23187. The ionophore was incorporated in the membrane to allow the loading of the cell with $^{45}Ca^{2+}$. The effect of Ca^{2+} was then measured after elimination of the ionophore.

8.2 METHODS

8.2.1 Preparation of membrane

Fresh blood, taken in CPD, or outdated blood was used and the membrane was prepared as described previously (section 7).

8.2.2 Preparation of IO vesicles

The vesicles were prepared as shown in fig 8.1a.

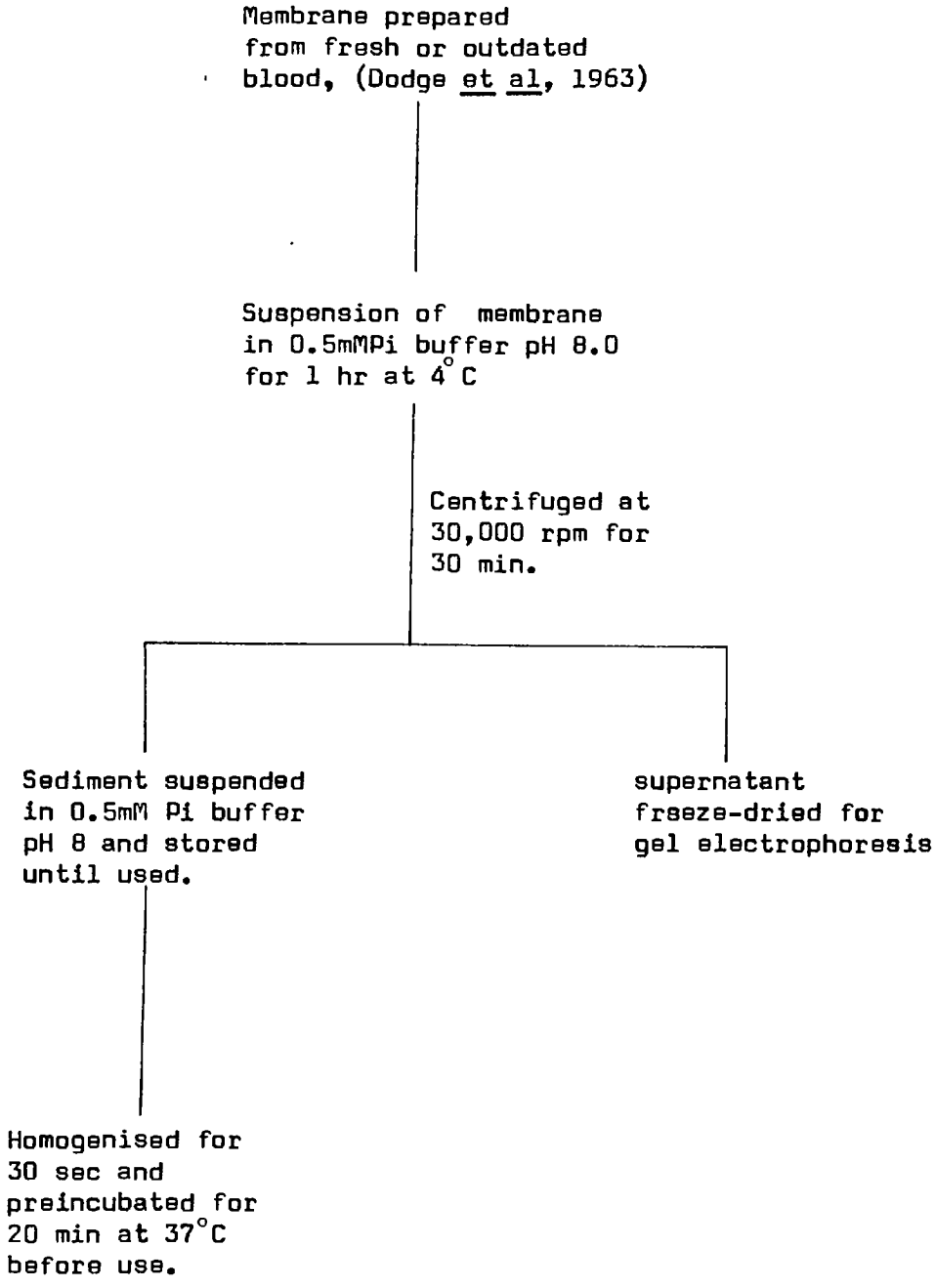


Fig 8.1a Flow chart showing the stages of preparation of membrane vesicles. Since the elimination of Mg^{2+} in the homogenisation step leads to a high proportion of the vesicles to be IO in orientation the prep is termed the IO prep.

Non-freeze-thawed membrane was suspended in x 10 its volume of 0.5mM phosphate buffer and the suspension was kept in an ice bath for 1 hr with gentle shaking occasionally. The suspension was then centrifuged at 30,000 rpm for 30 minutes using MSE superspeed 75 centrifuge in 8 x 50 ml rotor. The supernatant was freeze-dried and the powder was dissolved in H_2O and subjected for electrophoresis as will be shown later (Section 15). The sediment was suspended in 0.5mM phosphate buffer pH 8.0 to give protein concentration 2 - 3 mg/ml as estimated by the method of Lowry et al (1951). This was kept at -20°C until required. The vesicle preparation was thawed and homogenised at 4°C using Warring blender homogeniser (MSE) for 30 seconds at one third of full speed and incubated for 20 minutes at 37°C before addition to incubation mixture.

8.2.3 ^{45}Ca uptake by the IO vesicles

The uptake of the Ca^{++} ions by the membrane IO vesicles was measured using labelled ^{45}Ca. The incubation mixture in each tube contained the following in final concentrations.

histidine/imidazole buffer pH 8.0	30mM	1.25ml*
$CaCl_2$	0.2mM	0.25ml
KCl	120mM	0.25ml
NaCl	100mM	0.25ml
ATP or H_2O	1mM	0.25ml
$MgCl_2$	5mM	0.25ml
IO suspension	(2mg/ml)	0.10ml

* Stock solution volumes

to 2.5ml
The volume was completed ⌊ ⌊ by addition of H_2O and the tubes were preincubated at 37°C for 5 min before starting the reaction. The IO suspension was thawed, homogenised and pre-incubated at 37° C for 20 min before addition to the reaction mixture. The reaction was started at 37° C by the addition of the vesicle suspension and left to proceed for the time periods selected. The reaction was then stopped by immersing the tubes in an ice bath and immediately adding 5 mls of ice cold 30mM histidine/imidazole buffer pH 8.0. The tubes were centrifuged at 40,000 rpm in MSE superspeed 75 centrifuge using 10 x 10 ml rotor for 30 minutes. The supernatant was discarded and the tubes were drained. The sides were wiped and the sediment was dissolved in 500µl of 1% triton-x-100. Aliquots were taken in 10 mls of triton-toluene cocktail and counted for ^{45}Ca using Intertechnique SL30 scintillation counter.

8.2.4 Protein estimation

The protein concentration of each membrane sample was determined using the method of Lowry et al (1951).

Standard curve was obtained using different concentrations (0 - 400µg) of BSA.

Reagents

Alkaline solution:

This was prepared and used in the same day. 50 mls of sodium carbonate solution (2% w/v Na_2Co_3 in 0.1M NaOH) was mixed with 0.5ml of copper sulphate $CuSO_4$ $2H_2O$ 0.5% w/v and 0.5ml of Na, K tartarate 1%.

Folin-Ciocalteu reagent

The commercial solution kept at 4 °C was diluted 1 : 1 with distilled water and used on the same day. The samples (20 to 50 µg) were made up to 1ml with distilled water and 5ml of the alkaline solution was added, the tubes were shaken and left for 10 minutes. 0.5 ml of Folin-Coicalteu reagent was added followed immediately by vigorous shaking.

The tubes were left to stand at room temperature for 30 minutes with occasional mixing. The blank tube contained all the above reagents except for the protein samples. The spectrophotometer was calibrated using water and the above blank. The O.D. values were determined at 750 nm using Perkin Elmer 142 spectrophotometer.

8.2.5 Efflux of Ca^{2+} from intact red blood cells

Freshly drawn human blood (in CPD) was washed 3 times by centrifugation with 0.15M isotonic saline at 4°C and the buffy coat was removed after each wash. The packed cells were kept at 4°C. The incubation medium was prepared in an Erlenmyer flask and kept on ice at 0° C. *The mixture contained 0.165 mls of 0.16M KCl in 0.15mM tris (pH adjusted to 8.0 using HCl); 0.2 mls of 2×10^{-4} M ionophore A23187 and 0.25 mls of a solution containing 40mM $CaCl_2$ and 100mM NaCl to which 2 µl of 0.94×10^{-4} Ci/ml of ^{45}Ca was added. The final $CaCl_2$ was 2mM. 3mls of packed cells were poured into the incubation mixture and the flask was transferred immediately to 37° C with vigorous shaking for 2 minutes. The loading was stopped by pouring the mixture in 200mls of ice

* The loading of Ca^{2+} by ionophore was according to Sarkadi et al (1976)

cold 0.5% BSA in 0.16M KCl solution (pH 7.4). The mixture was rapidly centrifuged and the sediment was washed 3 times with the same buffer. The pellet was made to a haematocrit of 30% with the same washing buffer and the tube was incubated at 37° C in water bath with vigorous shaking for the time periods of

0, 3, 5, 10, 20, 30, 40, 50, 60 minutes. After each time period an aliquot of 1.2 mls was taken out of the tube and transferred to another test tube which was on ice. From this 500µl aliquots were layered gently on 7mls of ice cold solution of 0.7 M sucrose in 0.16M KCl and centrifuged at 3000 rpm for 15 minutes at 0°C on MSE Mistral 4L centrifuge. The supernatant was aspirated carefully and the pellet was dissolved in 500µl of 1% triton-x-100 solution. 50µl aliquots were taken for counting using either dioxane cocktail or triton cocktail scintillation fluid. In experiments where recovery of the label was studied, the supernatants were also saved and aliquots were taken for counting.

Initially quench correction was carried out using the external standards ratio. However, since the haemoglobin colour produced constant quenching in each batch of experiments, quenching could easily be determined by addition of unlabelled lysed cells to the standard after initially determining unquenched activity.

8.2.6 Uphill transport

Experiments were carried out as described above with the addition of 2µl of ^{45}Ca and 0.25 mls of 40mM $CaCl_2$ in 100mM NaCl solution to the cell suspension and incubated as above. A time course was carried out as described above.

8.2.7 Haemoglobin estimation

The amount of haemoglobin in each tube was measured according to the method of Dacie & Lewis (1975). Ten microlitres of the triton haemolysate was added to 4mls of Van Kampen solution containing (per litre) 200 mg of potassium ferricynide, 50 mg of

potassium cyanide,140 mg of potassium dihydrogen orthophosphate and 1ml of Nordite P40. The tubes were left at room temperature for 10 minutes and the developed yellow colour was meaeured at 540nm. The concentration of haemoglobin was determined against standard haemoglobin reagent (55.7 g/ml) by this equation:

$$\text{Hb conc (g/dl)} = \frac{\text{O.D}^{540}\ \text{std HiNC} \times 64{,}500 \times \text{dilution factor}}{44.0 \times d \times 1000 \times 10}$$

where $\text{O.D}^{540}\text{HiNc}$ = absorbance of std solution at 540

64,500 = molecular weight of haemoglobin

d = cuvette thickness in cm

1000 = conversion factor of mg to gm

10 = conversion factor of 1 to dl

8.2.8 $^{45}Ca^{2+}$ flux in IO prep

Experiments were carried out to measure the efflux of ^{45}Ca from previouely loaded inside-out (IO) prep. The vesicles were prepared from freshly drawn blood as described previouely and freeze-thawed once before homogenisation. The incubation mixture (total volume 38 mls) contained the following at the final concentrations shown:-

30mM Histidine/Imidazole buffer pH 8.0 containing:	
0.5mM EGTA + 0.1mg/ml ouabain	19mls
0.55mM Ca^{2+} total + ^{45}Ca (100 Ci)	3.8 mls
100mM choline chloride	1.52mls
veeicle suspension - 1.5mg/ml	9.12mls

The mixture was homogenised and incubated for 20 minutes before etarting the reaction as described previously. The reaction tubes

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8.3 RESULTS AND DISCUSSION

8.3.1 Ca^{2+} efflux measurements from intact cells after Ca^{2+} loading with the aid of ionophore A23187

The intact red cells were loaded with Ca^{2+} by briefly exposing washed red cells to the ionophore A23187. The ionophore was removed from the cells by rapid washing with BSA and KCl solution after the cells were loaded briefly with labelled $^{45}Ca^{2+}$ as a tracer.

The efflux of Ca^{2+} was monitored for various time intervals shown in fig 8.1, this being calculated by the amount of $^{45}Ca^{2+}$ remaining in the cells. Determination of Ca^{2+} levels by flame photometery on the lysate of such cells (with the membrane removed) showed no measureable changes in the specific activities of the tracer $^{45}Ca^{2+}$ from the initial incubation medium over the time course studied. The specific activity of the tracer in the incubation medium was therefore used for calculation for Ca^{2+} flux measurements.

The initial rate of efflux of Ca^{2+} was calculated by measuring the slope of the tangent drawn to the efflux curves as shown in fig 8.1. The initial rate of efflux was found to be in the range of 0.041 to 0.133 mmoles Ca^{2+} per litre of packed cells per min. The mean value was 0.080 $\pm$ 0.035 mmoles Ca^{2+} per litre of packed cells per minute (n = 5) as shown in table 8.1 .

The results were found to be variable even when only one

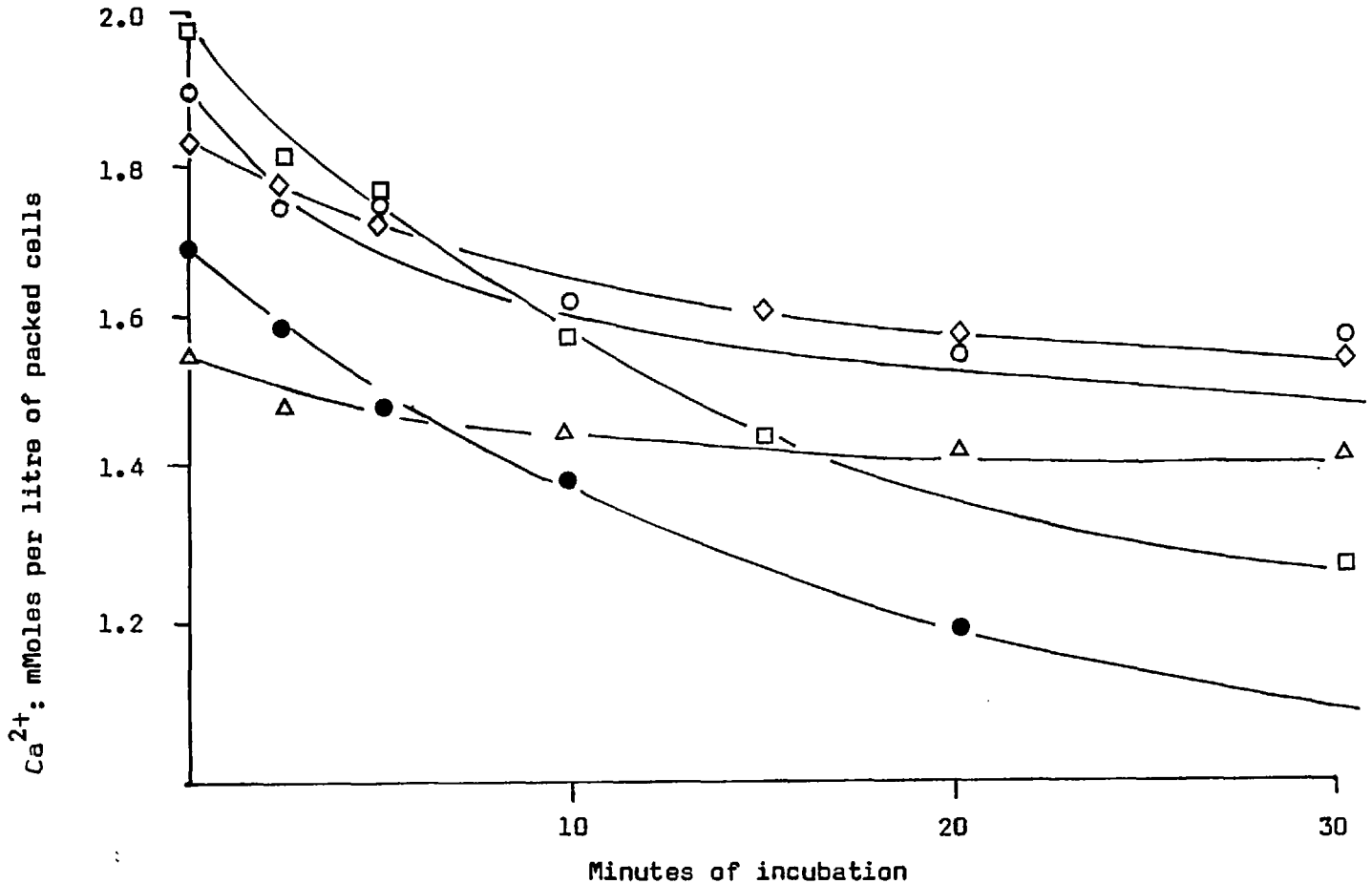

Fig 8.1 Efflux of Ca^{2+} in intact red cells loaded with Ca^{2+} after brief exposure to the Ca^{2+} ionophore, A23187. Initial rate of efflux was calculated by determining the slope of the tangent drawn at zero time. Values for initial efflux rates are shown in table 8.1 on the next page. Each curve is a mean of two determinations of different cells except for the open and closed circle symbols, which are of one subject repeated under identical conditions.

Table 8.1 Efflux of Ca^{2+} from intact red blood cells after initial rapid loading of Ca^{2+} with the aid of ionophore A23187. Each value is a mean of three determinations.

Time (mins)	mMoles Ca^{2+} per litre of packed cells at the time of determination.				
	1	2	3	4	5
0	1.40	1.12	1.6	1.80	1.98
3	1.20	1.00	1.66	1.56	1.64
5	1.09	0.98	1.56	1.44	1.48
10	0.80	0.91	1.26	1.49	1.16
15	0.61	0.89	1.35	1.18	0.92
20	0.39	0.87	1.26	1.13	0.84
25	0.27	0.85	1.17	1.11	0.68
30	0.19	0.83	1.15	1.11	0.60
Initial rate of efflux	-0.072 mMole/L/min	-0.063	-0.041	-0.092	-0.133

Total mean of Initial rate of efflux -0.080 (± - 0.035)

batch of the cells was investigated. The variability appears in the loading of Ca^{2+} as shown in fig 8.1. This is clear from the zero time values.

Experiments were also attempted in which the medium Ca^{2+} concentration was made approximately equal to the intracellular Ca^{2+} concentration. The reason behind this experiment was that the component of Ca^{2+} efflux due to passive diffusion would be cancelled if the outside medium contained Ca^{2+} at the same concentration as that inside the cell. However, of the few experiments attempted only one succeeded in having a medium concentration almost equal to that of the intracellular concentration. The difficulty was that the intracellular Ca^{2+} loading was variable, as shown, and this intracellular level could not be determined before the period of efflux experiment.

The one experiments in which a passive flux was cancelled had an active Ca^{2+} extrusion rate of 0.076 mMole Ca^{2+} per litre of packed cells per minute, which is within the mean value of the previous experiment in which passive Ca^{2+} flux could not be compensated for.

8.3.2 <u>Uptake of Ca^{2+} in IO prep. using incubation medium without ATP as a control for passive diffusion</u>

The problem of passive diffusion in the intact cells can be circumvented in the IO prep by two means:

(a) Control experiments in which only passive efflux takes place;

(b) A control experiment in which both passive diffusion and active flux occurs but in which no net accumulation of Ca^{2+}

is allowed. The experiment of type (a) is discussed in this sub-section and that of type (b) in the next.

As mentioned earlier, an IO prep is a preparation in which a mixture of IO and RO vesicles are present, the IO vesicles in this prep being greater than that of RO prep obtained by homogenisation in the presence of Mg^{2+}.

When Ca^{2+} is added to the incubation medium in an IO prep the cation will passively leak into the IO and RO vesicles (other membrane structure, such as the sediment of fig 8.1a, is not a sealed structure since it does not act as an osmometer. In these experiments this does not contribute to Ca^{2+} accumulation). On addition of ATP only the IO vesicles will accumulate Ca^{2+} actively since the ATP is accessible to the Ca^{2+} pump active site. Therefore the difference between the accumulation of Ca^{2+} in presence and absence of ATP (neglecting any binding effect of Ca^{2+} induced by ATP) is equal to active Ca^{2+} transport.

The results of such experiments are shown in figs 8.2 a, b and c where EGTA buffer system was not employed and where the Ca^{2+} concentration was 0.2mM. Three general types of Ca^{2+} accumulation occured with time. In all experiments 0.4 to 0.5 nmole of Ca^{2+} are bound to the membrane per mg protein at zero time of incubation. The difference between the +ATP and -ATP control exists and is equal to 0.15 nmoles per mg protein in each of the three experiments as shown in figs 8.2 a, b and c. The control without ATP being consistently lower than the

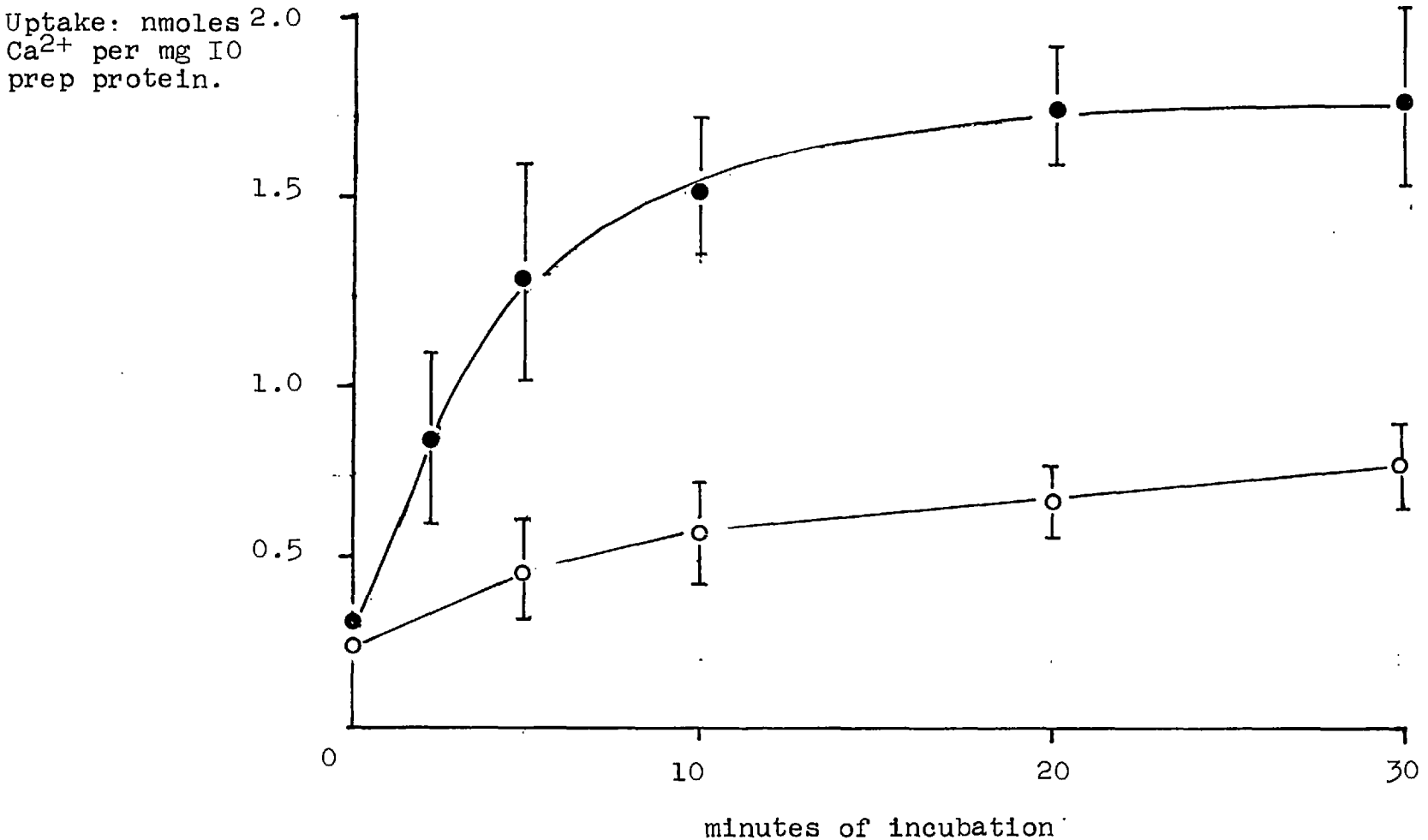

Fig 8.2a Ca^{2+} uptake into IO prep. Incubation medium contained Ca^{2+} at 0.2mM concentration (without EGTA). Active transport and passive diffusion was measured in presence of 2mM ATP (solid circles). Passive diffusion was obtained in absence of ATP (open circles) Note that zero time values show that Ca^{2+} is bound to the membranes and that a difference exists in presence and absence of ATP.

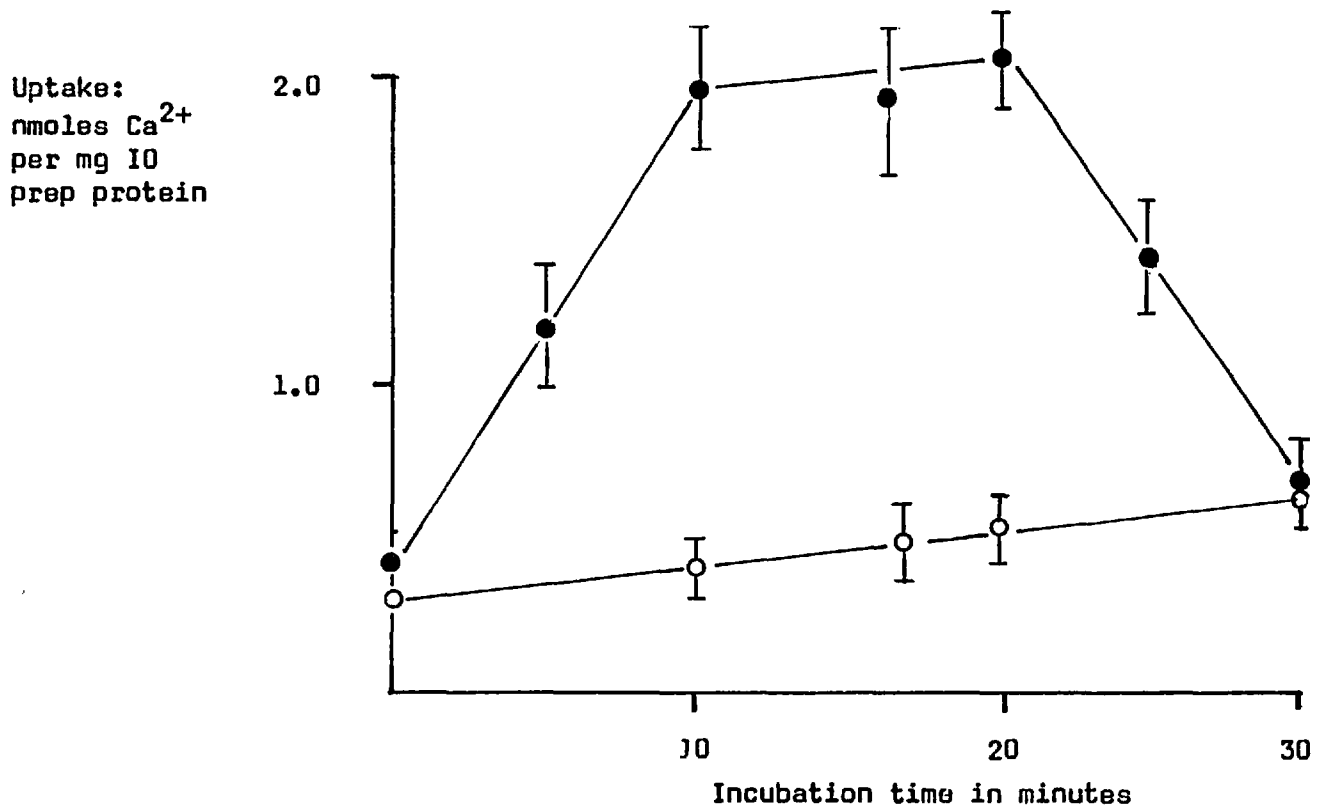

Fig 8.2b Uptake of Ca^{2+} into IO prep showing the instability of the accumulated Ca^{2+}. Conditions as described in fig 8.2a.

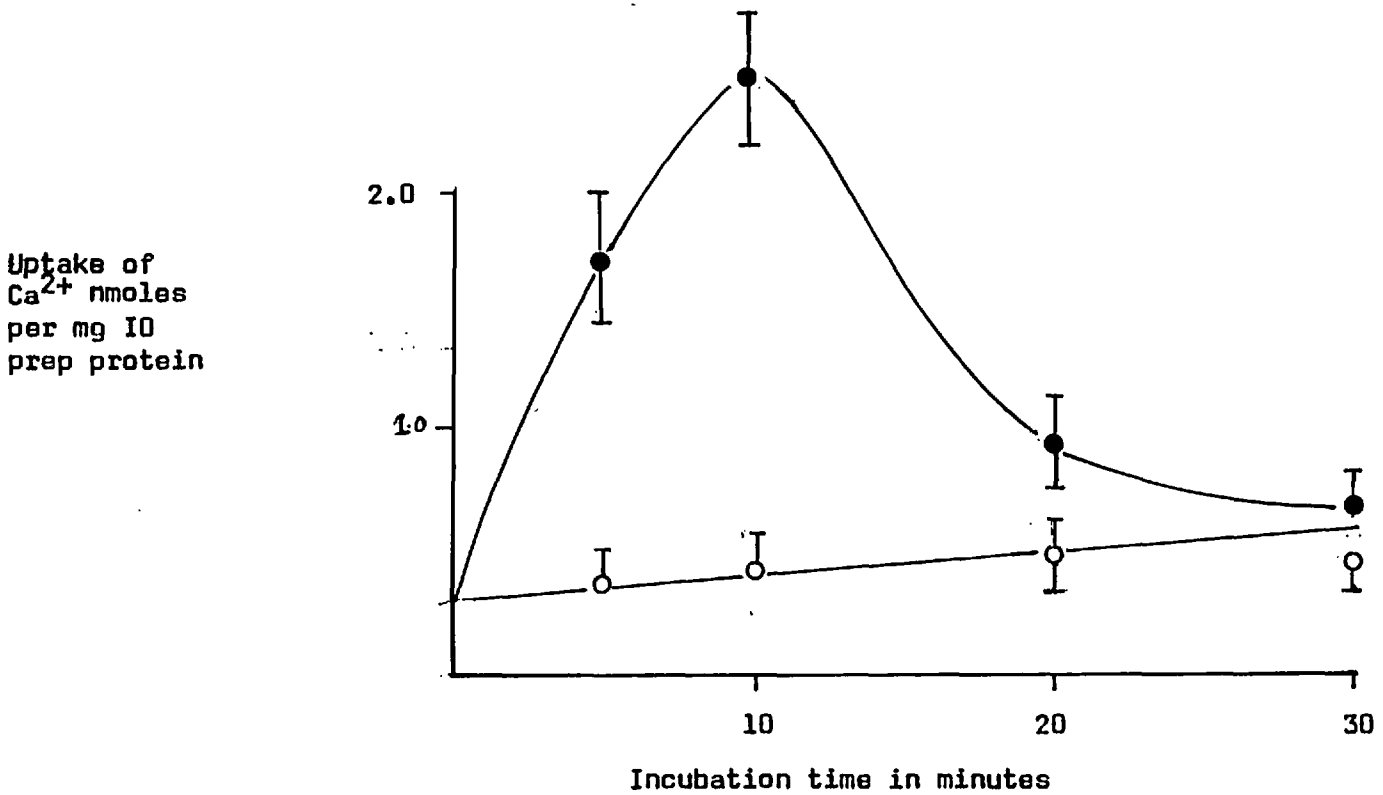

Fig 8.2c Uptake of Ca^{2+} into IO prep. The accumulation of Ca^{2+} in these preps was slightly in excess of the previous two figs 8.2a and 8.2b. Note the rapid loss of Ca^{2+}. Conditions same as for fig 8.2a.

experimental value with ATP in the incubation mixture.

The initial rate of influx of Ca^{2+} in the IO prep measured over the first five minutes of uptake with ATP was for 8.2a 0.14 nmole per mg protein per min, for 8.2b 0.10 nmole per mg protein per min and for 8.2c 0.11 nmoles per mg protein per min. Statistical analysis for differences in gradients for the three curves (taken a pair at a time) showed no difference at the $p = 0.05$ level. Analysis carried out according to Documenta Geigy (see appendix for further details).

The mean values of Ca^{2+} flux in presence of ATP was taken to be 0.119 ± 0.013 nmole Ca^{2+} per mg protein per min.

Similarly the values for incubation medium without ATP did not differ (fig 8.2 a, b and c) significantly and the mean value was 0.009 ± 0.004 nmoles Ca^{2+} per mg protein per min.

A difference in the three types of uptake was, however, observed when Ca^{2+} was accumulated to greater than about 2.0 nmoles per mg protein. In fig 8.2a, the Ca^{2+} concentration remained below this value for up to 90 minutes. In other experiments 8.2b and c this critical value was exceeded and a rapid loss of accumulated Ca^{2+} occured. In fig 8.2b it is seen that this value was approached in these experiments gradually and the loss of Ca^{2+} did not occur until well after 60 minutes of incubation. However, in other experiments, as shown in fig 8.2c, the critical value was exceeded rather faster and a similar loss of accumulated Ca^{2+} also occured (as in fig 8.2b) but rather more early.

Contd from p 110

were prepared beforehand containing 0.25ml of 20mM ATP (or phosphate Pi) and 0.05mls of 5mM $MgCl_2$. The reaction was started by addition of 2.2mls of the vesicles mixture to the preincubated tubes. The tubes were mixed and incubated for the time period selected and the reaction was stopped by immersing the tubes in an ice bath and immediately adding 10mls of ice cold 30mM histidine/ imidazole buffer (pH 8). The tubes were centrifuged at 30,000 rpm for 30 min using Beckman centrifuge (model L5 - 65 and 50.2 Ti rotor). The supernatant was aspirated after taking aliquots for counting and the sediment was dissolved in 1% w/v of triton-x-100. Aliquots were taken for counting in 10mls of Insta-gel (Packard) Scintillation fluid and counted for $^{45}Ca^{2+}$ in Beckman LS355 scintillation counter.

This phenomena is further discussed in the general conclusion. The effect of Ca^{2+} and ATP on the stability of these vesicles is discussed in section 9.

Entrapment of free Ca^{2+} level up to 50 μmole inside the vesicles did not lead to such Ca^{2+} losses as shown in the next sub-section. Since the accumulated Ca^{2+} in the IO vesicle is on the side of the membrane which correspond to the outside surface in the intact cell, it is not likely that this surface would respond to slight increases in Ca^{2+} levels.

8.3.3 Uptake of Ca^{2+} against a concentration gradient in IO prep using EGTA buffer system

Experiments were carried out in which the free level of Ca^{2+} was maintained at 0.05 mM in place of 0.2 mM, by use of the EGTA buffer system as described in the section 7.1.

The level of Ca^{2+} inside the vesicle prep was made approximately 0.004 mM greater than that in the incubation medium by homogenieation of the freeze-thawed vesicle preparation in the presence of 0.051 mM free Ca^{2+}. The incubation concentration of Ca^{2+} was reduced to 0.047 mM by addition of Mg ATP or Mg^{2+} and equivalent amount of inorganic phosphate after the preincubation step at which stage the vesicles had stabilised and sealed towards Ca^{2+}.

Fig 8.3 shows that the level of Ca^{2+} in the IO prep was 3.2 nmole per mg protein. Also no difference was noted in the presence or absence of ATP.

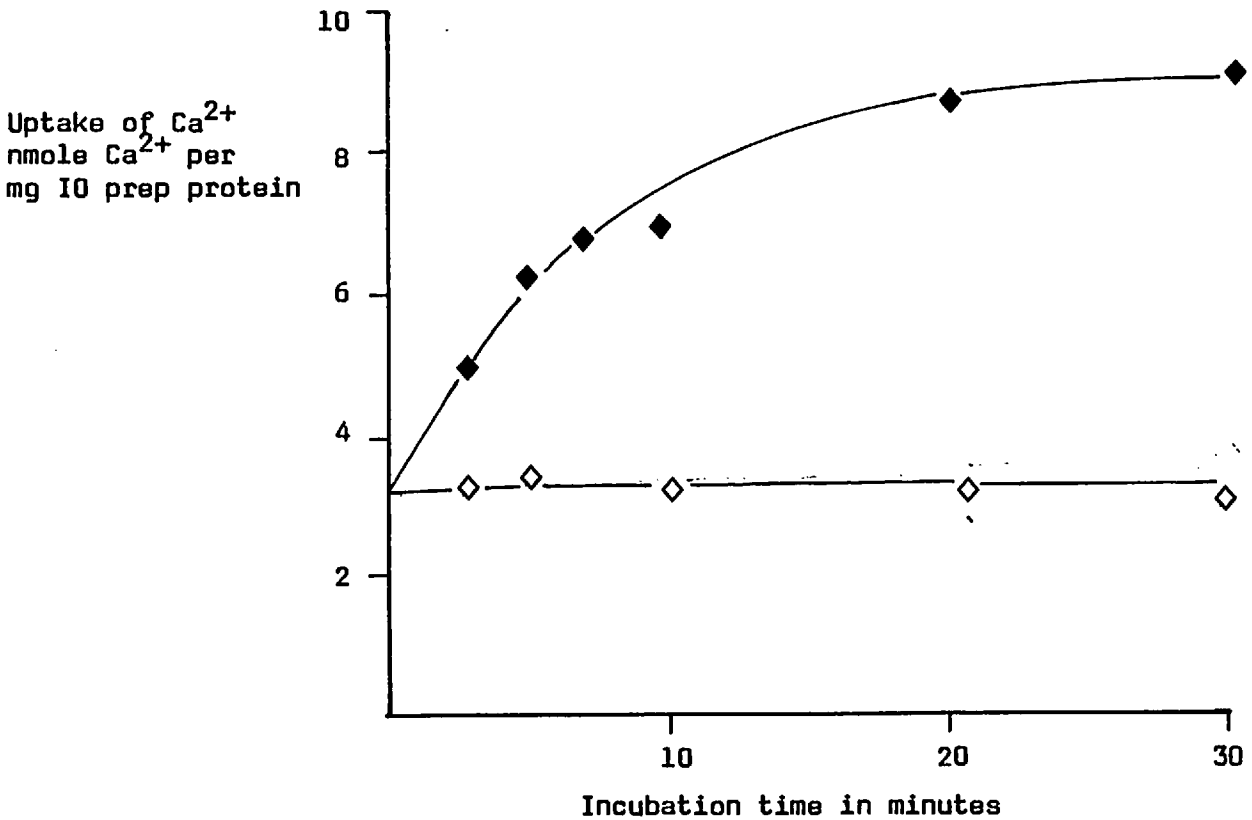

Fig 8.3 Uptake of Ca^{2+} in IO prep against concentration gradient. Solid symbols: + ATP Open symbols: -ATP in incubation medium. EGTA buffer system was used to make a Ca^{2+} concentration of 0.051 mM inside the vesicles.

Assuming a total Ca^{2+} concentration of 0.20 mMolar and that the binding of any Ca^{2+} does not significantly affect the results, this gives the total volume of intravesicular compartment to be 16.0 µl per mg protein. If the same amount of Ca^{2+} is bound to the membrane as in fig 8.2a, b and c, e.g. 0.45 nmole/mg protein) then this value is reduced to 13.8 ± 6.3 µl per mg protein. This value is not in agreement with ^{14}C-inulin exclusion experiments which gave a value of 59.2 ± 4.9 µl per mg protein. However, see Section 9 for stability effects of inulin with IO prep.

The initial rate of uptake of free Ca^{2+} was found to be 0.58 ± 0.06 nmole per mg protein per min. This velocity is approximately 4.9 times that in presence of 0.2 mM Ca^{2+} without the use of EGTA buffer system. The maximum level of Ca^{2+} accumulated occured after 30 minutes incubation and was found to be 9.10 ± 0.2 nmole per mg protein. If EGTA was not present inside the vesicles and all the vesicles were of IO type, then the Ca^{2+} concentration inside these vesicles would be 9.1 nmoles per 59.2 µl or 0.154 mMolar. However, under the experimental conditions used most of the Ca^{2+} is complexed to EGTA.

The passive diffusion was found to be less than 0.002 nmoles per mg protein per min and is less than one-fifth the value shown in figs 8.2 a, b and c. This indicates that the complex [Ca-EGTA] has a finite permeability since the value 0.002 nmoles per mg protein is greater than that expected for a chemical gradient of 0.004 mM of free Ca^{2+} present. Furthermore the direction of flux is

towards the outside of the vesicles into the medium, which is opposite to the previous experiments.

8.3.4 Uptake of Ca^{2+} against a concentration gradient using the ionophore A23187 as the control

Instead of using an incubation medium devoid of ATP as control, the experiments were carried out using ATP plus the ionophore A23187. The ionophore dissipates the Ca^{2+} concentration inside the cell within 8 minutes of incubation (fig 8.4) and gives an accurate estimate of the difference in concentration across the vesicle membrane. It also does not interfere with ATP induced Ca^{2+} binding and acts as a perfect control. Since the difference of free Ca^{2+} across the membrane is about 0.004 mmoles and the total vesicular volume is 59.2 µl, 0.232 nmoles of Ca^{2+} per mg protein should be released from the vesicles on addition of ionophore. However, the value is almost twice this (0.45 nmole per mg protein) and the result could be due to a shift in EGTA, Ca equillibrium position as must occur upon addition of the ionophore.

The ATP induced Ca^{2+} binding (if it occurs) is cancelled in this experiment since both the control and the experimental sample contained ATP.

8.4 Conclusion

The rate of efflux of Ca^{2+}, in red blood cells was found to be 0.080 ± 0.035 mmole per litre of packed cells per min using the ionophore for loading the cells with Ca^{2+}.

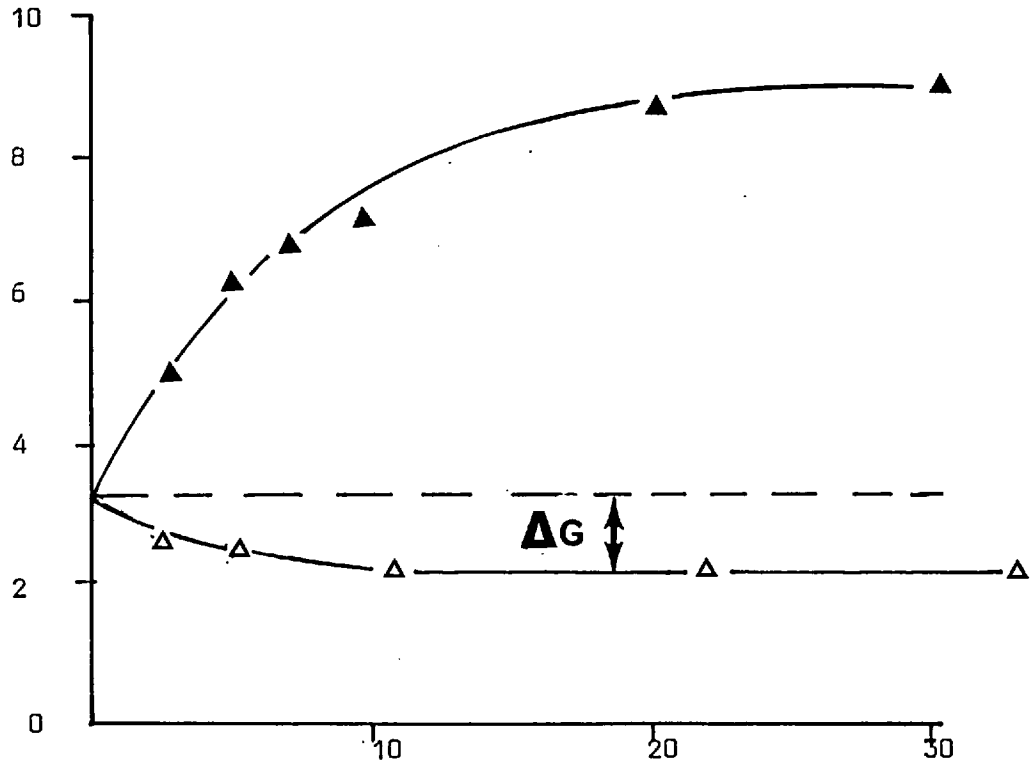

Fig 8.4 Uptake of Ca^{2+} against concentration gradient as described in fig8.3. Note the ATP (at 2mM) was also present in the controls (open symbols) as well as in the experimental samples (closed symbols). However, ionophore A23187was also present in control samples. The value ΔG indicates the value of Ca^{2+} gradient across the vesicles.

Active transport component was measured as 0.076 mmole per litre of packed cells per min in only one successful experiment. Difficulty was found in establishing a constant initial Ca^{2+} concentration across the cells.

In presence of 0.2 mM Ca^{2+} IO vesicles showed an initial uptake of 0.119 $\pm$ 0.013 nmoles Ca^{2+} per mg protein per min, in presence of ATP. In the absence of ATP this was 0.009 $\pm$ 0.004 nmole Ca^{2+} per mg protein per min. However, the vesicles were unable to retain the accumulated Ca^{2+} above 2.0 nmoles per mg protein. A rapid loss of Ca^{2+} occured when this value was exceeded in these experiments.

Uptake of Ca^{2+} against a concentration gradient (with free Ca^{2+} concentration of 0.05mM) showed an initial uptake rate of 0.10 nmole per mg protein per min.

Using ionophore A23187 it is possible to correct for ATP induced Ca^{2+} binding and also for estimating the gradient of Ca^{2+} formed across the vesicles and to demonstrate conclusively that gradient existed at the beginning of such an experiment.

SECTION 9

VESICLE STABILITY

In experiments described in the previous section it was noted that the IO prep could not retain accumulated Ca^{2+} above approximately 2 nmoles per mg protein. These experiments were carried out in absence of EGTA buffer system and in presence of 0.2mM Ca^{2+}.

In a few experiments it was also noted that after this loss of Ca^{2+} the final Ca^{2+} in the IO prep was even less than that of the control value. This result could be explained by occurance of IO vesicle fragmentation and conversion to RO vesicles upon resealing.

A striking similarity exists between the type of instability of membranes derived from the red cell and that from the sarcoplasmic reticulum (Martonosi & Feretose, 1964; Duggan & Martonosi, 1970; Martonosi & Halpine, 1971). This is discussed further in section 17.

Experiments were carried out to study the nature of the instability of the IO prep in presence of ATP and Ca^{2+}. Density gradient and 5'-nucleotidase techniques were found to be unsuitable. Entrapment of ^{22}Na or ^{14}C-inulin and the acetylcholine esterase assay gave results which were difficult to interpret.

9.1 METHODS

9.1.1 Preparation of the vesicles

The vesicles were derived from red cell membranes by suspension in low ionic strength buffer as described in the previous section. Mg^{2+} was omitted to obtain predominantly inside-out vesicles in the preparation. In the following this is referred to as the IO prep.

9.1.2 Assay mixture

The stability of vesicles was measured under the same conditions as those used for the measurement of (Ca^{2+} Mg^{2+}) - ATPase and the Ca^{2+} transport experiments. The experiments were carried out using the same incubation mixture as in section 7. Different Ca^{2+} concentrations (0.2,0.5 and 2mM) were employed in the presence and absence of 2mM ATP and the reaction stopped after 45, 90 and 135 minutes (unless otherwise stated) using ice-cold 30mM histidine/imidazole buffer. The following experiments were carried out to detect the stability of the vesicles.

9.1.3 Dextran density gradient centrifugation of membrane vesicles

Dextran gradients were made in each centrifuge tube. This was carried out by supporting the tube in vertical position and 6 mls of each gradient were applied carefully to the bottom of the tube starting from the least dense gradient, with the aid of a plastic tube connected to a long needle. Approximately 3mg of IO prep were added carefully to the top, assuring the balance of all tubes by addition of light gradient. The tubes were centrifuged

at 25,000 rpm for 16 hours in Beckman centrifuge model L5-65 using swingout rotor type SW27.1. The fractions were cerefully collected using a J-shaped pasteur pipette. The protein concentration of each fraction was estimated according to the method of Lowry et al (1951) and corrected for the variable background colour in each fraction. The density of the dextran in each fraction was determined from the cofresponding refractive index values, measured using refractometer (Bellingham & Stanley), the measurement being made with Na-light source at 4°C. When the refractometer was not available the density of each fraction was estimated by drying to constant weight an accurately measured volume of fraction of the gradient. Corrections were made for the weight of the salts and protein. Values obtained by this method were in reasonable agreement with values obtained by the refractometer.

9.1.4 Coupled optical assay for 5'-nucleotidase

This enzyme catalyses the hydrolysis of the adenosine monophosphate (AMP) to adenosine. The activity of this enzyme is measured indirectly by measuring the amount of inosine produced from the product adenosine due to its deamination by adenosine deaminase. The nucleotidase is located on the outer surface of the erythrocyte membrane and can, therefore, serve as a marker for the sidedness of the vesicles. The assay was carried out at room termperature (20°C) in 3ml cuvette; the reaction mixture contained 1.5ml of 5mM Tris buffer (pH 7.4), one IU of adenosine deaminase, 0.1ml (20µg protein) of vesicle preparation (homogenised and preincubated as above) and varying concentrations

of adenosine monophosphate. The volume was made up to 2.5mls with water. The suspension was mixed and the concentration of inosine was measured at 265nm using Perkin Elmer 124 spectrophotometer linked to a chart recorder.

9.1.5 Entrapment of ^{22}Na or ^{14}C-inulin

^{22}Na (10μCi/ml specific activity adjusted to 1μCi/nMole) or ^{14}C-inulin (2μCi/ml specific activity adjusted to 1μCi/2.5μMole) was added to the frseze-thawed membrane before the homogenisation step in the preparation of IO vesicles. After preincubation for 20 min at 37°C the homogenate was centrifuged at 50,000 rpm for 30 min using MSE superspeed 75 centrifuge and 10 x 10ml rotor. The supernatant (containing the extravesicular radioactivity) was discarded. The centrifuge tubes were drained and the sides wiped carefully. The sediment was suspended in 7.5mls of ice-cold 5mM phosphate buffer (pH8.0) and the above centrifugation procedure was repeated twice more to ensure the removal of traces of radioactivity. The final sediment was resuspended in the same buffer to give protein concentration of 2mg/ml. The reaction mixture used in these experiments was exactly as that of the Ca^{2+}-transport experiments. The reaction was started by addition of aliquots of the vesicles preparation and the amount of ^{22}Na entrapped was measured after 0, 45, 90 and 135 minutes of incubation at 37°C. The reaction was stopped by the addition of 5mls ice cold 30mM histidine/imidazole buffer (pH 8.0) and the tubes were centrifuged for 30 min at 50,000 rpm using the 10 x 10ml rotor as mentioned above. The supernatant was discarded after taking an aliquot for recovery of isotope estimation and the sediment was dissolved in 0.5ml of 1% triton-x-100. 0.1ml of this was taken for counting in Packard Tri-carb gamma counter.

9.1.6 Acetylcholine esterase assay

The enzyme acetylcholine esterase is located at the outer surface of the red cell membrane. Therefore it can also be used as a marker for detecting the orientation of the membranes. The substrate used was acetylthiocholine which is hydrolysed by the enzyme to yield thiocholine. This produces a yellow colour upon reaction with 5,5'-dithiobisnitrobenzoic acid and production of the anion 5-thio-2-nitrobenzoate. Therefore the enzyme activity could be measured spectrophotometrically at 412 nm by the increase in the intensity of this colour.

$$\text{Acetylthiocholine} \xrightarrow[\text{esterase}]{\text{acetylthiocholine}} \text{Thiocholine} + \text{acetate}$$

$$\text{Thiocholine} + \text{dithiobisnitrobenzoic acid} \longrightarrow \text{yellow colour}$$

The assay procedure:-

The assay was carried out according to Jarrett & Pennington (1976). 3mls of 0.4mM 5,5'-dithiobis-2-nitrobenzoic acid (Nbs) in 0.1M sodium phosphate buffer (pH 8.0) was mixed with 25µl of 75mM acetylthiocholine chloride and 25-50 µg protein (IO prep) in 3ml cuvette and the production of the yellow colour was measured at 412nm at room temperature for 4 min using Perkin Elmer 124 spectrophotometer and Perkin Elmer 516 chart recorder. The activity was expressed as nmole of the thiocholine produced per min per mg protein using a mMolar extinction of 13.61. The activity of this enzyme was measured after each step of the preparation of the IO vesicles. The measurement was carried out as follows:-

1. After thawing the suspension, before homogenization

2. After homogenization

3. At 5 min intervals after incubation up to 1 hr.

9.2 RESULTS AND DISCUSSION

9.2.1 Dextran density gradient centrifugation

The vesicles were separated on the density gradient. Two types of dextrans were used. When dextran 110T from Pharmacia was used the range of densities was from 1.01 to 1.07 and the pattern of the vesicle distribution on the gradient was similar to that obtained by Steck et al,(1970a) as shown in fig.9.1. When dextran grade C from BDH Chemical (molecular weight range 60,000 to 90,000) was used the density of a value beyond 1.044 could not be reached with the methods used. However, this did not affect the distribution of the first two bands shown in fig 9.1 but at the bottom of the tube the sediment was a mixture of the RO and the residual membrane. These fractions were collected and assayed for acetylcholine esterase activity.

Table 9.1 shows the acetylcholine esterase activity as an estimation of the proportion of IO and RO as separated by density gradient and that present before the 16 hour centrifugation. It is clear that the percentage of IO decrease on this prolonged centrifugation from 59.3 to 36%.

Further experiments were carried out under the same conditions but in the presence or absence of ATP and different concentrations of Ca^{2+}. A time period of 135 minutes was used to allow incubation of the IO prep at 37°C. The subsequent centrifugation was carried out at 4° C for 16 hours to separate the various fractions.

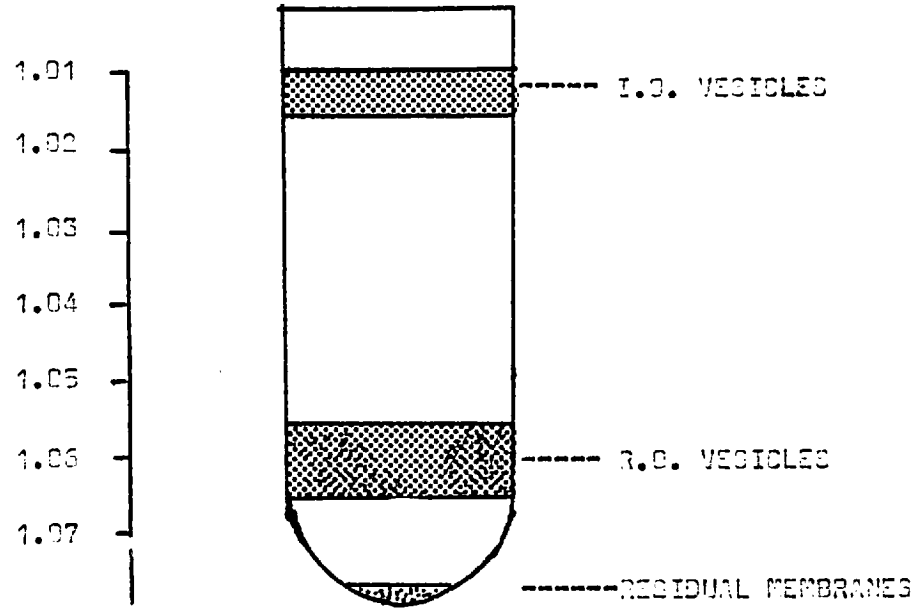

Fig. 9.1 Diagramatic representation of the dextran density separation of homogenised membran samples. $MgSO_4$ was omitted at the homogenisation step.

Table 9.1 Estimation of percent of IO and RO vesicles before and after density centrifugation use acetyl choline esterase activity.

Sample	% estimated by Dextran density gradient centrifugation ± SD (n = 3)	% estimated by acetylcholine esterase assay ± SD (n = 3)
IO	36% ± 12	59.3% ± 18
RO	46% ± 15	41.7% ± 8
Sediment	18% ± 5	

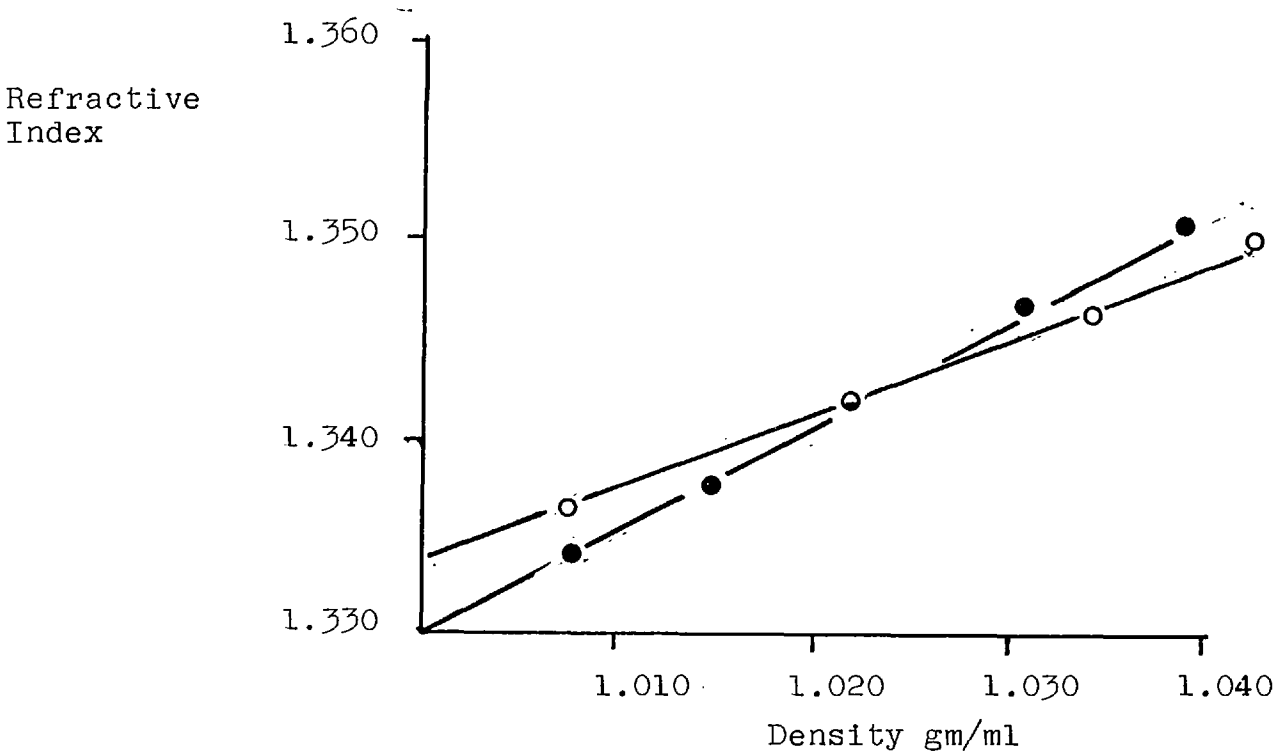

Fig 9.2 Calibration curve used for calculating the density of a gradient by use of refractive index.
O—O Refractive index for various density for dextran M Wt 72,000 as presented in Handbook of Chemistry and Physics, vol. 54.
●—● Values for dextran grade C (BDH) used determined by measuring refractive index and density.

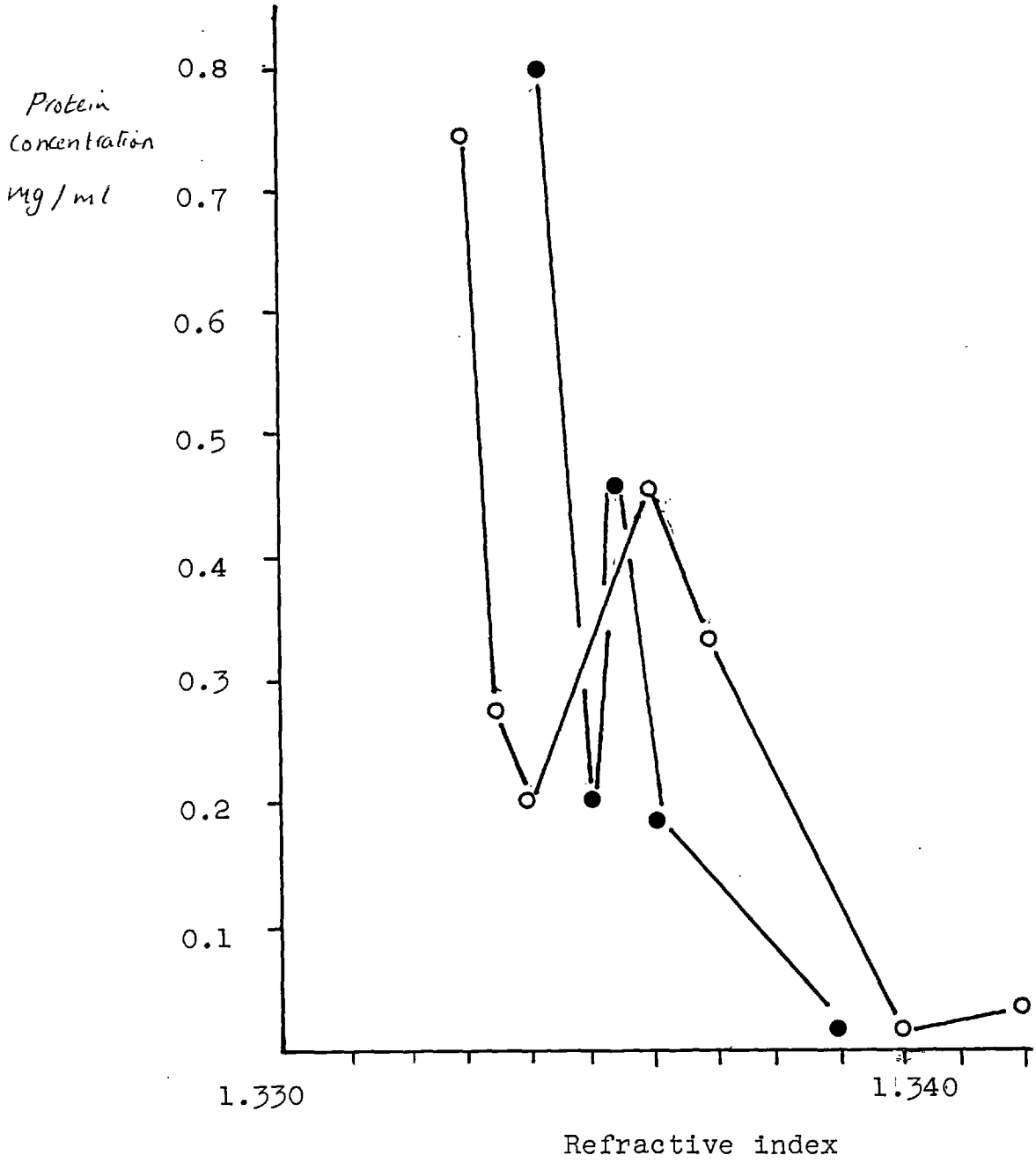

Fig 9.3 An example of variability of fractionation of IO vesicle. Both samples were analysed under identical conditions using the same IO prep. Both samples contain no added ATP or Ca^{2+}.

After numerous experiments it was evident that consistent results were not obtainable. An example of a duplicate control experiment under identical conditions is presented in fig 9.3. It is clear that even under these conditione consistent results were not tenable. This method for studying the stability of IO prep in presence and absence of ATP and Ca^{2+} was not used any further.

It is important to mention here that the IO fractions obtained after density gradient centrifugaion did not show any Ca^{2+} traneport capability when assayed as described in section 8.1. The vesicles however still retained both the high affinity (assayed at $\lceil Ca^{2+} \rfloor = 0.7 \mu M$) and low affinity ($\lceil Ca^{2+} \rfloor = 0.2 mM$) $(Ca^{2+} + Mg^{2+})$ - ATPase. The permeability of IO vesicle to Na^{+} and K^{+} was reported to be low (Kant & Steck, 1972). Since the vesicles generally seal rather more rapidly towards Ca^{2+} than monovalent cations, it is difficult to underetand why Ca^{2+} transport did not take place in these vesicles.

9.2.2 <u>^{22}Na and ^{14}C-inulin entrapment in IO prep as an indicator of vesicle stability in presence of ATP and Ca^{2+}</u>

The entrapment of labelled monovalant cation was initially used as an indicator for the detection of conversion of IO* to RO. The labelled $^{22}Na^{+}$ was added to the membrane suepension before homogenization and the vesicles were incubated for up to 135 minutes in the presence and absence of ATP and different concentrations of Ca^{2+} as shown in fig 9.4 and 9.5. The control

* Where IO and RO are written without mention of prep, read inside-out vesicle and right-side-out vesicle.

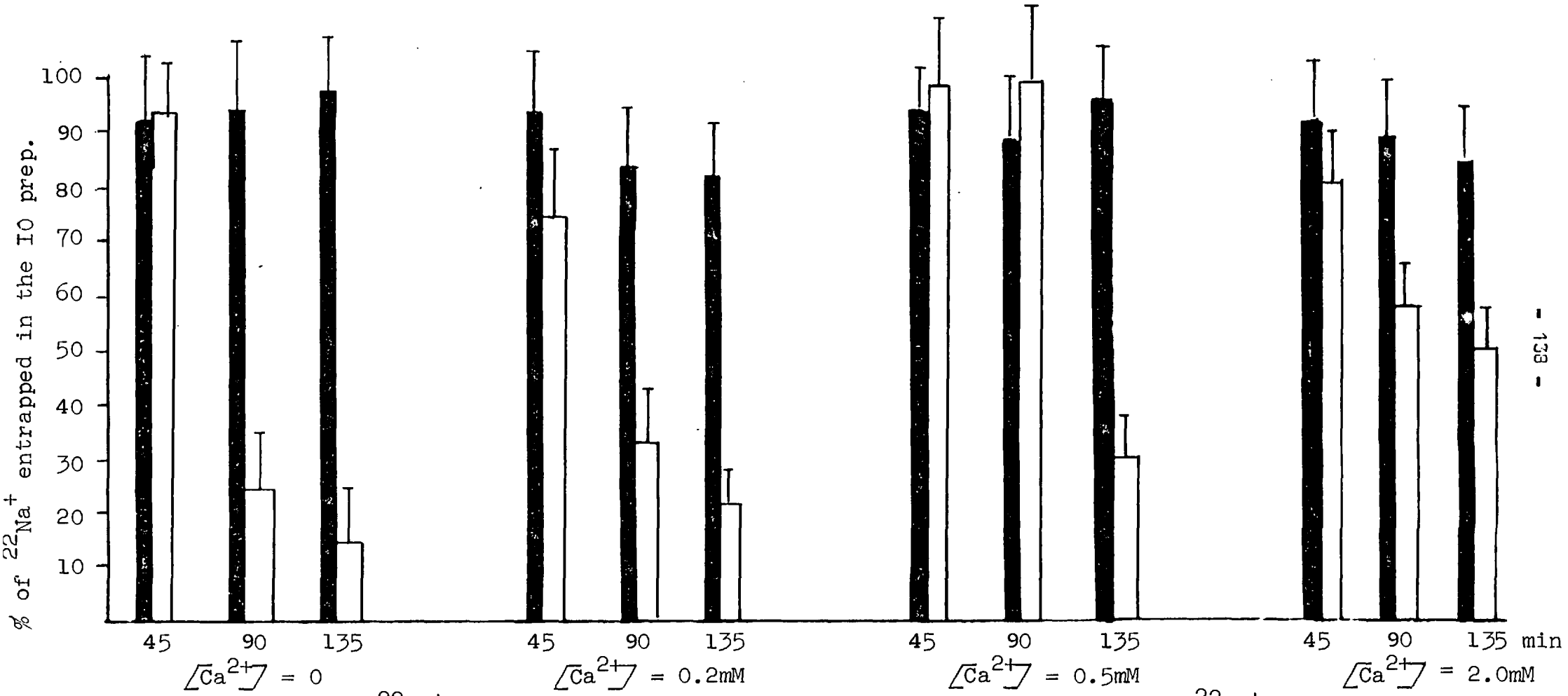

Fig 9.4 Histogram for the $^{22}Na^{+}$ entrapment experiment. Y-axis shows the percentage of $^{22}Na^{+}$ entrapped in the IO prep after 45, 90 and 135 minutes of incubation. The value for $[Ca^{2+}]$ = 0 without ATP is taken as 100%. Closed bars: control without ATP Open bars: Experimental deterimin. with 2mM ATP Each value is a mean of three duplicate experiments. Outdated blood used for preparation of membrane ghosts.

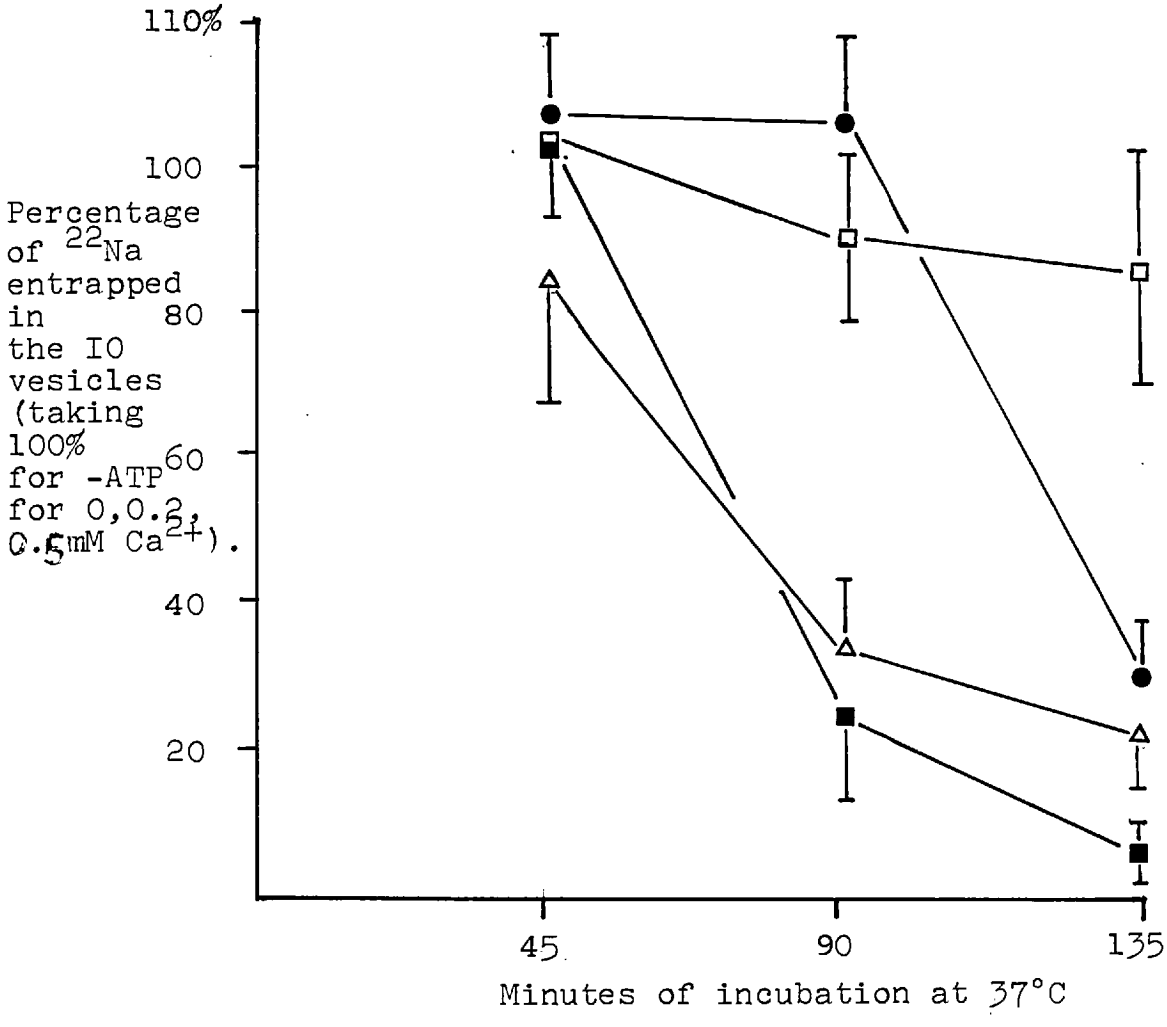

Fig 9.5 Same as Fig 9.4. Only one control value for 0.2mM Ca^{2+} shown for clarity. Others with 2mM ATP.

Ca^{2+} = 0mM; Ca^{2+} = 0.2mM

Ca^{2+} = 0.5mM; Control without ATP

samples containing no ATP were taken as 100% of the activity in the sediment. When ATP was present the loss of the counts from the sediment was much greater in the absence of Ca^{2+} than in its presence. As the Ca^{2+} concentration was increased this loss was more gradual and as it is noticed in fig 9.4 that the counts are retained in the vesicles for up to 90 min of incubation at concentration of Ca^{2+} as high as 2mM. The counts in the supernate reflected the amount lost from the vesicles and the total recovery from supernate and sediment was practically equal to the calculated value,(i.e. 100% $\pm$ LSD = .5%, n = 6).

The experiment indicates that the loss of entrapped $^{22}Na^{+}$ occurs in the presence of ATP. This is prevented by elevated Ca^{2+} concentration. Ca^{2+} may have these actions in two ways: by complexing with ATP and rendering ATP ineffective or the Ca^{2+} may act directly at the membrane level. If the latter case is correct, then the loss of $^{22}Na^{+}$ may take place by increased membrane permeability or by membrane vesicle destabilisation or both.

That Ca^{2+} forms a complex with ATP is a well documented fact and requires no further investigation. However, the second hypothesis was investigated by repeating the entrapment experiment using ^{14}C- inulin.

Studies on Ca^{2+} flux have shown that at high accumulation of Ca^{2+} rapid loss of the cation can be induced (see fig 8.2 b and 8.2 c). In some cases it was observed that the final value of

the accumulated Ca^{2+} drops below the passively accumulated Ca^{2+}. This can be explained by IO (loaded with Ca^{2+}) changing to an RO and in the process losing all the accumulated Ca^{2+} and, eventually, the RO pumping out the Ca^{2+} into the medium against a concentration gradient.

When ^{14}C-inulin was used the IO prep did not behave in the same manner as for $^{22}Na^{+}$. The control experiments in which ATP and Ca^{2+} were absent demonstrated a low inulin loss (fig 9.6) whereas the control for $^{22}Na^{+}$ entrapment showed no such loss. Hence inulin itself may have an effect on the stability of vesicles. It was further noticed that ATP with or without Ca^{2+} at the concentrations used did not alter the results significantly from that of the controls without ATP or Ca^{2+} (see fig 9.6).

When the $^{22}Na^{+}$ entrapment studies were initiated, the excess radio-label in the incubation mixture was removed by filtration of the IO prep through coarse Sephadex G-25. Although the method was more rapid than centrifugation no $^{22}Na^{+}$ was found in the void volume. Clearly the Sephadex can cause destabilisation of vesicles and loss of entrapped material from erythrocyte membranes but not from artificial membranes liposomes. Chemically Sephadex is similar to inulin, and the results obtained with inulin should, therefore, be expected.

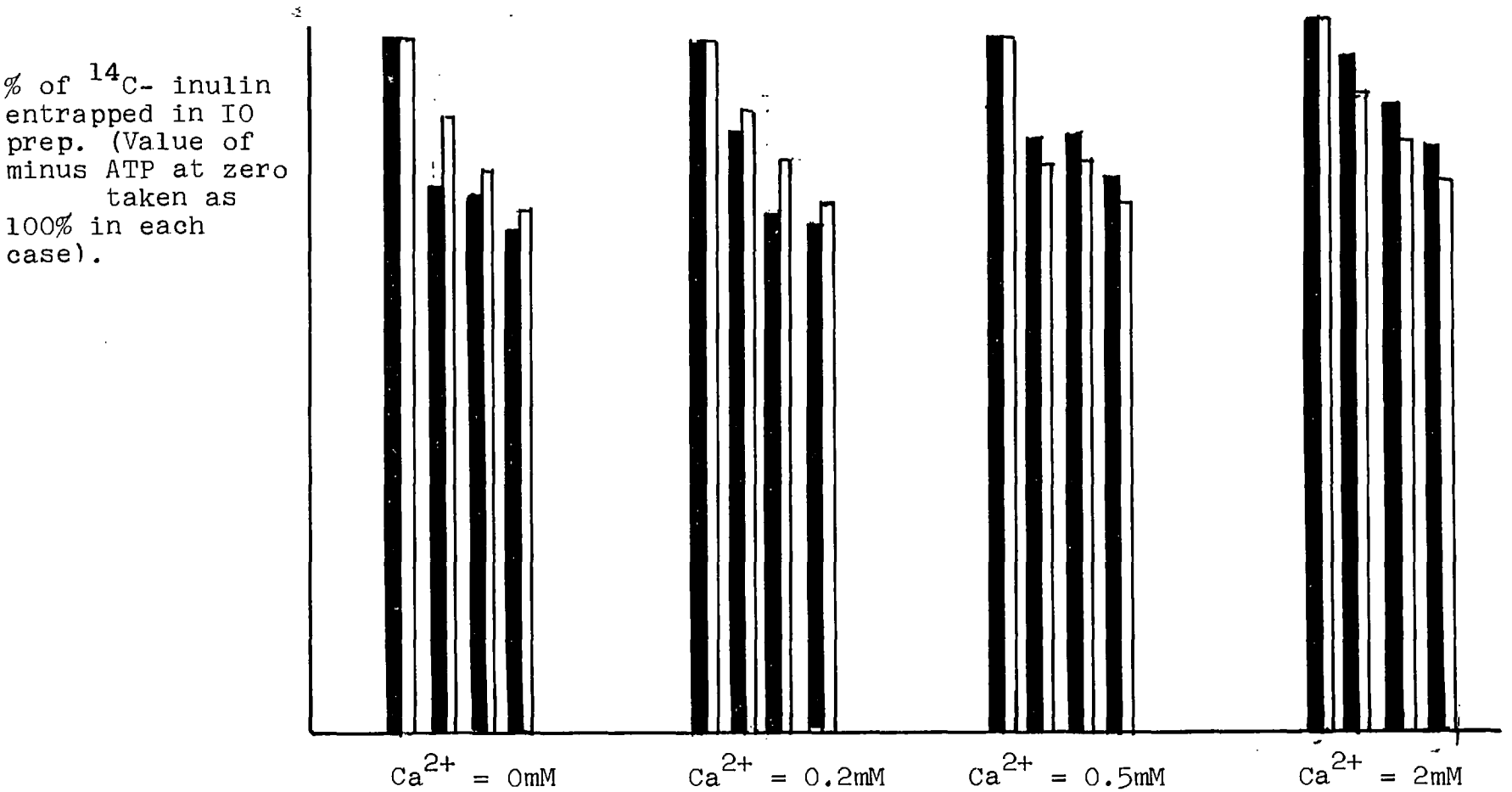

Fig 9.6 Entrapment of inulin. Conditions same as shown on fig 9.4. Note that each set of values for Ca^{2+} concentration of 0, 0.2, 0.5 and 2mM has a time course of 0, 45, 90 and 135 minutes from left to right of each set of histogram shown. Mean of three duplicate experiments. Closed bars : no ATP; Open bars : 2mM ATP

9.2.3 Use of enzyme markers for estimation of vesicle stability

Enzyme marker techniques were used for further investigation of the stability of vesicles towards ATP and Ca^{2+}.

The criteria for the marker was that it should be a membrane bound integral protein, which does not easily dissociate or elute from the membrane at any of the various steps of homogenisation or incubation at high or low ionic strengths used. Also the substrate for the enzyme must show a low membrane permeability and the assay should be sensitive.

Furthermore, it would be an advantage to have a continuously monitoring system e.g. colorimetric techniques. Finally, the assay must be very rapid in order to avoid interference with the stability and permeability properties of the IO prep.

9.2.3.1 5'-nucleotidase assay

The assay of adenosine-5'-monophosphate by 5'-nucleotidase satisfies all the above criteria and attempt was made to use this assay. However, at the time of the assay the author was unaware that the enzyme is absent from human erythrocyte. The following results show conclusively that the enzyme activity is absent. According to the method used, the activity of this enzyme was measured by coupled spectrophotometric determination of the rate of the deamination of adenosine to inosine, the reaction steps involved could simply be represented as follows:-

$$\text{Adenosine monophosphate} \xrightarrow{5'\text{-nucleotidase}} \text{Adenosine} + \text{inorganic phosphate}$$

$$\text{Adenosine} + H_2O \xrightarrow[\text{Deaminase}]{\text{Adenosine}} \text{Inosine} + NH_3$$

The extinction coefficient of adenosine and inosine was measured by use of standard solutions in the range of 0 to 40 μMoles. Fig 9.7 and 9.8 show linear regression for a straight line which best fits these points. The difference in extinction (ΔE265) for one mmolar solution at 22°C was found to be 7.80×10^3 at pH 7.5.

The reaction velocity of adenosine deaminase with adenosine as a substrate is shown in fig 9.9. Saturation of the enzyme was obtained at 0.026mM of substrate.

When the assay was used with the human red cell IO prep and ghosts as the source of 5'-nucleotidase, zero activity was recorded for up to 30 minutes, even when the membrane protein was increased to 1mg per assay.*

9.2.3.2 Acetylcholine esterase as a membrane marker in IO prep stability study

Although this assay fulfilled the criteria required for membrane marker for IO prep, it has been reported (Jarrett &Pennistone, 1976) that the initial rate of the reaction can only be measured after

* Lymphocyte membrane were found to show activity at less than 5μgm membrane protein using this assay condition.

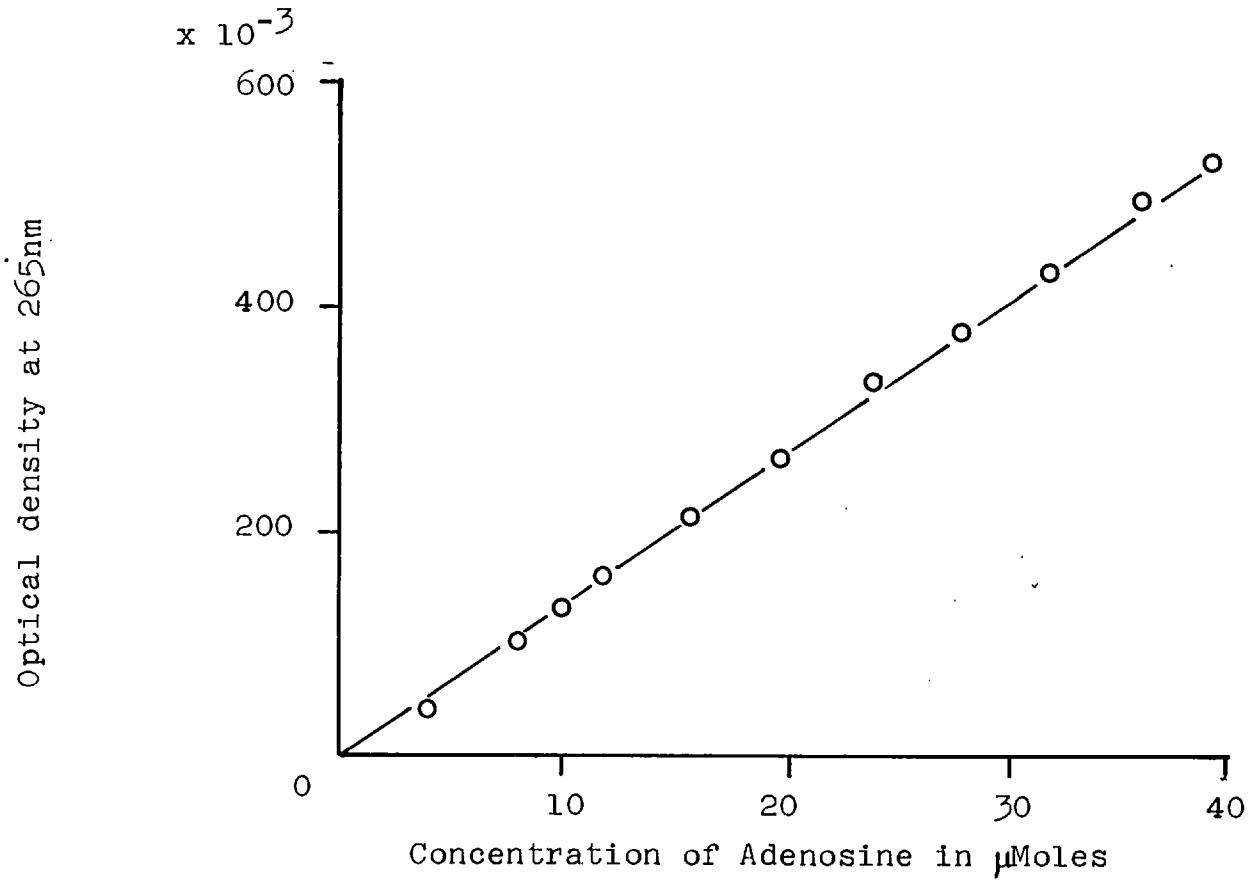

Fig 9.7

Calibration curve for adenosine. Equation of the line by linear regression is $y = 0.00013 + 0.01340_3(x)$. For 1 millimolar x = 1000. This gives a y value of 13.40×10^3.

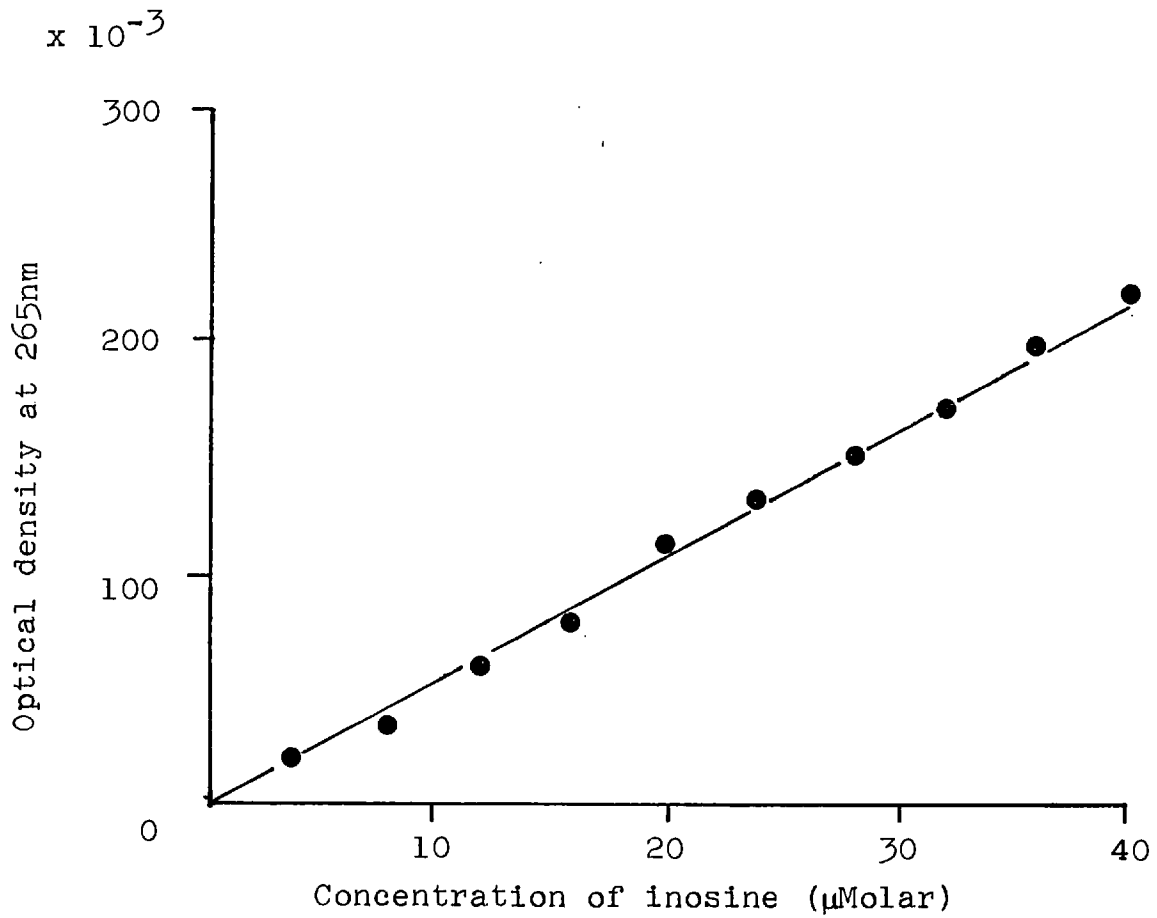

Fig 9.8 Calibration curve for inosine. Equation of the straight line is
$y = -0.00547 + 0.00561\ (x)$
For 1 mMolar solution $y = 5.61 \times 10^{3}$

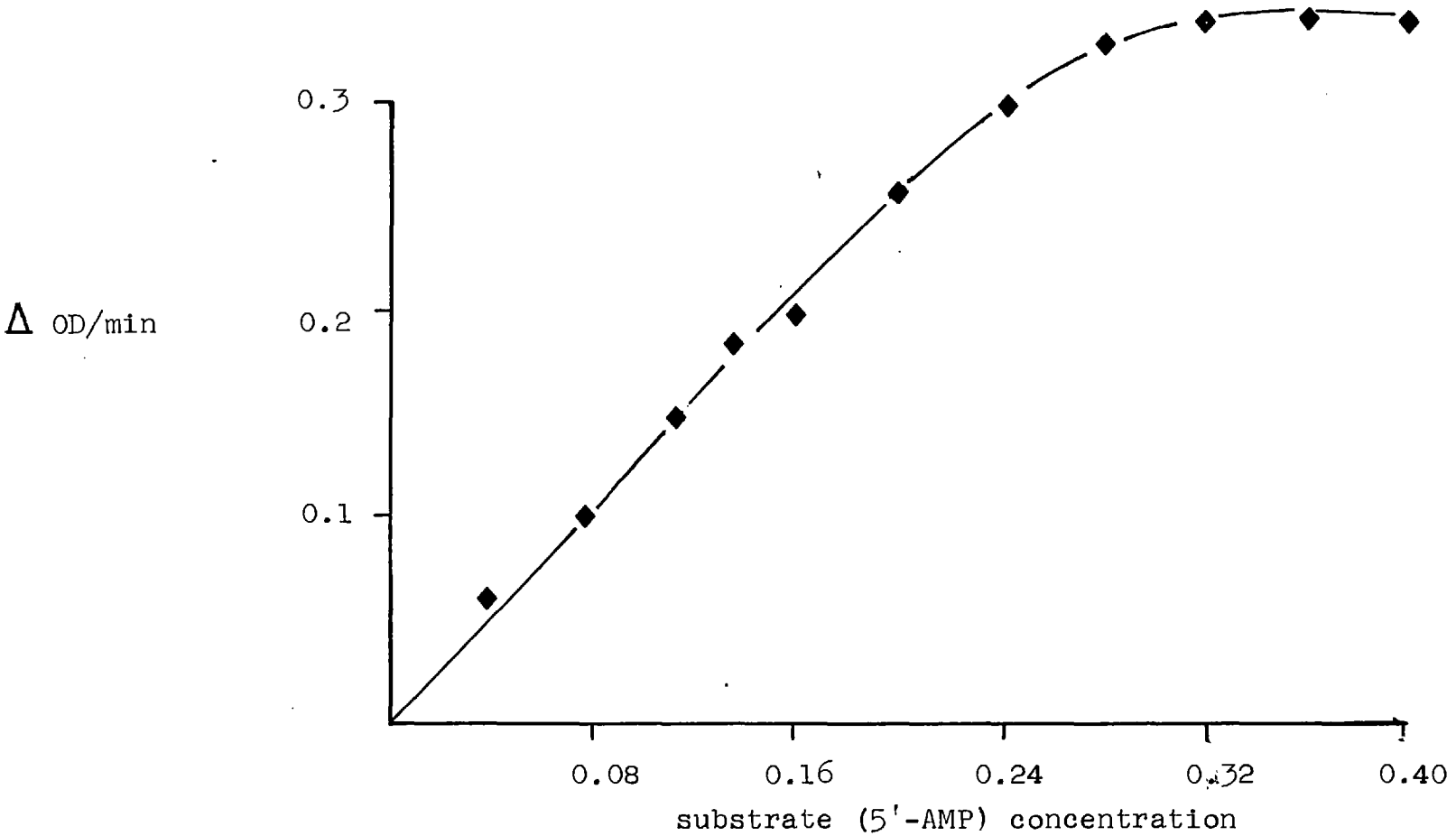

Fig 9.9 Saturation value for the assay conditions using adenosine deaminase at a fixed level and varying the concentration of 5'-AMP.

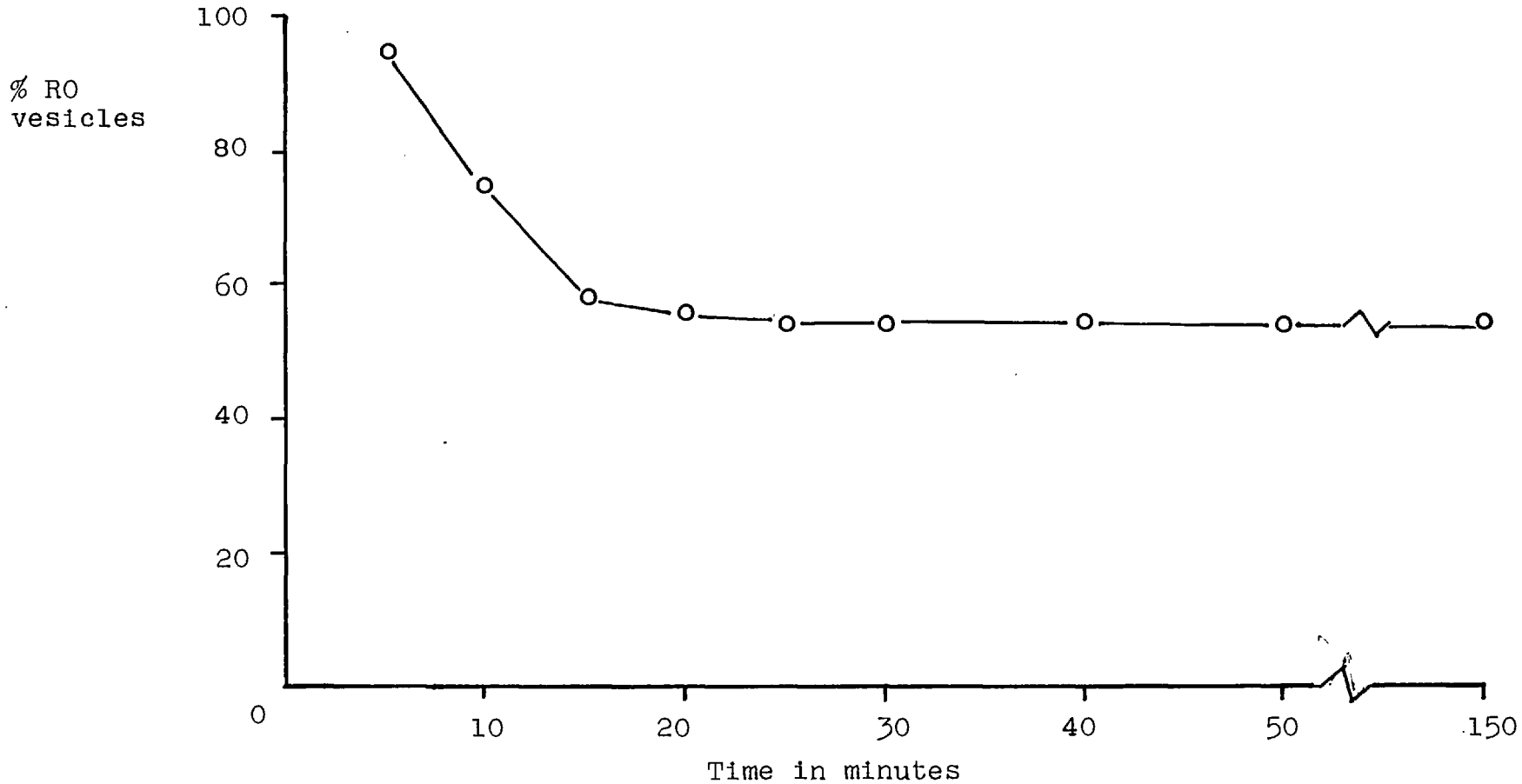

Fig 9.10 Estimation of percent RO vesicles after preincubation for 20 minutes at 37°C. The acetylcholine activity in presence of 1% triton-x-100 was taken as an estimate for 100% RO vesicle.

Table 9.2 IO prep was preincubated for 20 minutes before assaying for acetylcholine esterase. Samples were incubated with and without 2mM ATP. The values of activity in presence of 1% triton-x-100 was taken as 100% (n = 6)

Time of incubation minutes	% RO	
	-ATP	+ATP
0	62.8	74.1
10	75.6	79.0
20	58.3	61.4
30	55.2	58.0

the first minute. For this reason alone the 5'-nucleotidase assay was initially preferred. This study was carried out with ghost membranes. With IO prep, however, initial linear rate was obtained within 5 seconds of initiating the reaction.

The assay was carried out for samples at different stages of the vesicle preparation i.e. before and after homogenisation and at several time periods of preincubation. The results as shown in fig 9.10 are expressed as a percentage of total activity in the presence of triton-x-100.

The activity of the membrane suspension in the presence of 1% triton-x-100 was considered as 100%. It is noticed from this fig. that at 20 min of preincubation lowest percentage of activity is obtained indicating greatest population of IO vesicles. In other experiments the effect of ATP was measured for the preincubated vesicle suspension as shown in table 9.2. No effect of ATP was noted on the % of IO vesicles when the IO prep was preincubated for 20 minutes before addition of 2mM ATP. Addition of Ca^{2+} at final concentratione of 0.2, 0.5 and 2.0mM was also without effect in presence or absence of ATP.

However, an effect of ATP was obtained when it was present in the preincubation medium (table 9.2). Again Ca^{2+} at the concentrations used did not significantly alter this relative small change compared with fig 9.4 and 9.5.

9.3 CONCLUSION

Density gradient techniques were found to be unreliable for estimation of the stability of IO prep. $^{22}Na^{+}$ entrapment results indicate that ATP may have an effect on the IO prep. This effect could result either from membrane permeability change or membrane destabilization such that vesicle orientation change. When enzyme marker techniques were used it was found that 5'-nucleotidase (a candidate for the assay of membrane vesicle sidedness) was without activity. Acetylcholine esterase assay showed that the membranes sidedness changes only in the preincubation stages with ATP. Ca^{2+} was found to inhibit the lose of entrapped $^{22}Na^{+}$ but was without effect when acetylthiocholine assay was used for detection of sidedness. Inulin appears to cause destabilization of the membrane in a manner probably somewhat similar to Sephadex. None of the methods used in this section could be considered as a reliable technique of measurement of the stabilisation of the vesicles.

SECTION 10

10.1 Phosphorylation of the red cell membrane

The biological significance of the membrane phosphorylation is represented by the role of the phosphorylated protein in the regulation of certain biological activities associated with the membrane. For example, the role of the phosphorylated intermediates in the transport of the Na^+ and K^+ across the biological membrane is well documented. The synaptic transmission was shown to be regulated by phosphorylation of certain polypeptides in the membranes of the nerve cells (Greengard & Kababian, 1974; Greengard, 1975). The phosphorylation of the red cell membrane protein may exhibit functional significance related to the structural stability of the membrane. The phosphorylation is catalysed by a protein kinase, approximately 80% of the total cell activity being located on the cytoplasmic surface of the red cell membrane. (Rubin et al, 1972, 1973). The enzyme consists of regulatory or binding subunit (R) and catalytic subunit (C). The modulation of the activity of the protein kinase is accomplished by cAMP as discussed previously. The cAMP binds to R, forming the complex R-cAMP. This complex dissociates leaving the free active catalytic subunit, C. The reaction is represented in this simple formula:

$$R - C + cAMP \rightleftharpoons R\text{-}cAMP + C$$

The active C will transfer the terminal phosphate group from ATP to the membrane protein receptor. The R and C reassociate forming the inactive holoenzyme.

In this section the membrane of the red cell was subjected to phosphorylation in the presence of $AT^{32}P$ and cAMP and the incorporation of the ^{32}P was measured and the phosphorylation of

spectrin was studied in more detail.

10.2 METHODS

10.2.1 Isolation of membrane

The membrane was isolated from fresh and outdated blood samples collected in CPD as anticoagulant by hypotonic haemolysis in phosphate buffer (section 7). The final sediment was suspended in the same buffer and stored at -20°C.

10.2.2 Phosphorylation of the membrane

The endogenous phosphorylation of the red cell membrane was carried out using the method of Guthrow et al (1972). The assay medium was made up as shown below.

Reagent	Final conc.
ATP	0.05mM
cAMP	0.01mM
$AT^{32}P$	10^6 cpm per tube
disodium hydrogen phosphate	0.41 mM (pH 6.5 with HCl)
NaF	0.1 mM
Theophylline	2.5 mM
MgAcetate	10.0 mM
EGTA	0.3 mM

The final incubation volume was 0.5 ml. The tubes were preincubated for 5 min at 35°C before starting the reaction. Reaction was started by the addition of the once freeze-thawed membrane (0.2 - 0.5 mg protein per tube) and stopped by immersing the tubes in ice followed by the immediate addition of 0.2 mls of ice-cold 0.63% BSA and 4 mls of 7.5% ice-cold TCA. The tubes

were centrifuged at 2000 rpm using MSE refrigerated centrifuge, Mistral 4L. The supernatant was aspirated and the sediment was washed by dissolving in 0.2 mls of 1M NaOH and then 2 mls of ice-cold 5% TCA was added and the sample recentrifuged. The washing procedure was repeated three times. The final sediment was suspended in 0.2 ml of 1M NaOH, 50µl taken for ^{32}P counting in 10 mls of dioxane cocktail scintillant using Intertechnique SL 30 scintillation counter.

Experiments in which cAMP effects were investigated contained no cyclic AMP in the control tube and 0.01 mM cAMP in the experimental tube. For studies in which the effect of exogenous protein kinase was investigated, 50 µl of 1 mg/ml enzyme protein was added to a final volume of 0.5 ml. The reaction for both experiments was stopped as described above.

10.2.3 Extraction and purification of cytoplasmic protein kinase from human red cells

The enzyme was prepared by salt extraction and purification on DEAE cellulose chromotography using a modified method of Tao (1974). The extraction procedure is summarized as follows:(see fig 10.1)500 ml of human outdated blood collected in CPD was mixed with 1 litre of 2.5 mM $MgCl_2$ solutions at 4°C and kept on ice for 30 min with constant gentle stirring. The solution was centrifuged at 12,000 rpm for 30 min using Sorvall centrifuge (8 x 50 ml rotor). The supernatant was collected as the crude lysate, saturated to 50% with solid ammonium sulphate and left for 30 min on ice. The suspension was centrifuged as above and the precipitated protein in the pellet was dissolved in 20 mM tris buffer (pH 7.5) containing 1 mM dithiothrietol. The suspension was dialysed overnight against the same buffer at 4°C. The dialysed solution was centrifuged further to remove any insoluble contaminants before applying to the column.

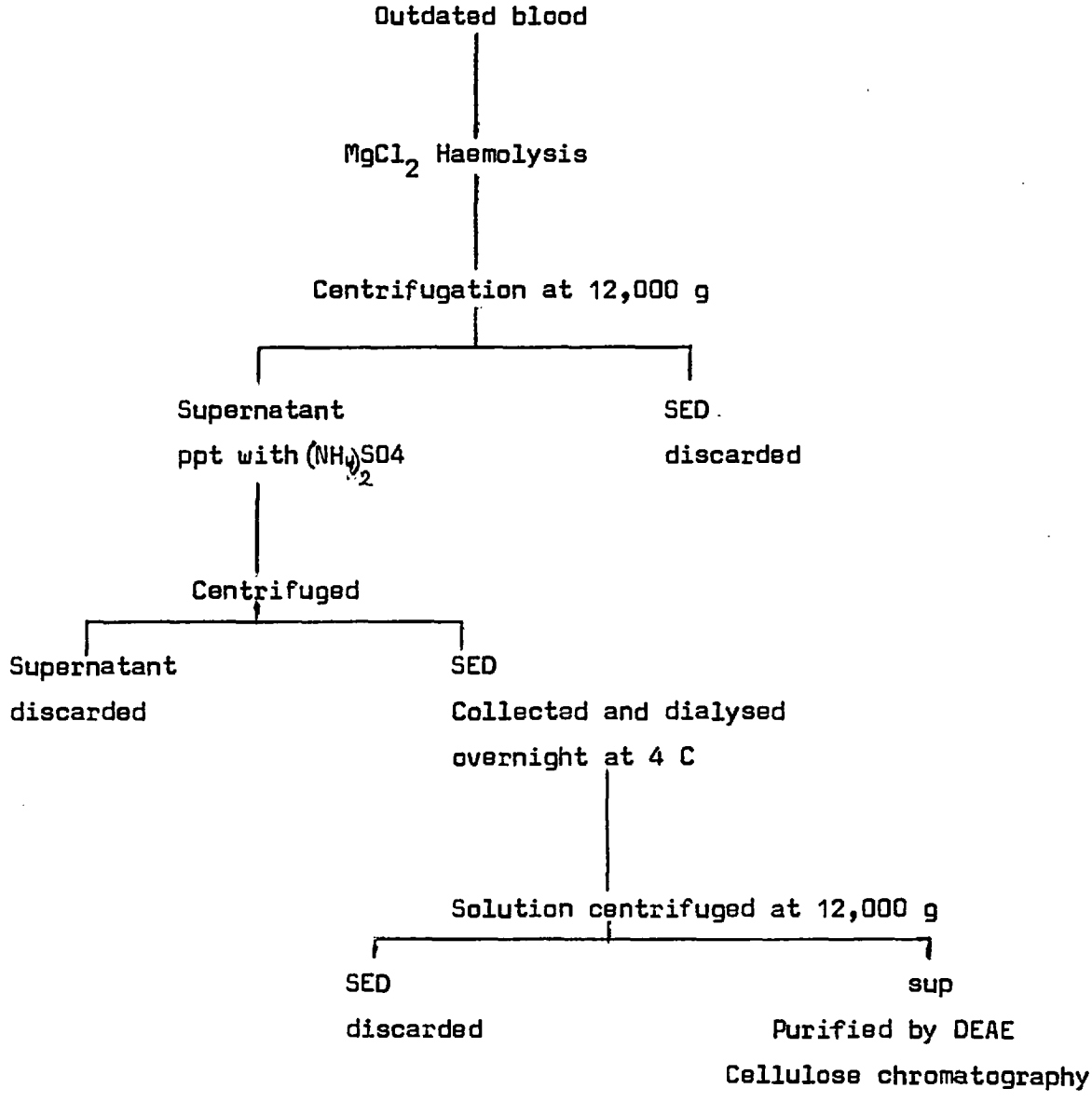

Fig. 10.1 Flow diagram of protein kinase extraction

10.2.3.1 Purification by DEAE cellulose column chromatography

A column of 0.95 x 9.2 cm of DEAE cellulose was used. The cellulose was suspended in the above dialysis buffer after washing twice and loaded in to the column and allowed to equilibrate with the dialysis buffer by elution with twice its bed volume. The procedure was carried out at 4°C. The sample was applied on the column and the column washed once again with the dialysis buffer. The protein was eluted using a linear gradient of 0 to 0.35 M KCl. Approximately one hundred fractions of 5 ml each were collected at elution rate of approximately 1.3ml/min. The O.D. was measured at 280 nm to detect the protein peaks I & II. All the collected fractions were divided sequentially into a total of 10 and pooled. The activity at peaks I & II was assayed for by the method used for the phosphorylation mentioned above using histon as the receptor protein.

10.2.4 Extraction of Spectrin

The extraction of spectrin was carried out according to the method of Marchesi & Steers (1968) as shown in fig 10.2. One volume of the membrane, prepared either from fresh or outdated blood, was suspended overnight at 4°C in 9 part volumes of 5 mM EDTA (pH 7.5) containing 5 mM β-mercaptoethanol. The suspension was centrifuged at 35,000 rpm in MSE superspeed centrifuge 75 using 10 x 10 ml rotor. The first supernatant was stored at 4°C. The sediment was resuspended (1 : 9) overnight in 5 mM glycine/NaOH pH 9.5 at 4°C. The suspension was centrifuged as above, and the second supernatant was kept at 4°C. Finally, the sediment was suspended (1 : 9) in distilled water for 6 hours at 4°C, the suspension centrifuged and the third supernate collected. The final sediment was discarded. The three supernatants

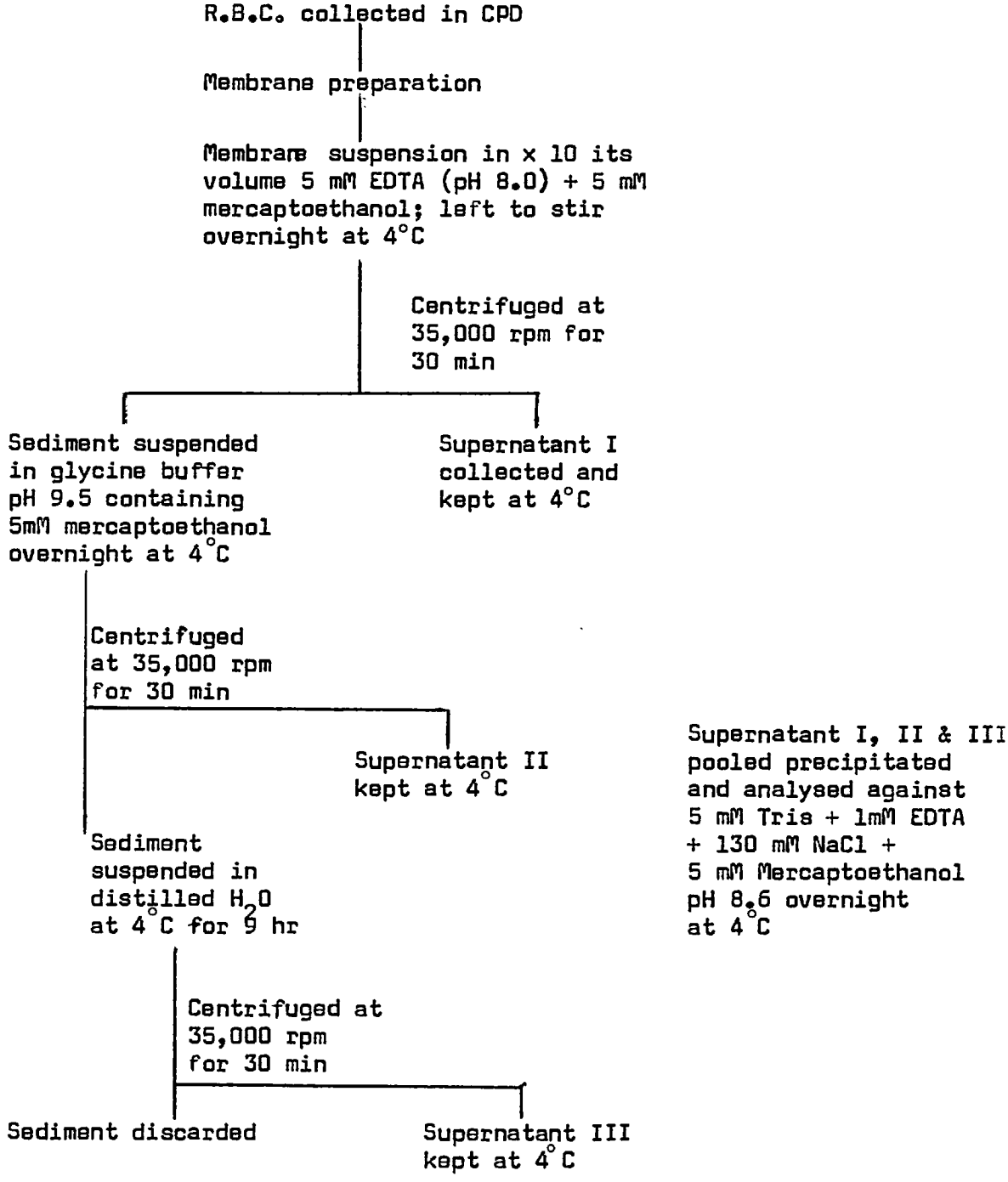

Fig. 10.2 Flow-diagram of method used for extraction of spectrin from red blood cell.

were pooled and the protein was precipitated at 50% saturation with solid ammonium sulphate with gentle stirring. The suspension was left on ice for 30 minutes and then centrifuged as above using 8 x 50 ml rotor. The sediment was suspended in a small volume of 5 mM tris buffer (pH 8.6) containing 1 mM EDTA, 150 mM NaCl and 5 mM mercaptoethanol. The suspension was dialysed overnight against the same buffer at 4°C with two changes of the buffer. The suspension was kept at 4°C until required. It was normally used within 48 hours of the last step.

10.2.5 Endogenous phosphorylation of extracted spectrin

The spectrin, extracted as described above, was phosphorylated in the presence of cAMP and $AT^{32}P$ as described in section 10.2.2. Each tube contained 0.2 to 0.3 mg protein and the phosphorylation was carried out for a time course of up to 100 minutes. Aliquots of 100µl each were taken from the final suspension in 10 ml dioxane cocktail and counted for the incorporation of ^{32}P in Intertechnique SL 30 scintillation counter.

10.2.6 Exogenous phosphorylation of extracted spectrin

This was carried out using 50µl of 0.1 mg/ml of purified extracted cytoplasmic protein kinase, the assay being carried out as above.

10.3 RESULTS AND DISCUSSION

10.3.1 Endogenous membrane phosphorylation

Membranes from human red cells exhibit the ability for endogenous phosphorylation in the presence of cAMP and ATP. Fig 10.3 shows time course for phosphorylation of red cell membrane. The phosphorylation was linear up to at least 10 minutes and over this period the phosphorylation velocity was 0.019 nmole phosphate bound per mg protein per minute. The maximum level of phosphorylation was 0.3625 $\pm$ 0.066 nmole phosphate bound per mg protein (n = 9; each value was made in duplicate). The values of phosphorylation reaction is shown in table 10.1.

After maximum level of phosphorylation was reached a gradual dephosphorylation process occurred as shown in fig 10.3. The reason for dephosphorylation could not be due to complete utilisation of ATP since it was present in access in the incubation tube (2.5 nmole), therefore ATP could not be a limiting factor for the phosphorylation. The rate of the dephosphorylation was much slower than that of the phosphorylation (-0.005) nmole phosphate bound per mg protein per minute. The negative sign indicates a decline in the phosphorylation activity. In few experiments two types of variability in the phosphorylation pattern was observed. In some samples the dephosphorylation process occured after 20 minutes of incubation at a rate which is similar to phosphorylation. After this period, rephosphorylation continues giving a peak value at 45 minutes as stated previously. In other cases dephosphorylation does not occur after the peak value is reached. However, the majority of the membrane samples

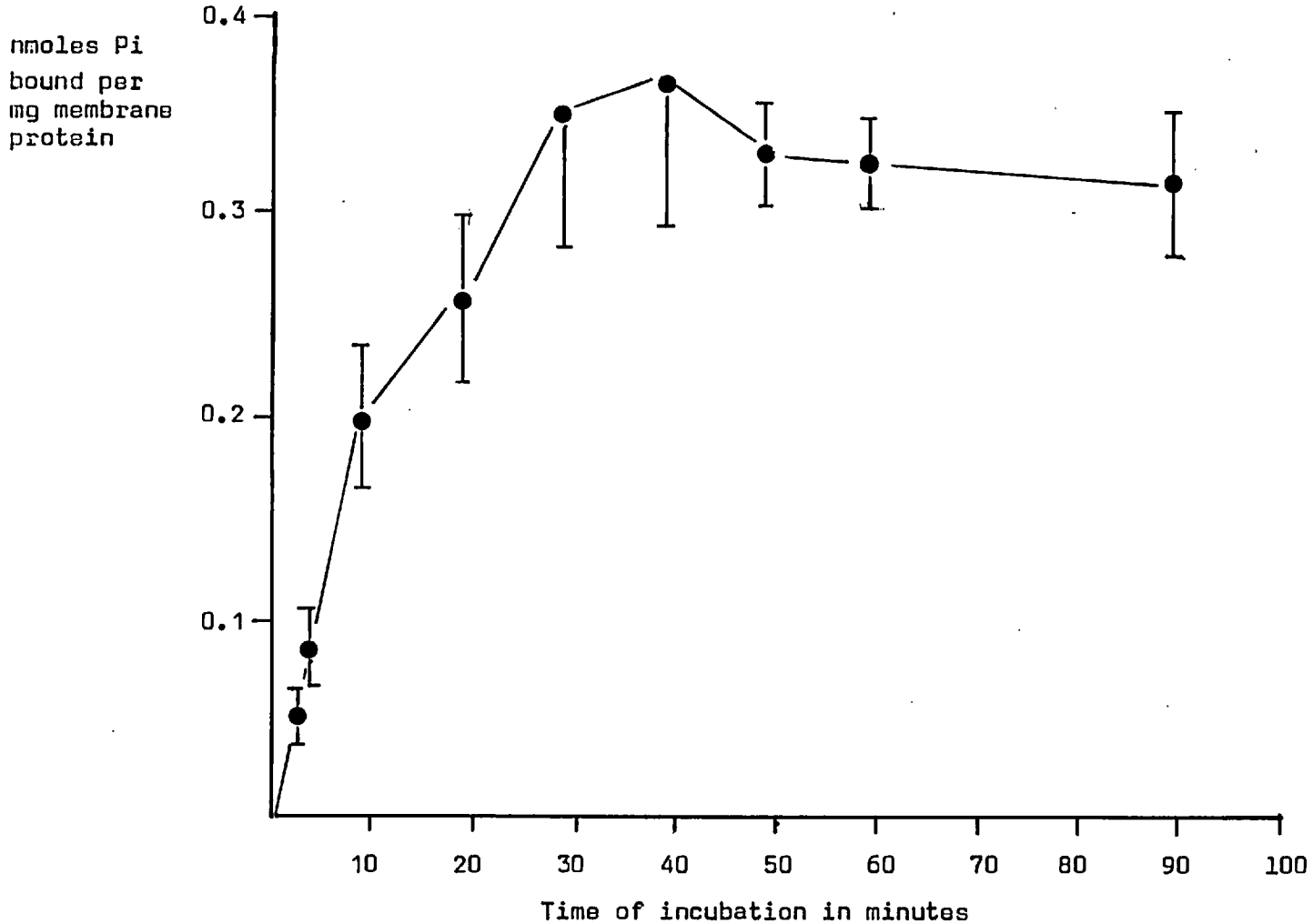

Fig 10.3 Time course of phosphorylation of red blood cell membrane. Note relatively high standard deviations at 30 and 40 mins due to dephosphorylation in some samples. Nine different samples were used. Each determination was made in duplicate.

Table 10.1 Time course of phosphorylation activity of membrane samples from six healthy normals. Each value is a mean of two determinations. Zero time values were taken as background and subtracted from each value.

Time (mins)	Activity: nmole Pi bound per mg membrane protein. subjects									
	1	2	3	4	5	6	7	8	9	Mean ± 1SD
3	0.045	0.065	0.052	0.053	0.036	0.058	0.052	0.072	0.059	0.055 ± 0.011
5	0.075	0.088	0.057	0.102	0.066	0.110	0.098	0.115	0.130	0.093 ± 0.024
10	0.209	0.225	0.103	0.241	0.166	0.215	0.193	0.127	0.148	0.181 ± 0.047
20	0.319	0.245	0.257	0.224	0.230	0.341	0.256	0.208	0.194	0.253 ± 0.049
30	0.389	0.476	0.278	0.207	0.320	0.355	0.309	0.405	0.297	0.337 ± 0.079
40	0.402	0.395	0.328	0.415	0.293	0.426	0.297	0.415	0.389	0.373 ± 0.053
50	0.330	0.391	0.305	0.357	0.285	0.409	0.293	0.389	0.267	0.336 ± 0.052
60	0.310	0.362	0.293	0.372	0.285	0.406	0.258	0.378	0.259	0.325 ± 0.055
70	0.332	0.392	0.312	0.309	0.271	0.343	0.396	0.352	0.255	0.323 ± 0.059

produced a pattern similar to that shown in fig 10.3 and when the rest of the dissimilar samples were combined, the overall general pattern was that shown in fig 10.3. This also explains the high standard deviation values shown in table 10.1.

10.3.2 Endogenous membrane phosphorylation and the effect of cAMP

The effect of cAMP was stimulatory for the endogenous phosphorylation of red cell membrane. In table 10.2 the initial rate of phosphorylation over the first 5 minutes period was measured. The experiment was carried out in duplicate using 3 normal membrane preparations in the presence or absence of 10 μmolar cAMP in the incubation mixture. Approximately 80% activation was observed in membrane samples in which cAMP was present. For the phosphorylation studies, cAMP was always present in further studies presented, unless otherwise stated.

10.3.3 Extraction and purification of cytoplasmic protein kinase

Protein kinase extracted and purified from human red cell cytoplasm was used to study the effect of protein kinase on the phosphorylation of extracted spectrin. Membrane bound protein kinase could not be used because satisfactory method for purification of membrane bound protein kinase after its extraction was not available at the time of the study. Membrane proteins which interfere with phosphorylation may also co-elute with the PK fractions.

The protein kinase was eluted in two distinct peaks as estimated by the O.D readings of all the fractions collected at 280 nm as shown in fig 10.4. When histone was used as the exogenous

Table 10.2 Effect of cAMP on the endogenous phosphorylation activity in normal red cell membrane. Each value is mean of two determinations.

Sample	Activity: nmole ^{32}P bound/mg protein/10 mins.		
	with^{out} cAMP	with + cAMP	% increase in activity
1	0.070	0.120	71.4%
2	0.067	0.111	65.7%
3	0.058	0.107	84.5%
Mean ±1SD	0.065 ± 0.006	0.113 ± 0.007	73.8% ± 9.6%

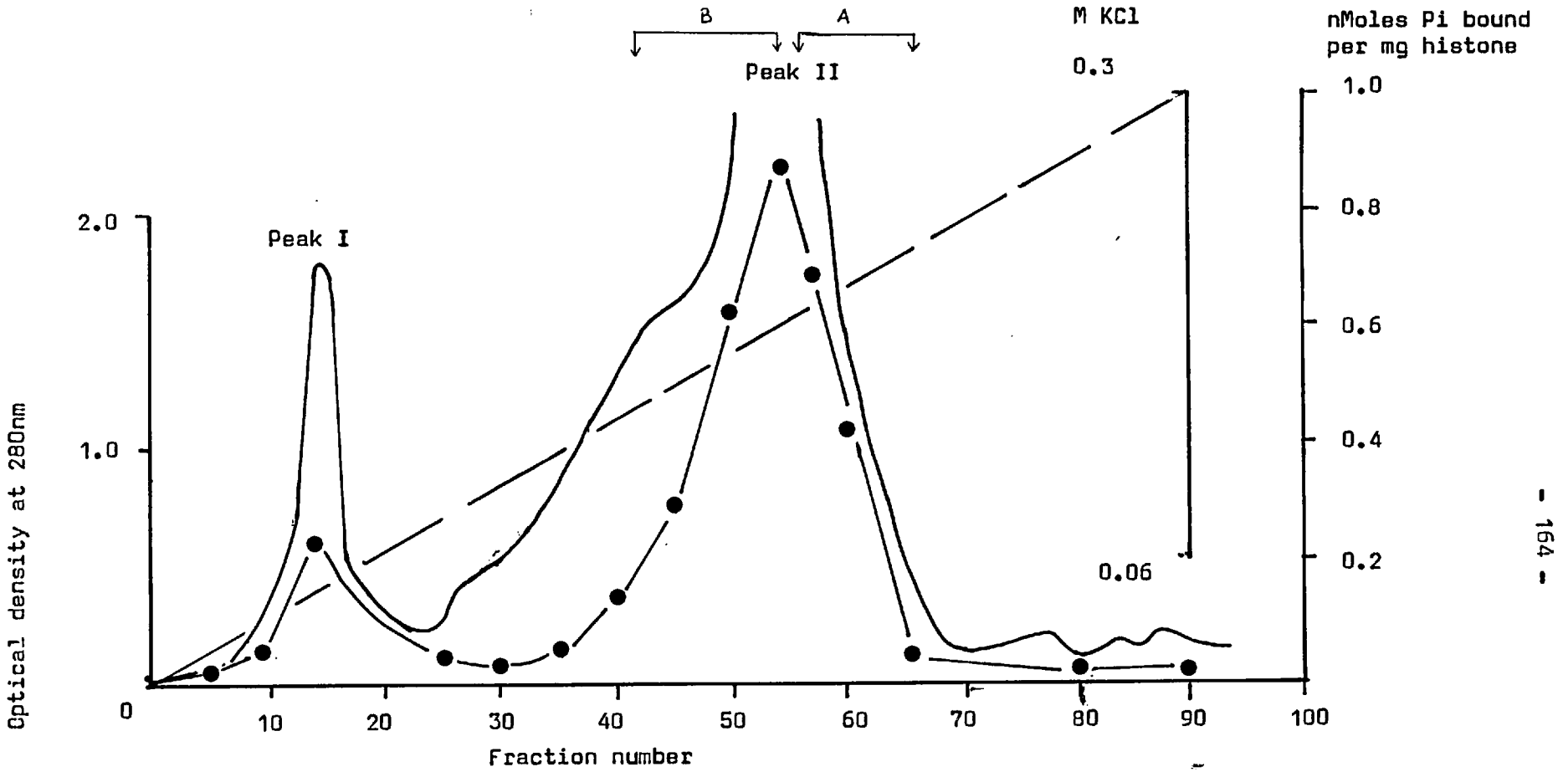

Fig 10.4 Elution profile for human cytoplasm red blood cell protein kinase. Protein was monitored at 280nm; the kinase activity (●——●) is shown on the right of the graph. Fractions between arrows shown at A of peak II was taken as the highest purity prep.

phosphate acceptor, peak I contained approximately one-fifth of peak II activity. Peak II containing the highest activity was therefore used. Fractions from the leading edge A (see fig 10.4) were collected and used since the trailing edge B showed some inflections in the O.D. values.

10.3.4 Phosphorylation of extracted spectrin

After 48 hrs of storage at 4 C the extracted spectrin showed no phosphorylation activity. Addition of purified protein kinase failed to produce any further activitation. Endogenous phosphorylation assay was carried out immediately after the overnight dialysis of spectrin. Fig 10.5 shows a general pattern of phosphorylation of extracted spectrin. The pattern is similar to that of membrane endogenous phosphorylation shown in fig 10.3. The initial linear rate of spectrin phosphorylation was found to to be 0.00153 nmole of phosphate bound per mg protein per minute and the linearity of the reaction was maintained up to 30 minutes. Maximal phosphorylation was 0.071 ($\pm$ 0.009) nmole phosphate bound per mg protein at 50 minutes incubations. Dephosphorylation process was also observed at rate of (-0.0011) nmole phosphate bound per mg protein per minute.

10.3.5 Effect of extracted protein kinase on spectrin phosphorylation

Spectrin phorphorylation was carried out at 20 minutes incubation period in the presence of cytoplasmic protein kinase as shown in fig 10.4. The experiment was carried out on spectrin extracted from six different normal membrane samples.

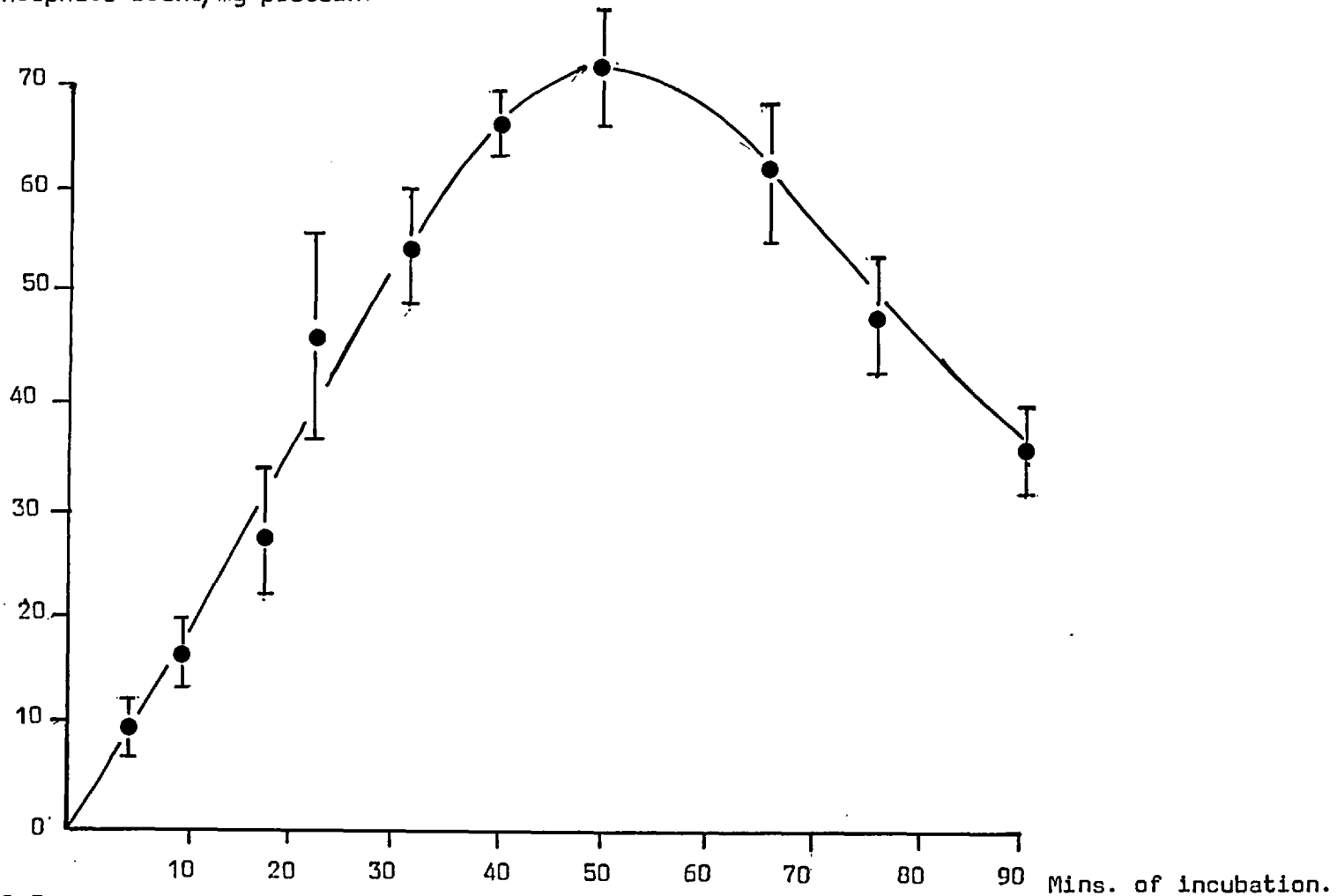

Fig 10.5

Time course of endogenous phosphorylation of freshly extracted spectrin.
Mean of 4 samples carried out in duplicate. ± 1SD shown.

Table 10.3 shows the activities of phosphorylation of spectrin with (A) and without (B) protein kinase and of the protein kinase with (C) and without spectrin (D). The calculation of ths background activity of spectrin was as follows:

1. Value D was subtracted from B to obtain the net value of spectrin phosphorylation activity.
2. Protein kinase alone showed some activity (C), therefore this value was subtracted from (A) to present the phosphorylation activity of spectrin after the addition of protein kinase.

Table 10.3 Effect of extracted protein kinase on spectrin phosphorylation. A typical analysis of the results presented in table 10.4 showing the control values for background subtraction.

Sample	Activity: nmole Pi bound/mg protein/20 mins.	
	With protein kinase	Without protein kinase
with spectrin	0.3310	0.0334
without spectrin	0.0180	0.0074

From table 10.3 the activity of protein kinase is 2.5 times the background value compared to 4.5 for spectrin. Step one of the calculation discussed above is followed to

Table 10.4 Extracted spectrin was phosphorylated in presence and absence of exogenous protein kinase. Each value is mean of two determinations. The increase in activity was calculated as the difference between A and B dividing by A. Values in B are corrected for background phosphorylation as shown in table 10.3.

	A	B	
Sample	Activity: nmole Pi bound per mg protein extracted spectrin only.	Activity: nmole Pi per mg protein extracted spectrin with purified protein kinase.	% increase in activity
1	0.023, 0.031	0.243, 0.179	956.5, 477.4
2	0.045, 0.043	0.213, 0.210	373.3, 388.4
3	0.028, 0.030	0.205, 0.202	632.1, 573.3
4	0.036, 0.025	0.194, 0.138	438.9, 452.0
5	0.035, 0.036	0.209, 0.212	497.1, 488.9
Mean ±1SD	0.033 ± 0.007	0.201 ± 0.027	524.8 ± 170.8

obtain the value of column A in table 10.4 and step two was used to obtain values in column B. The mean value for spectrin phosphorylation at 20 minutes of incubation was 0.0295 ± 0.011 nmole phosphate bound per mg protein per minute, whereas that of spectrin with the protein kinase was 0.193 ± 0.033 nmoles phosphate bound per mg protein per minute, an increase of 6.9 fold.

It can be concluded from the above study that the low activity of phosphorylation of extracted spectrin is due primarily to the presence of low levels of the endogenous enzyme, or to inhibition of its activity. Since spectrin kept for over 48 hrs at 4 °C shows practically no phosphorylation activity even when exogenous protein kinase is added, it is possible that spectrin loses its phosphorylation activity due to partial denaturation. The possibility that the loss of activity is due to action of proteolytic enzymes is partially excluded since immediate storage of spectrin at -20 °C or -193 °C after dialysis overnight showed similar loss in activity.

SECTION 11

Age dependent studies

Phosphorylation of membrane proteins of young and old cells separated by ultracentrifugation

The circulating blood contains heterogenous population of cells according to their age and maturity. As the cells age, different biochemical and biophysical alterations in the cell as well as in its membrane proteins occur. Several studies concerning these alterations have been carried out. Separation of the cells according to their age was achieved either by ultra-centrifugation (Regas & Koler, 1961) which separates cells according to their altered buoyant density with age or by density gradient separation (Leif & Vinograd, 1964). Studies were carried out in an attempt to characterize the metabolic alterations that accompany the ageing process (Bernstein, 1959). As mentioned in section 5, these studies showed that alteration in the enzyme activities during cell ageing process leads eventually to cell destruction by the spleen. Since the splenic sequestration is dependent probably on the alteration of the membrane surface and since phosphorylation is important in regulation of membrane function, it is of interest to measure the activity of the enzyme, protein kinase, and of the membrane protein phosphorylation in cells of different maturity.

11.1 METHODS

11.1.1 Separation of the cells

Normal fresh blood was collected in CPD and washed with cold isotonic saline (0.15M) three times to separate the white cells

and the plasma. The packed red cells were centrifuged at 40,000 rpm for 1 hr using MSE super speed 75 centrifuge and 10 x 10 ml rotor. The tube was divided into four layers. The top layer (A) was considered as the young cells and the bottom layer (D) as the old cells. The middle layers (B and C) represented the mature red cells. Only layers A and D were investigated. Slides were prepared for each layer and stained with bromocreasol blue for reticulocyte count. The activity of G-6-PD was measured in each layer immediately after centrifugation and was taken as an indication of age since the activity of this enzyme was shown to decline during ageing. G-6-PD activity was estimated as in Beutler (1975). The assay was carried out in a three ml cuvette at 340 nm using Perkin Elmer 142 spectrophotometer linked to chart recorder. The cuvette contained: (final concentrations) Tris-HCl 100 mM (pH 8.0), EDTA 0.5 mM, $MgCl_2$ 10 mM, NADP 0.2 mM. The substrates Glucose-6-phosphate and 6-phosphogluconate were present at 0.06 and 0.03 mM respectively. The packed red cells were haemolysed in distilled water (1 : 20) immediately before assay, centrifuged at 2,500 rpm in Mistral 4L for 10 mins and 50 µl of lysate taken for assay. The assay was carried out at 37°C for 5 min, the reaction being started by the addition of the lysate. Reference contained the same mixture as the sample except for ommission of the substrate. The initial linear velocity was calculated. The results are expressed in I.U. per gm Hb. The Hb. was estimated by Drabkins method as described in section 8. The activity obtained with 6-PG was subtracted from that obtained from G-6-P to give the activity of G-6-PD. Membrane preparation was carried out on each

layer as described previously (section 7.1) and stored at -20°C until used.

11.1.2 Endogenous phosphorylation of membranes from young and old cells

Freeze-thawed membrane samples were subjected to phosphorylation in the presence of cAMP and $AT^{32}P$ as described previously (Section 10.2.2). Time course for the reaction was carried out and the amount of ^{32}P bound to the membrane was determined.

11.1.3 SDS gel electrophoresis

The final sediment of the phosphorylated membrane samples was suspended in 1% SDS with sucrose solution for electrophoresis (section 15.1). Samples were incubated for 30 minutes at 37°C and 20 µl (10 µg membrane protein) was applied carefully to the top of the gel. Electrophoresis was carried out as described (section 15.1) using a current of 8 mA/tube at constant voltage for 30 - 45 minutes or until the tracker dye had migrated at least two-thirds the length of the gel. Gels were stained with coomasie blue. Spectrin bands (I + II) were cut, dissolved in H_2O_2 and counted in 10 mls of dioxane cocktail using Intertechnique SL 30 for ^{32}P incorporation.

11.1.4 Exogenous phosphorylation of membrane and spectrin bands of the layers A and D

The same procedure as for the endogenous membrane phosphorylation was carried out except that the tubes were incubated in presence and absence of extracted cytoplasmic human red cell protein kinase. The membrane samples were also subjected to electrophoresis

to separate spectrin bands (I + II). The gels were stained, cut and counted as above for ^{32}P incorporation.

11.2 RESULTS AND DISCUSSION

The cells were separated according to their age by ultra-centrifugation. The top layer was considered to contain the younger cells in the circulation. The reticulocytes were found to comprise 7 to 8% in the young cell population while the bottom layer contained approximately 0.2 to 0.5% reticulocytes and was considered as the oldest cells in the circulation. To confirm this further, the activity of G-6-PD was measured in both layers as shown in Table 11.1. The activity was 5 times higher in the top layer than that of the bottom. Since the activity of this enzyme was shown to decline during ageing (Turner et al ,1974) the high activity in the top layer indicates that it contains young cells.

Table 11.1 G-6-PD activity of upper and lower layers of RBC

	G-6-PD IU/gm Hb ± 1 SD
Upper layer	26.72 ± 4.76
Lower layer	5.01 ± 0.80

11.2.1 Phosphorylation of top and bottom layer membrane

The membranes prepared from these layers were assayed for endogenous phosphorylation in the presence of cAMP and $AT^{32}P$. Table 11.2 a and b show the level of phosphorylation of the top and bottom layers respectively with time. The corresponding figure (Fig 11.1) shows the pattern of phosphorylation of these membranes which resembled that of the general total membrane phosphorylation (see fig 10.3). The mean maximum value for the phosphorylation of the bottom layer was 0.451 nmole ^{32}Pi bound/mg membrane protein (n = 4) as shown in table 11.2b, which was reached after 45 minutes of incubation. The maximum level of phosphorylation of the top layer was 0.308 nmole Pi bound/mg membrane protein (n = 4) as shown in table (11.2 a), indicating a decreassd level of phosphorylation of these membranes compared to that of the bottom layer. This is also clearly shown in fig 11.1. These results may appear to be in contrast with the study of Pfeffer & Swislocki (1976) which showed a decreassd activity of protein kinase during ageing. The reason for increased phosphorylation level in the bottom layer is not known. It could however be due to several reasons. Firstly a change in the behaviour of the enzyme may occur. Either all or a fraction of this enzyme may not be stimulatsd by cAMP in early stages of maturation and the activity shown here is due to the cAMP independent fraction of the enzyme. cAMP may also bs inhibitory rather than stimulating the enzyme (see section 4). Secondly the effect of the fluoride which is present at low concentrations in the assay msdium may be activating to the phosphodiesterase, which bas besn demonstrated to show this anomalous behavious at early stagss of dsvelopment (Schmidt et al,

Table 11.2 a: Phosphorylation of membranes derived from red cells of the top layer. Each value is a mean of two determinations.

Time (mins)	Activity: nmole ^{32}P bound per mg protein Subjects							
	1	2	3	4	5	6	mean	± 1SD
0	0.018	0.023	0.017	0.019	0.109	0.016	0.020	0.004
5	0.139	0.123	0.128	0.148	0.23	0.216	0.164	0.047
10	0.151	0.142	0.135	0.183	0.34	0.329	0.213	0.095
20	0.163	0.172	0.153	0.193	0.359	0.411	0.242	0.113
30	0.148	0.202	0.177	0.540	0.365	0.417	0.308	0.157
45	0.183	0.180	0.157	0.163	0.498	0.511	0.282	0.173
60	0.156	0.159	0.139	0.161	0.471	0.359	0.241	0.140

Table 11.2b Phosphorylation of red cell membranes from bottom layer cells Mean of two determinations shown.

Time	Activity nmole ^{32}P bound per mg protein							
	Subjects							
	1	2	3	4	5	6	mean	± 1SD
0	0.012	0.0110	0.0109	0.0115	0.0106	0.0109	0.011	0.001
5	0.159	0.334	0.301	0.289	0.249	0.194	0.254	0.067
10	0.271	0.358	0.322	0.389	0.293	0.270	0.317	0.049
20	0.279	0.387	0.385	0.408	0.420	0.427	0.388	0.057
30	0.2895	0.413	0.432	0.489	0.507	0.444	0.429	0.077
45	0.3686	0.414	0.503	0.508	0.360	0.553	0.451	0.081
60	0.3142	0.352	0.408	0.515	0.454	0.489	0.420	0.081

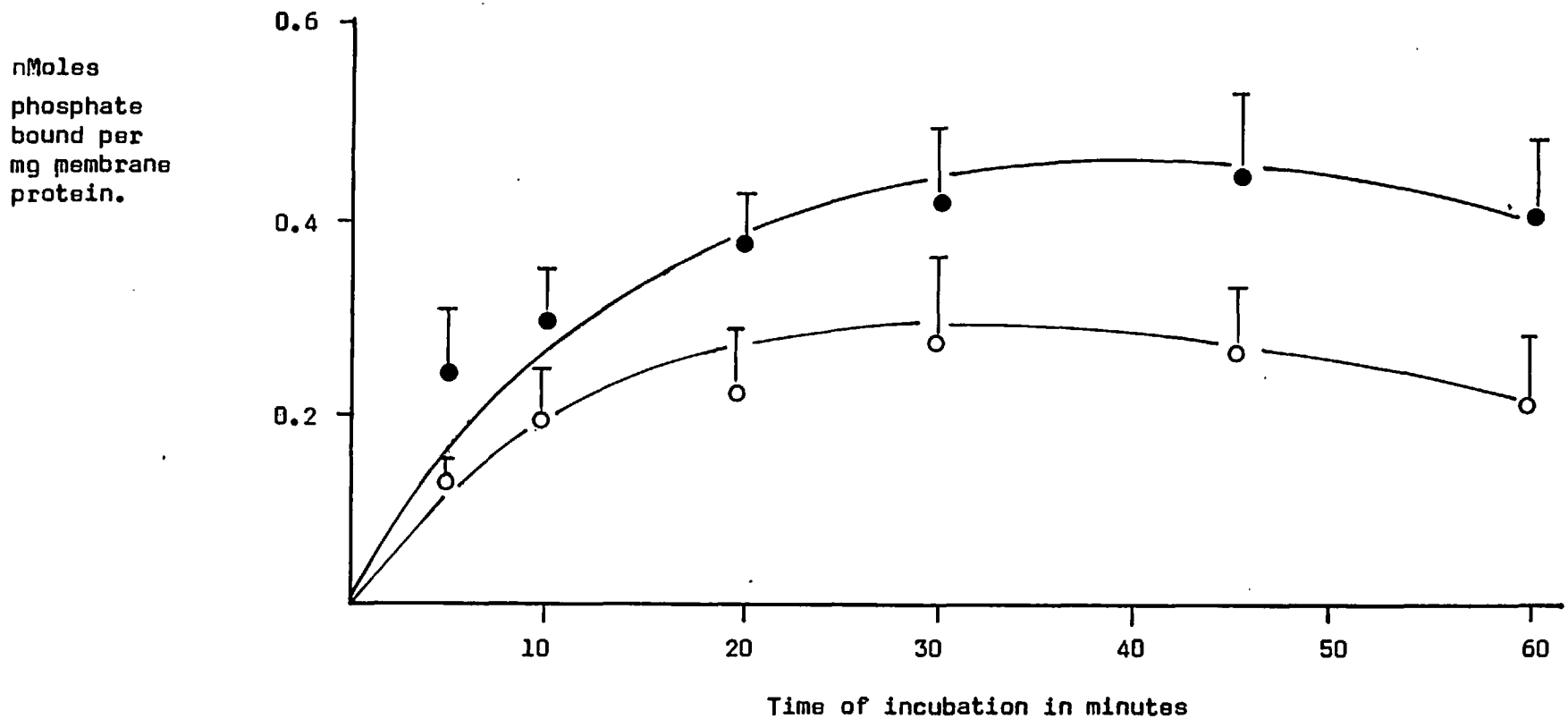

Fig 11.1 Phosphorylation of red cell membrane derived from top (open circles) and bottom (closed circles) layers of cells after high speed centrifugation. Each determination is a mean of four preparations assayed in duplicate.

1970). If this is true, then this would lead to the hydrolysis of cAMP in the assay mixture. Another possibility is the appearance of an additional protein (shown as band in SDS gel electrophoresis by Kadlubowski & Harris (1974)) in the membrane upon ageing. This band (4.1 according to the classification by Fairbanks et al (1971)) was also shown to be phosphorylated under similar experimental conditions to that used in this study (Roses & Appel, 1973). Therefore the increase of the phosphorylation may be attributed to this band. This was substantiated by the finding that the level of phosphorylation in spectrin bands of young cells was low as will be shown later.

When cytoplasmic protein kinase (extracted from human red cells) was added to the assay medium further decrease in activity was noticed as shown in Table 11.3a and b and the corresponding Fig 11.1 a and b. This decrease was more dramatic in the young cell membrane and it was not very significant in the old cell membrane. The maximum level of phosphorylation of old cells was 0.358 nmole of Pi bound/mg membrane protein at 45 minutes compared to 0.135 nmole of Pi bound/mg membrane protein of the top cell at the same period (n = 4 for each layer). The reason of suppression in the phosphorylation of both layers is not known. Since exogenous protein kinase does not increase the level of phosphorylation in either the young or old cells, it is possible that decrease in protein kinase activity with ageing is not responsible for this phenomena.

11.2.2 Phosphorylation of band (I & II)

However, similar patterns of phosphorylation were obtained when the membrane proteins were isolated on gel electrophoresis.

Table 11.7. Phosphorylation of red cell membranes from top layer cells with exogenous protein kinase.

Time (mins)	Activity: nmoles ^{32}P bound per mg protein + Protein kinase				
	Subjects				
	1	2	3	mean	± 1SD
0	0.015	0.010	0.017	.014	0.004
5	0.138	.087	.072	0.099	0.035
10	.110	0.120	0.145	0.125	0.018
20	0.124	0.143	0.120	0.129	0.012
30	0.130	0.115	0.160	0.135	0.023
45	0.122	0.150	0.130	0.135	0.014
60	0.121	0.189	0.131	0.147	0.037

Table 11.3b Phosphorylation of red cell membranes from bottom layer cells with exogenous protein kinase.

Time (mins)	Activity: nmoles ^{32}P bound per mg protein + Protein kinase				
	Subjects				
	1	2	3	mean	± 1SD
0	0.012	0.026	0.013	0.017	0.008
5	0.173	0.236	0.200	0.203	0.032
10	0.384	0.213	0.240	0.279	0.092
20	0.313	0.394	0.304	0.337	0.05
30	0.331	0.340	0.403	0.358	0.039
45	0.401	0.312	0.298	0.337	0.056
60	0.298	0.279	0.350	0.309	0.037

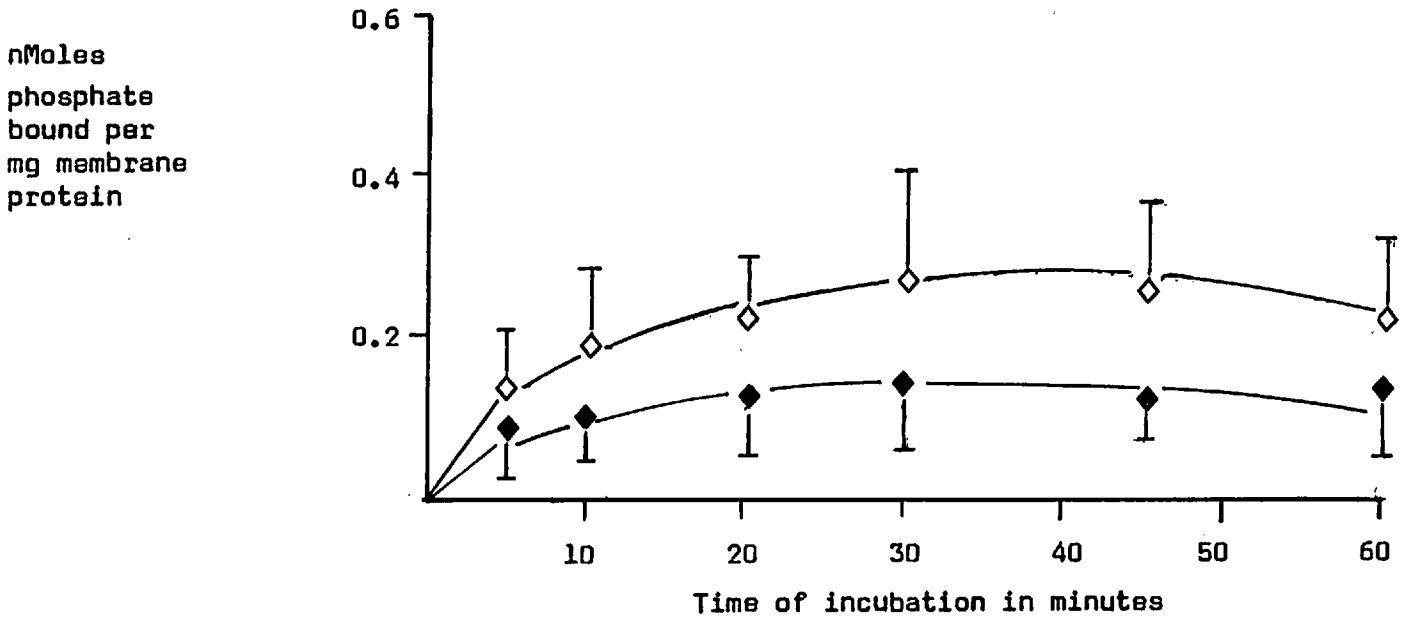

Fig 11.1a Phosphorylation of red cell membranes derived from the top layer of cells after high speed centrifugation. Open symbols: no exogenous protein kinase added. Closed symbols: exogenous cytoplasmic protein kinase present. Each point is a mean of four determinations using different membrane samples; each value is in duplicate. ± 1SD shown.

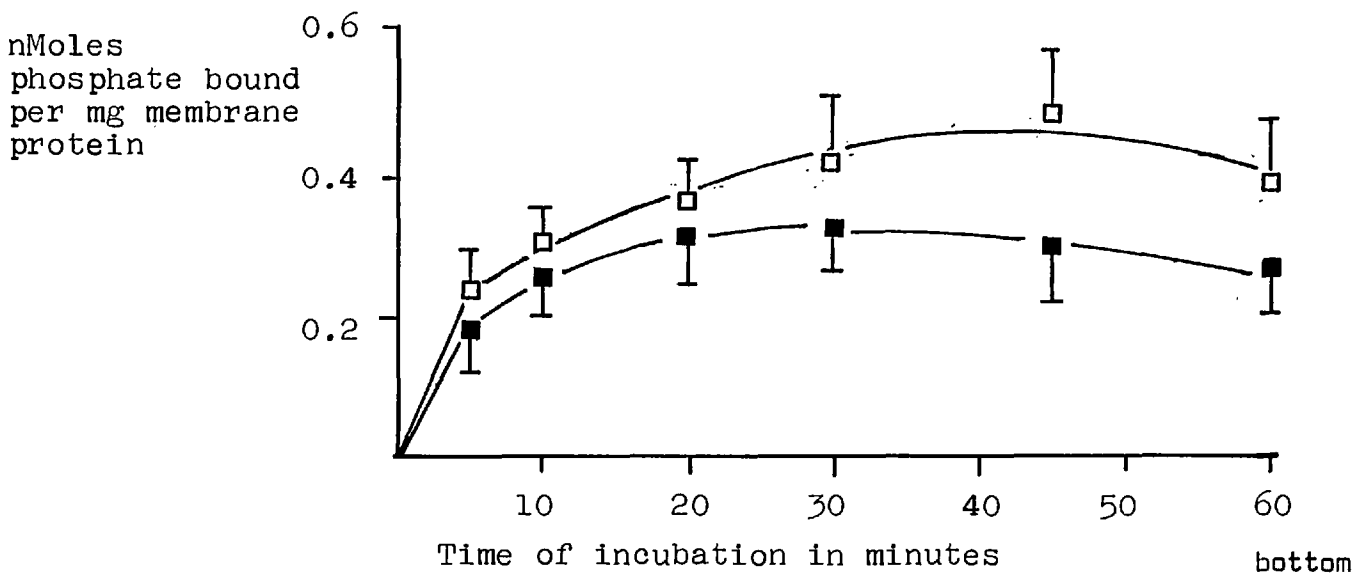

Fig 11.1b Phosphorylation of red cell membranes derived from the bottom layer of cells after high speed centrifugation.
Open symbols: no exogenous protein kinase added.
Closed symbols: exogenous cytoplasmic protein kinase present.
Each point is a mean of four determinations using different membrane samples: each value is in duplicate. ± 1SD shown.

Table 11.4 a and b shows the values of phosphorylation of band (I + II). Maximum level of phosphorylation of the bottom layer was 0.023 nmole Pi bound/mg membrane protein applied to the gel at 45 minutes compared to that of the top layer (0.016) at the same period. When cytoplasmic protein kinase was added a slight decrease in the phosphorylation level occurred. It is possible that the decreased phosphorylation level of top-layer membrane may be due to decreased number of phosphorylation sites on the phosphate acceptor protein and that this number increases during maturation. This is poseible since it could be argued that no further synthesis of protein could occur during maturation of the erythrocytes in the circulation since in anucleated cells further protein synthesis is absent. Conformational changes in the membrane protein during ageing may bring about the increased level of phosphorylation by exposing greater number of phosphorylation sites. The interaction of the protein with the lipids may also be altered in a way affecting the phosphorylation activity of the membrane proteins.

Table 11.4a : Time course of phosphorylation of membrane derived from the top layer of cells. Activity in band (I & II) determined without and with exogenous protein kinase.

Time (mins)	Activity: nmoles ^{32}P bound per mg protein of the original membrane									
	No PK					+ PK				
	Subjects					Subjects				
	1	2	3	mean	± 1SD	1	2	3	mean	± 1SD
5	0.008	0.013	0.009	0.010	0.003	0.007	0.005	0.0035	0.005	0.002
10	0.0094	0.012	0.011	0.011	0.002	0.0062	0.009	0.007	0.007	0.001
20	0.015	0.019	0.013	0.016	0.003	0.0075	0.011	0.0089	0.009	0.002
30	0.009	0.012	0.015	0.012	0.003	0.0089	0.015	0.012	0.012	0.003
45	0.012	0.019	0.018	0.016	0.004	0.0109	0.019	0.020	0.017	0.005
60	0.011	0.009	0.015	0.012	0.003	0.0079	0.009	0.018	0.012	0.006

Table 11.4b: Time course of phosphorylation of bottom layer membrane. Activity in band I&II determined with and without exogenous protein kinase.

Activity: nmoles Pi bound per mg protein used for electrophoresis.

	No PK					+ PK				
	Subject					Subject				
Time (mins)	1	2	3	mean	± lSD	1	2	3	mean	± S.D.
5	0.006	0.004	0.0089	0.006	0.002	0.006	0.017	0.009	0.005	0.0016
10	0.0071	0.0099	0.010	0.009	0.002	0.0075	0.009	0.010	0.009	0.001
20	0.0089	0.010	0.0150	0.012	0.003	0.0089	0.010	0.012	0.010	0.002
30	0.0125	0.0210	0.0200	0.018	0.005	0.0092	0.019	0.013	0.014	0.005
45	0.0921	0.0260	0.0210	0.023	0.003	0.017	0.023	0.019	0.020	0.003
60	0.0112	0.0230	0.0150	0.016	0.006	0.0098	0.015	0.008	0.011	0.004

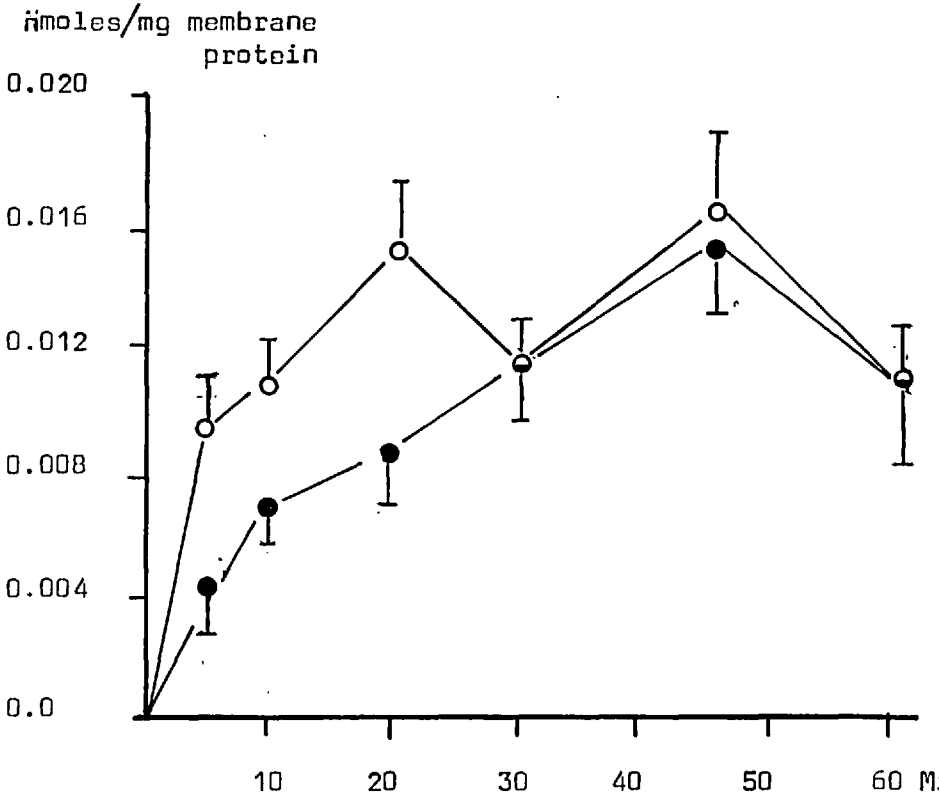

Fig 11.4a. Time course of phosphorylation of band(I+II) derived from top layer of RBC. Open circles: without protein kinase; closed circles with exogenous protein kinase. Mean of three samples in duplicate.

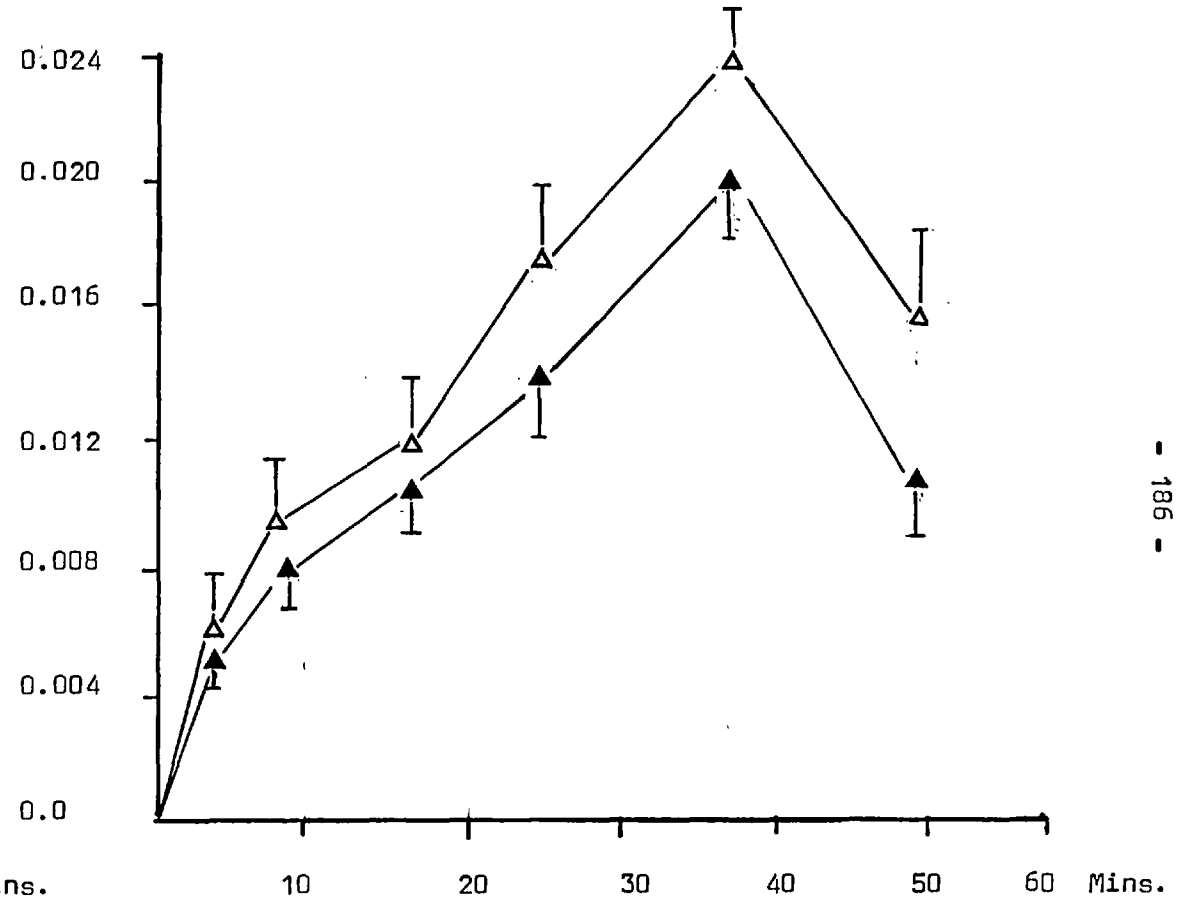

Fig 11.4b. Time course of phosphorylation of band(I+II) derived from bottom layer of RBC. Open triangles without exogenous protein kinase added; closed triangles with exogenous protein kinase. Mean of three different samples in duplicate.

SECTION 12

12.1 PHOSPHORYLATION OF SPECTRIN BAND (I & II) AND MEMBRANES OF HEREDITARY SPHEROCYTOSIS AND NORMAL SUBJECTS

As already shown in section 10 the membranes of the red cells exhibit an ability to undergo endogenous phosphorylation and dephosphorylation in the presence of ATP and cAMP. It was shown that when spectrin was extracted from these membranes, a similar pattern of phosphorylation and dephosphorylation was observed. In hereditary spherocytosis, a type of anaemia characterised by smaller than normal volume of the cells and spherocytic shape, abnormalities of the membrane components may be present. Many investigators have proposed that the red cell membrane is the site of such abnormalities (Harris and Frankerd, 1953).

In this study the rate of the phosphorylation of the membrane from patients with H.S. is studied and compared with the rate of phosphorylation of normal subjects. The pattern of phosphorylation of spectrin bands (I & II) from SDS gel electrophoresis, is also investigated.

12.2 METHODS

12.2.1 Endogenous phosphorylation of normal and H.S. membranes

Freeze-thawed membrane samples were phosphorylated in the presence of ATP and cAMP as described in section 10, for a period up to 120 minutes. The reaction was stopped at various time intervals as described previously and the final sediment was dissolved in 1% SDS and 50µl aliquots was taken from each tube for determination of radioactive incorporation of ^{32}P. To 20µl sample of this

sediment, 5 µl aliquot of sucrose solution was added and 12.5 µl of this was subjected to electrophoresis as described in section 14. After staining in Coomasie blue band (I & II) was identified, cut and dissolved in 30% H_2O_2 at 37°C overnight. The radioactivity was measured in dioxane cocktail using Intertechnique SL 30 spectrometer.

In some experiments, staining of the gels with Coomasie blue was not carried out. The final sediment was labelled with the fluorescent dye (dansyl chloride) before the electrophoresis and bands (I & II) were immediately visualised under the U.V. light, cut and dissolved in 30% H_2O_2 before counting. The disadvantage of this technique is that the bands tend to diffuse when fixation with acetic acid and isopropanol is omitted, therefore the need to cut the bands immediately on visualisation is important.

12.2.2 Dansylation of the membrane protein

Dansylation was carried out according to Talbot & Yphantis (1971) except that dansylation by-product was not removed with sephadex-25 before electropheresis, since these are of low molecular weight and did not effect or interfere with bands (I & II).

To 0.5 to 1 mg of sediment protein the following was added: 100µl of 0.1mM tris (pH adjusted to 8.1 with acetic acid); 50 µl of 20% SDS; 50 µl of 10% dansyl chloride in acetone. The mixture was left for 1 minute at room temperature and then transferred to a boiling water bath for 5 minutes. For general purposes 10 µl of this mixture was added to the top of gel tube and electrophoresis was carried out. When the protein concentration was low, the final reaction mixture was added to a small volume of 7.5% TCA and the precipitated protein was dissolved in 1% SDS for further analysis.

12.3 RESULTS AND DISCUSSION

12.3.1 Phosphorylation activity of normal and H.S. membrane

Membranes derived from H.S. and normal red cells were assayed for endogenous phosphorylation in the presence of ATP and cAMP. The phosphorylated membrane proteins were isolated by SDS polyacrylamide gel electrophoresis and bands (I & II) were cut into one piece, dissolved and counted for ^{32}P incorporation.

Time course studies showed that the phosphorylation of H.S. membrane (fig 12.1 and table 12.1) gives a similar pattern to that obtained with normal membrane (see fig 10.3). The results of H.S. phosphorylation were highly variable represented by different levels of phosphorylation among H.S. patients and a consistent pattern could not be achieved constantly as indicated by the elevated standard deviation values as shown in table 12.1. The maximum level of phosphorylation of H.S. membrane was reached at 40 to 45 minutes of incubation and it was 0.302 $\pm$ 0.090 nmole phosphate bound per mg protein. The maximum level of phosphorylation in the H.S. tends to be generally lower but because of the large deviations involved, it is not significantly different from normal (0.373 $\pm$ 0.053) at the $P = 0.05$ level (student t-distribution for the values of phosphorylation at 30, 40, and 50 minutes for normal and H.S. compared).

The initial linear velocity was similar to that of the normal at concentration of ATP used (5 μM) in this experiment. All patients investigated in this study had typical type of H.S. and were all splenectomised. Therefore lowered phosphorylation

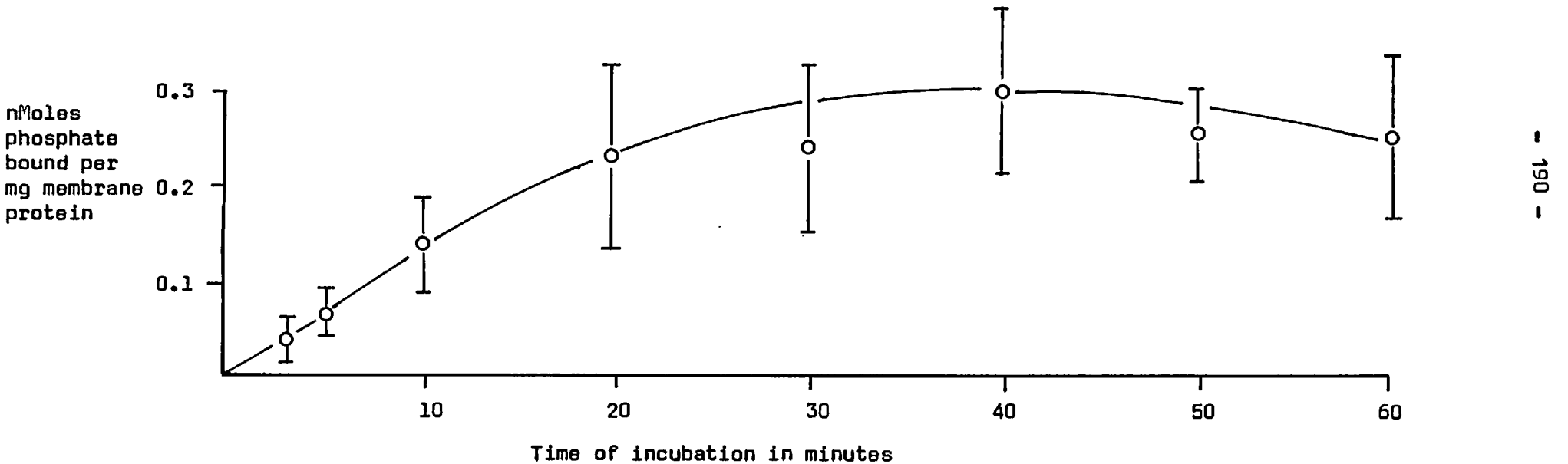

Fig 12.1 Time course for phosphorylation of red blood cell membranes from HS patients. Results were variable particularly in the region of 20 to 60 minutes. (Nine different subjects; each determination in duplicate). Compare with normal, fig 10.3.

Table 12.1 Phosphorylation of HS red cell membrane.

Time (mins)	Activity: nmoles Pi bound/mg membrane protein. subjects						
	1	2	3	4	5	6	Mean ± 1SD
3	0.052	0.029	0.035	0.038	0.046	0.041	0.040 ± 0.008
5	0.082	0.052	0.068	0.099	0.065	0.043	0.069 ± 0.20
10	0.203	0.198	0.166	0.130	0.083	0.088	0.145 ± 0.053
20	0.222	0.283	0.368	0.166	0.257	0.096	0.232 ± 0.095
30	0.281	0.321	0.348	0.150	0.229	0.107	0.240 ± 0.096
40	0.418	0.218	0.395	0.263	0.311	0.205	0.302 ± 0.090
50	0.244	0.195	0.344	0.252	0.308	0.197	0.257 ± 0.059
60	0.224	0.262	0.335	0.156	0.312	0.170	0.243 ± 0.073
90	0.351	0.325	0.276	0.187	0.309	0.158	0.269 ± 0.080

maxima could not be due to elevated reticulocytes (see section 11 for phosphorylation activity in young and old red blood cells).

12.3.2 SDS polyacrylamide gel electrophoresis of phosphorylated membranes from H.S. and normal red cells

The incorporation of ^{32}Pi in band (I & II) represents the total activity of both bands cut from the gel in one piece. Table 12.2 and 12.3 show the values of Pi incorporation in band (I & II) for normal and H.S. respectively during the time period of incubation selected. The results are expressed as pmoles of phosphate bound per mg protein.

The patterns of phosphorylation of band (I & II) of normal and H.S. are shown in figs 12.2 and 12.3 respectively. The initial rate of phosphorylation of H.S. band (I & II) is approximately 1.1 pmole phosphate bound per mg membrane protein per minute whereas that for normal the value is 2.92 pmole phosphate bound per mg membrane protein per minute.

Linear regression analysis for the initial velocity was not carried out since the H.S. gel pattern clearly showed non-linear behaviour. It is noticed that for the time periods of 3, 5 and 10 minutes the standard deviation values for each group do not overlap to any extent (n = 6 for each point). Dephosphorylation was shown to occur at 30 minutes of incubation in both normal and H.S. samples followed by rephosphorylation which reached a peak at 45 minutes and continued to dephosphorylate thereafter. Therefore two maximal phosphorylation levels are observed as shown in figs 12.2 and 12.3 with the level of H.S.

Table 12.2 Phosphate incorporated in gel slices of band (I & II) of HS red cell membrane. Results are expressed as mg protein of original membrane loaded onto the gel. Each value is a mean of two determinations.

Time (mins)	Activity pmole Pi bound/mg membrane protein						
	subjects						
	1	2	3	4	5	6	Mean ± 1SD
3	2.2	1.7	2.9	3.2	5.2	1.9	2.8 ±1.3
5	6.7	3.9	10.2	14.3	7.3	5.8	8.1 ±3.8
10	6.3	8.3	11.7	15.6	14.5	8.9	10.9 ±3.7
20	16.0	16.0	22.6	30.2	25.5	24.3	22.5 ±5.6
30	6.8	3.5	3.8	2.6	5.4	4.1	4.4 ±1.5
40	31.0	29.0	23.7	25.1	23.3	21.2	25.7 ±3.7
50	18.3	21.3	15.3	12.3	10.9	14.1	15.4 ±3.8
60	13.2	15.0	10.1	9.2	4.5	7.3	9.9 ±3.8
90	8.4	9.3	4.4	6.0	2.6	6.8	6.3 ±2.5

Table 12.3 Phosphate incorporated in gel slices of band (I & II) of normal membrane. Results are expressed as per mg protein of original membrane loaded onto the gel. Each value is a mean of two determinations.

Time (mins)	Activity: pmoles Pi bound/mg membrane protein						
	subjects						
	1	2	3	4	5	6	Mean ± ISD
3	6.3	8.4	9.19	8.9	5.9	7.3	6.7 ±2.5
5	11.1	10.9	17.4	13.3	15.4	19.3	14.6 ±3.4
10	17.6	15.6	21.4	17.3	23.3	20.2	19.2 ±3.0
20	22.3	19.9	41.3	29.7	32.3	28.5	27.3 ±5.05
30	5.2	8.3	15.3	10.62	16.4	12.3	11.34 ±4.3
40	28.4	32.9	29.6	34.2	35.7	39.3	33.4 ±4.0
50	21.2	28.5	35.7	32.1	28.3	25.3	28.5 ±5.1
60	19.3	25.4	28.8	29.4	25.8	22.3	25.8 ±5.0
90	8.4	14.4	23.4	17.3	15.4	9.7	14.7 ±5.4

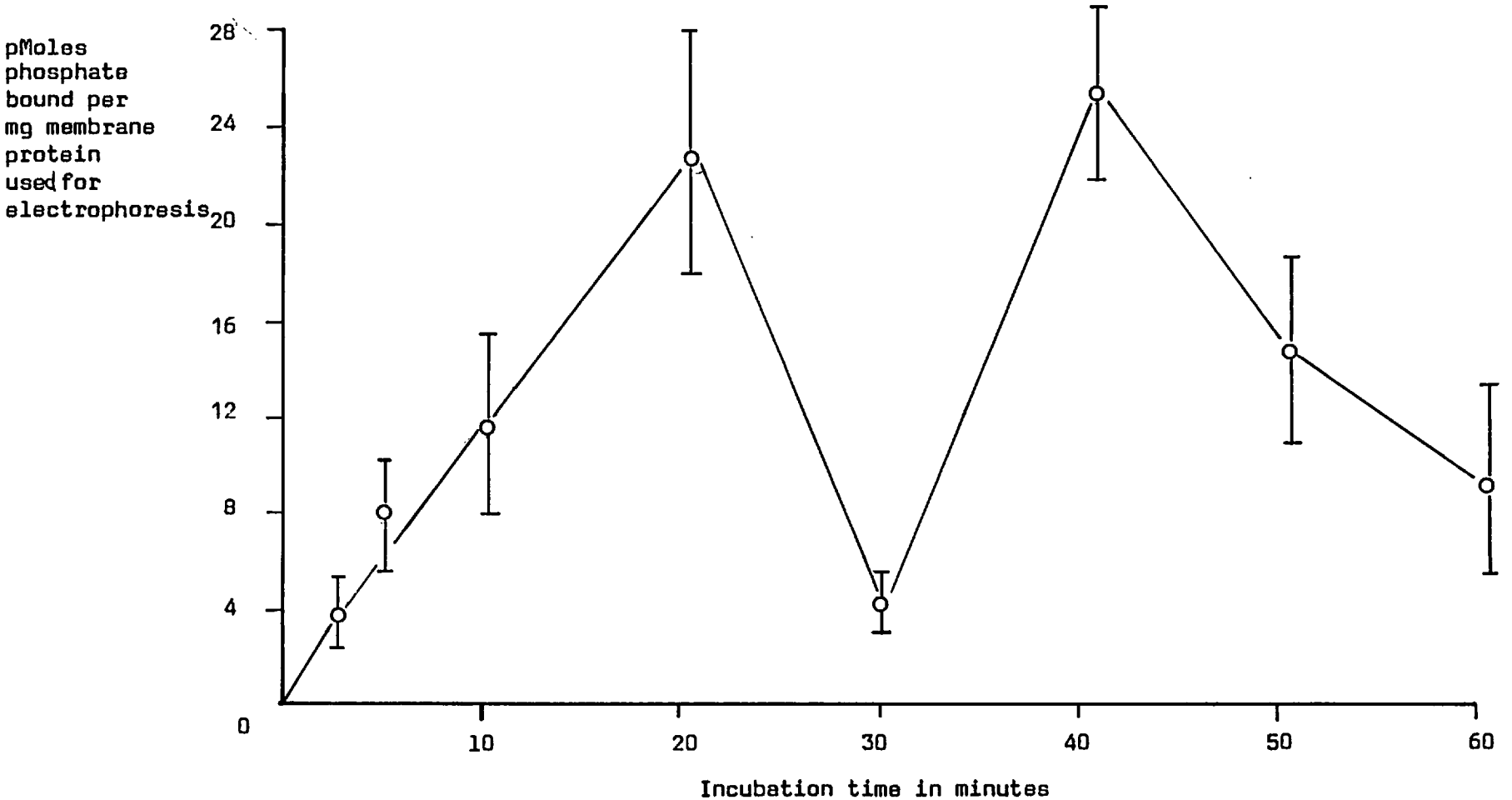

Fig 12.2 Phosphorylation of band (I & II) from SDS polyacrylamide gel. Membranes derived from samples of HS red blood cells (six subjects, single determinations).

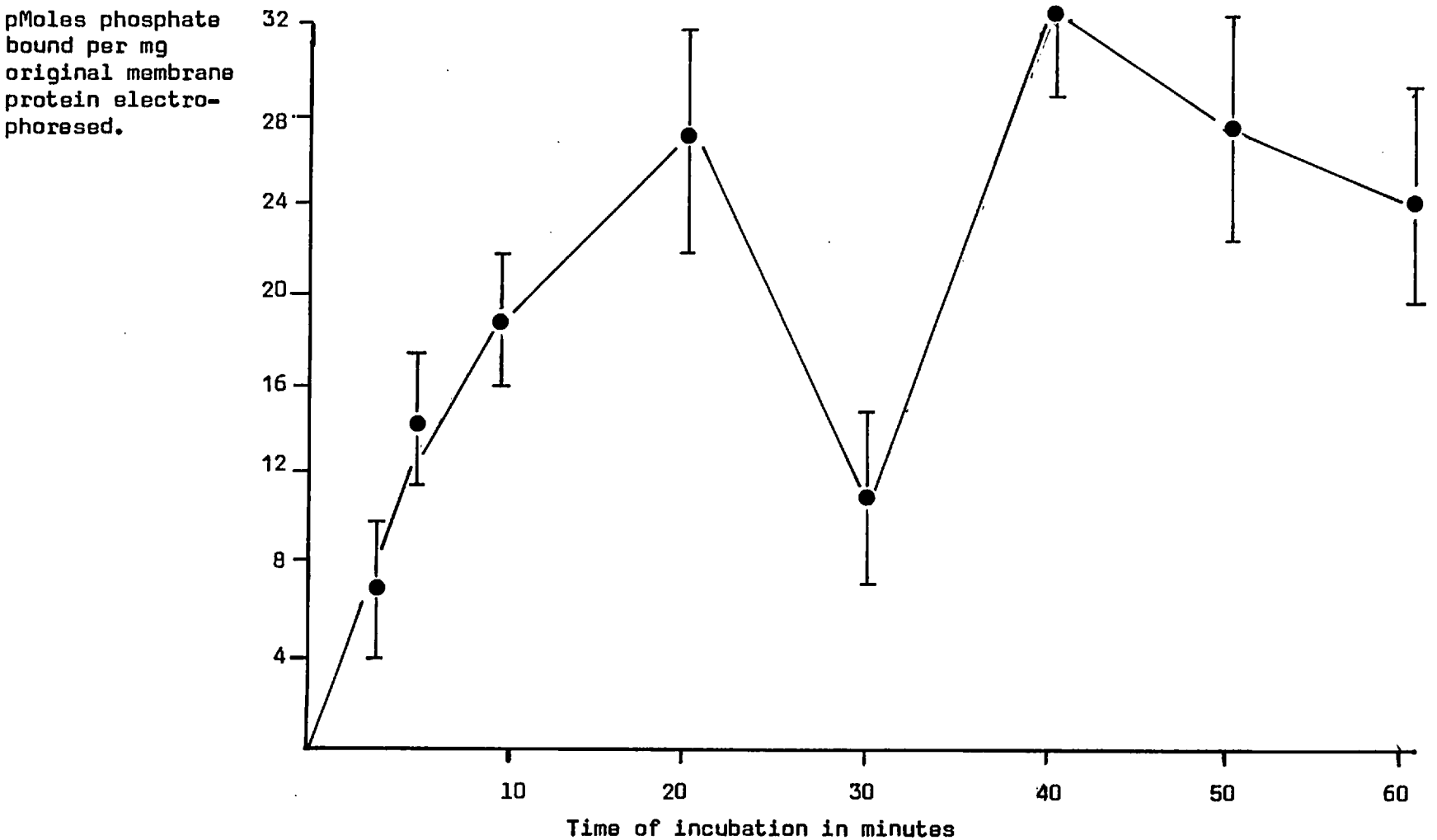

Fig 12.3 Phosphorylation of band (I & II) in normal red cell membranes. (Six subjects, single determination).

being consistently lower.

Since the activity measured was only greater than background by a factor of 2 or 3, it was important to find an alternative procedure to that of the Coomasie blue described (section 15.2) which normally requires 3 to 4 days. Further, dissolution of the gel slices with H_2O_2 may require additional day or two. Since the half-life of the isotope ^{32}P is approximately 14 days, the rapid method of labelling protein with the fluorescent dye (dansyl chloride) was used. This gave immediate visualisation under the u.v. light. Visualisation and band isolation were carried out immediately after electrophoresis since diffusion of bands was observed to take place when gels were left to stand for 2 to 3 hrs.

SECTION 13

13 PROTEIN KINASE KINETICS

The membranes of the red cells were shown to exhibit the ability for endogenous phosphorylation in the presence of cAMP and ATP. This phosphorylation is catalysed by the enzyme protein kinase. The enzyme protein kinase responsible for the phosphorylation of the membrane protein component is located on the inner surface of the membrane and approximately 70% of the total cAMP-dependent activity could be extracted by suspension in high salt concentrations (Rubin, et al, 1972). However, this enzyme when extracted, could catalyse the phosphorylation of exogenous substrates, particularly histone (Section 10.3). In this section the phosphorylation behaviour of the membranes of H.S. and normal subjects are investigated and the Lineweaver-Burk plots showed interesting and distinctive kinetic features for H.S. individuals as well as for one family of H.S. patients compared to that of normal. The membrane-bound protein kinases of these subjects were extracted and their kinetics were studied using histone as the phosphate acceptor.

13.1 METHODS

13.1.1 Preparation of the membrane

Blood was drawn from H.S. patients and normal subjects in CPD bottles. The membrane was prepared the same day according to Dodge et al (1963) as described in Section 7.1. The final sediment was suspended in cold 5mM phosphate buffer pH 8.0 to a protein concentration of 2 - 3 mg/ml and the samples were stored

at -20°C until required. The samples were used within the first two weeks of storage.

13.1.2 The phosphorylation assay

The membranes were thawed once and phosphorylated in the presence of ATP and cAMP as described in section 9.1. The activity was measured by the amount of the ^{32}Pi bound to the membrane components. When protein kinase was extracted from the membrane, histone was used as the phosphate acceptor protein and the phosphorylation procedure was carried out exactly as for the membrane samples.

13.1.3 Extraction of the protein kinase

The extraction of this enzyme from the membrane was carried out by modification of the procedure described by Rubin et al (1972) as shown in fig 13.1. The membranes were thawed once before use and 1ml was suspended in 2.7mls of 1M NH_4Cl containing 1% BSA for 2 hrs at 4°C with gentle stirring. The suspension was centrifuged for 30 mins at 30,000 rpm in MSE superspeed 75 using 8 x 50 ml rotor. The supernatant was dialysed against 500 volumes of 50mM phosphate buffer (pH 7.0) at 4°C overnight with two changes of the buffer. The supernatant was kept at -20°C after dialysis until required. The activity of this enzyme was stable under such storage conditions over the period it was used.

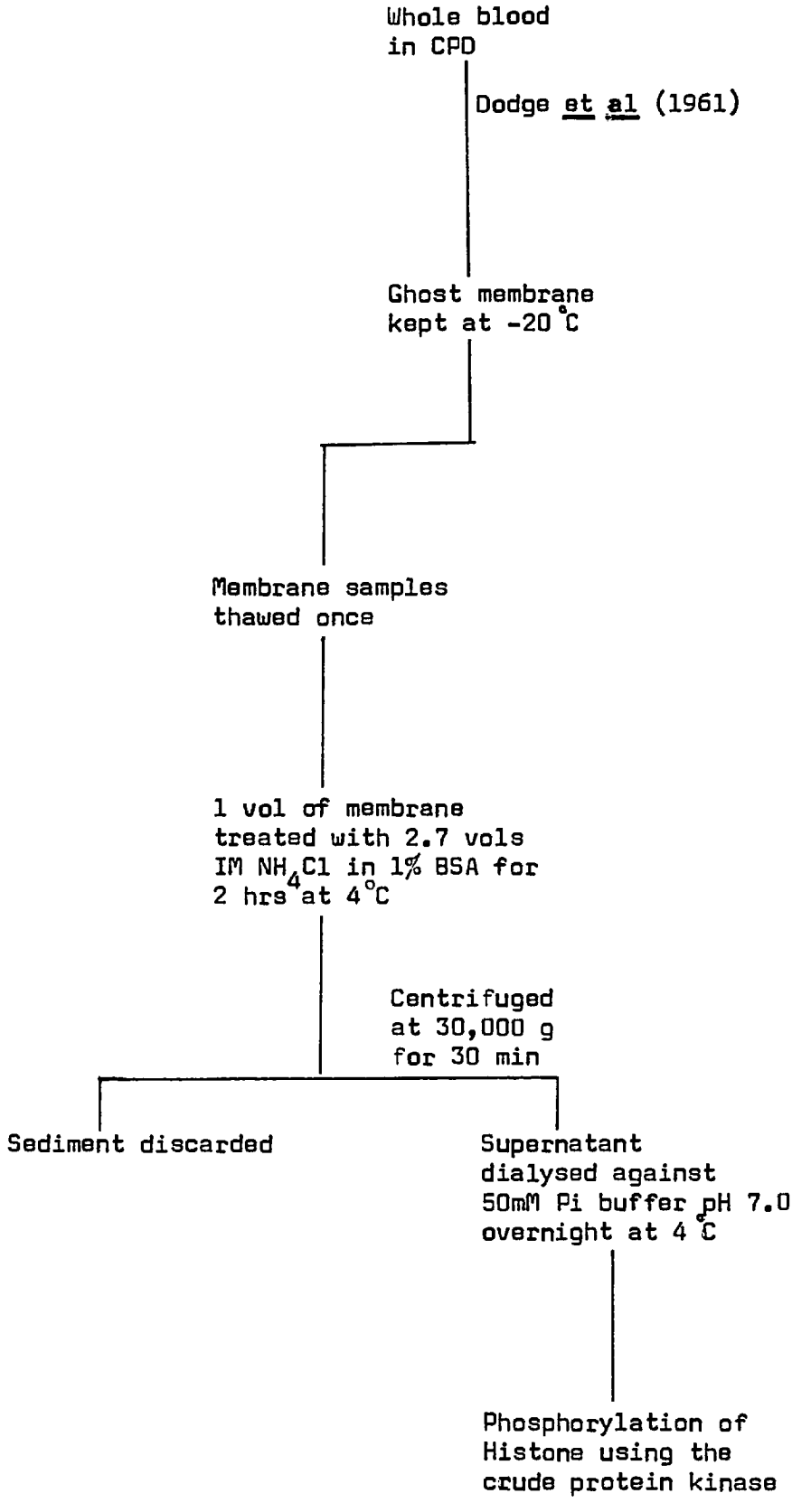

Fig 13.1 Flow diagram for the extraction procedure of membrane bound protein kinase from the red cell membrane.

13.2 RESULTS AND DISCUSSION

13.2.1 Assay conditions

Attempt was made to ensure that only linear rate of phosphorylation was measured.

A time course of phosphorylation for both normal and H.S. red cell membrane is presented in fig 10.3 and 12.1. It is observed that the reaction rates were normally linear in both instances for up to at least 10 minutes. Many readings were rejected due to non-linearity of the reaction.

For these studies the commercially available gamma-labelled $AT^{32}P$ was used. Each batch was tested on a dowex column for purity and level of ^{32}Pi as described in section 7.2.2. Although the level of labelled $AT^{32}P$ was very low, it was included in the calculations for the total ATP concentrations in the medium.

13.2.2 Kinetics of normal and H.S. red cell membrane phosphorylation

The velocity was calculated as the amount of phosphate (in nmoles) bound per mg protein per minute. The line fitted to the plots of $^{1}/v$ versus $^{1}/s$ was determined by linear regression analysis on Hewlett Packard calculator model 10A. The programme used and also the various statistical parameters are shown in appendix. Using these values, the variance and slope of the regression line was compared according to the statistics presented in Documenta Giegy, p. 176 - 178. These values are presented in tables 13.1 and 13.2. The lineweaver-Burk plots for normal and H.S. membrane are shown in fig 13.2 and figs 13.3 and 13.4 respectively.

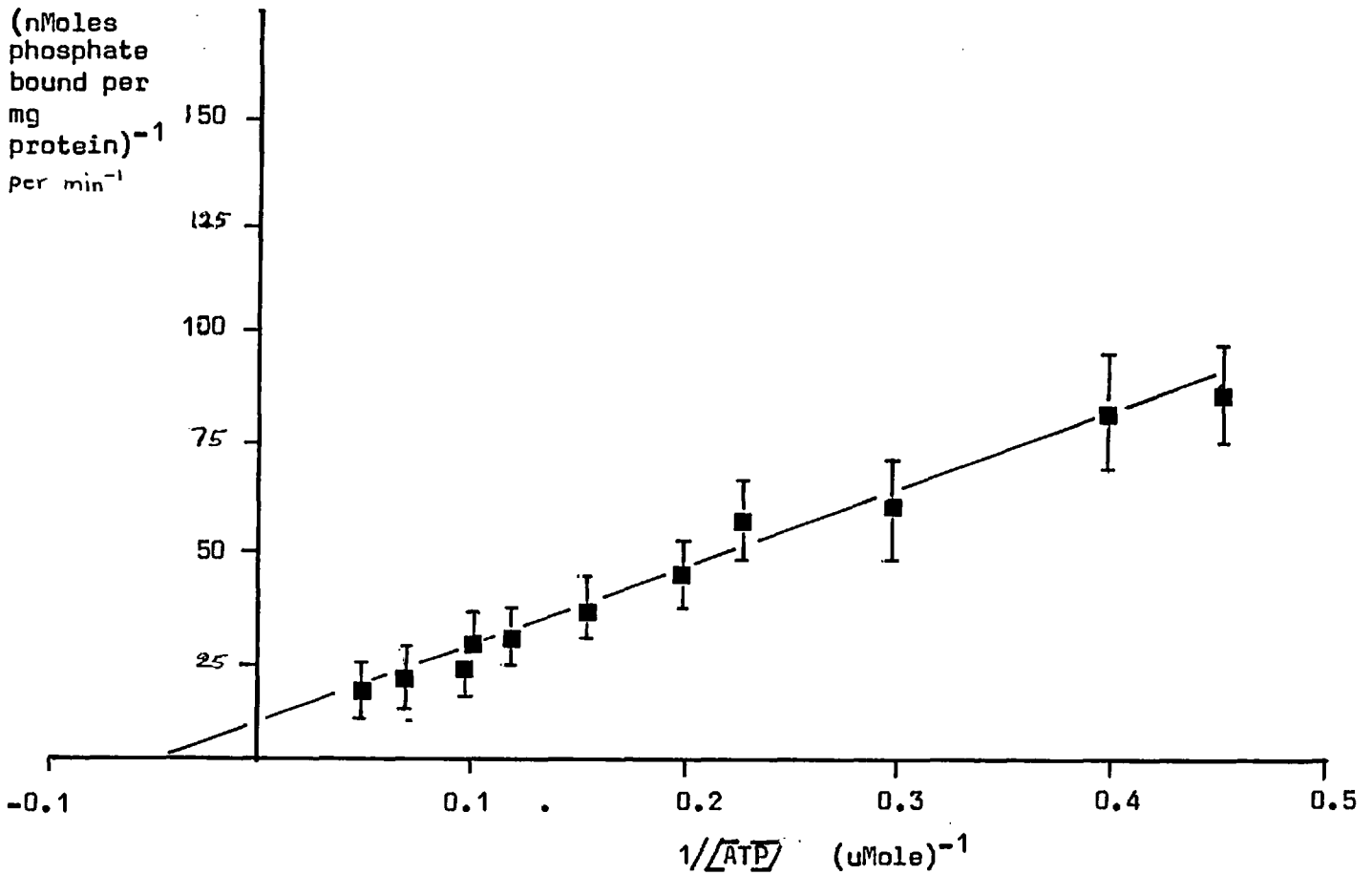

Fig 13.2 Double reciprocal plots for red cell membrane from normal subjects. The Km is 24.75 μMole. The Vmax 0.120 nmoles bound per mg protein per min. Each point is a mean of at least eight determinations. Six normal subjects studied.

Table 13:1 Reciprocal values of ATP concentration used and velocity of normal cell membrane. Each value is a mean of two determinations.

$\frac{1}{ATP}$ μM	(nmoles phosphate bound per mg membrane protein per min)$^{-1}$										
	Subjects										
	1	2	3	4	5	6	7	8	9	MEAN	± 1SD
0.430	88.1	100.2	110.1	95.3	85.7	108.4	88.5	80.3	75.9	92.5	11.9
0.400	85.1	70.8	69.3	87.4	111.9	91.9	68.3	98.4	91.8	86.1	14.7
0.300	66.3	68.5	70.0	79.2	85.2	62.7	73.9	78.8	81.8	74.0	7.9
0.233	37.4	53.4	60.8	64.2	39.8	36.4	67.9	51.3	56.1	51.9	11.7
0.200	41.7	43.9	56.0	40.5	54.6	50.3	44.9	48.1	39.8	46.6	6.1
0.160	35.2	24.8	39.3	43.8	24.3	38.5	47.7	37.3	40.4	36.8	7.9
0.120	37.8	33.6	21.1	32.7	20.5	39.7	40.9	20.4	34.3	33.1	8.3
0.100	38.4	20.2	18.4	22.1	32.6	34.3	15.9	24.3	11.8	24.2	9.0
0.099	31.3	35.4	24.3	36.4	34.2	29.7	22.9	21.2	21.8	28.7	6.0
0.070	18.4	31.3	22.1	18.4	34.5	16.9	34.2	19.3	21.9	24.1	7.2
0.050	26.1	14.4	18.3	27.9	15.0	20.3	19.4	13.2	26.7	20.1	5.6

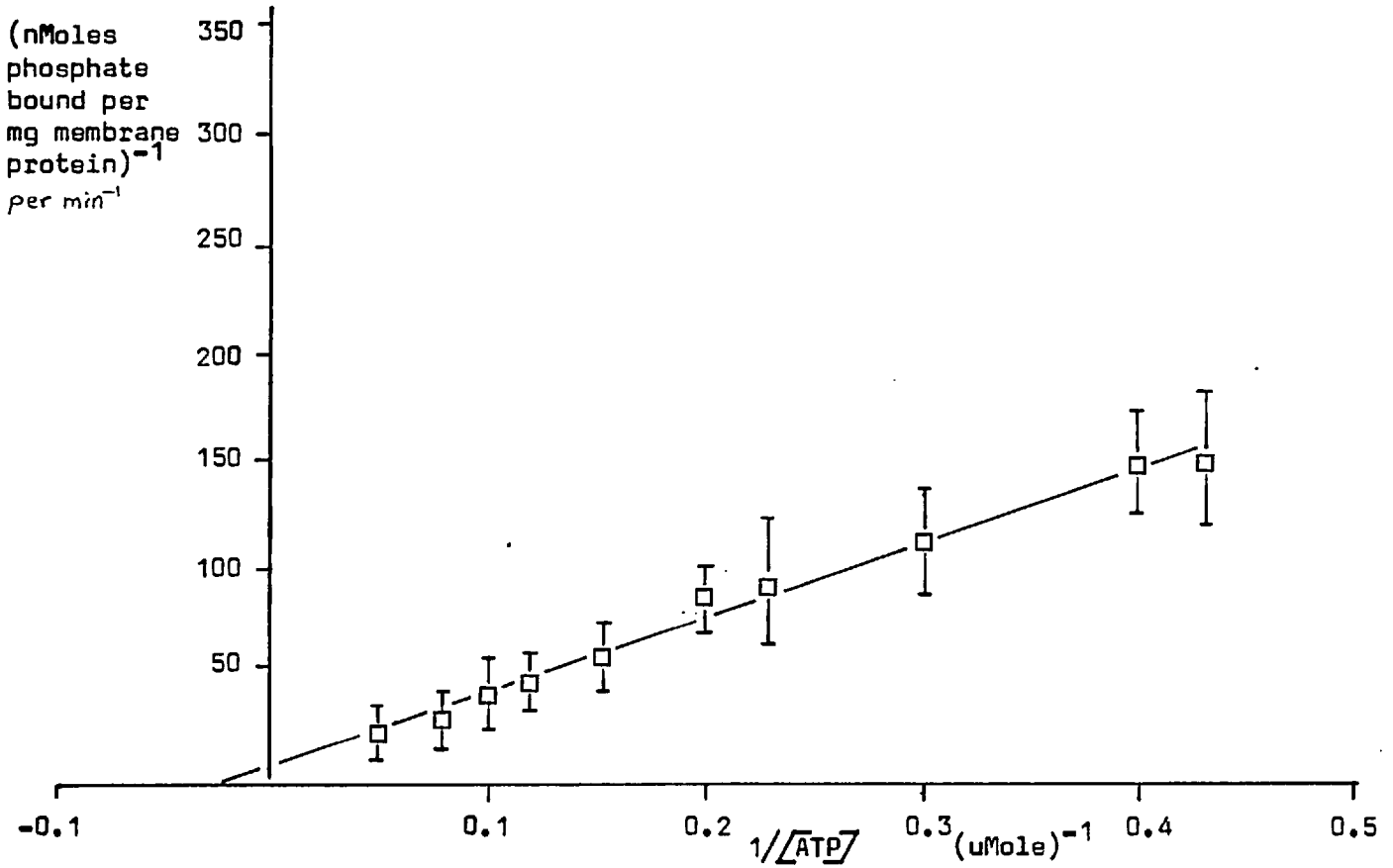

Fig 13.3 Double reciprocal plots using red cell membrane from H.S. patients. The Km value is 39.0uMoles and Vmax 0.118nM/mg/min. Each point is a mean of at least six determinations. (± 1SD shown). Seven typical splenectomised patients studied.

Table 13.2 Reciprocal values of substrate ATP and velocity for red cell membrane phosphorylation of H.S. subjects. Each value is a mean of two determinations.

$\frac{1}{ATP}$ μM	(nmoles phosphate bound per mg protein per min)$^{-1}$								
	Subjects								
	1	2	3	4	5	6	7	MEAN	± 1SD
0.430	155.8	163.2	131.1	142.0	150.8	125.4	178.3	149.5	18.4
0.400	131.8	140.5	140.7	133.6	128.3	170.9	143.2	141.3	14.8
0.300	84.9	101.9	90.4	120.1	91.0	110.7	115.6	102.1	13.8
0.233	71.2	83.4	75.5	95.7	81.2	65.3	88.1	80.9	9.7
0.200	61.6	84.4	71.4	81.0	68.3	60.9	56.4	69.1	10.6
0.160	62.3	63.1	75.0	62.7	49.8	58.9	53.6	60.8	8.1
0.120	35.7	33.1	42.4	38.3	48.1	54.4	39.1	48.2	7.4
0.100	33.3	40.6	42.4	38.9	37.7	50.1	48.5	41.6	6.0
0.099	51.5	39.8	52.5	42.9	30.4	36.2	41.0	42.0	7.9
0.070	32.2	24.9	28.7	38.0	33.6	41.9	32.0	32.2	6.2
0.050	28.2	22.2	25.1	24.8	22.1	24.8	29.3	25.1	4.5

The value of V max for normal membrane phosphorylation was found to be 0.120 nmole phosphate bound per mg protein per min and that of the H.S. was 0.118 nmole phosphate bound per mg protein per minute.

The Km values for normal and H.S. membrane phosphorylation was 24.7 and 39.3μM respectively. Analysis of the variances showed that the two regression lines were different at the P = 0.05 level.

A family of H.S. patients (mother and son) showed kinetics which were different from the rest of the H.S. patients etudied. A positive co-operativity of the phosphorylation was noticed (fig 13.4). The Km and Vmax values of curvilinear regression analysis was close to that of the rest of H.S. studied.

This indicates the existence of more than one interacting phosphate receptor site on these H.S. (family) red cell membrane.

Double reciprocal plots for histone phosphorylation , using extracted protein kinase, gives very similar values for normal and H.S. The Km value is 20.3 & 19.4μM ATP and the Vmax 0.121& 0.120 respectively. The plots are shown in Figs 13.5& 13.6.

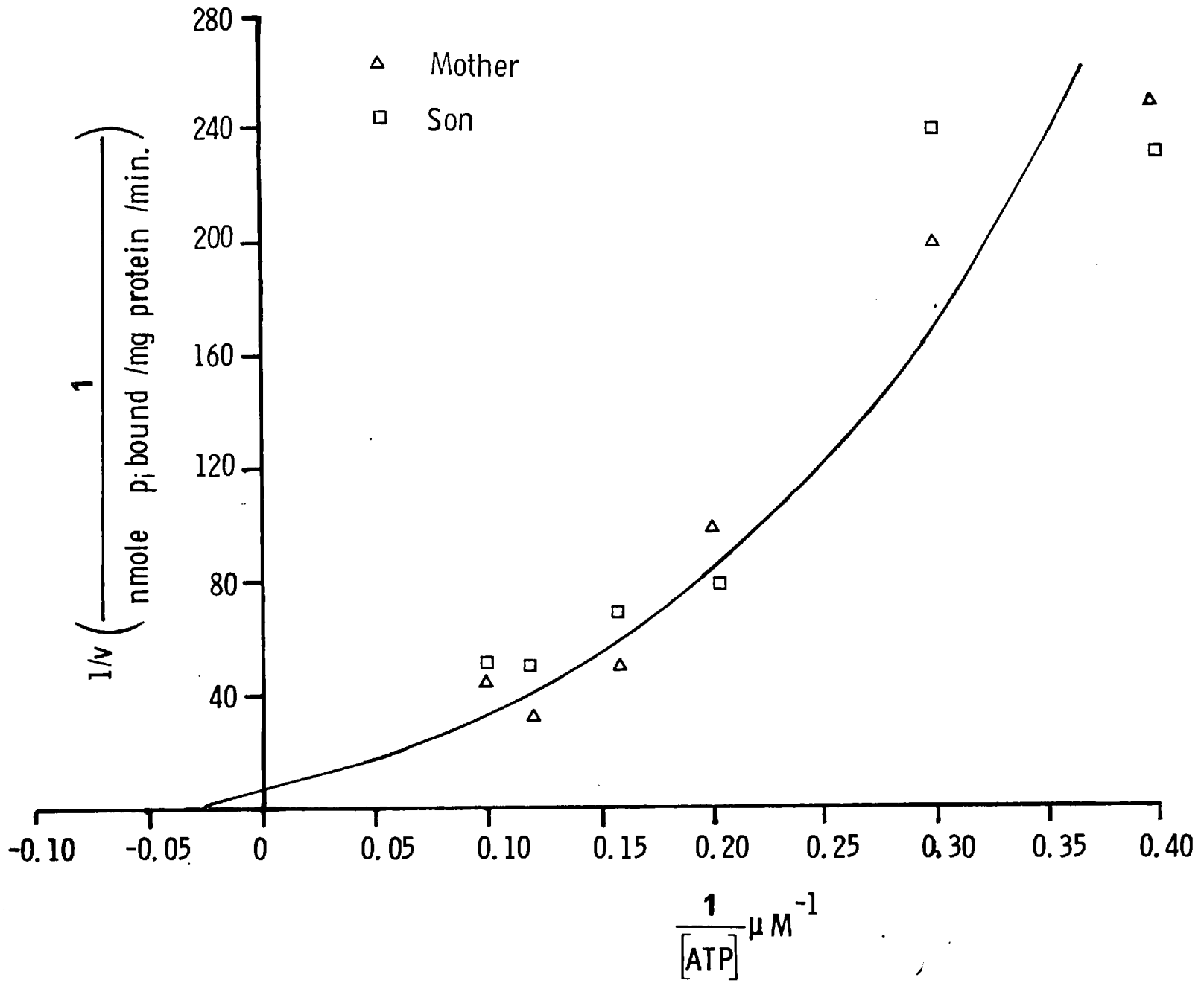

Fig 13.4 Lineweaver-Burk plots for membrane ghosts. A familial group of mother and son showing positive co-operativity effect. A mean of four determination in duplicate is shown. Mother (△); son (□). The Km value is 30 uM ATP and Vmax 0.110 nmoles phosphate bound per mg protein per min.

Table 13.3 Red cell membrane phosphorylation of family of H.S. using varying concentrations of the substrate ATP. Each value is a mean of four determination

$(ATP\mu Mole)^{-1}$	$(nmoles\ Pi\ bound\ per\ mg\ protein\ per\ min)^{-1}$		Mean
	Subjects		
	1	2	
0.433	112.5	218.0	165.3
0.400	125.8	125.0	125.4
0.300	96.9	97.4	97.2
0.233	110.8	103.3	107.1
0.200	64.0	68.1	66.1
0.160	34.3	44.6	39.5
0.120	25.1	34.7	29.9
0.100	33.8	42.2	38.0
0.099	21.4	46.4	33.9
0.070	17.1	28.6	22.9

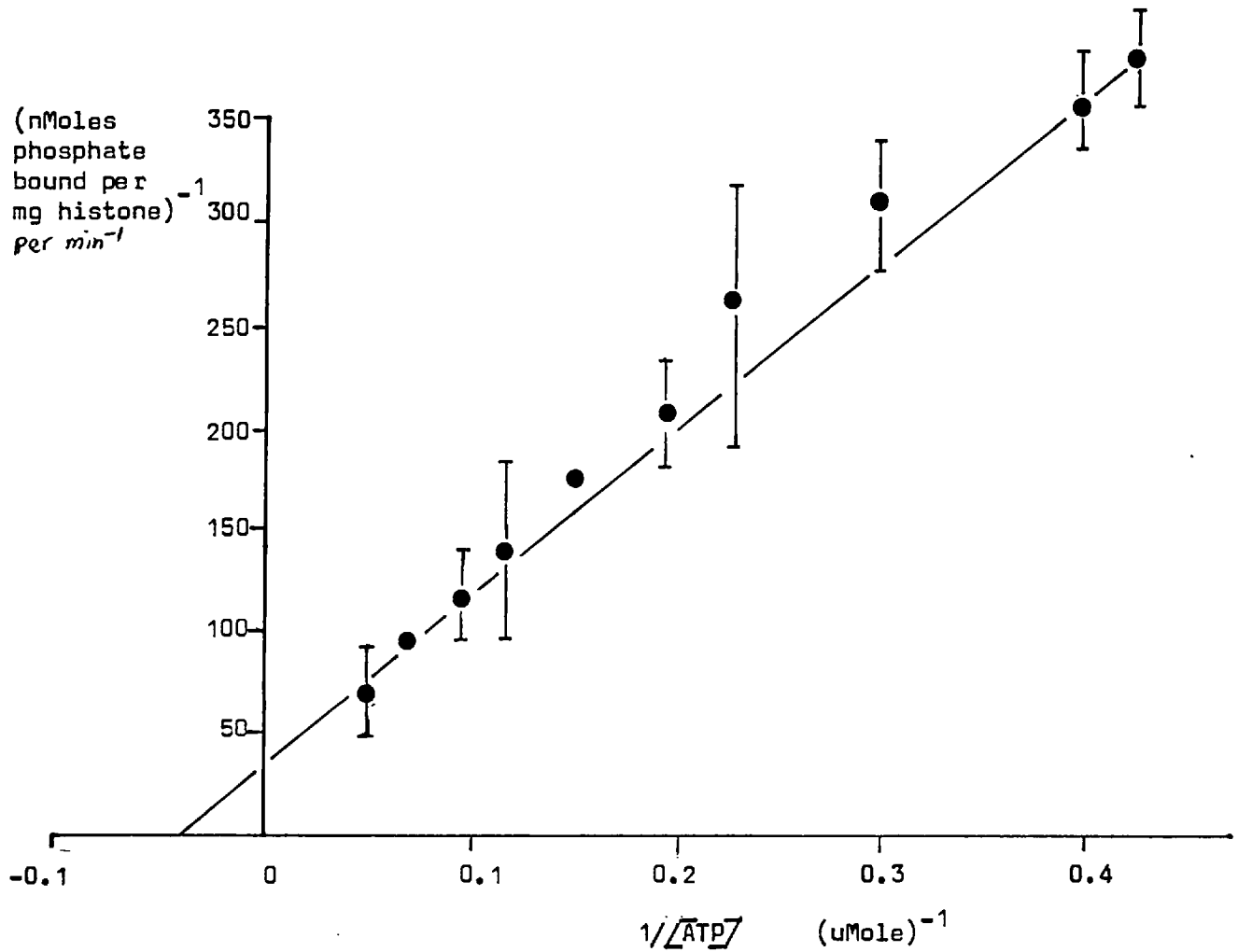

Fig 13.5 Lineweaber-Burk plot of histone phosphorylation using crude protein kinase extracted from the red cell membrane of normal subjects. Each point is a mean of at least eight determinations. Six different membrane samples used.

Table 13.4a Phosphorylation of histone by crude protein kinase fraction extracted from normal red cell membrane using varying concentrations of ATP. Each value is mean of two determinations.

ATP µMoles	nMoles Pi bound per mg protein per min				Mean ± 1SD
	Subjects				
	1	2	3	4	
0.100	110.9	109.4	107.5	139.4	114.3 ± 10.7
0.200	201	186.2	193.5	235.6	204 ± 21.8
0.300	335.9	308.7	286.2	354.1	321.3 ± 30
0.400	340.5	382.7	351.6	326.2	350.3 ± 21.6

Table 13.4b Phosphorylation of histone by crude protein kinase extract from normal membrane using varying concentration of ATP. Each value is a mean of two determinations.

ATP µMoles	nMoles Pi bound per mg protein per min		Mean ± 1SD
	Subjects		
	1	2	
0.433	392.2	364.9	378.2
0.233	196.1	114.0	155.1
0.160	189.2	165.2	177.2
0.120	154.9	103.8	129.3
0.099	134.2	95.2	114.7
0.070	127.3	89.4	108.3

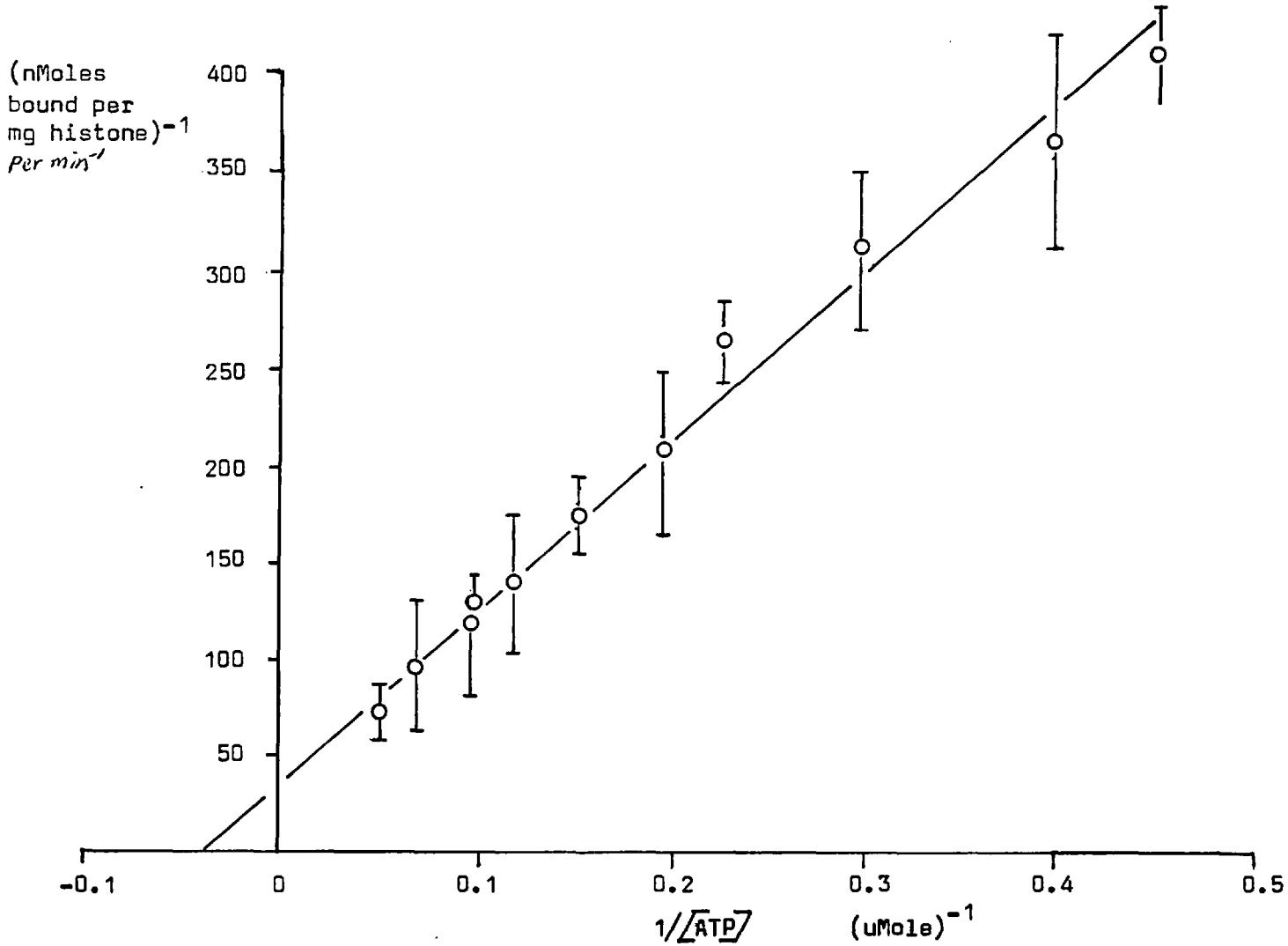

Fig 13.6 Lineweaber-Burk plot of histone phosphorylation using crude protein kinase extracted from red cell membrane of subjects with H.S. Determinations include the samples of protein kinase from mother and son (see fig 13.4). Nine subjects studied. Each point is a mean of at least ten determinations. Best fit line using linear regression analysis shown.

Table 13.5a Phosphorylation of histone by crude protein kinase fraction extract of H.S. membrane using varying concentrations of ATP. Each value is a mean of two determinations.

ATP µMoles	nMoles Pi bound per mg histone per min				Mean ± S.D.
	Subjects				
	1	2	3	4	
0.100	116.3	128.0	119.1	151.9	128.8 ± 16
0.200	169.5	208	181.0	268	206.7 ± 44.1
0.300	256.0	313.2	297.0	360.5	306.9 ± 43
0.400	287.3	383.9	318.7	419.5	352.4 ± 60.2

Table 13.5b Phosphorylation of histone by crude protein kinase extract from H.S. membrane with varying concentrations of ATP. Each value is a mean of two determinations.

ATP µMoles	nMoles Pi bound per mg protein per min			Mean ± 1SD
	Subjects			
	1	2	3	
0.433	397.2	378.4	431.4	402.3 ± 26.8
0.233	296.4	234.1	248.8	259.7 ± 32
0.160	210.5	145.6	167.2	174.4 ± 33
0.120	183.8	114.8	117.2	138.4 ± 38
0.099	155.3	87.5	100.2	114.2 ± 36
0.070	135.1	72.8	70.5	92.8

SECTION 14

14 IODINATION STUDIES: ENZYME CATALYSED IODINATION

The labelling of proteins with ^{125}I, catalysed by the enzyme lactoperoxidase, is widely used in the studies of the vectorial arrangement of membrane proteins since its first demonstration (Phillips & Morison, 1971). The specificity of the labelling allowed it to be used as a tool to detect the conformational changes of the proteins as they undergo chemical reactions with other substances. For example, the conformational changes of the horse heart cytochrome c upon oxidation and reduction were detected due to the inaccessibility of one of its subunits to the lactoperoxidase catalysed iodination, due to the shift of these subunits upon reduction (Dickerson et al, 1971; Takao et al, 1973).

Therefore it is important to understand the actual mechanism of halogenation in general and iodination in particular. Briefly, the halogenation process involves several steps during which intermediate complexes are formed between the enzyme and the halogen ions, the protein acceptor and the enzyme-halogen complex, (Morris & Hager, 1966; Hager et al, 1966). The general reaction steps can be represented as follows:

$$E + H_2O_2 \longrightarrow E' + H_2O$$

$$E' + X^- \longrightarrow E' - X^+$$

$$E' - X^+ + A \longrightarrow E' - X^+ - A$$

$$E' - X^+ - A \longrightarrow A - X + E$$

Where E is the enzyme, E' is the oxidised enzyme, X is the halogen ion; and A is the acceptor protein. In this study, the X and E

are iodine and lactoperoxidase respectively. E' appears as a common intermediate in all peroxidase reactions being derived from the interaction of the native protein E with hydrogen peroxide. The peroxidases show broad specificity with respect to halogen acceptor molecule; the most important halogenated amino acid being tyrosine in peptides. Since the halogens themselves are good nucelophiles, they can compete with tyrosine for the reaction.

Preliminary studies on the lactoperoxidase mechanism (Phillips & Morison, 1970) showed that the enzyme has two roles in the reaction. The first is the oxidation of the halogen ion, i.e. converting the iodine I^- to iodine I_2 which can react non-specifically with lipids or proteins non-enzymatically. The second role is in the interaction of the enzyme - halogen complex with the protein acceptor (A), resulting in the ionisation of (for example) the phenolic group of tyrosine to phenolate ion which will react with the iodide I^-. The enzyme lactoperoxidase has a molecular weight of 78,000 daltons and does not dissociate into subunits (Phillips & Morison, 1970), therefore it cannot penetrate the membranes of intact cells. However, as mentioned above, I_2 produced by the enzyme can penetrate the membranes labelling the proteins on either sides as well as the lipids causing errors in the labelling estimation. This can be prevented by lipid extraction prior to protein analysis.

In this study the lactoperoxidase catalysed labelling with ^{125}I was employed to detect the conformational changes of the protein spectrin in the phosphorylated and non-phosphorylated form. Since these changes could not be detected effectively when the intact membranes were used, the protein was isolated by SDS gel electrophoresis before determination of iodination. The most

successful study was, however, when spectrin was iodinated in the purified state.

14.1 METHODS

14.1.1 Phosphorylation of membrane and extracted spectrin

The phosphorylation of the membrane and isolated spectrin was carried out as described in section 10.1. The incubation period was 20 minutes except for the time course studies where the reaction was carried out for the time period selected. The iodination procedure was carried out at room temperature (22°C) immediately after this incubation.

14.1.2 Iodination of membranes and extracted spectrin

The membrane and extracted spectrin samples were labelled with ^{125}I following a modified procedure of Phillips & Morison (1971). The labelling was carried out at room temperature in a fume cupboard as follows. To one volume of the sample, two volumes of the labelling solution were added. The labelling solutions contained 150 mM NaCl, 10mM Tris, 10μMKI, 50 - 100 μCi ^{125}I per ml, (17mC/MgI^-) and 100μg/ml lactoperoxidase (specific activity = 120 units/min). The pH of this solution was adjusted to 7.4 with dilute HCl before the addition of ^{125}I and the enzyme. Immediately after the addition of the labelling solution 12 aliquots of 20μl each of 2.3 mM H_2O_2 were added to each tube at 30 second intervals, shaking the tubes after each addition. This mixture was then left for 5 min before the addition of 200μl of 100 mM KI as a "cold chase". The reaction was stopped as follows; the membrane and isolated spectrin samples being stopped differently.

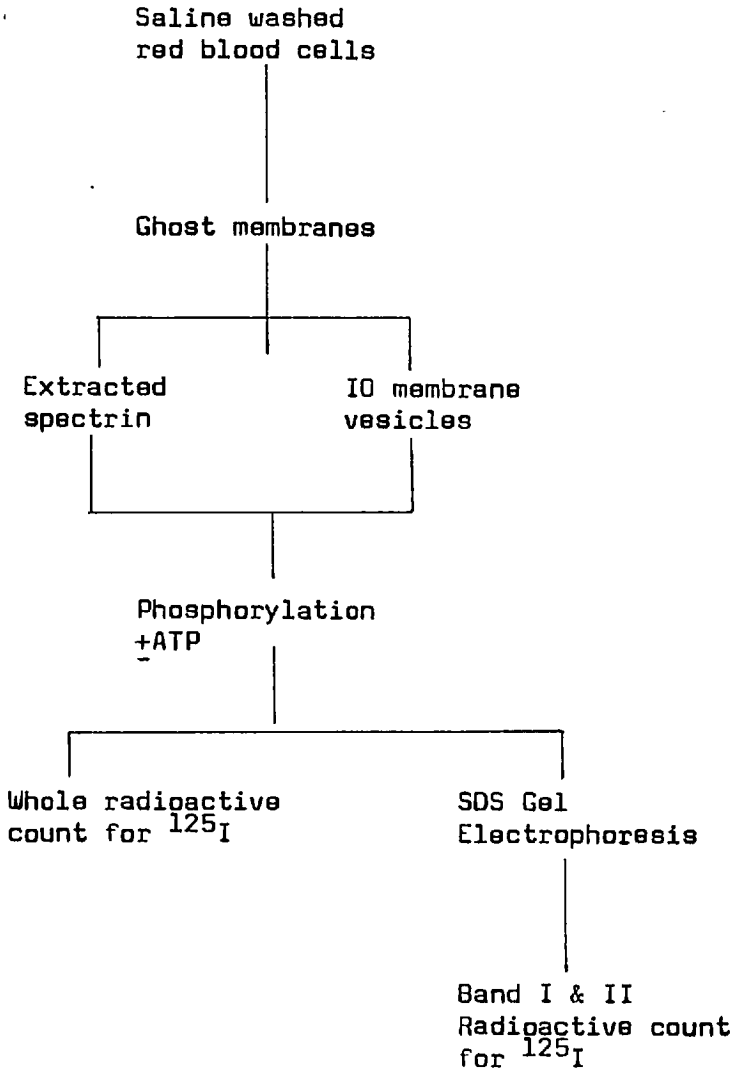

Fig. 14.1 A flow chart for experiments carried out

14.1.3 Stopping the iodination reaction for membrane samples

The reaction was stopped by addition of 3 mls of ice cold buffered saline (155 mM NaCl in 7 mM Na_2HPO4 buffer pH 7.4) and subsequently centrifuged at 4°C using MSE superspeed 75 in 10 x 10 mls rotor at 40,000 rpm for 20 min. The membranes were washed free of excess ^{125}I by repeating the centrifugation procedure in the buffered saline three times. The final sediment was suspended in 0.2 ml of 1% SDS and aliquots of the suspension was taken for counting in Pakard Tricarb-gamma spectrometer model 5898. The rest of the sample was kept for electrophoresis.

14.1.4 Stopping the iodination reaction for spectrin

When extracted spectrin was used the reaction was stopped at 4°C by the addition of 0.2 ml of 0.63% BSA followed immediately by the addition of 2 ml of 7.5% TCA. Further 2 ml of 7.5% TCA made up in 10% sucrose were pumped gently into the bottom of the tubs via a fine plastic tubing linked to a plastic syringe (the tubing was washed by shaking in cold TCA solution after each addition.) The sucrose solution forms a clear lower layer and the sample suspension was left in the top layer. The tubes were centrifuged at 4°C on MSE Mistral 4 L at approximately 2000 rpm for 20 minutes. The supernatant was aspirated and the sediment was dissolved in 0.2 ml of 1M ice cold NaOH. The solubilised material was reprecipitated by addition of 2 ml of cold 5% TCA and recentrifuged. This procedure was repeated three times and the final pellet was suspended in 0.2 ml of 1M NaOH. 50µl aliquots were taken for counting in Packard Tricarb-gamma spectrometer model 5898.

14.1.5 Gel electrophoresis of membrane and extracted spectrin

Twenty microlitre of the final suspension from either the membrane or the extracted spectrin was added to 10μl of solution containing: 1 mM EDTA, 10 mMTris pH 7.4, 5 mM β-mercapto-ethanol, 5% sucrose and 1% pyronin Y; 20μl of ghost membrane (4mg/ml) was added as a marker and the solution was incubated at 37 C for 25 - 30 min. Ten microlitre of this sample was applied carefully on top of each gel and electrophoresis and staining were carried out as described (section 15). Two methods were used for cutting the gels: either by staining and cutting the bands by razor blade or by serial sections made with stacked razor blades, 0.8 mm apart.

The gel slices were placed in the bottom of the counting vials and counted with 1ml of H_2O in the gamma counter.

14.2 RESULTS

14.2.1 Estimation of the degree of interference of iodination procedure with phosphorylation reaction of membrane and extracted spectrin

In most experiments the samples were phosphorylated before the iodination. It is therefore important to determine whether or not the iodination procedure interferes with the phosphorylation activity of the membrane proteins and extracted spectrin. The phosphorylation was carried out for 10 min at 35^{o}C and the sample tubes were immediately iodinated at room temperature. For each sample, the experiment was carried out using gamma-^{32}P labelled ATP and unlabelled iodine and repeated again using unlabelled ATP and radioactive iodine * 125. The samples were counted according to the type of isotope used. The results are presented in table 14.1. Approximately 10% change in the phosphorylation of

Table 14.1 Change in phosphorylation activity on iodination. Background was measured in absence of membrane and spectrin and the result expressed as the Pi bound to BSA. Isolated spectrin was used without exogenous protein kinase. Results are expressed with the appropriate background subtraction. Mean of three determinations. None of the paired values are significantly different at 5% level.

Sample	Phosphorylation activity: nMoles phosphate bound per mg protein mean	± lSD
Background (iodinated)	0.004	0.002
Background (non-iodinated)	0.006	0.003
Isolated spectrin (iodinated)	0. 21	0.004
Isolated spectrin (non-iodinated)	0.22	0.004
IO prep (iodinated)	0.180	0.013
IO prep (non-iodinated)	0.193	0.018

the protein in the presence of iodine compared to the control which contained no iodine was observed. This may be due to acceleration of the dephosphorylation process upon iodination or it may be due to interference with the phosphate binding sites on the protein, spectrin. However, the change is not significant as to prevent the use of iodination for detection of conformational changes of phosphorylated protein. All the samples were tested for their phosphorylation activity using gamma-^{32}P - ATP before starting iodination; this was done specially for spectrin which tends to loss activity during 48 hours of storage at 4°C.

14.2.2 Iodination of the phosphorylated and non-phosphorylated membrane vesicles using freeze-thawed and non-freeze-thawed IO preparations

Two different types of IO preparations were used. The IO vesicles were prepared as described in section 8.1 and for some preparations the phosphorylation and iodination procedure was carried out immediately, whilst the rest of the preparations were frozen and thawed before the phosphorylation and iodination procedures were carried out. This was done to observe the effect of the freeze-thawing (which render the vesicles leaky to macromolecules such as lactoperoxidase) on the extent of the labelling of the proteins with ^{125}I. Polyacrylamide gel electrophoresis in SDS was carried out for each sample and serial gel slices (0.8 mm thick) were made and counted for ^{125}I as described in the text.

Table 14.2 represents the amount of ^{125}I bound per mg protein of IO prep or extracted spectrin; it also represents the percentage of labelling in the freeze-thawed and non-freeze

Table 14.2 Iodination of freeze-thawed and non-freeze-thawed IO prep. Iodination value in samples not phosphorylated taken as 100%. Mean of five single determinations ± 1SD.

Sample	5μM ATP	nmoles I bound per mg membrane protein	% change	Student's t test for unequal means
Freeze-thawed IO prep	-	4.355 ± 1.196	100.0	$P < 0.05$
	+	4.793 ± 0.505	110.1	
IO prep	-	3.218 ± 0.733	100.0	$P < 0.05$
	+	3.526 ± 0.376	109.6	

thawed preparation. The samples which were not phosphorylated were taken as control for determination of the amount of iodide bound to the membrane. The phosphorylated samples were compared with the controls for estimation of the difference in iodide bound, and hence used as an indicator of extent of difference in the two states. When all freeze-thawed IO prep results were compared with that of the non-freeze thawed prep, the latter showed significantly higher iodination ($P = 0.05$). This is probably due to access of labelling medium to both sides of the membrane in such a prep. This also shows that the normal IO prep is sealed. However, with either type of prep, when the iodide bound in the phosphorylated membrane is compared to its appropriate control, no significant difference was found.

14.2.3 SDS polyacrylamide gel electrophoresis for phosphorylated and non-phosphorylated preparations

This was carried out on the freeze-thawed preparations only. The gels were sliced and counted for radioactivity as described in the text. Fig 14.2 represents the cpm of ^{125}I incorporated in each slice. It was found that spectrin bands I & II which comprise slices 3 to 6 had incorporated low label compared to the lactoperoxidase and the BSA where most of the ^{125}I was incorporated. This applies to all samples, although slight difference in the extent of labelling in the phosphorylated and the non-phosphorylated form of the freeze-thawed preparations was noticed. When the other method of gel slicing was followed i.e. by cutting

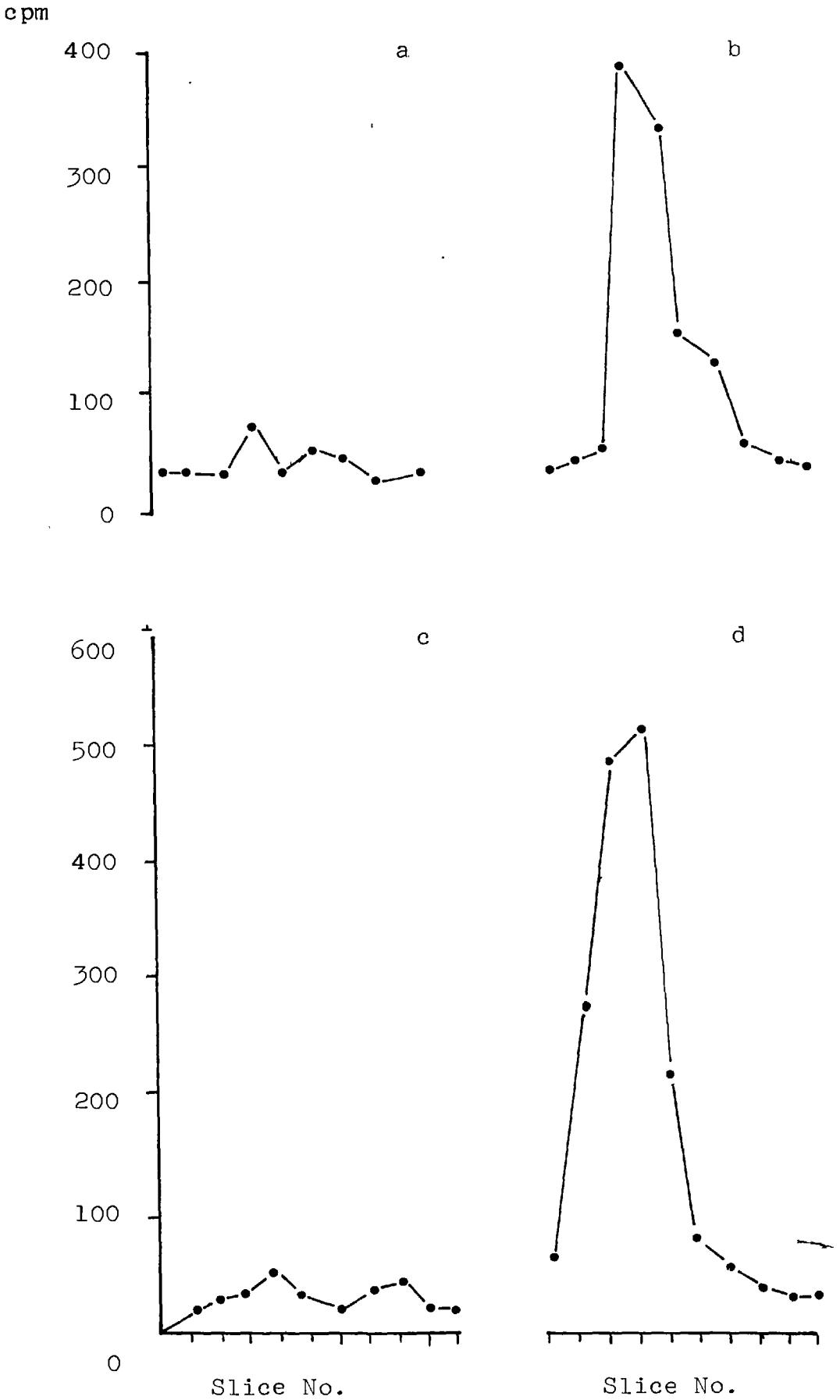

Fig 14.2 Incorporation of ^{125}I into gel slices of band (I & II).
a control no sample; b with membrane without ATP
c control no sample; d with membrane with ATP in phosphorylation buffer.

the first two bands from top of the gel after staining, the difference in the extent of labelling of the phosphorylated and non-phosphorylated spectrin was more evident.

These results are in agreement with Selhi and White (1975) in that bands are weakly labelled by ^{125}I. The high incorporation of ^{125}I in the spectrin bands observed by Phillips & Morison (1971) could be explained as due to overloading of the gels which led to immobilisation of the proteins and accumulation in the spectrin region, as is apparent from the staining and counting procedure presented by these authors.

14.2.4 Effect of calcium ions on the iodination of membrane proteins

The labelling experiments were carried out for the freeze-thawed preparations only, as described in the text in the presence of Ca^{2+} at final concentrations of 0.2, 0.5 and 2mM. The samples were counted and subjected to electrophoresis. Gels were stained and bands I & II were cut as a single slice and counted. Table 14.3 shows the percentage change in the labelling of ^{125}I of the phosphorylated and non-phosphorylated IO preparations, taking the samples which contain no ATP as 100%. The table also shows that the percentage of labelling decreased as Ca^{2+} concentration increases up to 0.5 mM then at 2 mM the labelling increases again. It could be explained that Ca^{2+} exerts an inhibitory effect. It either interferes with the phosphorylation of spectrin or it induces conformational changes in the protein in a way which makes it less accessible to the label. However, at Ca^{2+} concentration of 2 mM the labelling increases again. It should be borne in mind that at this concentration spectrin Ca^{2+} ATPase is activated (Rosenthal et al, 1970). Therefore the increase in the labelling

Table 14.3 Effect of phosphorylation on iodination of freeze-thaw IO prep at different Ca^{2+} concentrations. - ATP indicates no ATP present in the phosphorylation stage. All pairs of values are significantly different at 5% level. Mean of four determinations.

$[Ca^{2+}]$ mM	ATP	nmoles iodide bound per mg protein mean ± 1SD	% change in labelling taking - ATP as 100%
0	-	0.016 ± 0.002	125
	+	0.020 ± 0.002	
0.2	-	0.018 ± 0.001	111
	+	0.020 ± 0.001	
0.5	-	0.023 ± 0.002	109
	+	0.025 ± 0.001	
2.0	-	0.019 ± 0.001	137
	+	0.026 ± 0.002	

of spectrin may be due to the activity of Ca^{2+} ATPase which induces conformational changes.

Fig. 14.3 shows the incorporation of ^{125}I in bands I & II at the Ca^{2+} concentration used, showing increased inhibition of the labelling by Ca^{2+} up to 0.5 mM.

14.2.5 Iodination of the phosphorylated and non-phosphorylated extracted spectrin

The spectrin was extracted from membranes prepared from fresh or outdated normal blood as described in section 10.1. The extracted spectrin was used within 48 hr following its extraction and storage at 4 C. The spectrin samples were phosphorylated over a time period of 0,3,5,10,20,40,60, minutes and subjected to iodination immediately. The samples were electrophoresed, stained and bands I & II were cut and counted. The number of nmoles of I bound to one nmole and one mg of spectrin was calculated as shown in Fig 14.4. The amount of spectrin that bound to one nmole of I was also calculated. From this table it is noticed that the amount of I bound per mg spectrin varies with the time. However, the maximum amount of ^{125}I was incorporated after 10 min incubation. Fig 14.4 represents the nmoles of spectrin that bind one mole of ^{125}I during the time course of phosphorylation. It is clear that incorporation was maximum at 10 min and decreases with time to be equal for the phosphorylated and non-phosphorylated spectrin. It increases slightly forming a plateau over the second 30 minutes. The conformational changes as monitored by the extent of ^{125}I labelling is apparently time dependent. The increase in labelling at the period of 10 - 20 minutes reflect the changes occurring when phosphorylation is also at its highest level (see fig 10.)

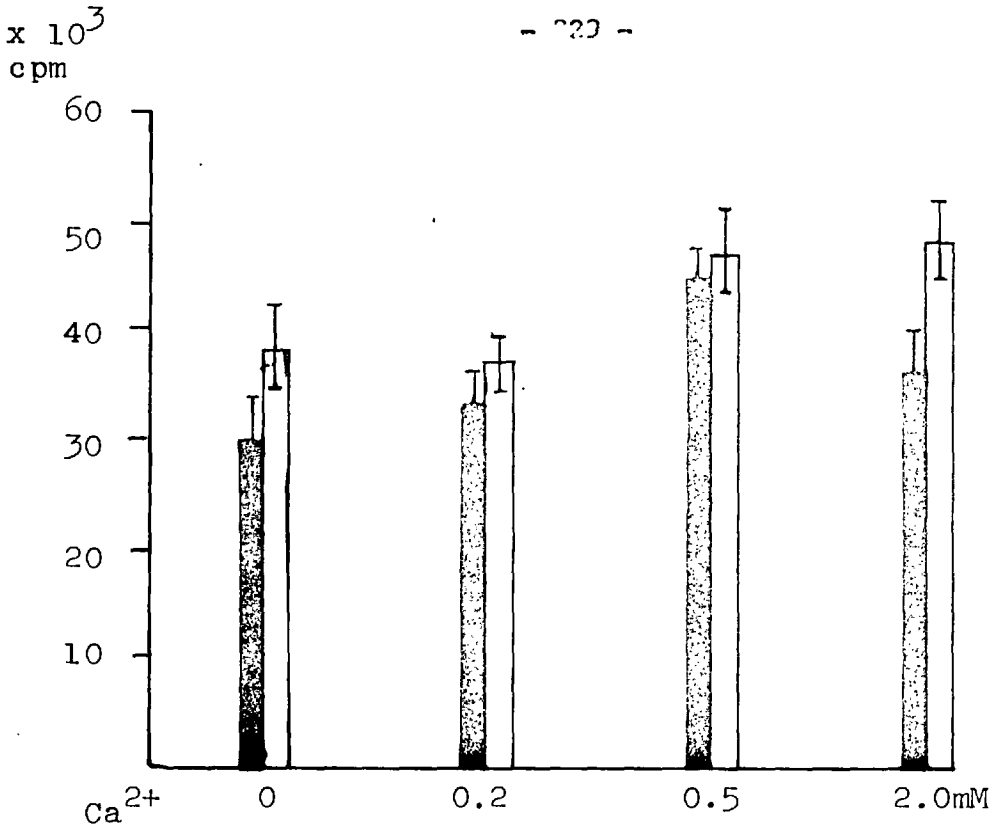

Fig 14.3 Effect of Ca^{2+} on iodination of phosphorylated and non-phosphorylated band (I & II). Mean of four determinations. EGTA was absent from phosphorylation buffer when Ca^{2+} was used. Closed bars - No ATP in phosphorylation buffer Open bars - 5mM ATP in phosphorylation buffer.

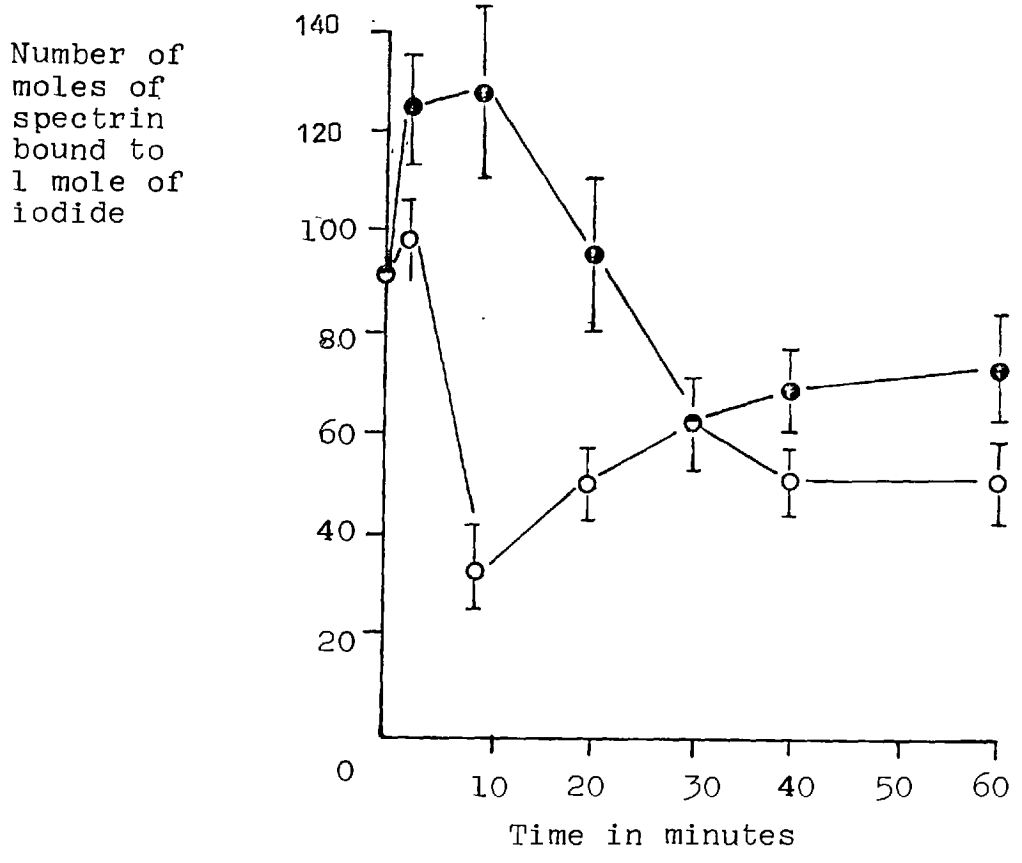

Fig 14.4 Time course of phosphorylation and subsequent iodination of band (I & II). Time is shown for phosphorylation· open circles in presence of ATP, closed circles controls in absence of ATP.

SECTION 15

15 GEL ELECTROPHORESIS

Sodium dodecyl sulphate gel electrophoresis is a widely used method for separation of different protein according to their molecular weight. Sodium dodecyl sulphate SDS causes breakdown of the protein into its subunits by attacking the hydrophobic interaction leaving negative net charge on the protein. When such protein is placed on a porous matrix such as polyacrylamide gels and a voltage is applied, it will migrate from the cathode to the anode. The rate of migration depends on the size of the protein and hence the molecular weight could be determined by comparing the relative mobility of a particular protein with that of a protein with known molecular weight. The size of the gel pores depend on the cross-linking between acrylamide and bisacrylamide.

In this section SDS polyacrylamide gel electrophoresis was used to obtain a characteristic pattern of migration of certain membrane proteins of the red cell. The method used is essentially that of Fairbank *et al*, (1971).

15.1 REAGENTS AND METHODS

Concentrated Bisacrylamide

Bisacrylamide	1.5 gm
Acrylamide	40 gm

Both reagents were dissolved with distilled water and made up to 100 ml.

x10 buffer

This was prepared as a stock solution and kept at room

temperature. It contained the following:

Tris	0.4M
Na acetate	0.2M
EDTA	0.02M

One litre of the solution was made up and the pH was adjusted to 7.4 with acetic acid.

Gel mixture

For 10 ml of gel mixture the following are added in the order indicated:

Concentrated bisacrylamide	1.4 ml
X 10 buffer	1.0 ml
20% (w/v) SDS	0.5 ml
distilled H_2O	5.6 ml
1.5% ammonium persulphate	1.0 ml
0.5% TEMED	0.5 ml

Overlay solution

This was made 0.1% in SDS; 0.15% in ammonium persulphate and 0.05% in TEMED using the above reagents.

Electrophoresis buffer

x 10 buffer	100ml
20% w/v SDS	50 ml

Made up to one litre with distilled H_2O.

Membrane suspension

0.5 to 1 mg membrane protein was suspended in a solution containing the following final concentration:

1% w/v	SDS
5 - 10% w/v	sucrose
10mM	tris/Hcl (pH 8.0)
1mM	EDTA
5mM	β -mercaptoethanol
100μg/ml	tracer-dye pyronin-y

A stock solution (x 10) of the above reagents was made and added to the membrane samples. The suspension was incubated at 37°C for 20 to 30 minutes before electrophoresis. Uniform bore glass tubes of 6mm internal diameter and 80mm long were used. The tubes were washed with water and soaked overnight with concentrated nitric acid. The tubes were rinsed several times with distilled water and either dried in the oven or rinsed with acetone and left to dry at room temperature. One end of each tube was sealed with parafilm and held in vertical position in a rubber holder. The gel mixture was made up and degassed under vacuum and 1.2 ml was added carefully to each tube using the 5ml Gilson adjustable pipett. 2μl of the overlay solution was added on top of each gel and the tubes were left to polymerise in vertical position for 45 minutes at room temperature. The overlay was drained by inverting the gel tubes. Electrophoresis buffer was added to the top of the gels and the tubes were left to polymerise for at least 12 hrs before use. The gels could be kept at room temperature with the buffer always on the top to prevent dryness and shrinkage. The membrane suspension containing 10 to 20μg of membrane protein was layered carefully on top of the gel and the electrophoresis was carried out at constant

voltage gradient of 10 v/cm with a current of 8mA/tube. The time required for electrophoresis was 45 minutes or the electrophoresis was allowed to continue until the tracer-dye had migrated two-thirds the length of the gel. The gels were taken out carefully from the tubes by injecting water between the gel and the side of the tube, taking care not to damage the gel. The position of the tracer-dye was marked by pricking the gel with fine needle dipped in ink.

15.A.2 STAINING PROCEDURE

The staining procedure can be summarised in three steps:

1. The gels were stained overnight with 25% w/v isopropyl alcohol, 10% v/v acetic acid and 0.025% w/v Coomasie blue.
2. The gels were transferred into a solution containing 10% v/v isopropyl alcohol, 10% v/v acetic acid and 0.0025% w/v Coomasie blue and left in this solution for 9 to 12 hours.
3. Destaining was carried out overnight with 10% v/v acetic acid only with two changes of the solution.

During the staining and destaining procedure the gels were put in plastic universal tubes and left to rotate gently to ensure complete staining and destaining.

15.2 RESULT AND DISCUSSION

15.2.1 Normal and H.S. membrane ghosts; extracted spectrin and protein kinase

Normal and H.S. red cell membranes were prepared as described in section 7.1. Figs 15.1 and 15.2 shows the SDS polyacrylamide gel electrophoresis for normal and H.S. membrane ghosts. Gel scans (using Giford Linear transport system linked to Gilford accessory and Pye Unicam monochromator at 540 nm) are also shown.

No qualitative difference between the normal and H.S. membrane scans were observed. When extracted spectrin is compared (Figs 15.3 and 15.4 respectively) it is observed that the only qualitative difference is in the level of protein extracted.

The level of extracted crude protein kinase appears similar in both normal and H.S. (fig 15.5 and 15.6). The intense band is due to BSA used in the extraction procedure.

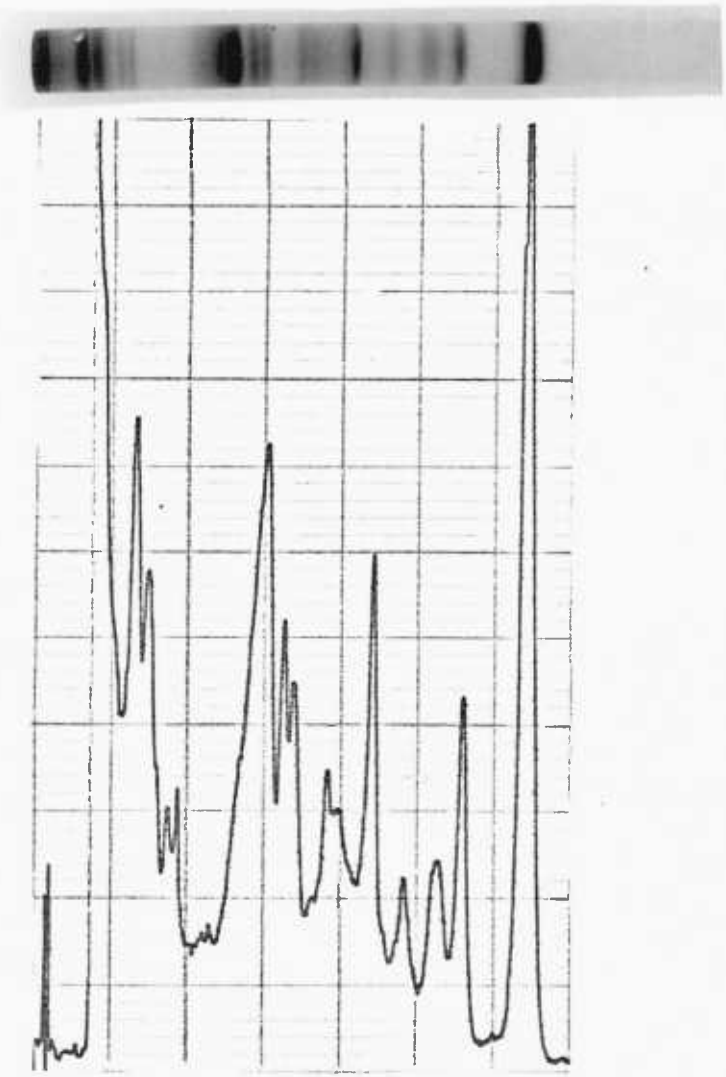

Fig 15.1 Normal membrane ghost

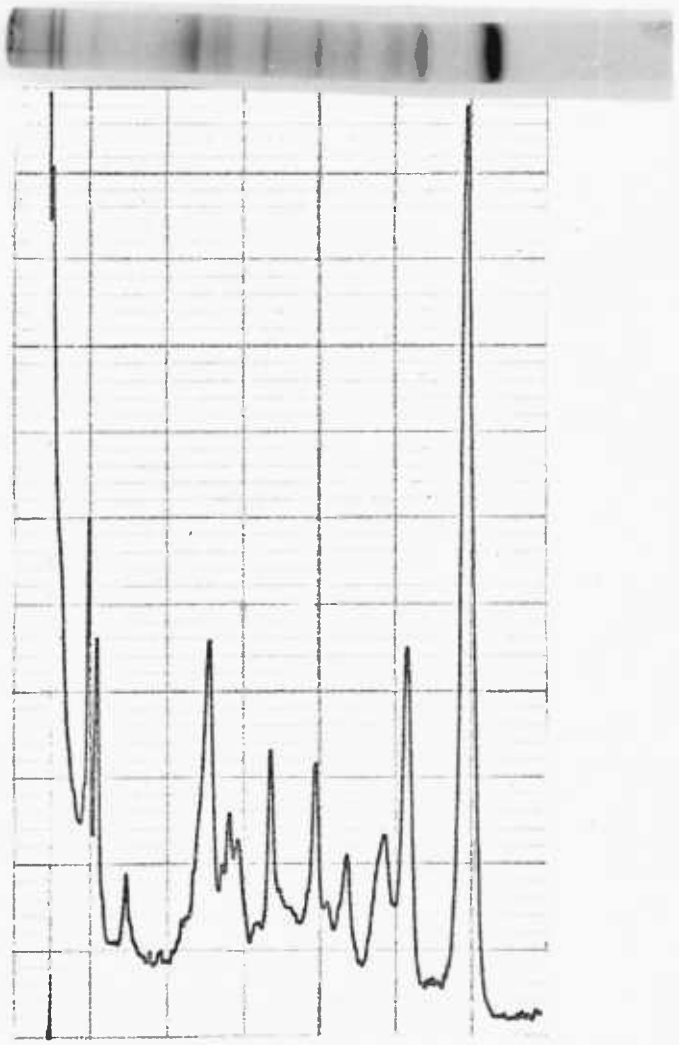

Fig 15.2 H.S. membrane ghost

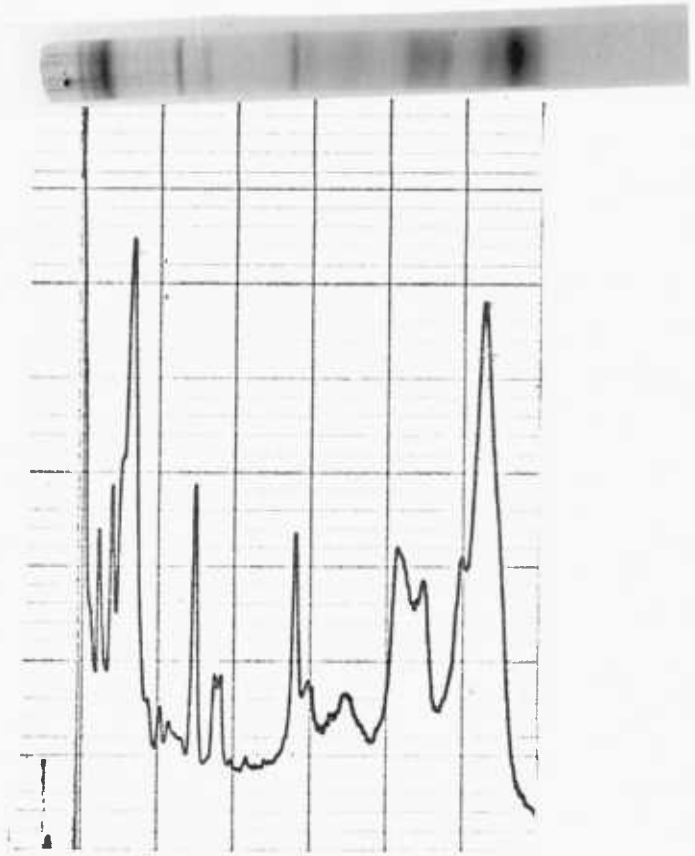

Fig 15.3 Extracted spectrin from normal membrane

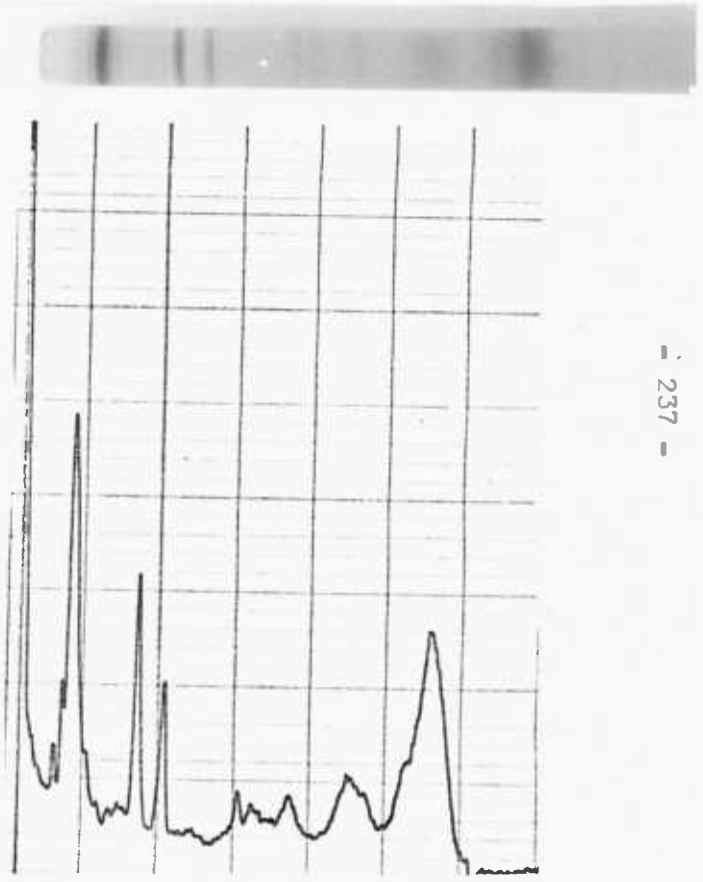

Fig 15.4 Extracted spectrin from H.S. membrane

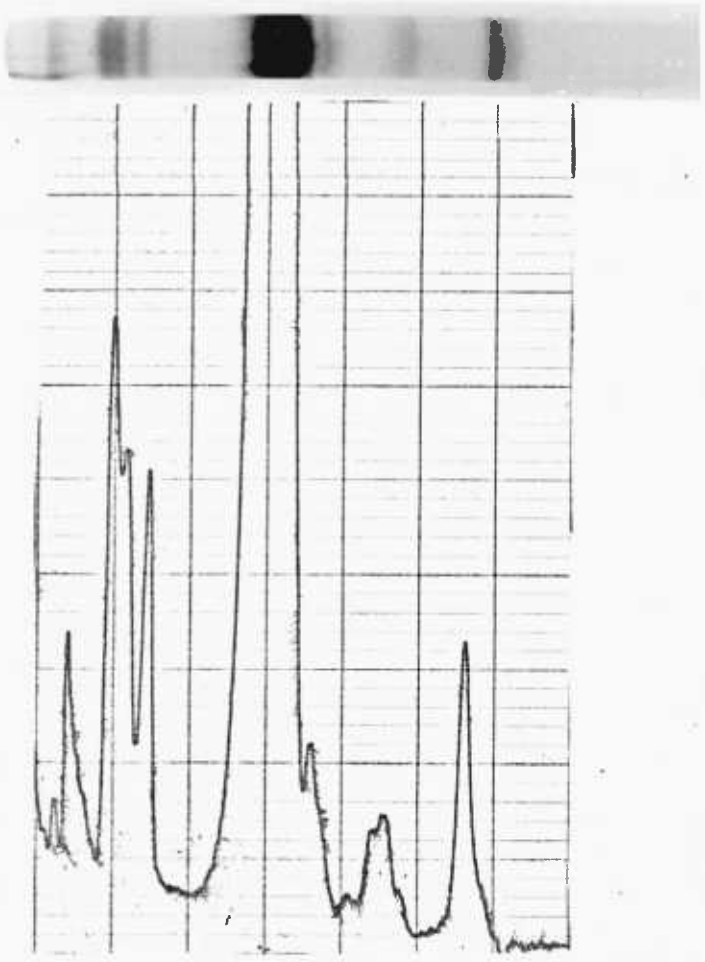

Fig 15.5 Crude extract of protein kinase from normal membrane

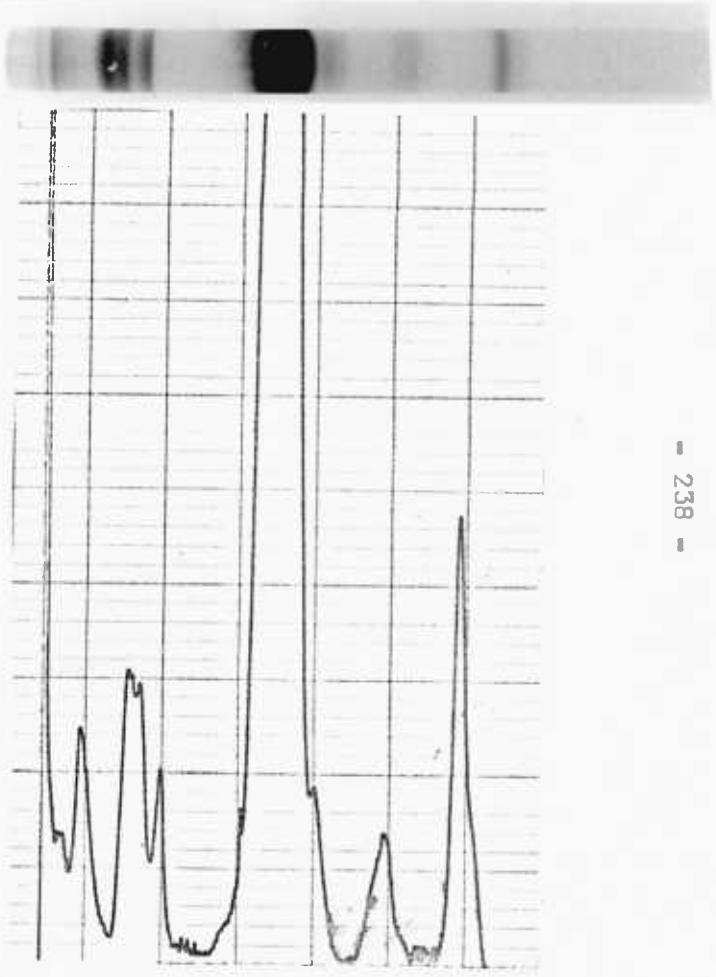

Fig 15.6 Crude extract of protein kinase from H.S. membrane

15.2.2 Extraction of spectrin

The supernates from spectrin extraction (fig 10.2) I, II and III are shown in figs 15.7, 15.8 and 18.9 respectively. All supernates were more than 48 hrs old. Fresh samples give similar pattern except for supernate I which is identical to supernate II. Although the spectrin may be degraded by action of proteolytic activity in fraction I, an alternative cause is discussed in section 17.

The final sediment of the spectrin extraction mostly showed an appearance on SDS polyacrylamide gel electrophoresis similar to that of normal membrane (fig 15.1), except for diminishes band I and II protein scans. Sometimes, however, appearance as shown in fig 15.10 was obtained. New bands were found at the position of band II.1 and II.2 which bear close resemblance to band I and II. The difference in molecular weight from band I and II is approximately 30×10^3 daltons. The reason for this is not clear.

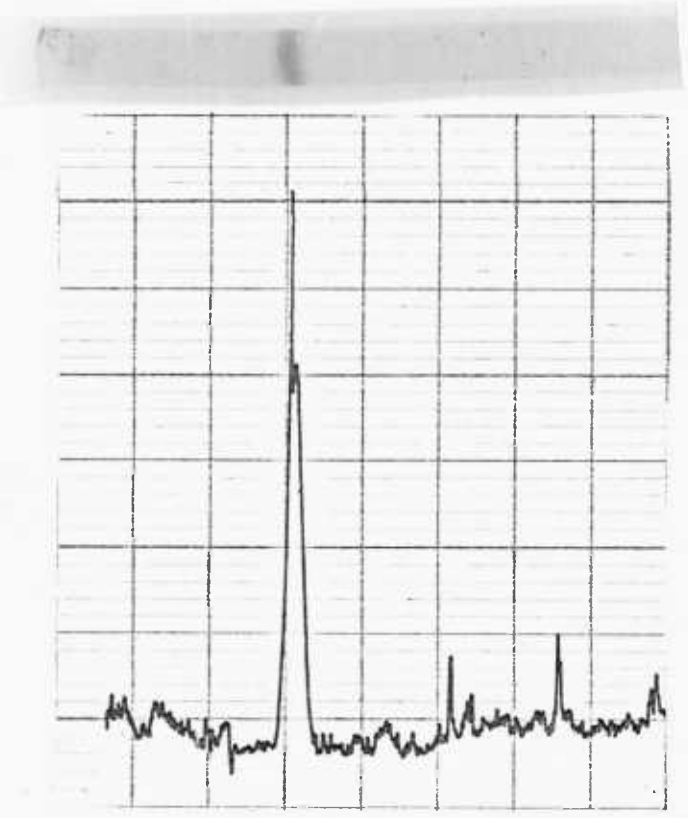

Fig 15.7 Supernate I of spectrin extraction. Note change in molecular weight of spectrin band I and II.

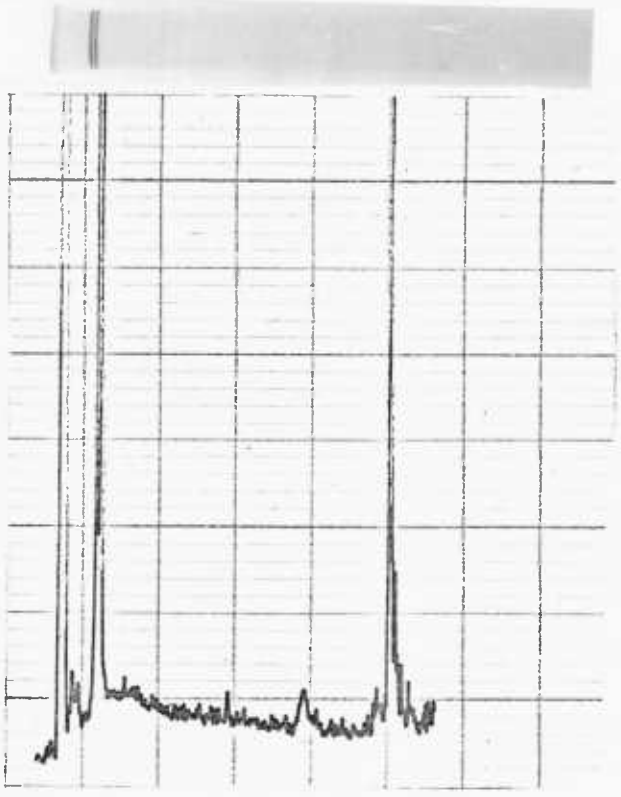

Fig 15.8 Supernate II of spectrin extraction. Sharp peak on RHS due to a tracker mark.

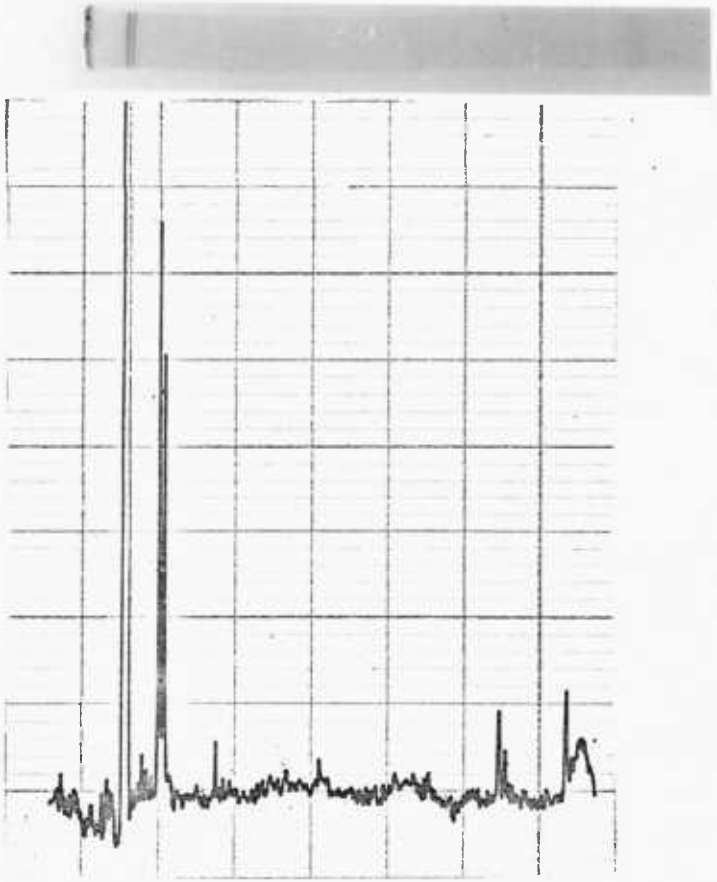

Fig 15.9 Supernate III of spectrin extraction.

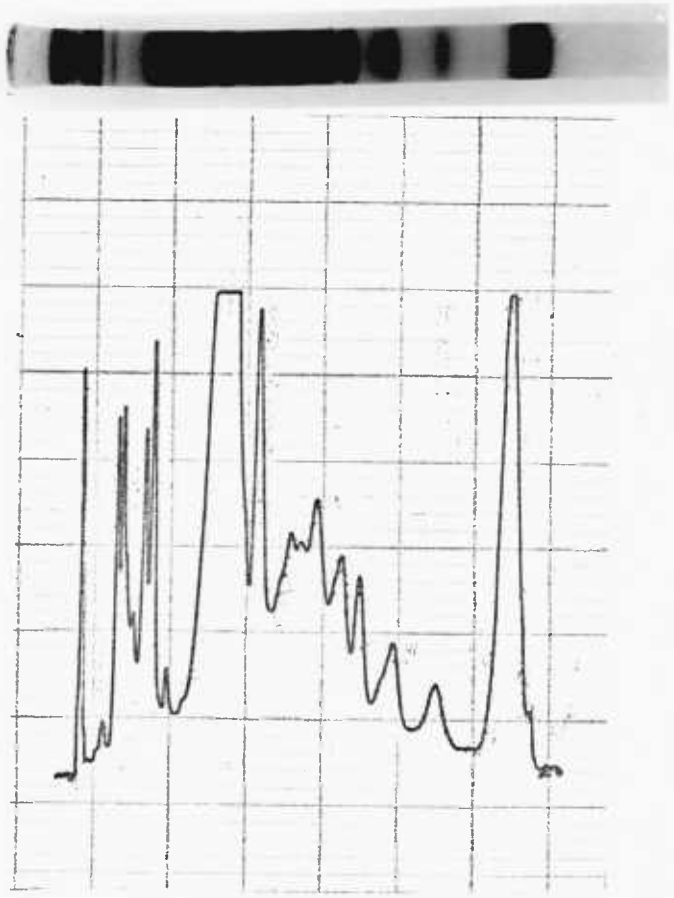

Fig 15.10 Final sediment of spectrin extraction.
Note appearance of a sharp band II.1 and II.2.

15.2.3 IO prep

Fig 15.11 shows the pattern for the final IO prep after homogenisation and preincubation. It is similar to normal ghost (fig 15.1) except for band III which appears rather more defined.

Using the supernate of the low ionic strength suspension (fig 8.1a) no bands can be detected clearly. However, on freeze drying and concentrating the supernate by 20 times, the pattern shown in fig 15.12 is obtained. Since majority of proteins present in the ghost are present it is possible that selective elution of spectrin does not take place, but the centrifugation procedure may be insufficient to remove small membrane fragments.

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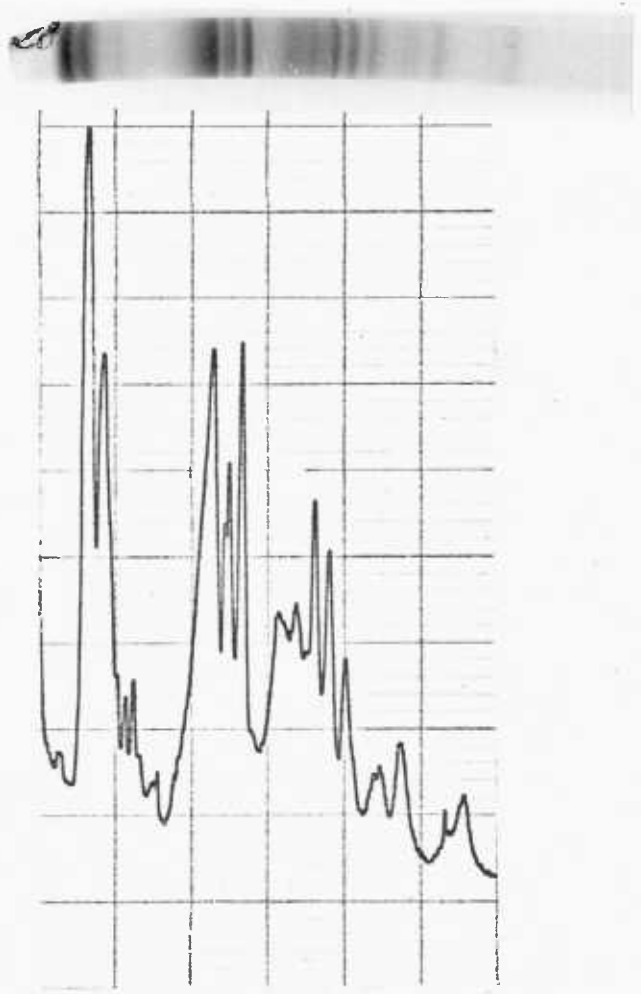


Fig 15.11 The final IO prep. Note proportion of spectrin to rest of the protein is similar to that of normal membrane (Fig 15.1).

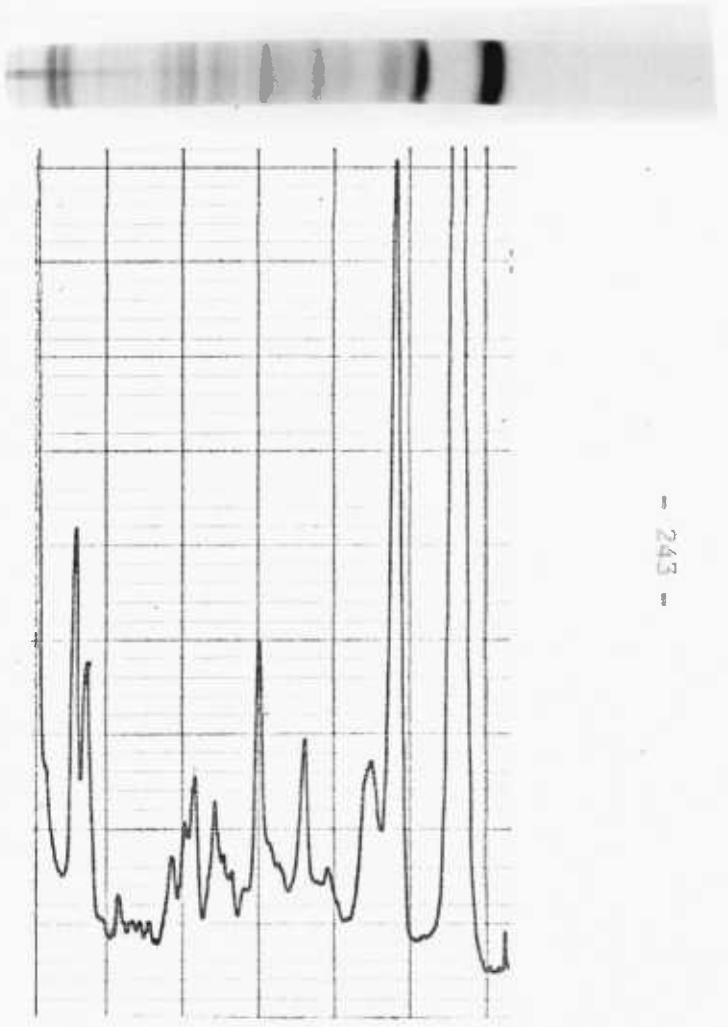


Fig 15.12 The supernate of IO suspension after centrifugation and freezing (see flow chart fig 8.1a). The supernate was concentrated twenty fold before application. Note spectrin does not appear to be the only protein present or selectively eluted.

SECTION 16

ELECTRON MICROSCOPE STUDIES

The process of endocytosis and formation of membrane vesicles has been studied by many investigators. It is possible that endocytosis can take place in two ways. Firstly, energised endocytosis requiring ATP and certain cations can take place. Penniston and Green (1968) have proposed a model in which ATP is thought to energise plasma membranes for endocytosis to take place. Secondly, endocytosis can also occur by reducing the ionic strength of the medium surrounding the cells. Kotsumata & Asai (1972) have discussed this type of endocytosis.

In this section electron microscopic technique was used to investigate the type of membrane fragments and vesicles produced during IO prep formation, and also whether endocytosis occure under experimental conditions, such as Ca^{2+} uptake.

16.1 METHOD

16.1.1 Reagents

Fixative:

2.5% glutaraldehyde in 0.2M phosphate buffer, pH 7.2

Resin 1:

Equal proportions of TAAB resin and DDSA

Resin 2:

Same as resin 1 but containing 2% of the accelator, DMP.

16.1.2 Fixation

At each step of IO prep formation, the membrane was centrifuged at 30,000 rpm for 30 minutes using MSE superspeed 75 centrifuge and 10 x 10 ml rotor. The resulting pellets were fixed in 2.5% glutaraldehyde for 3 hours. The pellets were left in 1 : 1 dilution of 0.2M cacodylate buffer, pH 7.2, and 0.5M sucrose for one hour after first three ten minute rinses in the same buffer.

16.1.3 Dehydration and embedding

The pellets were dehydrated stepwise by suspending in 30, 50, 70 and 100% ethanol for at least 10 minutes each. The 100% step was repeated three times. The dehydrated pellets were then suspended in propylene oxide twice for 15 minutes each. Equal amounts of propylene oxide and resin 1 for 15 minutes. The pellets were then left overnight in resin 1 at 37 C with constant gentle shaking. After blotting the pellets they were left in resin 2 for 9 hours.

The pellets were transferred to capsules, reein 2 poured into the capsules, avoiding formation of air bubbles, and left to polymerise at 60 C for 48 hours.

16.1.4 Sectioning and staining

Ultrathin sections were made using Reichert OMU3 microtome. The sections (silver to gold in colour) were transferred to

copper grids (400 mesh). The specimen was stained in uranyl acetate for two minutes, washed with ethanol, and two drops of lead citrate added. After leaving for 10 minutes, the grids with the specimens were thoroughly washed with distilled water.

16.1.5 Negative staining

This was normally carried out on samples of ghost membranes or the IO prep selected at random. This provided a quick means of checking the contamination of ghosts with debris or bacteria. It also gave an indication of the type of IO prep obtained.

A drop of 4% phosphotungstate (pH 6.0) was added to a drop of the membrane suspension. After 30 seconds a part of the suspenetion was transfered to a copper grid , excess removed with filter paper, and the sample allowed to dry. The specimen was then examined in the electron microscope.

16.2 RESULTS AND DISCUSSION

The IO prep was prepared as described previously (section 8.1) and at each step the membrane was pelleted for electron microscopy.

Plate I shows a negatively stained preparation of red cell membrane ghost which was normally obtained. At higher magnification the double layer membrane can be observed with a "fuzzy" layer at the outside surface.

On suspension of the ghosts in low ionic strength phosphate buffer, non-energised vesiculisation (endocytosis) can be detected. This endocytosis is more evident after 1 hr suspension and freeze-thawing the sediment membranes as shown in plate II. The ghosts have changed to small vesicles. Some intact membrane ghosts (with vesicles contained inside) are also seen. On homogenisation these entrapped vesicles are readily released.

Plate III shows a higher magnification of plate II, showing vesiculisation of ghost membrane. Entrapped vesicles are shown in plate IV. Penniston & Green (1968) also demonstrated a similar endocytosis in presence of divalent cations and ATP. Care must however be taken, since it is difficult to distinguish between entrapped vesicle and attached endocytic formation. On homogenisation such entrapped vesicles are normally not observed and it is concluded that homogenisation may cause a release of these vesicles from the parent membrane.

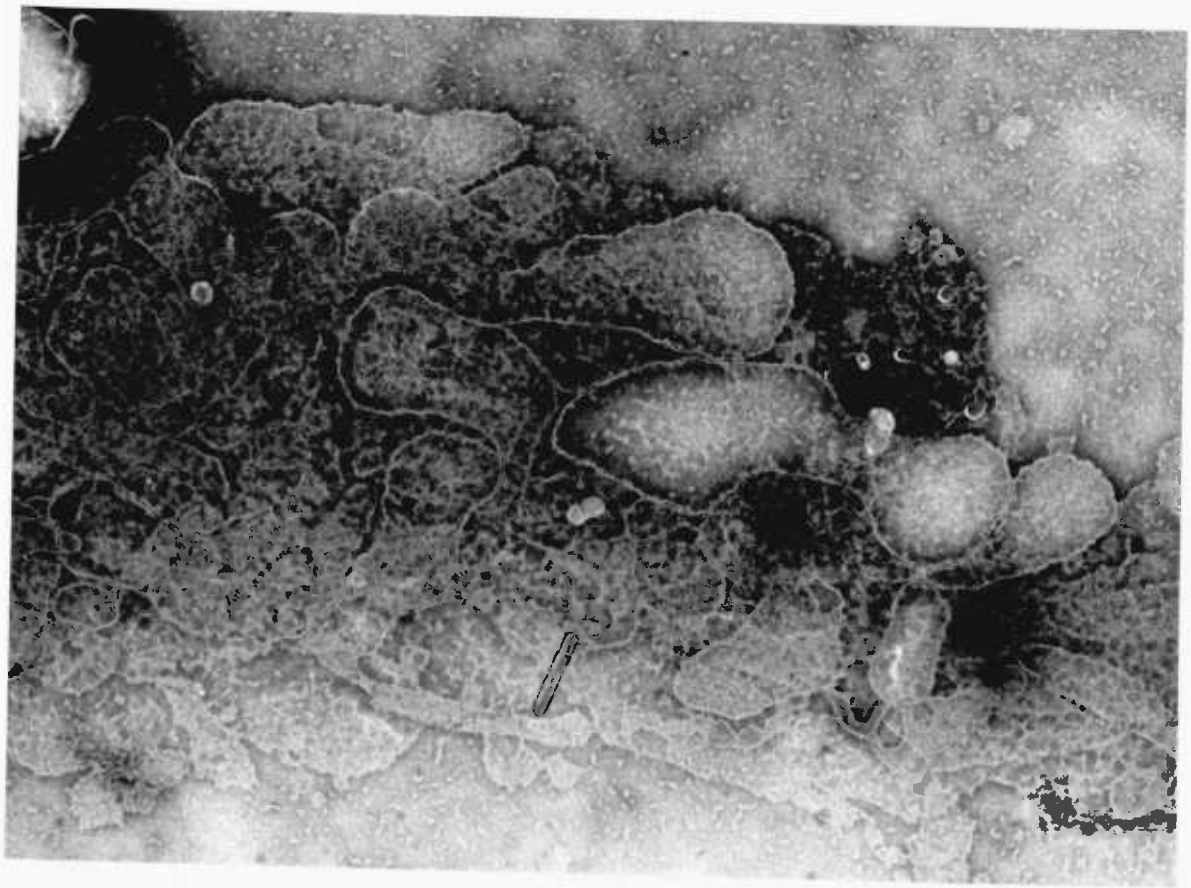


Plate I Negatively stained preparation of ghosts in 5mM phosphate buffer as prepared by method of Dodge et al (1963). Magnification, 20520

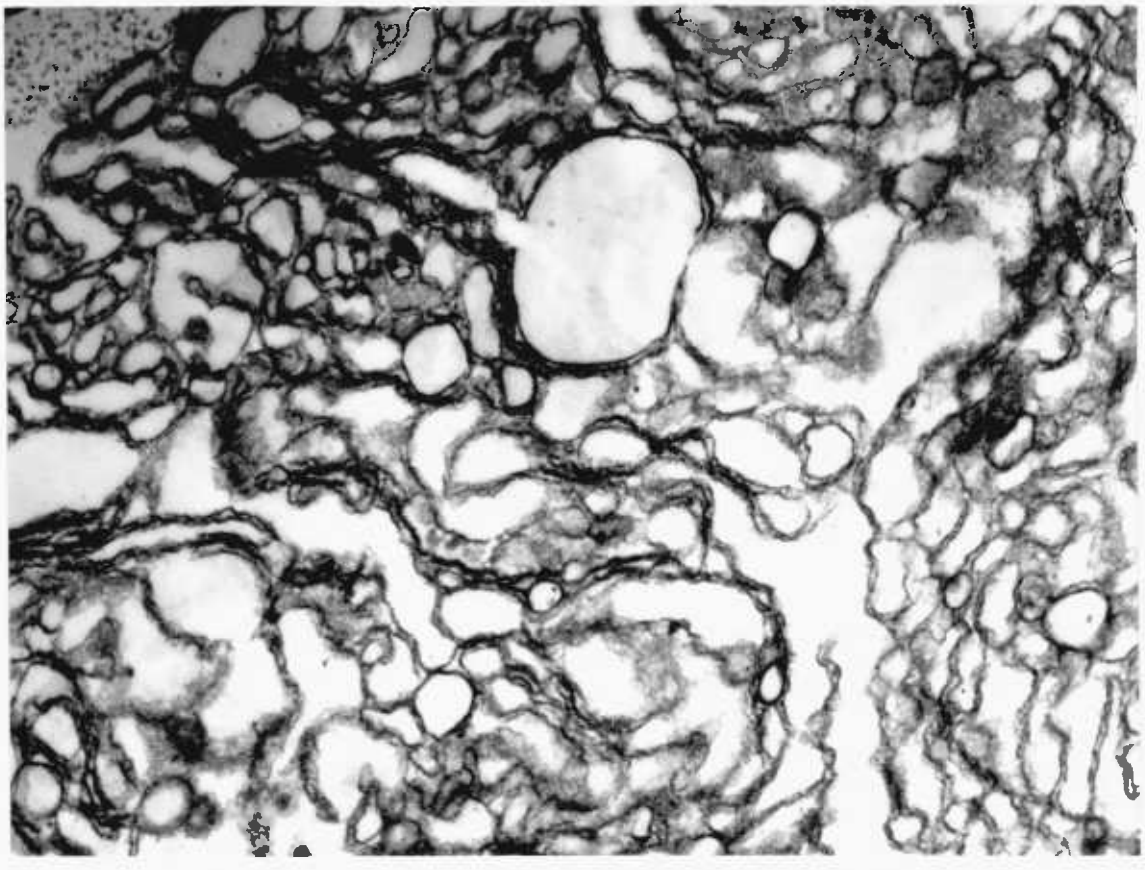


Plate II Non-energised vesiculation of the ghosts using ghosts diluted to 1 : 9 m 0.5 mM phosphate buffer. The sediment was freeze-thawed. Magnification, 19416.

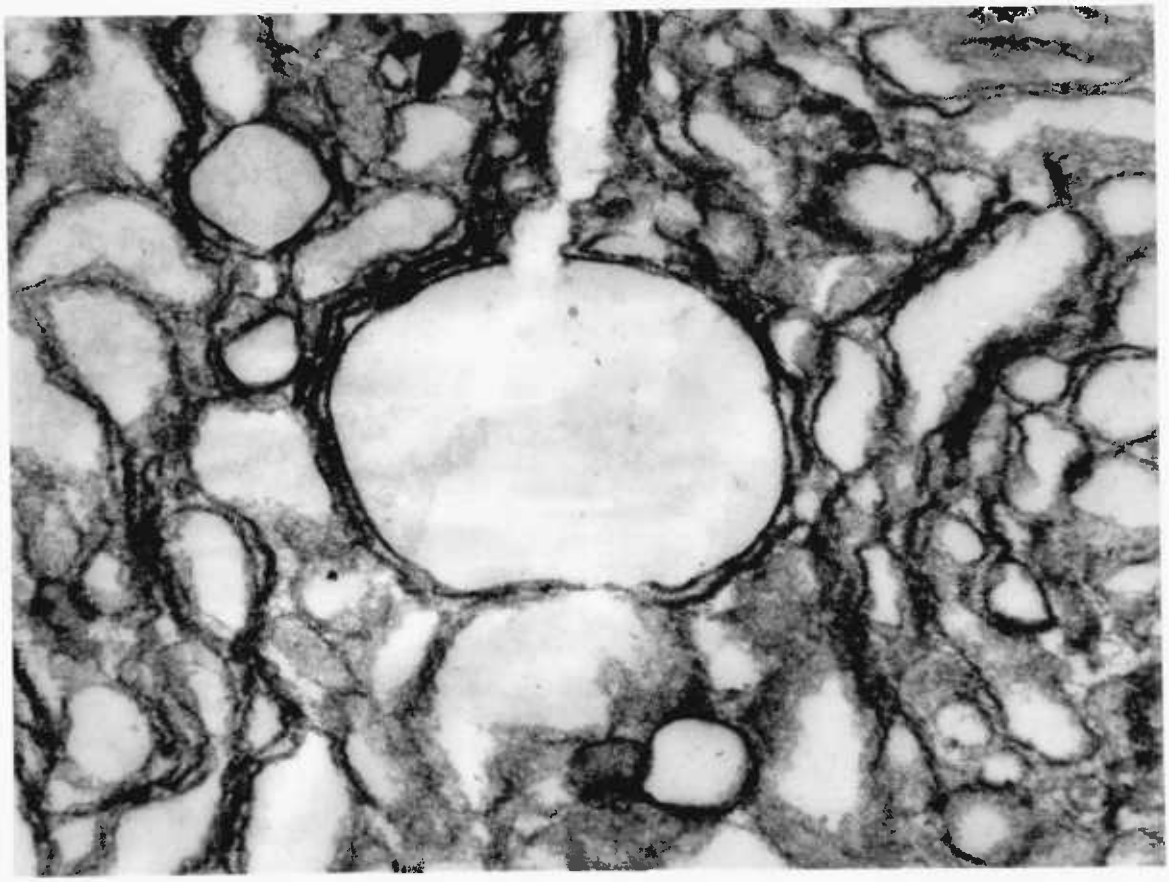


Plate III Higher magnification of plate II showing vesiculisation of ghost membrane. Magnification,57420.

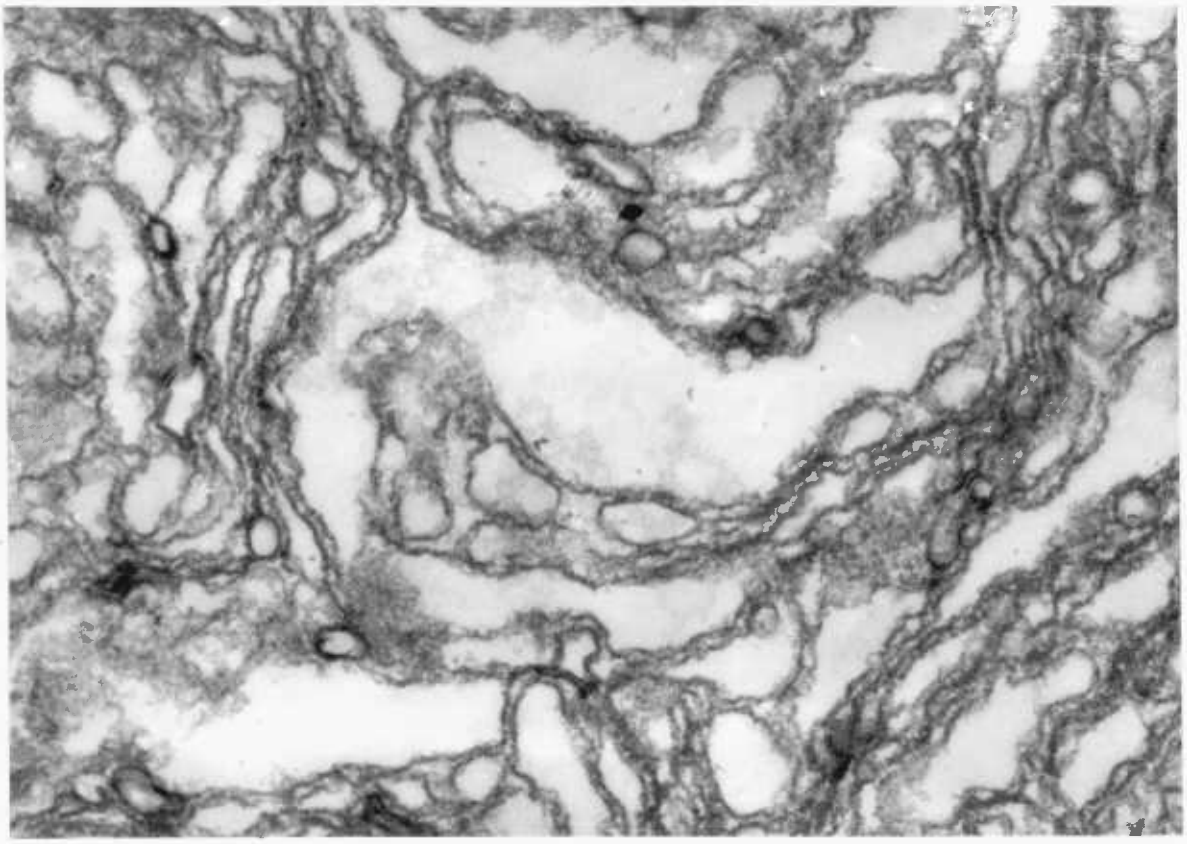


Plate IV Entrapped vesicles formed before homogenisation. Magnifcation, 37620.

Preliminary studies on homogenisation, investigated either by density gradient centrifugation or by use of marker enzymes, showed variable level of IO vesicle formation. For example sonication even at low Watt output was found to be too severe; hand-operated, loosely fitting dounce homogenisation and passage of the membranes through various gauge needles, was found to give low IO ve sicle yields. Homogenisation in MSE waring blender at $\frac{1}{3}$ full output (with medium size blades) was found to be most satisfactory.

Plate V shows the effect of such homogenisation. Although variable size vesicles are produced, fragmentation of membrane is not extensive as when sonication is used. Homogenisation may stimulate further vesicle formation from larger structures at this stage. Plate VI indicates that such vesiculation may be accelerated and completed rapidly when the preincubation at 37 C is carried out.

Examination of the IO prop after the preincubation period indicates that the percentage of vesicles with the fuzzy carbohydrate coat inside the vesicles is high. Precise calculation of the percentage of IO vesicles formed using this technique requires more critical investigation.

Incubation for up to 1 hour after the preincubation step appears to show no detectable difference in either vesicle population or structure from that shown in plate VI.

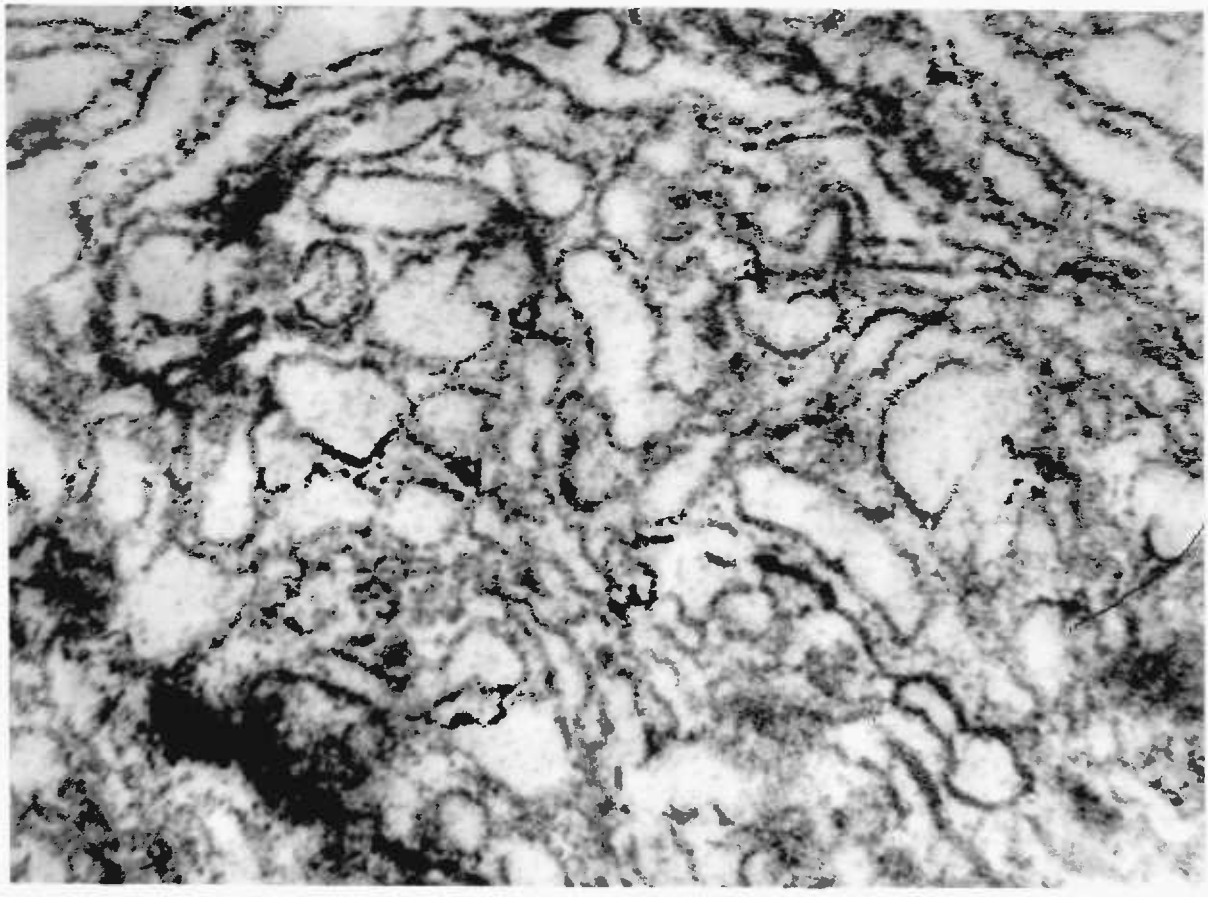


Plate V Effect of homogenisation on the freeze-thawed prep using MSE waring blender at $\frac{1}{3}$ full power.
Magnification, 63800.

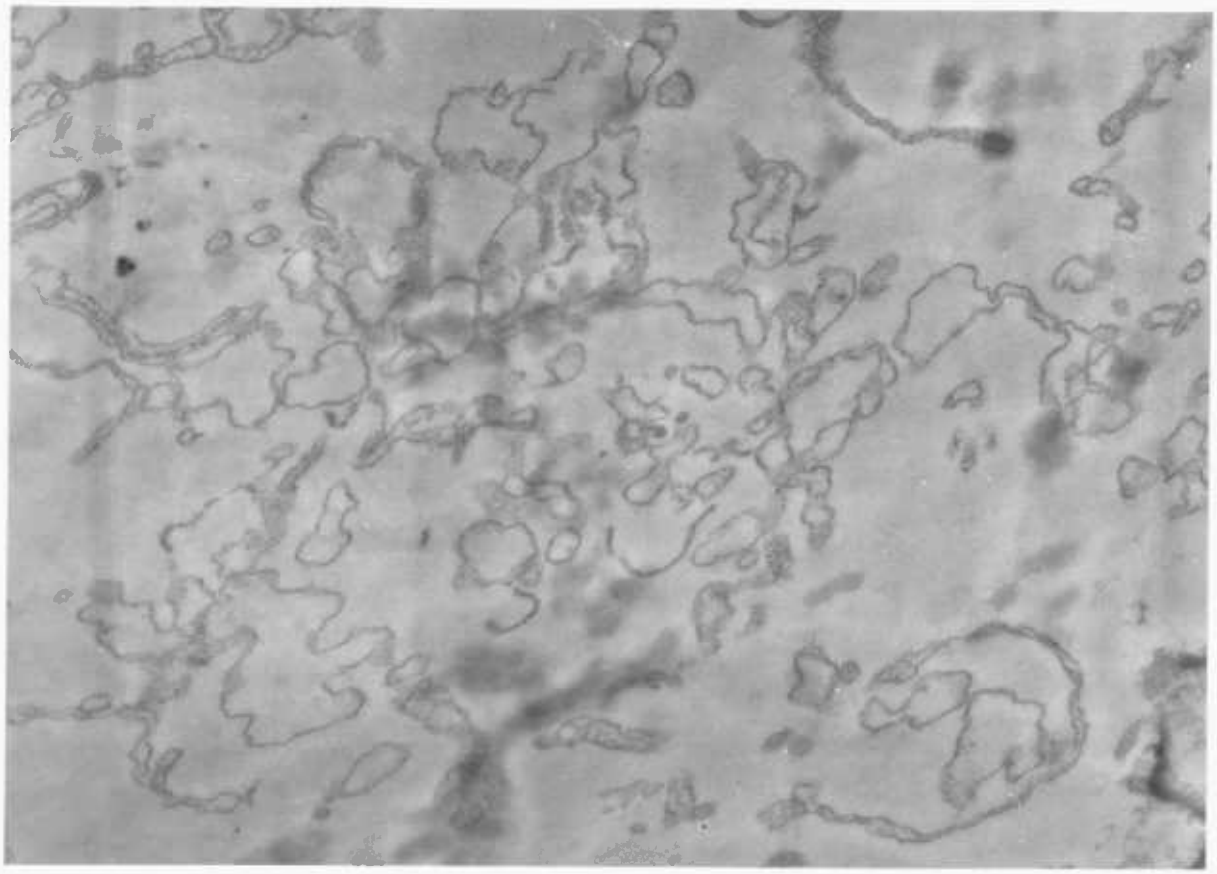


Plate VI Preincubation at 37 C of the homogenised IO prep. Further incubation does not alter the vesicle (as viewed under the electron microscope) for up to 1 hour. Magnification, 30780.

GENERAL CONCLUSION AND DISCUSSION

Reasons for study of Ca^{2+} transport and $(Ca^{2+} + Mg^{2+})$-ATPase in the red cell membrane

Very few abnormalities have been demonstrated that conclusively link the abnormality to a defect in the Ca^{2+} pump system of the human red cell. Even the existence of variant forms of Ca^{2+} pump activities have not been reported. Another protein, spectrin, which exists in abundance on the red cell membrane inner surface (Marchesi & Steers, 1968) has been implicated in various forms of abnormalities. However, as yet no study has conclusively found a defect of the protein related to the abnormality.

This vast gap in clinical understanding of such abnormalities can only be bridged by thorough biochemical investigation of the properties of such a system. H.S. is a disease in which the abnormality is thought to reside either in a component of the Ca^{2+} transport system or in the membrane protein spectrin.

That the disease cannot be linked conclusively to either or both (i.e. Ca^{2+} transport or spectrin) is due to two facts. Firstly, very little is known of the properties of the transport processes and the biochemical structure and function of the protein spectrin. Secondly, the molecular organisation of the system is not sufficiently understood to be able to locate the nature of the abnormality.

In the studies presented in this thesis, it was clear that biochemical functions of both the Ca^{2+} transport system and spectrin must be understood or at least attempted to form a basis for a more refined molecular investigation at some other stages.

Ca^{2+} transport activities and its associated ATPase were initially investigated. The studies involving the use of ionophore were not attempted until towards the end of the study. Excluding the use of the ionophore, the studies generally gave unacceptable tools for investigation of Ca^{2+} transport activities. The results being so variable from one batch of normal membrane to the next, it was decided that studies using blood from patients suffering from H.S. disease was unwarranted. The use of ionophore A23187 at very late stages in the studies (presented together with the transport in the IO vesicles for reasons of completeness) altered this situation. A tool for fairly accurately investigating abnormal membrane transport of Ca^{2+} has been outlined.

The situation of studying spectrin was rather more complex. The only biochemical reaction this protein demonstrates is that of phosphorylation by endogenous protein kinase in the presence of ATP and cAMP. The situation is made complex by the fact that other components in the membrane also show an ability to phosphorylate besides spectrin. It was decided to investigate this general membrane phosphorylation behaviour with particular reference to spectrin.

Membrane phosphorylation appeared to give results which were more consistent than Ca^{2+} transport studies. The phosphorylation behaviour of membrane isolated from H.S. patients was etudied. Although a consistent pattern of a decreaeed phosphorylation with time of H.S. membrane was noted compared with normal, this decrease was not statistically significant due to coneiderable overlap of standard deviations at the plateau values of the phosphorylation. These high standard deviatione were obtained due to dephoephorylation proces, which did not characteristically occur at a particular time period, but was distributed over a range of time.

It wae noted that the initial velocity of phosphorylation was linear for both the H.S. and normal membrane. Also as the dephoephorylation etep seldom occured at this phase, the etandard deviation valuee were relatively small. The phosphorylation etep also appeared to show a single eubstrate-enzyme type of reaction at this phase.

$(Ca^{2+} + Mg^{2+})$ - ATPase studies

Uee of a particular method of membrane preparation eeeme to have a great effect on the expression of many membrane bound enzyme activities. Inclusion of EDTA in the haemolyeing medium led to the increase of most enzyme activities, but membrane fragmentation also occured (Weed et al, 1963). Also haemolyeie in ieotonic buffer produced eealed membrane preparation but any

expression of the $(Ca^{2+} + Mg^{2+})$ - ATPase activity could not be obtained unless the haemolysis occured at 44°C, (Hanahan, 1973). The method of Dodge et al (1963) was found to be more rapid and yielded a sealed membrane preparation. This preparation showed no activity for $(Ca^{2+} + Mg^{2+})$ - ATPase until after freeze-thawing once.

The use of EGTA buffer system was essential particularly when very low Ca^{2+} concentrations were required. Accurate amount of free Ca^{2+} could be obtained by the use of this system, after the calculation of the concentration of the Ca-EGTA complex.

Ouabain is commonly used as an inhibitor of $(Na^{+} + K^{+})$ - ATPase, in most investigations on $(Ca^{2+} + Mg^{2+})$ - ATPases. In some instances in this study, particularly when assaying for the $(Ca^{2+} + Mg^{2+} + K^{+})$ or $(Ca^{2+} + Mg^{2+} + Na^{+})$ - ATPases, it was useful to use ouabain to suppress the $(Na^{+} + K^{+})$- ATPase activity totally so that a more realistic control value could be obtained. If this is not followed then the free Ca^{2+} ion (especially at high concentrations) may inhibit the$(Na^{+} + K^{+})$- ATPase to a considerable extent. Since the Ca^{2+} is absent from the control then the $(Na^{+} + K^{+})$ - ATPase activity may be actually higher than the Ca^{2+} samples. Subtraction of the control values from the Ca^{2+} samples leads to abnormally low $(Ca^{2+} + Mg^{2+})$ - ATPase activity.

This is more evident at high Ca^{2+} concentrations since the effect of Ca^{2+} is not significant on the $(Na^{+} + K^{+})$ - ATPase at a micromolar level in the presence of millimolar quantities of ATP. Mg^{2+} is essential for the activity of $(Ca^{2+} + Mg^{2+})$ - ATPase and it alone exhibited an ATPase activity (see fig 7.8). Therefore this activity must be subtracted from the total activity. Ca^{2+} only in the control could be used without the presence of Mg^{2+} but negative results were obtained specially at high concentration of Ca^{2+} (up to 2mM). This could be due to the activation of the Ca-ATPase activity described by Rosenthal et al (1970). This activity is not expressed in the experimental samples where Mg^{2+} is present, since Mg^{2+} was shown to inhibit this type of ATPase activity.

As discussed in section 3, there are various types of the ATPases which are stimulated by Ca^{2+}. For example the measurement of the $(Na^{+} + K^{+})$ - ATPase is relatively simple in that ouabain is available as a specific inhibitor of this enzyme, even though extreme care must still be exercised in interpreting such results (e.g. at various pH and ouabain concentration). The situation with Ca^{2+} is different. Many types of "Ca^{2+}- ATPases" exist. A list of these may be as follows (section 3 provides review of their existance):

(a) low affinity $(Ca^{2+} + Mg^{2+})$ - ATPase operating maximally at Ca^{2+} concentrations of 0.1 to 0.2mM and requiring Mg^{2+} for its activity; once thought as an artifact of membrane preparation (Schatzmann, 1973; Scharff, 1972).

(b) High affinity (Ca^{2+} + Mg^{2+}) - ATPase operating at maximal at levels of Ca^{2+} in the range of 1 to 10 μM (and probably much less). This high affinity is believed to be responsible for Ca^{2+} transport (Schatzmann & Rossi, 1971). It is thought that the low affinity part of the enzyme may also participate in the Ca^{2+} transport mechanism (Quirst & Roufogalis, 1975).

(c) Low affinity Ca^{2+} - ATPase operating maximally at Ca^{2+} concentration of 2mM and inhibited by Mg^{2+}. It is believed to be associated with spectrin (Rosenthal et al, 1970).

(d) Low affinity (Ca^{2+} + Mg^{2+}) - ATPase which showed different activities depending on the type of monovalent cation present. Two types of enzyme therefore exist (Ca^{2+} + Mg^{2+} + Na^{+})- ATPase and (Ca^{2+} + Mg^{2+} + K^{+}) - ATPase, both operating at 0.1 to 0.2 mM Ca^{2+}. These enzymes have not been proven to participate in the Ca^{2+} transport and are thought to be a result of uncoupling of the (Na^{+} + K^{+}) - ATPase in the presence of Ca^{2+} (Schatzmann & Rossi, 1971).

In the above a, b and d all activities are dependent on the presence of Mg^{2+}. In the absence of Ca^{2+}, Mg^{2+} itself shows an activity. This background activity is normally subtracted from that in the presence of Ca^{2+} to give the (Ca^{2+} + Mg^{2+}) component of ATPase. However, it is tacitedly assumed that the presence of Ca^{2+} has no effect on the Mg^{2+}-ATPase activity because, at the present, there is no way to prove this or allow for it.

In this study the possibility of existence of different $(Ca^{2+} + Mg^{2+})$ - ATPases is investigated. It was found that such types do exist. Na^{+} enhances the activity of the $(Ca^{2+} + Mg^{2+})$ - ATPase more than K^{+} does as it is clear from the greater initial velocity of the $(Ca^{2+} + Mg^{2+} + Na^{+})$ - ATPase reaction. This activity is non-linear after about 10 minutes and decreases rapidly and becomes less than that of $(Ca^{2+} + Mg^{2+} + K^{+})$ - ATPase after 20 minutes of incubation. These obaervations disagree with that of Schatzmann & Rossi (1971) who showed lower reaction velocity of the $(Ca^{2+} + Mg^{2+} + Na^{+})$ - ATPase. This could be due to measurement of the activity after 1 hr of incubation in their study.

In this field of research it is imperitive to have a specific inhibitor of the type of ATPase investigated. Although in section 7 Ru^{3+} and La^{3+} were used, these are far from specific and fail in suppressing the activity completely. The Ki value (fig 7.10) indicates that for full supression Ru^{3+} would have to be used at many orders of magnitude higher than that for instance of ouabain. At present no inhibitor is as well characterised for the $(Ca^{2+} + Mg^{2+})$ - ATPase as ouabain is for $(Na^{+} + K^{+})$ - ATPase.

In section 8, fig 8.2 a, b and c shows that the red cell membrane bind 0.45 nmole Ca^{2+} per mg of membrane protein. In the incubation tubes the binding would be 0.068 nmoles for 0.15 mg protein. In a reaction mixture of 2.5 ml total volume and at Ca^{2+} concentration of 50 μ Molar, the change in Ca^{2+} due to binding is negligible. For Ca^{2+} concentration of 0.1μ Molar, however, the inaccuracy in Ca^{2+} level is 23% due to binding.

Schatzmann & Rossi (1971) in their studies used a time period of 60 minutes for incubation to measure the (Ca^{2+} + Mg^{2+}) - ATPase activities. This is necessary when using release of inorganic phosphate from ATP as an assay method. It was shown that under experimental conditions used in this study, the reaction is no longer linear after 30 minutes of incubation. Very short time could be used in this study for the estimation of the (Ca^{2+} + Mg^{2+}) - ATPase activity since a radio labelled substrate was used (further advantage over the colorimetric method is that background phosphate problems are not encountered). Phosphate was constantly present since the membrane was prepared in phosphate buffer. The release of the labelled phosphate into a pool of unlabelled phosphate also has an advantage that the released phosphate reutilisation or binding is not a problem.

Ca^{2+} TRANSPORT STUDIES

Since investigations of (Ca^{2+} + Mg^{2+}) - ATPase were directed towards establishing the characteristics of the Ca^{2+} pump activities, it was considered that a more direct approach may be more fruitful in face of the previous difficulties.

If uncertainties are involved in determination of normal values of transport ATPase, then it is unwarranted to draw conclusions about the activities of abnormal membrane.

It must be clearly pointed out that the most successful method of Ca^{2+} transport studies, that of use of EGTA buffers using IO prep with and without the ionophore A23187 in the presence and absence of ATP, is capable of fairly accurate transport measurements. This is suited for studies of abnormal membranes. Unfortunately, the technique was evolved, after refinement, much too late in the study for this to be possible.

The homogenisation method which gave the highest level of IO vesicles (as measured by separation on dextran density gradient, see fig 9.1), in absence of Mg^{2+} was used throughout the study. Of the various homogenisation procedures used for the freeze-thawed IO prep, the Warring blender (MSE) homogeniser was found to be most suitable (at one third full output). The orientation of the membrane vesicles was determined by dextran density sedimentation or by estimating the activity of acetyl-choline esterase activity. Homogenisation and preincubation at 37°C for 20 minutes (see table 9.1) were also investigated in a similar manner.

Uptake experiments using IO prep from the density gradient separation gave no or very little accumulation of Ca^{2+} in the presence of ATP, although the high affinity (Ca^{2+} + Mg^{2+}) - ATPase activity was present. It is possible that the prolonged centrifugation or contact with dextran renders these vesicles highly permeable. Although Kant & Steck (1972) have shown that during the centrifugation these vesicles can retain Na^{+} or K^{+}, they have not conclusively shown that the permeability does not change after the vesicles have reached equilibrium position in the gradient.

Since the Ca^{2+} was always added to the incubation mixture and accumulation of Ca^{2+} was measured, it was unnecessary to separate the IO vesicles from the other membrane structures in order to study Ca^{2+} transport. Fig. 9.1 shows the percentage of IO vesicles present in the incubation medium under the experimental conditions. Using this value calculation of accumulation of Ca^{2+} due to mg of IO vesicles protein is possible.

The uptake studies were performed with the incubation mixture lacking ATP as a control for passive diffusion. It was found that the initial rate of flux was constant for approximately 10 minutes. If the accumulated Ca^{2+} exceeds a value of 2.2 nmole per mg protein, then a rapid loss of the accumulated Ca^{2+} was observed. Figs 8.2a, b and c show the possible modes of accumulation and loss of Ca^{2+}

Theee studies used Ca^{2+} at a concentration of 0.2 mM to avoid uncertainties of low level of Ca^{2+}. Studies using 50μ M Ca^{2+} ehowed that at this concentration the vesicles were accumulating Ca^{2+} at a rate four times higher than that obtained at 0.2mM Ca^{2+}. Thie indicates that some inhibition of Ca^{2+} transport occured at 0.2mM Ca^{2+}.

Variability of the efflux studies on the whole cells after loading with Ca^{2+} (with aid of the ionophore A23187) to a level of between approximately 0.9 to 2 mM may also be due to Ca^{2+} inhibition of the transport (fig 8.1). Preliminary results have shown, however, that this variability existed even with intracellular Ca^{2+} concentration as low as 50 to 70 μMolar.

Use of EGTA buffer system proved to be useful in preventing loss of accumulated Ca^{2+} from the IO vesicles. Using this system linearity of the uptake could be extended to about 15 minutes. Accumulated Ca^{2+} was found to be over four fold in excess of experiments carried out at 0.2mM in absence of EGTA (fig 8.3).

Furthermore, in these experiments the Ca^{2+} was accumulated againet its concentration gradient. Howe ver, this system would not allow estimation of ATP induced Ca^{2+}binding to the membrane. That ATP does cause an increased Ca^{2+} binding to the membrane was shown in figs 8.2 a, b and c (also see Schatzmann, 1975).

The initial gredient against which Ca^{2+} was being transported in not accurately known. An additional control experiment in

which ATP was present with the IO prep was used in presence of ionophore A23187. This allowed the calculation of the initial Ca^{2+} gradient across the vesicles and the level of ATP induced Ca^{2+} binding was same as the sample with ATP but without ionophore.

Calculation of the stoichiometric ratios

The stoichiametric ratio of the number of ions of Ca^{2+} transported for one ATP molecule hydrolysed can be calculated. For the intact cell this could be

$$SR = \frac{\text{Moles of } Ca^{2+} \text{ extruded from 1 litre of packed cells}}{\text{Moles of ATP hydrolysed by the total cell membrane}}$$

Where SR is the stoichiometric ratio. The number of cells per litre of packed cells was calculated from the blood indices. The amount of membrane protein per cell was determined by measuring the amount of membrane protein from 1ml of blood. The Hb in various lysates were determined and from the blood indices the number of cells per mg membrane protein was calculated. Using this method average mg protein per cell was 0.3956×10^{-9}. An average 1 litre of cells contained 1.2×10^{13} cells i.e. 4.7472 gm membrane protein per 1.2×10^{3} cells (or 1 litre).

From table 8.1a the value of efflux from whole cells loaded with Ca^{2+} with the aid of ionophore A23187, was 0.08 ± 0.035 mMole/ litre of packed cells / minute.

$$\therefore \text{ } Ca^{2+} \text{ efflux} = \frac{0.08}{4747.2} \text{ mmole/mg protein/min.}$$

$$= 16.85 \text{ nmole/mg protein}$$

Taking the $(Ca^{2+} + Mg^{2+})$ - ATPase activity at 0.2mM Ca^{2+} to be 0.0485 μM/mg protein/min (section 7.2.5),

$$SR = \frac{16.85}{48.5} = 2.93 \pm 1.28$$

A stochio metric ratio of 1Ca^{2+} ion transported per 2.93 $\pm$ 1.28 molecules of ATP hydrolysed is obtained. The range is therefore 1 Ca^{2+} ion transported per average of 3 ATP molecules hydrolysed. Considering that the Ca^{2+} transport may be severely inhibited by high intracellular Ca^{2+} (and therefore the value of the ATPase used in the calculation is high), the SR valus obtained appears reasonable. However, in this experiment the passive Ca^{2+} diffusion was not allowed for in the calculation, and does not significantly alter the results. Repeating similar calculations for the method used in section 8.2 i.e. using IO prep without a concentration gradient in absence of EGTA gives a value of the stochiometric ratio of one Ca^{2+} ions transported per 182 $\pm$ 28 mole of ATP hydrolysed (calculations shown in appendix).

However, when against the concentration gradient experiment was carried out in presences of EGTA buffer system at free Ca^{2+} concentration of 50 μM, the SR was found to be 0.424, i.e. one Ca^{2+} ion transported per 2.4 moles of ATP hydrolysed.

These stochiometric ratios clearly point out the difficulty in transport ATPase activity measurements. This is that a selective inhibitor of transport ATPase is not available. Therefore to attribute the total ATP hydrolysis in a system to the Ca^{2+} pump is an error which is commonly made in the literature and such data should be treated with caution.

Stability of the vesicles

It is imperative to know how the IO prep behaves under experimental conditions especially in the Ca^{2+} uptake studies. Results in section 8 have indicated that the vesicles are probably unetable, and can revert from one form to the other. For example, it is important to know whether these vesicles show uptake of incubation mixture by further pinocytosis or not. In most cases (especially the experiments against concentration gradient), such pinocytotic behaviour would not cause any alteration of final results, if the IO vesicle volume remains constant. However, in experiments in which the vesicles are accumulating Ca^{2+} without the presence of a concentration gradient, such pinocytotic behaviour would lead to erronous results.

Initial studies in obtaining a pure fraction of IO vesicles (using dextran sedimentation technique) was hampered by the observation that the vesicles showed no ability to accumulate Ca^{2+}. The (Ca^{2+} + Mg^{2+}) - ATPase activity (both the high and low affinity) was found to be prsent in these vesicles. The work of Kant & Steck (1972) showing that thsse vesicles are impermeable to Na^{+} or K^{+} may be correct in that both radioactive tracers were found in position of IO and RO vesicles. However, having attained this equilibrium position, it is necessary to sediment the various fractions to examine if the tracers are truly sealed. The inability of Ca^{2+} accumulation after the

vesicles attain the equilibrium position in dextran density gradient show that most probably the vesicles do not retain their impermeability. Further, it was found that dextran - like componds, such as inulin and sephadex, interfered with the vesicle stability.

Acetylcholine esterase measurements of the IO prep showed that after preincubation for 20 minutes at 37°C, the percentage of the enzyme activity remained essentially constant with time. ATP only had an effect in the preincubation period and both ATP with and without Ca^{2+} had no effect on this level of IO vesicles after the initial preincubation. In studies using $^{22}Na^{+}$ for entrapment, it was found that when ATP was present at 2mM in the incubation medium, the IO prep lost about 90% of the entrapped Na^{+} over 135 minutes. This Na^{+} loss was delayed by the presence of Ca^{2+} in the incubation mixture with ATP. When Ca^{2+} was present at 2mM concentration, virtually no loss of Na^{+} was detected for the first 90 minutes in presence of 2mM ATP (see fig 9.4).

The Na^{+} is lost in the presence of ATP without any major membrane alterations (as judged by acetylcholine measurements). Therefore, it is probable that channels are created in the vesicles to allow passage of Na^{+}. Since the loss of Na^{+} is as high as 80 to 90% of the entrapped Na^{+}, the process must involve both IO and RO vesicles.

The entrapment of ^{14}C-inulin partly failed in deciding whether these channels are large enough to allow the passage of Na^+ only because inulin showed a destabilising effect on the vesicles (see fig 9.6). However, it is seen that although difference in the amount of inulin trapped exists in the absence and presence of ATP at various Ca^{2+} concentrations, this difference is not significant. Since most of the inulin is not lost, the experiment shows that inulin is probably not released in the presence of ATP and (or) Ca^{2+}. Hence the channels formed are only sufficient to allow passage of Na^+ and not inulin. The loss of accumulated Ca^{2+} in figs 8.2 a, b and c also show that these channels may be non-specific in that Ca^{2+} is also able to leak from the vesicles. That this accumulated Ca^{2+} can be lost in presence of 0.2mM Ca^{2+} and not in 50 μM Ca^{2+} is contrary to the results obtained by Na^+ entrapment and cannot be explained.

That acetyl choline esterase show no changes in the IO to RO conversion (or RO to IO) support the hypothesis that channels are only sufficiently large enough to accommodate Na^+ or Ca^{2+} but not the enzyme substrate, Nbs.

Electron microscope study (section 16) have demonatrated that after the initial preincubation period, very little or no "budding" or pinocytosis of vesicles is noted. When ^{14}C-inulin was added to the vesicle preparation after homogenisation and preincubation, very little radioactivity was found in the sediment of these vesicles, after centrifuging through a 8% sucrose layer to remove

excess ^{14}C inulin.

However, as mentioned earlier, in some Ca^{2+} uptake experiments it was noted that after the loss of accumulated Ca^{2+} the level of Ca^{2+} in the IO prep was found to be less than that in the controls. This indicated that an IO to RO conversion of the vesicles must have taken place to a small, but limited, extent. Since the acetylcholine esterase assay is capable of measuring such small changes (accuracy of about ± 1%) an equivalent amount of RO vesicles must have changed to IO to avoid this detection. If this residual interconversion is taking place throughout the uptake experiments, it has little effect on the initial velocity of Ca^{2+} uptake.

Phosphorylation of the red cell membrane

In order to study the phosphorylation of spectrin in membranes (band I & II of SDS gel electrophoresis), it is necessary to study the general properties of phosphorylation of the whole membrane. The phosphorylation experimente were carried out as described in section 10.1. The incubation mixture contained theophylline to inhibit the phosphodiesterase activity; NaF at low concentrations inhibited the adenyl cyclase activity and prevented further formation of cAMP at the expense of ATP.

EGTA was necessary to chelate any contaminating Ca^{2+} and prevent the (Ca^{2+} + Mg^{2+}) - ATPase activity. K^{+} salts were eliminated to prevent activity of the (Na^{+} + K^{+}) - ATPase activity.

Under these conditions the reaction was linear for up to at least 10 minutes (fig 10.3). The reaction was studied over 60 minutes. The level of ATP decreased by approximately 30% in the first 10 minutes and 65% at the end of 60 minutes. The ATP is not a limiting factor in this reaction as evident in some experiments where rephosphorylation occured after an early dephosphorylation and since the maximum level of phosphorylation (0.06 nmole phosphate bound per 0.2 mg protein) was much less than the level of ATP (2.5 nMoles) in the incubation medium.

The phosphorylation of membrane proteins is catalysed by cAMP dependent protein kinase. In table 10.2 it can be seen that cAMP stimulates the membrane phosphorylation by 80%.

Rate of linear phosphorylation under the standard assay conditions was found to be 19.5 pMoles/min. The dephosphorylation can be due to several processes. A phosphoprotein phosphatase could cause the liberation of the phosphate as in the synaptic membranes. The phosphate could also be released as a phosphopeptide. Also the phosphate may exchange from one position to another, i.e. from an acid stable phosphate receptor to an acid unstable receptor.

These various activities could also be occurring simultaneously. The assay was stopped with 7.5% TCA and then the resulting pellet was redissolved in NaOH. Therefore any acyl phosphate bonds that are formed will not be detectable since the phosphoacyl is unstable under such extremes of pH.

The phosphorylated intermediate of the $(Na^+ + K^+)$ - ATPase has been shown to be a phosphoacyl compound. This is not assayed for since it is destroyed in the sample washing procedure with extremes of pH. The types of phosphate bonding which are stable to such treatment are the phospho-serine and phospho-threonine type of bonding. Roses & Appel, (1973a)have also shown that the bonding is mainly of the phosphoserine type for the red cell membranes under the conditions of the experiment. If in the phosphorylation reaction the phosphate were to be transferred from, say a serine residue to an acyl residue enzymatically, this would show as a dephosphorylation reaction, since for example, the phosphoacyl bond would be destroyed. It must be mentioned that, as in the carbohydrate metabolism, transfer of a phosphate from a serine or threonine group to an acyl group is energetically unfavourable and requires further use of ATP.

That such acid labile transfer must be regarded seriously, is indicated by the phosphorylation of band (I & II) from normal membrane with time. A characteristic dephosphorylation

is evident at about 20 to 30 minutes of incubation. (In some samples another dephosphorylation was noted at about 5 minutes). If the membrane phosphorylation of fig 10.3 is superimposed on band (I & II) phosphorylation, it is noticed that the difference between the two would give an extra peak of phosphorylation to the membrane minus band (I & II) proteins. Such analysis makes the case of transfer of phosphate from one protein to another seem possible.

For band (I & II) the maximum level of phosphorylation was in the region of 20 pmoles phosphate bound per mg original membrane protein. It appears that spectrin band (I & II) incorporates less than 10% of the phosphate of the total membrane protein.

If band (I & II) constitute about $^{1}/_{5}$ of the membrane protein and has a molecular weight of 430,000 daltons, then 0.065 M phosphate is bound per mole of spectrin. This is twice the number of moles of iodide bound per mole of spectrin (as will be discussed later in this section). Assuming each phosphate is bound to an identical peptide of spectrin, this would give a molecular weight of 6.45×10^{6} daltons for the spectrin phosphopeptide. (However, using extracted spectrin the figure is $13. \times 10^{6}$ daltons).

Phosphorylation of H.S. membrane

When SDS polyacrylamide gel electrophoresis of normal and H.S. membrane is compared, it is evident that band (I & II) do not show a decrease in the gel scans relativity to other membrane components. Although this is a semiquantitative method, it shows no reason for suspecting that total protein band (I & II) of H.S. red cell membrane (relative to the rest of membrane proteins) is any different from normal.

H.S. membrane phosphorylation showed lower level of phosphorylation compared to that of normal (see fig 12.1 and 10,3). This difference is not significant due to large standard deviation values in the plateau region. When band (I & II) are compared, the H.S. samples showed a decreased level of phosphorylation at the two peaks and the difference at these points is significant at a $P = 0.05$ level (using Student t test).

The study also ruled out the possibility that, in the free state, the protein kinase is altered for membrane phoephorylation. Results indicated that the membrane proteins receptor for the phosphate may be altered.

An alternative that the abnormality lies in some other component (rather than the phoephate receptor proteins) which alter membrane bound protein kinase activity is remote. Extracted spectrin from H.S. and normals still showed difference in the phoephorylation activity (the H.S. spectrin showad immeasurably

low level of phosphorylation under the experimental conditions used with three different samples extracted from different H.S. membrane samples even when exogenous protein kinase was used.

Phosphorylation of membrane derived from red cells in different stages of maturity

Endogenous phosphorylation activity of membranes prepared from the bottom layer cells was found to be 1½ folds higher than that of membrane prepared from the top layer cells. This appears to disagree with the results of Peffefer and Swimlock (1976) who reported a decline in the activity of membrane bound protein kinase during cell ageing. The reason for the descrepancy is not known, although it could be speculated that the enzyme is not fully activated at early stages of maturity. Also cAMP may have an inhibitory effect at this stage.

It was shown that high level of fluoride inhibits the phosphodiesterase activity during development in the brain tissue (Rall & Sutherland, 1958). Such observations may also apply on the red cell phosphodiesterase.

An endogenous protein kinase inhibitor which is shown to be present in the rabbit skeletal muscle (Walsh et al, 1971) (see section 4) may be present in the red cell at high levels at early stages of maturation and could be responsible for the reduced level of phosphorylation in the young cell population.

The high level of phosphorylation in the old cell membranes could be due to decline in the level of this inhibitor during maturation (see section 4 for further discussion).

Addition of extracted cytoplasmic protein kinase showed no significant alteration in the phosphorylation level in the old cell membrane while a decline in the phosphorylation activity in the young cell membrane is clearly noticed (see figs 11.3 and 11.4). The reason for the inhibitory effect of the extracted protein kinase is not clearly understood.

STUDIES ON EXTRACTED SPECTRIN FROM NORMAL RED BLOOD CELLS

The extraction of spectrin was carried out using the low ionic strength elution procedure rather than the technique of butanol extraction, which elutes practically all membrane proteins (Maddy, 1966).

The supernate I of spectrin extraction on SDS gel electrophoresis (see fig 15.7) shows that proteolytic activity may be present in this fraction. This fraction was electrophoresed after 48 hrs of standing at 4°C and would normally give the same pattern as shown in fig 15.8 and 15.9 (which are also used after 48 hr) if used immediately after dialysis. Based on this evidence it was reasonable to assume that the loss of phosphorylation activity may be due to proteolytic action.

Arguments against this are that extracted spectrin still loses phosphorylation activity even when stored at -20°C or in liquid nitrogen. Also, the H.S. extracted spectrin loses its activity much more rapidly even when exogenous protein kinase was added. On prolonged standing (about 60 hrs at 4°C) spectrin precipitates out of solution as straight white fibres, 2 to 3mm in length. Electrophoretic pattern of this precipitate on SDS gels also produces multiple bands. Spectrin may, therefore, be composed of multiple polypeptides which can organise themselves into different geometrical structures which depend on the medium conditions and time. Hence formation of lower molecular weight peptides is not necessarily an evidence for proteolytic activity.

Another interesting observation, which occurred a sufficient number of times to discount it as an artifact, is shown in fig 15.10. In the final sediment of spectrin extraction, a doublet band was found in the position where bands II.1 and II.2 normally appear. This doublet bears a resemblance to band I and II. Molecular weight of this new band is approximately 40×10^3 to 50×10^3 daltons less than band I and II.

When freshly dialysed spectrin is electrophoresed in SDS, clear spectrin bands I and II are noticed with little other banding except at about 15×10^3 daltons (possibly globin). On standing at 4 C for about a day the pattern changes to that shown in fig 15.3 (and 15.4 for H.S. spectrin).

It is tempting to speculate that the band I and II of spectrin are composed of lower molecular weight peptides. If they do exist, then it is difficult to understand why SDS is incapable of separating these on electrophoresis. It is possible that these peptides exist as a tightly interacting unit, as was observed when spectrin was centrifuged in an analytical machine. In presence of 4M urea a single peptide peak was observed with a molecular weight of approximately 30×10^3 daltons (fig 17.1). This peak did not "broaden" in the usual manner on extended centrifugation. This indicates that the polypeptides have a strong inter-molecular bonding even in presence of 4M urea. The result must be treated with caution

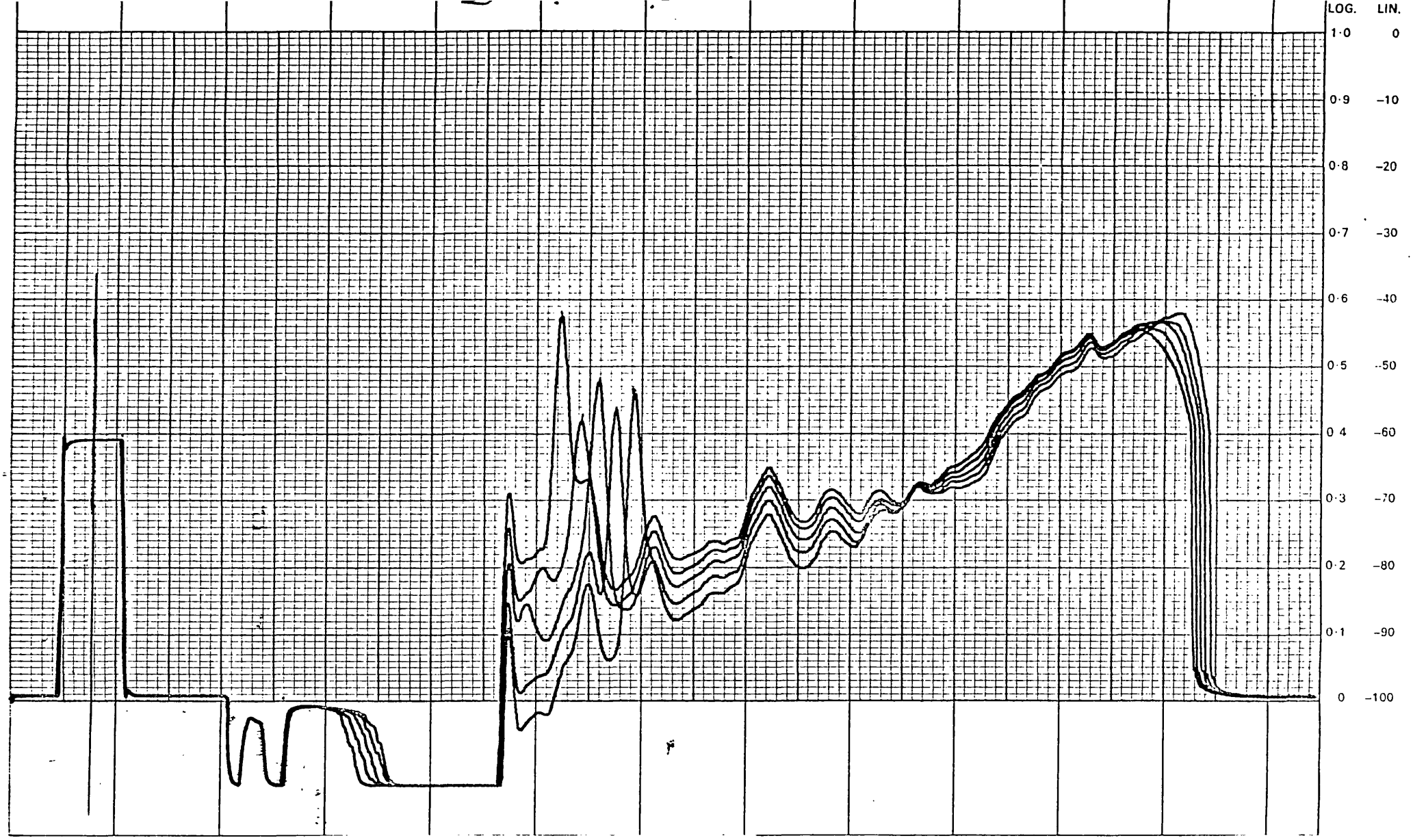


Fig 17.1 Analytical ultracentrifugation of extracted spectrin in 4M urea using MSE Centriscan 75 centrifuge. Note that the protein peaks do not spread as the protein migrates from left to right. Schliern optics used. Scan time 8 mins interval; knife angle 80 ; temperature 20 C; rotor speed 55,000 rpm.

since this experiment was carried out twice only.

Interesting results were also obtained with over 48 hr old extracted spectrin in tris buffer (see fig 17.2). A peak was observed with approximate molecular weight 10^5 daltons initially. On continued centrifugation, small peaks with higher molecular weight appear to "bud" from the main peak. It is possible that an equilibrium situation exists in the main peak. When high molecular weight components are removed from this "polypeptide pool", reorganisation takes place to re-establish the equilibrium and form another batch of aggregated protein and so on. Again, since this is a result of two experiments, great care must be exercised in interpreting the data. Spectrin extracted from H.S. membranes was not sufficiently concentrated for this experiment.

Phosphorylation of spectrin

Fresh sample of spectrin, in absence of exogenous protein kinase, incorporates a maximum of approximately 33 pMoles phosphate per mg protein. Assuming a molecular weight of band (I & II) to be the least molecular weight of spectrin (4.3×10^5 daltons), a molar ratio of spectrin to phosphate (S/Pi ratio) can be calculated. This is approximately 70. In the presence of exogenous protein kinase spectrin phosphorylation is stimulated by approximately seven fold (approximately 0.230 nMoles phosphate bound per mg protein, giving the new S/Pi ratio of 10).

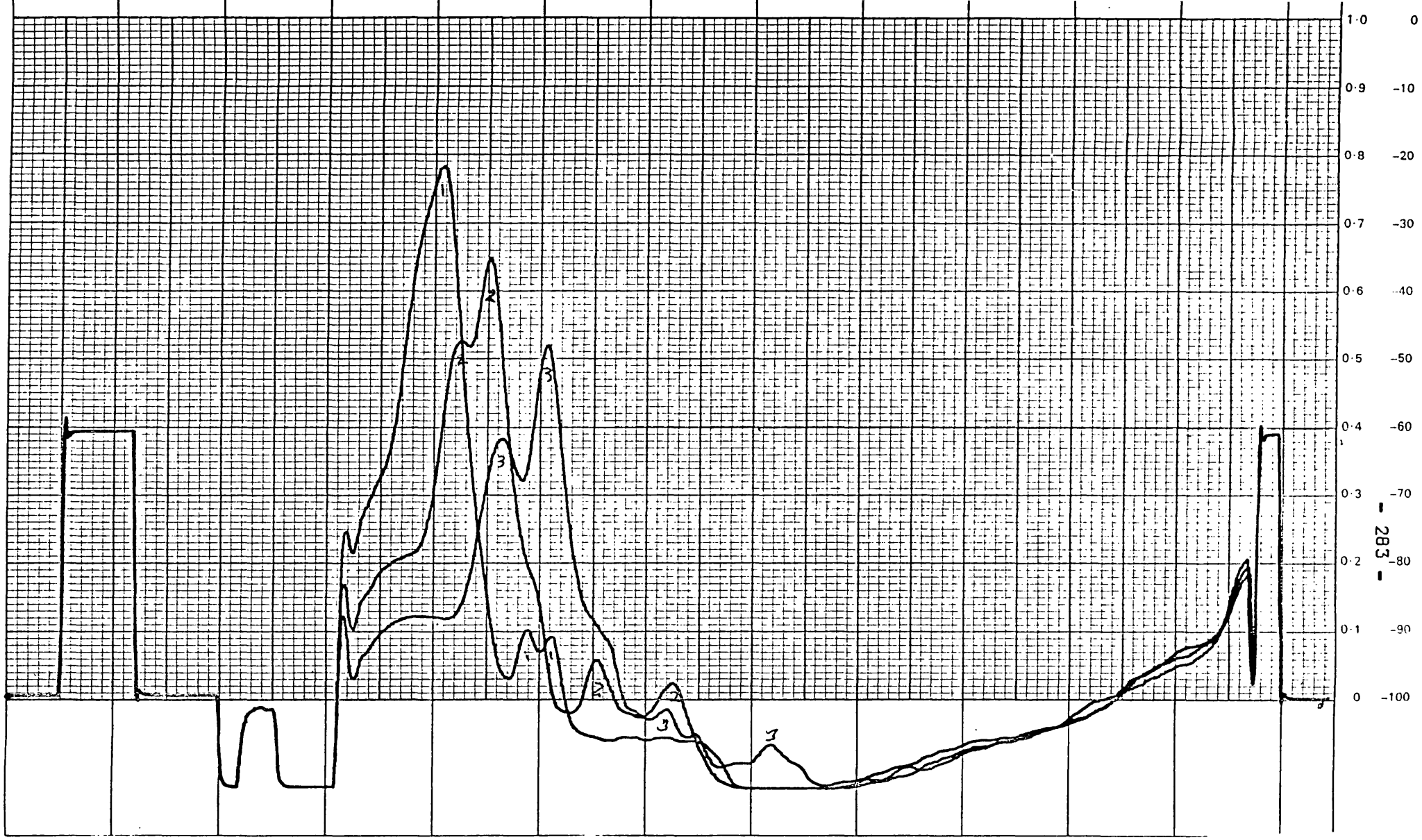


Fig 17.2 Analytical ultracentrifugation of spectrin in dialysis buffer (see fig 10.2). Note that multiple leading bands form on continued centrifugation. Conditions used as described for Fig 17.1.

Band (I & II) demonstrated a maximal phosphorylation of approximately 30 pMoles per mg original membrane protein (fig 12.3). Assuming that only 25% of total membrane protein is present in band (I & II), this gives a value of 0.120 nMoles phosphate bound per mg spectrin. The S/Pi ratio for this is 19.0.

Similar calculations can be used for spectrin band (I & II) from H.S. membrane. Using the data from fig 12.2 and fig 12.3 the S/Pi ratio with time are shown below in table 17.1.

	Molar ratio	
Time (mins)	S/Pi for Normal	S/Pi for H.S.
3	64	208
5	55	73
10	30	53
20	21	26
30	51	132
40	17	23
80	20	34
60	23	58

The S/Pi ratio may be useful when comparing phosphorylation of spectrin in various conditions such as H.S., sickle cell etc. Particularly useful are the values at maximum phosphorylation (20 and 40 minutes) and minimum phosphorylation (30 minutes).

IODINATION STUDIES

Exactly what role spectrin has in membrane regulation and cell shape control is not known with any certainty. Circumstantial evidence indicates that it could have a central regulatory and structural function. The difficulty is based on the lack of knowledge of its biochemical and molecular structure.

Since the phosphorylation studies have been investigated, it was of interest to note how the conformation of this protein altered during the various phases of phosphorylation. Physical techniques such as optical rotary dispersion and circular dichorism in this instance would only record the event as it occurred. Iodination studies, however, could record the events as they occurred and subsequent fractionation of proteins by SDS electrophoresis can indicate if conformational changes in such proteins have taken place. Physical studies on isolated spectrin would have been valuable but access to such techniques was not readily available.

Lactoperoxidase catalyses the iodination of tyrosine (and to a lesser extent histidine) residues of proteins with iodide. As shown in table 14.1 the procedure of iodination has only a relatively small effect on phosphorylation of membrane or extracted spectrin. Iodinated samples under these conditions still continue to exhibit phosphorylation activity, although at a slightly reduced rate.

Using IO prep it was noticed that the level of incorporation of ^{125}I in the freeze-thawed prep was greater than the non-freeze-thawed prep (table 14.2). Although this shows that the IO prep is intact, previous study on the stability of the IO prep indicates that a thorough investigation of stability should be made under every new condition used. Since such investigations are a lengthy procedure, the IO prep was rendered leaky by freeze-thawing.

The disadvantage in freeze-thawing is that selective iodination of spectrin of the IO vesicle is lost. The reason for using an IO prep rather than freeze-thawed ghost membrane is that band (I & II) poorly incorporate ^{125}I using ghosts. Why this should be is not quite understood. Perhaps the lactoperoxidase has only a limited capacity to diffuse into these fractured ghosts over the iodination period. However, even when IO prep is used, iodination of band (I & II) is relatively low compared with rest of the membrane proteins, as also shown previously by Selhi & White (1975).

Iodination of freeze-thawed IO prep which have been phosphorylated show no significant difference in labelling of ^{125}I when compared with non-phosphorylated samples. When these samples were subjected to SDS gel electrophoresis and band (I & II) isolated, a difference of approximately 25% in iodination was noticed (fig 14.2). This is also shown in fig 14.3.

The effect of Ca^{2+} (during the phosphorylation step) on subsequent iodination was examined. It was noticed that the difference in iodination of the phosphorylated and non-phosphorylated band (I & II) decreased when Ca^{2+} concentration up to 0.5 mM was used (see fig 14.3). Total level of iodination of band (I & II) of the phosphorylated and non-phosphorylated sample was increased by approximately 45% at 0.5 mM Ca^{2+} when compared with samples in which the Ca^{2+} was absent. When Ca^{2+} was present at 2 mM it was found that the level of iodination of the non-phosphorylated band (I & II) returned towards the value of zero Ca^{2+} concentration, whereas the phosphorylated band (I & II) remained elevated.

The experiment demonstrates that a difference in conformation of spectrin in the phosphorylated and non-phosphorylated form exists ($P = 0.05$). In presence of Ca^{2+} this difference diminishes, until at 0.5 mM Ca^{2+} it is insignificant at a $P = 0.10$ level. At this level, although the spectrin does undergo change in conformation, little difference in conformation exists between the phosphorylated and non-phosphorylated form.

It is possible that at a Ca^{2+} concentration of 0.5 mM the breakdown of ATP by Ca^{2+} ATPases takes place. Since ATP is present at a very low concentration (5 μMolar per 0.2 mg protein) in these experiments, the effect of Ca^{2+} on phosphorylation was not initially investigated. In fact 0.3mM EGTA was present in

every experiment not involving Ca^{2+} to supress any Ca^{2+} ATPase activity.

The difference in iodination between the phosphorylated and non-phosphorylated band (I & II) at Ca^{2+} concentration of 2mM reflect a large difference in conformation between the two spectrin samples. This change may have its origin in the stimulated spectrin Ca^{2+} ATPase activity as proposed by Rosenthal et al (1970).

Time course studies on isolated spectrin

The spectrin for these studies was used immediately after the final dialysis step (see fig 14.6) to avoid changes of spectrin band (I & II) as shown in fig 15.7.

Taking the molecular weight of band (I & II) as 430,000 daltons, the moles of spectrin bound to one mole of iodide (the S/I ratio) can be calculated. This is shown in fig 14.4. The smallest ratio was found to be approximately 30. If iodide is bound to an amino acid residue, then in the limiting case, one spectrin molecule must bind at least one iodide molecule. On this basis the least molecular weight of this is approximately 30 x 430,000 daltons (approximately 13 x 10^6 daltons). Since most electron micrographs of spectrin show existence of fibrils of undefined length (Marchesi & Palade, 1967; Marchesi & Steers, 1968) such a figure may have a real existence.

For the extracted spectrin in phosphorylation buffer in absence of ATP, the S/I ratio was 90 at zero minutes of incubation. This increased to about 120 at 10 minutes. Over the next 20 minutes this value decreases to between 60 and 80. It remains more or less constant at this level for up to 60 minutes (see fig 14.4).

The samples in which ATP is included in the phosphorylation medium shows a different S/I ratio with time. At zero time the unit is 90 as previously. However, a sharp decrease to 30 is noticed after about 5 minutes. Over the next twenty minutes this number reaches about 60 and remains at about 50 to 60 for the remainder period of incubation.

MODELS FOR SPECTRIN STRUCTURE USING THE S/Pi RATIOS

Table 17.1 shows the values of S/Pi ratio for normal and H.S. band (I & II). Since the exact molecular weight of spectrin is not known, it is reasonable to assume that it would be some multiple of band I or II (or even both). The S/Pi ratio of table 17.1 was obtained by adding the molecular weights of band I and II together (4.3×10^5 daltons). No S/Pi value less than unity was obtained, indicating that this estimation of molecular weight is still incapable of showing a mole per mole binding of phosphate to spectrin. S/Pi ratios for normal band (I & II) indicate that the least molecular weight of spectrin subunit may be $15 \times (4.3 \times 10^5)$ or 6.5×10^6 daltons.

Before proceeding further, this fairly high molecular weight must be corroborated by other studies. Marchesi & Steers (1968) have reported that spectrin forms fibrils which can be identified under the electron microscope. These fibrils are readily observed under fairly low magnification and the images are obtained with relatively simple techniques. Normally, fairly high magnification and great care is required to obtain an image of a protein with molecular weight of, for example, 0.25×10^6 daltons. Kirkpatrick *et al* (1976) have also reported that the hexamers and other torus protein molecules observed

Table 17.2 S/Pi and S/I ratios for the time course studies for band (I & II) of SDS gel electrophoresis. For membrane samples it was assumed that 25% of total membrane protein is present in band (I & II) for S/Pi calculation. (Values shown to the nearest integer).

Time (mins)	S/Pi Ratio		S/I Ratio	
	Normal membrane	H.S. membrane	Phosphorylated spectrin	Non-phosphorylated spectrin
3	64	208	100	120
5	55	73	115	120
10	30	53	30	120
20	21	26	50	100
30	51	132	60	60
40	17	23	50	70
50	20	34	50	70
60	23	58	50	70

under the electron microscope may be spectrin.

That these high values for molecular weights are not obtained when specimen of spectrin is analysed using analytical centrifugation, may be due to the instability of the extracted spectrin. This is shown in fig 15.3,15.4 and 15.7 using SDS gel electrophoresis. Furthermore, as previously discussed, spectrin is not stable in solutions and tends to precipitate out as fibres on standing. Normally samples used for analytical studies are centrifuged to obtain a clear solution. The pH also may be adjusted for this reason.

Returning to table 17.1, it is possible that the lowest S/Pi value obtained forms a basic subunit of spectrin. Other S/Pi values may reflect a multiple of this subunit. For the normal band (I & II) 17 is the lowest figure for S/Pi ratio. Similarly, fig 14.4 indicates the lowest value for S/I is 30. In the appendix, values ranging from 15 to 26 are used for dividing the S/Pi ratio (table 18.1). Since proteins are normally stable in the form of hexamers, tetramers, dimers and monomers, such pattern was sought for in table 18.1.

Taking 15 to be the least S/Pi ratio for normal membranes, the change in subunit arrangement with time is shown in fig 17.3a. Similarly for H.S. a S/Pi of 23 was taken to be the lowest value. The scheme of arrangement of subunits with time is shown in fig 17.3b.

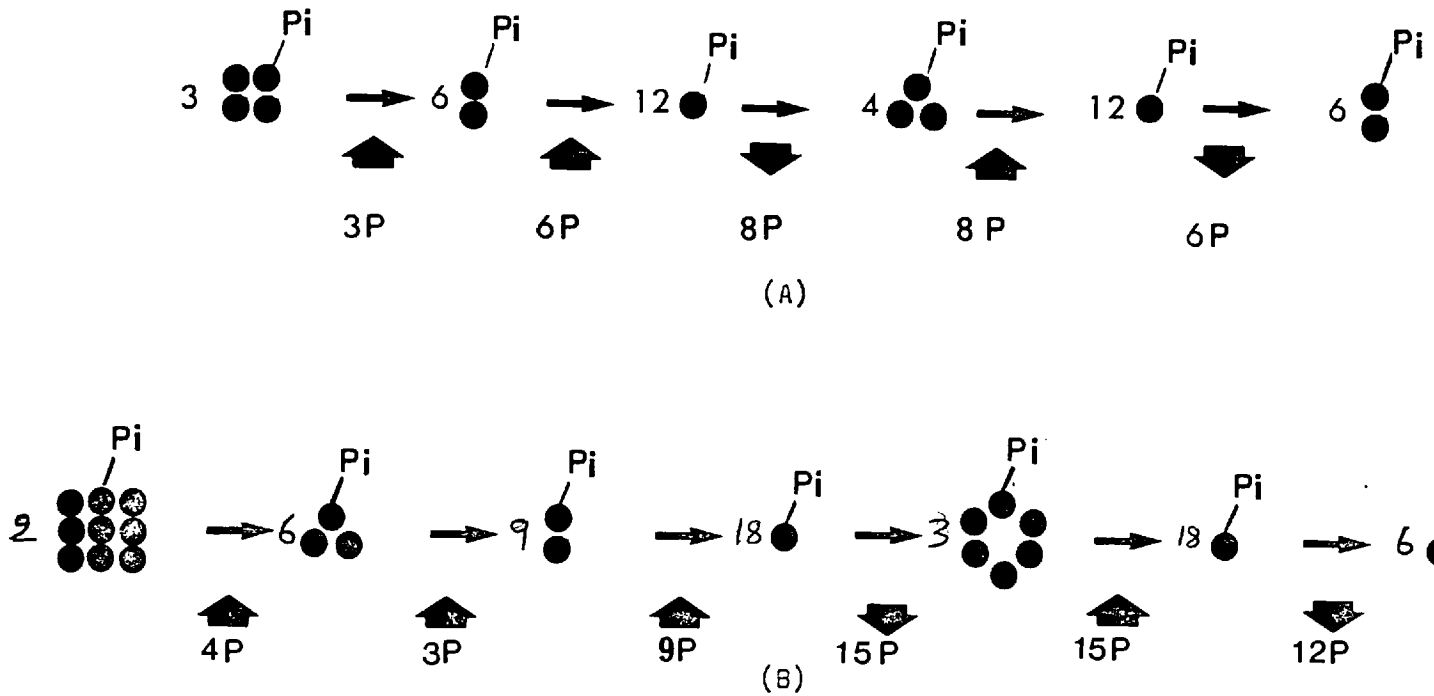


Fig 17.3 Structure of spectrin based on S/Pi ratios. (A): for band (I+II) of normal membrane. (B): for band (I+II)of HS membrane. Each circle represents a molecular weight of 6.5×10^6 daltons.. P indecates binding or loss of inorganic phosphate , phosphoprotein or both from band (I+II). Conformational change is possible at each step.

From table 18.1 it can be seen that at the maximum level of phosphorylation of band (I & II) a monomer is present for normal membrane, whereas a monomer or dimer is most probable for H.S. sample. At the minimum level of phosphorylation of band (I & II) (see figs 12.2 and 12.3) a trimer is the most probable state for normal spectrin and a hexamer for H.S. Also, at the next phosphorylation maximum a monomer for both H.S. and normal is shown in table 18.1. Although monomers are expected for at least one maximum value of phosphorylation of band (I & II), by virtue of the definition of S/Pi ratio, the value at the minimum level is not involved in such definition.

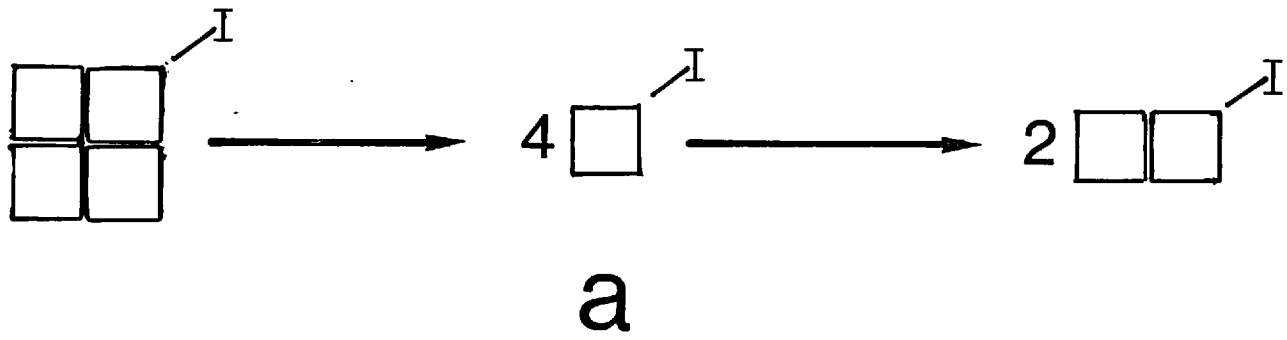
Using the S/I ratio for spectrin model

Assuming the results of the least subunit molecular weight obtained with S/Pi ratio to be correct (6.5×10^6 daltons), a model for spectrin can be constructed using S/I ratio values.

S/I value of 30 is taken as the least molecular weight of spectrin (see fig 17.4). The value of 30 is taken because it is the lowest value obtained and also because four subunits are more probable than eight for proteins.

Combining the S/Pi model for subunits with S/I model for total spectrin molecule at each time interval, a final model of spectrin is obtained.* The squares represent the basic spectrin molecule. It is composed of subunits represented by circles.

* See fig. 17.5.



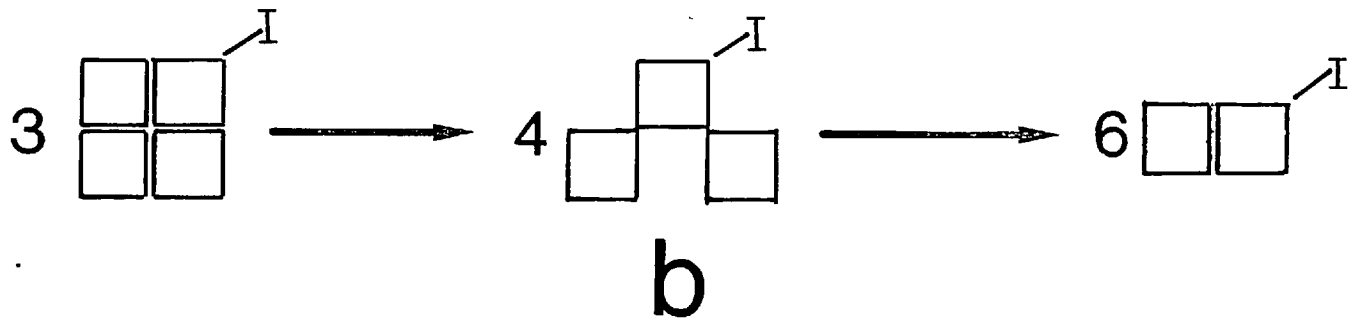


Fig 17.4 Supermolecular forms of spectrin on basis of S/I calculations. Each square represents a molecular weight of 13×10^6 daltons. (a): phosphorylated spectrin; (b): non-phosphorylated spectrin.

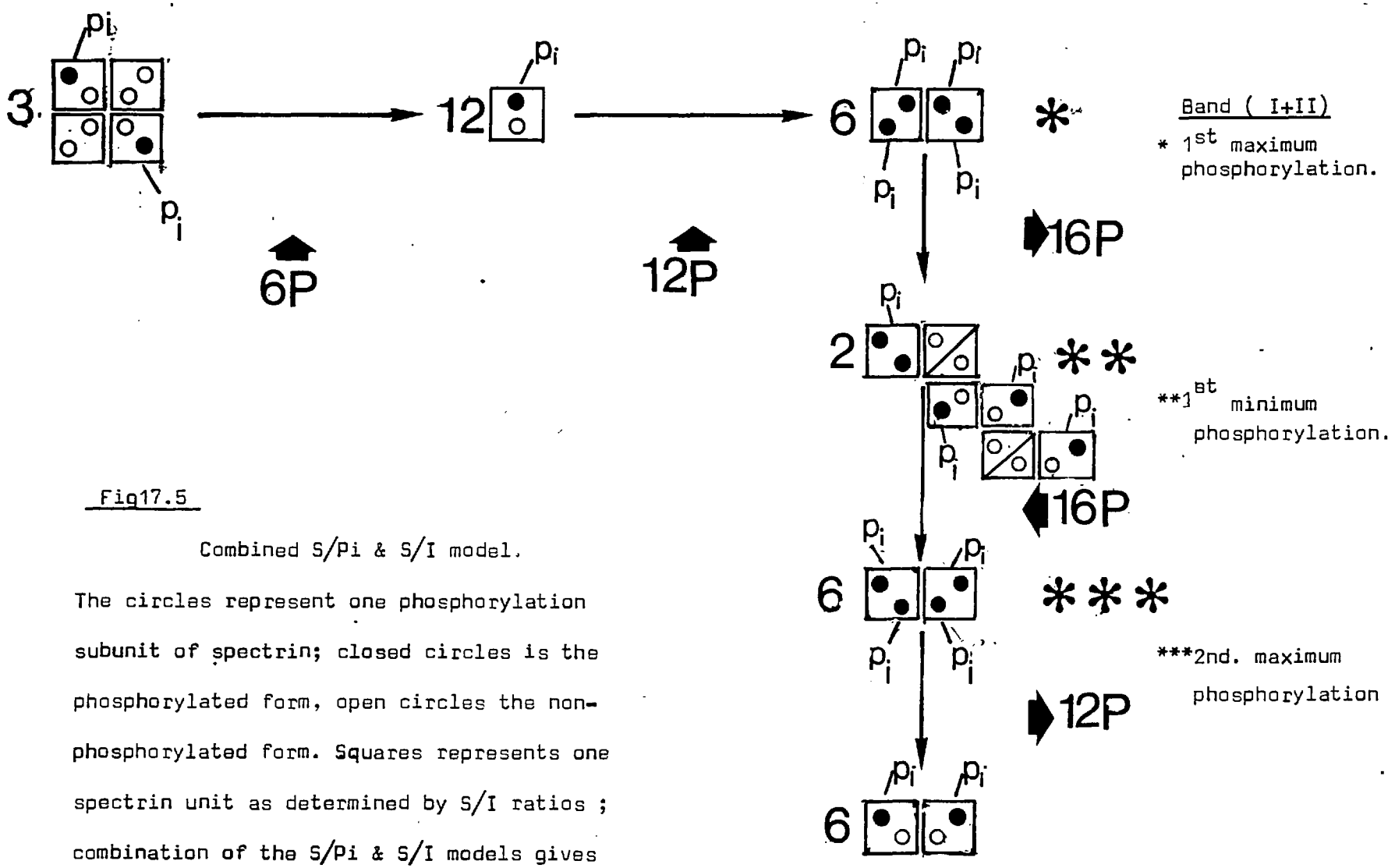


Fig17.5

Combined S/Pi & S/I model.

The circles represent one phosphorylation subunit of spectrin; closed circles is the phosphorylated form, open circles the non-phosphorylated form. Squares represents one spectrin unit as determined by S/I ratios ; combination of the S/Pi & S/I models gives supermolecular structure of spectrin as shown.

P represents inorganic phosphate , phosphoprotein or both.

Open circles indicate subunits not involved in phosphorylation. The closed circles are subunits actively involved in phospho-rylation, sharing the available Pi equally.

The final model predicts that spectrin is present in the normal red cell membrane probably as a dimer of molecular weight 26×10^6 daltons. This is composed of four subunits with molecular weight of 6.5×10^6 daltons. In the presence of ATP the subunits are able to phosphorylate, with consequent conformational changes represented as a transition from tetramer to monomer to dimer. In absence of ATP a dimer is also produced, but from a trimer, and may represent a different form of the dimer from the phosphorylated type. It is possible that the conformation of subunits change on phosphorylation. This phosphorylation may be regulated by the presences of ATP, cAMP, Ca^{2+} etc.

Spectrin may control the physical properties of the red cell membrane (e.g. shape changes, permeability changes) by modulation of its structure, induced by phosphorylation, either directly or indirectly by interacting with other membrane proteins.

A P P E N D I X

Regression Analysis

Two variable linear regressions were fitted to the following equation:

$$y = b_0 + b_1 x$$

where

$$b_1 = \frac{(x_k - \bar{x})(y_i - \bar{y})}{(x_i - \bar{x})^2}$$

$$= \frac{x_i y_i - \frac{x_i \; y_i}{n}}{x_i^2 - n\bar{x}} \qquad \text{(Gradient)}$$

$$b_0 = \bar{y} - b_1 \bar{x} \qquad \text{(Intercept)}$$

$$\bar{x} = \frac{x_i}{h}$$

$$\bar{y} = \frac{y_i}{n} \qquad \text{(Means)}$$

$$s_x = \frac{x_i^{\,2} - \frac{(\;x_i)^2}{n}}{n - 1}$$

$$s_y = \frac{y_i^{\,2} - \frac{(\;y_k)^2}{n}}{n - 1} \qquad \text{(Standard deviations)}$$

$$r = \frac{(x_i - \bar{x})(y_i - \bar{y})}{(x_i - x)^2 \; (y_i - y)^2} \qquad \text{(Regression)}$$

$$\text{Reg SS} = \frac{\left[(x_i - x)(y_i - y) \right]^2}{(x_i - x)^2} \qquad \text{(Regression sums of squares)}$$

$$\text{Total SS} = (y_1 - \bar{y})^2$$

The equation was solved with aid of Hewlett Packard 10A desk top calculator. The following table shows the values for the statistical parameters obtained for section 13 kinetics.

Statistical parameters	Normal membrane ghosts (Table 13.1)	HS membrane ghosts excluding the family (Table 13.2)	Normal extracted PK (Table 13.4, A & B)	HS extracted PK (Table 13.5 A & B)
Gradient b_1	198.0813	326.4135	799.5410	824.1886
Intercept b_0	8.0014	7.3345	39.8093	43.2657
Number of determination h	99.0	77.0	28.0	34.0
Sx	0.1255	0.1257	0.1227	0.1234
$\bar{x}$	0.1965	0.1965	0.2225	0.2160
Sy	26.4721	42.2625	103.9192	107.8449
$\bar{y}$	46.9333	71.4896	217.7071	221.3147
Reg SS	60546.5	127877.4	260042.4	341478.1
Residual SS	8129.0	7867.8	31535.9	42329.2
Total SS	68675.5	135745.2	291578.4	383807.3
Regression mean square	60546.4	127877.4	260042.4	341478.1
Residual mean square	83.8	104.9	1212.9	1322.8
F Ratio	722.4	1218.9	214.3	258.2

continued .....

Statistical parameters	Normal membrane ghosts (Table 13.1)	HS membrane ghosts excluding the family (Table 13.2)	Normal extracted PK (Table 13.4, A & B)	HS extracted PK (Table 13.5 A & B)
Standard error	9.15	10.24	34.82	36.37
Degrees of freedom for Student's "t" test	97.0	75.0	26.0	32.0
correlation, r	0.9390	0.9706	0.9444	0.9432
Upper confidence limits for b_1	210.3	341.9	892.6	911.1
Lower confidence limit for b_1	185.8	310.8	706.4	737.3
t value from Student's "t" table	1.6635	1.6655	1.7056	1.6939

Calculation of stochiometric ratio (S/R) of the number of calcium ions transported per ATP molecules hydrolysed.

A) Method used in section 8.2 using IO prep in presence of 0.2 mM Ca^{2+} in absence of EGTA buffer system; downhill transport.

Initial linear uptake	=	0.119 ± 0.013 nM/mg prot./min
Passive diffusion	=	0.009 ± 0.004 "
Net active uptake	=	0.110 ± 0.017 "
% IO vesicle in IO prep	=	41.2
Therefore actie uptake per mg IO vesicles	=	0.267 ± 0.041 "
$(Ca^{2+} + Mg^{2+})$- ATPase at 0.2mM Ca^{2+}	=	48.5 "
Therefore S/R	=	0.267 /48.5
i.e		1 Ca^{2+} transported per 181.65 ATP hydrolysed.

B) For transport against concentration gradient using 50μM Ca^{2+} levels(see Figs 8.3 & 8.4) :

Initial linear uptake	=	3.2 nM/ mg protein / min.
Correcting for % IO	=	7.767 "
$(Ca^{2+} + Mg^{2+})$-ATPase at 0.05mM	=	18.3 "
Therefore S/R	=	7.767/18.3
i.e		1 Ca^{2+} transported per 3.4 ATP hydrolysed.

Table 18.1 The S/Pi is divided by integers 15 to 26. Values to the nearest integer are calculated.

		S/Pi values divided by:																				
mins	S/Pi Normal	15	16	17	18	19	20	21	22	S/Pi H.S.	15	16	17	18	19	20	21	22	23	24	25	26
3	64	4	4	4	4	3	3	3	3	208	14	13	12	12	11	10	10	9	9	9	8	8
5	55	4	3	3	3	3	3	3	3	73	5	5	4	4	4	4	3	3	3	3	3	3
10	30	2	2	2	2	2	2	1	1	53	4	3	3	3	3	3	3	2	2	2	2	2
20	21	1	1	1	1	1	1	1	1	26	2	1	1	1	1	1	1	1	1	1	1	1
30	51	3	3	3	3	3	3	2	2	132	9	8	8	7	7	7	6	6	6	6	5	5
40	17	1	1	1	1	1	1	1	1	23	2	1	1	1	1	1	1	1	1	1	1	1
50	20	1	1	1	1	1	1	1	1	34	2	2	2	2	2	2	2	2	1	1	1	1
60	23	2	1	1	1	1	1	1	1	58	4	4	3	3	3	3	3	3	3	2	2	2

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