CHEMICALLY-INDUCED ALTERATIONS IN ERYTHROCYTE DEFORMABILITY

EFFECT OF ATP AND ALKALINE PHOSPHATASE ON

ERYTHROCYTE DEFORMABILITY

by

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ABSTRACT

The effect of altered spectrin phosphorylation on the deformability properties of human erythrocyte membranes was studied using the nucleopore filter method. The shear modulus of elasticity was found to decrease and increase as a result of the incorporation of ATP or alkaline phosphatase, respectively. Purified spectrin was used to immunize rabbits, in order to obtain anti-spectrin antibodies for future experiments.

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SECTION 1

INTRODUCTION

1.1 Introduction

Erythrocyte deformability has been under study for the last two decades. Initially the experiments were attempting to understand the biconcave shape of the red cell. Since then research has been done to examine the effect of metabolic changes in cells, and changes in pathological cells with respect to their cell deformability. The establishment of sensitive techniques to measure whole cell deformability as well as cell membrane deformability has prompted many researchers to use them in their studies of cell membrane structure and function.

It is now believed that the erythrocyte membrane has a cytoskeleton composed of protein components. This cytoskeleton has the ability to change its configuration and organisation in response to external influences. Physiologically this means that as the cell travels through the circulatory system, it can adjust with the changing environment and so maintain an optimal state of membrane integrity.

The changes in configuration of the cytoskeleton are related to the phosphorylation and polymerization properties of the protein components. These proteins have been studied in solution and on the cell membrane. However, the research has not yet yielded sufficient information to understand completely how the red cell deformability is controlled by the cyctoskeleton.

In the next section, the research to date will be reviewed in order to give the reader a better understanding of the red cell membrane

structure, its components, and its organization.

1.2 Background

1.2.1 Cell Membrane Structure

For a long time biologists had assumed that cells were bounded by a membrane that served to contain the cell contents. They explained many of the characteristics of cells in terms of properties that they attributed to this membrane. However, the membrane was not visible under light microscopes and so it was difficult to prove its existance, let alone study its structure and function.

Since lipids and lipid-soluble substances could move between cells and the surrounding medium, it was deduced that the membrane must be composed of lipids. The movement of water-soluble substances across the "lipid membrane" indicated that pores or non-lipid patches should also exist. But still another observation had to be accounted for. Small water-soluble ions did not all move freely across the membrane. This suggested that the cell membrane possesses charge and tends to attract or repel ions.

Gorter and Grendel[1] extracted the lipids from red cells and compared the area occupied by the lipids when oriented as a monomolecular film with the area of the membranes from which they were extracted. The area of the lipid monolayer was found to be twice that of the membrane area. They concluded that the red cell membrane must consist of a two-dimensional array of lipids, two molecules thick.

Gorter and Grendel were later shown to have made certain technical errors in their experiments.[2] Engleman,[3] using a different approach, determined the volume of membrane occupied by the hydrocarbon residues of phospholipid molecules and cholesterol. The final value obtained for the red cell membrane volume was too large for a membrane consisting of a monolayer of lipids yet too small for the entire surface to be in a bilayer configuration. He concluded that the 10-20% remaining hydrophobic region of the membrane must consist of non-lipid components.

Since the membrane is bounded on both sides by water solutions, it was concluded that the most likely structure for the lipids was in the form of a bilayer with the hydrophilic ends on the outside and the hydrophobic chains pointing to the centre of the bilayer.

In 1935, Danielli and his coworkers[4] measured the surface tension of cell membranes and found that it was much lower than the measured surface tension of lipid bilayers. They suggested that there was a layer of globular proteins on each side of the lipid bilayer that was responsible for this difference.

With the advent of the electron microscope, more information could be obtained about the cell membranes. Electron micrographs made by Robertson[5] showed that the cell membrane was composed of two electron-dense layers separated by a wider lighter area. The overall thickness of the membrane was measured to be close to 80 $\overset{\circ}{A}$. Due to the close agreement between the electron micrographs and the model, Robertson proposed that his model described a "unit membrane", the basic structure of all cellular membranes.
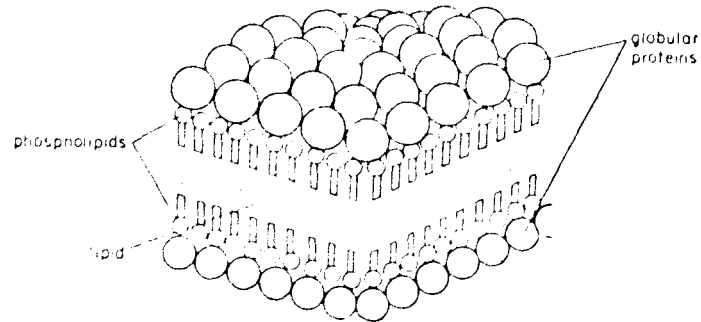
Fig. 1.2.1: The model of the molecular arrangement in cell
            membranes according to Danielli and Davison
            (copied from Quinn[6]).
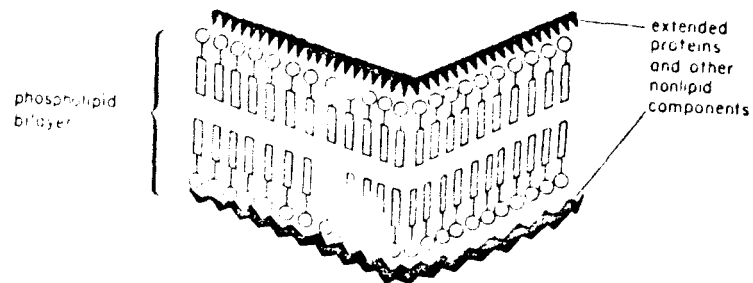

Fig. 1.2.2: The model of the molecular arrangement in cell
            membranes according to Robertson (copied from
            Quinn[6]).

The unit membrane consisted of a continuous lipid bilayer with extended proteins and other non-lipid components interacting with the hydrophilic groups of the lipids. Later researchers questioned this idea since membranes were found that had various thicknesses, and various lipid and protein contents. Also the necessity for drastic procedures to disrupt membranes and the fact that hydrophobic interactions must be weakened before all the proteins could be removed, indicated that the protein did not react only with the hydrophilic lipid groups through polar forces.

A number of alternatives to the "unit membrane" model had been suggested; that the proteins formed a matrix within the bilayer rather than coating the surface; or that the lipid was in the form of lipid globules rather than a bilayer.

The most satisfactory model was presented by Singer and Nicolson[7] in 1972. Their model arranges the lipids in a bilayer forming a fluid, liquid-crystalline matrix. This lipid matrix gives the membrane its high electrical resistance and relative impermeability and also its fluidity since the lipids were allowed lateral mobility. The proteins in the membrane were envisioned as globular due to their high α-helix content. Some were thought to penetrate the lipid bilayer from one side or the other, while others completely spanned the membrane. The proteins were also allowed lateral mobility.

Those proteins penetrating the membrane accounted for the remaining 10-20% volume of the membrane after the lipids had been accounted for. The membrane proteins would either be absorbed by predominantly polar
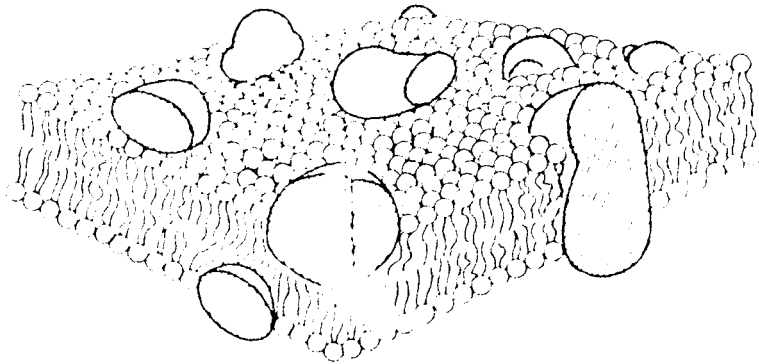
Fig. 1.2.3: The fluid mosaic model of membrane structure proposed
by Singer and Nicolson[7] (copied from Quinn[6]).

forces or be interpolated into the bilayer in direct contact with the hydro-
phobic region of the membrane. The proteins were thought to be arranged
in an amphipathic manner so that the polar groups protruded from the mem-
brane while the non-polar groups were buried in the hydrophobic region
of the membrane.

This fluid-mosaic model of the cell membrane accounts for many
of the differing properties observed. The differing protein and lipid
content amongst cell membranes explains the various thicknesses, electrical
and permeability properties found.

The fluid-mosaic model allows assymetry of the components of the
cell membranes to occur. With the exception of Golgi vesticles, the car-
bohydrate components are present on the outer surface of the cell membrane.
Labelled proteins cleaved from the outer membrane surface by proteases
are different from those cleaved from the cytoplasmic surface. The posi-
tioning of a certain component is determined by its site during synthesis
and by its polar and non-polar groups. Similarly, labelled phospholipids
show differences from one side of the membrane to the other. This assym-
etry in the membrane allows the antigens and receptor sites to be present
on the outer cell surface where their function is required. The fluid-
mosaic model allows this assymetry to occur.

1.2.2 Red Cell Membrane Proteins

Although the fluid-mosaic model describes the general cell mem-
brane, researchers have generally chosen the red cell membrane for study.
The red cell lacks a nucleus and has a relatively simple internal structure

and even more important, it is readily available.

New techniques have enabled the membrane proteins to be isolated in water-soluble form and their properties and inter-relationships studied. Those proteins that are firmly anchored in the membrane have been called intrinsic or integral membrane proteins. Those that are easily extracted are called extrinsic proteins.

Human red cell membranes contain eight major polypeptides, as shown by the technique of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The nomenclature used to describe them is based on the order of the bands of proteins in the gel as measured from the origin, as determined by Fairbanks et al.[8]

Figure 1.2.4 gives the densitometric scan of a gel stained for protein with Coomassie blue and of a gel strained for carbohydrate with PAS reagent. The numbers refer to the bands of protein, H is hemoglobin and TD is the tracking dye.

The polypeptides corresponding to bands 1 and 2 are readily solubilized at low ionic strength, form 30% of the membrane protein and have molecular weights of approximately 240,000 and 215,000 respectively. They were named spectrin by Marchesi and Steers[10] and are located on the inner surface of the membrane. Spectrin is believed to form a network or cytoskeleton with the band 5 protein (actin)[11] and so play a role in determining cell shape and deformability.

The band 3 protein is the most numerous protein in the membrane.[12] It spans the membrane and is considered to contain the anion transport channel.[12]
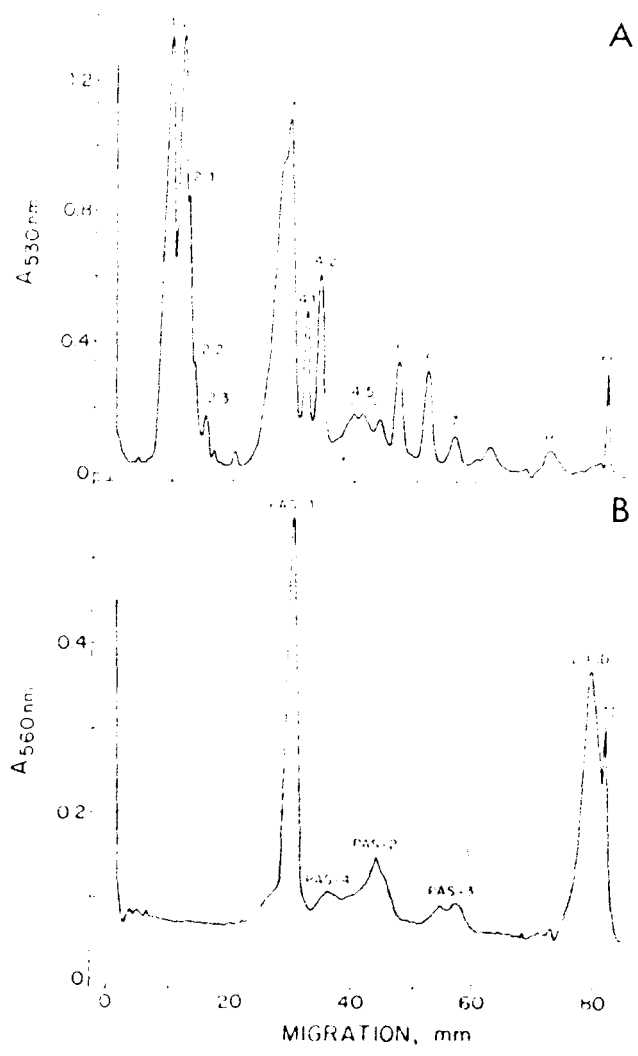
Fig. 1.2.4: Erythrocyte membrane polypeptides and glycoproteins (A).
shows a densitometric scan of a gel stained for protein
and (B) shows that of a gel stained with PAS reagent.
H stands for hemoglobin, TP for tracking dye (copied
from Steck[9]).

Band 5, called actin, has been shown to be similar to muscle actin.[13] It is positioned on the inner membrane surface,[14] is readily extracted at low ionic strength, and interacts with spectrin to possibly form a cytoskelton for the red cell membrane.[11]

Polypeptide 6 has been identified as the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase and is relatively loosely associated with the inner surface of the membrane.[15]

Staining the SDS polyacrylamide gels with the periodic acid Schiff (PAS) technique reveals more bands, the principal component being PAS 1, the major glycoprotein in the membrane.

Many other proteins are present in the erythrocyte membrane. Those described above form approximately three-quarters and so the bulk of the membrane proteins.

1.2.3   Spectrin and Red Cell Membrane Structure

Spectrin was first discovered by Marchesi and Palad[16] in 1967 when they were trying to separate ATP-ase from the membrane using tryptic digestion. The red cell membranes quickly inactivated the trypsin and fragmented. The structural breakdown of the membrane and the enzyme inactivation could be prevented by preincubation in a medium containing ATP and Mg^{++}. Ghosts, that had been protected, showed coiled filaments associated with the inner membrane surface.

Marchesi and Steers[10] in 1968 were able to extract 20% of the ghost membrane protein by dialyzing the membranes against ATP and β-mercapto-ethanol. This method was known to depolymerize and solubilize fibrous actin of muscle. Antibodies to the protein extracted reacted with red cells

and ghosts but not with serum, hemolyzate, platelets and leucocytes. They names the protein Spectrin (Latin: derived from ghosts).

Other workers[17] extracted similar protein fractions and named them Tektin. It has since become clear that these fractions consisted of spectrin.

In a subsequent study[18] the similarities between muscle actin and the proteins were examined. It was found that although the two proteins could both form filaments, and had similar solubility properties, they were very much different. Spectrin did not bind ATP or require ATP for solubilization. This led to the replacement of ATP by ethylenediaminetetraacetic acid (EDTA) to serve as a chelating agent in the extraction procedure.

Marchesi et al., in 1969[19] showed spectrin from guinea pigs to be homogeneous. Using acrylamide gel electrophoresis, spectrin was found to migrate as a single broad band and have a rather high molecular weight of 120,000 to 140,000. Electron micrographs of thin sections of red cell ghosts showed fine filaments on the cytoplasmaic surface of the membrane. Ghosts, whose spectrin had been removed fragmented into small vesisles of less than one micron in diameter which did not show these filaments. This seemed to indicate that spectrin formed a fibrous network along the inner surface of the membrane and it was suspected that spectrin plays a role in maintaining cell shape and stability.

Marchesi et al. in 1970[20] showed the protein polymerized into two major components with different sedimentation rates. The molecular weight of the single monomeric subunit was shown to be 140,000 - 150,000.

Fairbanks et al.[8] in 1971 used acrylamide gel electrophoresis

to separate the major polypeptides of the red cell membrane. Six major bands were shown to make up over two-thirds the ghost protein. Bands 1 and 2, containing 25% of the ghost protein resembled spectrin and their molecular weight was estimated at 250,000.

By 1976, it was recognized that bands 1 and 2 were indeed spectrin but the molecular weights of the polypeptide chains were not yet determined accurately.[17] It is now accepted that bands 1 and 2 have molecular weights of 250,000 and 225,000, respectively.[21] The uncertainty about the molecular weights of the bands comes from two problems. Spectrin is very sensitive to protease action and has a tendency to form aggregates. As well, SDS-PAGE is a relatively inaccurate method for determinations of molecular weight, especially high molecular weights.

The two polypeptides of spectrin differ slightly in molecular weight but this does not mean that they are similar or that band 2 is a smaller version of band 1.[21] Band 2 can be phosphorylated by enogenous protein kinases[22] and binds to the inner membrane surface,[22] whereas band 1 is not pohsphorylated to any extent and does not bind to the membrane.[23]

Maddy and Dunn[24] in 1978 prepared peptide maps of the two polypeptide chains of spectrin by chromatography followed by electrophoresis in the second dimension. A marked similarity between the peptide maps of the two polypeptides was found. Bands 1 and 2 were found to share many common peptides which suggests that they share certain common amino acid sequences. This is consistent with their similar amino acid compositions[25] and their immunological cross-reactivity. The chains are not identical however, since the maps of the polypeptides also possess peptides unique to themselves.

Anderson[25] also found that the subunits are chemically very similar and actual sequence homology is shared in some regions. A repeating structure was found to exist within band 2 at about 8,000 dalton intervals. Over 40,000 daltons of band 2 are believed to be composed of repeating sequences. Anderson suggests that the repeating structure could be the basis for multiple binding to other proteins in the red cell. This could explain the varoius types of interactions of spectrin with the membrane.

Spectrin binds to many membrane proteins, e.g. glycophorin,[26] actin,[11] band 3 and band 7,[27] band 3',[28] and band 2.1.[29] Although these various interactions are possible, it is now believed that spectrin binds predominantly to one integral membrane protein and to one extrinsic membrane protein, in vivo.

Tilney and Detmers[11] in 1975 showed that spectrin inhibits actin polymerization. However, if actin was already polymerized the addition of spectrin increased the viscosity of the solution, presumably by cross-linking the actin filaments. The addition of trypsin to erythrocyte ghosts results in filament formation. The effect of trypsin was interpreted as causing the removal of spectrin and thereby allowing the actin molecules to polymerize.

Erythrocyte actin is similar to muscle actin in its polymerization and depolymerization properties.[13] Changes in red cell membrane deformability were observed when agents known to effect the polymerization properties of muscle actin were incorporated into red cells. Using an ektacytometer, increases in cell deformability were found when a depolymerising agent

was incorporated into red cells, while decreases in cell deformability corresponded to the incorporation of a muscle actin-polymerising agent. These results support those of Tilney and Detmers, by showing that actin may also function as a membrane structural element.

Under controlled proteolysis, the spectrin-binding protein in the membrane can be cleaved so as to release the fragment to which spectrin binds. This 72,000 dalton fragment binds to spectrin in solution, inhibits the association of spectrin with inside-out vescicles and causes rapid dissociation of spectrin from vescicles.[30]

Luna et al.[31] in 1979 used two-dimensional peptide maps to show that the map of 72,000 dalton fragment is similar to maps of bands 2.1, 2.2, 2.3, and 2.6. Band 3' was very similar to band 2.1 and it is suggested that it is a proteolytic fragment of 2.1.

Bennett and Stenbuck[29] in 1979 produced antisera to the fragment and showed that spectrin binding was inhibited by it. Of the proteins mentioned above only band 2.1 reacted with the antisera. This was conclusive evidence that band 2.1 is the spectrin-binding protein and so they named Ankyrin (Greek: anchor).

Incubation of "leaky" ghosts with this fragment increases the laterial mobility of fluorescein isothiocyanate-labelled integral membrane proteins.[32] Also incorporation of anti-spectrin antibody inside ghosts gauses aggregation or clustering of the colloidal iron hydroxide binding sites of glycophorin on the outer membrane surface.[26] Conditions that promote spectrin and actin precipitation favour the clustering of particles

in partially-spectrin-depleted membranes.[33]   Haest et al.[34] showed
that the removal of spectrin enabled the phospholipids phosphatidylserine
and phosphatidylethanolamine to move from the inner to the outer lipid
layer of the membrane. Spectrin must therefore stabilize the orientation
of these lipids in the inner surface of the membrane.

These experiments show that the lateral mobility of the integral
membrane proteins and certain lipids are restricted by the interaction of
spectrin with the membrane.

Variations or alterations to the description of the red cell mem-
brane have been made to take into account all this new data. In 1974
Steck,[9] while ignoring the size,number, and function of the principal
polypeptides, illustrated their position and interactions within the mem-
brane as in Figure 1.2.5. Spectrin and actin are shown on the inner
membrane surface to constitute a self-associated network of microfilaments.
The glycoproteins are shown to be all on the outer membrane surface and
the PAS 1 and band 3 polypeptides span the membrane anisotropically. The
carbohydrate portions of these proteins are detectable only at the outer
membrane surface.

Other representations of the red cell membrane are shown in
Figures 1.2.6 to 1.2.8. Ralston's representation,[35] shown in Figure 1.2.7,
is based on his findings that spectrin appears to be a highly expanded but
basically symmetrical molecule. This differs from the other model's view
of spectrin forming microfilaments. Ralston believes that spectrin in
the membrane to be predominantly in tetramer form. Each tetramer is
joined to the next via an actin link and is attached to the bilayer by
the interaction of an integral membrane protein. This protein was now
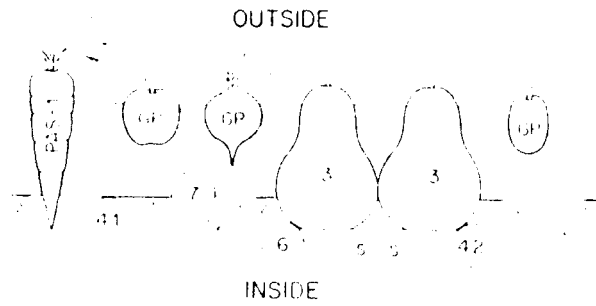
Fig. 1.2.5: A possible arrangement of the major red cell membrane
proteins. (Copied from Steck(9)).
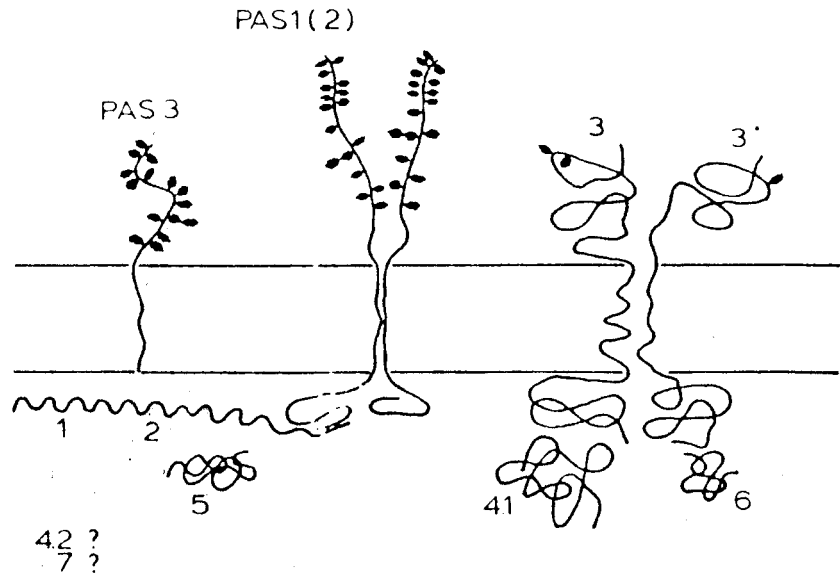

Fig. 1.2.6: A schematic representation of the red cell membrane
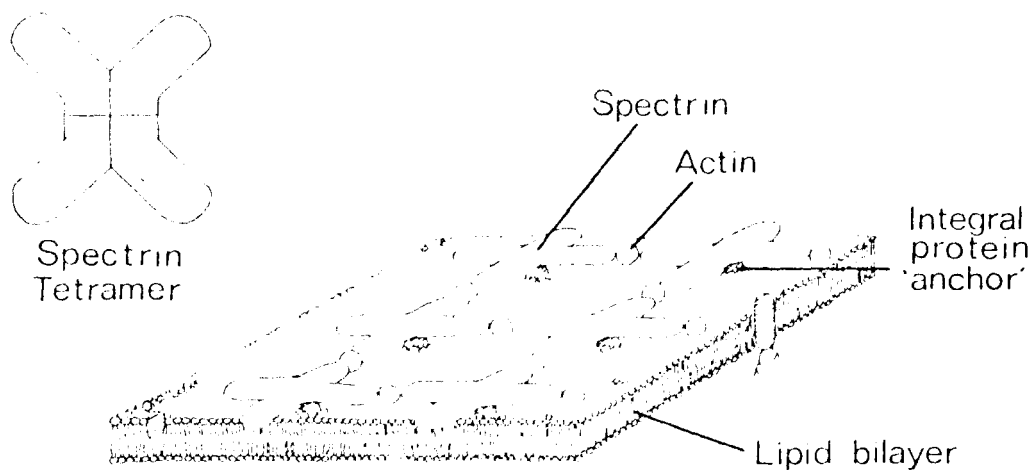(copied from Marchesi and Furthmayr(36)).

Fig. 1.2.7: A schematic representation of the red cell cytoplasmic membrane surface, illustrating possible interaction of spectrin with actin and the membrane (Copied from Ralston[35]).
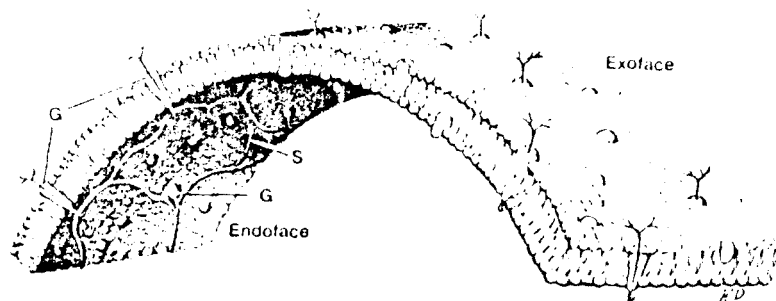

Fig. 1.2.8: A schematic representation of the red cell membrane (copied from Chien[37]).

been named ankyrin.

Recently, Shotton, Burke and Branton[38] have examined spectrin heterodimers and tetramers by electron microscopy after low angle shadowing. The spectrin heterodimers formed long flexible molecules of mean shadowed length of 97 nm. Often the two polypeptide chains were separated from one another or loosely coiled around one another. Strong bonds between them existed at the ends of the molecule.

Tetramers, formed by the association of two heterodimers, had a mean shadowed length of 194 nm. The heterodimers probably associate head-to-head, since if head-to-tail binding occurred larger oligomers should be present but these were never observed. The extended length of the spectrin heterodimer is sufficient to allow interactions with several integral membrane proteins. Whether spectrin in the membrane is present in this extended form has yet to be shown.

The various models described in the previous section all depict spectrin as part of the cytoskeleton of the red cell membrane. As such it seems quite possible that various substances present in the red cell, such as ATP, free ions, etc., might influence the structure or function of spectrin. This in turn might alter the cytoskeleton structure and change cellular properties. The next sections will deal with the role of spectrin in red cell membrane deformability and its dependence on ATP.

1.2.4 Cellular ATP and Membrane Deformability

Over the past decade many studies have been performed on metabolically-depleted cells. It was found that 24 hour incubation of red cells in serum at 37°C results in metabolic and cell shape changes. Alterations in cell membrane deformability properties have been reported by various

authors[39,40,41] however, other researchers[42,43] have demonstrated that no change in membrane deformability occurs with ATP-depletion. The work done in this field will be described in greater detail below in an attempt to understand the effect of cellular ATP-depletion in red cell deformation.

Weed and coworkers[39,44] in 1969 were the first to examine the role of ATP in red cell deformability. The metabolic state of red cells was studied during incubation in serum at $37^\circ C$ for periods up to 28 hours. It was found that the ATP levels fell during the incubation period to less than 15% and the Ca^{++} concentration increased 400%. Concomitant with these metabolic changes, the viscosity of red cell suspensions increased 500-750%, membrane deformability decreased and filterability decreased to zero. These changes were accompanied by an increase in ghost residual hemoglobin and non-hemoglobin proteins and by disc-crenated sphere transformations in cell shape. Regeneration of ATP by the addition of adenosine produced significant reversal in these changes. Addition of ATP, EDTA, or Mg^{++} to depleted ghosts could also reverse the changes. Ca^{++} addition to fresh ghosts however, mimicked the depleted state. It was suggested that these results showed that ATP and Ca^{++} determined the sol-gel balance at the interface between the insoluble cell membrane and soluble cell interior. In ATP-depleted cells or Ca^{++}-rich cells, the gel layer consisting of insoluble proteins lining the cell membrane is thickened. This has the effect of "hardening" the membrane and increasing the internal viscosity, thereby altering the cell's deformability properties.

Weed's model of ATP-modulated Ca^{++}-membrane interactions was
used by LaCelle et al.[45] in 1973 to explain the reduced deformability
of senescent red cells. In old cells, a five fold decrease in $(ATP)/(Ca^{++})$
levels was found. The ATP concentration within old cells is lowered
by 10% due to increased binding of ATP to hemoglobin, caused by decreases in
DPG concentrations with age. The resulting decrease in available ATP and
increases in Ca^{++}, as in ATP-depleted cells, was suggested to cause the
decrease in senescent cells' deformability.

Shohet and Haley[40] in 1973 incubated red cells for 24 hours and
showed that ATP levels were stable up to 8 hours and then fell to zero
by 24 hours. At 16 hours, the lysophosphatidyl choline (LPC) concentration
rose and the acylation capacity fell.   LPC is a strongly surface active
agent that is echinocytogenic. Addition of LPC to fresh red cells mimicks
the results of the 24 hour incubation. It was concluded that LPC may con-
tribute to cell shape changes in metabolically-depleted cells.

Subsequent studies of ATP-depleted cells have been contradictory.
The results of Weed were verified by Tadano et al.[41] in 1977, who used
a viscometer to study cell deformability and hemolytic response to shear
stress of ATP-depleted cells treated with various chemicals. As before
these properties were found to be inversely related to the intra-cellular
ATP concentrations.

The contradictory results were obtained by Heusikveld et al.[42]
in 1977 and Meisselman et al.[43] in 1978, who used micropipette and
flow chamber techniques for determining cell membrane deformability. In
these experiments ATP-depleted red cells deformed the same as fresh red
cells. The discrepancy between these results and those of Weed was

explained as being due to the methods of analysis and instrumentation. The increased volume/surface area ratio of ATP-depleted cells would decrease cell deformability but not necessarily membrane deformability. It was suggested that the lower deformability measurements in the earlier experiments could be due to the sphericity of the cell and not due to changes in the membrane itself.

Both Heusinkveld et al. and Meisselman et al. believed that their experiments avoided measurements of the contribution to deformability due cell shape. Thus showing that the physical properties of the cell membrane were maintained despite cellular ATP-depletion.

However, LaCelle demonstrated that sphering alone accounted for less than half the measured cellular deformability of ATP-depleted red cells[46] and that membrane deformability decreased during red cell storage before any discernable change in cell shape occurs.[47]

Feo and Mohandas[48] in 1977 put forward the hypothesis that ATP depletion was not the primary cause of cell shape changes. Using an ektacytometer to measure deformability, it was shown that red cells and echinocytes produced by ATP-depletion had identical deformability properties. Changes in deformability could only be detected after the sphero-echinocyte stage had been reached. Sphero-echinocytes produced by ATP-depletion however deformed the same as sphero-stomatocytes produced from fresh discocytes treated with chlorpromazine. This indicates that the change in deformability is due to the shape change and not the decrease in cellular ATP.

The experiments described in this section dealt with cellular ATP-depletion and they showed conflicting results regarding red cell

membrane to become more rigid,[39,40,41] while subsequent researchers demonstrated that no change in deformability occurred.[42,43,48] In comparing the various studies it became necessary to differentiate between cellular deformability and cell membrane deformability. Cellular deformability depends not only on the membrane deformability properties, but also on cell shape and the cell's internal viscosity. Evidence[48] indicates that cellular ATP-depletion causes a decrease in cellular deformability due to cell shape changes while the membrane deformability properties are unaltered.

1.2.5 Cellular ATP and Spectrin

Even though the latter experiments[42,43,48] indicated that the membrane's physical properties remain unchanged with ATP-depletion, biochemical studies showed that at the molecular level the membrane is indeed altered. Lux et al.[49] in 1978 found that spectrin extractibility decreases with ATP-depletion and correlates with the cell shape and deformability changes reported by Weed.

Spectrin extractibility was also related to pH. At low pH, where spectrin extractibility decreases and microspheres are formed. Cell deformability and shape have been reported as showing a corresponding pH dependance by Smith and LaCelle[50] and Crandall et al.,[51] and Weed and Chailley[52] respectively.

When the red cells were heated to 49°C, the temperature at which a subcomponent of purified spectrin is denatured and precipitated, similar results were obtained. A decrease in deformability under these conditions

had been reported earlier by Mohandas et al.[53] in 1976.

The decreased extractibility could be the result of increased spectrin-spectrin or spectrin-actin interactions or increased binding of spectrin to the membrane. Palek et al.[54] in 1978 showed that ATP-depleted cells spontaneously formed large spectrin-rich complexes. Using two-dimensional polyacrylamide gel electrophoresis these complexes were found to be either heterodimers of bands 1 and 2 and of bands 2 and 4.9 or a large molecular weight ($>10^6$ daltons) complex with a high spectrin to band 3 ratio.

These complexes could be dissociated with DTT and could be prevented by anaerobic incubation or maintenance of the ATP levels. It was suggested that two separate processes can occur to form these large spectrin-rich complexes in anaerobically ATP-depleted red cells. In the first process, ATP depletion alters the spectrin network so that closer contacts between the individual polypeptides are formed. A greater ease in crosslinking in this state has been shown using gluteraldehyde, where similar high molecular weight aggregates were formed.[55] The second process is the spontaneous oxidative crosslinking of the re-arranged spectrin network through intermolecular disulfide bridges.

Palek and Liu[56] suggested that the closer contacts between spectrin and the membrane proteins in ATP-depleted cells may contribute to the discocyte-echinocyte transformation. Addition of Ca^{++} into ghosts produces a non-reducible polymer of spectrin, band 3, and other proteins, whose formation stabilizes the speroechinocytic shape.

In studying ATP-depleted red cells, Lutz et al.[57] have possibly

found a process that mimicks in vivo aging of red cells. They found that
ATP-depleted cells release spectrin free vescicles. The process is inhibited
by maintaining the ATP levels within the cells. The vescicles contain
the same proportion of the integral membrane proteins as intact cells;
however, a two-fold increase in the concentration of the enzyme acetylchol-
inesterase was found.

Increased spectrin and decreased acetylcholinesterase concentra-
tions are found in aged cells. It was concluded that regions of the
membrane that lack extrinsic proteins and are enriched in acetylcholinest-
erase are selectively released by red cells aged in vitro and this is
part of the physiological aging process.

1.2.6 Phosphorylation and Dephosphorylation of Spectrin

The experiments discussed up to this point dealt with ATP-depleted
cells. In order to understand the role of spectrin and ATP in cell deform-
ability it is necessary to examine the effects of phosphorylation and
dephosphorylation of the spectrin in the membrane.

Red cell membrane proteins can be phosphorylated by endogenous
protein kinase.[22] More than half of [^{32}P] incorporated into ghosts is
taken up by band 2; the rest is distributed amongst the other membrane
proteins. When intact red cells are incubated in inorganic [^{32}P] phosphate,
polyacrylamide gel electrophoresis shows a similar pattern of phosphory-
lation to that of ghost membranes incubated with [γ-^{32}P] ATP.[58] This
indicates that ghost cells can be used as a model for membrane phosphoryla-
tion in intact cells.

The labelling of band 2 with $[\gamma-^{32}P]$-ATP is favoured at longer times and higher Mg^{++} or ATP concentrations. Monovalent ions and cAMP increased the incorporation of labelled phosphate from $[\gamma-^{32}P]$-ATP whereas Ca^{++} acts as an inhibitor.[17]

Dunbar and Ralston[59] examined the incorporation of $[^{32}P]$ into spectrin and spectrin aggregates. Covalent incorporation occured in all while non-covalent incorporation occured only in a higher aggregates of spectrin, that also contained actin and three other polypeptides. This non-covalent incorporation was shown to be due to the association of ATP with the higher aggregate. Chromatography of the aggregate led to the discovery that the ATP molecule was bound to an actin molecule. A constant stoichiometry between spectrin and actin was indicated since the molar ratio between spectrin and actin in the aggregate was the same as that found in the membrane; 5:1. This experiment leads to the conclusion that spectrin and actin are cross-linked in the membrane and supports the view of Tilney and Detmers.[11]

Pinder et al.[60] have shown that phosphorylation of the spectrin changes the spectrin-actin interaction without causing polymerization. A new type of intermolecular contact is established in the cytoskeleton that allows extensive cross-linking to occur. Figure 1.2.9 gives the schematic representation of the structure of the cytoskeleton and the effect of phosphorylation as proposed by Pinder et al.[60]

Cohen and coworkers[14,61] found results that contradict those of Pinder. They found that pure spectrin, phosphorylated or dephosphorylated, did not affect actin polymerization in solution or actin binding
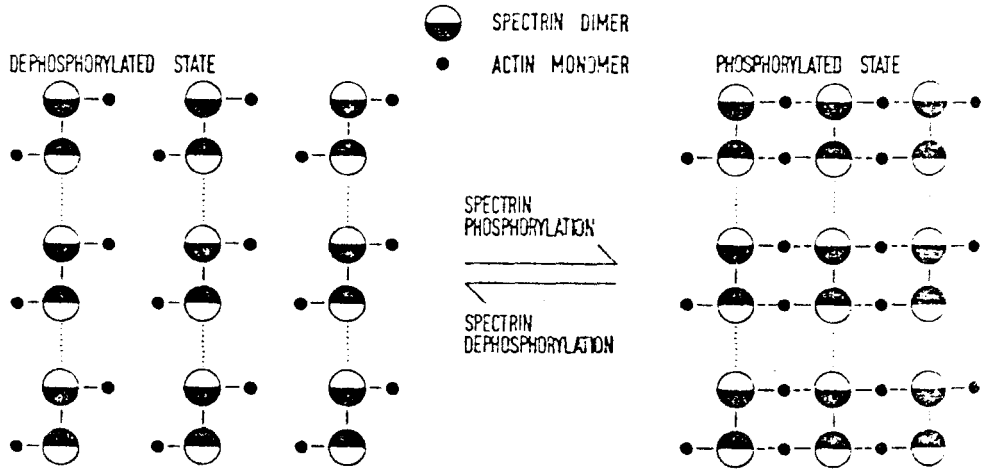
Fig. 1.2.9: A schematic representation of a possible mechanism for the action of phosphorylation on the structure of the spectrin-actin matrix (copied from Pinder et al.[(60)]).

to spectrin-actin-depleted inside-out vescicles. A higher molecular weight

aggregate of spectrin that contains actin, that is present in crude

spectrin extracts, did however stimulate actin polymerization and bind-

ing. It is possible that polyermization is nucleated by the aggregate

in solution or on the membrane.

Fairbanks et al.[62] have studied the mechanisms of phosphory-

lation and dephosphorylation properties of spectrin in the membrane.

Figure 1.2.10 presents a model that shows how spectrin phosphorylation

might affect the cellular properties. The equilibrium between phosphory-

lated and dephosphorylated forms of spectrin is regulated not only by

protein kinase and phosphoprotein phosphatase; it is possible that

1) a direct feedback mechanism exists from the cytoskeleton, 2) the cellu-

lar environment, causing for example deformation of the cell or chemical

signals from the medium, might regulate the equilibrium, and 3) the inter-

nal state of the cell might regulate the equilibrium of the spectrin

states.

Their experiments showed that the spectrin band 2 phosphorylation

reaction in the membrane resembles the cAMP-independent salt-stimulated

case in kinase phosphorylation reaction. Dephosphorylation of isolated

membranes occurs at the rate of 50% per hour whereas the rate of phosphory-

lation is 20 times greater.

It is shown that spectrin-actin lattice of the red cell may be

rearranged while passing through the capillaries. Changes in the levels

of free ATP, 2,3-DPG, and Mg^{++} on the venous side of the circulation,
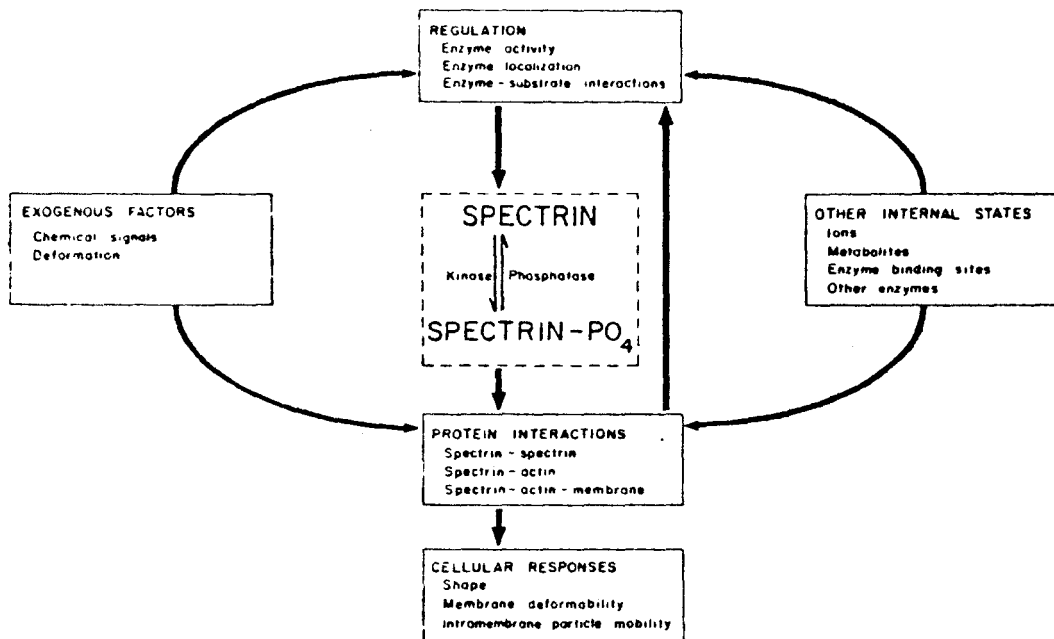
1.2.10: A heuristic model of the spectrin phosphorylation-
         dephosphorylation system, illustrating the variety of
         factors that affect the state of spectrin. (Copied from
         Fairbanks(62)).

can activate both spectrin kinase and phosphospectrin phospatase. This could cause a rearranging of the cytoskeleton. Fairbanks et al. suggest this may be the mechanism by which the red cell maintains an optimal state of membrane integrity and deformability.

Since the protein kinase and phosphatase, and spectrin are all semi-immobilized in the membrane, regulation of their interactions must necessarily be complex. It is suggested that physical stress on the membrane may alter the inter-molecular distances to either promote or inhibit the phosphorylation reaction. This model provides the spectrin-phosphorylation system to sense membrane deformation and adjust the cyctoskeleton to the optimal state.

This view is supported by the experiments of Chien et al.[63] who found that deformation of red cells by the micropipette method consisted of an initial rapid phase and a later slow phase. During the rapid phase the membrane viscosity varied inversely with the degree of deformation while no such correlation occured during the slow phase. It was postulated that for high rates of deformation the cell membrane is transformed to a "loaded configuration" with lower membrane surface viscosity. Under low deformation rates or after prolonged deformation, the membrane viscosity is higher and can be assumed to be similar to that for the membrane at rest. Thus it was concluded that the membrane can undergo molecular rearrangement depending on the extent and duration of deformation and so affect the rehological properties of the membrane.

Sheetz and Singer[64] have examined the effect of ATP on the shape of ghost cells. Crenated ATP-depleted cells were converted to disc

shape then cup shape by the addition of Mg-ATP. Anti-spectrin antibodies, present in amounts less than equivalent to spectrin, accelerated the effects of the Mg-ATP.

In a subsequent paper, Birchmeier and Singer[65] examined the biochemical effects of ATP on ghost cells that underwent the shape changes described above. They showed that of all the membrane proteins, band 2 is the most significantly phosphorylated under the conditions where cell shape changes areproduced. This phosphorylation occurs at a single site on the molecule according to cyanogen bromide peptide cleavage experiments. This site is phosphorylated by the action of a membrane-bound protein kinase, using ATP as the substrate.

The presence of a membrane-bound protein phosphatase was also shown using $[^{32}P]$Na phosphate pre-labelled ghosts. When incubated under shape change conditions without ATP, these ghosts showed a significant loss of the label from band 2 within 30 minutes. The addition of ATP would inhibit the loss of label. It was then shown that the shape of the red cell ghosts was directly related to the degree of phosphorylation of the band 2 components of spectrin.

Red cell shape changes did not occur provided the degree of phosphorylation was low, i.e. if the ATP concentration was less than 0.2 mM. Exogenously added phosphatase inhibited the shape changes that would occur with ATP provided the concentration of phosphatase was high enough.

In another experiment, crenated ghosts were made with the use of exogenously added alkaline phosphatase. Chlorpormazine, which would

normally convert the crenated ghosts to disk shape when added, had no
effect. If however the alkaline phosphatase holoenzyme, in the presence
of its inhibitor P_i or the inactive apoenzyme was used, then the addition
of chlorpromazine caused the crenated ghosts to convert to the disk shape.
Thus the persistance of the crenated shape with the active alkaline phosph-
atase and the added chlorpormazine was a specific result of a dephosphyrla-
tion reaction by the alkaline phosphatase.

The bilayer couple hypothesis[66] was used to explain the shape
changes. According to the hypothesis, the two lipid layers of the membrane
can respond differently to a perturbation of the membrane. An increase
or decrease in the surface area of one layer relative to the other, would
affect the shape of the cell. For example, a decrease in the surface
area of the inner layer relative to the outer layer would cause crenations
to form in the membrane.

Birchmeier and Singer hypothesized that the dephosphorylation
of the spectrin band 2 produces a depolymerization of the spectrin complex.
The depolymerization in turn causes a decrease in the surface area of the
inner layer of the membrane relative to the outer, and the membrane cren-
ates. Conversely, phosphorylation of the membrane would involve the poly-
merization of the spectrin complex, and increase in the surface area of
the inner layer relative to the outer and cupping of the membrane occurs.

Nakashima and Beutler[13] examined the effect of MgATP, exogenous
alkaline phosphatase and anti-spectrin antibody on the deformability of
resealed red cells. Using an ektacytometer they found that MgATP incorpor-
ated red cells were more deformable while the antibody and alkaline phospa-
tase additions decreased the red cell deformability.

Using the bilayer couple hypothesis these results seem confusing at first. The addition of ATP to fresh cells should cause the spectrin network to polyermize. The deformability of the cell under shear stress will be limited by the attachment of the spectrin network to the integral proteins of the membrane. When MgATP caused the cells to become more deformable, then the number of these attachment sites must decrease. Thus the effect of ATP is to polymerize the spectrin network and remove it from the membrane.

Alkaline phosphatase, on the other hand, causes deploymerization of the network and allows it to become better anchored to the integral proteins. This explains the decrease in deformability after incorpora- tion of alkaline phosphatase and the decreases in spectrin extractibility with ATP-depletion.

The anti-spectrin antibody, on the other hand, would cross-link spectrin and limit its movement. This would reduce the elasticity of the network. The interactions of the thus crosslinked network with the membrane is unaltered.[13] Thus the restrictions placed on the spectrin lattice by the antibody result in a decrease in cell deformability under the action of a shear stress.

The results of those experiments that showed ATP-depletion to have no effect on cell deformability,[42,43,48] are still compatible with the hypotheses of Birchmeier and Singer.[65] Those studies showed that the lack of cellular ATP does not change cell shape or deformability, whereas Birchmeier and Singer have shown that changes do occur when the cell membrane is dephosphorylated. Cellular ATP-depletion may not modify

the integrity of the membrane, so ATP depletion without spectrin dephosphory-lation would have no effect on the membrane properties.

It is hoped that researchers can, in the next few years, eluci-date the role of ATP and spectrin in red cell deformability. A better understanding of the normal erythrocytes deformability properties would have clinical applications and greatly aid research of abnormal red blood cells.

1.3 Project Aims

In this project the deformability properties of erythrocyte membranes and red cells incorporated with ATP and alkaline phosphatase will be studied. This will involve establishing the incorporation tech-nique and the application of the filter technique for the deformability studies.

Also methods will be learned to extract and purify spectrin from red cells. The purified protein will be used for immunizing rabits, for the production of anti-spectrin antibodies. These antibodies will be stored for later purification and incorporation into red cells.

SECTION 2

MATERIALS AND METHODS

2.1 Spectrin Extraction

Blood was obtained from the donor under sterile conditions and
mixed with ACD (85 mM Na_3 citrate, 65 mM citric acid, 2% dextrose). It
was washed three times with TBS (10 mM Tris, 150 mM NaCl, pH 8.0) at
1000 g (2500 r.p.m.) in a Sorval RC2-B centrifuge for 10 minutes at 4°C.
The buffy coat was aspirated after each centrifugation. This will have
removed all white cells, platelets, and plasma. A certain amount of
red cells must be sacrificed to ensure this. In most cases twice washed
red cells were obtained from Dr. Haslam's laboratory and these were subse-
quently washed two more times at least.

One milliltre of the packed red cells was mixed thoroughly by
inversion into 40 ml of ice cold 10T8 (10 mMTris, pH 8.0) in a capped
centrifuge tube. Within seconds, the suspension clarified, signaling
lysis. This was centrifuged at 30,000g (15,000 r.p.m.) for 10 minutes
at 4°C. The clear red supernatant was aspirated off carefully so as not
to disturb the pellet. The tube was then tilted to allow the ghosts
to slide away from the creamy white button. The button, rich in leuko-
cyte proteases, could then be removed by aspiration. The wash was repeated
two more times until the ghosts appeared creamy white.

The ghost pellet was resuspended in 9 volumes of 1 mM Tris, 1mM
dithiothreitol (DTT), 5 mM ethylenediamine tetracetric acid (EDTA), pH 8.0

-34-

and incubated for 30 minutes to produce vescicles. According to Steck[16] the "spontaneous communition of ghosts to small vesicles, perhaps one-third of which are inside out and sealed, ... seems to be related to the prompt and extensive release of spectrin at this temperature".

The resulting suspension was homogenized and centrigued at either 30,000 g for 20 minutes or at 41,000g for 10 minutes. The supernatant was carefully aspirated off so as not to disturb the vescicles and was centrifuged again. The final supernatant should be rich in spectrin.

2.2 SDS-Polyacrylamide Gel Electrophoresis

2.2.1 Gel Preparation

Components:

    I Buffer: 0.4 M Sodium phosphate buffer, pH 7.2 containing 0.4% SDS and 20% glycerel (40 ml of 1 M phosphate buffer pH7.2 and 0.4 g SDS and 20 ml glycerol to 100 ml, check pH).

    II 24% acrylamide, 0.64% N, N'-methylene-bis-acrylamide (24 g acryl and 0.64 g bisacryl per 100 ml) CAUTION: NEUROTOXINS.

    III 0.24% (v/v) N,N,N',N', - tetramethylethylenediamine (TEMED) (50 μl of TEMED to 20.8 ml with H_2O)

    IV 0.18% (w/v) ammonium persulfate (AMP) (0.036 g AMP per 20 ml).

Equal volumes of components I,II,III, and IV are mixed. Before adding IV, the mixture of I, II, and III is degassed and then IV added and gently mixed. Transfer the mixture to clean dry gel tubes. The tubes should be marked 1 cm and 8 cm from the bottom. They should be filled

∿3 mm above the higher mark. When the gel sets, it will shrink slightly and so should reach the mark. The gels should immediately be covered with a small layer of water. This must be added carefully so as not to promote mixing. This will ensure that the upper gel surface will be flat, i.e. no meniscus will be formed when the gels have set. The gels can be stored provided a layer of water is present above them to prevent them from drying out.

2.2.2 Electrophoresis

One litre of Electrolyte buffer is needed. The stock buffer (10X) is 1M phosphatase buffer pH 7.2 containing 1% SDS. It is made up by dissolving 141.96 g of Na_2HPO_4 in 1 litre of water, titrate to pH 7.2 with 1M H_3PO_4 (6.8 ml concentrated H_3PO_4 diluted to 100 ml with water - Note: add the acid to the water). To 1 litre of phosphate buffer add 10 g SDS.

700 ml of the electrolyte buffer is put in the lower reservoir of the electrophoresis tank. The gel tubes are inserted in the upper portion of the tank and the water above the gel is rinsed and replaced with electrolyte buffer. The sample is gently layered above the gel and below the electrolyte buffer either with a syringe or a λ-pipette.

The sample contains 10 μl sample buffer (11% SDS with 4mM disodiumethlyendiaminetetracate (Na_2EDTA)and 6 mM β-mercaptoethanol (β ME) (2.2 g SDS and 0.037 g EDTA and 8.4 μl ME to 20 ml with water), 10 μl 50% sucrose (w/v), 2.5 μl Pyronin Y (1 mg/ml) and 100 μl of sample.

When the sample has been layered on, the remaining electrolyte buffer is used to fill the upper reservior. The gels are run at constant voltage and 7 mA per gel. Initially, until all the sample has entered the gel, the current is 1/2 the final. When the Pyr Y tracking dye has reached the lower mark on the gel tubes the electrophoresis is complete.

The gels are removed from the tubes by injecting water with a syringe needle connected to a faucet. The water loosens the gel from the tube and allows it to slide out. The gels are placed in test tubes and stained overnight with 0.1% Amido Black in 7.5% (v/v) acetic acid. They are then destained with 7.5% acetic acid. The gels can then be scanned using a Gilford Spectrophotometer (Dr. Chui's laboratory) and a Linear Transport mechanism (Dr. Chan's laboratory) at 600 nm.

2.3 Protein Assay

The procedure for the protein assay was taken after the methods of Sutherland et al.[67] Bovine serum albumen is used as the standard at final concentrations of .2, .4, and .6 mg/ml for comparison with the sample. To 250 µl of the protein solution is added 2.5 ml of a solution consisting of three parts, as follows:

I   100 parts 3% sodium carbonate in 0.1 N sodium hydroxide
     (30 g Na_2CO_3 and 4 g NaOH per litre)

II   1 part 4% sodium tartrate (4 g Natartrate per 100 ml)

III 1 part 2% copper sulphate (2 g $CuSO_45H_2O$ per 100 ml)

This solution must be made fresh daily.

The mixture is then incubated for 10 minutes, and 250 µl of 0.5 N Phenal Reagent (Folin-Ciocaltean) is added. After a further 10 minute incubation the absorption is read on a Bausch & Lomb Spectronic 700 spectrophotometer (Dr. M. Rathbone's laboratory) at 660 nm.

2.4 Spectrin Purification

The crude spectrin extract obtained in Section 2.1 was purified using Sephadex G200. A column, 2.5 cm inside diameter and 90 cm high from Fischer and Porter Company was used. 15-20 g of Sephadex G200 was slowly added to 1 litre of buffer (50 mM Tris, 0.3 M NaCl, 1 mM EDTA, 3 mM DTT). The sephadex slurry was placed in a 37°C water bath and allowed to swell overnight.

The column was then filled approximately one quarter full of buffer and the air bubbles were removed from the outlet tubing. The column was filled with the slurry and the outlet opened to allow for very slow flow of buffer. The pressure head never exceeded 10% of the height of the column bed.

When the sephadex had settled the buffer above was aspirated off and more slurry added until the column bed reached the desired height.

The column was set up as shown in Figure 2.4.1 and 1 litre of buffer was allowed to run through. This was to ensure proper packing of the column had occurred.

To add the sample of crude spectrin, the buffer reservoir is removed and the buffer above the column bed was allowed to run into the
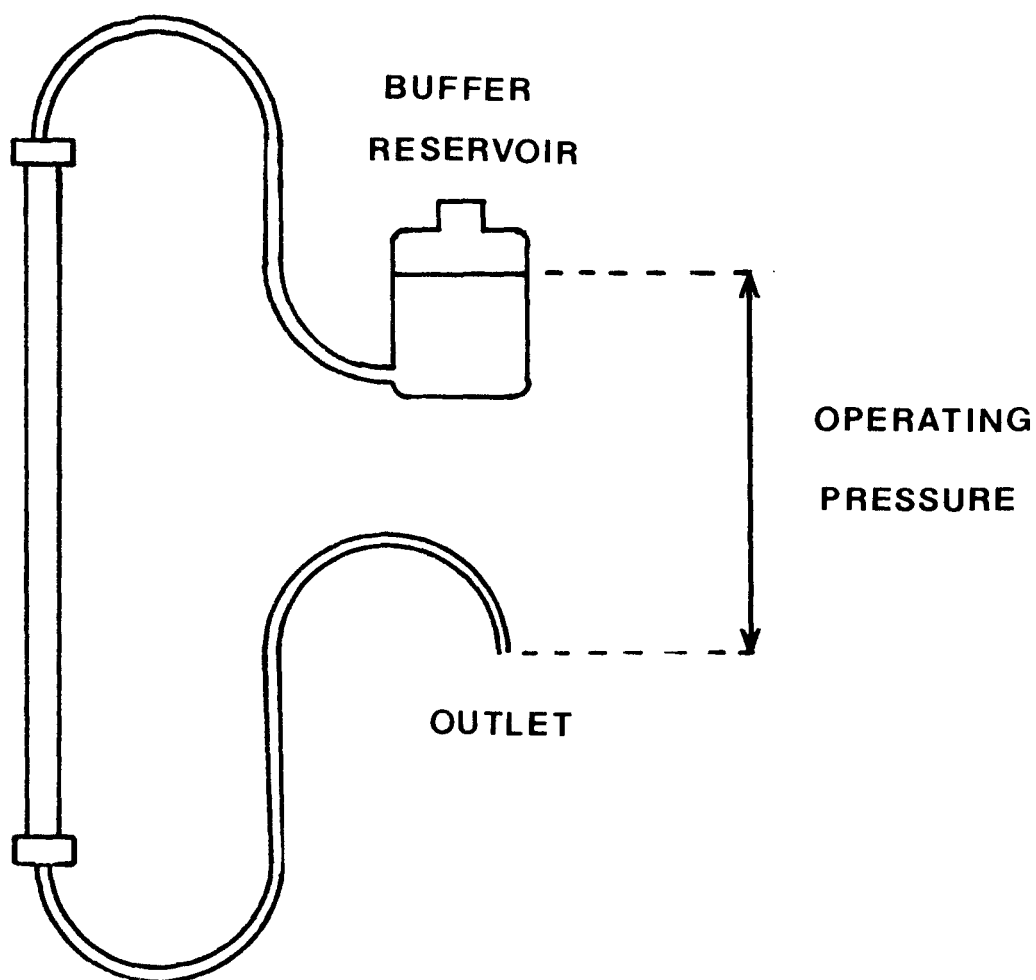
Fig. 2.4.1: The column used for gel filtration was mounted vertically
and fitted with tubing from the buffer reservoir containing
the eluant. The operating pressure is the difference between
the height of the eluant and the outlet tubing.

column bed and allowed to run in. A small amount of buffer was added next and run into the column to ensure that the sample was properly in the column bed.

The buffer reservoir was replaced and 5-10 ml fractions were collected from the outlet tubing. The fractions were covered and stored at 4°C. Using a Gilford Spectrophotometer the optical density of the fractions was measured at 260 and 280 nm. Figures 2.4.2 and 2.4.3 give the plots of optical density versus fraction number. The peak corresponds to purified spectrin.

Electrophoresis of the purified spectrin gives a gel scan such as that shown in Figure 2.4.4 and Figure 2.4.5 shows a gel scan of spectrin extract. Some of the polyacrylamide gels show not only bands 1 and 2 but a band of higher molecular weight corresponding to the heterodimer. In a few cases a faint band of still higher molecular weight was seen. This has been found to be an aggregate of spectrin and actin.[61]

2.5 Antibody Preparation

Two New Zealand white rabbits were each injected with 2 ml of a solution containing 1 ml of Freund's adjuvant. They were injected at five areas of the body; the thighs, the forelegs, and the nape of the neck. Subsequent injections of the spectrin solution only occurred at four week intervals.

The rabbits were bled 30 ml from the ear one week after the injection. The blood was allowed to clot. The clot was gently removed from the sides of the test tube. The tubes of blood were then incubated at
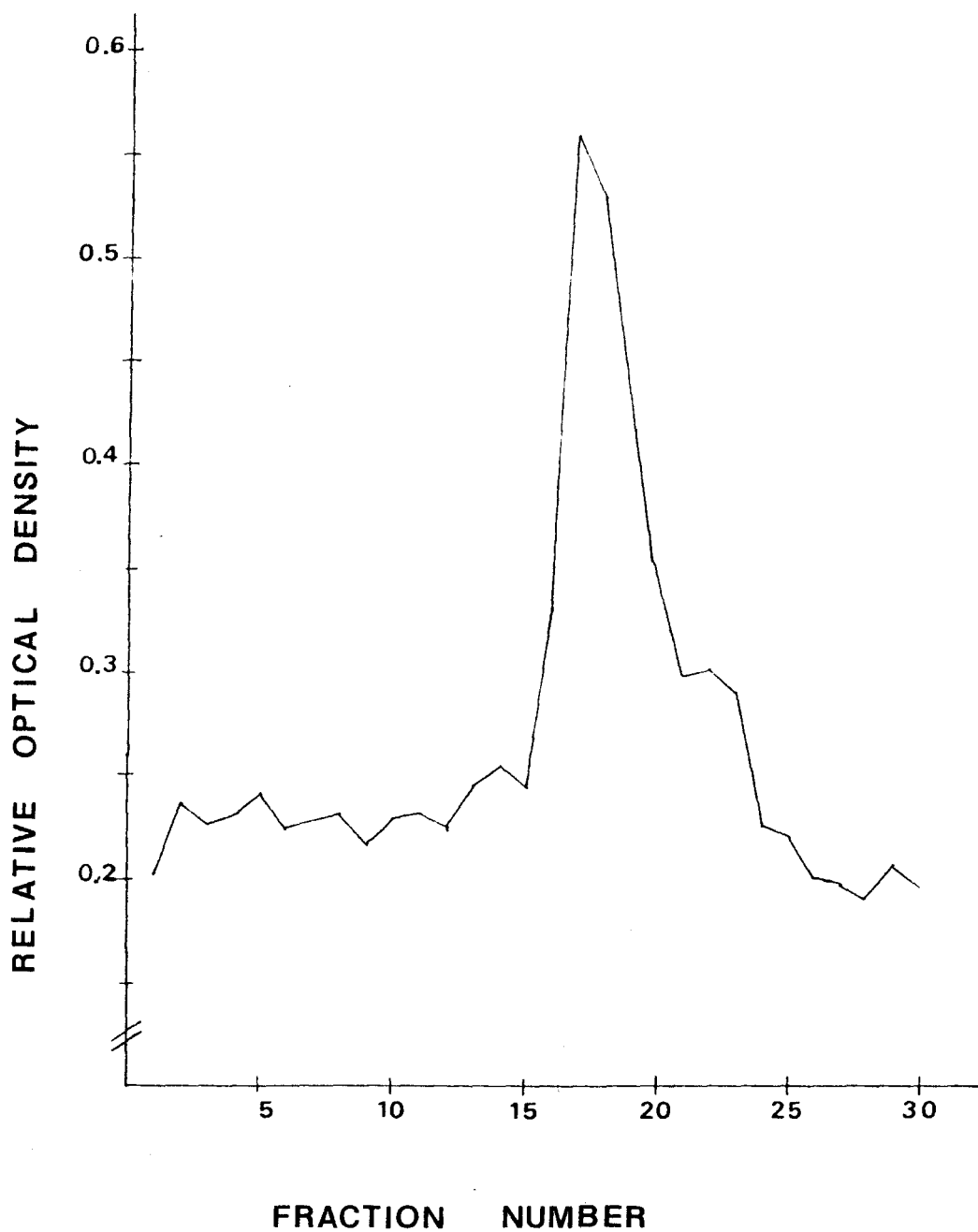
Fig. 2.4.2: A plot of relative optical density at 280 nm versus fraction number. Purified spectrin is present in fractions 16-19.
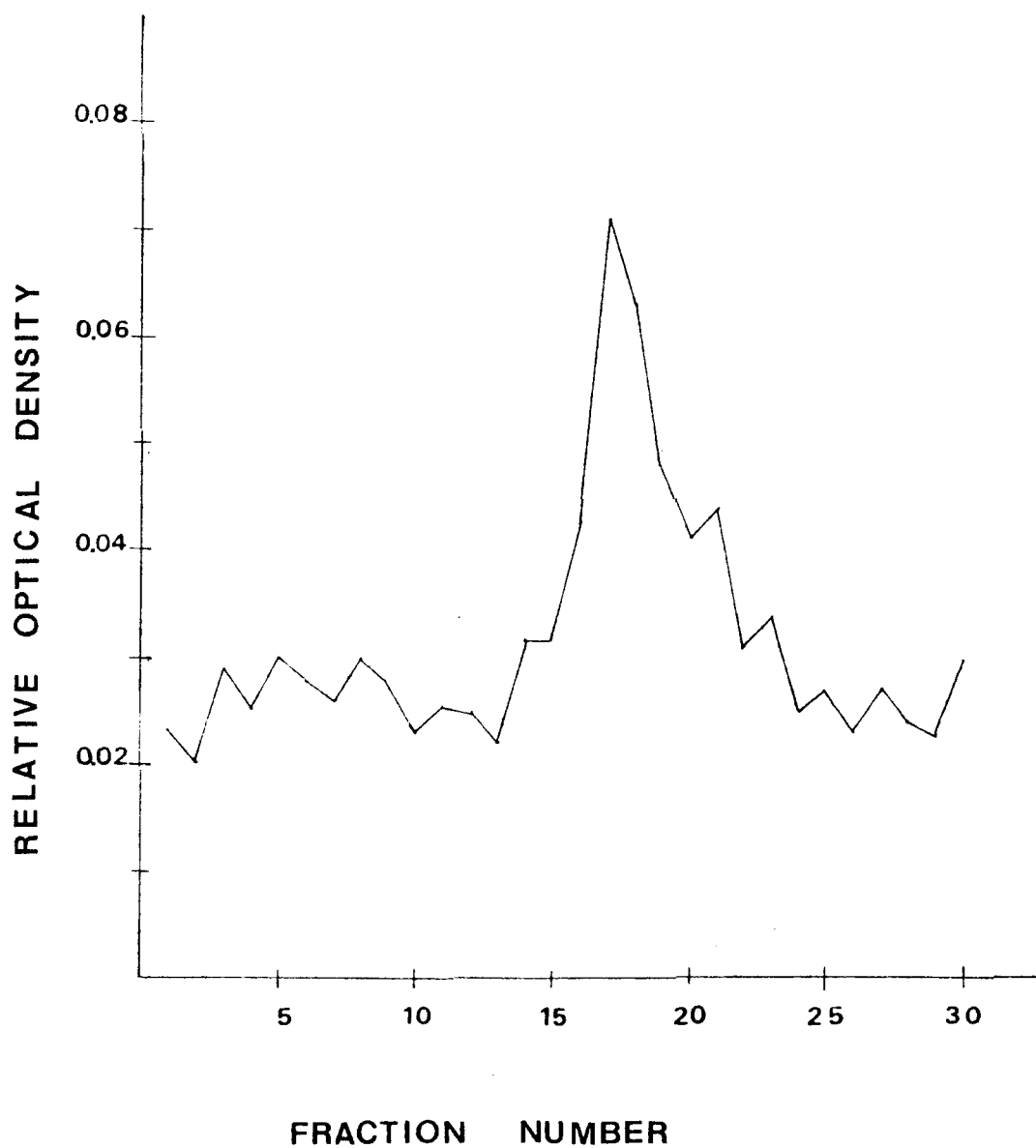
Fig. 2.4.3: A plot of relative optical density at 260 nm versus
fraction number. Purified spectrin is presented
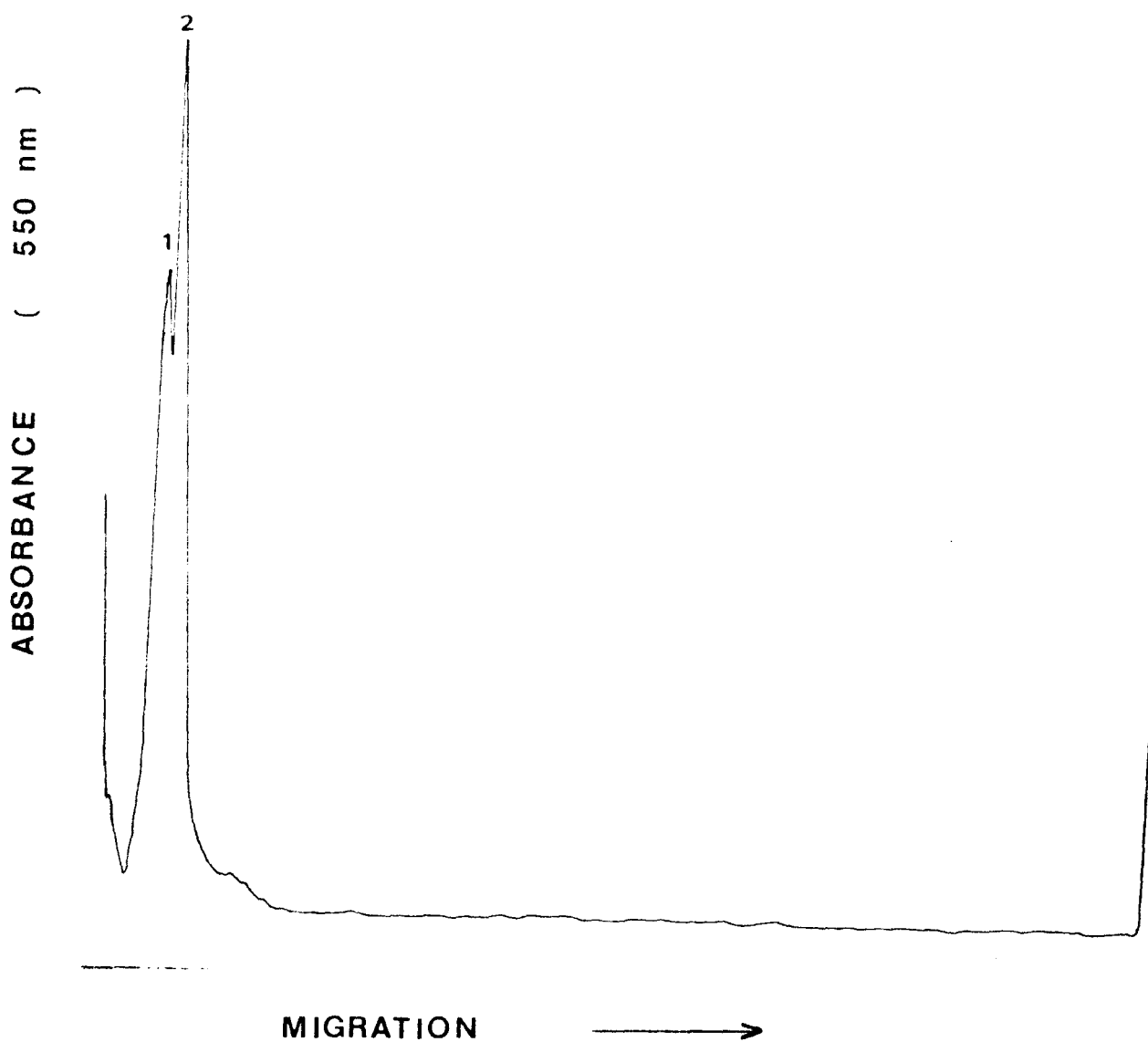in fractions 16-19.

Fig. 2.2.4: A densitometric scan of a gel containing purified spectrin stained for protein. The direction of migration of the sample through the gel is indicated on the abscissa. The actual gel length is 7 cm.
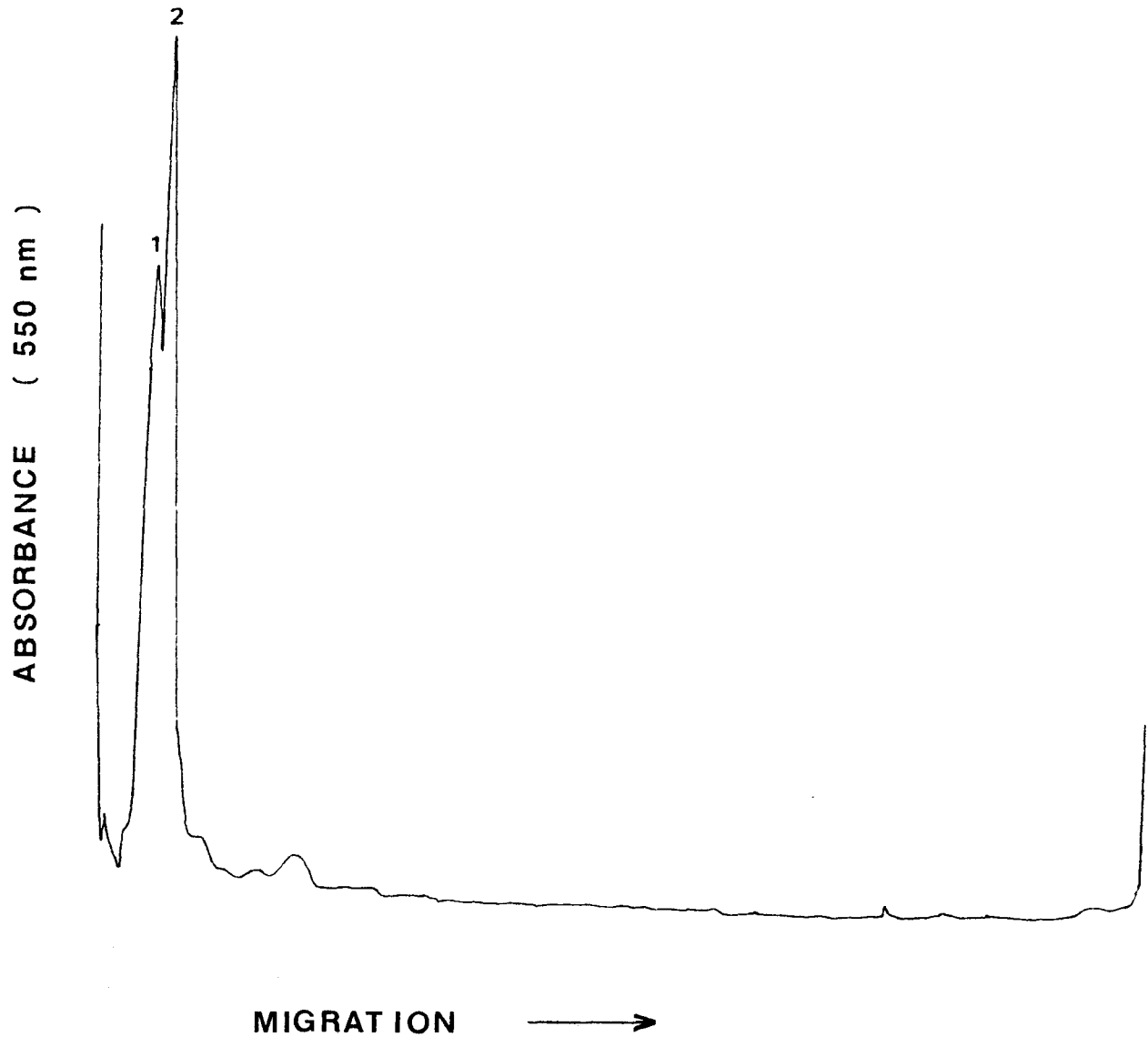
Fig. 2.2.5: A densitometric scan of a gel containing spectrin extract strained for protein. The direction of migration of the sample through the gel is indicated on the abscissa. The actual gel length is 7 cm.

37°C for 30 minutes, then at 40°C for 4-5 hours, to allow the clot to retract. The tubes were centrifuged for 10 minutes at 500 xg at 4°C. The serum was pipetted off and centrifuged again at 1000 xg. The serum was again pipetted off and stored frozen.

To test the serum for the presence of anti-spectrin antibodies an Ouchterlony (Immunodiffusion) Test may be performed. One ml of heated 1% agar in saline is allowed to form a thin gel on a microscope slide. Three wells are punched in the gel. One well is filled with the purified spectrin solution used for the injections while the other two wells are filled with serum from each rabbit. The slide was kept in a closed wet container for 1-2 days. In this time the solutions in the wells will diffuse through the agar and precipitant lines should form if antibody is present.

In order to see the precipitant lines better, the slide may be stained. First the agar must be washed free of protein by placing the slide in distilled water for 2 days. When it is lifted out care must be taken since the agar may be separated from the slide.

The agar and slide are then covered with lens paper and allowed to dry overnight. The lens paper is removed and the slide can now be stained in 1% amido black in 7.5% acetic acid for 10 minutes and destined in 7.5% acetic acid. The slide is then rinsed with distilled water and allowed to dry.

2.6 Ghost Preparation

Resealed biconcave erythrocyte ghost cells were made using two methods outlined by Steck.[68]

TBS Method

One ml of washed red cells is mixed thoroughly by inversion in to 40 ml ice-cold 10T8 in a capped centrifuge tube. The cells are centrifuged at 15,000 rpm (30,000 x g) for 10 minutes at 4°C and the supernatant removed. The cells are resuspended in 40 ml of TBS prewarmed to 37°C. The suspension is incubated at 37°C for 60 minutes and centrifuged. The sealed ghosts are washed twice more in chilled TBS and stored at 4°C in TBS.

Mg^{++} Method

0.5 ml of washed red cells is mixed thoroughly by inversion into 40 ml of ice-cold 10 T8 with 1 mM $MgSO_4$ added. This causes immediate resealing to the ghosts, trapping hemoglobin in a roughly inverse proportion to the degree of dilution. The cells are then washed three times in 40 ml of 10T8 - 1 mM $MgSo_4$ and stored in the presence of 1 mM $MgSO_4$ at 4°C.

2.7 Incorporation Into Erythrocytes

8 mls of washed red cells and 2 mls of saline containing the material to be incorporated are placed into a dialysis tubing. The bag is filled so that an air bubble represents 10-30% of the internal volume. The dialysis bag is placed in a graduated cylinder containing 20-100 volumes

of the lysing buffer, 5 mM K_2HPO_4/KH_2PO_4, pH 7.4. The buffer in the
cylinder is mixed with a magnetic stirrer for 75 minutes at 4°C.

The solution outside the dialysis bag is then changed to the
resealing buffer, .15 M KCl, 5 mM K_2HPO_4/KH_2PO_4, pH 7.4. The resealing
buffer is mixed with a magnetic stirrer for 30 minutes at room temperature.
The cells are then washed two times in .12 M NaCl, 20 mM Na_2HPO_4/NaH_2PO_4,
5 mM K_2HPO/KH_2PO_4.

This is essentially the method of Dale et al.[69] but using the
same buffers as Beutler and Nakashima.[13] According to Dale et al.
this procedure will give maximum incorporation. They found the necessary
conditions to be 80% hematocrit in the dialysis sac, 75 minutes lysis
time, and 30 minutes resealing time. Under these conditions, the mean
incorporation was found to be 38%.

2.8 Filter Technique

The Nucleopore filters (0.6 - 0.8 μm pore size, 2×10^6 pore
density) are applied to clean dry filter holders. A thin layer of sili-
con seal is applied to the ground edge of the holder and pressed onto the
filter. The filter membrane must be free of wrinkles and allowed to dry
at least 30 minutes. The filter membranes have a dull and shiny side. The
dull side has a very rough surface and cannot be used. Care must be taken
to ensure that the shiny side is the one to which the cells will settle.

The filter is wetted by filling the holder with buffer and apply-
ing suction. This verifies flow of fluid through the filter and that no
leaks exist. The holder is then filled up to 3/4 full with buffer and placed
in its holder and on a beaker containing buffer (see Figure 2.8.1). A
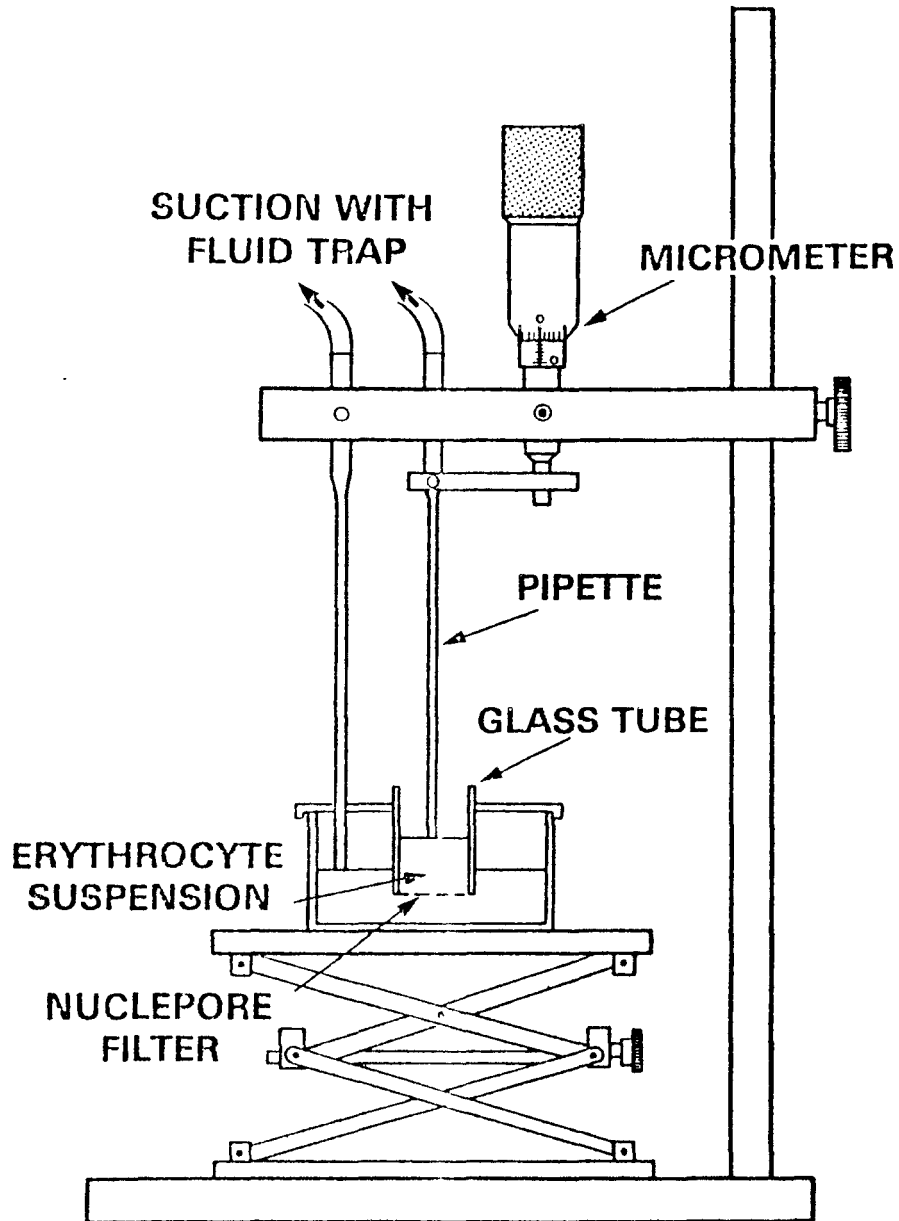
Fig. 2.8.1: Diagram of the apparatus used to aspirate erythro-
cytes into a Nucleopore filter under position hydro-
static pressure.

small amount of red cell suspension (light pink in colour) is layered close to the filter and the cells are allowed to settle for 5-10 minutes (ghosts require longer settling times). While layering the cells suction is applied inside and outside the holder maintaining a 1 mm pressure difference.

After the cells were added, the suction may be removed and the pipettes changed to the desired final height. After the settling time, the pipettes are lowered until both sides are down to the proper level, with suction applied. The outer pipette should be aspirating buffer at ∿.5 cm above the level of the filter. 10 ml of 1% glutaraldehyde in buffer is added inside the filter holder over a 10 minute period using a Sage Instruments syringe pump, Model 341.

After fixation with glutaraldehyde, the buffer levels are equalized and the pipettes lowered so as to remove most of the buffer inside the filter holder. The filter is removed from the holder and stored in glutaraldehyde buffer for short periods, or sodium cacadylate for long periods, i.e. overnight. The buffer used was the same as the final wash the red cells received as per Section 2.5, with 2.5 mg/ml bovine serum albumen added.

2.9 SEM Preparation

The filters prepared as per Section 2.8 must be further fixed with Osmium Tetroxide. This is a very toxic substance and so this fixation must be performed in a fume hood and care taken not to spill any of it. The filters are just covered with the Osmium Tetroxide solution and

the container covered with aluminum foil. After a 30 minute fixation period, the osmium tetroxide is removed into a special container in the E.M. Unit and the filters immersed either in water or 50% sodium cacatylate solution. For long term storage, they must be kept in sodium cacatylate.

The filters next must be dehydrated. This is performed by soaking them in 50%, 70%, 90%, 100% and 100% absolute ethyl alcohol for 2 minutes each. It was found that the initial 50% wash was not required; no difference in red cell appearance occurs if it is omitted.

The cells on the filters are now ready to be transferred onto glass coverslips (Corning, 22 x 22 mm, no. 1 size). A drop of a solution made of 1 ml absolute ethyl alcohol and .04 ml of .5% parlodian (obtained from E.M. Unit) is applied to the coverslip and allowed to dry.

A small portion of the filter is applied to the coated coverslip with the cell side down (shiny side has the cells). To ensure adhesion of the cells to the glass very slight pressure is applied by rolling an applicator stick over the filter.

The coverslip is then immersed in chloroform to dissolve the filter away. The red cells will remain on the glass coverslip. Agitation for at least 5-10 minutes is necessary to remove all the filter material.

The portion of the coverslip with the red cells on it is then cut out with a diamond-tipped scriber and put on an SEM stub freshly covered with silver paint. The sample is now ready for gold-coating with the Polaron Instruments Inc. SEM Coating Unit E5100 in the EM unit.

To coat the samples, the specimens are put in the coater, the top closed, and the argon gas cylinder turned on. The vacuum pump is turned on and when a pressure of .1 torr is reached, the argon leak valve is opened one full turn for 30 seconds to flush the system. If the silver paint on the stubs is not quite dry, flushing may be required earlier. The system is flushed again at .08, .06, and .04 torr. Then the HT knob is set, the amperage knob adjusted to 2.5, and the meter should show about 10 mA. The argon leak valve is then opened until 20 mA is reached on the meter, the timer button pushed, and the knob turned to timer. This will coat the specimens for the set time of 1.5 minutes.

When the coating is finished the amperage knob is turned completely down, the machine turned off, and the argon leak valve opened four full turns. It takes about 5 minutes for the pressure to increase. If this is not done gradually the cells will crack due to thermal shock. When the pressure has returned to normal, the specimens may be taken out. The system must be left with a vacuum of .1 torr and the argon gas cylinder turned off.

2.10 Cell Measurement

Using a Philips PSEM 510 scanning electron microscope it was possible to examine the fixed red cells. Those cells that had single tongues protruding from or near the dimple region were photographed using an Asahi Pentax camera and TX135 film.

Each cell was photographed two times. The first photograph showed the tongue from above, i.e. at 0° to its vertical axis while the

second photograph was taken at 45° to the tongues vertical axis.

The negatives of the film were placed in a Durst enlarger and the measurements of tongue diameter and length were made using venier calipers. The true length of the tongue, D_p, is given by

$$D_p = \frac{H - R_p}{\sin \theta} + R_p (1 - \cotan \theta) \qquad (2.10.1)$$

where R_p is the measured radius of the tongue and H is the measured length of the tongue at angle θ. Figure 2.10.1 shows how this equation is derived.

When all the cell measurements have been made from a particular sample, the mean R_p and D_p/R_p are calculated at each pressure, ΔP. Linear regression of the $(\Delta P . R_p, D_p/R_p)$ data points yields the slope of the line of best fit. The shear modulus of elasticity, μ, of the cells is then given approximately by

$$\mu = 0.75/\text{slope} \qquad (2.10.2)$$
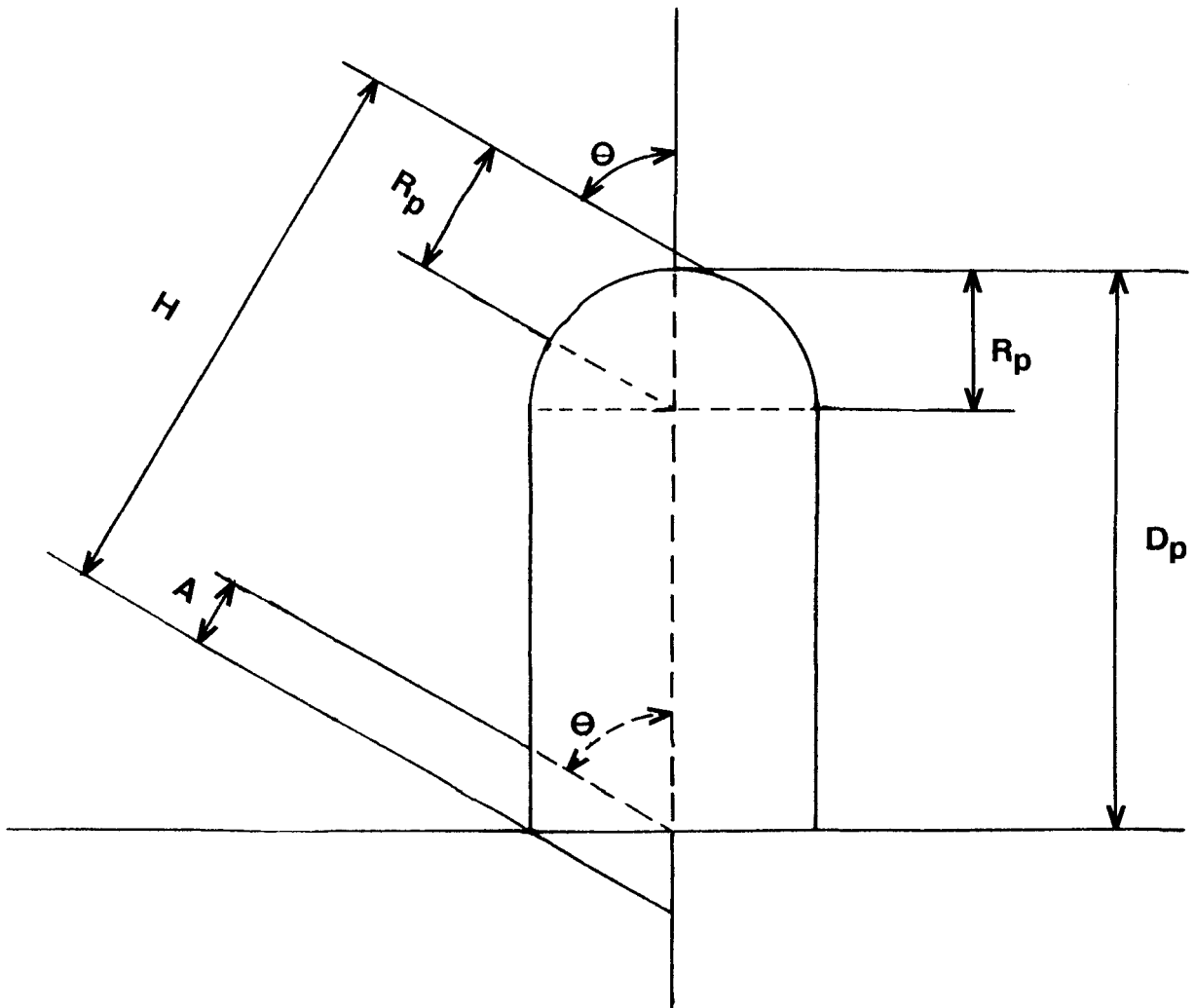
$$A = R_p \, \cos \, \Theta$$

$$H = R_p + (D_p - R_p) \, \sin \, \Theta + R_p \cos \, \Theta$$

Fig. 2.10.1: A schematic representation of a cell tongue to illustrate how equation (2.10.1) is derived.

SECTION 3

RESULTS

3.1 Resealed Erythrocyte Ghosts

Erythrocyte ghosts with low hemoglobin content, prepared by
either method described in Section 2.6 appeared as biconcave disks under
a light microscope. Due to the difficulties involved in examining ghost
cells under light microscopy, a small sample was given to Dr. I. Feuerstein's
laboratory where the cells were labelled with fluorescein isothiocyanate.
These labelled ghosts could be seen using equipment in Dr. Feuerstein's
lab and the ghosts biconcave shape was verified.

When filters were prepared however, no cells could be found on
the coverslip. Biconcave cells could be seen on the filters under SEM,
as shown in Figure 3.1.1, indicating that the osmium tetroxide and
alcohol dehydration steps were not responsible for the ghost cell disappear-
ance. Thus it was determined that the chloroform treatment to dissolve
the filter must be destroying the structural integrity of the membranes.

Methylene chloride was the only other substance shown to dissolve
the filters but the membranes were still not intact after its use. Using a
light microscope, the ghosts appeared wrinkly, as if echinocytic, but
under SEM, they appeared as a fibrous mass bearing no likeness at all to
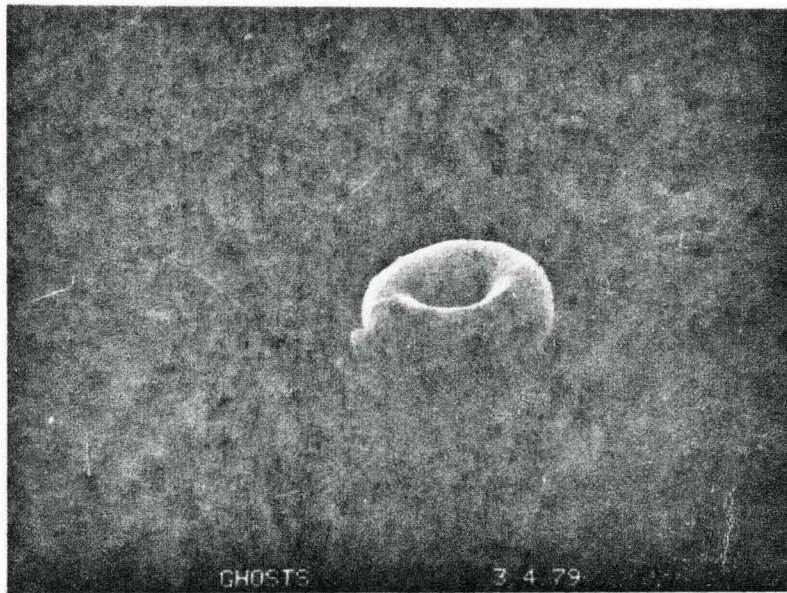red cells.

Fig. 3.1.1: A photograph of a hemoglobin-free ghost cell prepared
by the method of Steck[68] is shown on the polycarbon-
ate filter.

3.2 Resealed Red Cells and the Incorporation Experiments

Resealed red cells made by the method of Dale[69],with saline incorporated,deformed similar to control red cells. The shear moduli of two experiments are given in Table 3.2.1 and the graphs of the normalized tongue length versus pressure are shown in Figures 3.2.1 and 3.2.2.

Incorporation of ATP or alkaline phosphatase into red cells has been shown to cause changes in cell deformability.[13] As shown in Table 3.2.1 and Figure 3.2.3 alkaline phosphatase decreased cell deformability. Two ATP incorporation experiments were done, each giving opposite changes in deformability. Figures 3.2.4 and 3.2.5 are the graphs of normalized tongue length versus pressure. In the first experiment ATP was added only to the resealing buffer using themethod of Nakashime and Beutler.[13] In the second experiment ATP was added to the saline in the dialysis bag, the lysing buffer and the resealing buffer. This method, it was hoped, would provide better incorporation of ATP into the cells.

Figures 3.2.6 - 3.2.11 show the appearance of those cells chosen for measurement.

3.3 Antibody Preparation

Immunodiffusion tests of the serum of the two rabbits against the purified spectrin that the animals were immunized with show the presence of anti-spectrin antibodies. Figure 3.3.1 shows the appearance of the stained slides. The precipitant lines from each rabbit are single joined with no spurs. This indicates that both rabbits have produced the same antibody to the same antigen.

Table 3.2.1: Shear Moduli Results of the Incorporation Experiments

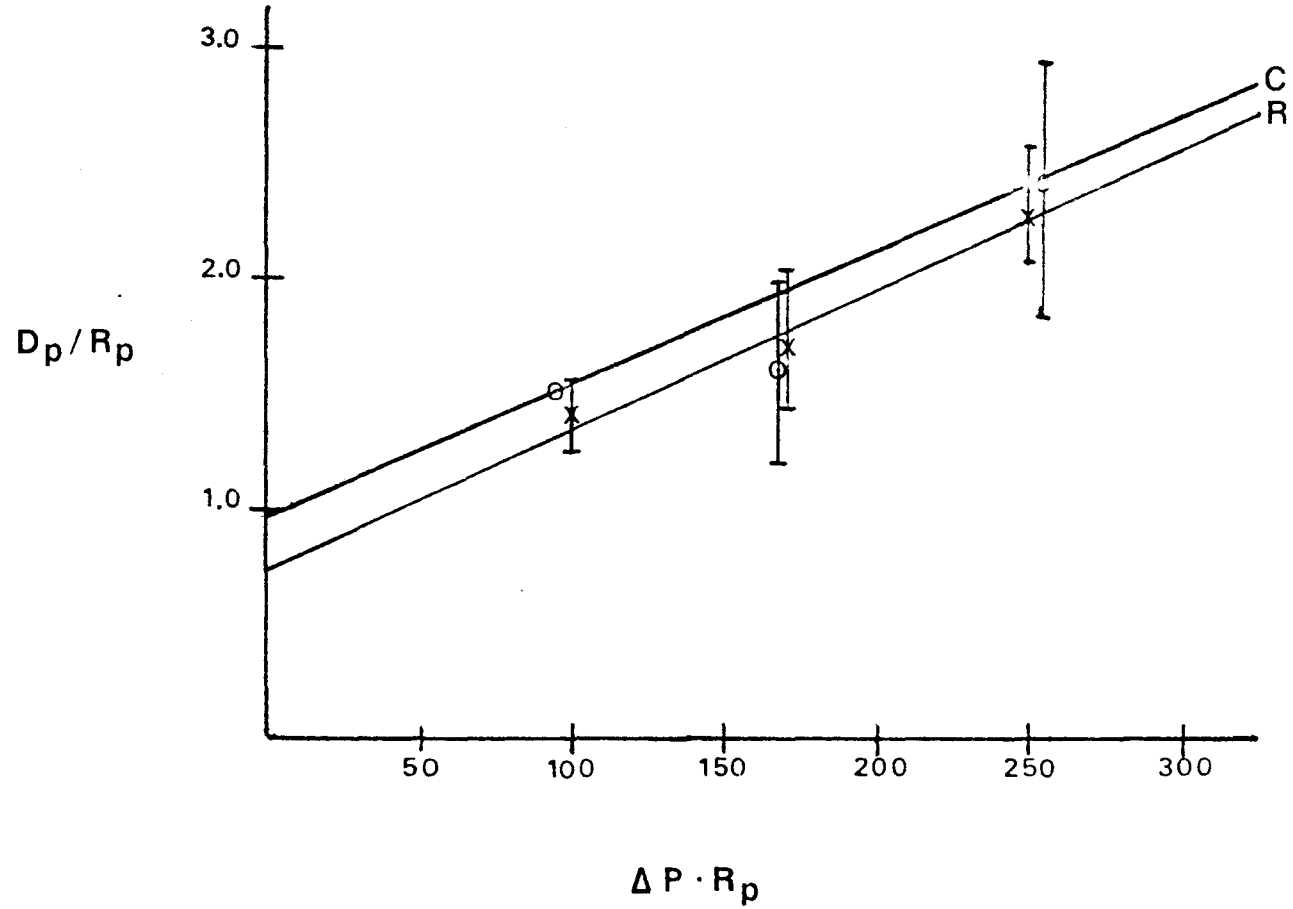|	Experiment 1	2	3
Control 1: red cells	.013	.010	
Control 2: saline incorpor- ated red cells	.013	.011	.013
ATP incorporated Red Cells	0.18		.010
Alkaline Phosphatase incorporated red cells		.019	

Fig. 3.2.1: A graph of the results from Experiment 1 for control 1, washed red cells (C-data points shown by O) and control 2, saline incorporated red cells (R-data points shown by X).
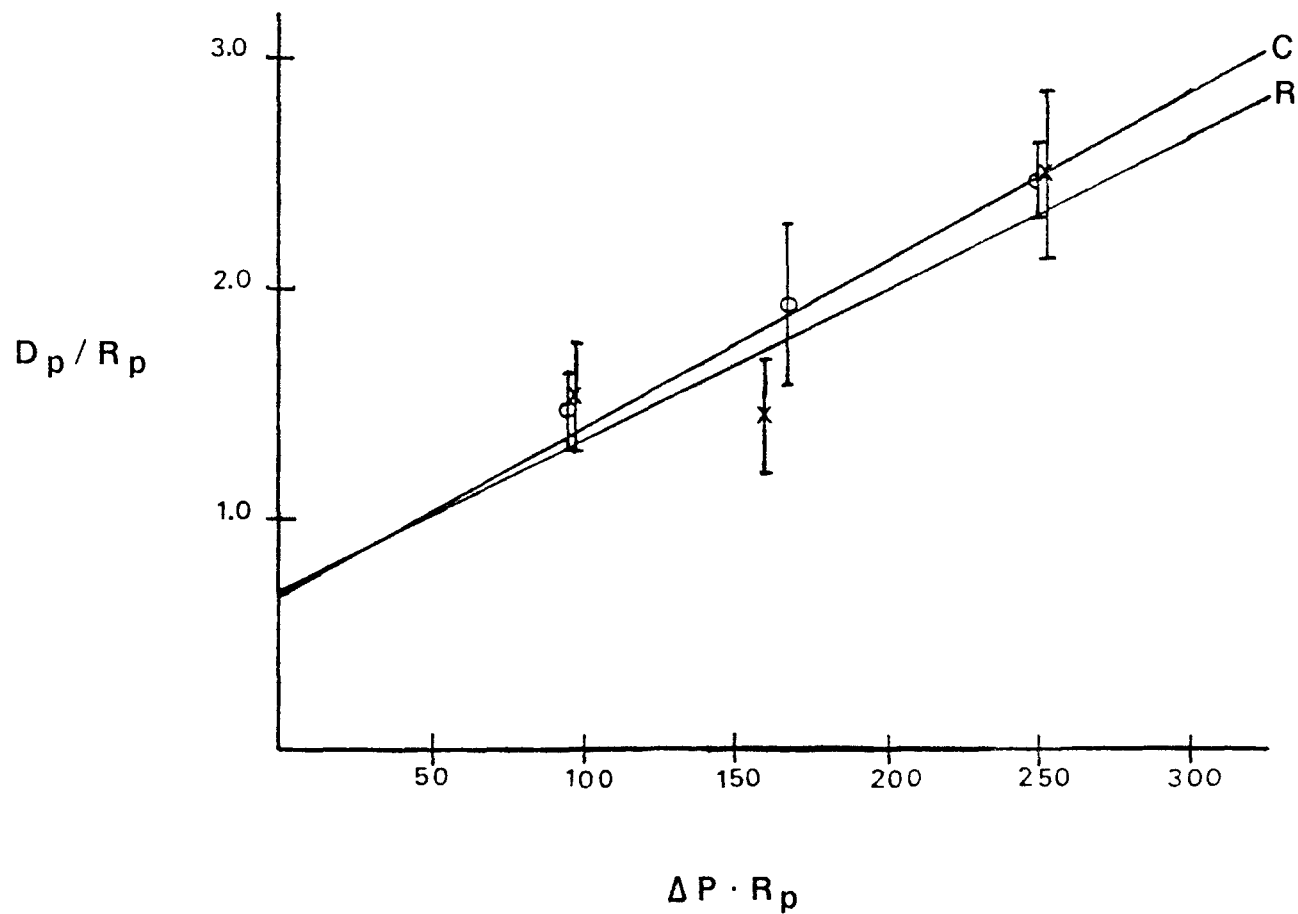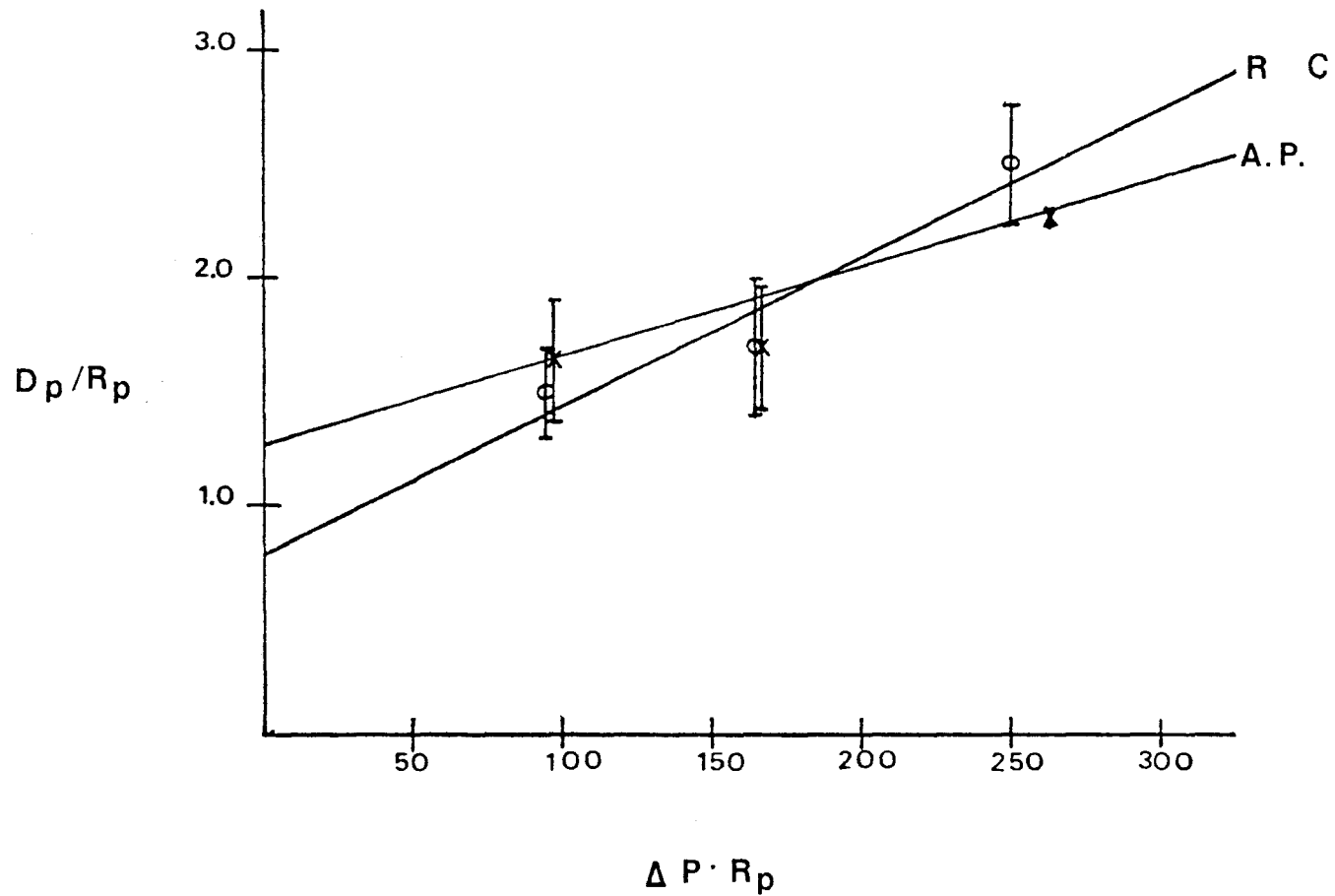
Fig. 3.2.2: A graph of the results from Experiment 2 for Control 1, washed red cells (C-data points shown by C) and Control 2, saline incorporated red cells (R-data points shown by X).

Fig. 3.2.3: A graph of the results from Experiment 2 for Controls 1 and 2 combined (R+C-data points shown by 0) and Alkaline Phosphatase incorporated red cells (A.P.-data points shown by X).
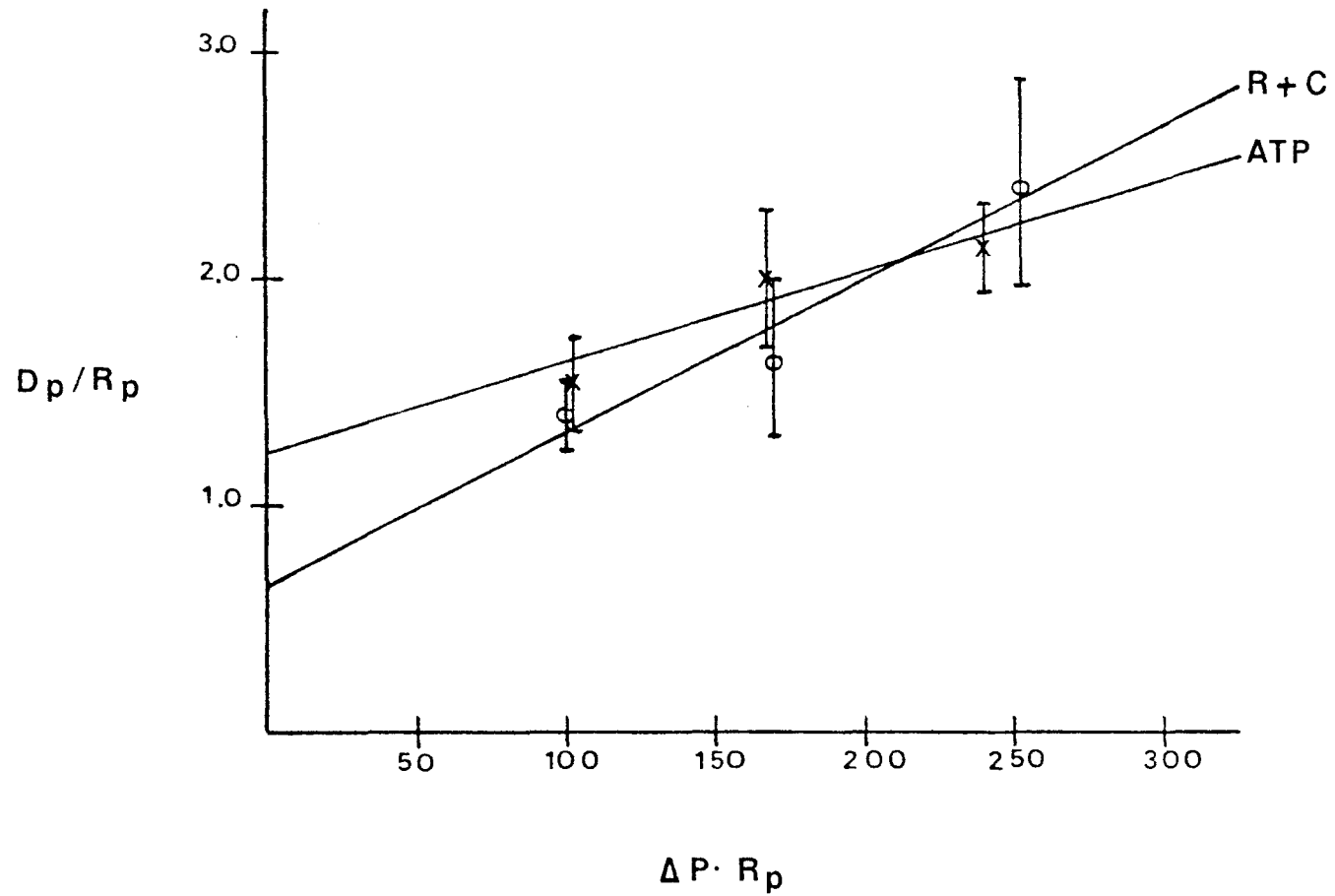
Fig. 3.2.4: A graph of the results from Experiment 1 for Controls 1 and 2 combined (R+C-data points shown by O) and ATP incorporated red cells (ATP-data points shown by X).
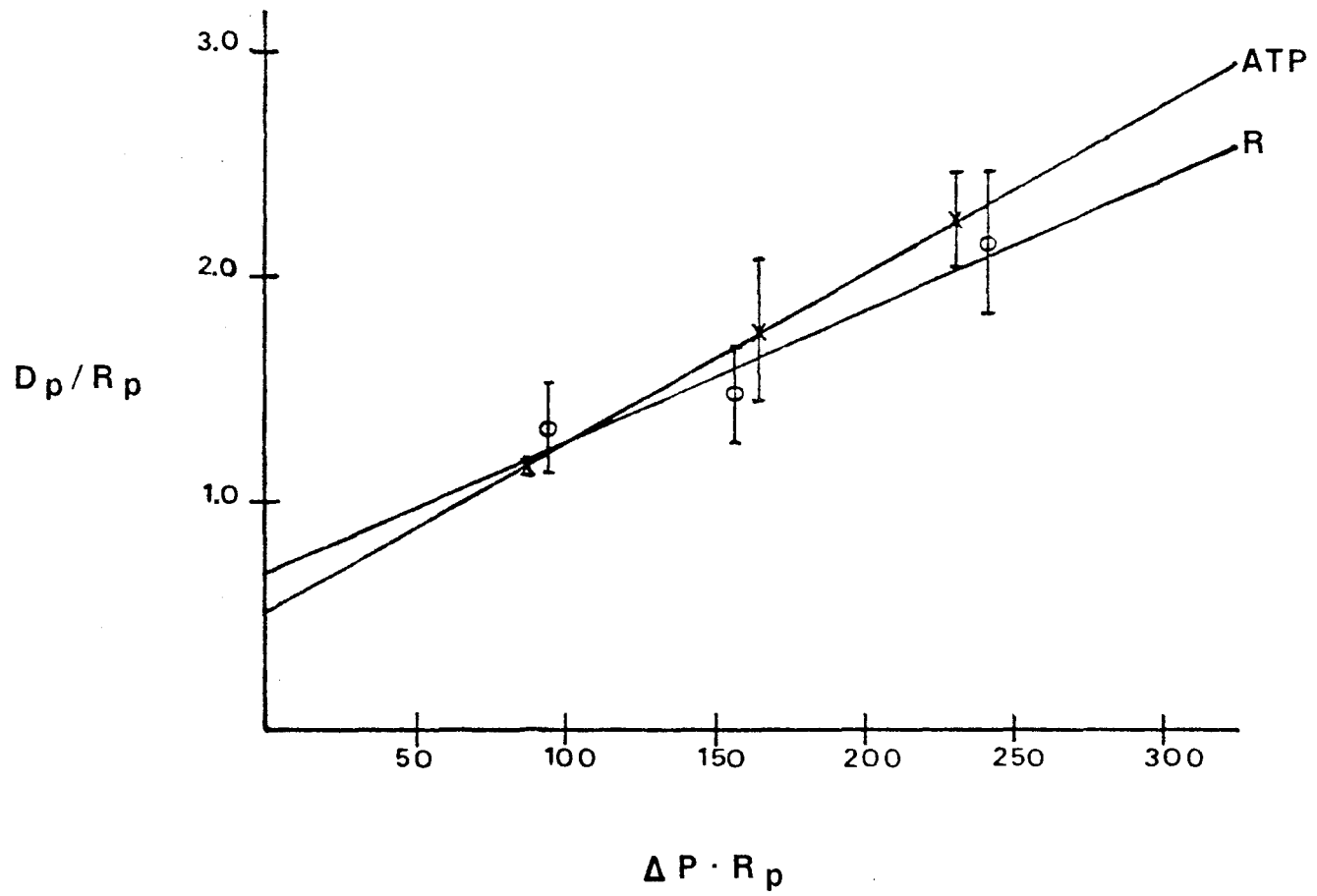
Fig. 3.2.5: A graph of the results from Experiment 3 for Control 2, saline incorporated red cells (R-data points shown by O) and ATP incorporated red cells (ATP-data points shown by X).
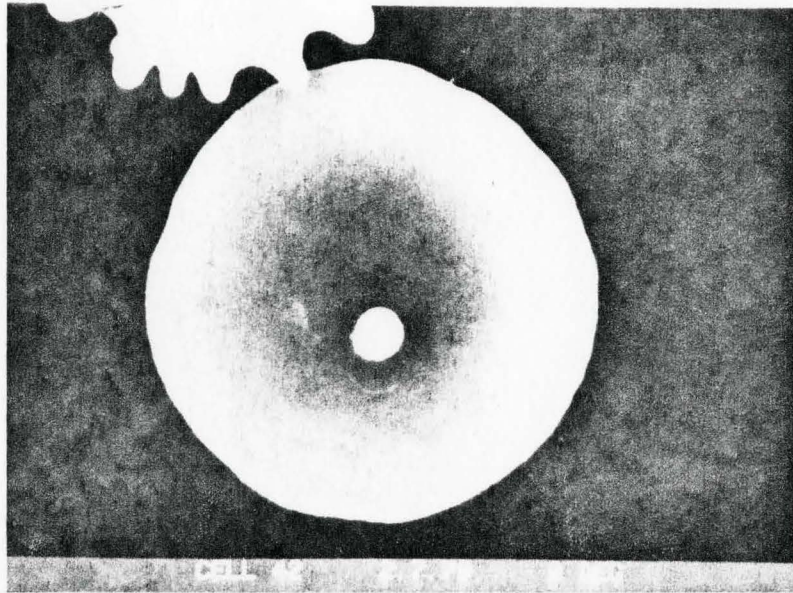
Fig. 3.2.6: A photomicrograph of a typical human red cell chosen for study seen at 0 degrees. The tongue was aspirated at 3mm H_2O pressure.
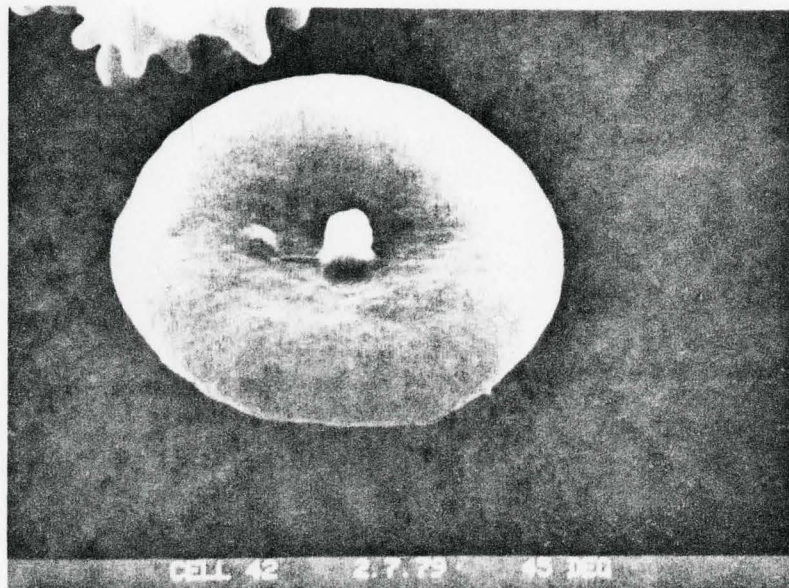
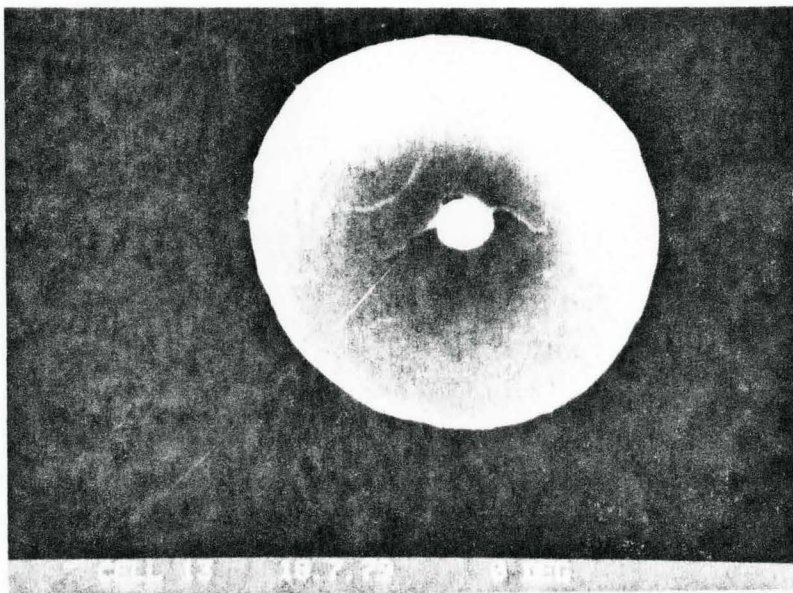Fig. 3.2.7: The same cell as Fig. 3.2.6 seen at 45 degrees.

Fig. 3.2.8: A photomicrograph of a typical human red cell   chosen
for study seen at 0 degrees. The tongue was aspirated
at 5 mm H_2O pressure.

Fig. 3.2.9: A photomicrograph of a typical human red cell - chosen for study seen at 45 degrees. The tongue was aspirated at 5 mm H_2O pressure.

Fig. 3.2.10: A photomicrograph of a typical human red cell chosen for study seen at 0 degrees. The tongue was aspirated at 7 mm H_2O pressure.
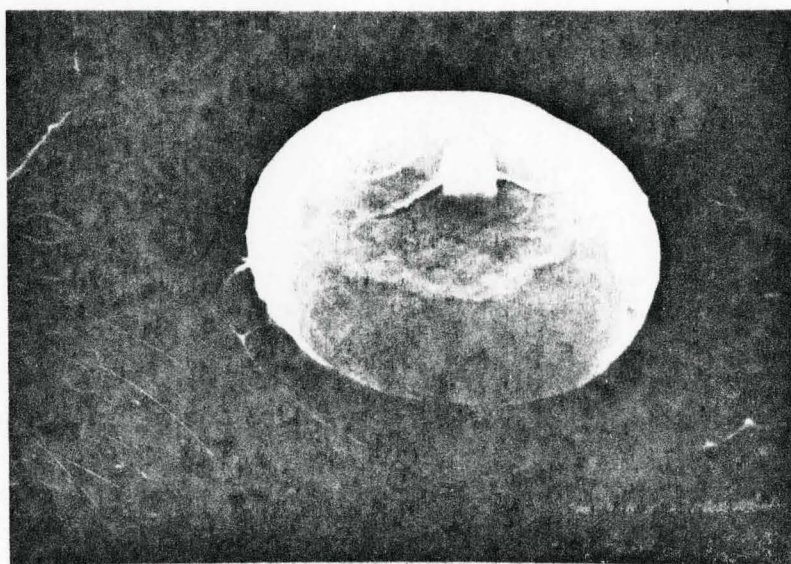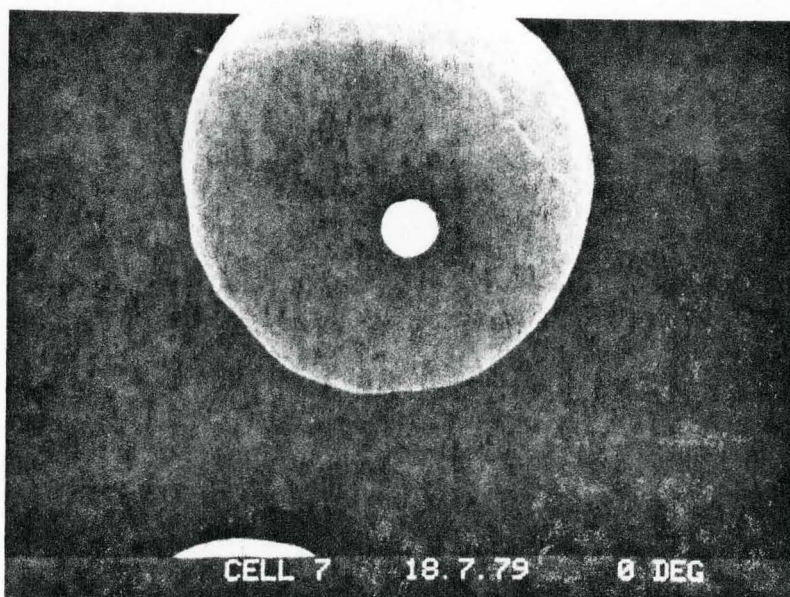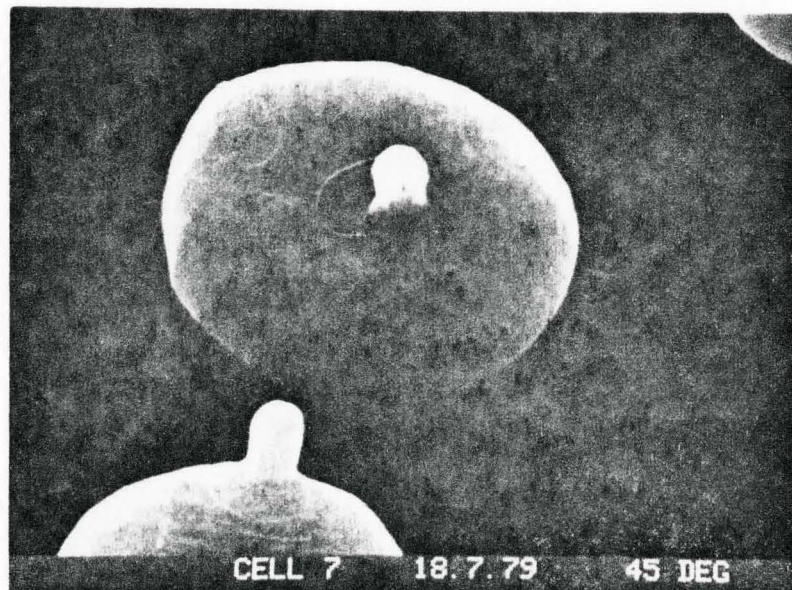
Fig. 3.2.11: A photomicrograph of a typical human red cell chosen for study seen at 45 degrees. The tongue was aspirated at 7 mm H_2O pressure.
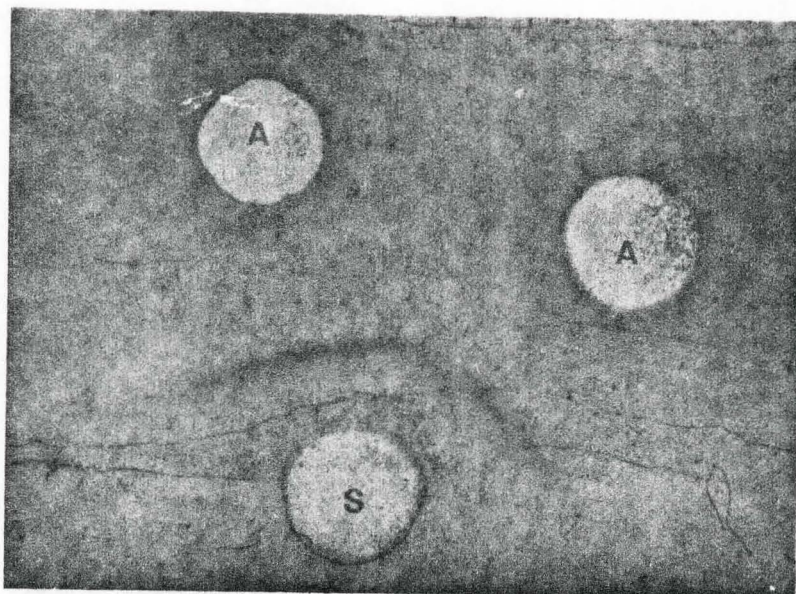
Fig. 3.3.1: Immuno diffusion test. Rabbit serum (A) and purified spectrin (S) present in the wells and diffused out to form a precipitant arc (stained curve inbetween wells).

A small sample of spectrin extract, from a patient with myotonic dystrophy obtained from J. Vickers, was tested against the anti-spectrin antibody present in the rabbit serum. As seen in Figure 3.3.2, the dystrophic spectrin is immunologically identical to control spectrin since the precipitant lines are single and joined.

The rabbit serum is presently frozen at -18°C. The anti-spectrin antibodies will be purified at a future date for use in deformability experiments with antibody-incorporated red cells.

Fig. 3.3.2:   Immunodiffusion Test.   Rabbit serum (A), purified control
spectrin (S) and spectrin extract from myotonic dystrophy
patient (D) were present in the wells and diffused out
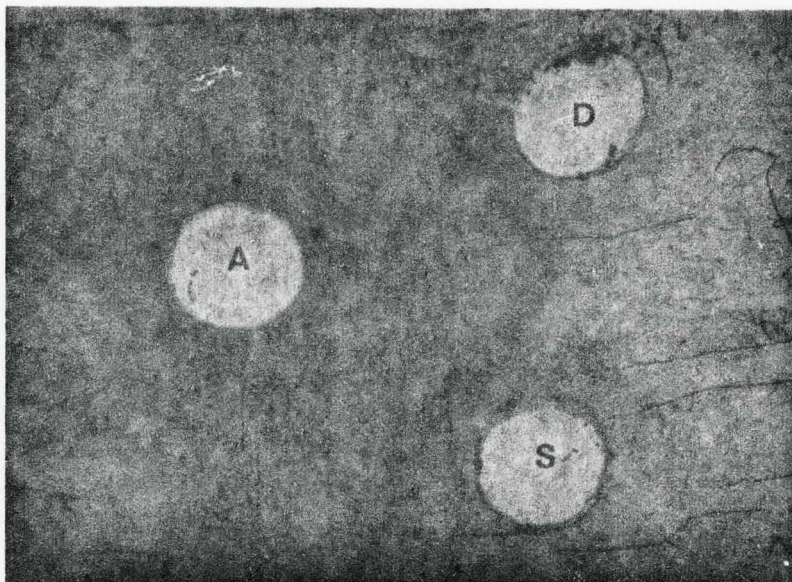to form precipitant arcs (stained curve inbetween wells).

SECTION 4

DISCUSSION

4.1 Experimental Techniques

4.1.1 Filter Technique

The improved method of the filter technique presented by
Missirlis and Brain[70] was used to determine the deformability proper-
ties of red cells. This technique provides a relatively rapid method of
deforming red cells when compared with the micropipette technique. A
large population of red cells can be deformed and fixed in their deformed
state in a short period of time. Subsequent examination of the cells by
SEM however, has proven to be tedious and costly due to the poor quality
of the filters. The relative scarcity of single tongued cells due to the
poor pore distribution in the filters increases greatly the amount of
time spent in finding suitable cells.

Care must be taken in choosing the cells for measurement since the
major source of error in the technique comes from the tongue length measure-
ments. The tongues chosen for measurement were circular when examined
along their vertical axis and were free of wrinkles. The cells were
normal biconcave discs.

It is very important that the tongue is near the centre of the
dimple. As seen in Figures 4.1.1 and 4.1.2 difficulties are encountered
in measuring the length of a tongue that is on the slope of the dimple since
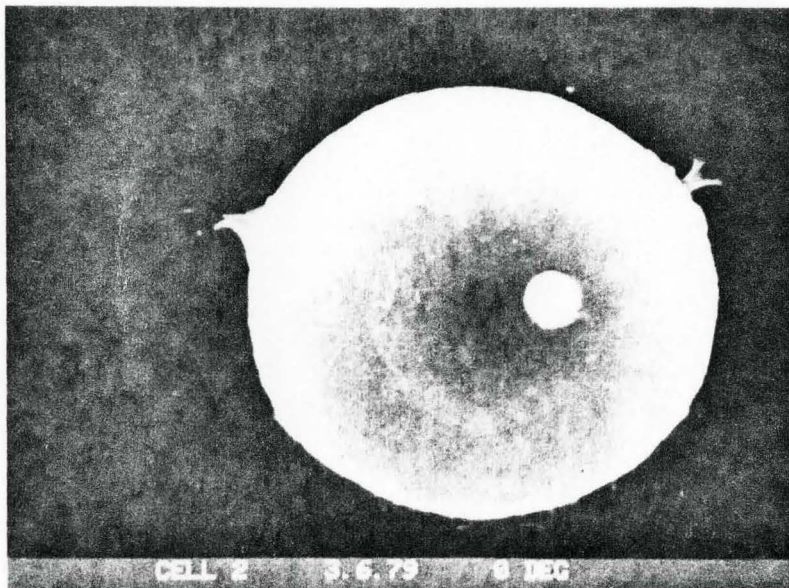it is not clearly defined.

Fig. 4.1.1: A photomicrograph of a human red cell not chosen for study since its tongue is not in the dimple region.
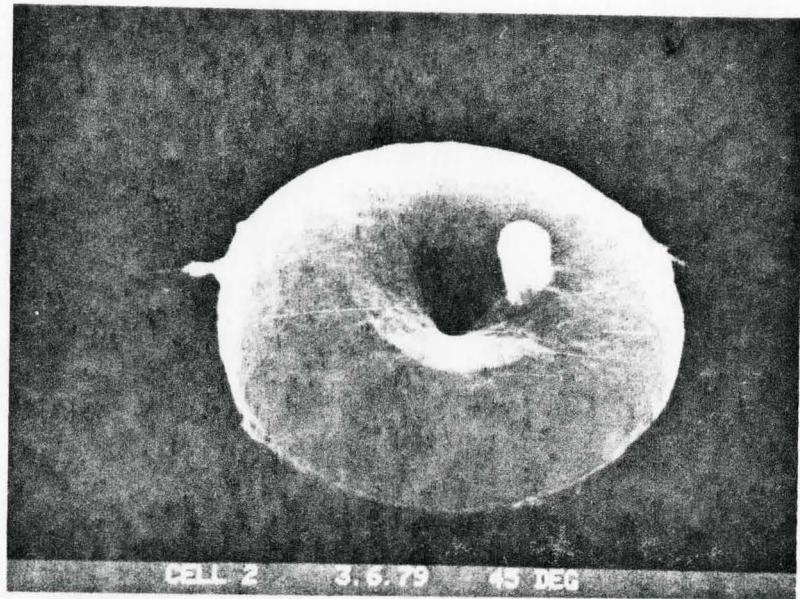
Fig. 4.1.2: A photomicrograph of a human red cell not chosen for study since its tongue is not in the dimple region. At this angle it is clearly seen that the length of the tongue is not well defined.

The tongues that were chosen were all from or near the dimple. This condition for choosing tongues is based on the findings of Evans;[71] that if a tongue is aspirated from an infinite plane membrane the tension in the plane around the tongue decreases with the square of the distance from the tongue's verticle axis. The dimple region of the red cell best approximates a flat plane whereas at the rim bending stresses must be taken into account. For small pore sizes, if a tongue is pulled from the dimple region, the effect at the rim will be negligible.[70]

Due to the poor pore distribution of the filter it was found that cells with a single tongue from the dimple were scarce whereas many multi-tongued cells with one tongue protruding from the dimple region could be found. It was decided that to reduce the tedious amount of work required to find several single tongued cells, that at low pressures cells with 2 or 3 tongues could be chosen provided the additional tongue(s) were "far" from the dimple region and the cell remained dis-coid. Statistical t-tests were done to see if there was a significant difference in tongue length due to the additional tongues. The t-test was performed on the D_p/R_p values obtained for a given sample at a given pressure. Of the 16 possible data sets (multi-tongued cells were not selected at the highest pressure) only 10 contained cells with multiple tongues.

The results of the t-tests on the 10 data sets are shown in Table 4.1. At the 95% confidence level 8 data sets showed no significant differences between single and multiple tongued cells while 2 data sets showed single tongued cells to be more deformable. Thus provided the cells are chosen with care under the conditions described above, it is

felt that multitongued cells will also provide a measure of cell membrane deformability.

When examining a particular cell under SEM, it is important that the verticle axis of the tongue can be determined. When looking along the perpendicular to the glass coverslip, defined as 0° by the SEM, this may not be the verticle axis of the tongue. In some cases the tongue is tilted. The microscope stage may be tilted in the y-direction (as defined in Figure 3.10.1) up to 60° and so those tongues that are tilted in this direction by <15° can be measured. Any tilt in the x-direction would cause the cell to be rejected since the vertical axis could not be determined accurately.

When looking along the vertical axis of the tongue, it should appear circular and have radial symmetry. Figure 4.1.3 and 4.1.4 demonstrate how a tongue that is tilted appears from above and at from the side. The shading of the tongue, as seen from above, is not symmetrical with respect to the vertical axis since the side of the tongue can be seen. Cells such as this were rejected.

Once the vertical axis of a particular tongue has been determined, a photograph at 45° to the axis can be taken, the tongue length measured and the true length determined by equation 2.10.1.

4.1.2 Spectrin Purification

It has been shown that "Purified" spectrin prepared from the void volume fractions of Sephadex G-200 or other large-pore gels also contains the heterodimer (spectrin bands 1 and 2) and aggregates of spectrin and
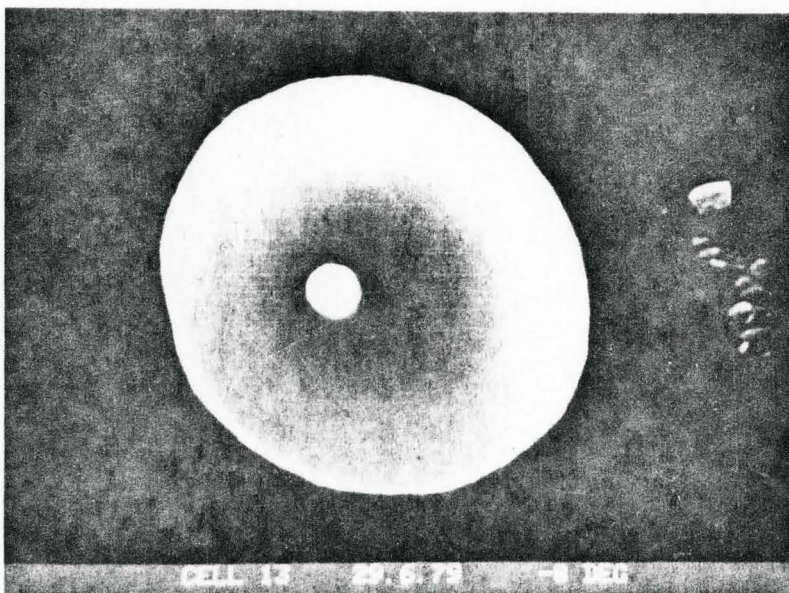
Fig. 4.1.3: A photomicrograph of a human red cell not chosen for study since its tongue is not radially symmetric at 0 degrees.
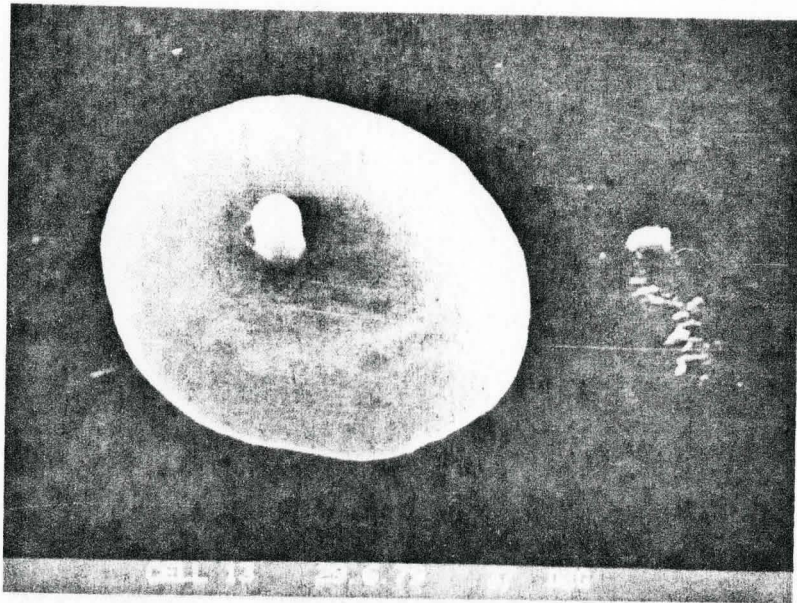
Fig. 4.1.4: A photomicrograph of a human red cell not chosen for
study since its tongue is not radially symmetric at
0 degrees. At this angle it is clearly seen that the
tongue is tilted to the left.

actin and possibly other membrane proteins.[59,72] Bands of higher molecular weight corresponding to these aggregates were found in some of the samples of purified spectrin. It has been shown that the spectrin can be extracted in either the dimer state or the tetramer state depending upon conditions.[72] However no methods have been found to obtain purified spectrin without aggregates containing other membrane proteins.

4.2 Experimental Results

4.2.1 Resealed Erythrocyte Ghosts

The disruption of the cell membranes of hemoglobin-free ghosts and not of red cells or the resealed red cells by chloroform or methylene chloride treatment must be due to the preparation methods. Removal of >95% of the hemoglobin appears to cause changes in the membrane structure that make it susceptable to the treatment. Gentler methods of lysing and resealing the cell, such as the method of Dale,[69] where 80% of the cytoplasm remains in the resealed cells, do now show this susceptability. This indicates that the removal of the cellular contents to the extent of the first method's alters the erythrocyte membrane structure significantly.

It was later discovered that cells could be removed from the filter without chloroform or methylene chloride treatment. As before, a portion of the filter is placed on a pardolian treated coverslip and slight pressure applied. If the filter is carefully peeled off the coverslip, in most cases the cells will remain intact on the glass. Using this method, it may be possible to examine the deformability properties

of red cell ghost membranes.

4.2.2 Resealed Erythrocytes and the Incorporation Experiments

Due to an unfortunate lack of time the effect of incorporating alkaline phosphatase or ATP in to resealed red cells could not be examined fully. Alkaline phosphatase, as discussed in the introduction is thought to cause the depolymerization of the spectrin network and reduce membrane deformability. This trend is seen in the experiment performed.

ATP, on the other hand, should cause polymerization of the cyto-skeleton components and increase cell deformability. This change in deformability was not seen in the first experiment and so another was performed under conditions that would allow for better incorporation of ATP. In the latter experiment the expected change in deformability was seen but it was not of the same magnitude as that seen by Nakashima and Beutler.[13] It is possible that the amount of incorporated ATP was insufficient to produce significant membrane phosphorylation and thereby significant changes in membrane deformability properties.

Let us consider the work of Birchmeier and Singer[65] who examined the dependance of spectrin band 2 phosphorylation on the concentration of ATP in the incorporation medium. In our experiments 2 mM MgATP was used. According to Dale[69] a maximum of 40% incorporation occurs, allowing only .8 mM MgATP to be incorporated. At this level of ATP approximately 0.2 moles of $[^{32}P]$ were incorporated per mole of spectrin band in an experiment by Birchmeier and Singer. Their experiment was performed with erythrocyte ghosts whereas here resealed red cells were used.

The presence of approximately 80% cytoplasm in the cells will also reduce the amount of membrane phosphorylation since many ATP-dependent processes may still be present in the cell.

It has also been shown that dephosphorylation of the membrane by endogenous phosphatases occurs at the rate of 50% per hour at 37°C.[62] After resealing of the membranes, the cells were washed two times before the filters could be prepared. Completion of the filters was as much as 5 hours after the cells were removed from the presence of ATP. Since the samples were blind coded in the first experiment it is possible that the level of membrane phosphorylation in the ATP-incorporated cells decreased somewhat.

In the second experiment only one control was used thereby decreasing the total time to prepare the filters to 3 1/2 hours after removal of the cells from the resealing buffer. Also the samples were not blind coded, in order to allow the ATP-incorporated sample to be completed first, reducing the time to 2 hours.

In summary, the presence of endogenous phosphatases and ∿80% cytoplasm in the resealed red cells will reduce the maximum possible incorporation of 0.2 moles phosphate per mole spectrin band 2 considerably. Even at this level of phosphorylation however, it has been shown that 10% spherical ghosts are converted to biconcave discs.[66] Thus at the level of ATP incorporated into the cells a significant change in cell deformability could not be expected. A trend was shown however, and further experiments will be necessary to determine the dependance of cell deformability on ATP.

In future experiments, it is recommended that for each red cell prepraration whose cell deformability properties are measured, the degree of phosphorylation of spectrin band 2 be determined as in Birchmeier and Singer.[65] This will hopefully verify the direct correlation of membrane phosphorylation with cell membrane deformability implied by Sheetz and Singer.[62]

SUMMARY

The technique of cell membrane deformation by the use of
nucleopore filters and the incorporation of substances into resealed
erythrocytes have been used to study the effect of ATP and alkaline
phosphatase on human erythrocyte deformability. These are believed
to change the membrane's deformability properties by altering the
state of phosphorylation of spectrin, a component of the membrane
cytoskeleton. The experimental results show that small alterations
in membrane deformability do occur as a result of the incorporation
of these chemicals. ATP-incorporated cells have a decreased shear modulus
of elasticity as compared to normal erythrocytes or saline-incoroporated
cells. The alkaline phosphatase-incorporated cells have an increased
shear modulus as compared to the controls. These results are in accord-
ance with those previously published[13] but the magnitude of the changes
observed is much smaller.

In addition, spectrin was purified from normal erythrocytes
and used to immunize rabbits. Serum containing anti-spectrin antibodies
has been obtained from them and is stored for future use.

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