AN INVESTIGATION OF THE EFFECTS OF ANTITUMOUR AND OTHER DRUGS ON CELL MORPHOLOGY AND THE CYTOSKELETON OF ERYTHROCYTES

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SUMMARY

Human erythrocytes were used as a model system for an investigation of the mechanism of action of the antiproliferative drug Adriamycin. Erythrocytes were induced to undergo a change in morphology by elevation of intracellular calcium. It was revealed that the widely used media employed for the study of morphological change were unsuitable; a new incubation medium was developed so that cells were metabolically replete. In this medium echinocytosis took place both in a calcium concentration- and time-dependent manner. Pretreatment of erythrocytes with Adriamycin (10 μM for 10 mins) protected the erythrocytes against calcium-induced echinocytosis at calcium concentrations <150μM.

SDS-PAGE analysis of the cytoskeletal proteins prepared from erythrocytes revealed the calcium-induced proteolysis of two main cytoskeletal proteins: band 2.1 and band 4.1. Only the rate of the proteolysis of band 2.1 correlated with the onset of echinocytosis. Adriamycin inhibited the breakdown of band 2.1 even when the cells formed echinocytes; this raises doubts concerning the importance of band 2.1 in the maintenance of discocyte morphology. Adriamycin only marginally inhibited the purified calcium-activated thio protease (calpain).

Calcium-loading of human erythrocytes increased the phosphorylation of several major cytoskeletal proteins including pp120, band 3, band 4.1 and band 4.9. The pattern of increase resembled that induced by 12-0-tetradecanoyl-phorbol-13-acetate. Pre-treatment with Adriamycin prior to calcium loading caused a general lowering of basal phosphorylation. Adriamycin had no effect on the activity of the calcium-activated phospholipid-dependent protein kinase (protein kinase C). A hypothesis is put forward that the morphological transition of erythrocytes might be dependent upon the activity of a contractile system.

KEYWORDS: Erythrocyte; cytoskeleton; ankyrin; proteolysis; phosphorylation; calcium; Adriamycin (Doxorubicin).

FOR MY PARENTS,

FOR ALL THEIR HELP

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ABBREVIATIONS

1,3 DPG	1,3 Diphosphoglycerate
2,3 DPG	2,3 Diphosphoglycerate
^{32}Pi	^{32}P-orthophosphate
A23187	Calcium Ionophore A23187
ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
APS	Ammonium persulphate
Arg	Arginine
ATP	Adenosine-5'-triphosphate
ATPase	Adenosine-5'-triphosphatase
BSA	Bovine serum albumin
cAMP	Adenosine-3',5'-monophosphate
CHAPS	(3-(3-Chloramidopropyl)-dimethylamino-ammonio)-1-propane sulfonate
CMP	Cytidine-5'-monophosphate
CPM	Counts per minutes
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
Conc	Concentration
DEAE	Diethylamino ethyl
DG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DPM	Disintegration per minutes
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether) N,N, N',N'-tetraacetic acid
G protein	Guanine nucleotide binding protein
GDP	Guanosine-5'-diphosphate
GMP	Guanosine-5'-monophosphate
G_i	Inhibitory Guanine nucleotide binding protein
Gly	Glycine
G_s	Stimulatory Guanine nucleotide binding protein
H-7	1-(5-Isoquinolinylsulfonyl)-2-methyl-piperazine
HCO_3^-	Bicarbonate
HPLC	High performance liquid chromatography
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
Ins	Inositol
InsP	Inositol monophosphate
$InsP_2$	Inositol diphosphate
$InsP_3$	Inositol trisphosphate
KCl	Potassium chloride
Kd	Kilodalton
KH_2PO_4	Potassium dihydrogen phosphate
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
Mw	Molecular weight

NAD	Nicotinamide adenosine dinucleotide
NADH	Nicotinamide adenosine dinucleotide - reduced form
Na	Sodium
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
NaF	Sodium fluoride
NaH_2PO_4	Sodium dihydrogen phosphate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
PAGE	Polyacrylamide gel electrophoresis
PDBu	Phorbol 12,13-dibutyrate
Phe	Phenylalanine
PI-PLC	Phosphatidylinositol-specific phospholipase C
PKC	Protein kinase C
PKM	Protein kinase M
PMSF	Phenyl methyl sulphonyl fluoride
PVP-40	Polyvinylpyrrolidone, average Mw 40,000
Pi	Orthophosphate
PtdIns	Phosphatidylinositol
PtdIns(4)P	Phosphatidylinositol 4-phosphate
PtdIns(4,5)P_2	Phosphatidylinositol 4,5-bisphosphate
PtdInsP_2	Phosphatidylinositol bisphosphate
PtdInsP	Phosphatidylinositol monophosphate
Ser	Serine
SDS	Sodium dodecylsulphate
TCA	Trichloroacetic acid
TEMED	N,N,N'N'-tetramethylethylenediamine

TFA	Trifluoroacetic acid
TPA	Phorbol 12-myristate 13-acetate
TRIS	Tris[(hydroxymethyl)aminoethane]
TRITC	Tetramethylrhodamine B isothiocyanate
UDP	Uridine-5'-diphosphate
UTP	Uridine-5'-triphosphate
V8	Staphylococcus aureus V8
W-7	N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide

CHAPTER ONE

INTRODUCTION

All living organisms possess a distinct spatial organization of their biological molecules. The pattern of this organization determines the events which are involved in cellular metabolism, mobility, differentiation, reproduction and evolution. Aberrations in the control of growth and differentiation, which lead to malignancy, may thus be associated with changes in the architecture of the cell. In this thesis, the interactions between an anticancer drug, Adriamycin, and the cytoskeleton of a "model" cell type, the human erythrocyte, have been studied. The cytoskeleton plays a pivotal role in determining cellular architecture, and much of the thesis is concerned with an investigation of the role of erythrocyte cytoskeletal components in the determination of erythrocyte morphology. Adriamycin modulates certain morphological transitions that may be induced in the erythrocyte, and the drug has been used as a pharmacological tool in an attempt to analyze the role of the cytoskeleton in cellular shape changes. Interestingly, although Adriamycin is a widely used antitumor drug in the clinic (Bonadonna et.al. 1970, Bonadonna et.al. 1975), there is a controversy regarding its precise mechanism of action; the results of the studies presented in this thesis support an existing hypothesis that it exerts selective effects upon the functions of the cytoskeleton and the cell membrane (reviewed by Tritton and Hickman, 1985).

The introduction to this thesis reviews the structure and function of the erythrocyte cytoskeleton and the role that it

may play in controlling cell shape. The biochemical mechanisms for control of the cytoskeleton are reviewed, and the modulation of erythrocyte shape by drugs is then surveyed, with particular respect to the effects of Adriamycin and other amphipaths upon this process.

In early studies of cell biology, it was widely believed that individual cells consisted of a capsule-like material which enclosed free-flowing cellular constituents. Later, the development of light microscopy and electron microscopy revealed that the cell consisted of a highly complex outer plasma membrane (reviewed by Bretscher 1985, Storch & Kleinfeld 1985) which encapsulated the cytoplasm and the nucleus. Electron microscopy further revealed that the cytoplasm not only consisted of an aqueous phase and distinct organelles, such as the mitochondria, but also of extensive and highly organised membranous structures. The perception of the cytoplasm as a complex solution of biochemicals containing `free floating' organelles was widely adopted, initially. With enhanced biochemical and electron microscopic techniques, it was revealed that what was believed to be a "free-flowing" cytoplasm, actually contained highly organised structural elements, mainly in the forms of filaments, and this was referred to collectively as the cytoskeleton. The term "cytoskeleton" was also used to refer to the insoluble protein aggregates which sedimented upon centrifugation after extraction of a cell suspension with an non-ionic detergent, such as triton X-100. Such aggregates were found to consist

exclusively of a complex mixture of proteins organised into these filamentary structures. There are three main classes of filaments present in the cytoskeleton of normal cells: the microfilaments, microtubules and intermediate filaments (Weber & Osborn 1985). These filaments are arranged into different networks and the presence of other accessory cytoskeletal proteins coordinate the interactions between and the functional characteristics of these networks. The cytoskeletal network also interacts with and anchors to both the plasma membrane and nuclear envelope (Gumbiner & Louvard 1985). Fluorimetric images obtained from immunological studies of cytoskeletal proteins in fibroblasts showed the high complexity of this cytoskeletal network (figure.1). This image depicts the localization of only three cytoskeletal components, and the degree of complexity at this relatively simple level is obvious. From figure 1, it can be seen that the cytoskeleton appears to spread over the cell mass, extending from the nuclear envelope to the cell surface, where substratum-cell contact is developed. The findings from recent studies that the nucleus also possesses an organised protein network (eg. Mckeon et.al. 1986), which may interact with the cytoskeleton, has supported the idea of a role for the cytoskeleton in the regulation of cell growth and differentiation.

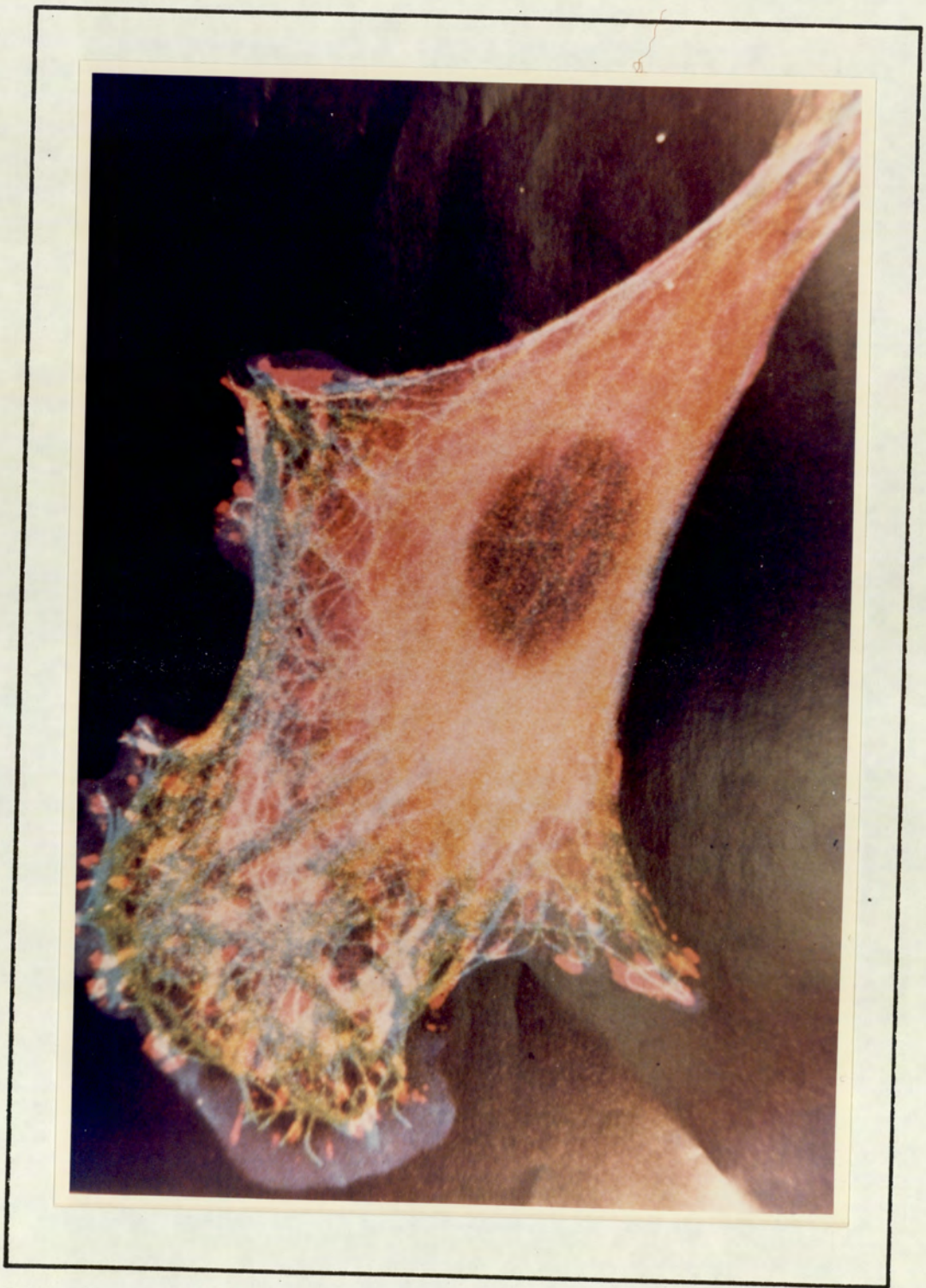


Figure.1 Illustration of cytoskeletal organization in fibroblast.
The cell was immunocytochemically stained with antibodies raised against actin (blue), vinculin (red), tubulin (green) and the yellow colour results from superposition of colours. (reproduced with permission from Dr. K. Weber, Sci.Am. 253 P.92-103 (1985)

In initiating studies of cytoskeletal function, the mechanism of its regulation and its possible role as a drug target, it is fair to state that little is known of the overall arrangement of the cytoskeleton of somatic cells. Thus, for the purpose of this study, a model system was sought. The human erythrocyte was chosen as a suitable model system, since it is a "simplified" non-nucleated cell consisting of a well developed plasma membrane and cytoskeleton, but little else, and could be obtained in large quantities. It is believed that the life span and proper functioning of the erythrocyte in vivo is determined by its deformability, which is believed to be regulated by the cytoskeletal network. Moreover, human erythrocytes are known to undergo distinctive morphological transitions under certain conditions (see section 3), a property which makes experimental manipulation of cell shape possible. The human erythrocyte membrane lipid composition is well documented and its cytoskeletal arrangement is the best understood amongst all cell types. Their lack of a nucleus, an intracellular membranous system and the presence of a well described cytoskeletal network makes them a suitable model for studies of drug-membrane-cytoskeletal interactions. Studies on the effects of Adriamycin and its analogues on human erythrocytes have been reported (Mikkelsen et.al. 1977, Goldman et.al. 1978, Henry & Bolard 1986). Investigations have been carried out in our laboratory on the effect of various agents, including Adriamycin, on the morphological changes induced by certain conditions such as intracellular ATP-depletion and

elevation of intracellular calcium level (see section 3 & 4).

1.1. The Human Erythrocyte Cytoskeleton and Its Relationship to Cytoskeletons of Other Cell Types

In the past two decades, many studies have been made which aim to elucidate the molecular organization and the mechanism of regulation of the human erythrocyte cytoskeleton (reviewed by Bennett 1985, Branton et.al. 1981, Branton 1983, Cohen 1983, Haest 1982). In the human erythrocyte, the underlying cytoskeleton, which is obtained after Triton X-100 extraction, is a less complicated structure when compared to other mammalian somatic cells. Thus the human erythrocyte cytoskeleton is sometimes referred to as a `membrane skeleton'. The human erythrocyte cytoskeleton differs from other cytoskeletons in several aspects: although it contains actin, it does not possess microtubules or intermediate filaments. Generally, somatic cell cytoskeletons interact with the plasma membrane at various adhesion points and the main mass of the skeleton is relatively remote from the plasma membrane. The cytoskeleton of human erythrocyte is mainly located within 10nm from the plasma membrane (Cohen 1983). Despite this major difference in the erythrocyte cytoskeleton from those of other cell types, some of which are discussed below, the presence of analogous cytoskeletal proteins to those of the erythrocyte at functionally important sites in other cells, (Low 1986, Black et.al. 1988, Regnouf et.al. 1985, Goldenring et.al. 1986, Howe et.al. 1985, Davis &

Bennett 1986, Bourguignon et.al. 1986, Nelson & Veshnock 1987, Harris et.al. 1986, Baines & Bennett 1986, Drenckhahn et.al. 1985, Cohen et.al. 1982) indicates that the erythrocyte cytoskeleton may be a suitable model for initial studies of cytoskeletal protein interactions, and their modulation by drugs.

Detailed studies of the human erythrocyte cytoskeleton over the past two decades have provided a detailed "map" of the probable in vivo arrangement of the cytoskeleton and the interactions between various cytoskeletal proteins and plasma membrane. Figure 2 shows a schematic drawing illustrating the transmembrane distribution and interactions of major erythrocyte cytoskeletal proteins. The names of most of the constituents are derived with reference to their relative mobility on SDS-polyacrylamide gel electrophoresis as shown in figure 3 (Fairbanks et.al. 1971).

1.1.1 The components of the human erythrocyte cytoskeleton

1.1.1.1 Spectrin

Spectrin is the major protein component of the erythrocyte cytoskeleton and constitutes approximately 75% of the cytoskeletal protein. It is believed that the coiled tetramer form is the native conformation in vivo. The spectrin tetramer is made up of two types of subunit in a configuration of $alpha_2$-$beta_2$ form (Morrow et.al. 1980, Morrow & Haigh 1983, Liu et.al. 1984). The unidentical subunits of spectrin alpha (band 1) and spectrin beta (band 2)

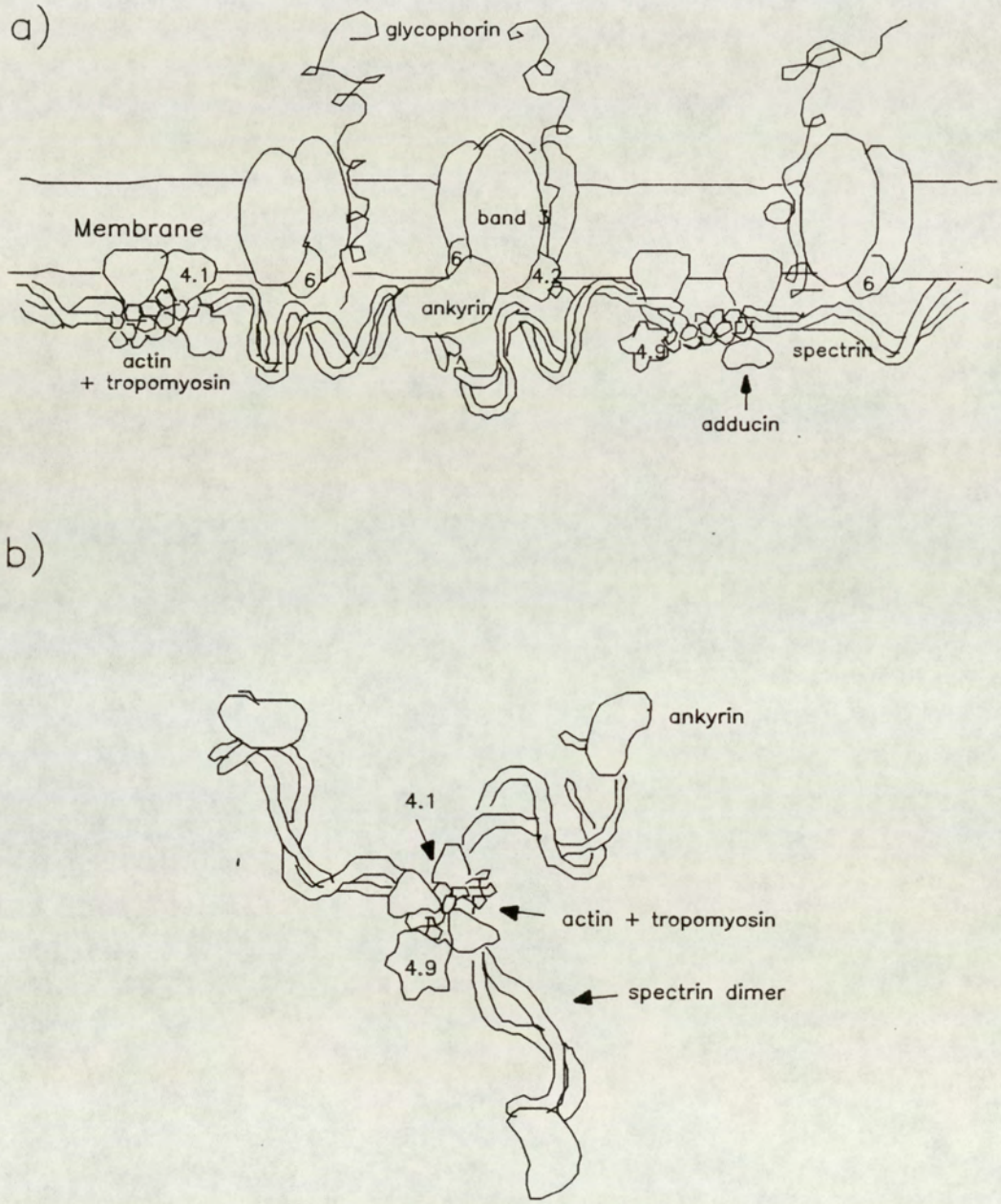


Figure.2 A schematic representation of the spatial arrangement of the human erythrocyte cytoskeleton.
The transverse view of the arrangement of the cytoskeleton (a) and the interactions between several major cytoskeletal proteins (b) are shown.

have molecular weight of 240,000 and 220,000 daltons respectively. Spectrin exists as 10^5 tetramer/cell and each alpha-beta subunit associates to form a double helical coil structure, which further interacts with another dimer to form a elongated structure in a head to head fashion. During *in vivo* assembly in avian erythroid development, there is evidence to suggest that spectrin association is a result of competition between heterodimer and homodimer formation (Woods & Lazarides 1986). Higher polymeric forms of spectrin oligomers can also be formed from spectrin monomers but it is believed that they are only formed under extreme conditions (Liu et.al. 1984). Spectrin beta chain is one of the major phosphoproteins isolated from the erythrocyte membrane. It is thought that spectrin beta phosphorylation is mediated by cAMP-independent kinase activity as well as cAMP-dependent and calmodulin-dependent protein kinases (Boivin et.al. 1981, Fairbanks et.al. 1978, Harris & Lux 1980, Nelson et.al. 1982, Speicher et.al. 1982).

1.1.1.2 Actin

Actin (band 5 on SDS-PAGE, see figure.3) is an ubiquitous protein (Pollard & Cooper 1986, Blikstad et.al. 1978) in eukaryotic cells as well as prokaryotic organisms. It has a molecular weight of approximately 43,000 daltons. Erythrocyte actin exists predominantly as the beta-actin isotype (Nakashima & Beutler 1979). It appears to have similar properties as striated muscle actin although additional properties which cannot be qualified by conventional assay

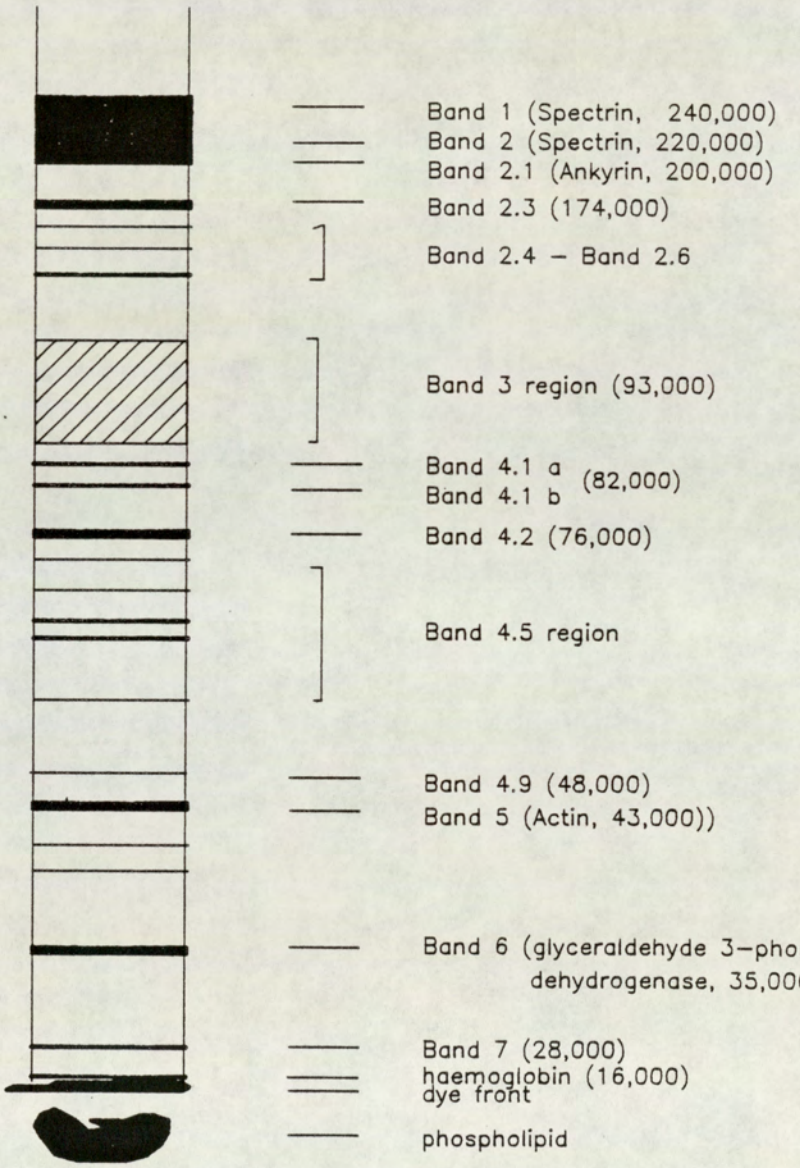


Figure.3 Diagrammatic representation of the relative mobility of erythrocyte membrane-cytoskeletal proteins separated on a 7.5% w/v SDS-polyacrylamide gel.

techniques cannot be ruled out. There is recent evidence to suggest that erythrocyte actin has a greater potential to activate a membrane-associated ATPase when compared to muscle actin (Schrier et.al. 1981).

As with actin which is present in other cell types, erythrocyte actin can associate into double helical, filamentous structures. But, instead of forming long microfilaments as in other cell types, erythrocyte actin only forms very short filaments (approx. 25nm) containing 10-13 monomers each (Pinder & Gratzer 1983). This restriction may be caused by the presence of actin binding proteins, which include capping and bundling proteins such as the spectrin/band 4.1 capping complex (Pinder et.al. 1984). By using a DNase assay to quantitate free actin monomers in erythrocytes, it has been shown that <10% of actin is present as monomers (reviewed by Marchesi 1985). Recently, tropomyosin has been identified in human erythrocytes (Fowler & Bennett 1984) and there are enough copies to saturate both grooves in each short actin filament. There are findings of longer actin filaments present in ghost preparations, after ghosts have been treated with an actin stabilising toxin (such as Phalloidin) and in Triton X-100 extracted ghosts (Atkinson et.al. 1982). The presence of endogenous actin-associated proteins in erythrocytes such as band 4.9 (actin bundling protein) and spectrin-band 4.1 complex (actin filament pointed end capping protein) further suggests that the possibility of the regulation of actin filament length by the cytoskeleton is an important component of the control of cell morphology

(Husain-Chishti et.al. 1988).

1.1.1.3 Ankyrin

Ankyrin (band 2.1, M_w 200,000) is a globular protein and it is believed that it plays a central role in linking the erythrocyte cytoskeleton to the plasma membrane (Jinbu et.al 1984, Jinbu et.al. 1982). Ankyrin was firstly identified as a high affinity spectrin binding protein. High affinity binding was observed between ankyrin and a specific segment on the spectrin beta subunit which links it to a specific site on the cytoplasmic segment of the major erythrocyte transmembrane protein, band 3 (Hargreaves et.al. 1980). However, the interaction of band 3 and ankyrin is still poorly understood (Hall & Bennett 1987). One significant discrepancy is that there are over 10^6 copies of band 3 present in erythrocytes but under 200,000 copies of ankyrin. It has been proposed that band 3 may exist as a tetramer in physiological conditions such that most band 3 binding sites for ankyrin would be occupied (Hargreaves 1980). However, there is substantial evidence to suggest that band 3 may exist as a dimer (Cuppoletti et.al. 1985). Other workers have suggested that the band 3 molecules which bind ankyrin may be different in their properties from the unassociated band 3 molecules (Bennett & Stenbuck 1980).

Recently, detailed studies of several ankyrin related polypeptides from human erythrocytes suggested an activated form of ankyrin, which may play an important role of interacting of the cytoskeletal network to the transmembrane

protein band 3 (Bennett & Stenbuck 1979a, 1979b, Hall & Bennett 1987). It was shown that one of the related polypeptides, band 2.2 (M_w 186,000), was capable to interact with twice as many high affinity sites in ankyrin-depleted inside-out membrane vesicles as compared to native ankyrin molecules (Hall & Bennett 1987). Another proteolytic form of ankyrin, which was generated through the in vitro proteolytic digestion by calpain, was also found to have twice the number of high affinity binding sites in ankyrin-depleted vesicles but only 1/8 of the affinity of band 2.2 (Hall & Bennett 1987). The importance of band 2.2 in the regulation of membrane-cytoskeleton interaction is as yet to be established.

1.1.1.4 Band 3

Band 3 (reviewed by Hargreaves et.al. 1980, Ideguchi et.al. 1982) is also one of the major cytoskeletal proteins of the erythrocyte cytoskeleton. In SDS polyacrylamide gel electrophoresis, band 3 appears as a broad and diffuse band. It is believed that the band 3 region consists of a major protein, which was characterised as an anion transporter. In addition, the region also contains minor proteins such as glycophorins. Band 3 is found to be highly glycosylated and it constitutes one of the major blood group determinant structures. Thus the diffuse appearance of band 3 may be explained by the slightly heterogeneous arrangement of the glycocalyx on the protein. The protein is securely anchored in the membrane bilayer by spanning the bilayer twelve times

(Kopito & Lodish 1985). Thus band 3 appears to provide a suitable anchoring site for the cytoskeleton.

1.1.1.5 Band 4.1

Band 4.1 includes two polypeptides (M_w 4.1a = 80,000 and 4.1b = 80,000), which are highly homologous and for which the cDNA sequence has been reported (Conboy 1986). Both polypeptides produced nearly identical peptide maps (Goodman 1982). It is widely believed that band 4.1 plays a central role in organizing and stabilizing the cytoskeletal network (Ohanian et.al. 1985). In vitro, spectrin and actin will only weakly associate and it was shown that the interaction between spectrin and actin could be strengthened by the addition of band 4.1 protein (Ohanian et.al. 1985). It has been suggested that band 4.1 may interact with calmodulin (Husain et.al. 1985) indicating it may be a calmodulin binding protein. Band 4.1 has been identified in a wide variety of cell types (Cohen et.al. 1982) but its expression is tissue specific (Granger & Lazarides 1984).

1.1.1.6 Adducin

Adducin is one of the recently characterized proteins which is associated with the erythrocyte cytoskeleton (Gardner & Bennett 1986). It was first identified in a low salt extract of the skeleton, after triton X-100 treatment. It is believed that native adducin exists in the form of heterodimers (alpha-beta) with an apparent molecular weight of 103K (alpha) and 97K (beta). The number of heterodimers was

estimated to be 30,000 copies/cell.

Adducin was first identified as a high affinity calmodulin binding protein with K_d approx. 230nM. The calmodulin binding site resides on the beta subunit of the heterodimer (Gardner & Bennett 1986). It was observed that adducin was able to bundle actin filaments by forming intrafilamental crossbridges (Mische et.al. 1987). Adducin preferentially associates with spectrin-actin complexes and binds to actin or spectrin alone with less affinity (Gardner & Bennett 1987, Mische et.al. 1987). As a result of adducin binding to the spectrin-actin complex, the capacity of further spectrin binding to the ternary complex is increased, with a maximum adducin : spectrin tetramer ratio of 1:2 (Ling et.al. 1986, Gardner & Bennett 1987). It was suggested that this increase is due to the creation of new spectrin binding sites (Gardner & Bennett 1987) in the ternary complex. This enhancement of spectrin binding is, however, antagonized by the presence of micromolar concentration of calmodulin and calcium (Gardner and Bennett 1987).

Adducin has been shown to be phosphorylated in intact cells and ghost membranes by endogenous or exogenous protein kinase C (Ling et.al. 1986). In metabolically labelled erythrocytes, it was shown that TPA treatment caused 3-15 fold increase in phosphorylation of both the alpha and beta subunits of adducin (Ling et.al. 1986). Increases in the phosphorylation of a similar protein after TPA treatment has also been reported (Palfrey & Waseem 1985, Cohen & Foley 1986). Each subunit of adducin can accept up to 3 mol of

phosphate/polypeptide (Ling et.al. 1986). Moreover, adducin was also found to be a substrate of cAMP-dependent protein kinase (Ling et.al. 1986). Nevertheless, the functional significance of the phosphorylation of adducin and the effect of phosphorylation on calmodulin binding has to be established.

1.1.1.7 Other cytoskeletal components

Apart from the major cytoskeletal proteins described above, minor constituents including band 4.2 (Korsgren & Cohen 1986), band 4.5 regions, band 4.9 (actin bundling protein?), band 6 and band 7 should not be neglected. Although the erythrocyte cytoskeleton has been under extensive investigation for more than two decades, little is known about the function of each of these constituents. Recently, it has been reported that band 4.5 is involved in nucleoside transport (Good et.al. 1987). All of the major constituents described above exist as phosphoproteins, both in vivo and in vitro. Alterations of their phosphorylation levels were observed under various conditions (see later sections). However, the precise role and effect of phosphorylation in the modulation of cytoskeleton interactions, and function of the cytoskeleton is still unresolved.

The interactions, and biochemical modulation of the major elements of the erythrocyte cytoskeleton are discussed in section 3 below.

1.1.2 Other Cytoskeletal Components not found in Erythrocytes

Investigations of cytoskeletal components and their functions were first carried out using human erythrocytes. The identification of various cytoskeletal components in human erythrocytes and the availability of information on the detailed arrangement of the cytoskeleton led to the beginnings of investigations of the cytoskeleton in higher cell types. Surprisingly, it was found that only actin, one of the major cytoskeletal proteins in erythocyte cytoskeleton, was found to be a major constituent in the "higher" cell types. In these cells, the main cytoskeletal mass was found to be located further away from the plasma membrane, when compared with that of human erythrocyte. Their cytoskeleton is composed of three main kinds of filaments; microfilaments, microtubules and intermediate filaments. These will be described.

1.1.2.1 The Microfilaments

Microfilaments are often referred to as actin filaments since they consist principally of actin (Geiger 1985). There are six different types of actin synthesized in vertebrate cells and they are mostly functionally interchangeable despite of their slight difference in amino acid sequence. Actin (M_w 41,800) normally exists in either monomeric (G actin) or filamentous form (F actin). The monomeric form consists of a single polypeptide which folds into a globular form. Under suitable conditions, G actin polymerizes into F actin, at the expense of ATP, forming double helical filaments which are

approximately 8nm in diameter. The presence of tropomyosin, which resides in the long pitched groove on either side of the actin filament, may modulate the function of the actin filament. Similar to skeletal muscle actin filaments, polymerization and depolymerization of actin monomers, as well as treadmilling, can occur at both ends of filaments in the cytoskeleton of non-muscle cells. These processes are normally regulated by actin filament-associated proteins, which have a wide spectrum of activity in determining the polymerization state of actin filaments. These proteins include various capping proteins, bundling proteins and filament crosslinking proteins. The actin binding protein, gelsolin, has been shown to bind actin filaments and block its elongation. The presence of phosphatidylinositides caused the release of gelsolin and the promotion of actin polymerization (Jammey et.al 1987). Thus actin filaments may also be interacting with or be regulated by a membrane/receptor linked mechanism (see section 1.2.4).

The role of actin filaments is mainly determined by the cell type they are associated with. In addition to its involvement in muscle contraction, actin has also been shown to be indispensable in other important functional structures (eg. Mooseker 1985, DeRosier et.al. 1980, Byers & Fujiwara 1982), and involved in cell proliferation (Schroeder 1973). In contrast to muscle cells, the roles of the contractile processes in non-muscle cells are concerned with cell survival and cellular responses to external stimuli rather than force generation. These often involve alterations of cell shape,

cell-substratum adhesiveness, cell motility and cell growth. It has been shown that the degree of actin polymerization may relate to the metastatic potential of the cancer cell (Zachary et.al. 1986). Actin thus presents itself as a potential target for anticancer drug action.

1.1.2.2 Microtubules

Microtubules are the largest amongst the three main types intracellular filaments. Microtubules are found in all eukaryotic cells, from amoebas to higher plants and animals, with few exceptions such as human erythrocytes. However, they are absent from all prokaryotic cells. Microtubules play vital role in intracellular organization. They are long fibres, about 24nm in diameter; in cross section, individual microtubules reveal a hollow centre. Crossbridges are found between adjacent microtubules, forming bundles of microtubules.

Microtubules are polymers of alpha- and beta-tubulin, each of M_w=55,000 and their structure has been well documented (reviewed by Dustin 1980). It is believed that `treadmilling' of microtubules is the main mechanism of the intracellular transport machinery. Microtubules may also be regulated by accessory proteins called microtubule associated proteins (MAPS) which regulate the formation of different forms of microtubules, thus providing differential functional characteristics, and a means of regulation. Since most of the MAPs are phosphoproteins, it is believed that microtubule assembly and their function could be regulated by

phosphorylation.

Microtubules were found to play an important role in the formation and operation of flagella and cilia (see Satir 1974 & Goodenough & Heuser 1985). In addition to the involvement of microtubules in biological motor assembly in flagella and cilia, microtubules are also found to be the major component of the intracellular transport systems (for both intracellular vesicles and organelles) (eg. Lasek 1981, Hayden & Allen 1984, Koonce & Schliwa 1985, Schnapp et.al. 1985, Vale et.al. 1985), in addition to the minor transport systems based on the actin filament system (Masters 1978). The involvement of microtubules in the regulation of cell proliferation is also well documented (reviewed by Alberts et.al. 1983, Darnell et.al. 1986)

1.1.2.3 Intermediate filaments

Intermediate filaments (Geiger 1987) are so named because their size is intermediate between actin filaments and microtubules. Intermediate filaments are generally 8-10nm in diameter and are distributed throughout the cytoplasm. Unlike actin filaments and microtubules, in which protomers are globular in nature and filament assembly is in dynamic equilibrium with a soluble pool of protomers, intermediate filaments are composed of fibrous polypeptides associated side by side to form rope-like structures, similar to that of collagen fibres, such that filaments with high tensile strength are formed. These are believed to be very important for their <u>in vivo</u> function. Intermediate filaments are

extremely insoluble and their assembly can be regarded as irreversible and so far intermediate filaments can only be dissociated by protease treatment. It is considered that *in vivo*, filament dissociation is mediated by the activation of Ca^{2+}-dependent proteases (Nelson & Traub 1981). The filaments are not in equilibrium with monomers and no monomer pool is found in the cytosol.

Intermediate filaments can be divided into five major classes: keratins (epithelial cells), desmin (Z line in skeletal muscle), neurofilaments (present in axon of nerve cells), glial fibrillary acidic protein (glial cells) and vimentin-containing fibers. Most of the filaments often terminate at the nuclear envelope and at desmosomes or adhesion plaques at the plasma membrane. In addition, intermediate filaments are often found associated with microtubules since fibrous bridges, with unknown composition, could be seen between microtubules and intermediate filaments (Hirokawa 1982) and disruption of microtubules by drug treatment often lead to the collapse of intermediate filaments to the perinuclear region (Lin & Feramisco 1981). Rapidly growing cells, such as those of early mammalian embryos, do not contain cytoplasmic intermediate filaments. With these properties and their insolubility, a structural role for the intermediate filaments is suggested.

The three filament types described above are the major constituents of the eurkaryotic cell cytoskeleton. There is much evidence that these different types of filament interact

with each other to form a continuous functional network (Hirokawa 1982) which regulates cell shape and motility, and coordinates intracellular regulatory processes. It appears that microtubules may be the major organizers of the cytoskeleton. In additional to their role in intracellular transport and a possible structural role, they also function as a temporary intracellular scaffolding for the assembly and organization of intermediate filaments and the proper functioning of actin filaments. The finding of various cytoskeletal accessory proteins and their susceptibility to covalent modification in vitro suggests that microtubules may play an important regulatory role in cytoskeletal function.

1.1.2.4 The cytoskeleton and its role in mediating morphological changes and cellular functions

Cell transformation, which often precedes tissue malignancy, has been a subject of extensive investigations. In view of the close correlation between alteration of cell shape, substratum-adhesion and motility with changes in differentiation state and cellular transformation (eg. Ungar et.al. 1986 and reviewed by Bissell et.al. 1982), recent interest has been focused on the changes in cytoskeletal-membrane functions and their relation to alterations of cellular regulation (eg. Lyass 1985). It has been shown that the extracellular matrix-induced differentiation of granulosa cells in vitro was associated with changes in cell morphology, and was accompanied by simultaneous alterations of the organization and expression of

cytoskeletal proteins that are involved in determining cellular structures (Ben-Ze'ev & Amsterdam 1986). The mitogenic hormone oestrogen and the anti-oestrogen drug Tamoxifen were shown to cause rearrangement of cytoskeletal and adhesive structure which may be related to their effects on the proliferation of the MCF-7 cells (Sapino et.al. 1986).

Detailed studies also showed that changes of cell shape, induction of differentiation, onset of cell growth and motility upon substrate contact involve modification of the cytoskeleton. Vinculin which is a component of the cytoskeleton in complex cell types, is assembled into the cytoskeleton as one of the earliest events prior to subsequent local assembly of actin-rich filament bundles in the development of substratum adhesion (Ungar et.al. 1986). These studies show that the cytoskeleton may also be a modulator of the intracellular response arising from external stimuli, such as a hormonal stimulus. Recent studies have shown that fibroblasts possess a surface receptor, the fibronectin receptor, which is responsible for the linkage between the extracellular matrix and the intracellular cytoskeletal network (Tamkun et.al. 1986, Horwitz et.al. 1986) and the receptor is subject to covalent modification by normal cellular mechanisms or products of oncogenes. (Hunter 1984 & 1985, Yarden & Ullrich 1988)

Recently, several studies, such as those described above (Lyass 1985, Ungar et.al. 1986, Ben-Ze'ev & Amsterdam 1986, Sapino et.al. 1986), suggested a close relationship between regulation of the cytoskeleton and the overall control of cell

growth and differentiation. The observation of the highly ordered protein shell of the nuclear envelope further suggested that cytoskeletal interactions may extend into the nucleus, where another skeletal protein network was documented (McKeon et.al. 1986), and which may serve as regulation of the transcriptional and mitogenic event in the nucleus as well as in controlling chromosome movement. Hence, an extracellular signal could, in theory, be transmitted through the cytoskeleton to reach the intra-nuclear protein network and/or the genome (Ingber & Jamieson 1985, Packard 1986 and reviewed by Bissell et.al. 1982). Therefore, any modification of these cytoskeletal interactions, by drugs for example, may perturb the organized network resulting in changes of cellular metabolism and/or cell behaviour. It has been observed that there are significant differences in the organization of cytoskeleton between normal cells and tumour cells (Lyass 1985). The cytoskeletal network is also under continuous regulation by means of covalent modifications (see later). It has been shown that the cytoskeleton-membrane and protein-protein interactions are subjected to metabolic regulation (Burn et.al. 1988) in vivo.

In order to study the mode of regulation of cytoskeletal interactions in vivo and the effects which may occur as a result of these modifications, a model system has to be chosen for preliminary experiments. In mammalian cells, despite the identification of several major cytoskeletal components (see above) and various accessory proteins, the function and the mode of regulation of the cytoskeleton is still not clear.

Some of these regulatory mechanisms will now be reviewed, with particular emphasis upon their possible role in the erythrocyte.

1.2. Regulation of Enzymic Activity or Protein-Protein Interactions by Covalent Modifications

In a living cell, enzymatic activities are under rigourous control in order to regulate cell function, growth and proliferation. As illustrated in the previous sections, cytoskeletal interactions may also be regulated in order to coordinate various cellular functions. Since the cytoskeleton is composed exclusively of proteins, it is likely that the intracellular regulation of cytoskeletal function may be mediated by one or more covalent modifications. Each of these will be reviewed briefly here.

1.2.1 Proteolytic modifications - calpain proteases

The proteolyic systems of human erythrocytes have been studied extensively. Proteolytic activities have been identified and located in both cytosolic and membrane fractions. At least three proteinases were identified from erythrocyte membranes (Pontremoli et.al. 1979). These proteinases belong to the acidic endopeptidase family. Recently, a significant degree of calcium activated neutral protease activity was also found associated with the membrane (Pant et.al. 1983). In contrast, the erythrocyte cytosolic fraction contains at least six proteinases, including three proteinases which have similar characteristics with those

found in the membrane fractions. The other proteinases include one neutral endopeptidase (calpain) and two dipeptidyl aminopeptidases (Pontremoli et.al. 1980).

Calpains (calcium activated neutral protease, CANP) are a family of proteases widely distributed in many tissues (reviewed by Pontremoli et.al. 1986). In general, there are two main types of calpain activity observed in various cell types: the mM species requires high calcium concentrations for its activation and proteolytic activity whilst the μM species require sub-micro molar concentrations of calcium. Recently, interest in these enzymes has been increased because they may play a significant role in controlling intracellular regulatory processes, via their actions on cytoskeletal function and other enzymic activities (Yoshida & Kimura 1985).

In the human erythrocyte, only one type of calpain is present. The purified enzyme shows a Ca^{2+} requirement, with a $K_{\frac{1}{2}}$ of 40-50μM. Thus it is belong to the mM form of calpain (Pontremoli 1986). Total recovery of the enzyme from erythrocyte cytosol can be achieved provided that the cell lysis was performed in the presence of a calcium chelator (EGTA or EDTA). Erythrocyte membranes prepared from calcium-loaded erythrocytes showed that 2% of the calpain activity was associated with the membrane fraction (Hatanaka 1984). This result suggested that calpain may transiently translocate to the inner leaflet of the plasma membrane after calcium activation.

Erythrocyte calpain is present in the cytosol as an proenzyme (M_w 110,000) and is believed not to belong to the

papain protease family (Metrione 1986). This proenzyme is composed of two subunits; one polypeptide of 80,000 as the catalytic subunit, the other polypeptide of 30,000 with unknown function. The proenzyme can be converted into the fully active form by two alternative mechanisms and they are summarised in figure 4. Firstly, at high calcium concentrations (in mM range), the activation is a two step process. The 110K complex is dissociated into the 80K and 30K subunits follow by autolytic activation of the 80K subunit into a 75K fully active form (Pontremoli et.al. 1984, Imajoh et.al. 1986). The alternative mechanism is the binding of the 80K subunit to the plasma membrane in the presence of low calcium (in μM range). Autolytic activation is followed by membrane binding and evidence suggests that membrane phospholipid, possibly phosphatidylinositol (Coolican et.al. 1984), is responsible for the lowering of the calcium requirement for the conversion of the 80K subunit to the 75K fully active moiety (Pontremoli et.al. 1985). The active moiety may finally be inactivated through self digestion, into a 36K fragment (Grasso et.al. 1986, Kuboki et.al. 1987).

A natural endogenous inhibitor of calpain (calpastatin) is also present in the erythrocyte cytosol. This inhibitor has a molecular configuration of 4x60K and it reversibly binds to calpain only at high calcium (approx. 1mM) (Melloni et.al. 1982) to form a inhibitor (60K) + calpain (80K) complex. Since the binding only occurs at high calcium, the possible role of the inhibitor may be to control free calpain proenzyme

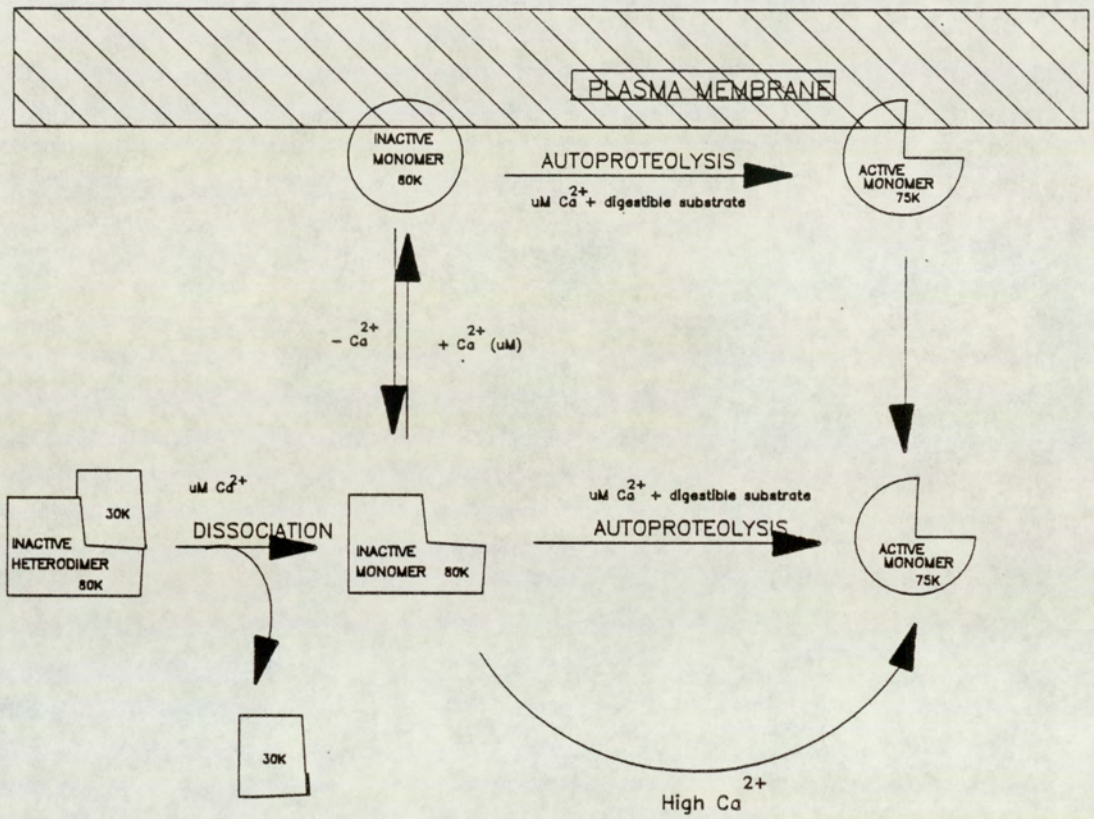


Figure.4 Diagrammatic representation of the translocation and activation of the calcium-dependent thio protease, calpain, and its regulation by an endogenous inhibitor complex.

levels during intracellular fluctuations of calcium, or in old erythrocytes where the calcium level is elevated.

It is believed that the presence of calpain in erythrocytes is responsible for the degradation of various intracellular proteins including excess haemoglobin (Pontremoli et.al. 1984) and cytoskeletal proteins (Grasso et.al. 1986). It was observed that in the presence of a digestible substrate, the calcium requirement for full activation (forming 75K species) is reduced to 1-5μM (Melloni et.al. 1984). This suggests that calpain may have a modulatory role in the control of limited proteolysis in vivo. Calpain was also found to act on cytoskeletal proteins (Verhallen et.al. 1987, Billger et.al. 1988, Inomata et.al. 1986, Yoshida 1985, Pontremoli et.al. 1987, Ruggiero et.al. 1985) and it enhanced the activity of a protein phosphatase activity (Waelkens et.al. 1985) in other cell types. Recently, alterations of calpain activity were also found associated with certain cellular abnormalities such as in subjects who suffer from hypertension (Pontremoli et.al. 1986, Pontremoli et.al. 1988, Morelli et.al 1987) and proteolytic modification of cytoskeletal proteins was observed. However, the role of calpain in the onset of various pathological symptoms has to be clarified.

1.2.2 Phosphorylation/dephosphorylation of intracellular proteins

Protein phosphorylation is one of the most common endogenous regulatory processes (reviewed by Krebs 1985) and

it has been under extensive research since the discovery of cAMP and adenylate cyclase. In general, protein phosphorylation involves the transfer of the gamma-phosphate group from ATP to a hydroxy group on a protein by the catalytic action of a protein kinase. In normal cells, phosphorylation is predominantly carried out at serine and threonine residues and tyrosine phosphorylation comprises only <0.3% of the total phosphorylated residues. The findings that various oncogene products and growth factor receptors possess tyrosine protein kinase activity has raised interest in the phosphorylation of this residue (reviewed by Hunter 1984 & 1985, Yarden & Ullrich 1988). Modulations by phosphorylation are subject to counterbalance by the activities of various protein phosphatases present in the cytosol (Ingebritsen & Cohen 1983). The net balance between the systems, of phosphorylation and dephosphorylation, determines the overall phosphorylation state of a particular site on a protein.

There are several protein kinases systems which have been identified in eurkaryotic cells, and these have been investigated extensively. These included the cAMP-dependent protein kinase (sometimes refer as protein kinase A), Ca-calmodulin-dependent protein kinases, Ca-dependent calmodulin-independent protein kinases, the protein kinase C family and other identified minor protein kinases with specific characteristic kinase activity. Each of these will be reviewed briefly.

1.2.2.1 cAMP-dependent protein kinase

cAMP-dependent protein kinase was the first protein kinase found in eukaryotic cells. As its name suggests, it is activated by the binding of cAMP, which is synthesized by adenylate cyclase associated with the plasma membrane (reviewed by Limbird 1981). Adenylate cyclase is the effector for certain extracellular stimuli. Activation of the cyclase is a result of agonist binding to certain receptors. The finding of an involvement of guanine nucleotide binding proteins (G proteins) (Bokoch et.al. 1984, Katada et.al. 1984a, Katada et.al. 1984b, Katada et.al. 1984c) in receptor-cyclase coupling has added a new dimension to receptor research. There are two G proteins, G_s (for stimulatory) and G_i (inhibitory protein). These couple receptor stimulation to cyclase activation or inhibition. (reviewed by Gilman 1987)

cAMP-dependent protein kinase is activated during cAMP elevation by the binding of cAMP to a regulatory subunit, releasing an active catalytic subunit. cAMP-dependent protein kinase phosphorylates predominantly serine and threonine residues in target proteins. In human erythrocytes, addition of a membrane permeable analogue of cAMP has been shown to increase the phosphorylation level of certain cytoskeletal proteins (Horne et.al. 1985).

1.2.2.2 The phosphatidylinositide pathway and protein kinase C

Phosphoinositides are phospholipids which are present in minor quantities in cell membranes, where they are mostly compartmentalized in the inner membrane leaflet. There is

overwhelming evidence to support the role of phosphoinositides (Nishizuka 1983, Berridge 1985, Kirk et.al 1985, Parthasarathy & Eisenberg 1986, Marx 1984) as intracellular signals and their role in the regulation of cell proliferation (Forer & Sillers 1987, Newmark 1985). In more complex cells, PtdIns(4)P and PtdIns(4,5)P2 are hydrolysed into InsP2, and InsP3 (Berridge & Irvine 1984, Berridge 1984) and diacylglycerol by a receptor-linked, PtdIns-specific phospholipase C (PI-PLC) as a result of receptor stimulation by an agonist (figure.5). The activation of PI-PLC by the receptor-agonist complex is believed to be mediated through guanine nucleotide binding proteins different to those associated with the adenylate cyclase system (Joseph 1985, Proud et.al. 1986). The InsP3 released is believed to be responsible for the Ca^{2+} release from the intracellular Ca^{2+} store (believed to be the endoplasmic reticulum) (Streb et.al. 1983, Joseph 1984, Prentki et.al. 1984). In addition, the diacylglycerol (DG) moiety released is capable of activating a calcium-activated phospholipid-dependent protein kinase (PK-C) in the presence of low $[Ca^{2+}]_i$. Interest in the phosphoinositides was heightened when several oncogene products and hormone receptors (Hunter 1984, Hunter 1985, Weinberg 1985, Van Obberghen et.al. 1985, Yu et.al. 1985, Goustin et.al. 1986, Ross et.al. 1986, Ullrich et.al. 1985, Ullrich et.al. 1986, Ellis et.al. 1986, Rosen et.al. 1983, Yarden & Ullrich 1988), which are tyrosine protein kinases, were also shown to possess possible phosphatidylinositol kinase activity (Macara et.al. 1984, Sugimoto et.al. 1984,

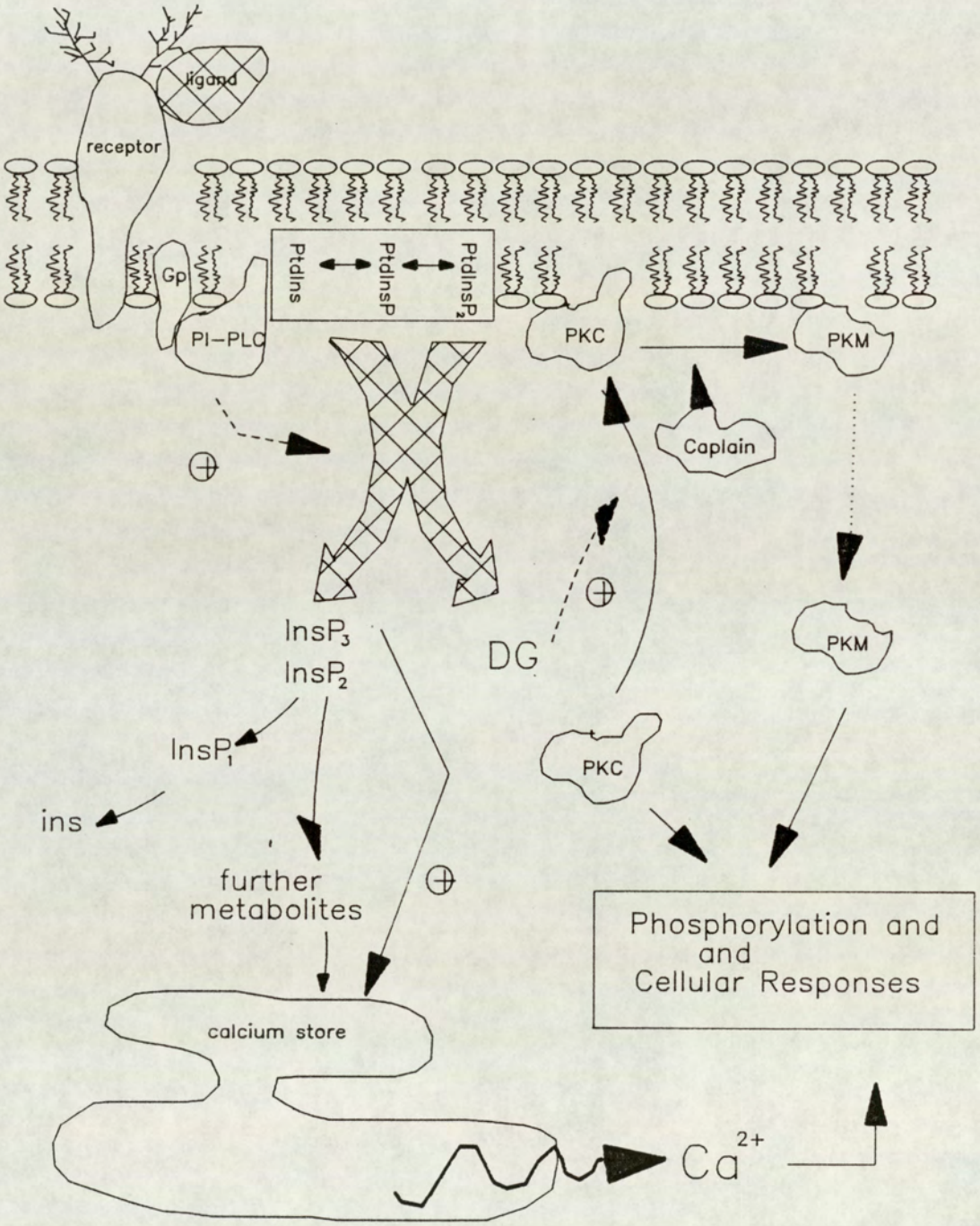


Figure.5 Illustration of a simplified pathway of the Phosphoinositides cascade and its contribution to other cellular regulatory processes.

Baldi et.al. 1986, Stoscheck & King 1986). This suggested that the presence of these proteins may accelerate and amplify the phosphatidylinositol cycle during receptor stimulation (see figure 5). However, doubts were raised regarding these findings and the association between certain oncogene product activity and inositide metabolism is still a controversy. (MacDonald et.al. 1985).

Protein kinase C (reviewed by Ashendel 1985, Schwantke et.al. 1985, Bell 1986, Ullrich et.al. 1986, Nishizuka 1984, Nishizuka 1986, Rosen & Koshland 1987, Nishizuka 1988) was found to be the receptor for the diacylglycerol arising from phosphoinositide hydrolysis. Recent interest in this pathway has arisen after the finding that protein kinase C was also the major receptor responsible for the binding of several tumour promoters (Castagna & Nishizuka 1982). Tumour promoters such as phorbol 12-myristate 13-acetate (TPA) and phorbol 12,13-dibutyrate in nanomolar concentration were found to compete with the binding of diacylglycerol to protein kinase C. This suggested that protein kinase C may be an important enzyme involved in the process of tumour promotion.

Protein kinase C was first described by Nishusuki et.al. as a calcium-activated phospholipid-dependent protein kinase (Nishusuki et.al. 1979). The activation process required phosphatidylserine as a co-factor, whose binding resulted in a dramatic reduction of the calcium requirement for activation of kinase activity. It was shown that activation of protein kinase C was an event which followed the adrenergic receptor

(alpha$_2$ subtype) binding of agonist on the cell surface. It is widely believed that protein kinase C activation is one of the consequences of receptor-agonist binding, certain ligand binding leads to the activation of phosphatidylinositol-specific phospholipase C (PI-PLC) possibly via other G proteins (reviewed by Birnbaumer et.al. 1987) which then results in phosphoinositide hydrolysis. One of the resulting products of this hydrolysis, diacylglycerol (see below and figure 5), causes the activation of protein kinase C. Protein kinase C normally resides in the cytosol. In the presence of diacylglycerol, the calcium requirement is dramatically reduced (Wolf et.al. 1985a). This results in the binding of the protein kinase C-diacylglycerol-Ca^{2+} complex to the plasma membrane (Wolf et.al. 1985b, Ganong et.al. 1986). This translocation process has been demonstrated in a wide variety of cell types (eg. Shoji et.al. 1986, Guy et.al. 1986). It is believed that the binding occurs at membranes which contain phosphatidylserine.

Protein kinase C is a widely distributed and ubiquitous enzyme found in both in animals and plants. The highly homologous amino acid sequence which exists between different species suggests the importance of this enzyme in cellular regulatory processes. Protein kinase C has a molecular weight of around 80,000 Dalton. Recently, the techniques of molecular biology have revealed that protein kinase C is a large family of enzymes, comprising a seven or more isozyme types (Knopf et.al. 1986, Kikkawa et.al. 1986, Kikkawa et.al. 1987, reviewed by Nishizuka 1988). There is evidence

which indicates that different subspecies of protein kinase C may be distributed differentially amongst different tissue types according to their functions (Ohno et.al. 1987). This may provide the degree of specificity for the proper metabolic coordination of the organism with respect to both enzyme diversity and substrate specificity.

Protein kinase C is found to phosphorylate only serine and threonine residues in target proteins (Kishimoto et.al. 1980). Autophosphorylation was also described for protein kinase C when it was studied in vitro (Rosen & Koshland 1987). However, the role of its autophosphorylation remains to be established. Extensive studies are underway to investigate protein kinase C mediated phosphorylation in vivo in several cell types. Protein kinase C was found to have a wide spectrum of substrate activity as well as interacting with other signalling pathways (Downward et.al. 1985). Early studies suggested that treatment of cells with either TPA or diacylglycerol resulted in the elevation of intracellular pH, which is thought to be an important signal in the initiation of cell proliferation (Moolenaar et.al. 1984, Moolenaar 1986). Several phosphoproteins were identified, which were thought to be phosphorylated as a result of protein kinase C activation, after cells were treated with particular receptor agonists or phorbol esters (Litchfield & Ball 1986). One of the substrates was identified as vinculin, a cytoskeletal protein in fibroblasts thought to be important in the control of the interaction and anchoring of actin filaments to plasma membrane (Wilkins & Lin 1986). Treatment of cells with

phorbol esters has also been shown to cause reorganization of actin filaments and a protein analogue to spectrin (Sobue et.al 1988). Thus, protein kinase C activation may have a significant role in the re-organization of the cytoskeletal network which takes place upon cell stimulation (Kellie & Bissell 1985, Hoshi et.al. 1987, Hoshi et.al. 1988). In addition, protein kinase C mediated nuclear matrix protein phosphorylation was also reported (Macfarlane 1986). It was also shown that protein kinase C activation led to a modulation of the activity of membrane calcium channels, which are believed to play a role in maintaining both the intracellular calcium and the response to certain cell stimuli (Harris et.al. 1986). Protein kinase C was also found to phosphorylate a calmodulin-dependent protein phosphatase (Tung 1986) and although phosphorylation did not result in significant changes in activity, alteration of enzymic characteristics such as substrate specificity could not be ruled out. Recently, the use of phorbol esters and related compounds, especially TPA and bryostatins (Kiss et.al. 1987), and several protein kinase C inhibitors, has permitted the elucidation of processes related to cell differentiation and proliferation with regard to the activation of protein kinase C (Schwantke et.al. 1985, Forsbeck et.al. 1985, Fontana et.al. 1986, Perrella et.al. 1986, Helfman et.al. 1983, Girard et.al. 1986, Angel et.al. 1987, Adamo et.al. 1986, Proud 1986). The involvement of protein kinase C in differentiation is further suggested by the finding that TPA, being a differentiation inducer in HL-60 leukemic cells,

caused protein kinase C translocation in sensitive cells but failed to do so in resistant cells (Honma et.al. 1986). There is also accumulating evidences to suggest that protein kinase C has regulatory role in gene expression (Kariya et.al. 1987) such as decreasing the in vivo translational inhibitor activity (Konno et.al. 1986) although a direct action of TPA is not ruled out. Protein kinase C was also found to increase DNA polymerase alpha (Sylvia et.al. 1986) and RNA polymerase II activity, suggesting that the phosphorylation of these enzymes may be involved in their action (Chuang et.al.1987).

Further studies of protein kinase C have provided another possible and alternative mechanism for its activity. Several reports have been able to show that protein kinase C is a substrate for the calcium-dependent thio protease, calpain (see above). The proteolytic cleavage of protein kinase C resulted in the generation of a second protein kinase, protein kinase M (M_w58000, see figure.5) (Girard et.al. 1987). Protein kinase M is also a serine/threonine protein kinase but its activity is no longer calcium activated nor phospholipid dependent and it may have a different substrate specificity. It has been shown that activation of protein kinase C by TPA or an appropiate agonist resulted in rapid down regulation of the kinase (Grove & Mastro 1988, Glynn et.al. 1986, May et.al. 1985) with a $T_{\frac{1}{2}}$ of approximately two hours when 400nM TPA was used (Ballester & Rosen 1985). It was shown that the effect of down regulation is mainly due to the proteolytic modification by calpain into protein kinase M (Tapley & Murray 1985, Pontremoli et.al. 1986, Melloni et.al. 1986,

Pontremoli et.al. 1987, Mizuta et.al. 1985, Young et.al. 1987). This is followed by further cleavage to lower molecular weight species, which are probably enzymatically inactive. Restoration of protein kinase activity is mainly accounted for by new protein synthesis. Thus this system provides a sophisticated pathway and controlled mechanism by which agonists which induce phosphoinositide hydrolysis lead to the elevation of diacylglycerol and intracellular calcium levels: the diacylglycerol moiety activates protein kinase C, and this is followed by its interconversion to protein kinase M. Finally, it is inactivated by calpain (Tapley & Murray 1985), which is itself activated as a consequence of the elevation of intracellular calcium. This implies a close relationship between the activity of calpain and protein kinase C. It was shown that calpain was mainly localized near the plasma membrane-cytoskeleton boundary in cells and there are difficulties in separating protein kinase C and calpain activities (Savart et.al. 1987)

1.2.2.3 Other protein kinase systems

Various other protein kinase systems have also been described. Apart from cAMP-dependent protein kinase and protein kinase C as described above, protein kinases activated by calcium and/or calmodulin are amongst the most extensively studied. They are also specific for threonine and serine residues. Calmodulin, being an ubiquitous protein, is widely involved in cellular control processes including muscle contraction and cellular metabolism (reviewed by Cheung 1980,

Cheung 1982). These regulatory activities are mediated by the binding of calmodulin, in the presence of an elevated calcium level, to the target protein with a specific function. Such a protein can be a regulatory subunit of an effector complex such as a protein kinase. Several protein kinases have been identified such as calmodulin-dependent protein kinases in various tissues, including human erythrocytes (Cohen & Foley 1986). It has been observed that calmodulin activated the calmodulin-dependent protein kinase II which resulted in the association of the kinase with cytoskeletal elements and autophosphorylation of neural tissue (Le Vine III et.al. 1986). Apart from calmodulin-dependent protein kinases, there are also various kinase activities in vitro which are calcium-dependent or independent. Although these kinases only constitute a minor proportion of the total intracellular kinase activity, the potential for their involvement in regulating intracellular processes should not be overlooked.

Another protein kinase activity which although constituting a minority of the total kinase activity, is attracting current interest is the tyrosine protein kinase family of proteins (Gammeltoft & Obberghen 1986). These kinases include the translational products of several oncogenes (Stiles 1985, Dean et.al. 1985). It has been shown that tumour cells contain a significantly higher level of tyrosine phosphorylation (Seki et.al. 1986). The oncogene product $pp60^{v\text{-}src}$ phosphorylates protein phosphatase I and inactivates phosphatase activity (Johansen & Ingebritsen 1986). Although tyrosine phosphorylation has been studied

extensively, especially since the discovery of the insulin receptor associated tyrosine protein kinase activity, the role of the activity and nature of the physiological substrates in vivo are as yet undetermined.

In human erythrocytes, the presence of protein kinase C in the cytosol has been reported. It was shown that treatment of human erythrocyte with calcium, diacylglycerol or TPA resulted in elevation of the phosphorylation level of several proteins (Raval & Allan 1985, Palfrey & Waseem 1985). There is evidence that pathological conditions such as sickle cell anemia are associated with elevated intracellular `free' calcium levels and the activation of membrane associated protein kinase C of human erythrocytes (Pershadsingh et.al. 1987, Johnson et.al. 1986). Alteration of the phosphorylation of proteins was observed when erythrocyte membranes were treated with other agents such as cAMP analogues and elevated intracellular calcium levels (Horne et.al. 1985). Although protein phosphorylation has been described as the most common covalent modification amongst all regulatory mechanism, however, the role of phosphorylation changes in cytoskeletal proteins in erythrocytes is controversial. Recently, it was speculated that phosphorylation may play a role in erythrocyte cytoskeletal assembly, but little role in its subsequent activity (Backman 1988).

1.2.3 Transglutamination

Transglutaminases are a family of enzymes often involved

in catalysing the covalent condensation between glutamine and lysine residues in proteins to form high molecular weight protein aggregates (reviewed by Gaffney 1985). The enzymes are found in both extracellular and intracellular sites and are often activated in the presence of elevated calcium level. The existence of the reaction in Ca^{2+}-loaded cells has been well documented (see review above). Although the enzyme has been identified for more than a decade, the physiological role of the intracellular enzyme is still undetermined. Recently, transglutamination has been suggested to be involved in cell activation and differentiation (Lee et.al. 1985). Transglutaminase reactions were also found to play a role in the inhibition of adenylate cyclase in pigeon erythrocyte membrane preparations (Porta et.al. 1986). In human erythrocytes, it has been shown that the elevation of cytosolic calcium concentrations resulted in the activation of the calcium-activated transglutaminase, which catalyses the formation of high molecular aggregates between several cytoskeletal proteins. These included spectrin, band 3, band 2.1 and band 4.1 (Lordland et.al. 1983). Similarly, the consequences of these reactions for the control of cytoskeletal function are still unclear.

1.2.4 ADP-ribosylation

Descriptions of the ADP-ribosylation of proteins has generated considerable interest in the biochemical community. It has been described in many cell types, especially concerning the activation/deactivation of guanine nucleotide

binding proteins in hormonal signalling. It has also been found that ADP-ribosylation is a normal covalent modification in vivo and the enzymes involved have been described (Gaal & Pearson 1986). Recently, an endogenous NAD-ribosylase was identified in human erythrocytes (Tanuma et.al. 1988) but its role in the control of cellular function remains to be established.

1.2.5 Fatty acylation of proteins

Recent evidence has shown that several proteins isolated from cells were covalently attached with fatty acid (Schmidt 1982, Towler & Glaser 1986a, Towler & Glaser 1986b, Wold 1986). The oncogene product pp60src was amongst the first to be described as fatty acylated. The attachment of a fatty acyl group may serve as a tool to anchor and stabilise the interaction between the acylated protein and the membrane lipid bilayer. It has been shown that the oncogene product, $pp60^{src}$, could transform cells into the malignant phenotype provided that it was fatty acylated and that the de-acylated form was inactive in transformation (Gibbs et.al. 1985). A similar finding was also reported for the actin binding protein, vinculin: it was shown that vinculin acylation was closely related to cellular transformation (Burn & Burger 1987).

The most common fatty acid found in acylated proteins are myristic acid, stearic acid and palmitic acid. There is evidence that myristic acid acylation only occurred at an extremely early stage in the life of the protein, possibly

co-translationally or shortly after. Both membrane and cytosolic proteins are susceptible (Olson & Spizz 1986) of myristoylation. In contrast, palmitic acid attachment occurs post-translationally and exclusively on membrane proteins. Palmitization is highly reversible and there is rapid turnover (Staufenbiel 1987) thus it is suitable as a mean of covalent regulation.

Recent evidence has shown that ankyrin (band 2.1) and several other membrane-cytoskeletal proteins in chicken erythrocytes are palmitoylated (Staufenbiel & Lazarides 1986, Staufenbiel 1987). Thus ankyrin acylation may also play a role in stabilising the membrane-cytoskeletal interaction and may be subjected to regulation. There are also reports that purified band 4.1 is associated with fatty acids. It was shown that each purified band 4.1 molecule contained a stearic acid and two palmitic acid molecules (Niggli & Burger 1987). Thus it was suggested that band 4.1 is capable of directly interacting with the membrane bilayer.

1.2.6 Methylation of proteins

Protein methylation has been studied in micro-organisms, and related to their chemotaxis ability, for a considerable period of time (eg. Springer et.al. 1979). Protein methylases transfer the labile methyl group from S-adenosylmethionine to hydroxyl groups on the target protein. Recently, protein methylation was also documented to have a regulatory role in eukaryotic cells. Its involvement in the chemotaxis behaviour of leukocytes may suggest possible related mechanism to micro-organism chemotaxis. Since

leukocytes do not possess flagella or related structures, their mobility is believed to be mediated by the mobility of the cytoskeleton. Thus effects of intracellular protein methylation on cytoskeletal function could not be ruled out.

It has been shown that the methylation of membrane proteins also takes place in human erythrocytes as a rapid event and the process may be subject to biochemical regulation (Maeda et.al. 1986). Several cytoskeletal proteins including band 2.1, band 3, band 4.1, band 4.5 and glycophorins (Kim et.al. 1980, Galletti et.al. 1986) were shown to be carboxy-methylated in vivo (Lou & Clarke 1986, Lou & Clarke 1987). Detailed studies in human erythrocyte have shown that upon Ca^{2+} treatment (40uM), there was a >50% decrease in membrane protein methylation which could be antagonized by EGTA (Galletti et.al. 1986). Such treatment also resulted in the alteration of the functional characteristic of membrane proteins (Lou & Clarke 1987).

1.2.7 Covalent attachment of phosphatidyl inositol

In addition to the phosphoinositides participating in the mediation of the generation of second messengers, recent studies have revealed that several membrane proteins are covalently linked to phosphatidylinositol (Low & Kincade 1985, Low et.al. 1986, Low & Saltiel 1987). Since the phosphatidyl inositol linkage is equally susceptible to PI-PLC action as in other phosphoinositides, similar events which cause the hydrolysis of phosphoinositides may also cause the hydrolysis of the glyco-conjugate. Recently, it has been proposed that

the hydrolysis of the glyco-conjugate may be the intracellular second messenger for insulin action and more work is require to clarify the second messenger role of the hydrolysed products (reviewed by Low & Saltiel 1987, Ferguson & William 1988). So far the phosphatidylinositol covalent linkages found were restricted to membrane proteins residing in the outer membrane leaflet of the plasma membrane. However, there is the possibility that cytoskeletal proteins also possess this linkage to the inner leaflet, hence a susceptibility to PI-PLC hydrolysis cannot be ruled out. Such an arrangement and PI-PLC action on the protein would cause the proteins to become detached from the membrane bilayer and may thus have considerable influence on cytoskeleton-membrane interactions.

1.3. Interactions within the Membrane-Cytoskeleton and Their Possible Role in the Control of Morphological Transformations.

Human erythrocytes normally adopt the discoidal morphology (discocyte). Under various conditions, human erythrocytes undergo morphological transitions to form one of several morphologies (for review see Bessis 1973). These include echinocytes, stomatocytes, spherocytes, and elliptocytes. Certain of these erythrocyte morphologies could be observed in blood samples from patients with genetic disease (Palek & Lux 1983, Schmid-Schönbein et.al. 1986a, Schmid-Schönbein et.al. 1986b). Most of the symptoms are associated with deficiencies or defects in one or more cytoskeletal proteins. This

suggests that the cytoskeleton may play an important role in determining erythrocyte morphology.

Under artificial conditions, erythrocytes undergo particular morphological transitions according to the conditions employed: ATP-depletion, calcium loading of erythrocyte in the presence of calcium ionophore, or treatment with certain anionic drugs or chemicals results in the formation of echinocytes. On the other hand, treatment of erythrocytes with certain cationic agents, such as the phenothiazines (e.g. trifluoroperazine), results in the rapid formation of stomatocytes followed by endocytosis (see review Bessis 1973). In 1974, Sheetz and Singer (1974) postulated the `bilayer couple hypothesis' which suggested that morphological changes are due to a shift of the equilibrium distribution of lipid molecules, and thus charge, in the two leaflets of the bilayer. This hypothesis can be used to account for the shape changes observed when erythrocytes are treated with amphipathic molecules (of different charge), which consequently have different affinities for the two leaflets. Since the inner leaflet of the plasma membrane possesses a net negative charge, this may facilitate the entry and determine the distribution of charged species between the bilayer leaflets.

For more than a decade, attempts have been made to put forward hypotheses which account for the mechanistic basis of these morphological transformations. Early suggestions put forward attempted to correlate spectrin phosphorylation and

the regulation of erythrocyte morphology under various conditions. The effect of a cAMP-independent protein kinase on spectrin phosphorylation was also examined (Birchmeier & Singer 1977, Sheetz & Singer 1977). According to their model, dephosphorylation of spectrin would result in rearrangement of the cytoskeletal network and the contraction of the inner membrane leaflet. This model was undermined by reports which showed that there was no correlation between spectrin phosphorylation and morphological changes (Anderson & Tyler 1980, Patel & Fairbanks 1981). They showed that echinocytosis largely preceded spectrin dephosphorylation in intact erythrocytes. Addition of MgATP to membrane ghosts could determine the morphological state of the ghost even when all the spectrin kinase activity has been extracted. However, there were also reports to suggest the possible involvement of spectrin phosphorylation in the regulation of cytoskeletal protein interactions (Maretzki & Lutz 1986, Anderson & Morrow 1987). Thus, further investigations are required to clarify the role of spectrin phosphorylation in the regulation of erythrocyte morphology.

The close resemblance in shape of membrane ghost and `triton shell' (prepared by treatment of erythrocytes with the non-ionic detergent, triton X-100) to the morphology of the cells they derived from regardless of the morphological state of the cell, has been interpreted as evidence to suggest a regulatory role of the cytoskeletal network in the control of cell shape (Yu et.al. 1973, Sheetz et.al. 1979). The findings that isolated triton shells of erythrocyte were able

to maintain the discoidal shape (Yu et.al. 1973, Sheetz 1979), and exhibited MgATP-dependent expansion that reverses the shrinkage induced by 0.1-1.0μM calcium (Jinbu 1984a, Jinbu 1984b) and the observation that isolated triton shells were still capable of undergoing morphological changes during preparation (Sheetz 1979) further suggested the possibility of additional or alternative mechanisms of morphological control under certain conditions.

Phospholipid turnover has also been suggested to be involved in the regulation of shape control. Allan and Michell (1974) showed that both ATP-depletion and calcium loading of human erythrocytes resulted in accumulation of diacylglycerol. It was subsequently found that the diacylglycerol was generated as a result of phosphoinositide hydrolysis (Allan & Michell 1978, Downes & Michell 1981, Allan & Cockcroft 1983). Recently, Ferrell and Huestis (1984) showed that ATP-depletion of human erythrocytes led to a decrease in the level of phosphatidylinositol 4,5-bisphosphate in the erythrocyte membrane. This was parallel to the progress of echinocytosis induced by ATP-depletion. They also showed that reversal of echinocytes to discocytes also correlated with the re-generation of phosphatidylinositol 4,5-bisphosphate. The role of phosphatidylinositol 4,5-bisphosphate - and thus a role for the lipid bilayer in shape control - was further supported by the report that treatment of cells with vanadate during ATP-depletion accelerate both phosphatidylinositol 4,5-bisphosphate breakdown and echinocytosis in a parallel manner (Backman

1986).

Various attempts have been made to account for the discoidal shape by using sophisticated theoretical, mathematical calculations regarding the physical properties of the lipid leaflets (reviewed by Brailsford et.al. 1976, Elgsaeter et.al.1986).

Other mechanisms have also been proposed by Jinbu and his coworkers. They observed that ATP-dependent shape change of resealed ghost occurred in two stages, a slow and a rapid step (Jinbu et.al. 1983, Jinbu et.al. 1984a, Jinbu et.al. 1984b). They proposed that membrane protein phosphorylation as well as a non-covalent ATP-dependent process were necessary for the morphological changes. They also suggested that the integrity of the cytoskeletal protein, ankyrin, may play an important role in modulating cell shape by interacting with the transmembrane protein band 3, thus affecting the anchoring of the cytoskeletal network to the membrane bilayer (Jinbu et.al. 1982, Jinbu et.al. 1984c).

The complexity of this morphological regulation was confirmed by the identification of several proteins which are members of the actomyosin system. These included myosin, tropomyosin and a tropomyosin binding protein (Fowler et.al. 1985, Wong et.al. 1985, Metovcik et.al. 1986, Fowler & Bennett 1984, Fowler 1987). This raises the possibility that a functional actomyosin system may be presented in human erythrocytes and it could be involved in the control of shape

change.

Various investigations have been made of the interactions between the different cytoskeletal elements of the erythrocyte. As described in section 2 above, ankyrin is thought to be crucial in the anchoring of the cytoskeletal network to the lipid bilayer via its interaction with the transmembrane protein, band 3. Recent studies have also proposed a possible additional anchoring site: it was shown that purified band 4.1 bound phosphatidylserine containing vesicles (Cohen et.al. 1988) suggesting a possible direct interaction between band 4.1 and the plasma membrane. Since band 4.1 plays an important role in stabilising the interaction between spectrin tetramers and actin oligomers (Correas et.al. 1986, Podgorski& Elbaum 1985), direct anchoring of the cytoskeletal network is possible with band 4.1. Further studies of band 4.1 revealed that it also directly interacts with other components of the plasma membrane. It has been shown that band 4.1 was capable of binding to the transmembrane proteins band 3 and glycophorin (Anderson & Lovrien 1984, Anderson & Marchesi 1985). In addition to its binding to phosphatidylserine containing membranes, it was also found that band 4.1 bound specifically to phosphatidylinositol-4,5-bisphosphate and the binding was greatly enhanced in the presence of glycophorins (Anderson & Marchesi 1985, reviewed by Niggli & Burger 1987). Further involvement of phosphoinositides in the regulation of cytoskeleton-membrane interactions was indicated by the increase in glycoprotein mobility within erythrocyte membrane

as a result of increased phosphoinositide levels (Sheetz et.al. 1982) and there are reports to indicate that phophoinositides may be involved in the regulation of membrane-cytoskeleton interactions (Burn 1988).

As described earlier, phosphorylation may also regulate various cytoskeletal functions. It has been reported that a decrease in the phosphorylation level of band 4.1 enhanced the interactions between spectrin tetramers and actin filaments (Cohen et.al. 1986). As a result of band 4.1-spectrin association, the complex may limit the mobility of proteins and lipids (Mohandas et.al. 1985, Forte et.al. 1985) in the lipid bilayer. There is evidence that band 4.1 and band 4.9 are substrates for both protein kinase C and cAMP-dependent protein kinase and it was shown that these protein kinases phosphorylated difference sites on the proteins (Horne et.al. 1985). A decreased phosphorylation level of band 2.1 (ankyrin) has also been reported to increase its interaction with the spectrin tetramer (Lu et.al. 1985), which may stabilise the anchoring of the network to the membrane. Recently, there are reports to suggest phosphorylation and dephosphorylation cycles are important in the control of shape change in other cells (Zhang et.al. 1988).

Until recently, most of the studies of phosphorylation concentrated on determining the role and regulation of serine/threonine phosphorylation in the erythrocyte cytoskeleton. It was then found that the transmembrane protein, band 3, also contained phosphotyrosine residues (Boivin et.al.1986). It has been shown that band 3 is a

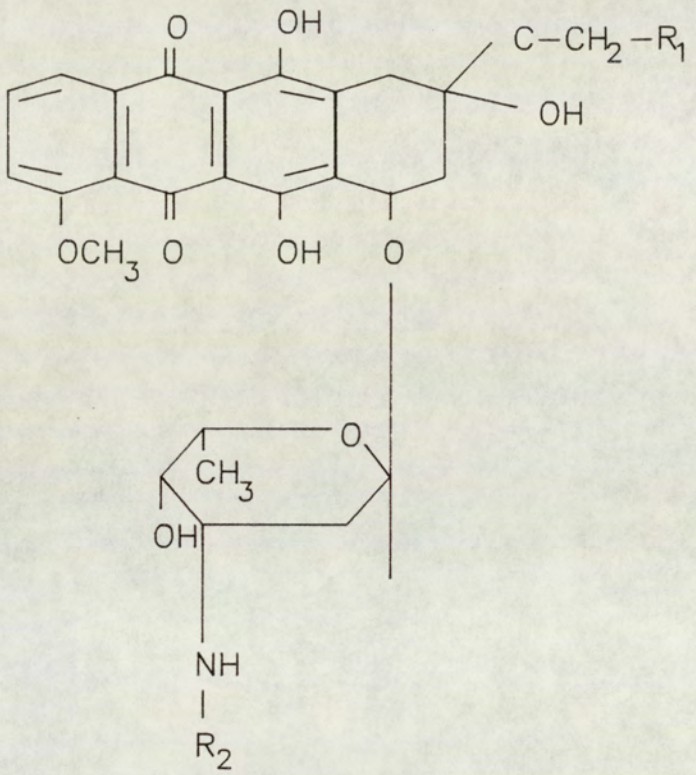
substrate for the EGF receptor and pp60^{src} *in vitro* (Shiba et.al. 1986). The tyrosine kinase responsible for *in vivo* tyrosine phosphorylation in the erythrocyte has been located at the membrane (Mohamed & Steck 1986) and its activity is not affected by insulin, epidermal growth factor or TPA. It was shown that the ratio of tyrosine to serine/threonine phosphorylation could be changed under various conditions. In isolated ghosts, tyrosine phosphorylation accounted for approximately 50% of the phosphoamino acids in band 3 (Vasseur et.al. 1987). As a result of tyrosine phosphorylation, the binding of protein, such as aldolase, to band 3 is inhibited (Low et.al. 1987) and the inhibition is reversed by dephosphorylation using acid phosphatase. Recently, it has also been shown that the acid phosphatase present in erythrocyte cytosol is a tyrosine phosphatase, which is capable of the dephosphorylation of tyrosine residues on band 3, near the amino terminus (Boivin & Galand 1986).

Micro-environment has also been suggested as a significant factor in determining erythrocyte morphology. It has been shown that alterations of the environment of cell suspensions may alter the mobility of the glycocalyx of the erythrocyte membrane; this may then interfere with the balance of interactions within the cytoskeleton or with the distribution of cell surface charges (Herrmann et.al. 1986). There is also evidence to suggest that the intracellular micro-environment may play an important part in spectrin-band 4.1 interaction rather than covalent modification in morphological changes (Podgorski & Elbaum 1985).

1.4. The antitumour drug Adriamycin: a probe to study the mechanism of morphological transitions in human erythrocytes

Adriamycin (doxorubicin) (figure 6) belongs to the anthracycline family of antitumour drugs. It is an aminoglycoside antibiotic, with a net positive charge at physiological pH. Although Adriamycin has been prescribed in cancer treatment for more than two decades, its mechanism of action is still poorly understood. Adriamycin was believed to be a DNA intercalating agent and it was suggested that it exerted its anti-tumour activity by preventing DNA replication and transcription. By some as yet undefined mechanism, this inhibition also results in cell death. Recently, evidence has been provided that Adriamycin causes protein-associated DNA-strand breaks, resulting from an inhibition of the complete cycle of activity of topoisomerase II. (Liu et.al. 1983, Rowe et.al. 1986, Tewey et.al. 1984a, Tewey et.al. 1984b, reviewed by Ross, 1985). However, there is little evidence which supports a direct correlation between DNA breakage, protein-DNA adduct formation and cytotoxicity.

Recently, it was reported that Adriamycin could be cytotoxic without entering cells (Tritton & Yee 1982). In addition, these workers also showed that low concentrations of Adriamycin had an early and profound effect on the order parameter of sarcoma 180 tumour cell membranes (Murphree et.al. 1981). Photolabelling studies using an Adriamycin



	R_1	R_2
Adriamycin	OH	H
Daunomycin	H	H

Figure.6 Chemical Structure of Adriamycin.

analogue also showed that the drug only crosslinked the cell surface proteins but not lipids or DNA (Yee et.al. 1984). At non-nuclear loci, Adriamycin was found to exert other effects on cellular functions. An Adriamycin analogue has been found to interact with tubulin (Na & Timasheff 1977). Adriamycin caused the up-regulation of EGF receptors (Zuckier & Tritton 1983). Increases in adenylate cyclase activity in cardiac muscle after chronic treatment of Adriamycin was also reported (Robinson & Giri 1986), and many studies have reported the effects of Adriamycin on the physical properties of membranes (reviewed by Tritton and Hickman 1985).

Adriamycin has been shown to bind preferentially to negatively charged phospholipids, particularly cardiolipin in membrane vesicles (Henry et.al. 1985, Fiallo & Garnier-Suillerot 1986b, Nicolay et.al. 1985, reviewed by Goormaghtigh and Ruysschaert, 1984) and the association is enhanced in the presence of calcium ions (Brenzaet.al 1985). Whether the interaction with negatively charged phospholipids is related to its cytotoxity to tumour cells is uncertain, although Tritton and coworkers suggested that the reported presence of cardiolipin in the plasma membrane of tumour cells may explain the selective cytotoxic action of Adriamycin (Tritton et al 1978).

Studies of the effects of Adriamycin and its analogues on human erythrocytes have been reported (Mikkelsen et.al. 1977, Henry & Bolard 1986, Goldman et.al. 1978, Chahwala et al 1982, Chahwala and Hickman, 1985a and b). It has been shown that Adriamycin enters human erythrocytes in a time- and

temperature-dependent manner (Mikkelsen et.al. 1977). Pre-treatment of human erythrocytes with Adriamycin had certain modulating effects upon morphology when erythrocytes were under conditions of ATP-depletion, so as to inhibit the formation of echinocytes. These studies identified that Adriamycin was capable of binding to the cytcskeletal protein spectrin. Recently, it has been shown that Adriamycin was able to protect human erythrocytes against echinocytosis under both conditions of ATP-depletion and calcium-loading (Chahwala & Hickman 1985a, Hickman et.al. 1986). It was shown that Adriamycin had no effect on calcium transport (Chahwala & Hickman 1985b) potassium efflux or membrane potential of cells (Chahwala & Hickman 1985a). In addition, Adriamycin has been shown to be only a very weak calmodulin inhibitor (Thompson et.al. 1987) and unlike the phenothiazines had no effect on discocyte morphology per se. Studies on phosphoinositides metabolism showed that pretreatment of human erythrocytes with Adriamycin before ATP-depletion protected cells from echinocytosis under conditions where phosphoinositide hydrolysis was inhibited. This supported the proposal (Ferrell & Heustis 1984) that phosphoinositides may play a role in the maintenance of discocyte morphology although mechanisms other than phospholipid metabolism could be involved.

In view of the importance of cytoskeleton in the control of various cellular processes as described above, and the possibility that Adriamycin may exert its modulating effects on red cell morphological changes via drug-cytoskeletal

interactions, the studies described in this thesis were performed. Adriamycin has been shown to alter the morphology and interfere with the organization of the cytoskeletal network in other cell types prior to cell death (Persky et.al. 1983), and it was considered important to investigate this type of interaction in detail. The use of the erythrocyte cytoskeleton as a model system in which to study drug-cytoskeletal interactions and morphological transitions, and interest in the role of the cytoskeleton per se, was supported by various studies (reviewed by Gratzer 1984). Recently, many of the erythrocyte cytoskeletal proteins have been identified as major cytoskeletal elements in other cell types (Low 1986, Black et.al. 1988, Regnouf et.al. 1985, Goldenring et.al. 1986, Howe et.al. 1985, Davis & Bennett 1986, Bourguignon et.al. 1986, Nelson & Veshnock 1987, Harris et.al. 1986, Baines & Bennett 1986) and immunological studies have suggested similar interactions may exist between these components, as in the human erythrocyte (Drenckhahn et.al. 1985, Cohen et.al. 1982). The results obtained in the present studies may provide more information for the initiation of studies of more complex cytoskeletal interactions in other cell types in relation to cell differentiation, proliferation and malignancy, and perhaps, lead to the design of more selective drugs for cancer chemotherapy.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

Adriamycin was kindly provided by Pharmitalia Carlo Erba, Stabilimento Di Nerviano, Via Milano 68, 20014 Nerviano, Italy. Bryostatin 1 was a gift from Dr.J.Baer, University of Aston, Birmingham. Leupeptin was obtained from Peptide Synthesis Inc., Japan via Scientific Marketing Associates, London. The following chemicals were obtained from Sigma Chemicals Co. Ltd., Poole, Dorset: Coomassie brilliant blue R250, BSA, aprotinin, bestatin, iodoacetamide, alpha-casein (protease free), butyl-agarose, fluoresamine, sodium cyanoborohydride, magnesium chloride solution (4.9M), ATP assay kit, adenine, inosine, PMSF, calcium ionophore A23187, histone H1,, histone 2A, dipalmitoylphosphatidyl serine, diolein, protamine sulphate, CHAPS, alpha-NAD, cAMP, saponin, trifluoroperazine, TPA, H-7, W-7, cAMP-dependent protein kinase, glyceraldehyde dehydrogenase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, 3-phosphoglycerate kinase, lactate dehydrogenase and L-alpha- glycerophosphate.

Calmidozolium was obtained from Jansen Scientific Products, Jansen Parmaceutical Ltd., Grove, Wantage, Oxon and Trisacryl DEAE cellulose was supplied by LKB Instrument Ltd., Croydon, Surrey. Dried DE32 DEAE cellulose and 17Chr chromatography paper were obtained from Whatman Labs Sales Ltd., Maidstone, Kent. Acrylamide (ultrapure) and glutaraldehyde were supplied by Fisons Scientific Equipment, Loughborough, Leics. SDS (specially purified),

bisacrylamide, all HPLC solvents and chemicals were supplied by BDH Chemicals Ltds., Poole, Dorset. Coomassie blue protein assay mix was obtained from Bio-Rad Laboratories Ltd., Watford, Herts. ^{32}P-orthophosphate (40mCi/ml in HCl), ^{32}P-gamma-ATP and Hyperfilm-MP were obtained from Amersham International Plc., Amersham Laboratories, White Lion Road, Amersham, Buck.. Unless otherwise stated, all the other chemicals used were at analytical grade and they were obtained from either Sigma, Fisons or BDH.

2.2 PROTEIN ASSAYS

The Coomassie blue protein assay was used due to its high sensitivity and the simplicity of the procedure. The assay was carried out as described in the Bio-Rad coomassie blue protein assay mix documentation using a `Microassay' technique.

2.3 SODIUM DODECYLSULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

A discontinous buffer system as described by Laemmli (1970) was modified and used throughout the study. Electrophoresis was performed in a Dual Protean Cell or Multi Protean cell system supplied by Bio-Rad Laboratory (Caxton Way, Watford, Hertfordshire), which was connected to a Bio-Rad power supply. The gel separation system

consists of a stacking gel which was formed above the preformed resolving gel.

2.3.1 Acrylamide/ bisacrylamide stock (30% w/v)

The acrylamide stock solution was prepared as a 30% w/v acrylamide and 0.8% w/v bisacrylamide. The solution was filtered before use and stored at 4°C.

2.3.2 Resolving gel (7.5% w/v)

7.5 mls of the acrylamide stock was added into 15mls of gel buffer A (0.75M Tris-HCl, 0.2% SDS pH 8.8) and 7.5 mls distilled water. The acrylamide mixture was deaerated for 30 mins under vacuum. 100ul of TEMED and 25ul of 15%w/v APS (freshly made) were added. The mixture was immediately introduced between the two glass plates in the gel casting unit, avoiding bubble formation. The gel surface was carefully overlaid with 2 mls of distilled water and the gel was left to polymerise for at least one hour before introducing the stacking gel.

2.3.3 Stacking gel (5% w/v)

5mls of the acrylamide stock was added into 15 mls of buffer B (0.25M Tris, 0.2% SDS pH 6.8) and 10 mls of distilled water. The overlay on the resolving gel was then removed and a teflon comb (with 15 teeth) was placed above the resolving gel, such that the distance between the teeth and the gel surface was approximately 1 inch. 100ul of TEMED and 50ul of 15% APS were added and the mixture was

introduced onto the resolving gel. The gel was left to polymerize for at least 10 hours before use.

2.3.4 Running conditions

After the gels were cast and left overnight for complete polymerization, the sample wells were rinsed with distilled water. The upper and lower buffer chambers were then filled with the electrode buffer (glycine 14.4 g/litre, SDS 1g/litre, Tris 3 g/litre pH 8.3).

After protein samples had been loaded into the sample wells, through a Hamilton syringe, the gel was electrophorezed, as detailed above, in an discontinous buffer electrophoresis apparatus containing the electrode buffer. The gel apparatus was then connected to the power supply and electrophorezed at either 20-40mA/gel at constant current (during day-time) or 55 V constant voltage (overnight).

2.3.5 Gel staining and destaining - coomassie blue stain

After electrophoresis, the polyacrylamide gel was carefully removed from the gel apparatus and glass plates. The gel was fixed with 10% TCA in 45 % methanol until the blue dye-front turned yellow (approx. 10 mins.)

The gel was then rinsed with water and stained with the gel stain (0.25%w/v coomassie blue R250, 50% methanol and 10% glacial acetic acid) for 60-120 mins. The stained gel was briefly rinsed with water and immersed into the gel

destain (45% methanol, 7% glacial acetic acid). The destain procedure was continued until the background of the gel was clear.

After destaining, the gel was left in 7% v/v glacial acetic acid to restore its size.

2.3.6 Gel drying

After the gels were destained, they were dried under reduced pressure in a Bio-Rad Slab Gel Dryer. Gels were dried between presoaked cellophane and Whatman 3mm chromatographic paper for permanent storage. If autoradiography followed, the gels were dried between two layers of presoaked cellophane sheets in order to give maximum resolution during autoradiography. Drying time was approximately 3 hours followed by 1 hour cooling time, during which time they remained under reduced pressure.

2.3.7 Densitometry

The intensity of protein bands on the coomassie blue stained gel or autoradiograph was quantitated by laser densitometry using LKB Laser Densitometer (Gel Scan). The results obtained after scanning was analysed with the a gelscan software (GSXL) provided by LKB Ltd.

2.4 AUTORADIOGRAPHY

Autoradiography was carried out using a Kodak X-ray cassette without the intensifying screen (by blocking the

screen with light proof paper) in order to maximize the resolution of the image.

For samples labelled with ^{32}Pi , the dried gel was exposed to Amersham Hyperfilm-MP for 3-5 days at room temperature.

The film was developed by treating it in Kodak D19 X-ray film developer for 5 minutes, then briefly rinsed with water and fixed in Kodak Fix fixing solution for at least twice the clearing time. The fixed film was washed with running water for at least 10 minutes before being air dried.

2.5 ADENOSINE TRIPHOSPHATE (ATP) EXTRACTION AND QUANTITATION

2.5.1 ATP extraction

2.5.1.1 from high cell density preparations

When ATP had to be extracted from cell suspensions of $2x10^8$ cells/ml or higher, the following procedure was used. 50µl of cell suspensions was withdrawn into a 1.5ml microcentrifuge tube, 500µl of ice-cold distilled water was added, mixed and followed by 50µl of 20% perchloric acid. The mixture was mixed thoroughly and left on ice for 5 minutes, then mixed again and centrifuged at 13,000xg for 1 minute in a Hereaus microcentrifuge. 450µl of supernatent was withdrawn and added to 10µl of universal indicator. The mixture was neutralized with a pre-determinated volume of a solution of triethanolamine/KOH (0.5M & 2M

respectively) such that the final pH of the mixture was pH 7-8. The mixture was immediately frozen at -20°C. The sample was stable at this temperature for at least one month.

2.5.1.2 from low cell density preparations

For suspension containing 10^7 cells/ml, 1 ml of cell suspension was transferred to a chilled centrifuge tube and cells were centrifuged at 13,000xg for 30 seconds. The supernatent was aspirated and the cells were lysed with 500µl of ice-cold distilled water. 50µl of 20% perchloric acid was added to the lysate and the sample was processed as described in previous section (2.5.1.1).

2.5.2 ATP quantitation

2.5.2.1 Ion-exchange HPLC

Nucleotide standards and ATP extracts were separated on a ion exchange HPLC system using Waters 510 pumps. The system was equipped with a Waters WISP 710B automatic sample injection module. The analytical column used was a Whatman Partisil-SAX anion exchange column (2.1 X 250mm). The following buffer system was used. Buffer A: 100mM KH_2PO_4 pH 2.7. Buffer B : 1.3mM KH_2PO_4 pH 2.7. The mobile phase was titrated to the required pH using concentrated phosphoric acid. Both mobile phases were filtered and they were degassed under vacuum prior to use and all separations were carried out with columns at ambient temperature, at a

flow rate of 1ml/min. Gradient elution was performed for the separation of various nucleotides: linear gradient from 0 - 100 buffer B over first 20 minutes then 100% B for further 5 minutes. Nucleotides were detected by monitoring at 254 nm with a Waters 480 detector. The HPLC system was controlled by the Waters 720 system controller and data was collected and analysed by the Waters 730 Data Module.

In the analysis, the frozen samples were thawed, mixed thoroughly and centrifuged at 13,000xg in a Heraeus microcentrifuge for 2 minutes to remove $KClO_4$ crystals. In HPLC separation, 150µl samples (placed in the WISP injection sample vials) were injected in each separation.

2.5.2.2 Reverse Phase Ion Pairing HPLC

In reverse phase HPLC separation, the Waters HPLC system described previously was used with a Merck Hibar Lichrosorb C18 reverse phase analytical column (2.1x250mm). Isocratic elution was performed. The mobile phase was a 70:30 mixture of a solution containing 100mM KH_2PO_4, 25mM tetrabutylammonium hydroxide pH 5.0 and methanol. Separation was carried out at a flow rate of 1 ml/min. Nucleotide elution was monitored at 254nm.

2.5.2.3 Luciferase/Luciferin assay

ATP extracted from low cell density samples (10^7 cells/ml) was measured by the luciferase / luciferin assay using the ATP assay kit from Sigma. The assay was

performed as described in the Sigma technical bulletin BAAB-1 supplied with the assay kit. Briefly, ATP extracts were thawed, mixed vigourously and centrifuged at 13,000xg for 2 minutes in a Heraeus microcentrifuge. 100µl of the ATP extract supernatent was transferred to a disposable cuvette in the luminometer, then 100µl of a 1 in 15 diluted luciferase / luciferin mixture was added without delay and the luminescence measured by a LKB Luminometer.

ATP standards (0-0.1 µmol/assay) were prepared using the ATP standard supplied with the assay kit.

2.6 ERYTHROCYTE PREPARATION

Blood was obtained from healthy volunteers using a 21G hyperdermic needle and disposable polystyrene syringe. The blood was finally collected in a lithium heparin tube, transferred to a universal tube and centrifuged at approx. 1000xg for 3 mins . The plasma, including the creamy, buffy layer of lymphocytes which sedimented on the surface of the erythrocyte layer, was discarded. The erythrocytes were washed twice with an equal volume of incubation buffer A (10 mM KCl, 130mM NaCl, 2mM $MgCl_2$ and 15mM Tris pH 7.4)(Chahwala & Hickman 1985a). This procedure was employed in preparing erythrocytes for ATP-depletion experiments and in the early series of Ca^{2+} loading experiments (see results 3.2 and 3.3). Unless otherwise stated, in all later experiments, the erythrocytes were

washed and maintained in incubation buffer B (123mM NaCl, 10mM KCl, 15mM Tris, 5mM Na pyruvate, 2mM Na gluconate, 10 mM glucose, 1mM adenine, 1mM inosine, 2mM $MgCl_2$, 1mM NaH_2PO_4, 0.75%w/v dextran-500, 0.002% dialysed BSA, pH 7.4)

2.7 CHARACTERISTICS OF HUMAN ERYTHROCYTES

2.7.1 Cell fixation and morphological studies

A sample (250 µl) of the cell suspension was placed in a 3 ml polystyrene tube and fixed with 1.25 ml 2% glutaraldehyde buffer (2% glutaraldehyde, 93mM NaH_2PO_4, 80mM NaOH pH 7.3). The cells were either pelleted down by centrifuging at 500xg for 5 mins or left to sediment overnight and the cell pellet was resuspended in approx. 250 µl distilled water. The cell suspension (approx. 10 µl) was introduced into a haemocytometer and examined under 400x magnification using a Jenalumar microscope fitted with Normaski optics. At least 100 cells were scored for morphology by the criteria of Bessis et.al. (1973).

2.7.2 Membrane proteins of whole cell membranes

Pelleted erythrocytes were lysed with lysing buffer A (5mM Tris, 7mM NaCl, 1mM EDTA, 0.1mM PMSF pH 8.0). The lysate was centrifuged at 9000rpm in 6x500mls aluminium rotor in an MSE Hi-spin 21 for 20 mins. The pelleted erythrocyte membrane were washed twice with the washing

buffer A (5mM Tris, 140 mM NaCl, 1mM EDTA, 0.1mM PMSF pH 7.4). 1ml of 20%w/v TCA was added into the erythrocyte membrane preparation and the mixture left on ice for 10 mins. The samples were centrifuged, and the protein pellets were blotted dry. 100ul of sample buffer A (6mM Tris-HCl, 5% SDS, 0.5% Triton X-100, 20%w/v sucrose, 1mM EGTA, 1mM EDTA, 0.001% bromophenol blue, and 10% glycerol pH 6.8 with 5% 2-mercaptoethanol were added immediately prior to use) was added to the protein pellets. The precipitated protein was resuspended in the sample buffer and 5ul aliquots of 1M Tris was added if the sample had turned yellow, until the blue colour reappeared. The sample was then centrifuged in a microcentrifuge for 10 mins. Aliquots of the supernatent, which were equivalent to 90ug of protein (see protein assay), were loaded into the sample well of the stacking gel. Electrophoresis was performed as described in section 2.3.

2.7.3 Membrane proteins of spectrin-depleted cell membranes

The pelleted membranes obtained after ultra-centrifugation as described in the previous section (2.7.2) were resuspended in 80 volumes of spectrin extraction buffer A (0.1mM EDTA, 0.03mM PMSF, 0.5mM DTT, pH 9.5)(Litman et.al. 1980). The membrane suspensions were incubated at 37°C for 30 mins and then centrifuged at 100,000xg for 1 hour at 4°C. The spectrin-depleted membranes were then mixed with sample buffer A, boiled and electrophorezed in 7.5% polyacrylamide gel as described

previously in section 2.3.

2.8 INDUCTION OF MORPHOLOGICAL TRANSITIONS

2.8.1 ATP-depletion

A cell suspension of 10^7 cells/ml was prepared from cells washed with incubation buffer A. The cells were then incubated at 37°C for 0 - 5 hours. At one hour intervals, duplicated samples were removed, fixed with 2% glutaraldehyde buffer and examined under the microscope as described earlier (8.a).

2.8.2 Elevation of intracellular calcium

A cell suspension of 10^7 cells/ml was prepared from cells washed with incubation buffer B and kept at 37°C throughout the experiment. $CaCl_2$ (100µM) and calcium ionophore A23187 (5µM) were added to the cell suspension to initiate calcium loading. The suspension was incubated for further 5 mins. Duplicate samples (250µl) were withdrawn, fixed with 1.25ml 2% glutaraldehyde and examined as described in section 2.8.1.

2.9 ATP-DEPLETION INDUCED ECHINOCYTOSIS IN HUMAN ERYTHROCYTES

2.9.1 Time course

A cell suspension of 10^7 cells/ml was prepared from cells washed with incubation buffer A. The cells were then

ATP-depleted for 0 to 5 hours. The incubation was carried out either at room temperature or at 37°C as stated. At one hour intervals, duplicated samples were removed and fixed with 2% glutaraldehyde buffer and examined under the microscope as described earlier (2.8.1).

2.9.2 Effect of Adriamycin

Procedures described in the previous section (2.9.1) were carried out as the control experiment as described in section 2.9.1. In parallel, Adriamycin was added to a similar cell suspension to give a final concentration of 10μM. Incubation was continued and duplicated samples were taken and treated as in control experiments.

2.10 MAINTENANCE OF HUMAN ERYTHROCYTE PREPARATIONS

2.10.1 Effect of incubation temperature

Fresh blood obtained from volunteer was washed with incubation buffer A or B at either ambient temperature or 37°C as described in section 2.6. The cells were then used to prepare suspensions with 10^7 cells/ml. Duplicated samples were withdrawn, and fixed at zero time and 5 mins after incubated at corresponding temperature. The samples wee then examined as described in section 2.8.1.

2.10.2 Effect of the addition of glycolytic substrate

Freshly obtained blood was washed and maintained at 37°C

in either incubation buffer A or B, which is a modification of a medium described by King & Michell (King & Michell 1987). Cell suspensions of 10^7 cells/ml were made. $CaCl_2$ and the calcium ionophore A23187 were added to a final concentration of 0 - 400µM and 5 µM respectively and incubated at 37°C. Duplicated samples were withdrawn, and fixed at zero time and after 5 mins incubation. The samples were then examined as described previously (2.8.1).

2.10.3 Effect of bovine serum albumin

Freshly obtained blood was washed and maintained at 37°C in either incubation buffer B with or without BSA. Cell suspensions of 10^7 cells/ml were made and divided into 5ml aliquots. Bovine serum albumin was added to each aliquot to give a final concentrations of 0 - 0.01%w/v. The suspensions were then incubated for 15 mins. $CaCl_2$ (100µM) and calcium ionophore A23187 (5µM) were added and incubated at 37°C for 5 mins. Duplicate samples were withdrawn, and fixed before ionophore addition and at the end of incubation. The samples were then examined as described previously (2.8.1).

2.10.4 Effect of temperature variation and the addition of glycolytic substrate on Adriamycin actions

A cell suspension of 10^7 cells/ml was prepared from cells washed with incubation buffer B and kept at either room temperature or at 37°C throughout the experiment.

Adriamycin was added to the suspension to give final concentrations of 10 and 50μM and incubated for 10 mins. $CaCl_2$ and calcium ionophore A23187 were then added to give final concentration of 0 - 400μM and 5μM respectively. The suspension was incubated for further 5 mins. Duplicated samples (250μl) were withdrawn, fixed with 1.25ml 2% glutaraldehyde and examined as described in section 2.8.1.

2.11 CALCIUM-INDUCED ECHINOCYTOSIS IN HUMAN ERYTHROCYTES

2.11.1 Time-dependent effects

30 mls of fresh human blood was centrifuged and washed with NaCl/Hepes buffer (154mM NaCl, 0.36g/l Hepes pH 7.4) as described in section 2.6.1. 0.5ml aliquots of packed erythrocytes were incubated in 4.5ml NaCl/Hepes buffer in the presence of 1mM $CaCl_2$. The reaction was started by the addition of calcium ionophore (5uM final) and incubated for 0 - 30 mins. Control experiments in which no $CaCl_2$ was added, were performed in parallel. The assay was terminated by adding 100ul of EDTA (200mM) and cooled on ice. The cell suspension was centrifuged at 1000xg for 10 mins. The sedimented cell pellets were lysed with 7 mls of lysing buffer A. The lysate was centrifuged at 54000xg for 20 mins to sediment the membranes. A further two washes with lysing buffer A were required to obtain haemoglobin-free membranes. The membrane pellets were

resuspended in 50µl of lysing buffer A and aliquots equivalent to 90µg protein was mixed with sample buffer A, boiled and electrophorezed as described before (section 2.3).

2.11.2 Calcium concentration dependence of the calcium-induced morphological transition

A cell suspension of 10^7 cells/ml was prepared from cells washed with incubation buffer B and kept at 37°C throughout the experiment. The suspension were divided into 8 ml aliquots. $CaCl_2$ (0-400µM) and calcium ionophore A23187 (5µM) were added to the cell suspension to initiate the calcium loading process. The suspension was incubated for further 5 mins. Duplicated samples (250µl) were withdrawn, fixed with 1.25ml 2% glutaraldehyde and the morphology of the cells was examined as described in section 2.8.1.

2.11.3 Intracellular ATP levels during echinocytosis

The experiment was performed in a similar manner as described in the above section (2.11.2). In addition to the sampling for morphological examination, 2 x 1ml aliquots of the cell suspension were also taken for the extraction of ATP as described previously (section 2.5.1.2) and the ATP was quantitated by the luciferase/luciferin method (section 2.5.2.3)

2.11.4 Preparation of phosphatidylcholine vesicles

30 mg of dipalmitoyl phosphatidylcholine (synthetic) was

dissolved in 200ul chloroform. The solution was injected into 30 mls of lysing buffer A (section 2.7.2) through a fine needle. The fine droplets suspended in the buffer were dispersed by sucking up and down through a 5ml pipette until no chloroform droplets remained. The suspension was then sonicated at high frequency with 6x10secs burst with 5 secs relaxation time. The resulting unilaminar vesicle suspension was then kept on ice and used within 1 hour.

2.11.5 Effect of calcium on membrane and cytoskeletal proteins in isolated human erythrocyte membranes

Erythrocyte membranes were prepared as described in section 2.7.2 except five washes were required to obtain haemoglobin-free membranes. The membranes were washed twice further with washing buffer B (120mM KCl, 5mM Tris pH 7.2) to remove EDTA. Aliquots of erythrocyte membranes (0.45ml) were treated with 1mM $CaCl_2$ for 0 - 30 mins. At the appropriate time, 200ul of 100mM EDTA was added and the samples were centrifuged at 41000xg for 20 mins in an MSE Hi-spin 21 centrifuge. Samples of the pellets were taken for assay of their protein content. An aliquot of each pellet was mixed with an equal volume of sample buffer A and boiled for 1 min. An appropriate volume of each sample, equivalent to 90ug of membrane protein, was loaded into each sample well and electrophoresis performed as described earlier (section 2.3).

2.11.6 Studies of the effect of calcium loading and other agents on membrane and cytoskeletal proteins in intact human erythrocytes

A cell suspension of 10^7 cells/ml was prepared from cells washed with incubation buffer B and kept at 37°C throughout the experiment. The erythrocyte suspensions were incubated in 8 mls aliquots at 37°C. Appropriate amounts of a $CaCl_2$ solution (40mM standard) was added to give a final Ca^{2+} concentration of 0-400 uM immediately prior to addition of the calcium ionophore A23187 (5µM). The cell suspensions were incubated further for 5 mins. 2x250ul samples were withdrawn and fixed with 1.25ml of 2% glutaraldehyde buffer for morphological examination (section 2.7.1). The rest was immediately lysed with 15ml of lysing buffer B (1.5mM NaH_2PO_4, 2mM EGTA, 1mM EDTA, 50ug/ml PMSF pH 8.0, and immediately before use, it was supplemented with 2ug/ml each of leupeptin, bestatin, aprotinin and 0.5mM iodoacetamide), mixed and immersed into liquid nitrogen for 10 secs before being kept on ice. Then 1 ml of the phosphatidylcholine vesicle suspension was added into each sample and the lysates were centrifuged at 54000xg as described earlier (section 2.11.5). The resultant pellets were resuspended in 1 ml of lysing buffer B and transferred to microcentrifuge tube. The samples were centrifuged in a bench microfuge for 5 mins and the resultant pellets were resuspended in 30ul of sample buffer B (12mM Tris, 10% SDS, 10% Triton X-100,40%w/v sucrose, 2mM EGTA, 2mM EDTA, 0.002% bromophenol blue, 20% glycerol and 160mM DTT pH 6.8). The

samples were then boiled for 1 min and the whole sample was loaded into each sample well in the stacking gel. Electrophoresis were performed at 55V constant voltage overnight as described earlier (section 2.3)

2.11.7 Effect of Adriamycin pretreatment on erythrocyte morphology and membrane and cytoskeletal composition

2.11.7.1 time-dependence changes

The experiment was carried out in incubation buffer A as described in section (2.11.1). $CaCl_2$ was added to the cell suspensions to give final concentrations of 200uM or 1 mM and incubations were terminated at 0, 30 secs, 1 min and 10 mins respectively after the addition of calcium ionophore A23187. Parallel experiments were also performed in which cell suspensions were pre-treated with 100uM Adriamycin for 10 mins before addition of calcium ionophore.

2.11.7.2 on the calcium-mediated morphological transition response

The experiments were carried out in a similar manner as described in section 2.11.2. In addition, parallel incubations were set up and pretreated with 10 or 50µM Adriamycin for 10 mins before $CaCl_2$ and calcium ionophore additions.

2.11.7.3 on ATP level

The experiments were carried out in a similar manner as

described in section 2.11.3. In addition, parallel incubations were set up and pretreated with 10 μM Adriamycin for 10 mins before $CaCl_2$ and calcium ionophore additions. ATP extraction was then carried out as in section 2.11.3 and quantified using the luciferase/luciferin assay (section 2.5.2.3)

2.11.8 Effect of leupeptin

The experiments were carried out in a similar manner as described in section 2.11.2. In addition, parallel incubations were set up and pretreated with 1mM leupeptin (a thio-protease inhibitor) for 30 mins before $CaCl_2$ and calcium ionophore additions.

2.12 PURIFICATION OF THE CALCIUM-DEPENDENT THIO PROTEASE (CALPAIN) FROM HUMAN ERYTHROCYTES AND THE DEVELOPMENT OF QUANTITATIVE ASSAY FOR THE PROTEOLYTIC ACTIVITY

2.12.1 Purification of calpain from erythrocyte lysate

Human erythrocyte calcium-dependent thio protease (calpain) was purified from human erythrocyte lysate using a method as described by Melloni et.al. (1982) with modifications.

2.12.1.1 Preparation of membrane free erythrocyte lysate

300ml of human blood was freshly drawn from healthy donor

and washed three times with 600ml of ice-cold isotonic NaCl solution. 100ml of packed erythrocytes were lysed with 500ml of ice-cold of a solution containing 2mM EDTA and 2mM EGTA. The lysate was then sonicated for 3x10 seconds at 4°C. The lysate was then centrifuged at 25,000xg for 30 minutes. The membrane pellet resulted after centrifugation was discarded and the supernatant (600ml) was used for the purification procedures described below. From this point onwards, all the purification steps were carried out at 4°C.

2.12.1.2 Preparation of crude calpain

150g of DE32 ion exchange resin (wet powder), which had been extensively washed with a solution containing 50mM sodium acetate, 1mM EDTA and 1mM EGTA pH 6.7 (equilibration buffer A), was added to the membrane free lysate. The final pH was re-adjusted to pH 6.7 in the mixture and was then stirred for further 10 minutes at 4°C. Under these conditions, most of the calpain activity had adsorbed onto the DE32 resin. The resin was collected on a Buchner funnel and further washed with one litre of equilibration buffer A. The washed resin was resuspended in equilibration buffer A and packed into a glass column (6cmx13.5cm) and washed with equilibration buffer A at a flow rate of 4ml/min until the absorbance (at 280nm) of the eluant reached below 0.02. The flow rate was reduced to 3ml/min and the enzyme was eluted with a 500ml linear gradient of NaCl (0-1.5M) in equilibration buffer A. Two minutes fractions were

collected and assayed for calcium-dependent protease activity as described below (section 2.12.2.2). The fractions containing calpain activity were pooled and concentrated to 4mls using an Amicon concentrator-B. The enzyme concentrate was dialysed overnight in 5 litres of equilibration buffer and the enzyme preparation obtained after the dialysis was referred as the crude calpain preparation.

2.12.1.3 Trisacryl DEAE ion-exchange chromatography

The dialysed crude calpain preparation was applied onto a trisacryl DEAE column (1cmx9cm), which has been pre-equilibrated with 10 volumes of equilibration buffer A. After the enzyme preparation has entered the resin, the column was further washed with 50ml of equilibration buffer A and the enzyme was eluted with 100ml of a linear NaCl gradient (0-200mM). The elution was performed at a flow rate of 0.4ml/min and 5 ml fractions were collected. The calpain activity from each fraction was assayed and active fractions were pooled. The combined mixture was then concentrated to 3ml using a Amicon concentrator-B followed by dialysis overnight in 5 litres of a solution which contained 50mM sodium borate, 1mM EDTA and 1mM EGTA pH 7.2 (equilibration buffer B). The resultant enzyme preparation was either used directly for other studies or further purified using hydrophobic chromatography as described in the following section.

2.12.1.4 Hydrophobic chromatography

3ml of the dialysed calpain preparation was applied onto a column of butyl-agarose (1x1.5cm) which has been pre-equilibrated with equilibration buffer B. The column was washed with 40ml of equilibration buffer B followed by a 100ml linear gradient of NaCl (0-100mM) at a flow rate of 2ml/min. Two minute fractions were collected and enzyme activity was assayed as described below.

2.12.2 Development of a quantitative assay for calpain

Both of the procedures described below are based on the use of heat treated alpha-casein (by heating a 1%w/v solution at 100°C for one hour) as a protease substrate. Although the use of denatured haemoglobin beta chain has been described (Pontremoli et.al. 1984) and is a preferred substrate compared with casein, casein is more readily available and reduces the worker's risk of handling excessive amount of blood. Casein is hydrolysed by proteases, including calpain, into acid soluble peptides or amino acids. The soluble products were then either quantitatively measured.

2.12.2.1 Fluoresamine method

In each assay, 200µl of enzyme preparation was added to 1.8ml reaction mixture, which gave a final concentration of 50mM sodium borate, 5mM DTT, 0.1% w/v heat inactivated casein, pH 7.5 with either 2mM $CaCl_2$ or 2mM EGTA. The mixtures were then incubated at 37°C for 30 minutes.

Reaction was terminated by the addition of 1ml 7.5% w/v TCA. The mixtures were then mixed vigourously and centrifuged at 3000xg for 10 mins. 1.25ml of the supernatant was withdrawn and neutralized with 250µl NaOH. The neutralized solution was then mixed with 500µl of fluoresamine solution (15mg/100ml in acetone). The fluorescence of the resultant product was measured at 390nm excitation and 475nm emission.

2.12.2.2 Radioisotopic method

In this method, the assay is similar to the basic assay as described above. Instead of using ordinary casein, ^{14}C-methylated-casein was used as a substrate. The ^{14}C-methylated-casein was prepared as described by Rice & Means (1971) with slight modification.

The reaction mixture was prepared by mixing 1M potassium phosphate pH 8.0 (600µl), distilled water (2.13ml), 1% w/v heat denatured casein (3ml) and ^{14}C-formaldehyde (90µCi, 180µl). The mixture was left for 15 minutes at room temperature followed by addition of 4x150µl of 0.2M sodium cyanoborohydride at 30 seconds intervals. The mixture was left for further 3 hours before a final addition of 600µl of 0.2M sodium cyanoborohydride. The reaction was left to proceed overnight.

Reaction was stopped by dialysis of the reaction mixture against 20 litres of 25mM potassium phosphate buffer at pH 8.0 for at least 24 hours. The dialysis process was repeated once more with potassium phosphate buffer and a

final dialysis with 20 litres of 25mM sodium borate pH 7.5. A typical preparation would yield a protein solution of approximately 3mg/ml with a specific activity of approximately 5000dpm/µg protein. The protein solution was stored in 0.5ml aliquots at -20°C.

In the assay, 250µl of enzyme preparation (including any test agents) was added to a reaction mixture containing sodium borate 50mM, DTT 10mM, ^{14}C-casein 10µg pH 7.5 with either 3mM $CaCl_2$ or 2mM EGTA. The assay mixture was then incubated at 37°C for 30 mins. To terminate the reaction, 50µl of a 11% w/v BSA solution was added immediately followed by 250µl of 22.5%w/v TCA. After centrifugation, 0.5ml of the supernatant was withdrawn for scintillation counting.

2.13 ^{32}P-ORTHOPHOSPHATE LABELLING OF INTACT HUMAN ERYTHROCYTES

2.13.1 Procedures of metabolic labelling

2.13.1.1 Standard method

Human erythrocytes were labelled with ^{32}P-orthophosphate using a modified buffer system described by Wolfe & Lux (1978). The blood was collected (section 2.6) and transferred to a universal tube and centrifuged at approx. 1000xg for 3 mins . The plasma, including the creamy, buffy layer of lymphocytes sedimented on the surface of the erythrocyte layer, was discarded. The erythrocytes were

washed twice with equal volumes of incubation buffer (NaCl 120mM, $NaHCO_3$ 20mM, KCl 5mM, glucose 10mM, $MgCl_2$ 1mM, BSA 1mg/ml, PMSF 0.1mM pH 7.4 at 37°C.). 0.5ml of washed packed erythrocyte was added to a mixture of 250µl ^{32}P-orthophosphate (and distilled water) and 250µl of 2X incubation buffer (with or without 4mM K_2HPO_4). The cell suspensions was further incubated for 3 - 3.5 hours in order to achieve the steady state isotopic labelling.

2.13.1.2 Development of a refined media

The blood was transferred to a universal tube and centrifuged at approx. 1000xg for 3 mins. The plasma, including the creamy, buffy layer of lymphocytes sedimented on the surface of the erythrocyte layer, were discarded (section 2.6). The erythrocytes were washed twice with equal volume of incubation buffer B supplemented with 0.02% BSA. 0.5ml of washed, packed erythrocyte was added to a mixture of 250µl ^{32}P-orthophosphate (and distilled water) and 250µl of 2X incubation buffer (with 4mM K_2HPO_4 and 4% BSA). The cell suspensions was further incubated for at least 3.5 hours in order to achieve steady state isotopic labelling.

2.13.2 Metabolic state of the erythrocyte during labelling

Human erythrocytes were prepared and labelled with ^{32}P-orthophosphate as described previously (section 2.6 and 2.13.1.2). The metabolic state of the erythrocytes was

monitored by the intracellular steady state level of ATP during the isotopic labelling period. Samples were taken from the incubation suspension between 0 - 6 hours from the beginning of labelling. ATP was extracted from the cell suspensions and quantitated as described in section 2.5.1.1 and 2.5.2.1.

2.13.3 Specific activity of the intracellular ATP during labelling

In order to determine the duration required for isotopic equilibrium during labelling of human erythrocytes with ^{32}Pi, human erythrocytes were prepared and labelled with ^{32}P-orthophosphate as described previously (section 2.6 and 2.13.1.2). Duplicate samples were taken from the incubation suspension between 0-6 hours from the beginning of labelling. ATP was extracted from the cell suspensions and quantitated as described in section 2.5.1.1.

The ATP extracts were defrosted, mixed and centrifuged to remove potassium perchlorate crystals. The supernatent was then used in the assay. The assay were carried out as described by Hawkins et.al.(1983). Briefly, 40µl of the supernatent was added to 50µl incubation medium (Histone 2A 0.4 mg/ml, cAMP 10µM, magnesium acetate 10mM, DTT 20mM, NaF 20mM, KH_2PO_4 24mM pH 6.5) and warmed up to 30°C. Reaction was started by the addition of 10µl of enzyme preparation, which contained cAMP Protein kinase (5.4 pmol Pi transferred/min into casein/10µl) in sodium citrate pH 6.5, in 20 seconds intervals. The mixtures were incubated at

30°C for further 140 mins. Reaction was terminated by transferring 2 X 45µl to separate 1.5cm x 1.5cm Whatman 3MM papers and immersed in ice-cold 10%W/v TCA for 15 mins. The papers were then washed four times (15 minutes each) with cold 10% TCA and finally washed in ethanol. The papers were then dried and counted by scintillation counting using a Packard Tricarb 2000CA scintillation counter.

2.13.4 SDS-PAGE analysis and Autoradiography of ^{32}P-labelled human erythrocyte cytoskeletal proteins

Human erythrocytes were washed and labelled with ^{32}P-orthophosphate as described in section 2.13.1.2. 25µl samples were withdrawn from the incubation suspension between 0 - 6 hours from the beginning of labelling and frozen immediately. At the end of the incubation period, the frozen cell suspensions were lysed with 8ml of lysing buffer B (section 2.11.6) supplemented with 1mM NaF. The lysate was centrifuged at 54,000Xg for 25 mins. The supernatent was discarded carefully and the membrane pellets were washed further with 8ml lysing buffer B with NaF. The resultant pellets were then dissolved in 50µl of sample buffer, boiled and electrophoresis and autoradiography were carried out as described earlier (section 2.3 & 2.4).

2.14 PHOSPHORYLATION OF THE HUMAN ERYTHROCYTE MEMBRANE AND CYTOSKELETON

2.14.1 Effect of calcium loading on the cytoskeletal protein phosphorylation in human erythrocytes

Human erythrocytes were prepared and labelled with ^{32}P-orthophosphate as described in section 2.6 and 2.13.1.2. After 3½ hours incubation, the labelled cells were washed twice with phosphate-free incubation buffer B to remove external phosphate. The washed erythrocytes were diluted to 10^7 cells/ml and divided into 8 ml aliquots and maintained at 37°C. Appropriate amounts of a $CaCl_2$ solution (20mM & 40mM standard) was added to give a final Ca^{2+} concentration of 0-400uM immediately prior to addition of the calcium ionophore A23187 (5µM). The cell suspensions were incubated further for 5 mins. 2x250ul samples were withdrawn and fixed with 1.25ml of 2% glutaraldehyde buffer for morphological examination (section 2.7.1). The remainder was immediately lysed with 0.5ml of permeabilizing buffer (20 mM each of EDTA, EGTA, Na Vanadate, NaF. 30µg/ml each of leupeptin, bestatin and aprotinin. 800µg/ml PMSF, 3.2 mg/ml saponin). The lysate was centrifuged at 54000xg for 25mins and the pellets were washed once with 10ml lysing buffer B with supplements (section 2.11.6) and 1mM each of NaF and Na Vanadate. The resulting pellets were then dissolved in 50µl of sample buffer, boiled and electrophoresis and autoradiography were carried out as described earlier (section 2.3 & 2.4).

2.14.2 Effect of various agents on cytoskeletal protein phosphorylation in human erythrocytes

The experiments were carried out as described in the previous section (section 2.14.1). Parallel incubations were carried out in which the cell suspensions were pre-incubated with Adriamycin (10μM), trifluoroperazine (10 - 50μM), TPA (100nM),or Bryostatin 1 (100nM) for 10 mins, or H-7 (50μM), leupeptin (1mM), W-7 (50μM), Calmidozolium (0 - 50nM) for 30 mins. $CaCl_2$ (0 - 400μM) and calcium ionophore were then added and continued as described in section 2.14.1.

2.14.3 concentration-dependence phosphorylation response to TPA

Human erythrocytes were washed, labelled with ^{32}P-orthophosphate for $3\frac{1}{2}$ hours as described previously (section 2.13.1.2). The suspensions were treated with TPA (0-100nM) for 10 mins, lysed, and membranes were prepared as described in section 2.14.1. The resultant pellets were then dissolved in 50μl of sample buffer, boiled and electrophoresis and autoradiography were carried out as described earlier (section 2.3 & 2.4).

2.15 SYNTHESIS OF ATP-GAMMA-P^{32}

ATP-gamma-^{32}P was synthesized from ADP and ^{32}P-ortho-

phosphate as described by Johnson & Walseth (1979) with some modifications. The reactions were catalysed by several coupled enzymatic reactions as shown below (figure 7). The procedure described below describes the incorporation of ^{32}Pi onto ADP, to give ATP, and its subsequent purification.

2.15.1 ATP synthesis

2.15.1.1 Enzyme mixture

All the enzymes were prepared and stored separately in 50mM Tris-HCl, pH 9.0 at -20°C. An enzyme mixture was prepared immediately before use. The final enzyme mixture contained glyceraldehyde dehydrogenase 6.0 U/ml, triose phosphate isomerase 5.0 U/ml, glyceraldehyde 3-phosphate dehydrogenase 8.0 U/ml, 3-phosphoglycerate kinase 45 U/ml, lactate dehydrogenase 27.5 U/ml, Tris-HCl 77.5mM and DTT 5mM pH 9.0. The enzyme mixture was kept on ice until use.

2.15.1.2 Reaction medium

The reaction medium was made freshly for each synthesis and kept on ice until use. The reaction medium consists of Tris-HCl pH 9.0 125mM, $MgCl_2$ 30mM, L-alpha-glycerophosphate 0.3mM, DTT 15mM, alpha-NAD 1.25mM, ADP 0.125mM and pyruvate 2.5mM.

The reaction was started by combining the various substances in the following ratio : ^{32}P-orthophosphate

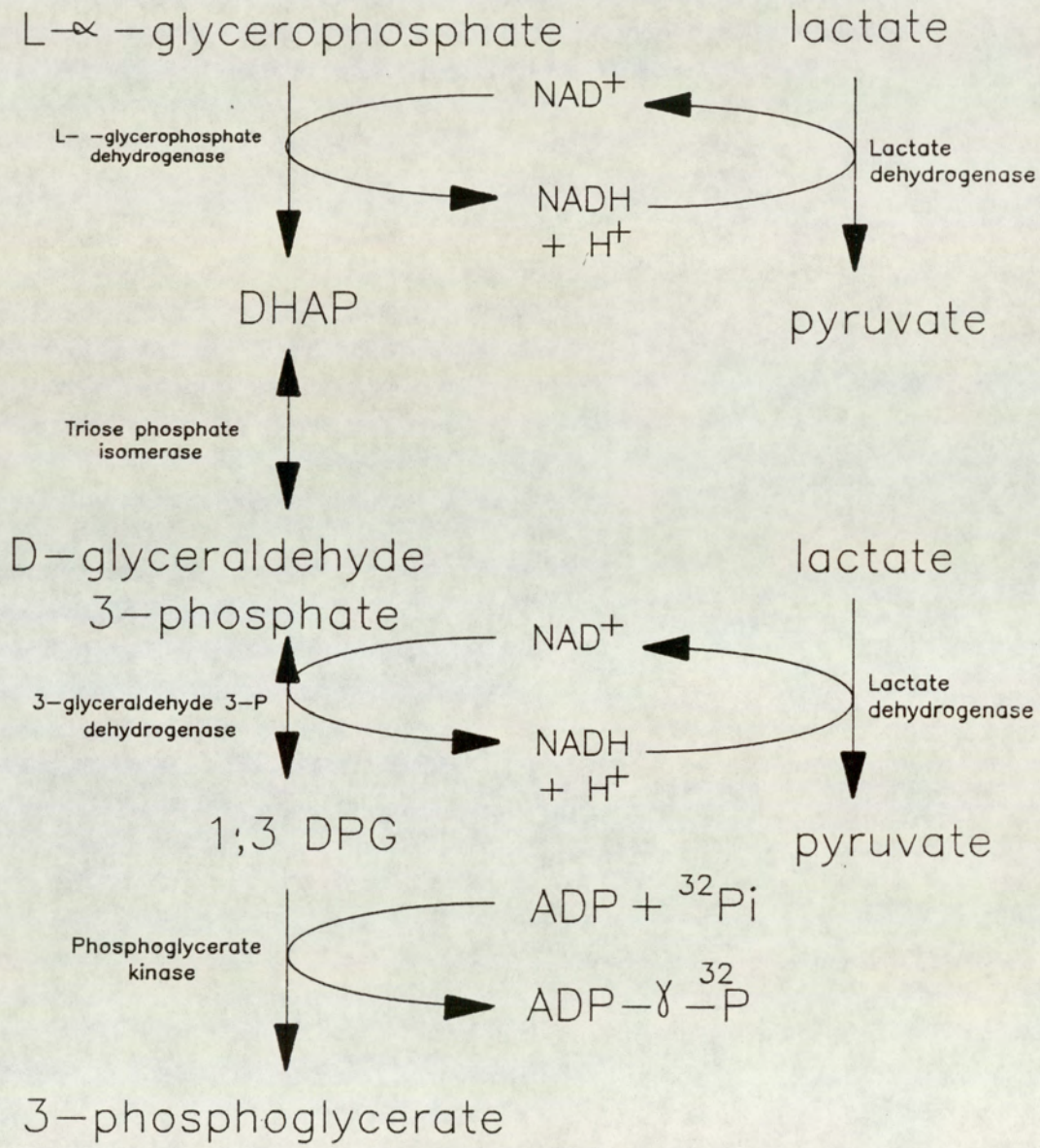


Figure.7 32P-gamma-ATP synthesis by Enzymatic coupled reactions.

(10mCi) : reaction medium : enzyme mixture of 5:4:1. Before the addition of the enzyme mixture, a small aliquot (50-500nl) was removed to monitor the zero time charcoal-absorptable radioactivity as described in the reaction monitoring procedure (see section 2.15.2). After the addition of the enzyme mixture, the reaction was left to proceed at 37°C until >95% ^{32}Pi was incorporated into ATP. The reaction was monitored at 5 mins intervals and the reaction should be completed within 15 - 30 mins. The reaction was stopped by immersion of the reaction mixture into water at approximately 95°C for 10 mins. The mixture was then either kept on ice for immediate purification or kept frozen.

2.15.2 Reaction monitoring

A small aliquot (5-500nl) was withdrawn from the reaction mixture by dipping a 200µl pipette tip into the reaction mixture and the pipette tip was withdrawn from the mixture and placed into 1 ml of 50mM KH_2PO_4. After mixing, 2 x 25µl were withdrawn for scintillation counting to give 'C_1' cpm. Approx. 10mg of activated charcoal was added to the remaining solution, mixed and centrifuge at 13000xg for 1 min. 2 x 25µl were withdrawn and counted to give 'C_2' cpm.

$$\% \text{ of } ^{32}Pi \text{ reacted} = (C_1 - C_2) * 100 / C_1$$

2.15.3 ATP-gamma-^{32}P purification

The ^{32}P-labelled ATP was purified from the mixture by separation on a column which contained Sephadex A25 in HCO_3^- form, (0.7 x 4cm) equilibrated with 100mM triethylammonium bicarbonate pH 8.6. Both elution buffers employed in the separation were prepared by adding the require amount of freshly distilled triethylamine into distilled water and pH the solution to pH 8.6 with CO_2 to give the triethylammonium bicarbonate buffers.

The denatured ^{32}P-ATP-enzyme mixture was loaded on the column and approx. 2ml fractions were collected. The column was then washed with 40ml 100mM triethylammonium bicarbonate pH 8.6 and followed by 80ml of a gradient from 100mM to 400mM triethylammonium bicarbonate pH 8.6. Finally, the column was eluted with 40ml 400mM triethylammonium bicarbonate. 5µl was withdrawn from each fraction (approx. 80 fractions) and the radioactivity was measured by scintillation counting. The fractions containing ^{32}P-ATP were identified by using the monitoring procedure as described in section 2.15.2. The fractions which contained ^{32}P-ATP were pooled into a round bottom flask and freeze-dried overnight. The residue was washed with 2 X 10ml high purity methanol and dried. The final residue was eventually dissolved in 2ml 50%v/v ethanol for 24 hours or longer. A typical preparation provided ^{32}P-labelled ATP solution containing 1.5 - 3.5 mCi/ml.

2.16 QUANTITATION OF PROTEIN KINASE C IN HUMAN ERYTHROCYTE MEMBRANES

2.16.1 Quantitative assay of protein kinase C by non-denaturing polyacrylamide gel electrophoresis

Quantitation and subcellular distribution of PKC was determined using the method of Fabbro et al (1985). Briefly, erythrocyte membranes were prepared from cell suspensions after calcium loading, followed by separation of the cytoskeletal proteins by non-denaturing polyacrylamide gel electrophoresis. Gels were then frozen and sliced into 1mm sections before eluting protein from the slices overnight. The eluates were assayed for protein kinase C activity by measuring the incorporation of ^{32}P from [^{32}P]ATP into lysine-rich histone or protamine sulphate.

2.16.1.1 Preparation of polyacrylamide gels

All gels were cast in borosilicate glass rods of length 14-15 cm, outer diameter 0.7 cm and inner diameter 0.5 cm. All gel casting was performed at 4°C. Unwashed tubes were sealed with parafilm at one end before the addition of gel mixes.

Gels were prepared only one day before sample application.

2.16.1.1.1 Resolving gel.

In all experiments resolving (lower) gels were prepared at a total monomer concentration of 10% acrylamide with 2% bisacrylamide crosslinks. A maximum of twelve gels could be run in an experiment.

For twelve 10% polyacrylamide gels, 6 ml resolving gel acrylamide stock (40% w/v acrylamide, 2% w/v bisacrylamide), 6 ml resolving gel buffer stock (18.16g/100ml Tris-HCl pH 8.83), 6 ml freshly made potassium persulphate/riboflavin solution (0.6mg/ml potassium persulphate, 20µg/ml riboflavin) and 6 ml distilled water were mixed in a brown bottle on ice. The gel mix was deaerated by stirring on ice for 5 min while passing nitrogen through the bottle. Triton X-100 (20%, 240 µl) and TEMED solution (10% v/v, 240 µl) were added in order to initiate polymerization. Gel mix (1.6 ml) was added to the glass tubes to form gels of approximately 6cm length. The top of the gels were overlaid with distilled water and photopolymerized by illuminating with fluorescent light at 4°C for 30min. After polymerization was complete, the gel surface was rinsed 2-3 times with distilled water, residual water removed by aspiration and the tubes dried carefully.

2.16.1.1.2 Stacking gel

Stacking (upper) gels were prepared at a total monomer concentration of 3.5% acrylamide with 20% bisacrylamide crosslinks. It was essential that the volume of the stacking gel (usually 700 µl) was at least twice that of

the sample volume loaded onto the gel (usually 200 µl).

For twelve gels, 6 ml stacking gel acrylamide stock (8%w/v acrylamide, 20% bisacrylamide), 3 ml stacking gel buffer stock (Tris 2.96g/100ml, pH 6.67 using phosphoric acid) and 3 ml potassium persulphate/riboflavin solution were mixed in a brown bottle on ice. The gel mix was deaerated as described for the resolving gel before adding Triton X-100 (20%, 120 µl) and TEMED solution (120 µl) to initiate polymerization. The gel mix (700 µl) was poured into the tubes, overlaid with distilled water and photopolymerized as described for the resolving gel.

2.16.1.2 Preparation of membrane-cytoskeleton from calcium loaded human erythrocytes

Human erythrocytes were washed and maintained in incubation buffer B, as described in section 2.6, at 10^7 cells/ml at 37°C. 100ml of erythrocyte suspension was required for each membrane sample prepared for the PKC quantitation. The suspensions were either treated with 0 and 100µM calcium in the presence of calcium ionophore for 5 minutes or 100nM TPA for 10 minutes. At the end of the incubation period, 100ml of ice cold incubation buffer B was added to the suspension and the mixture was centrifuged at 4°C for 5 minutes. The supernatent was removed and 5 mls of cold lysing buffer (5mM KH_2PO_4, 2mM EGTA, 2mM EDTA, 10µg each of leupeptin, bestatin and aprotinin pH 8.0) was added. The suspension were then sonicated at setting 24 (3 x 5 seconds) in a MSE

sonicator. The cell lysate was centrifuged at 54,000xg at 4°C for 25 minutes. The membrane pellets were further washed twice with 5 ml of lysing buffer. The final pellets were then supplemented with 15% (w/v) glycerol and immediately frozen at -20°C.

2.16.1.3 Sample application to gels

The membrane samples were rapidly thawed and 270µl was transferred into a 1.5ml tube. 30µl of cathode buffer (Tris-HCl 42.6 mM, glycine 46.1 mM, Triton X-100 0.2% v/v, pH 8.9) containing 2% (v/v) Triton X-100, 2% (w/v) CHAPS and Amaranth front dye (sufficient for the dye to be visible when loading onto the gel) was added. After removal of Parafilm from the ends of the glass tubes and rinsing of the gel surface 2-3 times with distilled water, 200 µl of sample was loaded onto the gel and overlaid with cathode buffer containing 0.2% Triton X-100 to the top of the tube.

2.16.1.4 Electrophoresis running conditions

Electrophoresis was performed using a Bio-Rad Model 175 tube cell gel apparatus. Anode (Tris 62.5 mM, pH 7.51) and cathode buffers had previously been cooled to 4°C overnight and the temperature of the system was maintained at approximately 4°C by circulating ethylene glycol antifreeze (precooled to -20°C using a Haake DK12 refrigeration unit) through the central cooling core of the gel apparatus.

Running conditions for the gels were as follows: voltage fixed at 100 V whilst the dye boundary moved through the stacking gel (running time approximately 4 h), and 160-180 V while the boundary moved through the resolving gel (total running time 7-9 h). Throughout electrophoresis, a current in excess of 0.8 mA per gel was avoided if possible.

2.16.1.5 Gel slicing and elution of enzyme activity from slices

Electrophoresis was complete when the dye boundary reached the end of the resolving gel. Gels were extruded from tubes by using a needle and syringe to force distilled water up the inner rim of the tube until the gel was free. After discarding the stacking gel, the resolving gel was carefully laid in a polystyrene centrifuge tube and frozen by placing on a block of dry ice. Gels were sliced into 1 mm sections using a Mickle gel slicer (Mickle Laboratory Engineering Co. Ltd., UK) and the gel discs transferred to 3.5 ml polystyrene centrifuge tubes (Starstedt) containing 300 µl elution buffer (Tris-HCl 20mM, EGTA 0.1 mM, NaCl 50 mM, beta-mercaptoetanol 50 mM, leupeptin 10µg/ml, aprotinin 2µg/ml, pH 7.4). Enzyme activity was eluted from slices overnight by gentle shaking at 4°C.

2.16.2 Protein kinase C assay

2.16.2.1 Protein kinase assay using protamine sulphate as substrate

PKC activity was assayed in gel eluates using a microassay technique derived from the method of Kikkawa et al (1983).

96-well U-form microtitre plates (Boehringer-Mannheim) were taped securely to the top of a perspex box designed such that water heated and circulated by a thermostirrer maintained solutions in the microtitre wells at 32°C. Gel eluate (150µl) was pipetted into wells and allowed to equilibrate to 32°C. The enzyme reaction was initiated by the addition of 50 µl protamine-assay mix (0.5 µCi ATP^{32}/assay) to the wells using a multichannel pipette (Flow Laboratories Ltd.). Final concentrations of assay components in the incubate were: 20 mM Tris-HCl, pH 7.4, 10 mM $Mg(NO_3)_2$, 24 µM ATP and 100 µg protamine. After 10 min incubation 150 µl of the reaction mix was removed using the multichannel pipette and immediately spotted onto Whatman 17Chr filter paper (approximate dimensions 20 mm x 6 mm) which had previously been numbered for identification purposes and arrayed in 96-well microtitre plates. The soaked papers were then plunged into ice-cold 10% (w/v) trichloroacetic acid to quench the reaction. All papers were washed by shaking gently in 500 ml of 10% (w/v) TCA for 1 h, the washing solution being changed every 15 min. They were then briefly soaked in methanol

(to aid drying) and placed in a hot oven until dry. Dried papers were transferred to 10 ml scintillation vials and 5 ml Luma gel scintillation fluid added. Radioactivity was counted using a Packard Tricarb CA2000 scintillation counter.

2.16.2.2 Protein kinase C assay using histone H1 as substrate

Phosphorylation of histone H1 by PKC is dependent on the presence of calcium and phospholipid and only activity of the holoenzyme is detected (protamine is a substrate for both the holoenzyme and PKM).

Assay of PKC by this method is similar to that described above for protamine with the following exception: gel eluate (100 µl) and phospholipid mix (50 µl) were pipetted into wells and allowed to equilibrate at 32°C. The reaction was then initiated by the addition of 50 µl histone assay mix. Final concentrations of assay components in the mixture were: 20 mM Tris-HCl, pH 7.4, 10 mM $Mg(NO_3)_2$, 300 µM $CaCl_2$, 24 µM ATP, 100 µg phosphatidylserine, 10 µg diolein and 50 µg histone H1. Basal activity was measured by incubating samples in the absence of Ca^{2+}, phosphatidylserine and diolein.

2.16.3 Effect of Adriamycin on Protein Kinase C activity

The protein kinase C assay was carried out as described in the above section (section 2.16.2.1) using the protamine sulphate as substrate. 5µl of a pure PKC solution

(purified from rat brain) was used in the assay such that approximately 150,000 cpm ^{32}P was incorporated into protamine sulphate was obtained. In other assays, 0-1mM of Adriamycin was added to the pure PKC solution for 10 minutes during the pre-incubation period and the assays were proceeded as described above.

2.17 PHOSPHOPEPTIDE MAPPING BY HPLC

The procedure was carried out as described by Aebersold & Leavitt et.al. (1987) with slight modification.

2.17.1 Sample preparation

Human erythrocytes were washed and labelled with ^{32}P-orthophosphate as described in section 2.13.1.2 except that a total volume of 2.5ml was labelled. After 3½ hours labelling, the cell suspension was lysed with 35 ml of lysing buffer B and centrifuged at 50000xg for 30 mins. The resultant membrane pellet was washed once more with 35ml lysing buffer B and centrifuged. The pellet was dissolved in equal volume of sample buffer A and boiled for 5 mins. The sample were then electrophorezed as described in section 2.3.

2.17.2 Transblot onto nitrocellulose and blocking

After electrophoresis, the SDS polyacrylamide gel was removed from the glass plates and immediately assembled in a sandwich of porous sponge and Whatman 3MM chromatography

papers. Inside the sandwich, the gel was in direct contact with a Bio-Rad transblot nitrocellulose membrane. All the components in the sandwich was pre-soaked in the blotting buffer (Tris-base 12.5mM, glycine 7.2 g/l, SDS 0.005%w/v, methanol 20%v/v) before assembly. The assembly was then immersed into the Bio-Rad Transblot cell filled with blotting buffer. Blotting was then carried out using approx. 25-50mA constant current for 24 hours.

At the end of the blotting period, the blot was removed from the sandwich and stained for 30 sec with a solution contained 0.1w/v Ponceau S dye in 1% aqueous acetic acid. Excess stain was removed by gently agitating in 1% acetic acid for several minutes to visualize the proteins. The stained regions, which contained the protein intended for analysis were cut out and transferred to a test tube. The strips were then washed with distilled water and destained with a solution containing Trisma 50mM, NaCl 150mM and NaN_3 0.05% until the strips were completely destained. The strips were then rinsed with distilled water and either stored wet at -20°C or proceeded to the next step.

The protein-containing strips were combined in a test tube containing 3ml of a solution of 0.5%w/v PVP-40 in 100mM acetic acid and incubated for 30 mins at 37°C. This step was essential to prevent the adsorption of the protease onto the nitrocellulose strips during the next digestion stage. Excess PVP-40 was removed by extensive washing with distilled water (approx. 5 - 10 washes) since PVP-40 has strong UV absorbance at short wavelength which

interfere the HPLC separation step.

2.17.3 Protease digestion

The nitrocellulose strips were then cut into approx. 1mm X 1mm and placed in a round bottom tube. The protein on the nitrocellulose was digested as follows:

2.17.3.1 Trypsin

Trypsin was stored as small aliquots (1mg/ml) in 1mM HCl at -20°C. The enzyme should be stable for approx. 3 months under these conditions. To the cut strips, 300µl of a solution containing Tris-HCl 100mM pH 8.2, acetonitrile 5%v/v and trypsin (enzyme:substrate approx. 1:20). The mixture was incubated at 37°C for 24 hours in a shaking water bath. After digestion, the whole reaction mixture was immediately frozen at -20°C and each reaction mixture only defrosted immediately before peptide HPLC.

2.17.3.2 Staphyloccus aureus V8 protease

V8 protease was stored in 50mM NH_4HCO_3 (1mg/ml) as small aliquots at -20°C. To the cut strips, 300µl of a solution containing Sodium phosphate 100mM pH 7.8, acetonitrile 5%v/v and Staphyloccus aureus V8 protease (enzyme:substrate approx. 1:20). The mixture was incubated at 37°C for 24 hours in a shaking water bath. After digestion, the whole reaction mixture was

immediately frozen at -20°C and each reaction mixture only defrosted immediately before peptide HPLC.

2.17.4 Peptide separation by HPLC

The enzymatic digest from the protein-containing strips were separated using a Reverse-Phase HPLC method. Separation of peptides were performed on a analytical column, which is a Vydac protein-peptide C18 column 4.6 x 250mm. The system was equipped with a Waters 480 detector and Waters 510 pumps. The system was controlled by the Water 720 system controller and data analysed by a Water 730 data module. The following buffer system was used. Buffer A: 0.06%v/v trifluoroacetic acid (sequencial grade, Sigma) in water. Buffer B: 0.052%v/v trifluoroacetic acid in water/acetonitrile 20:80 (v/v). The optical density of buffers A and B were matched at 214nm by titrating with trifluoroacetic acid. Both buffers were degassed under vacuum before use. All separations were performed at ambient temperature and the elution was monitored at 214nm. The gradients were run as shown in figure 8.

The defrosted protease digest was acidified with trifluoroacetic acid to a final concentration of 1%v/v. The mixture was mixed quickly and centrifuge in a microcentrifuge at high speed for 2mins. The supernatent was then separated and immediately injected into the HPLC system. At the same time, fractions were started to be collected at the detector outlet and measured by scintillation counting.

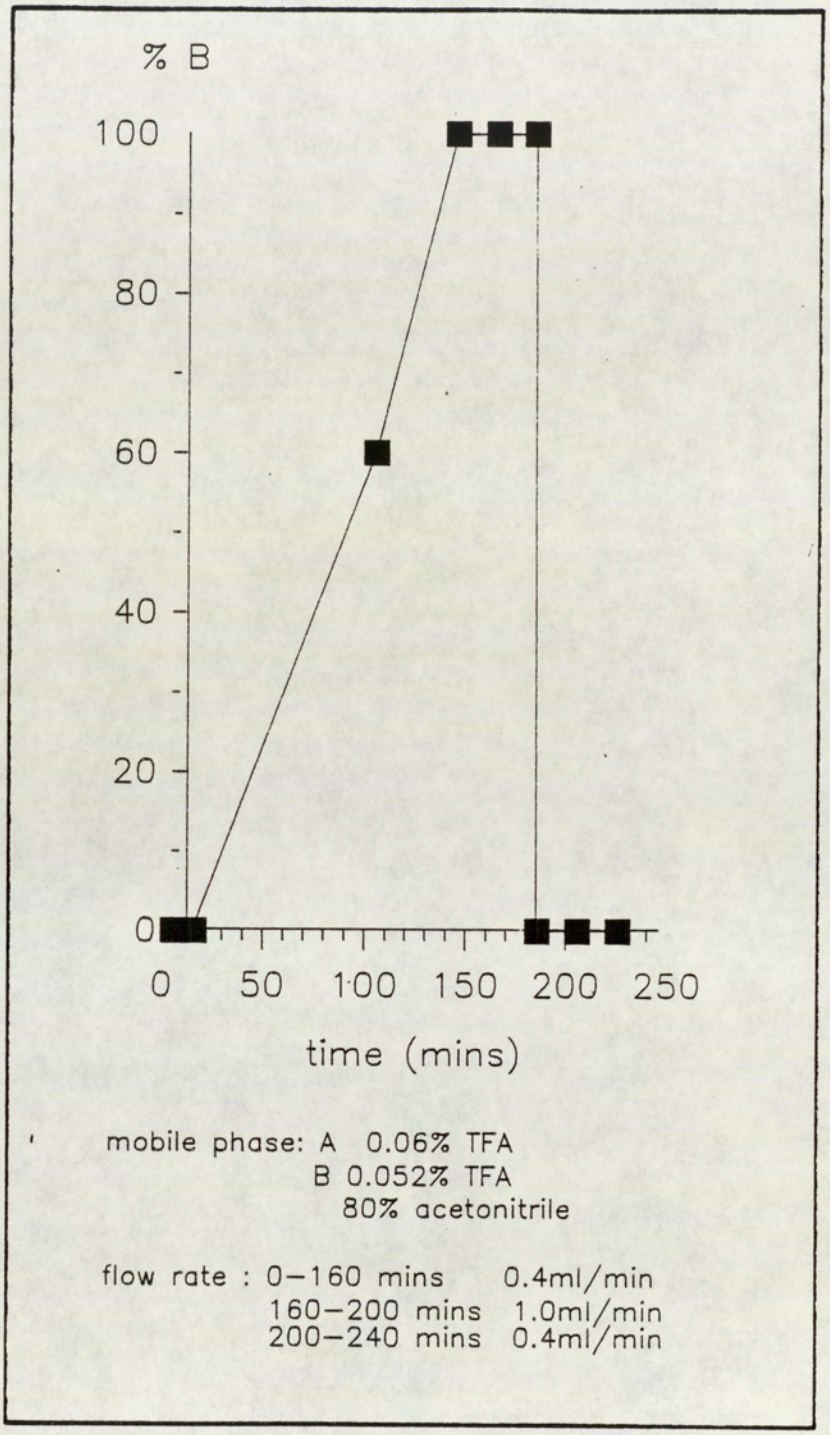


Figure.8 Gradient profile for the separation of phosphopeptides by reverse phase HPLC

2.18 CHEMICAL MODIFICATION OF ISOLATED PEPTIDES PRIOR TO PEPTIDE SEQUENCING

Peptides were modified at phosphoserine residue by the method of Meyer et.al. (1986) with modifications as described below. A model peptide Synapsin, which is a analogue of protein 4.1, was used in the study.

2.18.1 Time course

1nmol of the synapsin peptide in aqueous trifluoroacetic acid was aliquoted into a screw cap tube and dried in a Gyrovac concentrator for 30 mins. The tube was flushed with nitrogen gas and capped. 150µl of a solution consists of DMSO 33%v/v, ethanol 13.22%v/v, 5N NaOH 10.74 %v/v and ethanethiol 10%v/v, flushed with N_2 again and capped. The mixture was incubated at 50°C on a heating block for 0 - 30 mins. After the reaction, the tube was placed on ice and the tube was centrifuged for 5 mins at 1000xg. The contents were transferred to a Waters HPLC sample injection vial and the tube was rinsed with 60µl 7M guanidine HCl and the solutions were combined into the sample vial. The samples were then subjected to Reverse-Phase HPLC separation as described in the previous section (2.17.4).

2.18.2 B-elimination conditions

The experiments were carried out as described in the previous section (2.18.1) except the reaction time was

fixed for 20 mins. NaOH in the reaction mixture was either varied from 0.5N - 5N or substituted with Na_2CO_3, trimethylamine or triethylamine.

2.18.3 Temperature effects

The experiment was performed in a similar manner as in section 2.18.1. In order to maximize the yield and obtain the cleanest reaction product, the effect of temperature on the reaction was investigated. The reagent used in section 2.18.1 was employed and the reaction was proceeded at 50°C for 5 mins, ambient temperature for 5 mins or at 4°C for 60 mins. The samples were then processed as described in section 2.18.1.

CHAPTER THREE

RESULTS & DISCUSSION

3.1 GENERAL CHARACTERISTICS OF HUMAN ERYTHROCYTES

3.1.1. MORPHOLOGIES OF HUMAN ERYTHROCYTES

Human erythrocytes were obtained from healthy volunteers and used immediately. The morphological transitions of human erythrocytes which were observed in this study encompassed three major forms of morphology, namely, discocytes, echinocytes and stomatocytes (figure 9). Human erythrocytes normally adopt the discocyte morphology in vivo and other morphologies only occur in pathological conditions or after artificial manipulations in vitro (reviewed by Bessis 1973, Branton et.al. 1981, Palek & Lux 1983).

In the work outlined in this thesis, the examination of erythrocyte morphology played a major role in determining the relevance of the biochemical changes which occurred in the erythrocyte membrane and cytoskeleton as morphology changed. Since human erythrocytes normally contain no intracellular structures, light microscopy showed only the outline of the cells which were under observation. In contrast, interference microscopy and electron microscopy were capable of revealing the surface architecture of the erythrocytes; this made the identification of early stage echinocytic erythrocytes possible.

It has been shown that when a diluted erythrocyte suspension was allowed to come into contact with a glass surface, the glass induced rapid echinocytosis of the erythrocytes, probably due to an electrostatic effect (Bessis 1973). In order to avoid the `glass effect' on the morphology

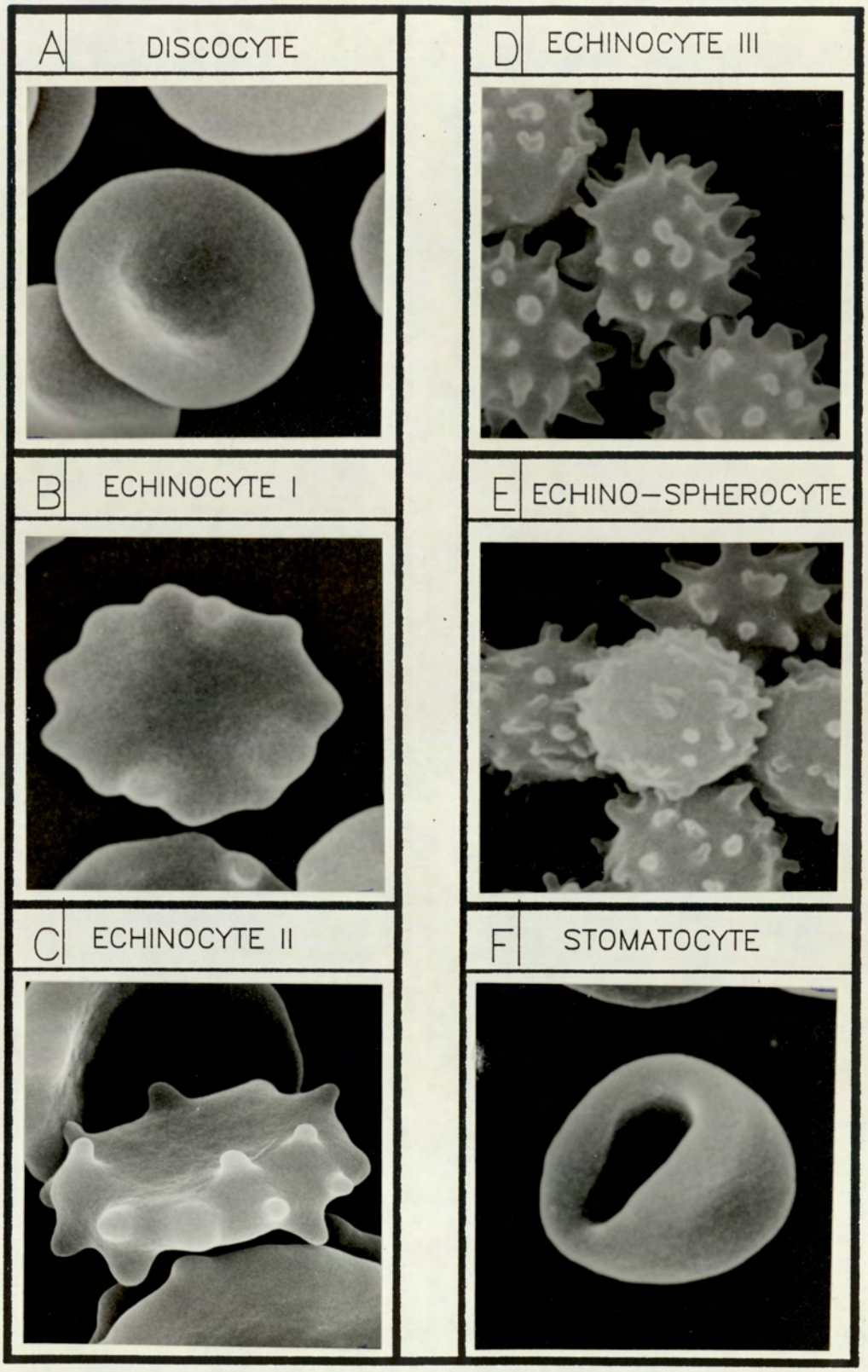


Figure.9 Morphologies of human erythrocytes under various conditions: eg. under calcium-loading or ATP-depletion (B-E).

of human erythrocytes, glutaraldehyde fixation was necessary for the study of morphological changes in vitro. It is generally accepted in hematological studies that fixed erythrocyte suspensions from venous blood of healthy volunteers normally contain 2-5% of stomatocytes. Detailed investigations, in the present study, has observed that when an erythrocyte suspension was added to the glutaraldehyde buffer, it resulted in the formation of stomatocyte-like cells and the process could be clearly observed by following the fixation process under light microscopy. The high reactivity (in milliseconds) of glutaraldehyde and the high deformability of erythrocytes caused immediate fixation while the cells were still in rapid motion. It was clear fromfurther studies that formation of stomatocytes during fixation of fresh, untreated blood could be avoided, provided that buffer which contained glutaraldehyde was added into the erythrocyte suspension slowly and not vice versa.

3.1.2. PROTEINS OF THE HUMAN ERYTHROCYTE MEMBRANE AND ITS CYTOSKELETON

When human erythrocytes were lysed and the resulting membrane ghosts were subjected to SDS polyacrylamide gel electrophoresis, using the discontinous buffer system described by Laemmli (1970), the resulting protein pattern was as shown in figure 10. This is similar to the previously published SDS-PAGE pattern of erythrocyte membrane proteins (Fairbanks et.al. 1971). The protein pattern obtained from the discontinous buffer system used in this study was

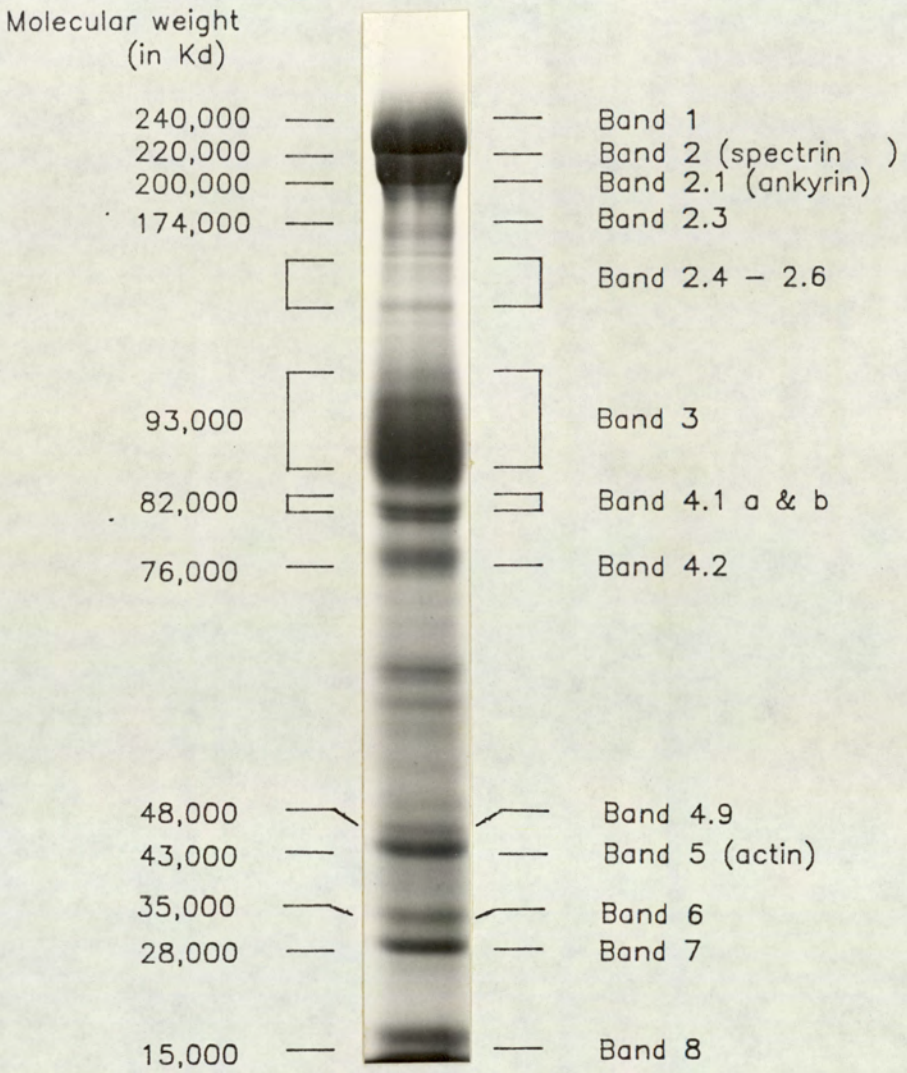


Figure.10 Electrophoretic separation of human erythrocyte membrane-cytoskeletal proteins by SDS-polyacrylamide gel electrophoresis.
The erythrocyte membranes were separated using a discontinous buffer system as described by Laemmli (1970).

different to that obtained by the continuous buffer system as described by Fairbank et.al. (1971), in which the "Fairbank system" was able to resolve band 2.1 (ankyrin) from band 2, while band 4.1 a & b remained as a single band. However, the discontinous buffer system offered the advantages that different volumes of samples could be applied onto the gel, and the protein bands which resulted from the separation were sharper and more discrete.

3.2 MORPHOLOGICAL TRANSITIONS OF HUMAN ERYTHROCYTES

3.2.1 ECHINOCYTOSIS DURING DEPLETION OF GLYCOLYTIC SUBSTRATE

Preliminary investigations were performed to examine the effect of glycolytic substrate depletion when human erythrocytes were incubated at 37°C, and the effect of Adriamycin treatment on the erythrocyte during the course of incubation without a glycolytic substrate. The results are summarized as shown in figure 11. In the absence of a glycolytic substrate, human erythrocytes gradually transformed from the discocyte morphology, through stage I and II echinocytes to the final stage III echinocytic form, at the end of an 5 hours incubation period. In the majority of experiments, 90% echinocytosis were obtained in the control sample after 5 hours incubation at 37°C.

When ATP-depletion of human erythrocytes was carried out in the presence of 10μM Adriamycin, Adriamycin was capable of suppressing echinocytosis. In figure 11 it is shown that the presence of 10μM Adriamycin offered approximately a 20% protection against echinocytosis. However, the course of echinocytosis was highly variable in both control and treated cell populations, although Adriamycin-mediated protection was observed in each experiment. The variability was possibly due to the variation in susceptibility of the erythrocytes from different donors towards ATP-depletion or may be correlated with the nutritional state of the donor at the time of blood donation. This degree of variability with respect to morphological changes which took place under ATP-depletion

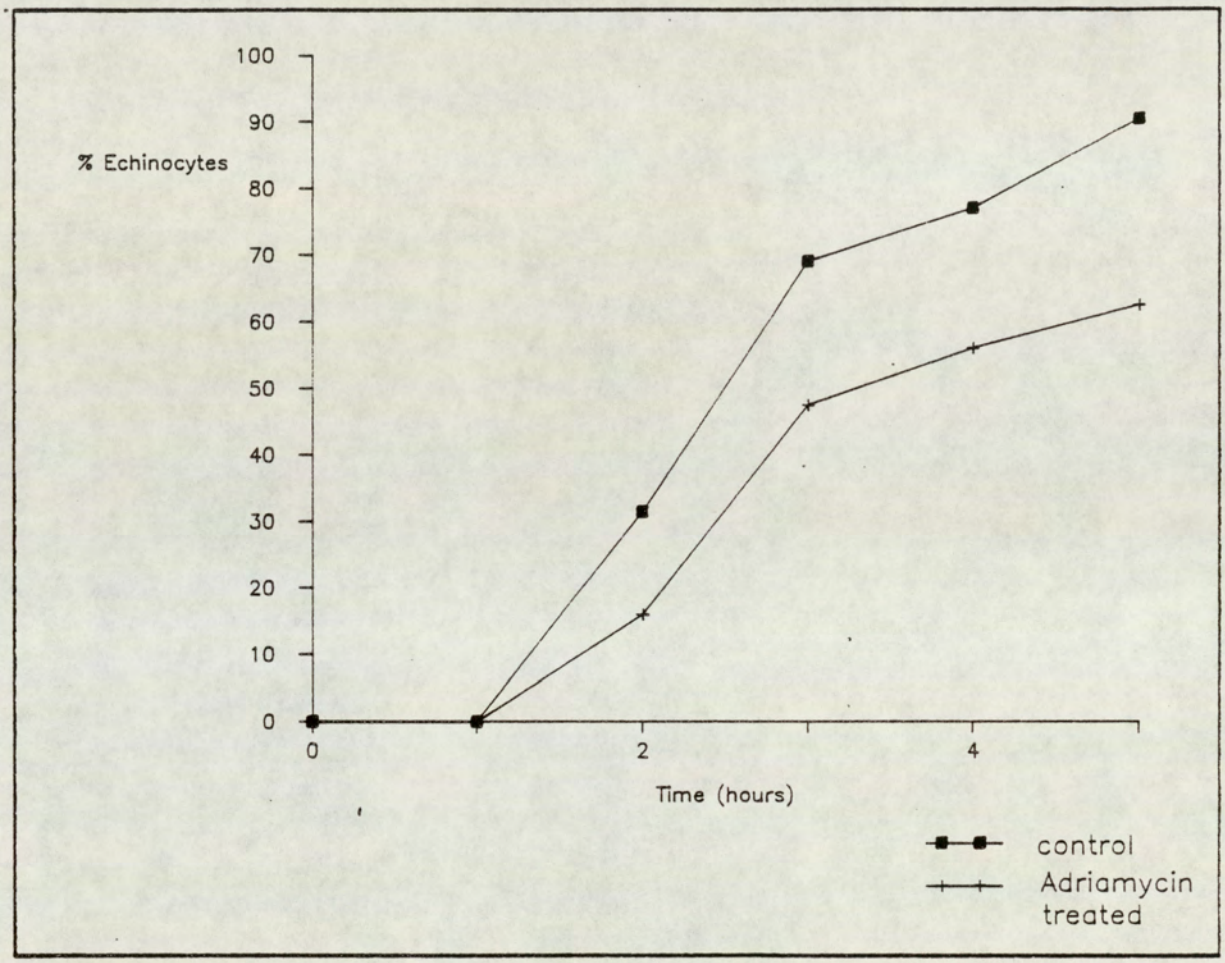


Figure.11 Echinocytosis induced by ATP-depletion in human erythrocytes and the effect of Adriamycin.
Human erythrocytes were ATP-depleted for 0 - 6 hours either in the absence (■) or presence (+) of Adriamycin (10µM). Data shown were taken from a representative experiment (n>5).

conditions led to the search for an alternative, and more reproducible method of the induction of morphological transitions. The method chosen was that of calcium-induced echinocytosis, which was found to be less variable under `defined' conditions, as described below.

3.2.2. CALCIUM-INDUCED ECHINOCYTOSIS IN HUMAN ERYTHROCYTES

Human erythrocytes were found to undergo echinocytosis when cytosolic free calcium levels were elevated, using the calcium ionophore A23187. However, it was found that fresh preparations of human erythrocytes from blood contained variable and high basal levels of the echinocytic morphology, and consequently the induction of echinocytosis was highly variable in preliminary studies. This variability then created difficulties of interpretation of the results from both morphological and biochemical experiments: the problems were overcome as the result of an investigation of the factors which affected the maintenance of erythrocyte morphology in vitro, and details of this will be described in detail in the following section (section 3.3).

The calcium-induced echinocytic morphological transition took place in both a Ca^{2+} concentration-dependent and time-dependent manner. Figure 12 shows the concentration-dependent morphological changes of human erythrocytes. At low calcium concentrations (<50μM), the echinocytes present in the suspension were mainly stage 1 and stage II (see figure 9 & Bessis et.al. 1973). At higher calcium concentrations, rapid

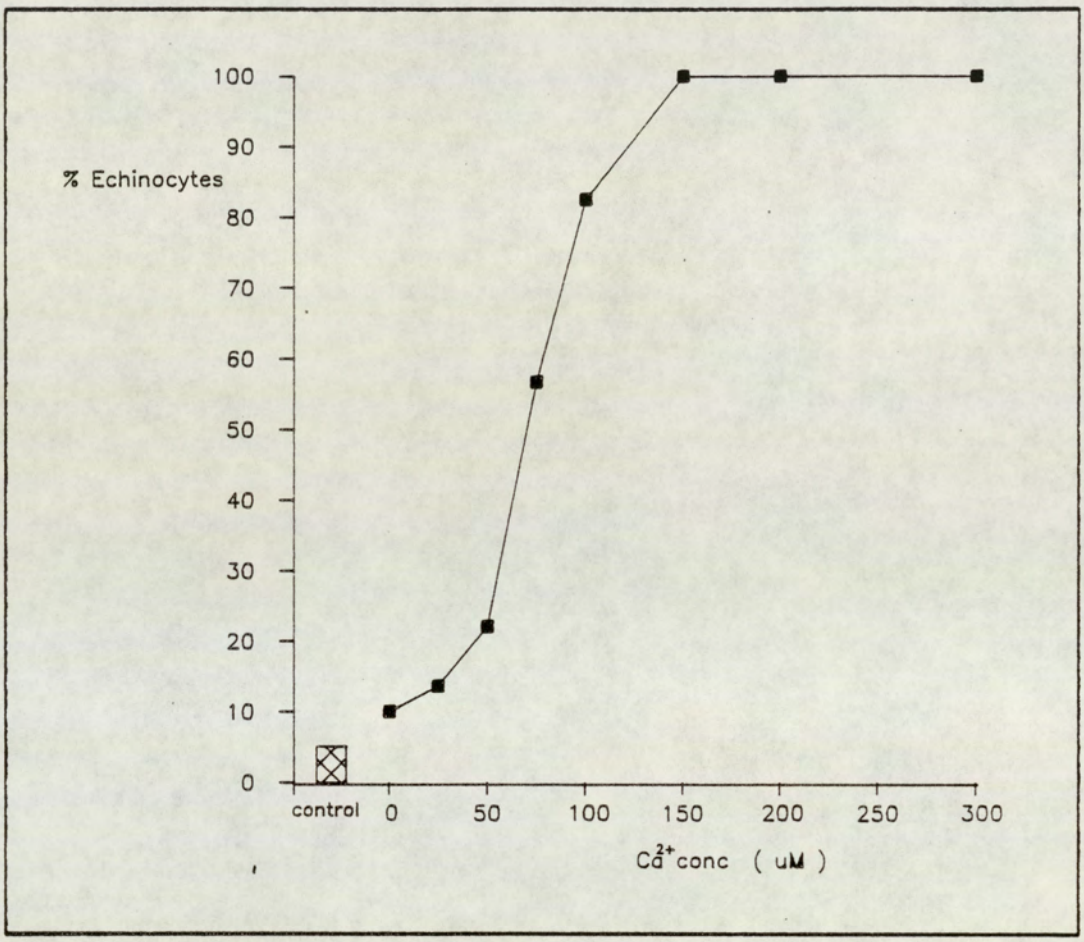


Figure.12 Echinocytic response of human erythrocytes to calcium. Human erythrocytes were incubated with various concentration of calcium for 5 minutes and their morphologies were accessed as described in method. Data shown were obtained from a representative experiment (n>30).

morphological transitions occurred and stage III echinocytes and echino-spherocyte were formed.

Figure 13 shows the time-dependent changes of the morphological state of erythrocytes when they were loaded with either 50μM or 1mM calcium. With 50μM calcium, erythrocytes were gradually transformed into various echinocytic forms such that all stages of echinocytic morphology were observed. Under conditions of loading with a high calcium concentration (1mM), the transformation was rapid (< 1 min) and all erythrocyte were at stage III echinocyte after less than 1 minute.

3.2.3 EFFECT OF ADRIAMYCIN ON CALCIUM-INDUCED ECHINOCYTOSIS

Under conditions where 50μM calcium was used for the calcium loading of human erythrocytes, pre-incubation of erythrocytes with Adriamycin (10-50μM) was found to have a protective effect, inhibiting echinocytosis. Figure 14 shows the echinocytic transition of human erythrocytes with various calcium concentrations and the inhibitory effects on morphological transition provided by a 10 minutes Adriamycin pre-treatment. It was observed that preincubation of erythrocytes with 50μM Adriamycin resulted in further protection against echinocytosis at higher calcium (>200μM). However, this effect was found to be variable with only 50% of the studies (n=8) conducted showed the extended protection and further investigation is required to clarify the protective effect at these calcium concentrations. As shown previously, exposure of untreated erythrocytes to increasing calcium

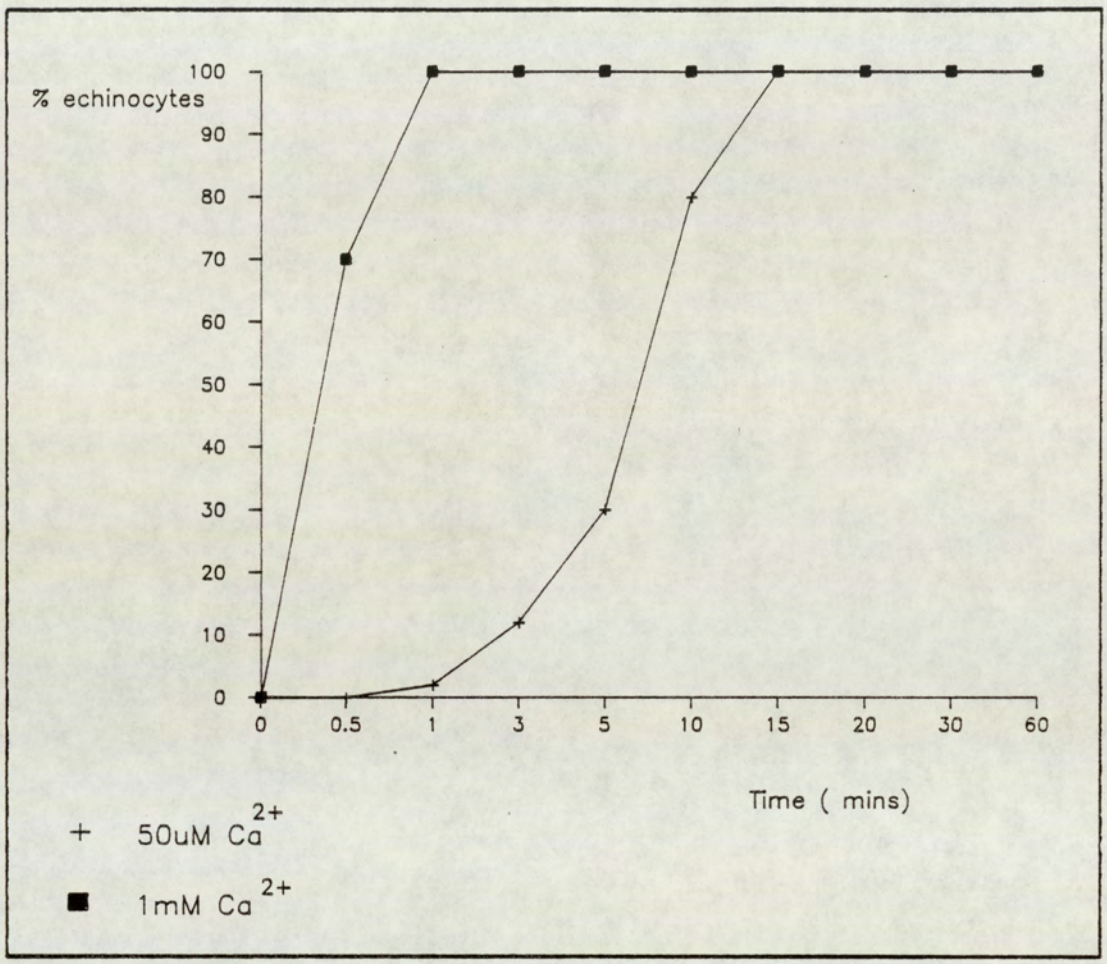


Figure.13 Time course of calcium-induced echinocytosis at two different calcium concentrations.
Human erythrocytes were incubated with either 50µM or 1mM calcium in the presence of calcium ionophore for 0 - 60 minutes. Data were shown from results obtained in a representative experiment (n=2)

concentrations (<200μM), in the presence of calcium ionophore, resulted in progression of echinocytic transformation. Pre-incubation of erythrocytes with 5μM Adriamycin did not alter the morphological response to calcium. However, 5μM Adriamycin was capable of reducing basal levels of echinocytosis seen prior to calcium loading. These results suggest that there may be distinct mechanisms in the induction of echinocytosis during the cell preparation procedures and calcium loading. In addition, Adriamycin may modulate both mechanisms to prevent echinocytosis but higher concentration of Adriamycin was required during calcium loading.

Adriamycin was shown to exert a potent protective effect against calcium-induced echinocytosis when erythrocytes were pre-treated with >= 10μM Adriamycin. As shown in figure 14, Adriamycin was capable of restraining echinocytosis at calcium concentrations of up to 150μM. Above this concentration of calcium, a sudden and rapid morphological transition occurred and studies of cellular morphology revealed that all the erythrocytes were stage III echinocytes or echino-spherocytes. Although figure 14 indicates that the transitional calcium concentration was approximately 150μM, the figure shows the results obtained from the majority of experiments carried out and extensive studies showed that the transition point varied from approximately 75 - 150μM calcium.

Preliminary studies on the effect of Adriamycin on calcium-induced echinocytosis have been performed in our laboratory as described by Chahwala and Hickman (Chahwala et.al.1985). In these studies, it was shown that Adriamycin

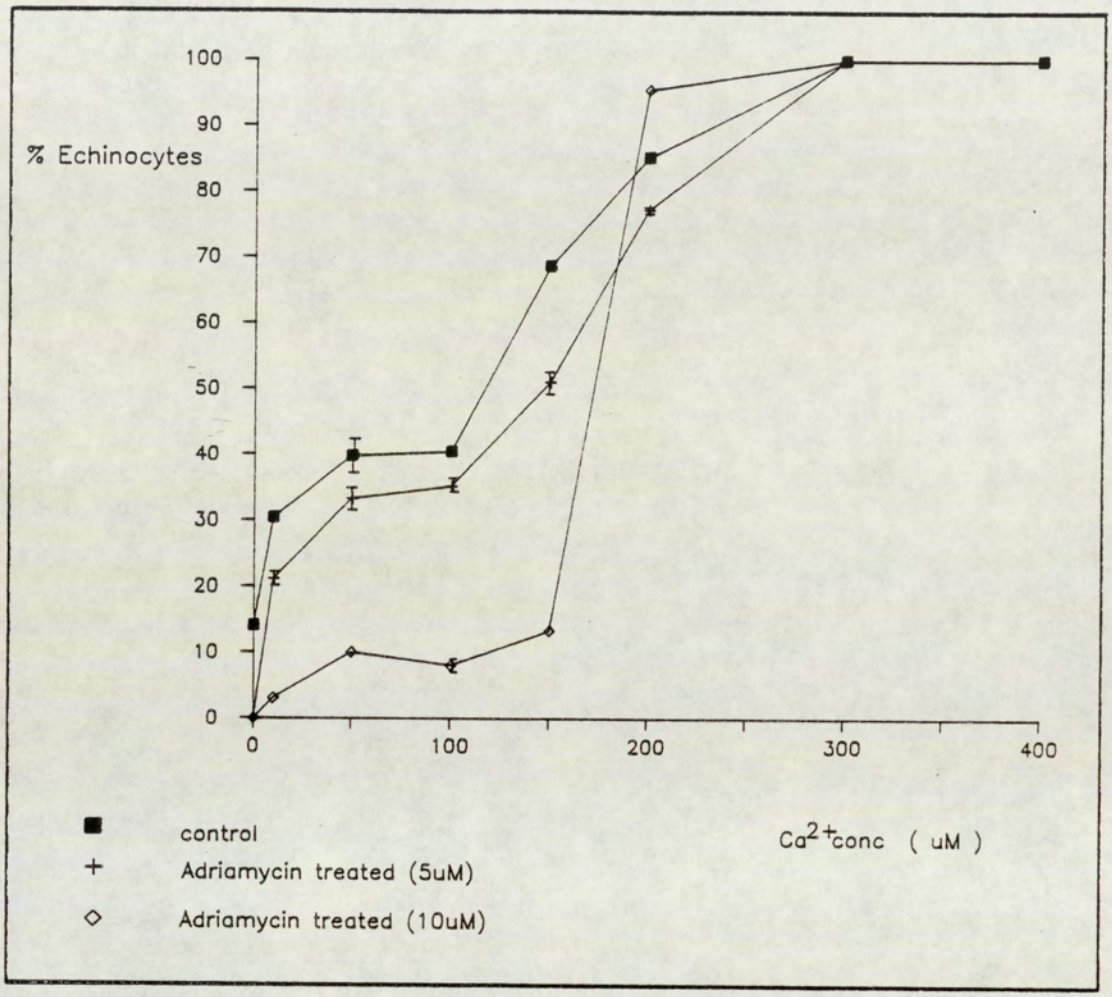


Figure.14 Effects of Adriamycin on calcium-induced echinocytosis in human erythrocytes.
Human erythrocytes were incubated with various concentrations of calcium (0 - 400μM) for 5 minutes in the presence of calcium ionophore. Incubations were carried out either in the absence of Adriamycin or Pre-treated with Adriamycin (5 or 10μM) for 10 minutes prior to calcium loading. Results were shown from a representative experiment (n>20).

was capable of protecting human erythrocytes against calcium-induced echinocytosis when erythrocytes were loaded with 200μM calcium. The protection was described as being in a concentration-dependent manner with approximately 50% protection at 0.5μM Adriamycin. However, such potent protective effects were not observed under the conditions described in this thesis. The difference may due to the variation in the criteria of determining what morphology constituted an echinocyte and the utilisation of interference microscopy, which greatly facilitate the identification of early echinocytosis, in stage I or II. It is likely that Chahwala and Hickman scored these early stage echinocytes as discocyte-like cells, whereas in the present study stage I echinocytes were scored as a significant morphological transition.

3.3 FACTORS INFLUENCING THE MAINTENANCE OF ERYTHROCYTE PREPARATION AND THE CONTROL OF ECHINOCYTOSIS

The experiments described so far in this thesis were carried out using a widely adopted methodology (Anderson and Lovrien 1981). Human erythrocytes were washed, maintained in incubation buffer A and kept at either room temperature or at 4°C. Since erythrocytes normally adopt the discocyte morphology *in vivo*, maintenance of discocyte morphology in erythrocyte preparations prior to manipulation was essential. However, despite the fact that most of the previous studies have intended to elucidate the role of erythrocyte cytoskeleton in morphological transformations, few studies have related these biochemical processes to any morphological transitions, which may have taken place during the course of the experiments. Little attention has been given to the maintaining erythrocytes in the discocytic form throughout the course of such experiments, or prior to manipulation of morphology.

In this study, experiments were designed to investigate various biochemical processes, which may be responsible for the onset of morphological changes in human erythrocytes. The experiments were designed such that parallel samples were prepared for the examination of biochemical changes and those of morphology. By the use of morphology modulating agents, such as Adriamycin, the biochemical changes observed could be correlated to the morphological changes observed.

As a continuation of the initial studies carried out in

our laboratories (Chahwala & Hickman 1985a, Hickman et.al.1986), conditions were used early in this study which were identical to these. However, difficulties were encountered in maintaining the discocyte morphology of erythrocytes prior to experiment during early stage of the studies. When experiments were performed using incubation buffer A, it was observed that a significant population of erythrocytes in the suspension were echinocytes. Although this morphological transition was not clearly noticeable by normal light microscopy, the presence of stage 1 echinocytes were clearly observed by interference microscopy. These experiments indicated that the extent of such echinocytosis ranged from 20-80 %, with mostly stage 1 echinocytes present in fresh erythrocyte preparations. Detailed investigations showed that these morphological changes took place as early as the erythrocyte washing process (result not shown). For the purpose of the other experiments which are recounted in this thesis, experiments were performed to investigate the conditions required in order that the discocyte morphology and the metabolic activity of erythrocytes be maintained for an extended period.

3.3.1. THE EFFECT OF TEMPERATURE ON THE MAINTENANCE OF DISCOCYTE MORPHOLOGY AND ON CALCIUM-INDUCED ECHINOCYTOSIS

Human erythrocytes were washed and the cell suspension was prepared at 10^7 cells/ml using incubation buffer A. The cells were washed at 24°C and used promptly. The suspensions

were divided into aliquots, pre-incubated for 10 minutes with and without Adriamycin, followed by calcium loading at either 24°C or 37°C. Figure 15 summarized the morphological state of erythrocytes prior to calcium loading and their response to calcium loading at different temperatures. When erythrocytes were prepared and maintained at 24°C, a high percentage of a basal echinocytic morphology was observed prior to calcium loading. After 5 minutes of calcium loading with various concentrations of calcium (0-200μM) in the presence of calcium ionophore A23187, the extent of echinocytosis was slightly increased with most of the echinocytes being at stage I of the echinocytic morphology. However, the increase in echinocytosis was only marginal, and there was no correlation between the extent of echinocytosis and calcium concentration. In fact, further studies, which will described later, show that the increase in echinocytes was simply due to the further incubation period during calcium loading rather than to the influx of calcium.

When the erythrocytes suspensions were incubated at 37°C for 10 minutes prior to calcium loading, extensive echinocytosis (up to 80%) was observed before the addition of calcium and ionophore. Most of the echinocytes observed were also at stage I (figure 15). Addition of calcium and ionophore promoted further echinocytosis; high percentages of the echinocytes were then observed to be at stage II or stage III in their morphology.

Incubation temperature also had effects upon the action

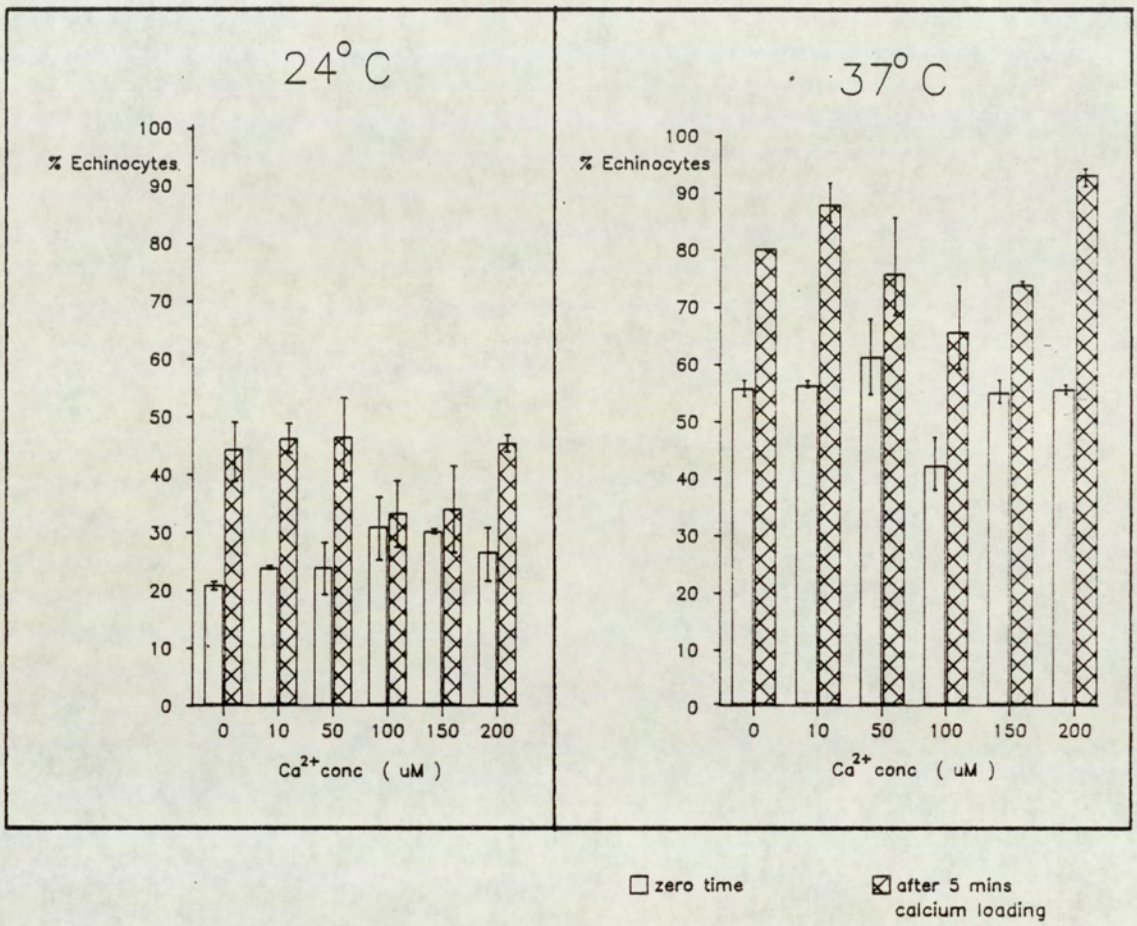


Figure.15 Effect of temperature on calcium-induced echinocytosis.
Human erythrocytes were loaded with calcium for 5 minutes either at room temperature (24°C) or 37°C. The morphology of the erythrocytes were examined both at zero time and at the end of the calcium-loading period and representative results were shown (n=2).

of Adriamycin on morphology. When erythrocytes were preincubated with 50μM Adriamycin for 10 minutes at 24°C prior to addition of calcium and ionophore, Adriamycin effectively reduced both the basal level of echinocytosis and that of calcium-induced echinocytosis (figure 16). When the Adriamycin preincubation was carried out at 37°C, the potency of Adriamycin to reduce basal echinocytosis was greatly enhanced. Upon the addition of calcium and ionophore, Adriamycin was found to exert a potent protective effect against calcium-induced echinocytosis up to 150μM calcium. Above this calcium threshold level, the ability of Adriamycin to protect against calcium-induced echinocytosis was greatly reduced. When erythrocytes were pre-treated with a lower concentration of Adriamycin (10μM), Adriamycin exerted similar potency in inhibiting calcium-induced echinocytosis at calcium concentration below the threshold level (<150μM) as shown in figure 14. This protective effect offered by Adriamycin was lost when calcium concentration was above the threshold level.

The differences in the extent of echinocytic transformation prior to calcium-loading and the response to calcium-loading at different temperatures may be explained by the lowering of metabolic activity of erythrocytes at the lower temperature. The laws of thermodynamics suggest that a temperature decrease of 10°C will cause a 50% decrease of any enzymatic process, including calcium-induced processes. Since the erythrocytes were maintained with incubation buffer A, which is only an isotonic medium without metabolic substrates, the erythrocyte preparation was relatively

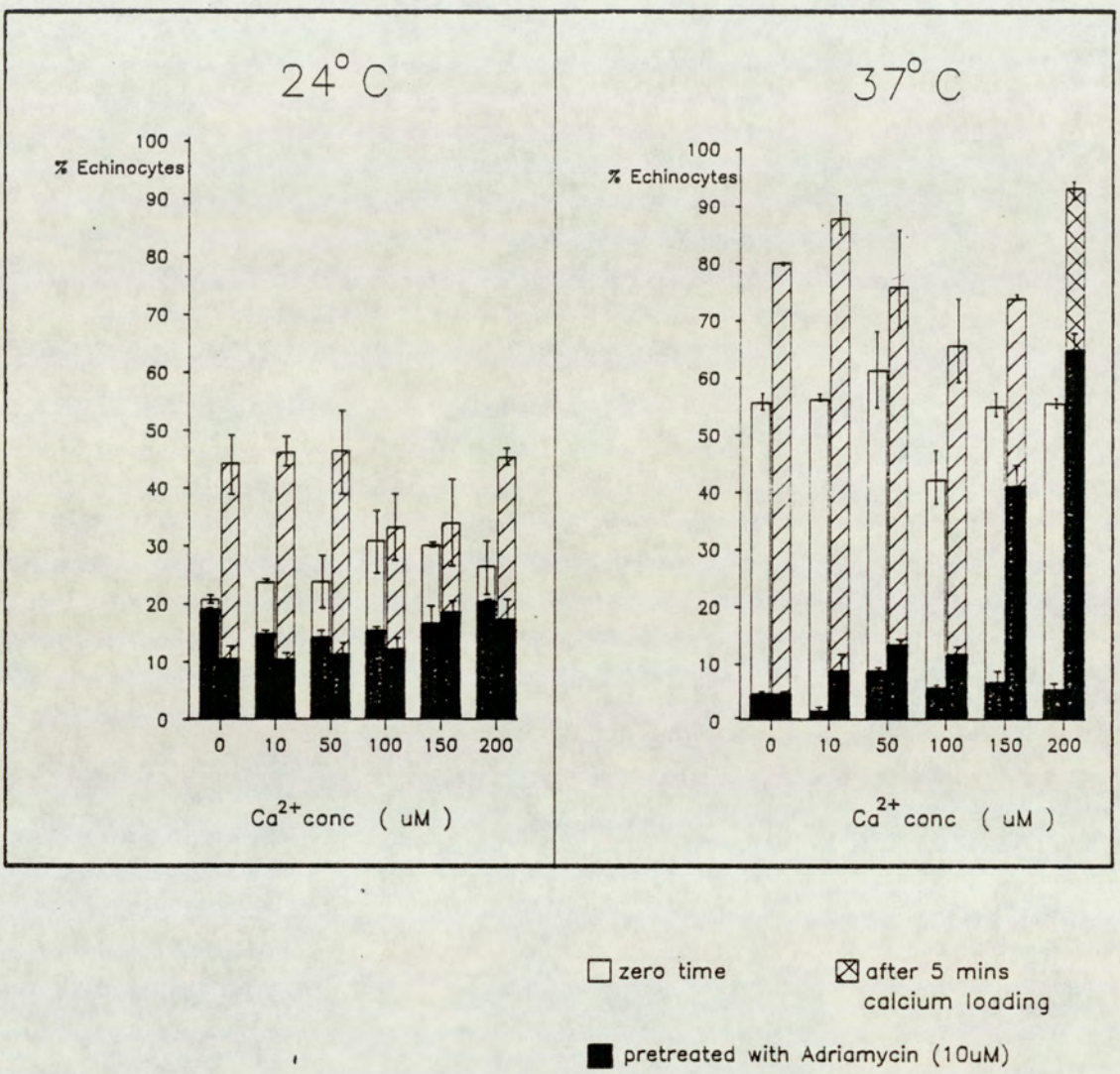


Figure.16 Effect of temperature on the efficacy of Adriamycin in the inhibition of calcium-induced echinocytosis.
Human erythrocytes were preincubated either in the absence (light bar) or in the presence of Adriamycin (10μM) for 10 minutes prior to calcium loading with various concentration of calcium. Parallel experiments were carried out to investigate the effect of incubation temperature (24°C or 37°C) on the calcium-induced echinocytosis process and representative results were shown (n=2).

depleted of a glycolytic substrate. When the erythrocytes were maintained in incubation buffer A at 24°C, the lack of a glycolytic substrate may decrease the activity of certain metabolic processes which may themselves be responsible for the onset of echinocytosis (discussed below). The lack of a glycolytic substrate may cause the onset of echinocytosis but the rate of this process may be decreased by the lowering of incubation temperature. After the addition of calcium and ionophore, the low temperature may also hinder the progress of various calcium-mediated processes; this may explain the lack of any consistent calcium response during calcium loading.

When the incubation temperature was increased, the intracellular metabolic rate was also increased, thus echinocytosis was accelerated as a result of substrate depletion, and not only because of calcium loading. Various calcium-mediated processes could also proceed more optimally as a result of the elevated temperature. Taken together, this gives rise to the higher echinocytosis observed prior to, and during, calcium loading.

Increased temperature also affected the efficacy of Adriamycin: it could be seen from figure 16 that Adriamycin possessed higher potency in preventing echinocytosis under both basal and calcium induced conditions. This could be explained, in accordance with Mikkelson et.al (1977), by an enhanced temperature-dependent membranous and intracellular uptake of Adriamycin.

3.3.2. EFFECTS OF GLYCOLYTIC SUBSTRATE ON MORPHOLOGY

As shown in the last section, the elevation of the incubation temperature to physiological (37°C), promoted calcium-induced echinocytosis. However, the temperature increase also caused a dramatic increase in the basal level of echinocytosis, prior to calcium loading. Such high basal levels of echinocytosis may obscure the effects of Adriamycin treatment, and consquently the interpretation of biochemical events accompanying their changes are difficult. The effect of glycolytic substrate replenishment was therefore investigated, in order to overcome these problems.

Figure 17 summarised the results obtained from such studies. Figure 17a shows the high basal level of echinocytosis, and the extent of calcium-induced echinocytosis when cells were washed and incubated with incubation buffer A, as described previously. Adriamycin was found to inhibit or reverse the high level of basal echinocytosis, and to inhibit calcium-induced echinocytosis at low (50μM) calcium loading, after 10 minutes of pre-incubation of the cells with the drug. When erythrocytes were prepared and incubated with a glycolytic replenished medium (incubation buffer B), the results were as are summarised in figure 17.b. In these experiments, the onset of basal echinocytosis was abolished and cells were maintained in a discocytic morphology for at least 6 hours. The cells also showed an increased and controlled calcium-induced echinocytosis which was in a Ca^{2+}-concentration dependent manner. Adriamycin inhibited this controlled process of echinocytosis. The level of

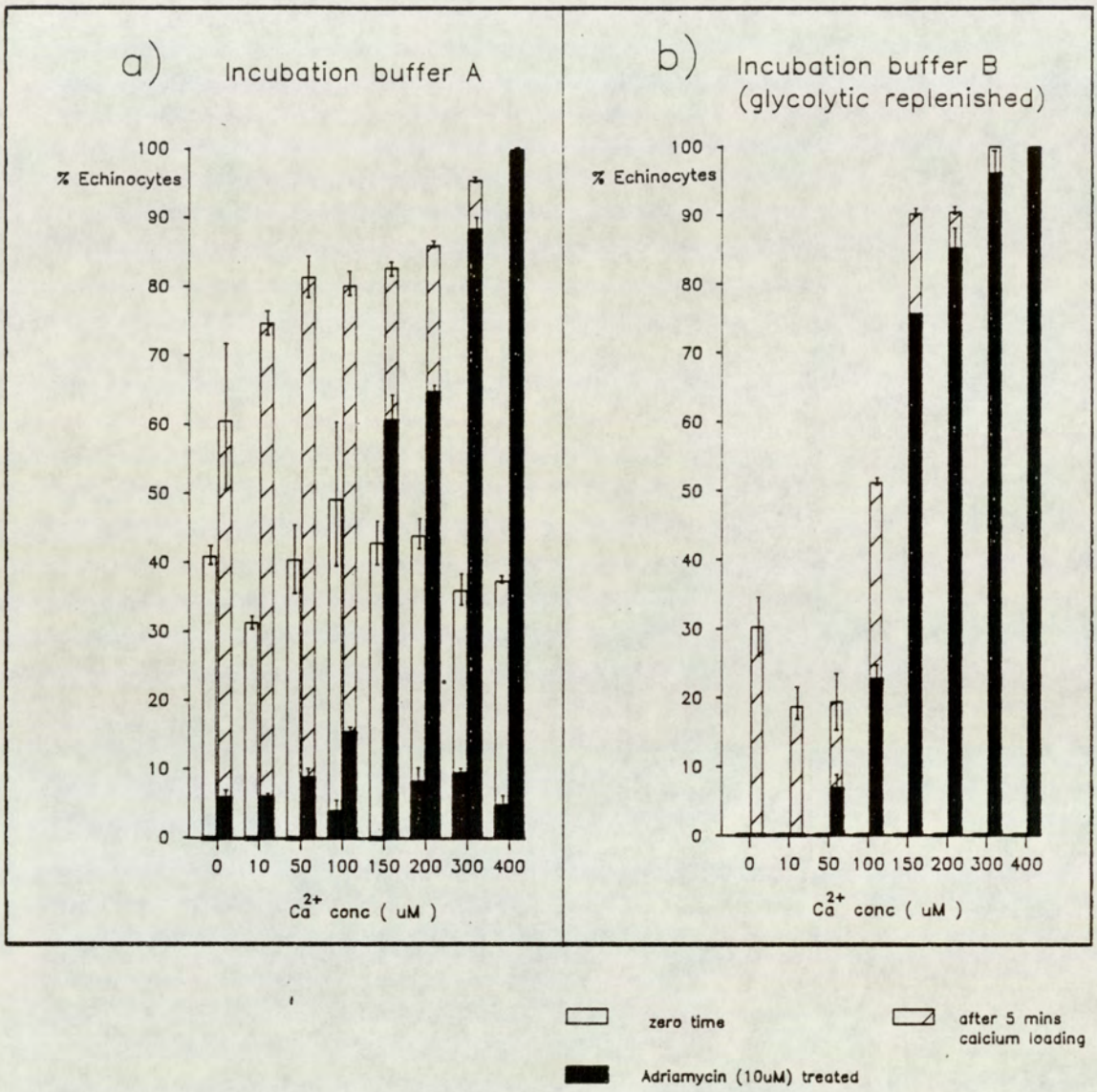


Figure.17 Effect of glycolytic substrate on calcium-induced echinocytosis and on the efficacy of Adriamycin in the inhibition of calcium-induced echinocytosis.
Human erythrocytes were either maintained in incubation buffer A or incubation buffer B (glycolytic replenished) followed by calcium-loading for 5 minutes at 37°C. Parallel incubations were prepared and pre-treated with Adriamycin (10μM) for 10 minutes prior to calcium loading.

echinocytes at low calcium (0 - 50μM) was considered, in early experiments, to be due to slight calcium contamination from other reagents, and this was eliminated in further experiments.

3.3.3. THE EFFECT OF BOVINE SERUM ALBUMIN

It has been noted that the presence of bovine serum albumin (BSA) in incubation buffer B was also essential in the maintenance of the discocyte morphology of human erythrocytes. However, an excess of BSA in the medium was considered potentially to buffer the calcium added during calcium loading. The albumin also presented problems in subsequent SDS-PAGE analysis: BSA has a molecular weight of 66,000, and the presence of excess BSA in the sample obscured the pattern of proteins seen in the SDS-PAGE analysis of cytoskeletal proteins. Hence investigations were made in order to determine the optimum concentration of albumin for maintaining discocyte morphology and calcium loading.

Figure 18 shows the effect of including various concentrations of BSA in the incubation medium. BSA with concentration higher than 0.0005% w/v significantly prevented basal echinocytosis. The extent of calcium buffering was found not to be significant at low concentrations (<0.002% w/v). The possibility that various agents such as calcium ionophore and drugs might also bind to the higher concentration of BSA was considered, and 0.002%w/v of BSA, which significantly prevented basal echinocytosis, was then used throughout.

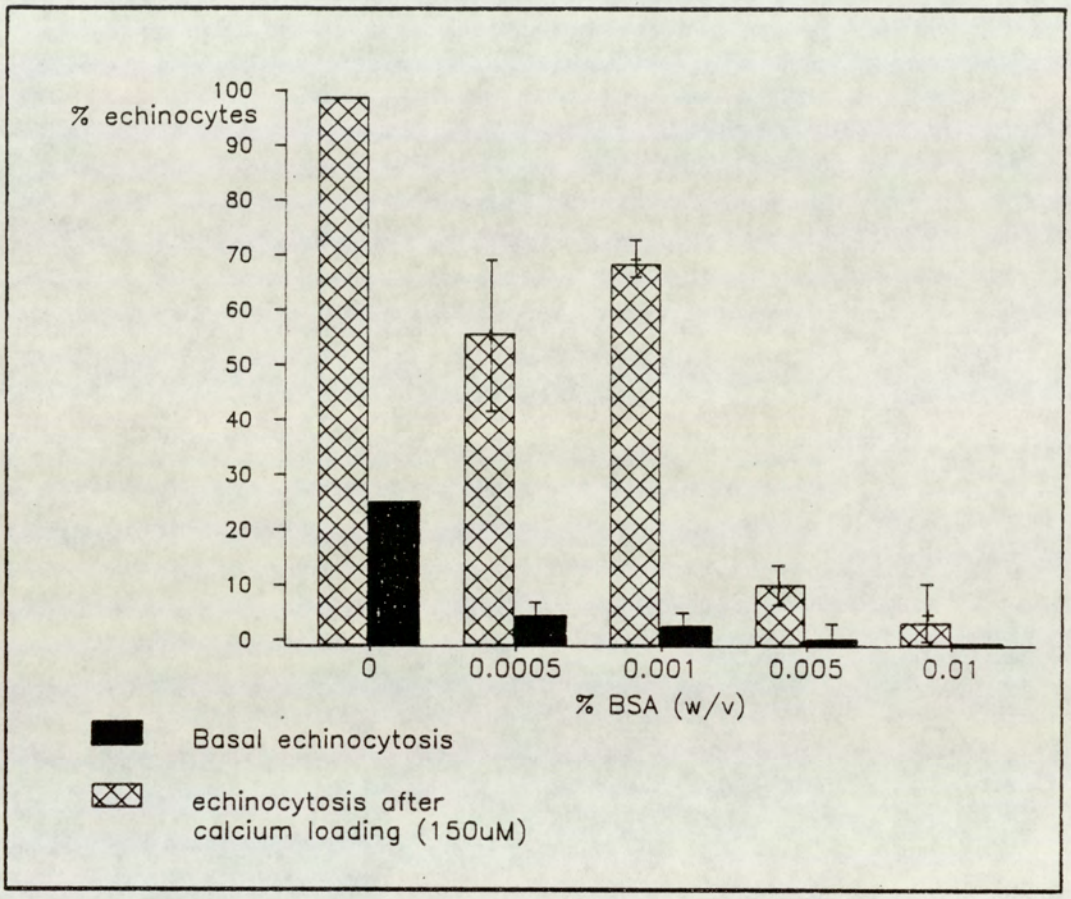


Figure.18 Effect of bovine serum albumin on the basal maintainance of human erythrocytes and calcium-induced echinocytosis.
Morphological examinations were performed on incubations where cells were washed and maintained in incubation buffer B contained various concentration of bovine serum albumin and the effect of BSA on calcium-induced echinocytosis was assessed.

The above results indicated that, in the past, relatively little effort has been made to undertake parallel studies involving both cell biological and biochemical evaluation of erythrocyte behaviour. Many of the studies have used incubation media which may cause echinocytosis, yet they intended to investigate the underlying mechanism of echinocytosis.

3.4 CALCIUM-INDUCED ECHINOCYTOSIS IN HUMAN ERYTHROCYTES - PROTEOLYTIC ACTIVATION

3.4.1. TIME-DEPENDENT PROTEOLYSIS

Chapter two described that the calcium loading of erythrocytes induced a controlled echinocytic transformation of human erythrocytes, in a calcium concentration- and time-dependent manner. Previous studies by other investigators, on the morphological transition of erythrocytes and its underlying biochemical mechanisms, had been performed with little regard to the metabolic state of the cells or to their morphological status. In experiments where changes in cytoskeletal proteins are to be analysed, it was important to be able to relate the status of the cytoskeleton to that of cell morphology. In the present study, all cell samples were prepared for morphological examinations, under the defined conditions described above, and biochemical studies were performed on the same samples.

The underlying mechanism by which erythrocytes undergo morphological transformation, under various conditions, is controversial (see introduction). Interest has been focused on the biochemical regulation of the cytoskeletal network, and in those processes which may cause the onset of cell shape change. It has been shown that when human erythrocytes were loaded with high concentrations of calcium, the proteolytic modification of several major components of the erythrocyte membrane and cytoskeleton was observed (Allan & Thomas 1981a &

b).

In the experiments described here, human erythrocytes were loaded with either 50μM or 1mM calcium at 37°C in the presence of calcium ionophore for 0 to 60 minutes. At appropriate time intervals, duplicate samples of cells were taken for both morphological examination and the remaining cell suspension was used for the preparation of membrane ghosts for SDS-PAGE analysis. The results are summarised in figure 19 (50μM calcium) and figure 20 (1mM calcium). When erythrocytes were loaded with 50μM calcium, morphological studies revealed that echinocytosis took place gradually and reached 100% completion 15 minutes after calcium and ionophore addition. The results of SDS-PAGE analysis of the membrane ghosts after staining with commassie blue are shown in figure 19, two major changes on the gel were observed: it could be seen that two protein bands with molecular weight 174,000 and 66,000 daltons were increased in the membrane ghosts after calcium loading. The 174K protein co-migrated with band 2.3, which is a proteolytic product of Ankyrin (band 2.1 Mw 200,000). Unfortunately, the breakdown and disappearance of band 2.1 could not be seen clearly on the gel since the band 2.1 band co-migrated with band 2, using this discontinous buffer system. However, the increase in the intensity of band 2.3 with time clearly reflected the proteolysis of band 2.1 after calcium loading. The time course also indicated that the proteolysis of band 2.1, which increased significantly 3 minutes after calcium loading, coincided with the onset of echinocytosis (see figure 19b & c).

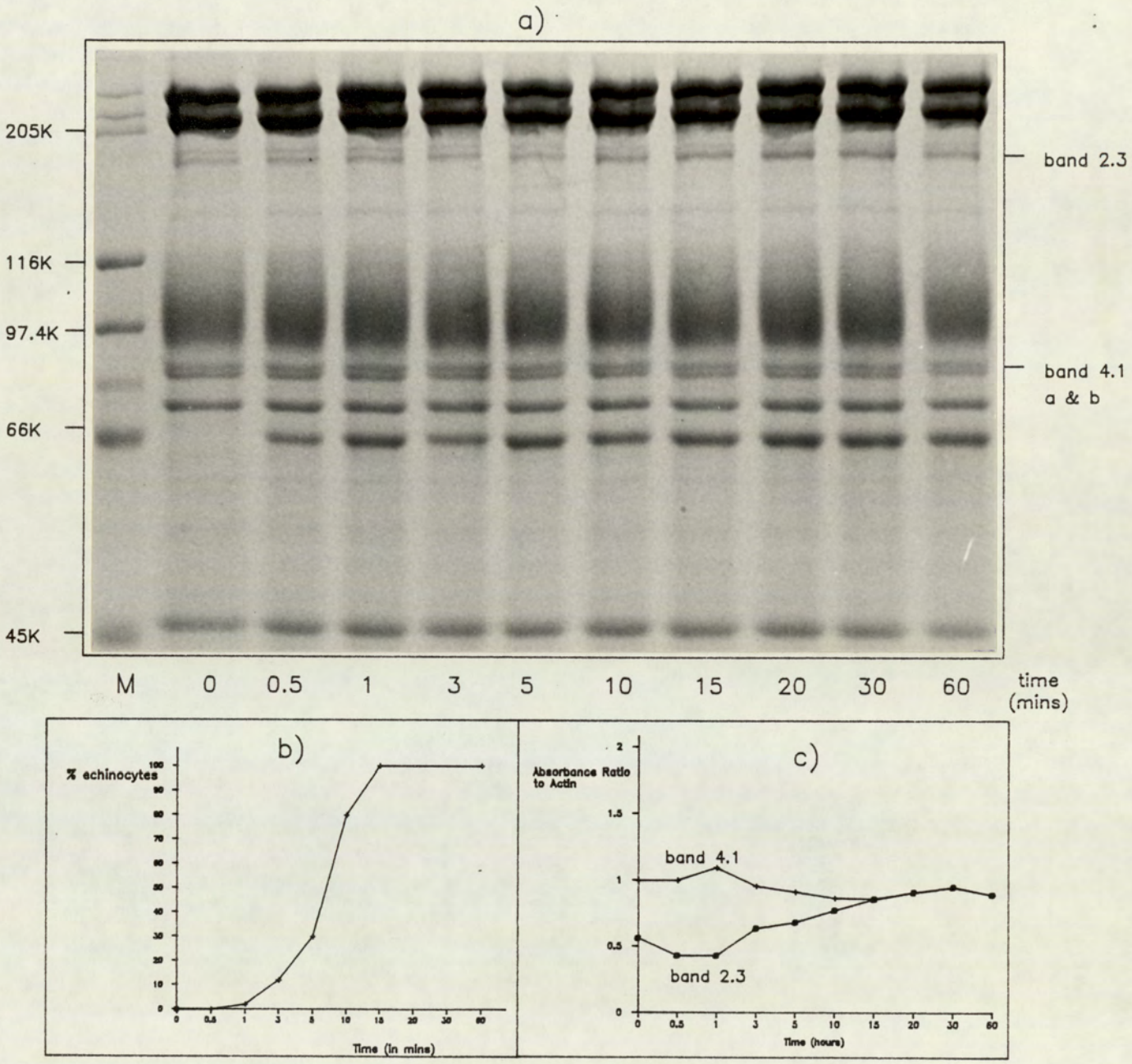


Figure.19 Time-dependent proteolytic modification of human erythrocyte membrane-cytoskeletal proteins - 50μM calcium.
Human erythrocytes were loaded with calcium (50μM) at 37°C. (a) erythrocyte ghosts were prepared and analysed by SDS-PAGE with a 7.5% gel. Lane M is the molecular weight marker. (b) morphological state of erythrocytes from same samples at different time intervals. (c) densitometric analysis of band 2.3 and band 4.1 as shown in (a).

The identity of the 66K protein was found to be bovine serum albumin, which also has a similar molecular weight. The fact that the absence of the 66K proteins on the gels from experiments, which BSA was omitted, tends to confirm the identity of this protein. Although addition of calcium to the cell suspension enhanced the association of BSA with the membrane, it is unlikely that BSA plays a major role in the regulation of morphological transformation during calcium-induced echinocytosis although it was shown to maintain the discocyte morphology in unloaded cells. The coincidence between band 2.1 breakdown and morphological transition suggested the band 2.1 may play an important role in the onset of calcium-induced echinocytosis as suggested by Allan et.al. (Allan et.al. 1982).

In contrast, when human erythrocytes were loaded with 1mM calcium, morphological studies showed that the erythrocytes underwent rapid echinocytosis and reached completion of the morphological transition within 1 minute. Greater than 95% of the cell population possessed a stage III or sphero-echinocyte morphology. SDS-PAGE analysis of the membrane ghosts prepared from the calcium loaded cells showed the increase in intensity of band 2.3 reflecting the proteolysis of band 2.1 (figure 20). In contrast to cells loaded with a low concentration of calcium (50μM), cells loaded with 1mM calcium caused a more rapid and greater degree of proteolysis of band 2.1: approximately two fold increase was suggested by the ratio of the commassie blue intensity of band 2.3 and actin, the latter which was constant throughout the incubation (see figure 19c &

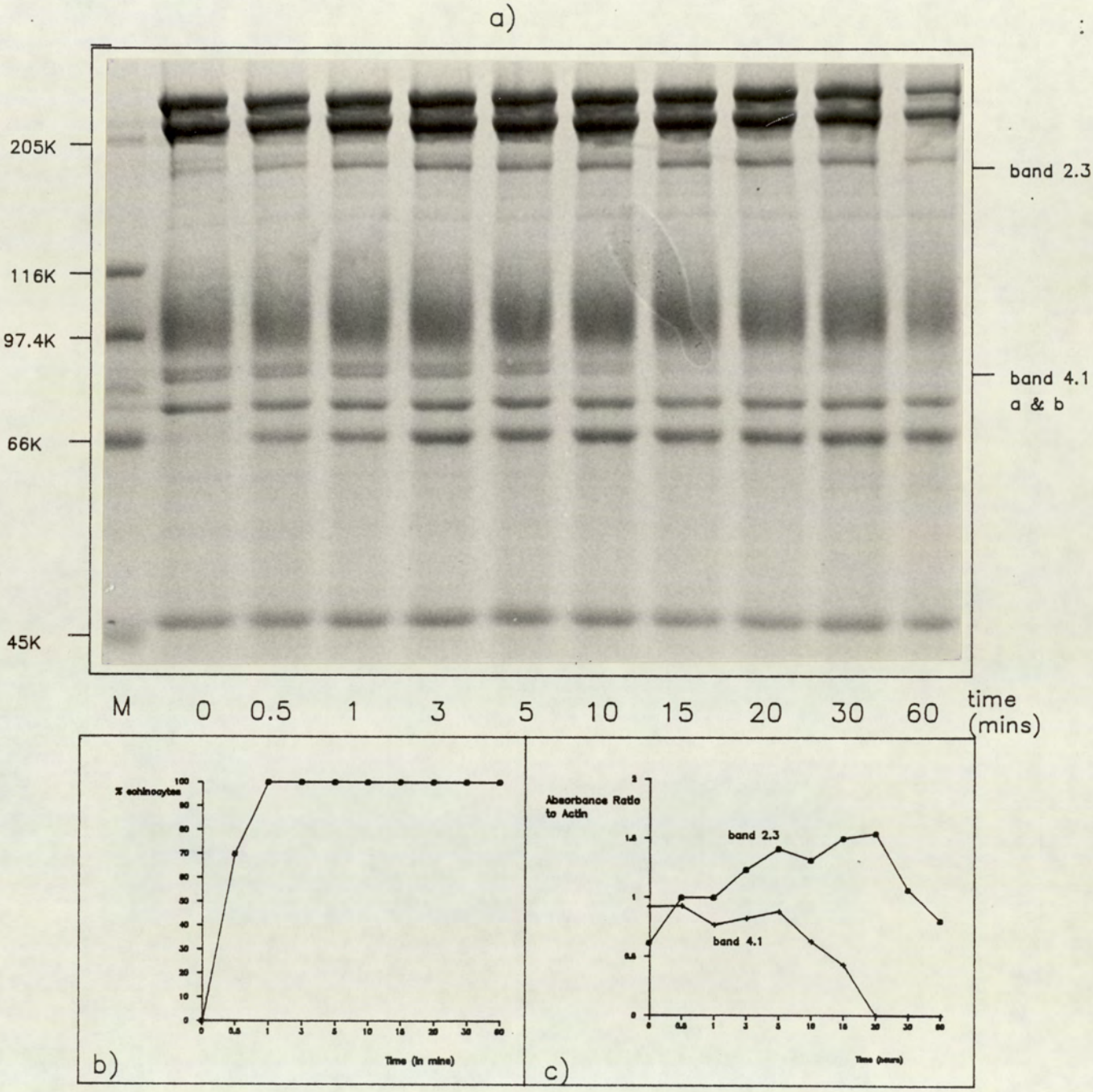


Figure.20 Time-dependent proteolytic modification of human erythrocyte membrane-cytoskeletal proteins - 1mM calcium.

Human erythrocytes were loaded with calcium (1mM) at 37°C. (a) erythrocyte ghosts were prepared and analysed by SDS-PAGE with a 7.5% gel. Lane M is the molecular weight marker. (b) morphological state of erythrocytes from same samples at different time intervals. (c) densitometric analysis of band 2.3 and band 4.1 as shown in (a).

20c). Thus, there was a good correlation between the time course of morphological changes and the rapidity of band 2.3 accumulation after calcium loading with both calcium concentrations.

In addition to the breakdown of band 2.1, loading of cells with high concentration of calcium (1mM) resulted in the breakdown of another two proteins, band 4.1 a & b (figure 20a). Band 4.1 a & b exist as a doublet in the coomassie blue stained SDS-polyacrylamide gel. It is shown in figure 20c that the breakdown of band 4.1 a & b did not commence until 5-10 minutes after the addition of the calcium and ionophore. After the breakdown of band 4.1 was initiated, the process was rapidly complete. Although band 4.1 a & b were completely proteolysed at the end of the incubation, echinocytosis was complete within the first minute of calcium loading. Thus it appeared the integrity of band 4.1 a & b was not important in the regulation of discocyte-echinocyte transition. However, the possible involvement of band 4.1 in processes which occurred during the later stage of the process of morphological change, such as microvesiculation, should not be overlooked.

Early investigations in the present study to examine the time-dependent calcium-induced changes were carried out using isolated human erythrocyte membrane ghosts. The ghosts were incubated with either 50μM or 1mM calcium at 37°C for 0-60 minutes followed by SDS-PAGE analysis. It was observed that the changes were similar to the results obtained from calcium loading of intact human erythrocytes. Band 2.1 was being

proteolysed in both conditions where 50μM or 1mM calcium was used. Similarly, band 4.1 was broken down at 1mM calcium but only 30-60 minutes after calcium addition (15-20 minutes when intact erythrocytes were used). This could be explained by the fact that the protease responsible for the proteolysis resides mainly in the cytosol, but is only weakly associated with the membrane-cytoskeleton. In experiments which isolated membrane ghosts were used for the calcium induction, the majority of the enzyme may be removed from the membrane during membrane preparation prior to experiments; thus a much lower activity was observed in membrane ghosts.

In addition to these proteolytic events, calcium loading of intact cells with 1mM calcium also resulted in the appearance of intensely commassie blue stained bands appearing on top of the stacking gel (figure 21). This band was only observed in samples which had been loaded with 1mM calcium for 10 minutes or longer (figure 21c). This band is referred to as high molecular weight aggregates, which represent the crosslinked products of various cytoskeletal proteins, catalysed by the calcium activated transglutaminase (Gaffney 1985). It can be seen from figure 21c that the intensity of band 2.3 in cells loaded with high calcium (1mM) started to fall after 20 minutes loading. This could be due to the proteolytic product band 2.3 becoming utilized in the crosslink aggregate formation since band 2.1 is also a major component in the calcium-dependent glutaminase mediated aggregate formation. The delay in the onset of the transglutamination process, compare to the timecourse of the

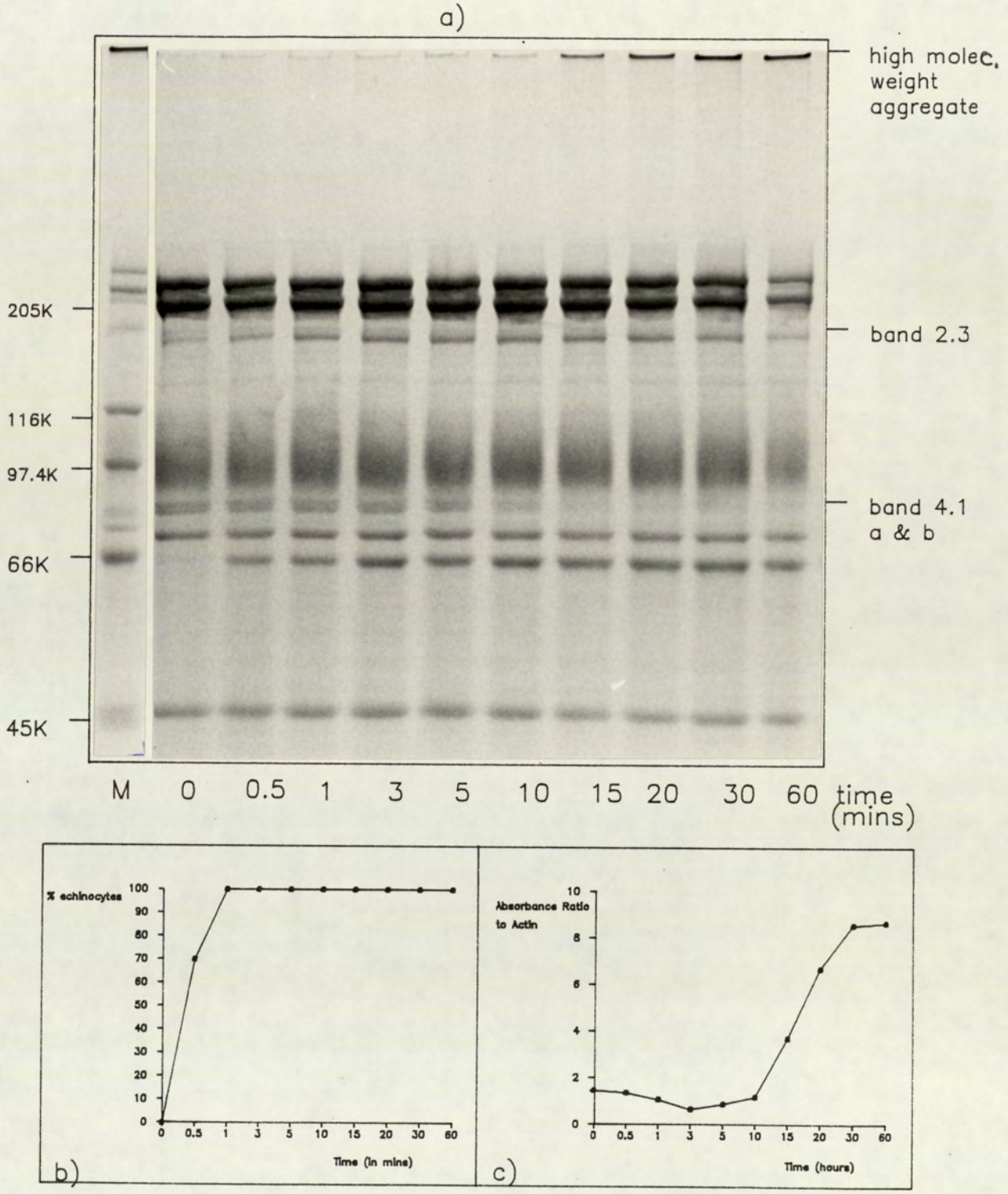


Figure.21 High Molecular weight aggregate formation during calcium loading.
Human erythrocytes were loaded with calcium (1mM) at 37°C. (a) erythrocyte ghosts were prepared and analysed by SDS-PAGE with a 7.5% gel. Lane M is the molecular weight marker. (b) morphological state of erythrocytes from same samples at different time intervals. (c) densitometric analysis of high molecular weight aggregate band as shown in (a). No aggregate formation was observed when human erythrocytes were loaded with 50µM calcium.

morphological change suggested a minimal involvement in the regulation of discocyte-echinocyte transformations.

3.4.2. CALCIUM CONCENTRATION-DEPENDENT PROTEOLYTIC ACTIVATION

It has been shown in the last section that band 2.1 may play an important role in the maintenance of the erythrocyte's discocyte morphology, since its breakdown was correlated with the extent of calcium-induced echinocytosis (figure 19). Further investigations were carried out to examine the calcium concentration dependence of this calcium activated proteolytic process. Figure 22a shows the extent of echinocytosis after loading with various concentrations of calcium (0-400μM) at 37°C for 5 minutes. It can be seen that echinocytosis increased, with 100% of cells reaching stage III of echinocytosis after 5 minutes of loading with 200μM calcium. SDS-PAGE analysis was carried out to study the parallel changes in the cytoskeletal proteins after calcium loading and the results are summarised in figure 23a. It can be seen that when erythrocytes were treated with increasing concentrations of calcium, proteolysis of band 2.1 (indicated by the increase in band 2.3) occurred in a concentration-dependent manner. All other major membrane proteins remained relatively unchanged up to a calcium concentration of 400μM. The extent of band 2.1 proteolysis was measured by performing densitometry analysis of the gel and the peak corresponding to band 2.3 was analysed. The peak height of the band 2.3 at each calcium concentration was measured and the results are summarised in figure 22b, which clearly shows that calcium-

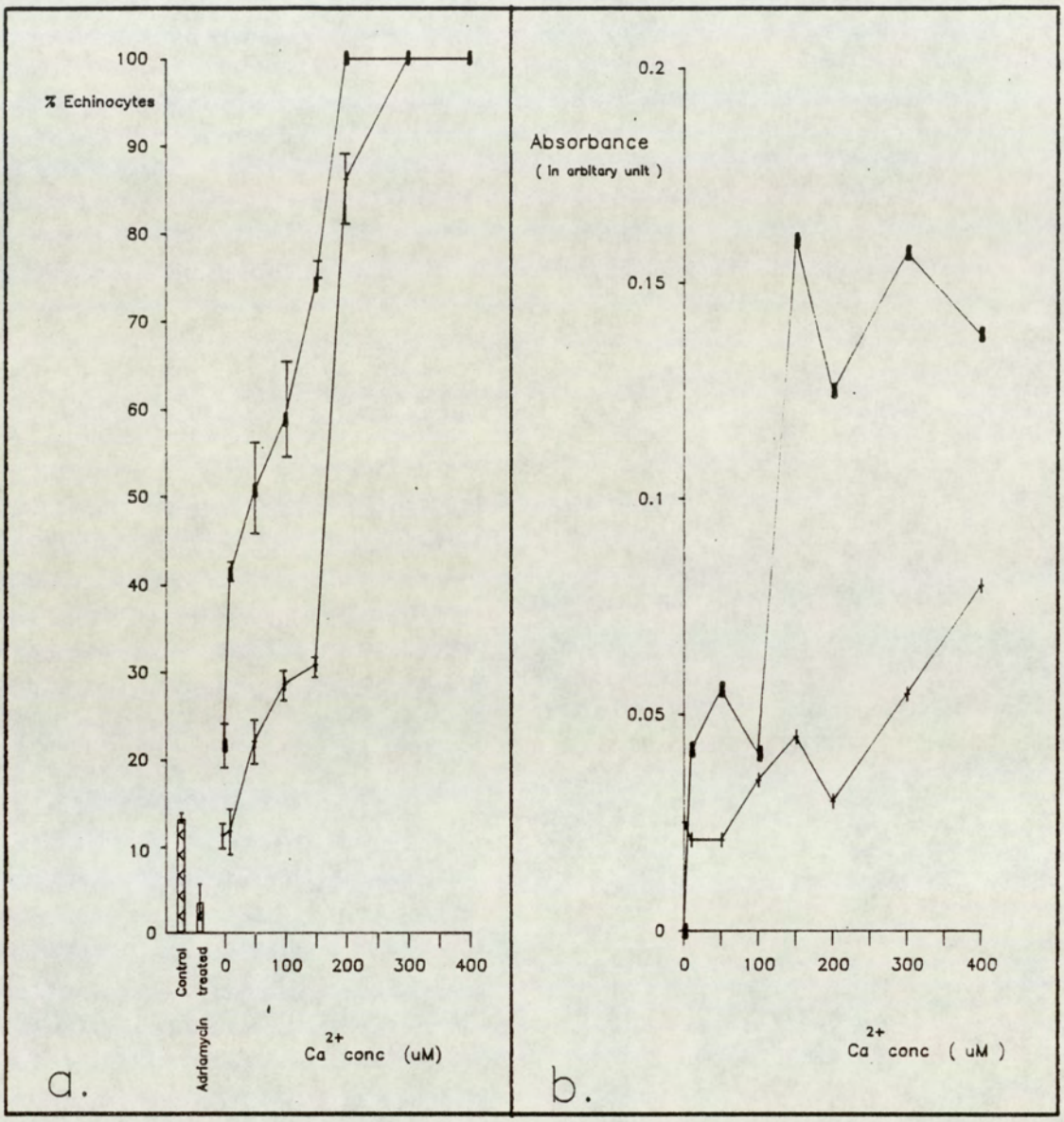


Figure.22 Calcium concentration-dependent proteolytic modification of human erythrocyte membrane-cytoskeletal proteins and the effect of Adriamycin pre-incubation.
Human erythrocytes were loaded with calcium (0-400µM) for 5 minutes at 37°C. Cell suspensions were either pre-incubated without (■) or with (+) Adriamycin (10µM) prior to calcium loading. (a) morphological state of erythrocytes at the end of the loading period. (b) densitometric quantitation of band 2.3 after calcium loading (SDS-PAGE is presented in figure.22).

induced echinocytosis is accompanied by the proteolysis of band 2.1.

When human erythrocytes were pre-incubated with Adriamycin at 37°C for 10 minutes followed by calcium loading with 0-400μM calcium, the results were as summarised in figure 22a. This shows that the protective effect of preincubation with Adriamycin against calcium induced echinocytosis occurred as described in chapter 2.3. Results from SDS-PAGE analysis of the cytoskeletal proteins prepared from these cells are shown in figure 23b. It can be seen that when erythrocytes were loaded with calcium after preincubation with Adriamycin, the proteolytic degradation of band 2.1 (i.e. the increase in band 2.3) induced by calcium loading was greatly inhibited. The densitometric analysis of the appearance of band 2.3 under these various conditions is presented in figure 22b. This shows that Adriamycin inhibited proteolysis at calcium concentrations up to 400μM.

The result of the study of the time-dependent and concentration-dependent events of echinocytosis from morphological studies and SDS-PAGE analysis of cytoskeletal proteins suggested that the proteolytic cleavage of band 2.1 correlated with the calcium-induced process of echinocytosis. Thus band 2.1 may be important in the regulation of the calcium mediated discocyte-echinocyte transformation, as suggested by Jinbu et.al. (Jinbu et.al. 1982, Jinbu et.al. 1984), consequently Adriamycin might have mediated this

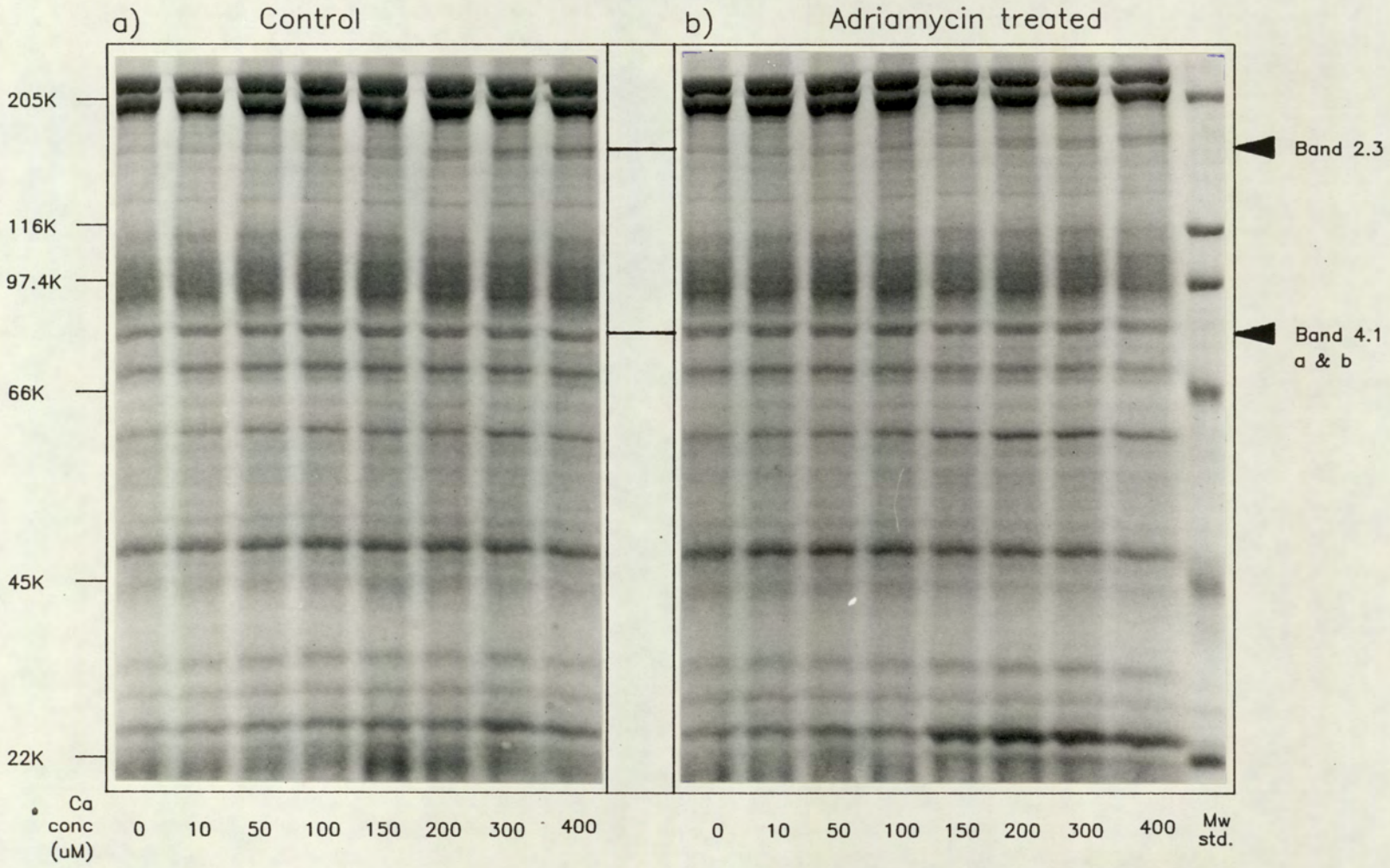


Figure.23 Calcium concentration-dependent proteolytic modification of human erythrocyte membrane-cytoskeletal proteins and the effect of Adriamycin pre-incubation - SDS-PAGE analysis.
Human erythrocytes were loaded with calcium (0-400µM) for 5 minutes at 37°C. Cell suspensions were either pre-incubated without (a) or with (b) Adriamycin (10µM) prior to calcium loading.

protective effect by inhibition of the proteolysis of band 2.1. However, the observation that morphological studies showed the loss of the protective effect of Adriamycin at high calcium concentrations (>=200μM) whilst Adriamycin was still capable of inhibiting band 2.1 proteolysis, raises doubts as to the role of band 2.1.

3.4.3. EFFECTS OF CALCIUM AND ADRIAMYCIN ON THE HUMAN ERYTHROCYTE CALCIUM-DEPENDENT PROTEOLYTIC SYSTEM

It has been described in the above section that calcium loading of human erythrocytes activated the calcium-dependent thio protease, calpain, which catalysed the proteolytic breakdown of band 2.1. The inhibition of band 2.1 breakdown down by Adriamycin under conditions of calcium loading suggests the possibility of a direct interaction between Adriamycin and the calcium-dependent enzyme, calpain.

Calpain has been found to present in a wide variety of cell types in a proenzyme form, procalpain and the enzyme mainly resides in the cytosol (see introduction section 2.1). In the present study, procalpain was partially purified from human erythrocytes as described earlier (see materials and methods section 2.13).

Prior to the purification of calpain, it is essential to obtain a reliable assay method to quantify enzyme activity during the purification procedure and further enzymatic studies. Initially, a fluorimetric method utilizing fluoresamine, so as to detect and quantify small TCA soluble peptides released from heat denatured casein after protease

treatment was used. The method was found to be highly sensitive provided that the acidity which arose from the added TCA (required to terminate the incubation), was neutralized prior to the addition of fluoresamine. However, the assay was later found unsuitable: firstly, it was observed that good reproducibility was not achieved, possibly as a result of a slight variability in pH after neutralization. Secondly, the spectral excitation and emmission spectrum of fluoresamine is partially overlapped by that of Adriamycin, which was to be used in further studies on the enzyme. The possibility of energy transfer between fluoresamine and Adriamycin indicated the requirement of alternative assay procedures.

An alternative method devised from a modification of the fluorimetric assay, was used. The enzymatic substrate, casein was isotopically labelled with carbon-14 by reductive methylation using ^{14}C-formaldehyde and sodium cyanoborohydride (see methods section 2.13). In the preparation of ^{14}C-casein, isotopic yield of approx. 50% were achieved with approximately 75% protein recovery. Typically, ^{14}C-casein with a specific activity of 5000 dpm/µg was obtained. The labelled casein was then substituted with casein in the enzyme assay and proteolytically released peptides were quantified by scintillation counting (see methods section 2.13). This was not open to interference by the presence of Adriamycin.

Human erythrocyte cytosolic fractions were prepared by hypotonic lysis and sonication followed by ultracentrifugation. The protease in the lysate was absorbed onto DE32 resin followed by NaCl elution (see method 2.13).

The distribution of the enzyme in the eluant is shown in Figure 24. It could be seen that a large proportion (approx. 57%) of proteolytic activity was refined into a single peak, which contained calcium-dependent activity, procalpain, followed by several peaks contained calcium-independent activities. The calcium independent activities may represent other proteases present in the erythrocyte cytosol, and small percentages of activated thio protease (calpain), which is no longer calcium dependent. It is noted that the total proteolytic activity obtained after purification was several orders of magnitude higher than estimated from the original erythrocyte lysate. This could be explained by the separation of procalpain from the endogenous inhibitor, which was also found in erythrocyte cytosol (Melloni et.al. 1982), in the initial DE32 separation. The pooled enzymatic activity was concentrated and further purified by chromatography on Trisacryl DEAE followed by butyl-agarose hydrophobic chromatography. The elution profiles from both separations are shown in figure 25. In both cases, a single peak was obtained. High yield was obtained from both DE32 and tris acryl DEAE cellulose (>90% recovery) separations. However, when pooled fractions obtained from the tris acryl DEAE chromatography were separated on butyl-agarose, this resulted in the loss of >90% proteolytic activity, and no further activity could be eluted with 2M NaCl. Since continuous monitoring of the eluant at 280nm had revealed the elution of protein at various stages, it is plausible to speculate that the protease may have been tightly interacting with the

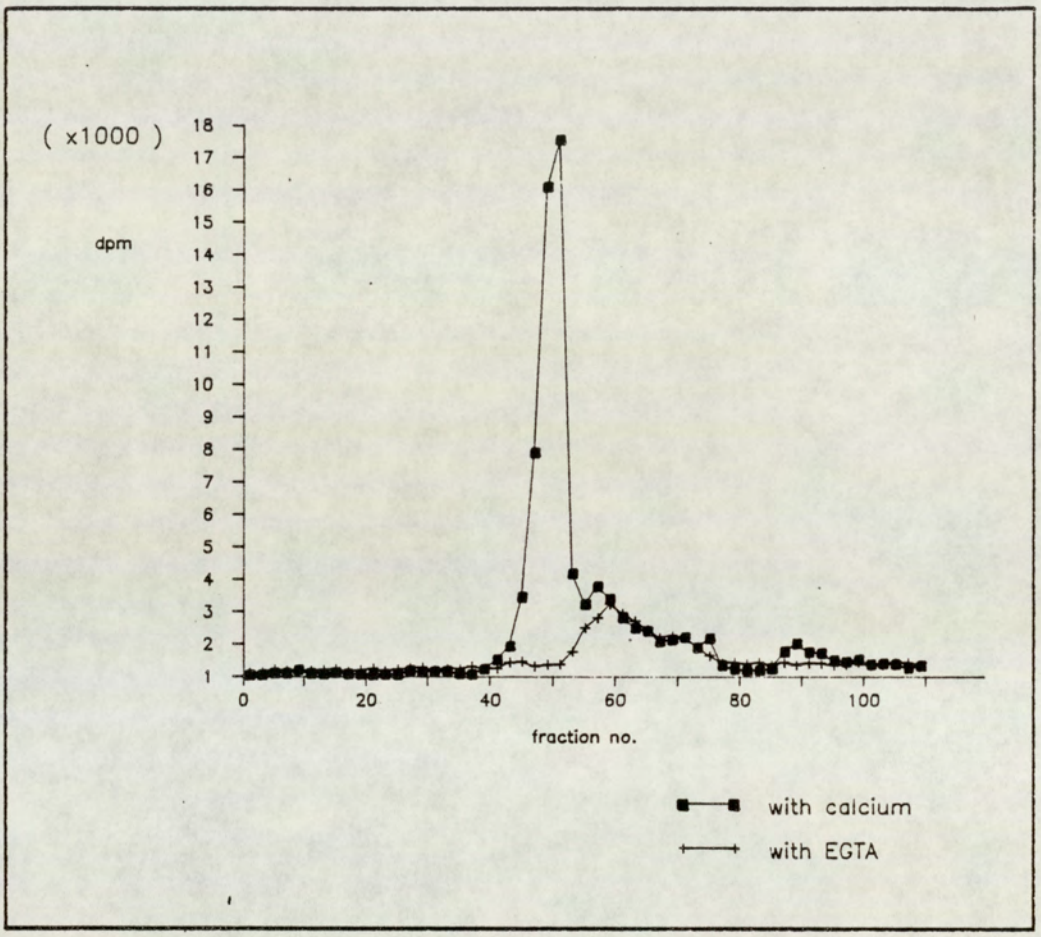


Figure.24 Purification of calpain from human erythrocyte - separation by DE32.
Human erythrocytes were lysed and pre-washed DE32 resin was added to the "membrane free" lysate (see methods). After extensive washes, the resin was packed into a column and calpain was eluted with a sodium chloride gradient (0-1.5M). Calpain activity was assayed by the ^{14}C-casein method as described in the method section either in the presence of calcium (■) or EGTA (+).

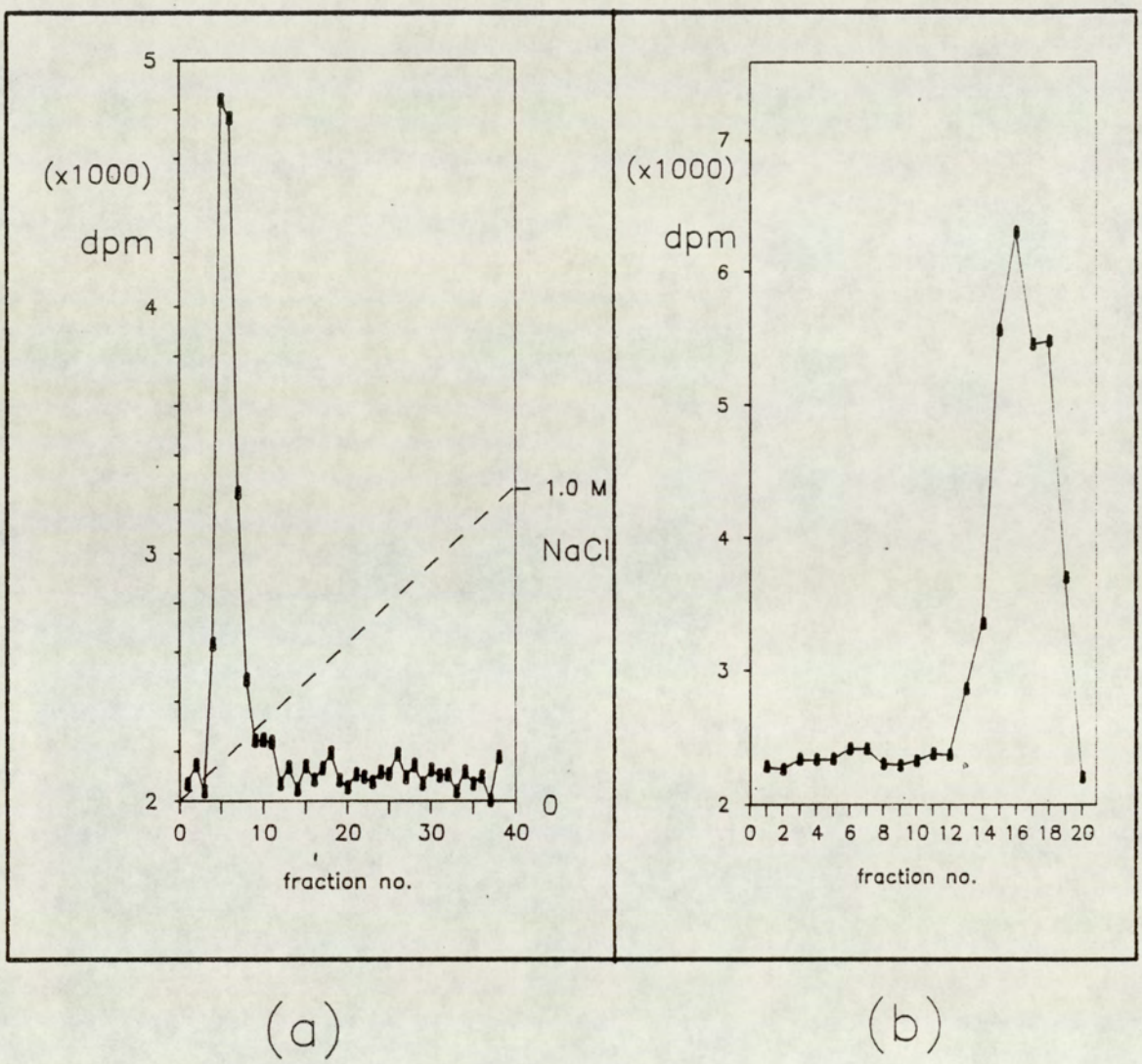


Figure.25 Purification of calpain from human erythrocyte - separation by trisacryl DEAE cellulose and butyl-agarose.
Crude calpain preparation obtained after DE32 separation was concentrated and further separated on a trisacryl DEAE column (panel a). Calpain was eluted within a linear sodium chloride gradient (0-200mM) and assayed for calpain activity. Active fractions were pooled and further purified by butyl-agarose chromatography using a linear gradient of sodium chloride (0-100mM) as described in methods section) (panel b).

butyl-agarose resin matrix. The alternative explanation, which would be more appropiate, was that denaturation of the protease by butyl-agarose came about as a result of a hydrophobic interaction with the resin matrix. Despite the advantage of further purification, the highly enriched protease fractions from tris acryl DEAE cellulose separation was used for further biochemical study of protease activity.

The protease was stored in 50mM sodium acetate buffer pH 7.5 in the presence of 1mM each of EDTA and EGTA. No loss of activity was found after one week storage at 4°C compare with the enzyme preparation obtained by Melloni et.al. (1982), which had a half life of less than 24 hours. It could be seen from figure 26 that 1mM leupeptin, which is a specific thio protease inhibitor, was capable of the inhibition of >95% of the protease activity. This inhibition by leupeptin confirmed the identity of the protease. Figure 26 also shows the extent of inhibition of calpain by Adriamycin (0-1mM). It shows that Adriamycin, at 1mM, was only slightly, but significantly inhibitory (approx. 15%) towards the proteolytic activity of calpain. The implications of these studies will be discussed in a later section (see discussion).

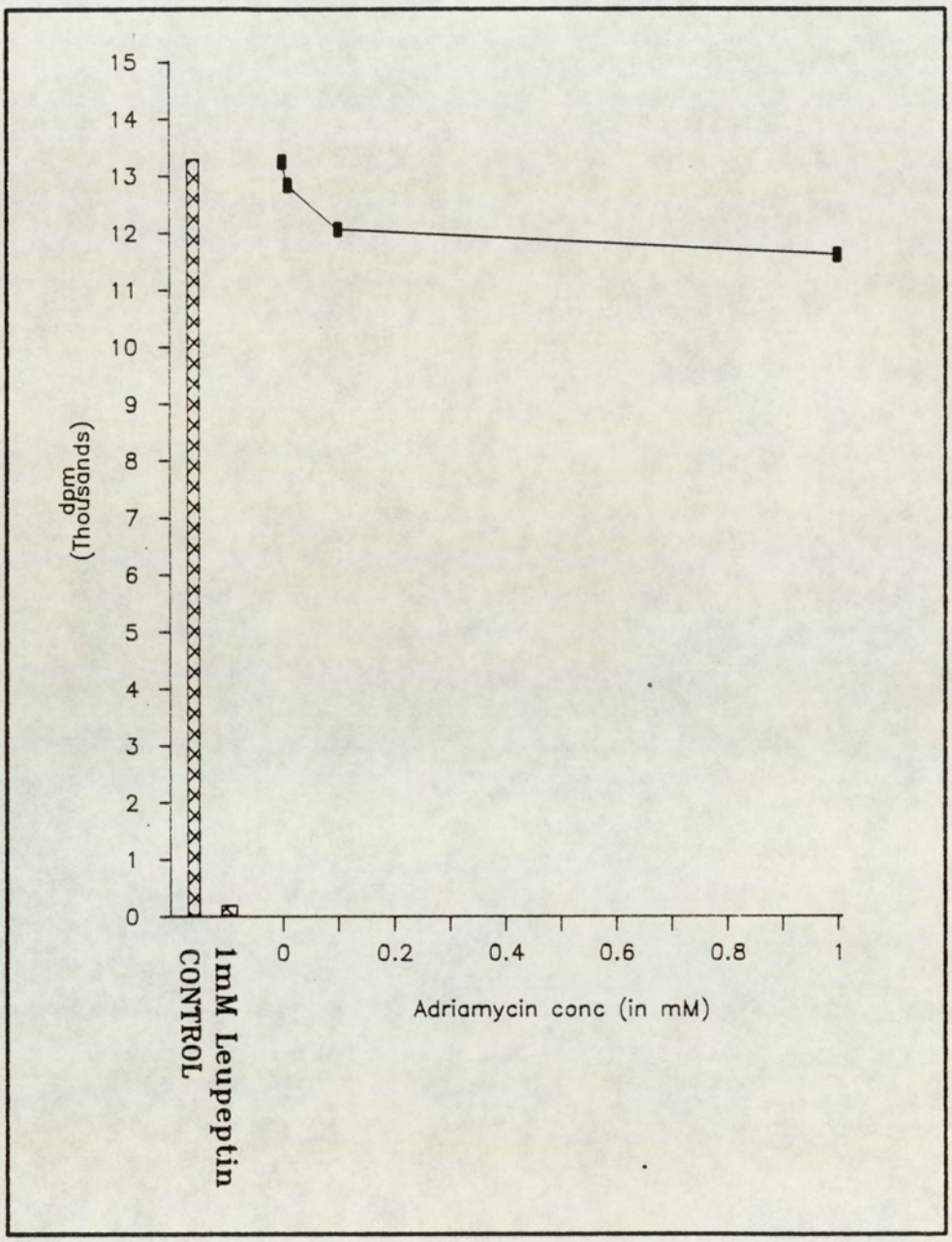


Figure.26 Inhibition of calpain activity by leupeptin and Adriamycin.
Purified calpain was pre-incubated with either leupeptin (1mM) or Adriamycin (0-1mM) for 10 minutes the calpain assay was commenced using the ^{14}C-casein assay.

3.5 PROTEIN PHOSPHORYLATION IN HUMAN ERYTHROCYTES - METHOD DEVELOPMENT

In the previous chapter, the results from the studies of one of the important proteolytic systems, which is highly regulated in vivo in human erythrocytes was discussed. It has to be noted that covalent modification by proteolysis is generally irreversible and is normally associated with long-term changes, until the protein which is proteolysed is replaced as a result of protein synthesis. Human erythrocytes are terminally differentiated cells and, as a result, their protein synthesis capability is lost. Therefore proteolytic modification as a method of intracellular regulation would not be a convenient mechanism for the reversible and rapid regulation processes required to modulate morphology, and other means of modification should not be overlooked. In the present study, alterations of protein phosphorylation activity was also investigated.

Studies of phosphorylation in intact cells had been carried out in various cell types, including human erythrocyte (Patel & Fairbanks 1986), by incubating the cells with ^{32}P-orthophosphate (^{32}Pi). Various different incubation media have been used in the metabolic labelling of cells including human erythrocytes. In general, these studies suffer several limitations. Firstly, experiments often utilized incubation media that were not able to maintain the viability of the cells during the metabolic labelling period.

Secondly, metabolic labelling of cells was often terminated (10-60 mins) before isotopic equilibrium was reached: this requires at least 3 hours (see below). Metabolic labelling with ^{32}Pi proceeded through the labelling of intracellular ATP, which is then followed by the transfer of the gamma-^{32}P from ATP to the hydroxy groups of target proteins by the action of protein kinases. In order to assess changes in phosphorylation which occur as a result of certain treatments, it is essential to investigate these changes under conditions in which any increase in ^{32}P incorporation is not due to the increase in specific activity of ^{32}P of the phosphates incorporated into proteins, arising as a result of an increase in specific activity of intracellular ATP-gamma-^{32}P. Thus it is essential that cells are metabolically labelled to such an extent that the distribution of ^{32}P in both ATP and proteins reaches isotopic equilibrium prior to experimentation. Any result which claims to show an increase in phosphorylation, arising from investigations using cells which have not been labelled to isotopic equilibrium, has to be treated with caution.

Thirdly, `phosphate free' medium has become a widely used incubation system for the metabolic labelling of various cell types. It was thought that the presence of physiological concentration of inorganic phosphate (1mM) may drastically reduce the final specific activity of the target proteins as a result of isotopic dilution. Previously, the radioactive isotope ^{32}P-orthophosphate has been used for labelling at 0.2-1.0mCi/ml, which corresponds to 0.2-1.0µM orthophosphate.

Therefore, there is a possibility that a 1000-fold decrease in the availability of phosphate may have a profound effect on the intracellular uptake and the utilization of phosphate in reactions such as ATP synthesis. This is supported by the report (King et.al. 1987) that when human erythrocytes were labelled with ^{32}P-orthophosphate in the presence of 1.8mM phosphate, >80% of the phosphate in the medium has been taken up by the cells after 2 hours of incubation. These results showed that, at least in human erythrocytes, there is a high demand for the presence of adequate amount of inorganic phosphate.

In the present study, investigations were carried out in order to obtain a suitable incubation medium for the isotopic labelling of human erythrocytes and comparison will be made to a commonly employed medium (see method section 14a) as described by Wolfe & Lux (1978). It could be seen from chapter three ,that a medium, which can maintain both erythrocyte morphology and metabolism, was obtained and this medium was used to initiate the study of protein phosphorylation in metabolically maintained erythrocytes.

Several modifications to the incubation buffer B (see chapter three) were made such that the concentration of certain constituents resembled that found in vivo. It was found that BSA was indispensable for the labelling of cells with ^{32}Pi. The absence of BSA during isotopic labelling resulted in poor ^{32}Pi incorporation and severe streaking of the autoradiograph image. Thus BSA was increased to 1% w/v, which is equivalent to the serum albumin level in the blood.

Figure 27 shows the results of the SDS-PAGE analysis of membrane-cytoskeletal proteins prepared from cells labelled with ^{32}Pi for $3\frac{1}{2}$ hours using the widely used labelling medium (medium A) and the medium developed in this study (labelled medium B). It can be seen from the figure that the inclusion of physiological levels of inorganic phosphate during labelling did not alter the coomassie blue staining pattern of the gel (lane a,b vs c,d & e,f vs g,h), i.e. there were no changes in protein-membrane associations. However, additional bands (arrows) were present in samples from cells labelled with medium B (lane a-d vs e-h). This indicated the presence of the additional glycolytic substrates and ATP precursors enhanced the association of various proteins with the membrane or cytoskeleton.

It was considered that medium A, which contained the serine protease inhibitor PMSF, might have prevented proteolysis from the action of plasma originated serine protease during labelling. Studies were then carried out to investigate the role of PMSF during the labelling period: it was found that the presence of PMSF during labelling has no effect on the protein pattern. The importance and the identity of the additional bands which appeared in samples from cells labelled with medium B was not clear and remain to be clarified.

Figure 28 shows the autoradiograph from the gel shown in figure 27. Firstly, it could be seen that cell labelling with ^{32}P in medium A was not significantly different, with respect to the pattern of phosphoproteins, to cells labelled with

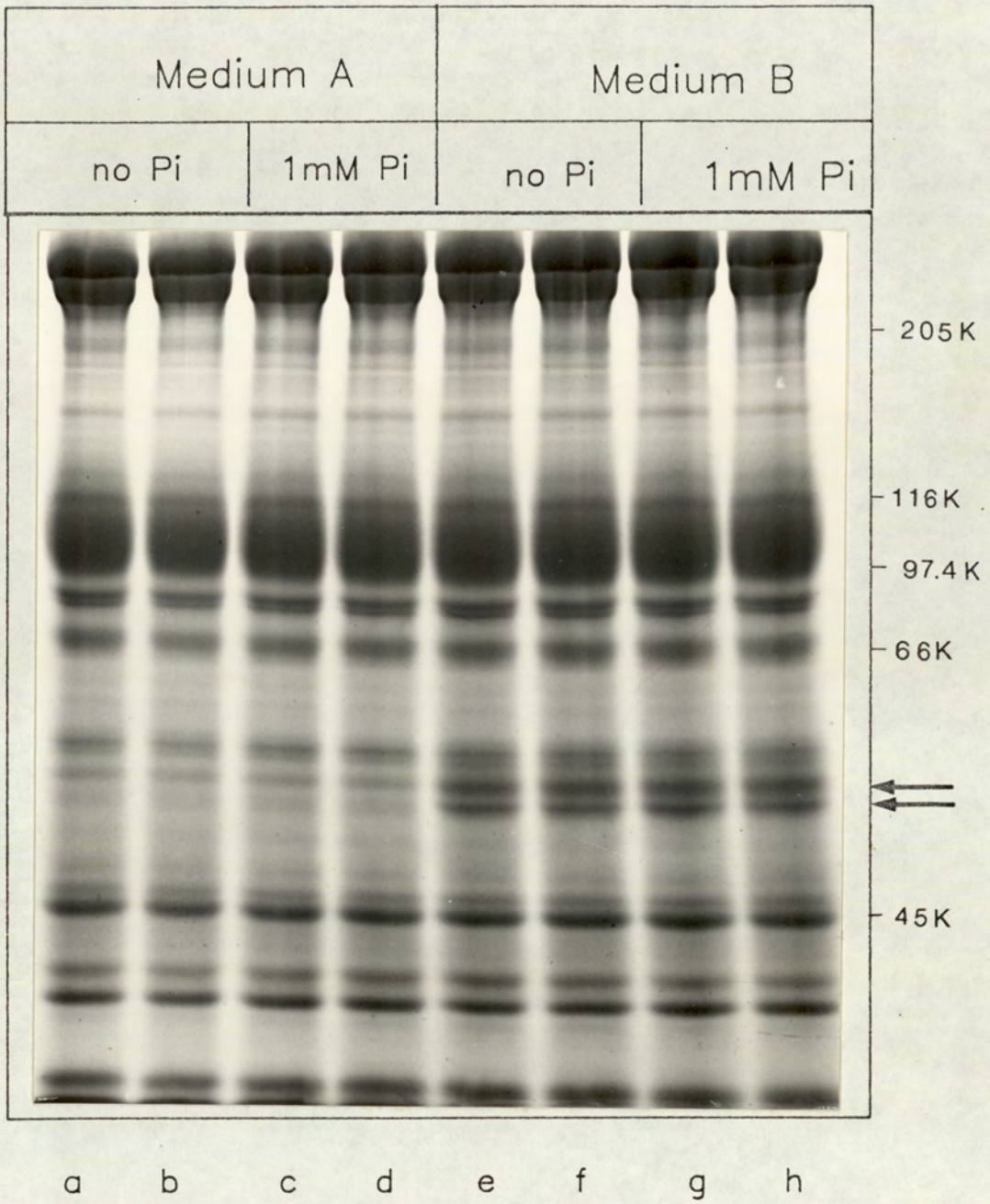


Figure.27 Metabolic labelling of human erythrocyte membrane-cytoskeletal proteins - coomassie blue pattern
Human erythrocytes were maintained and labelled with either medium A or medium B (see method 2.13.1) in the presence of ^{32}P-orthophosphate for 3½ hours. In each case, parallel incubations were performed which either contained only ^{32}P-orthophosphate or supplemented with 1mM potassium phosphate. At the end of the labelling period, membrane ghosts were prepared and analysis by SDS-PAGE.

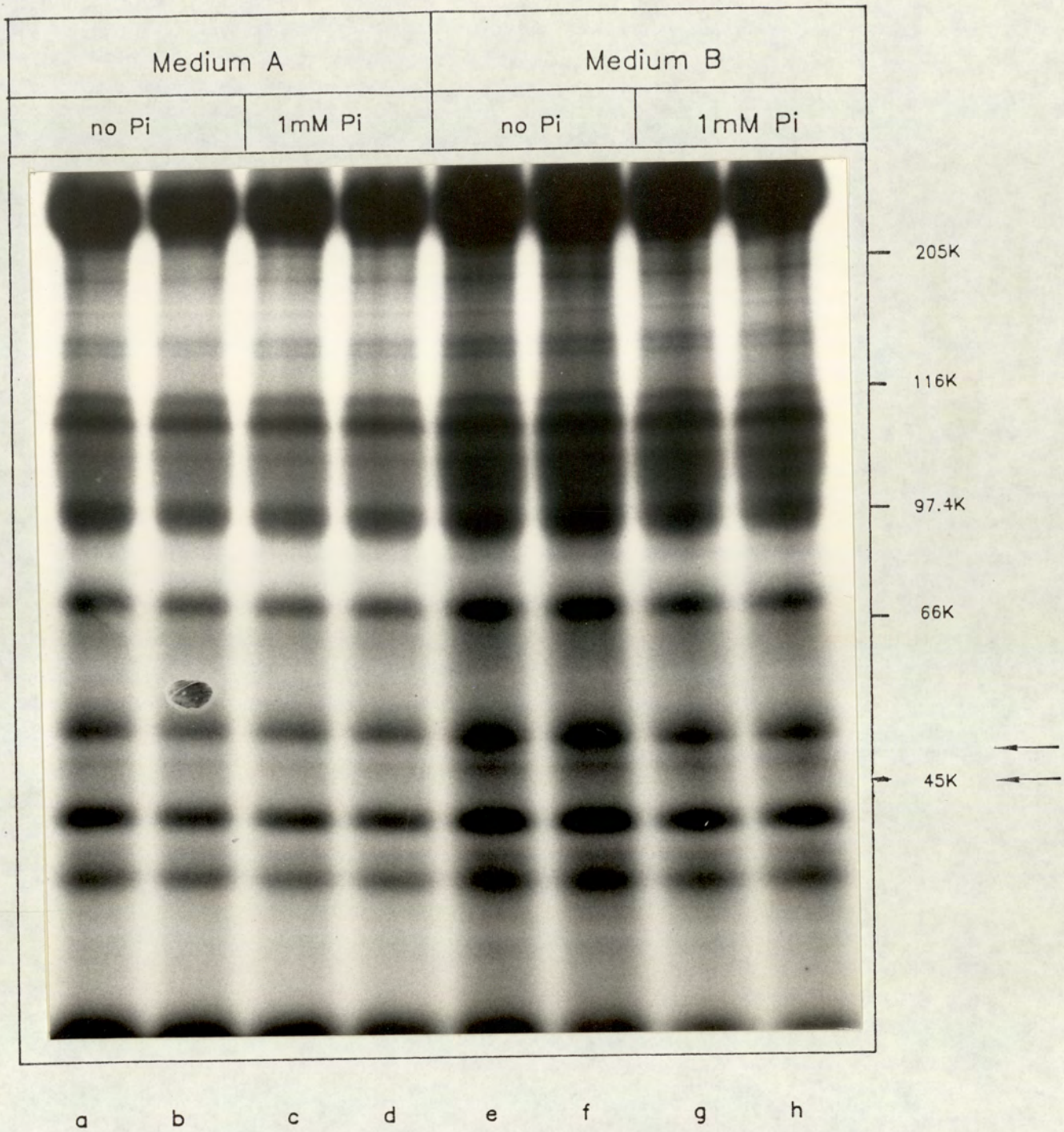


Figure.28 Metabolic labelling of human erythrocyte membrane-cytoskeletal proteins - autoradiography.
The SDS-polyacrylamide gel as shown in figure.27 was dried and subjected to autoradiography for 3 days (without intensifying screen) and developed.

medium B. However, the latter was found to incorporate significantly more phosphate into proteins (lane a-d vs e-h) under the same conditions. It should also be noted that the additional bands which appeared in membrane preparations from cells labelled in medium B, were also phosphoproteins and the possibility arises that the enhancement of protein-cytoskeleton interactions results from an increase in protein phosphorylation levels. Secondly, previous methods which suggest the use of `phosphate free' medium to avoid isotopic dilution were found possibly to be limited.

It has been mentioned above that inclusion of 1mM inorganic phosphate in the labelling medium may effectively dilute the ^{32}P-orthophosphate by 1000-fold. In order to mimic physiological conditions, 1mM inorganic phosphate was also included in the incubation medium during labelling with ^{32}P (1μM).

Figure 28 shows the effects after the inclusion of 1mM inorganic phosphate in medium A & B. It shows that, in both media, the level of ^{32}P-orthophosphate incorporation into proteins was only marginally decreased when 1mM phosphate was used (lane a,b vs c,d & lane e,f vs g,h). Despite the slightly lower intensity obtained as a result of the inclusion of physiological levels of inorganic phosphate, the actual phosphorylation level was at least several orders of magnitude higher when the isotopic dilution factor was considered. In addition, the autoradiographic image was not very much lower than that using phosphate free medium and it did not affect the time required to perform the analysis. This suggested

that the proposals for the use of `phosphate free' medium are not justified.

The studies described above suggested that medium B, which is a modified medium of incubation buffer B, may be used to perform ^{32}P-Pi labelling of human erythrocytes and would be compatible with incubation buffer B, which will be used in the calcium loading studies later. It has also been noted that when erythrocytes were labelled with medium A, considerable cell lysis was observed after the 3 hours labelling period whilst no apparent lysis was observable in cells labelled in medium B. These results further support the use of medium B for the labelling procedure.

Although the studies above indicated the erythrocytes were able to incorporate ^{32}P into proteins, the erythrocytes obtained have to be viable and metabolically active after the labelling period. This could be investigated by measuring the ATP level associated with erythrocytes during the labelling procedure. ATP extracted from cell suspensions obtained during metabolic labelling was measured by ion exchange HPLC. Figure 29a shows the separation of various nucleoside phosphates standards. Because of the requirement for the use of high phosphate concentrations for the elution of nucleoside phosphates, particularly the nucleoside triphosphates, the base line absorbance gradually increased as the phosphate concentration increased. Nonetheless, the separation of nucleoside phosphates was clear and quantification was achieved. Human erythrocytes were labelled from 0 to 6 hours and duplicate samples were withdrawn for analysis. The

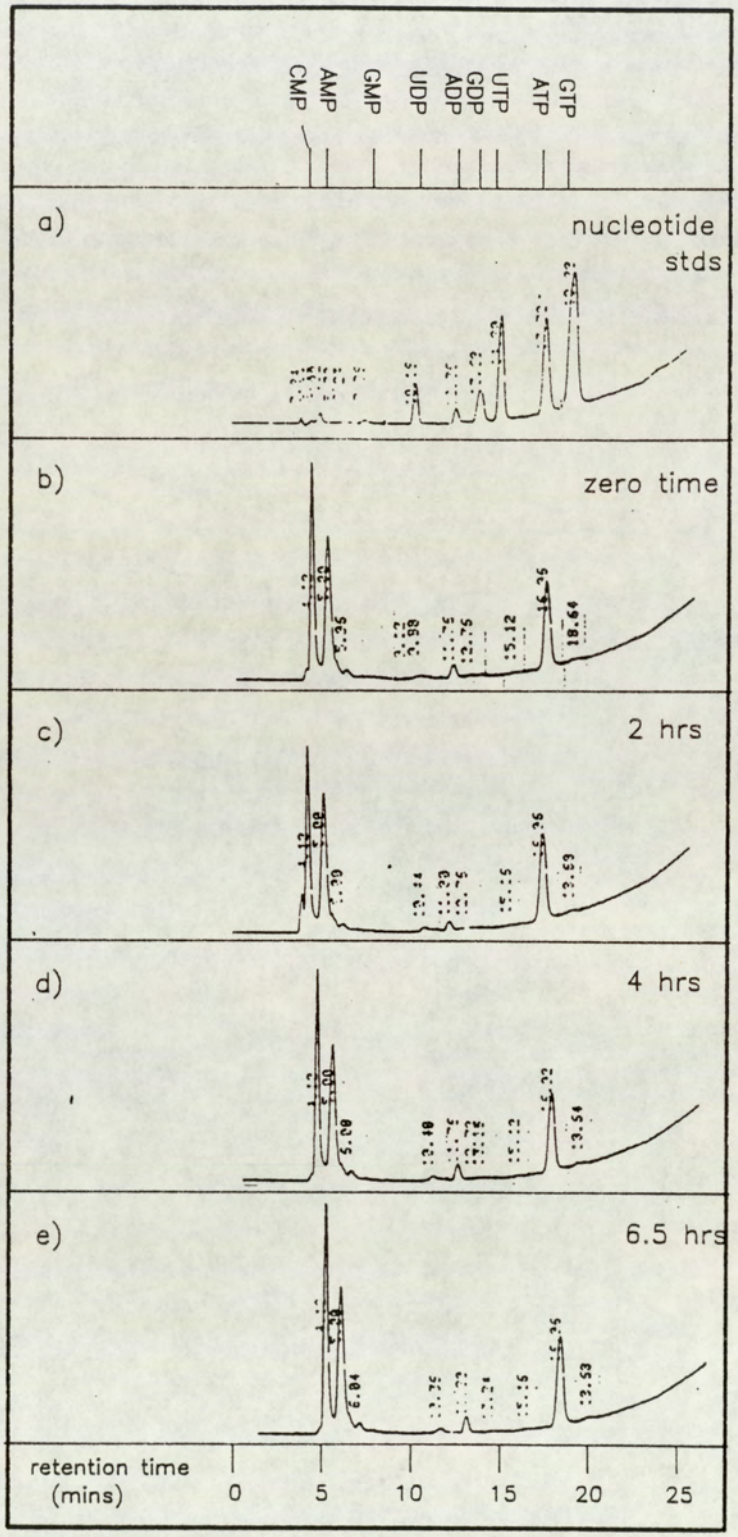


Figure.29 HPLC analysis of human erythrocyte intracellular ATP level during metabolic labelling.
Human erythrocytes were labelled with medium B in the presence of 1mM potassium phosphates from 0 - 6½ hours. Samples were drawn at regular intervals and ATP was extracted followed by HPLC analysis.

results are summarised in figure 29 (b-e). The nucleoside phosphate peak corresponding to ATP was integrated using the data module (see methods) and the results are summarised in figure 30. It can be seen that the ATP level of the human erythrocytes was constantly maintained and this indicated the cells were still metabolically active throughout the incubation period.

In the past, the majority of the studies of the phosphorylation of proteins in intact cells was carried out using cells which were labelled with ^{32}Pi for only short periods of time (10-60 mins). In order to assess changes of phosphorylation under certain conditions (such as morphological transition), it is essential to investigate these changes under conditions in which any increase in ^{32}P incorporation is not due to the increase in specific activity of ^{32}P of the phosphates incorporated into proteins, arising as a result of an increase in specific activity of intracellular ATP-gamma-^{32}P. ATP was extracted from human erythrocytes as described in materials and methods (section 2.5). The specific activity of the ATP was measured by incorporation of the gamma-phosphate from ATP into histone using cAMP protein kinase. The reaction was left to proceed until 100% completion, i.e. until all histone has been phosphorylated. The results are shown in figure 31. Figure 31a shows the linearity of the ^{32}P incorporation into histone with different specific activities of ATP-gamma-^{32}P, which were obtained by the isotopic dilution of commercially available high specific activity ATP-gamma-^{32}P. Figure 31b

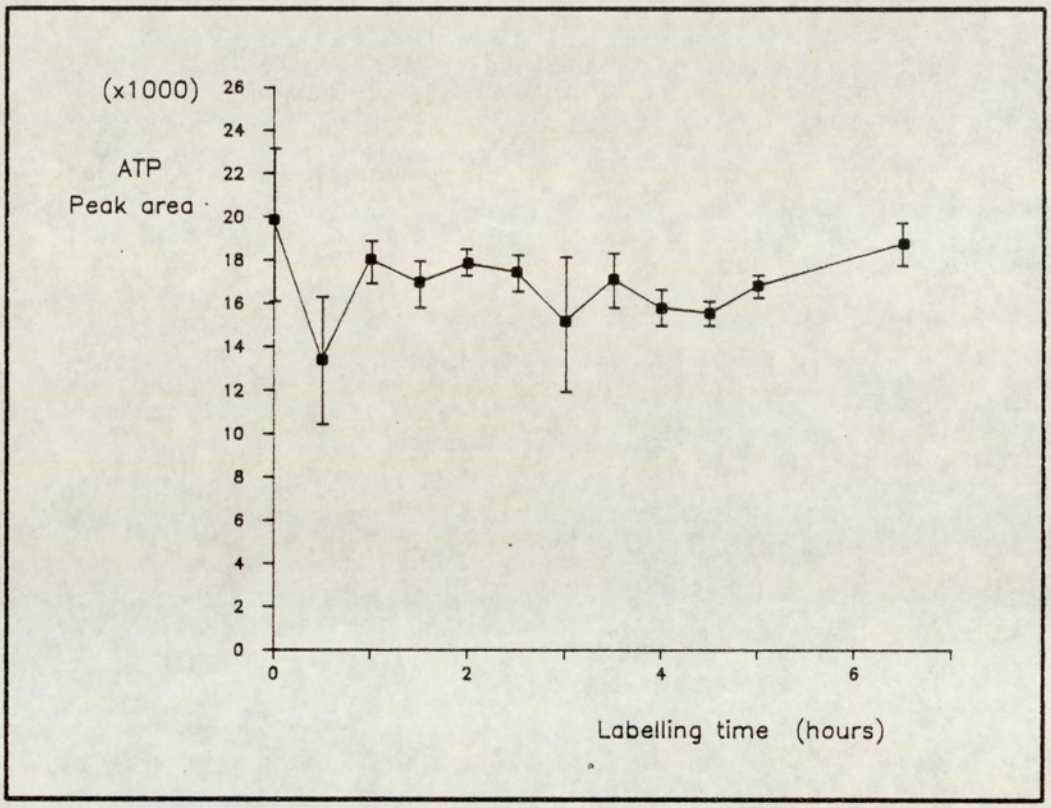


Figure.30 HPLC analysis of human erythrocyte intracellular ATP level during metabolic labelling - peak quantitation. ATP was measured as shown in figure.28 and result from three injections were quantitated.

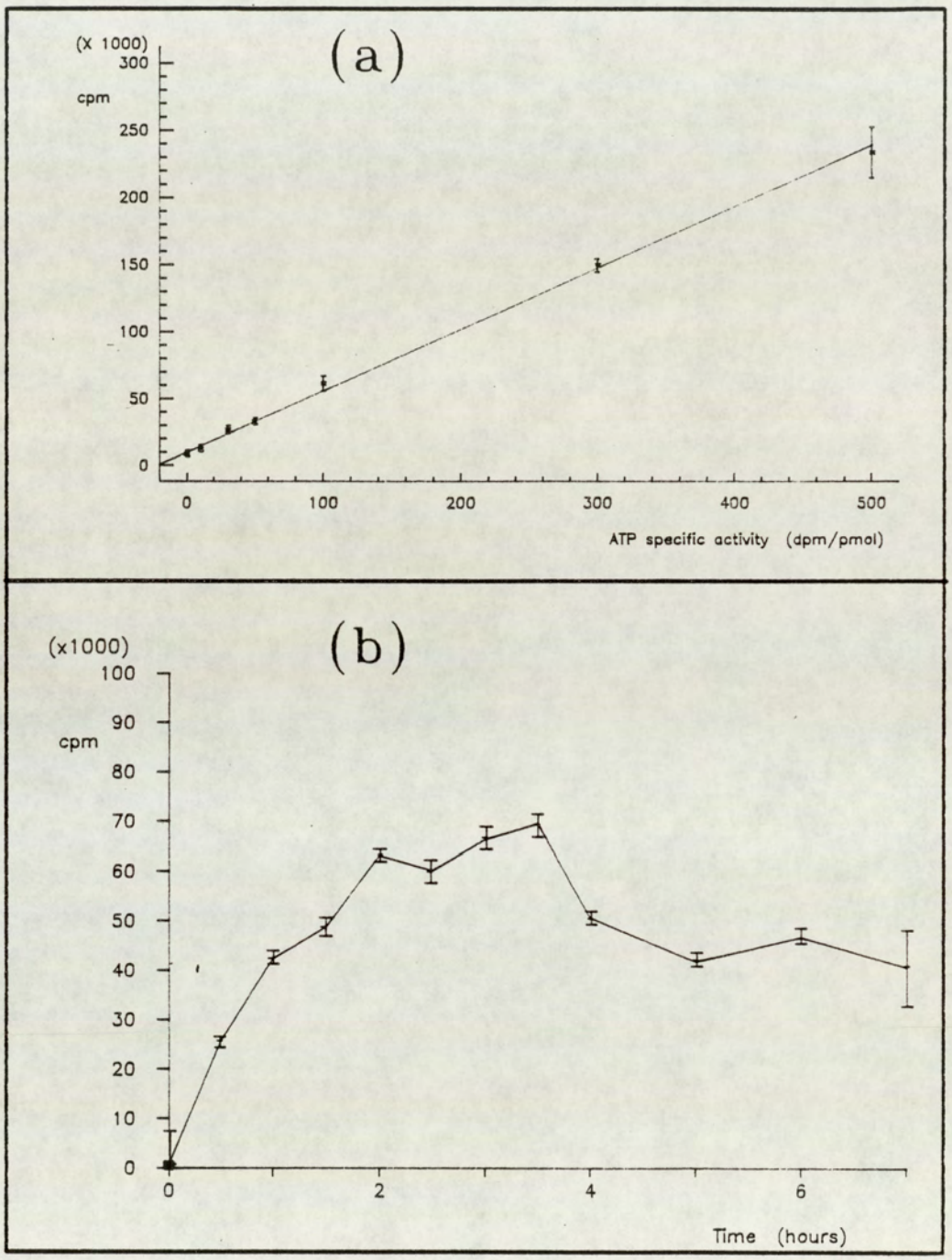


Figure.31 Specific activity of the intracellular ATP of human erythrocytes during metabolic labelling.
Human erythrocytes were labelled with medium B in the presence of 1mM potassium phosphates from 0 - 6½ hours. Samples were drawn at regular intervals and ATP was extracted. The specific activity of the ATP was measured by the incorporation of ^{32}P into histone by cAMP-dependent protein kinase. Standard curve (a) was constructed using ATP with known specific activity and the specific activity of the extracted ATP was evaluated (b).

shows the changes in specific activity of the intracellular ATP during the course of ^{32}Pi labelling. It could be seen that the specific activity increased gradually and reached the maximum, plateau level only after 2-3 hours. However, the specific activity started to decreased as the incubation period was prolonged. This might be explained by two possible mechanisms: firstly, the lowering of the scintillation counts obtained from the histone phosphorylation assay (see above) may be caused by dramatic decreased in ATP concentration in the ATP extract obtained. It has been shown that in this ATP assay, 100% histone phosphorylation could only be achieved if the ATP concentration was at least three times higher than the histone concentration (King & Michell 1987), the lowering of the ATP concentration from the ATP extract may well reflect the lowering of intracellular ATP, arising from a decrease in cell viability. However, this mechanism was unlikely, as it has been shown earlier that the labelling of human erythrocytes, under these conditions, did not cause any change in intracellular ATP level for up to 6 hours. Secondly, therefore the reduced counts obtained may be truly reflecting the lowering of the specific activity in intracellular ATP. This explanation is supported by similar observation from other investigators (King & Michell 1987). They found that the lowering of the specific activity of ATP after the labelling human erythrocytes for more than 4 hours was due to the sudden exchange of the intracellular ^{32}Pi with the unlabelled Pi, which is released in the turnover of intracellular 2,3-diphosphoglycerate (2,3-DPG). 2,3-DPG exist

in extremely high concentration in human erythrocytes. As a result, the specific activity of the ATP-gamma-^{32}P reached a second, but final plateau level after 6 hours.

The results described above have shown that human erythrocytes labelled with ^{32}Pi in medium B were maintained in a metabolically active state for at least 6 hours, and that the discocyte morphology was also maintained. The intracellular ATP reached the first and second isotopic equilibrium at 2-3 hours and 6 hours respectively. Figure 32 shows the autoradiograph of the SDS-polyacrylamide gel after separation of cytoskeletal proteins prepared from erythrocytes labelled with ^{32}Pi for 0-6 hours. It can be seen from the autoradiograph that ^{32}P was incorporated rapidly into proteins, and that the incorporation reached isotopic equilibrium after 3 hours. The level of incorporation did not change significantly after 6 hours. This may be due to the relatively slow turnover rate of the phosphate groups in proteins with respect to that of intracellular ATP.

As a result of the above studies, a final set of conditions was obtained for the ^{32}P-labelling of human erythrocytes prior to all phosphorylation studies performed for this thesis. It is worthwhile to note that erythrocytes, due to their fragility and high metabolic rate, should be used without delay for any investigation. Therefore, human erythrocytes were labelled with ^{32}Pi in medium B at 37°C in the presence of 1 mM orthophosphate for 3-3½ hours. The cells were washed and further manipulations or experiments were performed promptly and ended within one hour such that any

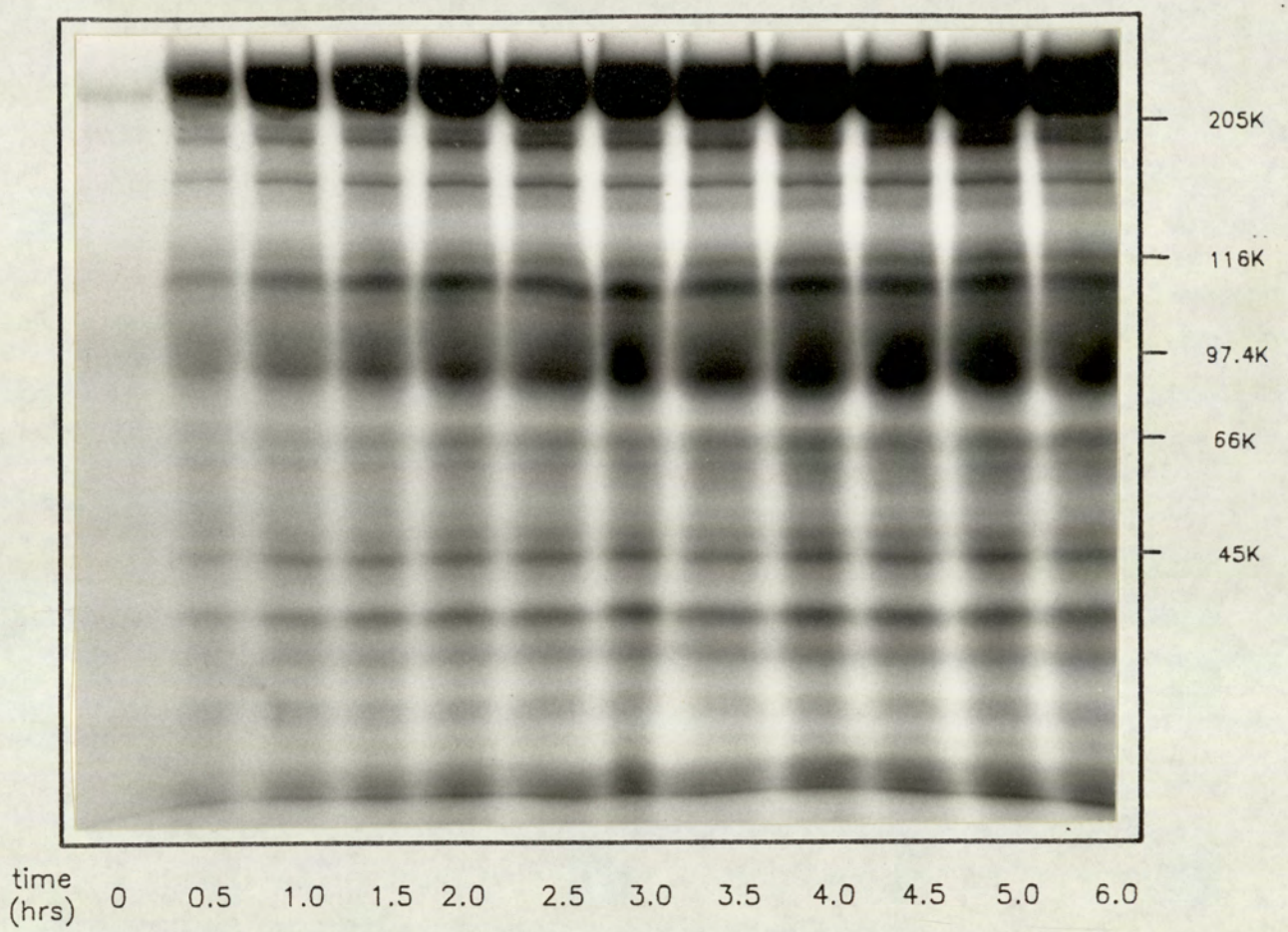


Figure.32 Autoradiography showing the progress of metabolic labelling.
Human erythrocytes were metabolically labelled with ^{32}P-orthophosphate in medium B in the presence of 1mM potassium phosphate.

change in phosphorylation level in proteins would be a direct consequence of the condition under study.

In comparison, several studies on erythrocyte phosphorylation (Cohen & Foley 1986, Palfrey & Waseem 1985) have used relatively simplified media, which are similar in composition to medium A employed in this study (see above), for the labelling of erythrocytes. In these studies, erythrocytes were labelled with ^{32}P-orthophosphate for 3-22 hours and there was no report on either the morphology or the metabolic state of the erythrocytes at the end of the labelling period. In addition, most of these studies have omitted serum albumin in the labelling medium, which appears to be indispensible for the efficient uptake of orthophosphate and the final resolution of the autoradiograph. The possibility of extensive hemolysis during labelling with these media, which has not been considered, further raising doubts on the viability of the erythrocytes and the integrity of intracellular biochemical processes. Thus data obtained from studies using erythrocytes, where their viability is not assessed, have to be treated with caution. The deficiencies of standard labelling methods, outlined above, are not limited to investigations of erythrocytes. Similar facets have also been overlooked in the labelling of other cell types. Moreover, numerous studies have also overlooked the importance of labelling cells to isotopic equilibrium, and they have often prematurely terminated cell labelling. The experimental design of much of this work requires scrutiny.

3.6 CALCIUM-INDUCED ECHINOCYTOSIS IN HUMAN ERYTHROCYTES - EFFECTS ON PROTEIN PHOSPHORYLATION

It has been shown in chapter four that the calcium loading of human erythrocytes caused the activation of intracellular proteases, which modified several major components of the erythrocyte cytoskeleton. Phosphorylation and dephosphorylation are the most common modifications of proteins. Consequently, the effect of calcium-loading on the level of protein phosphorylation of the erythrocyte has also been investigated (Pontremoli et.al. 1988). However, most of the previous studies have been performed such studies using either isolated membranes or resealed erythrocyte ghosts and no studies of intact erythrocytes have been reported. In view of the importance of cytosolic protein kinases, phosphatases and other essential intracellular interactions, experiments were designed to investigate the phosphorylation changes in intact erythrocytes. Erythrocytes were prelabelled with ^{32}Pi to isotopic equilibrium as described in the previous chapter. The cells were washed and loaded with various concentrations of calcium (0-400μM). The cytoskeletal proteins from these cells were separated by SDS-PAGE and autoradiography was performed. The coomassie blue stained gel also showed the presence of native band 2.1 and the proteolysis of band 2.1 after calcium treatment indicating the integrity of the cytoskeletal elements and the protease activity did not deteriorate after the cells were labelled with ^{32}Pi for $3\frac{1}{2}$ hours. figure 33a shows the extent of echinocytosis of the ^{32}P-labelled human erythrocytes and the inhibition of the

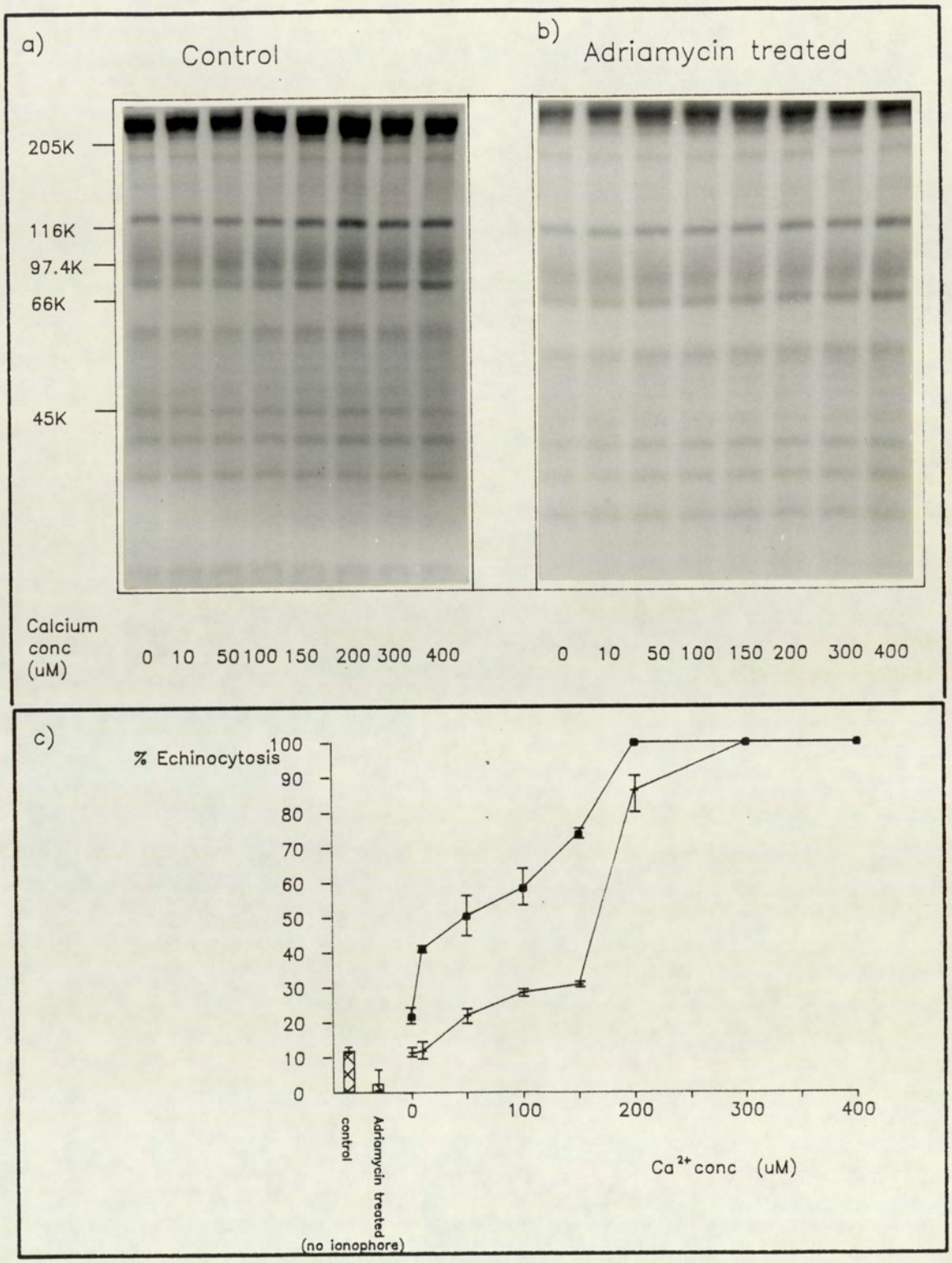


Figure.33 Effects of calcium loading on the phosphorylation of human erythrocyte membrane-cytoskeletal proteins and the effect of Adriamycin pre-treatment.
Metabolically labelled human erythrocytes were pre-incubated without (a) or with (b) Adriamycin prior to calcium loading. The erythrocytes were loaded with calcium (0 - 400µM) for 5 minutes. Morphological examination (c) was taken and membrane ghosts were prepared. The isolated membranes were then analysed by SDS-PAGE followed by autoradiography (a & b).

morphological transition by Adriamycin (10μM) pretreatment. It indicates that the morphological response of these erythrocytes were comparable to erythrocytes prepared immediately from fresh blood as described earlier.

3.6.1. CALCIUM MEDIATED CHANGES IN PHOSPHORYLATION OF CYTOSKELETAL PROTEINS AND THE EFFECT OF ADRIAMYCIN PRETREATMENT

Figure 33b shows the results of an autoradiograph obtained after separation of the cytoskeletal proteins obtained from calcium loaded cells on SDS-PAGE. It can be seen from the figure that calcium loading of human erythrocytes caused dramatic increases in the phosphorylation level of several major constituents of the cytoskeleton, including pp120, band 3, band 4.1 and several proteins with lower molecular weight. Among these proteins, phosphorylation of pp120 and band 4.1 was increased most.

The nature of pp120 was still uncertain. however, a phosphoprotein, adducin, which has subunits with similar molecular weight has been described recently (Gardner & Bennett 1986, Ling et.al. 1986). Although pp120 appeared as a single band on the autoradiogram shown in figure 34, its separation into doublet has also been observed in the present study. This could be the result of slight variation in the polyacrylamide gel concentration during SDS-PAGE separation.

Adducin has also been described as a spectrin-actin binding protein (see section 1.1.1.6). It was observed that the formation of the ternary complex resulted in the

generation of a new spectrin binding site which was antagonized by addition of micromolar concentration of calmodulin and calcium (Gardner & Bennett 1986). This provided an additional mechanism by which elevation of intracellular calcium could modulate cytoskeletal protein organization. Adducin has also been shown to be a protein kinase C substrate as well as for cAMP-dependent protein kinase (Ling et.al. 1986). However, the functional consequences of such a modification and the effect on the cytoskeleton organization and cell morphology has yet to be established.

It has been reported that a decrease in phosphorylation level of band 4.1 enhanced the interactions between spectrin and actin filaments (Cohen 1986). Thus it is possible that an elevation of the phosphorylation level of band 4.1 after calcium loading may cause a weakening of cytoskeletal protein interactions. The findings that band 4.1 was also involved in other cytoskeleton-membrane interactions (Mohandas et.al. 1985, Forte et.al. 1985) suggests further possible effects of the increase in the level of band 4.1 phosphorylation. In contrast, it was shown recently that calcium loading of resealed ghosts, which were resealed in the presence of ^{32}P-gamma-ATP, caused an increased in phosphorylation of band 3 but no other cytoskeletal proteins (Pontremoli et.al. 1988). Although the phosphorylation level of band 3 was marginally elevated after calcium loading as described in this study, other phosphorylation increases were more profound.

These discrepancies may be accounted for, as results from the use of resealed ghosts (i.e. no cytosolic constituents), with exogenous ^{32}P-gamma-ATP for the labelling of cytoskeletal proteins and the relatively short labelling time (10 mins) may introduce artefacts. The validity of results obtained from such studies has to be clarified.

When the calcium loading experiment, as described above, was repeated with cytoskeletal protein samples prepared from calcium-loaded cells which were pre-treated with 10μM Adriamycin prior to calcium loading, the results were as summarised in figure 33a. It shows that calcium loading also caused an increase in the phosphorylation level of several proteins, similar to that obtained from erythrocytes without Adriamycin pretreatment. Despite a similar increase in phosphorylation levels after calcium loading, the autoradiograph image shows that the overall phosphorylation level of proteins in samples which have been pretreated with Adriamycin was reduced compared with the corresponding controls (see figure 33 b & c). The results of the densitometric analysis of the autoradiographs are summarised in figure 34. Figure 34 b-h show the peak height, which is proportional to the phosphorylation level, of each major individual protein band on the autoradiograph at variouscalcium concentration (0-400μM). It could be seen that although the phosphorylation level of each protein band was increased as a result of calcium loading, the phosphorylation level of the Adriamycin treated samples was always lower than the untreated samples. This may be caused by the inhibition

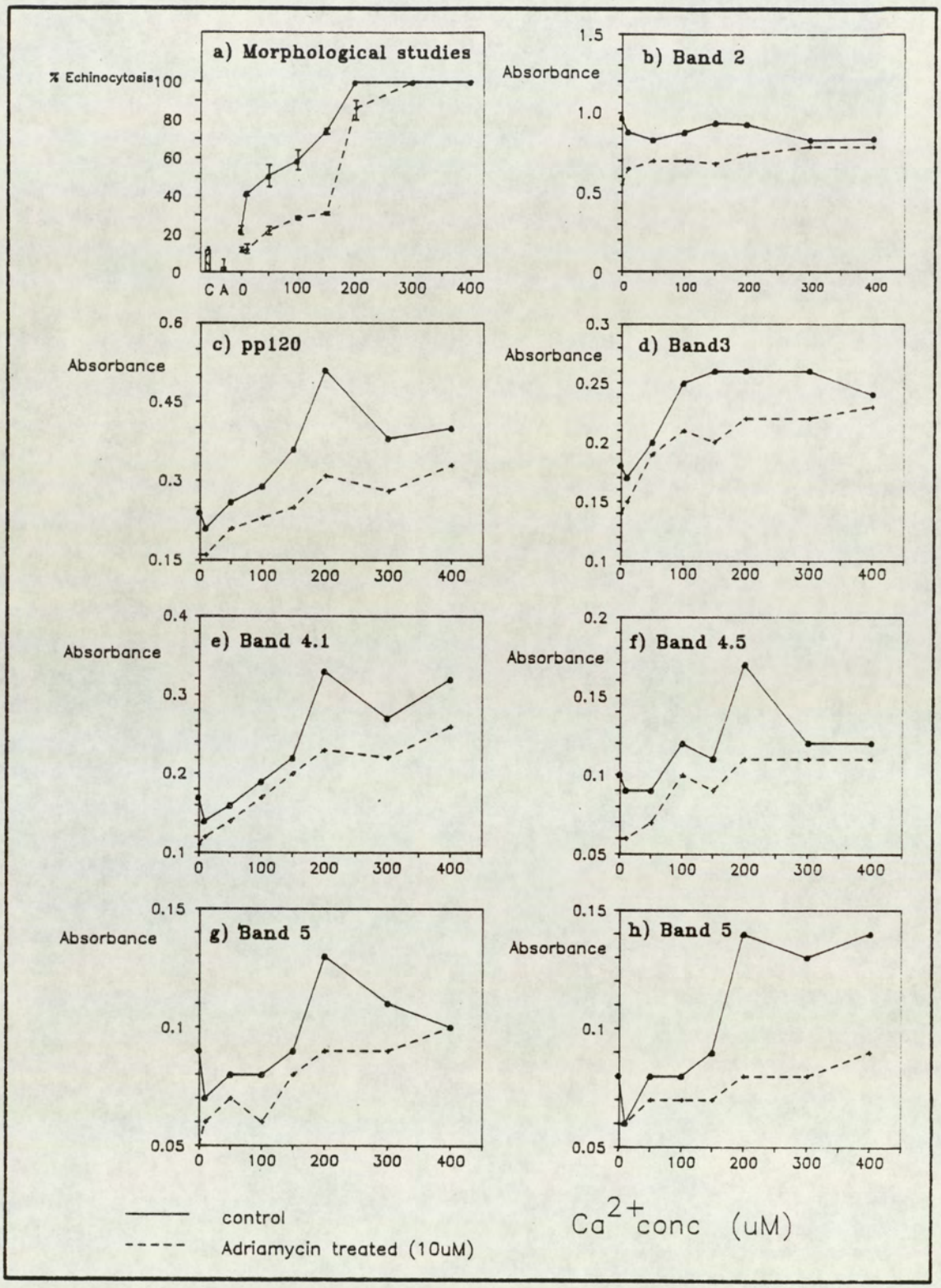


Figure.34 Effects of calcium loading on the phosphorylation of human erythrocyte membrane-cytoskeletal proteins and the effect of Adriamycin pre-treatment - densitometry analysis.

of one or more of the protein kinase systems which resulted in the lowering of phosphorylation. However, there is a possibility that Adriamycin may also activate phosphatase activity.

It has been reported previously that calcium loading of human erythrocytes resulted in a dramatic decrease of intracellular ATP levels due to the activation of the membrane Ca-ATPase (Anderson & Lovrien 1981). However, most of these studies was performed at high calcium concentrations (>1mM) for a prolonged period of incubation (1-24 hrs). Nevertheless, the possibility that Adriamycin may lower the phosphorylation level of proteins by lowering the intracellular ATP level could not be discarded. Thus experiments were carried out to study the ATP level in human erythrocytes during calcium loading and the effect of Adriamycin treatment. Figure 35a shows the morphological studies of calcium-induced echinocytosis and the protective effect exerted by Adriamycin pretreatment. Figure 35b shows the ATP level measured at the end of the incubation period of calcium loading. It could be seen that the ATP level did not change significantly at concentration between 0 and 300μM calcium whilst 100% stage III echinocytes were already obtained below 150μM calcium.

Thus these results suggested that, in calcium loading, there is no significant correlation between the onset of calcium-induced echinocytosis and changes in intracellular ATP level. On the other hand, figure 35c shows the level of ATP

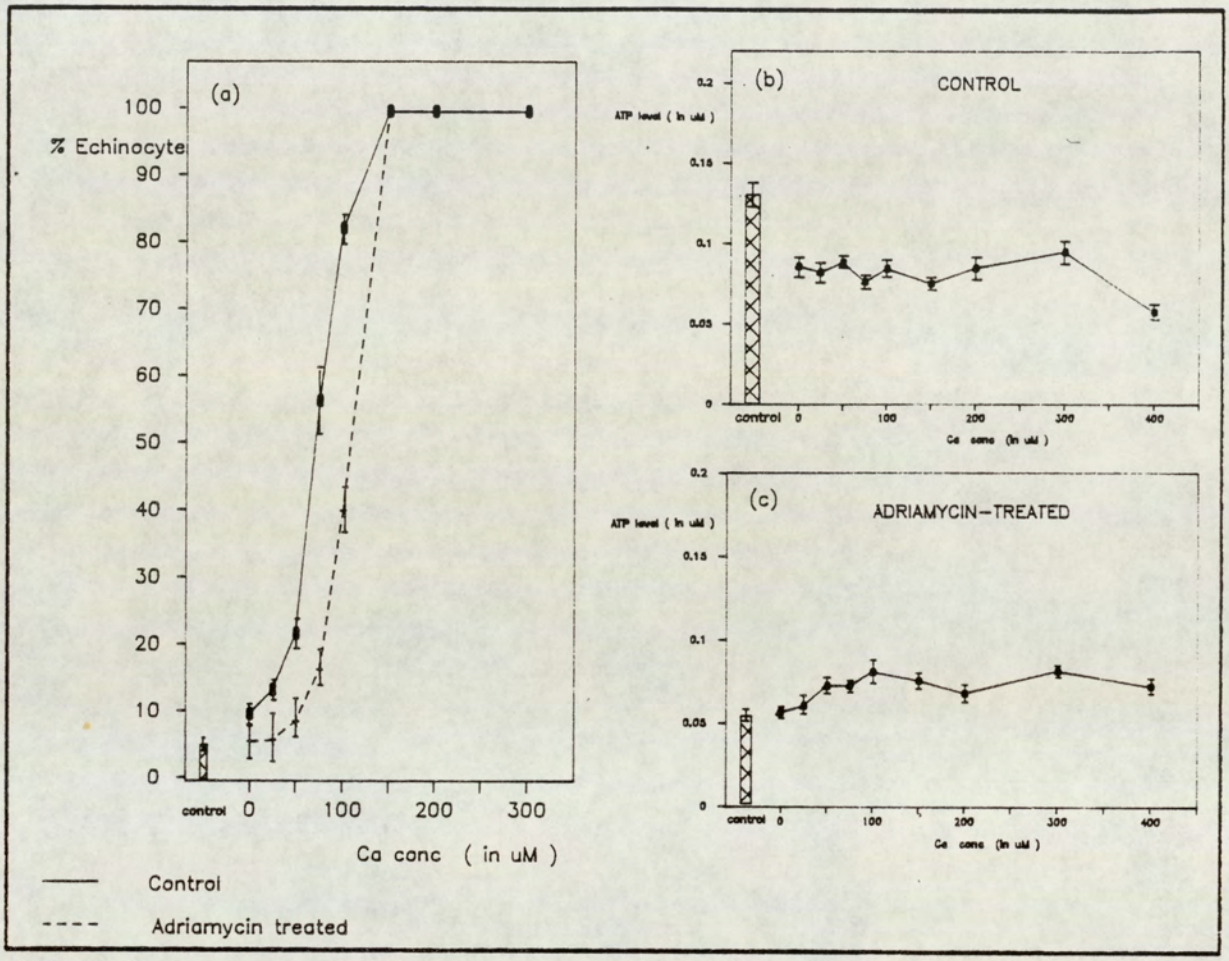


Figure.35 Effect of calcium loading on the intracellular ATP level of human erythrocytes and the effect of Adriamycin pre-treatment.
Human erythrocytes were loaded with calcium (0 - 400µM) for 5 minutes. Morphological examination (a) were carried out and ATP was extracted. The ATP was quantitated, using a luciferase/luciferin assay, for both control (b) and Adriamycin treated (10µM) (c) cells.

in human erythrocytes, which had been pre-incubated with Adriamycin (10μM) for 10 minutes prior to calcium loading. Similarly, there is no correlation between intracellular ATP level and morphological changes. There is no evidence to suggest Adriamycin may exert its protective effect against calcium-induced echinocytosis by the lowering of intracellular ATP level. It is also seems unlikely that Adriamycin may reduce protein phosphorylation by affecting the intracellular ATP level.

Similar studies on the effect of calcium loading of erythrocytes on intracellular ATP levels have been undertaken previously (Anderson & Lovrien 1981). It was reported that calcium loading caused the activation of the membrane calcium pump, which utilizes a large proportion of the intracellular ATP and resulted in ATP depletion. As a consequence, the decrease in ATP level caused the onset of echinocytosis. They were able to show that calcium echinocytosis could be reversed by incubating calcium loaded cells in a glucose/inosine containing medium in order to replete the intracellular ATP level. They claimed that the reversal process could take place provided that ATP repletion was carried out not later than 15 minutes after calcium loading with less than 500μM calcium. However, the report did not show any data on the intracellular ATP levels at each stage of the manipulation. Thus experiments were designed in the present study to investigate the reversibility of calcium loading- induced echinocytosis. In this study, erythrocytes were loaded with 50-200μM calcium for 5 minutes in incubation buffer B at 37°C.

At the end of the calcium loading period, EGTA was added to reduce the free calcium level well below 1μM and the cells were allowed to recover for 2 hours or longer. Since incubation B already contains the necessary constituents for ATP synthesis (it was shown earlier that ATP level did not alter significantly even under calcium loading conditions) the cells should be able to revert to discocytes if the claim was valid. However, no such morphological reversal was observed. Although the cause of this discrepancy is unclear, it was noted that, in their experiments, the incubation temperature was too low and the medium may not be suitable for the proper maintenance of erythrocyte metabolism. This may have caused several artifacts in the experiments. Firstly, the low incubation temperature and unsuitable medium may have caused a decrease in ATP synthesis which resulted in calcium-unrelated echinocytosis. Secondly, a low incubation temperature would also reduce the rate of, or even halt all calcium-induced biochemical events. As a result, the lowering of calcium followed by ATP repletion may have enabled the partial recovery of the discocyte morphology since little or no calcium-induced events had actually taken place.

3.6.2. EFFECTS OF PROTEIN KINASE INHIBITORS ON CALCIUM-INDUCED ECHINOCYTOSIS AND IN VIVO PROTEIN PHOSPHORYLATION.

Investigations were carried out in an attempt to identify the phosphorylation system(s) which were responsible for the increase in phosphorylation level observed during calcium loading and the possible inhibitory effect exerted by Adriamycin on each of these. The protein kinases that may possibly take part in the response are calcium-calmodulin-dependent and independent protein kinases, protein kinase C/protein kinase M system or other as yet unidentified kinases. Various protein kinase inhibitors have been used as probes in these investigations. The results are summarised in figure 36.

Trifluoroperazine has been extensively used as calmodulin inhibitor. However the role of calmodulin in the control of erythrocyte morphology is the subject of controversy. It has been proposed that calmodulin was required to maintain the discocyte morphology of erythrocytes (Nelson et.al. 1983). However, there was no direct biochemical proof for the involvement of calmodulin in the morphological regulation of the human erythrocyte. In the studies of Nelson et al, erythrocytes were treated with the so-called calmodulin inhibitors and they observed that the potency of each agent to produce stomatocytes was related to the reported potency, in various biological systems, to inhibit calmodulin. It has to be noted that most of these drugs are amphipathic, including trifluoroperazine, and affect erythrocyte morphology simply

AGENTS	MORPHOLOGICAL TRANSITION	PHOSPHORYLATION LEVEL
Adriamycin (5–10uM)	protected	decreased
Trifluoroperazine (20–50uM)	stomatocytosis due to mechanical effect	N.A.
Calmidozolium (10–50nM)	cell lysis ocurred at lowest cocentration	N.A.
H–7 (50uM)	–ve	?
W–7 (50uM)	–ve	–ve
Leupeptin (1mM)	–ve	decreased
TPA (0–100nM)	?	increased
Bryostatin 1	–ve	increased

Figure.36 Effects of protein kinase inhibitors & activators on cell morphology and protein phosphorylation in human erythrocytes.

due to the alteration of charge distribution between the two membrane leaflets. Thus these agents have to be used with caution.

Incubation of human erythrocytes with effective concentration of trifluoroperazine (20-50μM) resulted in complete stomatocytosis of the erythrocyte morphology as early as two minutes after addition of trifluoroperazine. Calcium loading (0-200μM), which caused echinocytosis, did not affect the stomatocytosis process. Recently, a more potent ($I.D._{50}$ 50nM) calmodulin inhibitor, calmidozolium, was described (Van Belle 1981) which claimed that the inhibitor is not cytotoxic to cells even at micromolar concentration. However, incubation of erythrocytes with calmidozolium, at concentration as low as 10nM, caused immediate cell lysis. Thus the role of calmodulin in the calcium-mediated increase in cytoskeletal proteins phosphorylation has to be clarified.

Leupeptin, which is an antibiotic inhibitor of proteolysis, has already been described in the previous chapter. Being a specific thio protease inhibitor, incubation of leupeptin (1mM) with ^{32}P-labelled erythrocytes for 30 minutes prior to calcium loading resulted in the lowering of both the basal level and the calcium-mediated increase in phosphorylation (n=3). However, leupeptin was not capable of protecting the erythrocytes against calcium-induced echinocyte formation.

Investigations carried out by Thompson et.al. (1987) showed that calcium loading of human erythrocytes also caused the breakdown of phosphatidyl inositol bisphosphate. Since

one of the breakdown products, diacylglycerol, was found to be the natural activator of PKC, the possible involvement of protein kinase C (PKC) in the calcium-mediated phosphorylation of the cytoskeleton was also investigated.

The inhibitor, 1-(5-Isoquinolinylsulfonyl)-2-methyl-piperazine (H-7), has been recently described as a specific PKC inhibitor (Matsui et.al. 1986). However, there are doubts of whether H-7 is a reliable inhibitor of PKC. It has been found that, in certain systems, H-7 did not have any effect on PKC mediated phosphorylation, yet the presence of PKC was demonstrated. In this study, erythrocytes were incubated with an effective concentration of H-7 (50μM) for 30 minutes prior to calcium loading. However, only in 1 of 8 experiments did H-7 show an effect of lowering the calcium-mediated phosphorylation as described earlier. Similarly, another protein kinase inhibitor, W-7, which is claimed to be a inhibitor for both PKC and calcium-calmodulin dependent protein kinase with higher potency against PKC (Matsui et.al. 1986) was also found ineffective in this study. Thus the possible involvement of PKC in the regulation of phosphorylation, at both basal level and during calcium loading, requires further investigation.

3.6.3. EFFECTS OF PROTEIN KINASE C ACTIVATORS ON THE PHOSPHORYLATION OF PROTEINS

Previous investigations in our laboratory (Jones et.al. 1987) showed that the phorbol ester, TPA, when pre-incubated with human erythrocytes, protected erythrocytes against

calcium-induced echinocytosis. Since TPA was recently found to be a potent activator of PKC (Ashendel 1985), experiments were undertaken to examine the protective effect of TPA against echinocytosis and its effect on protein phosphorylation. However, these experiments were not able to validate the protective effects observed by TPA preincubation (100-500nM) and the causes of this discrepancy with the earlier report are not clear.

Detailed studies of phosphorylation by autoradiography showed that treatment of erythrocytes with TPA resulted in the increase in phosphorylation of several cytoskeletal proteins (figure 37). Interestingly, proteins which were increased in phosphorylation level as a result of TPA treatment were also increased during calcium loading (figure 33 & 37). This increased the possibility of involvement of PKC in these calcium mediated processes. In figure 37, it can be seen that the protein band pp120, which was described earlier (see figure 33), was resolved into a doublet. This might have been caused by a slight difference in polyacrylamide gel concentration between experiments. Figure 38 shows the autoradiograph of the TPA concentration-dependent phosphorylation response of the human erythrocytes. It can be seen that the maximum response of phosphorylation varied between 0.5-3nM TPA dependent upon the identity of the protein. In addition, a decrease in the phosphorylation of several proteins was also observed (figure 38 arrows). However, it is not clear what the consequence of these dephosphorylations areand whether this is a direct effect of

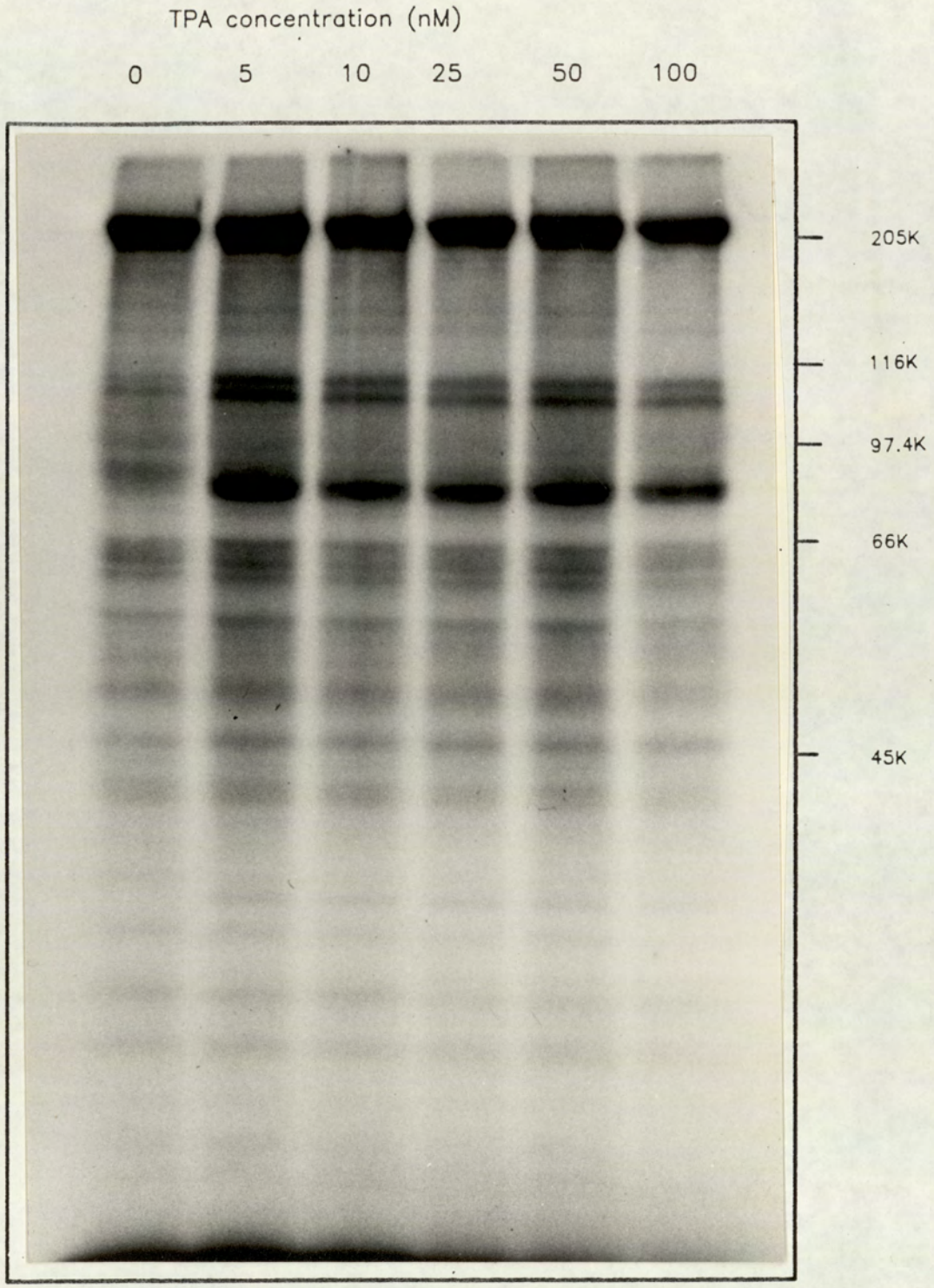


Figure.37 Effects of TPA on protein phosphorylation in human erythrocytes.
Metabolically erythrocytes were treated with various concentration of TPA (0-100nM) for 10 minutes. Membrane ghosts were then prepared and analysed by SDS-PAGE followed by autoradiography.

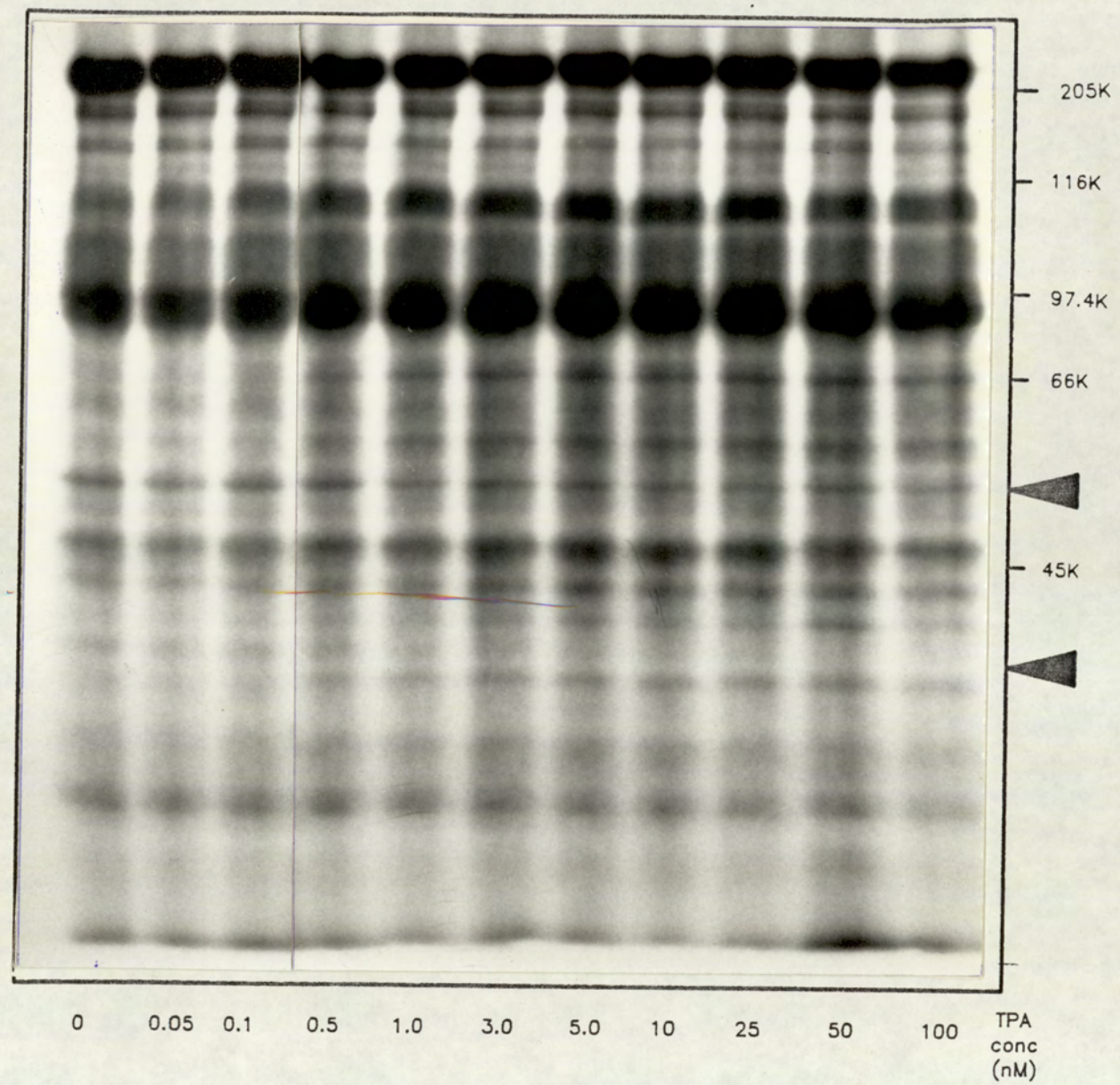


Figure.38 TPA dose response of metabolically labelled human erythrocytes.
Metabolically labelled human erythrocytes were treated with TPA (0 - 100nM) for 10 minutes. Isolated membranes ghosts were then subjected to SDS-PAGE analysis followed by autoradiography for 5 days. Note the autoradiograph was over-exposed in order to show possible dephosphorylated proteins (arrows).

TPA acting on intracellular phosphatases or through protein kinase C activation of phosphatases. It has been shown in previous experiments that TPA (100-500nM) was not found to offer any significant protective effect against morphological change at concentrations well above its optimum concentration for the promotion of protein phosphorylation. It is considered that it is unlikely that it would inhibit morphological change at higher concentrations.

The effect of protein kinase C on human erythrocyte cytoskeletal proteins has been reported elsewhere (Palfrey & Waseem 1985, Cohen & Foley 1986, Ling et.al. 1986). Interestingly, it was shown that treatment of ^{32}P labelled erythrocytes only caused an increase in the phosphorylation of two approx.110K proteins, band 4.1 and band 4.9 (Cohen & Foley 1986, Ling et.al. 1986); this is fewer than that observed in this study. A similar labelling pattern was also reported by Palfrey & Waseem (1985); they also showed that an elevated calcium level caused a lowering of phosphorylation, which contradicts the normal characteristics of the elevation of phosphorylation by calcium as observed elsewhere. Similar studies have been undertaken in this study (results not shown) and they have shown that although calcium and TPA increase phosphorylation level in a similar set of cytoskeletal proteins, they did not work synergistically. Calcium loading of cells which have been pre-treated with TPA did not significantly alter the phosphorylation response to TPA. In addition, there has been no report of specific dephosphorylation of erythrocyte cytoskeletal proteins as a

results of TPA treatment. There is a possibility that these contradictions were caused by the improper labelling of erythrocytes with ^{32}P-orthophosphate and prolonged maintenance of the cell suspension, under adverse conditions as described above.

Recently, several other families of tumour promoting protein kinase C activators have been described (Kiss et.al. 1987). One of these activators, bryostatin 1, was also found to dramatically increase protein phosphorylation in the erythrocyte, with target proteins similar to those described with TPA treatment. Preincubation with it was found not able to offer a protective effect against calcium induced echinocytosis.

3.6.4. CALCIUM-INDUCED ECHINOCYTOSIS AND PROTEIN KINASE C TRANSLOCATION

It is widely believed that the calcium-sensitive phospholipid-dependent protein kinase (PKC) translocates to the plasma membrane under conditions where diacylglycerol production is increased, with a minimal elevation in calcium concentration. Thus, investigations were carried out to study the possible PKC translocation in human erythrocytes after calcium loading, where calcium acts as both a PKC activator and a mediator of diacylglycerol production.

Since this study required the use of large quantities of ATP-gamma-^{32}P, which is very expensive commercially, the Salt-free ATP-gamma-^{32}P used in this study was synthesized using the coupled enzymic reactions as described by Johnson &

Walseth (1979) with modifications (methods section 2.15). The rate of ATP synthesis is illustrated in figure 39a. The reaction was incubated until more than 95% of the ^{32}Pi was incorporated into ATP. The reaction mixture was then separated on a sephadex A25 equilibrated with triethylammonium bicarbonate and eluted with a gradient of triethylammonium bicarbonate. The elution profile of radioactive materials is shown in figure 39b. The fractions containing ATP were pooled and freeze dried. The residue was washed and finally dissolved in 50% ethanol, left at -20°C for at least 24 hours in order to dissolve the residue such that maximum yield could be obtained. Generally, an isotopic yield of approximately 70% could be achieved.

In the investigation of PKC translocation in human erythrocytes, erythrocyte membranes were prepared and tested for PKC activity as described in materials & methods (section 2.16). Unfortunately, no PKC activity could be recovered after gel electrophoresis. However, the large increase in phosphorylation level after treatment with low concentrations of TPA suggested the presence of PKC in human erythrocytes. Since samples obtained from other nucleated cell lines showed high activity in the same experiment, so that there was no defect in the techniques involved in the investigation (results not shown). Surprisingly, addition of purified PKC (from rat brain) to the human erythrocyte membrane samples prior to gel electrophoresis also failed to allow the isolation of any PKC activity whilst the pure PKC alone was still highly active after gel electrophoresis. This indicated

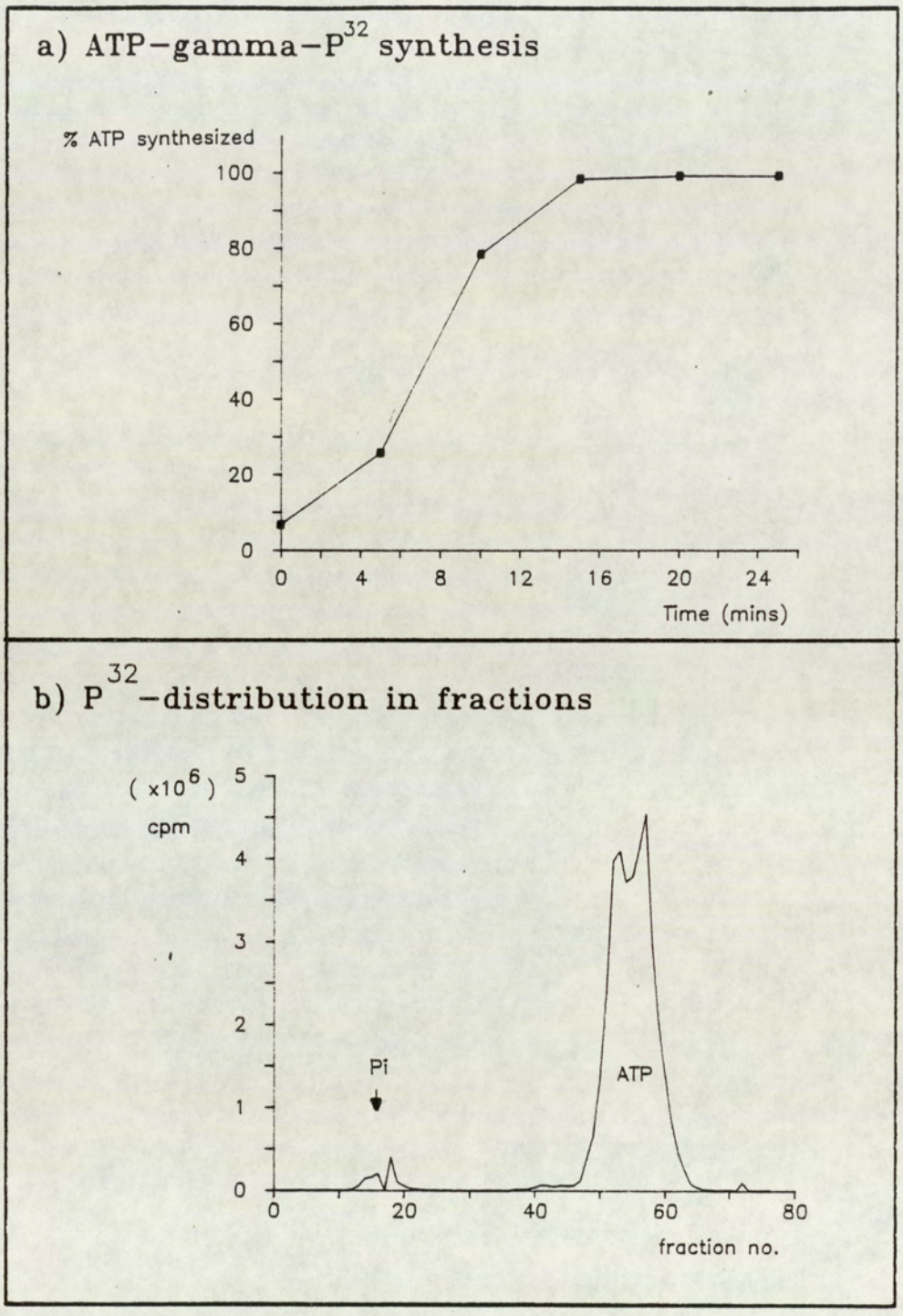


Figure.39 ^{32}P-gamma-ATP synthesis.
(a) rate of synthesis of ^{32}P-gamma-ATP in the coupled reactions. (b) ATP-elution profile from DEAE sephadex column eluted with a linear gradient of triethylammonium carbonate solution (100 - 400mM).

either there is a very potent endogenous inhibitor presented in erythrocyte or the added purified PKC was being rapidly broken down by proteases present in the samples even in the presence of various protease inhibitors.

Other approaches were also used in this study, which intended to clarify the role of PKC. Experiments were performed to examine the ability of the erythrocyte membranes to bind ^{3}H-Phorbol dibutyrate (PDBu), which is a phorbol ester analogue. Despite the high level of PDBu binding to the membranes, the level of non-specific binding, determined by a several thousand fold isotopic dilution with non-radioactive PDBu, was exceptionally high such that no significant specific binding was obtained and the cause is as yet unclear.

So far the role of PKC in the calcium-induced echinocytosis and the phosphorylation increase in cytoskeletal proteins is undetermined. It has been reported by Katoh et.al.(1981) that Adriamycin was capable of inhibiting a calcium-sensitive phospholipid-dependent kinase activity from skeletal muscle. Therefore, preliminary experiments were carried out using purified PKC to examine the effect of Adriamycin on PKC activity. The results are summarised in figure 40. They indicated that Adriamycin, at concentration which exerted protective effects against calcium induced echinocytosis, did not significantly inhibit protein kinase C activity. Results from several studies actually suggested marginal stimulation of protein kinase C activity at the concentration of Adriamycin used. This is supported by the report from Palaez et.al. (Palaez et.al. 1986) that

Condition	Protein Kinase C activity (cpm)	% control
Protein kinase C	144364 ± 29383	100
Protein kinase C + 5uM Adriamycin	162144 ± 10557	112
Protein kinase C + 10uM Adriamycin	166384 ± 30418	115

n = 3

Figure.40 Effect of Adriamycin on purified protein kinase C activity.

Adriamycin at concentration up to 200 and 400μM did not show any inhibitory effect towards PKC activity. However, one has to aware that the purified protein kinase C used in this study originated from rat brain and several isozymes of protein kinase C have now been described (Knopf et.al. 1986, Kikkawa et.al. 1986, Kikkawa et.al. 1987). In view of the existence of multiple form of protein kinase C and the species difference involved, the results obtained from the above study using the purified protein kinase C (from rat brain) has to be treated with caution when the results are extrapolated to system such as human erythrocytes.

3.7 ALTERNATIVE TECHNIQUES IN STUDYING PROTEIN PHOSPHORYLATION

In the last chapter, various results involving studies on cytoskeletal proteins phosphorylation in human erythrocytes have been discussed. It has been shown that calcium loading resulted in increased phosphorylation of several cytoskeletal proteins. On the other hand, pre-incubation of human erythrocytes with Adriamycin caused the lowering of basal phosphorylation levels with little effect on the calcium-mediated elevation of phosphorylation. However, these experiments were performed using a 1-dimensional gel separation system and each individual coomassie stained band is unlikely to represent more than one protein. Theoretically, the cytoskeletal proteins could be separated according to their charge and molecular weight on a

2-dimensional gel separation system (isoelectrofocusing followed by SDS-PAGE) such that proteins with similar molecular weight could be separated from each other. Using this technique, changes of phosphorylation during calcium loading and Adriamycin treatment can be examined in order to determine whether the phosphorylation changes undergone were general or were in specific proteins. However, difficulties have been encountered in separating erythrocyte membrane-cytoskeletal proteins by 2-dimensional electrophoresis. Limited success has been reported by other groups (Rouyer-Fessard et.al. 1987) but no satisfactory and reproducible pattern was achieved. The possible cause may be the insolubility of the spectrin network present in erythrocyte cytoskeleton which renders it insoluble, even in urea-triton solution.

In a study visit to Dr.W.C.Horne in Yale University School of Medicine, a rapid method was developed to study phosphorylation by using SDS-PAGE followed by peptide HPLC. The method was developed using ^{32}P-labelled erythrocyte membrane proteins. The proteins were separated by 1-dimensional SDS-PAGE followed by blotting onto nitrocellulose and protease digestion (see method). The digested mixture was then separated by C_{18} reverse phase HPLC. The cytoskeletal protein band 4.1 was studied since it is a major component in erythrocyte cytoskeleton. Figure 41 shows the HPLC peptide map (monitored by absorbance at 214nm) from samples digested with either trypsin (figure 41a) or Staphyloccus aureus V8 protease (figure 41b). Fractions were

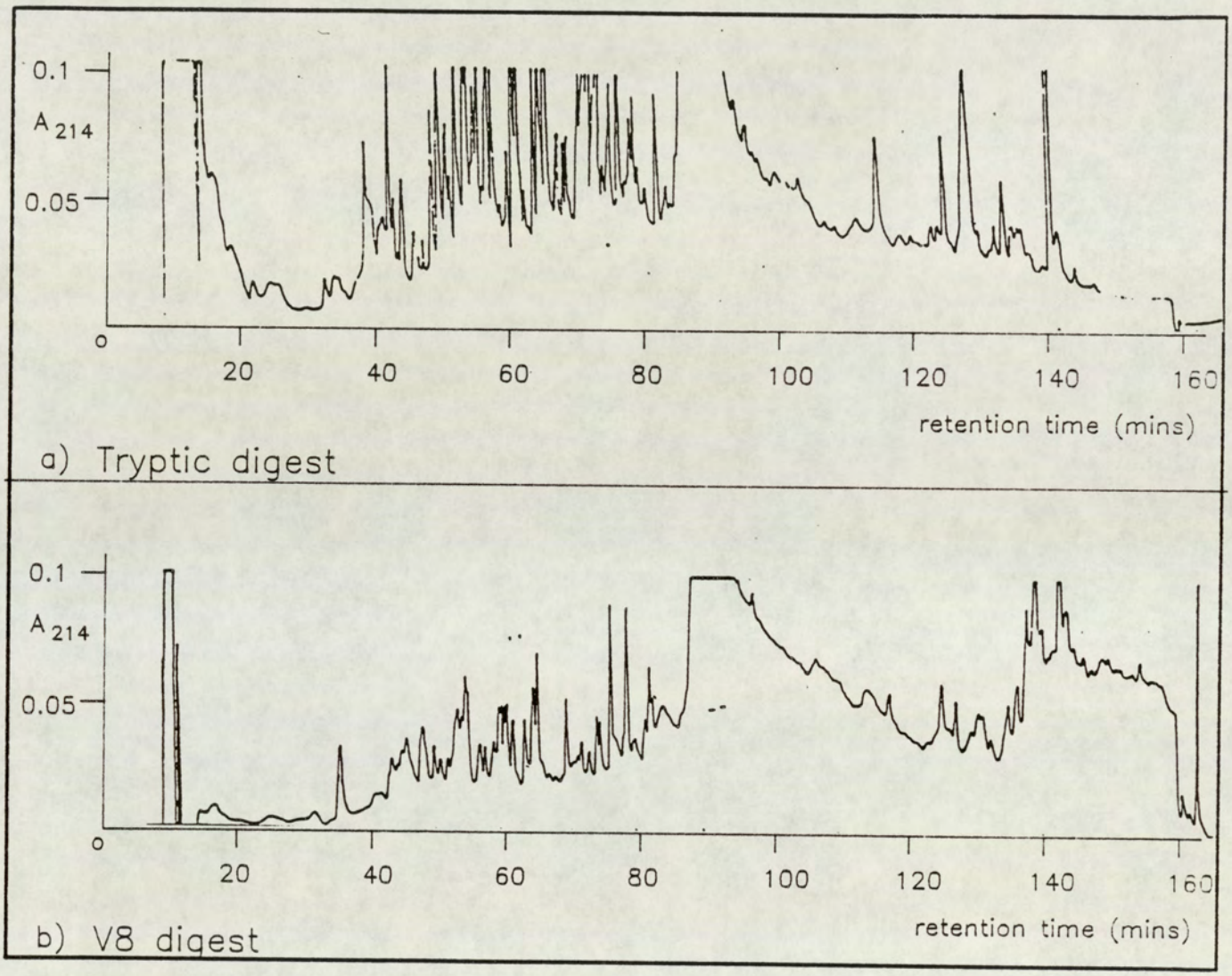
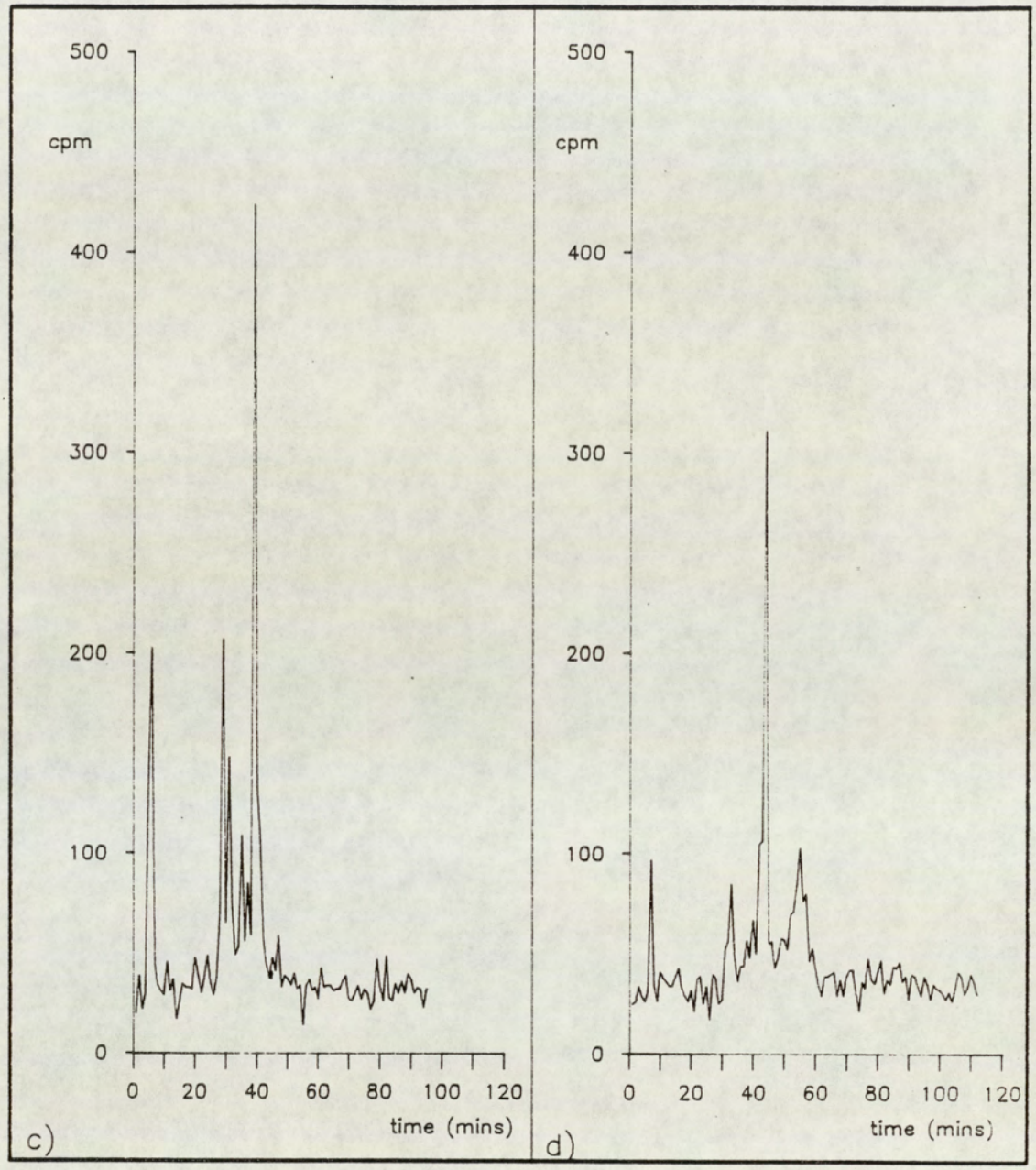


Figure.41 Phosphopeptide mapping of erythrocyte cytoskeletal protein band 4.1 by reverse phase HPLC.
Human erythrocytes were metabolically labelled and cytoskeletal proteins were separated by SDS-PAGE followed by blotting onto nitrocellulose. Band 4.1 was located by staining, excised and digested with either trypsin (a & c) or V8 (b & d) proteases. The digests were then separated by reverse phase HPLC (a & b). Fractions were collected and ^{32}P was measured by cerenkov counting (c & d).


500
cpm
400
300
200
100
0
0 20 40 60 80 100 120
time (mins)
c)
500
cpm
400
300
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0
0 20 40 60 80 100 120
time (mins)
d)

collected during HPLC and subjected to scintillation counting. Figure 41 c & d show the ^{32}P-labelled peptide distribution during the elution in samples from trypsin and V8 digestion respectively. It could be seen that, for band 4.1 protein, trypsin digestion generated several more ^{32}P-positive peptides than V8 digestion indicating band 4.1 possessed more cleavage sites for trypsin than V8.

Preliminary investigation was also carried out to study the effects of TPA on phosphorylation changes in band 4.1. Human erythrocytes were labelled with ^{32}Pi and treated with TPA. ^{32}P-labelled band 4.1 was obtained and processed as described above. Figure 42 shows the results from scintillation counting of fractions from the HPLC separation. The ^{32}P-peptide map of band 4.1 from untreated erythrocytes (solid line) was similar as shown in figure 41a. After TPA (100nM) treatment, it could be seen that TPA caused dramatic increase in phosphorylation in at least 11 peaks (dotted line) in the HPLC phosphopeptide map. It is noted that several of these peaks were not present in control samples. Fractions which contained each of these peaks were pooled and rechromatographed on a different C_{18} reverse phase column and fractions were collected. Scintillation counting results indicated that most of the peaks which appeared in the first chromatogram consisted of multiple peptides and further separation was obtained on second chromatography. Since Cerenkov counting was used for the quantitation of ^{32}P in fractions, further manipulation of the peptides contained in the fractions was possible. As a result, sequence information

was obtained for peak 6, which was found to contain one major ^{32}P-labelled peptide in second chromatography, as shown in figure 42. The sequence was found to be Phe-Arg-Tyr-Ser*-Gly-Arg which corresponds to residues 298-303 in the band 4.1 sequence reported by Conboy et.al.(Conboy et.al. 1986). The serine residue in the sequence was found to exist solely as serine derivative during the sequencing procedure indicating this residue was 100% phosphorylated in the protein as a result of TPA treatment.

In addition, studies were also carried out to define conditions for the pre-sequencing modification procedure, such that adjacent partially phosphorylated serine/threonine residues could be identified and quantitated during protein sequencing. Since pure erythrocyte cytoskeletal proteins were only in limited supply, a band 4.1 analogue protein, synapsin, was used in the study. Modification was carried out as described by Meyer et.al. (1986) to attach a S-ethyl moiety onto serine residue after beta-elimination reaction (in the presence of NaOH) of phosphoserine residues (figure 43). The peptide used was 100% phosphorylated at the serine residue. Figure 44 a-d shows the progress of the reaction as monitored by separation of the mixture by C_{18} peptide HPLC. It was observed that modification was successfully initiated and products were formed as early as 5 minutes after the addition of reagents. The reaction resulted in the formation of peptide products which appeared as two groups of peaks on the HPLC chromatogram (see figure 44 a-d). The results indicated the modification of the synapsin peptide resulted in the

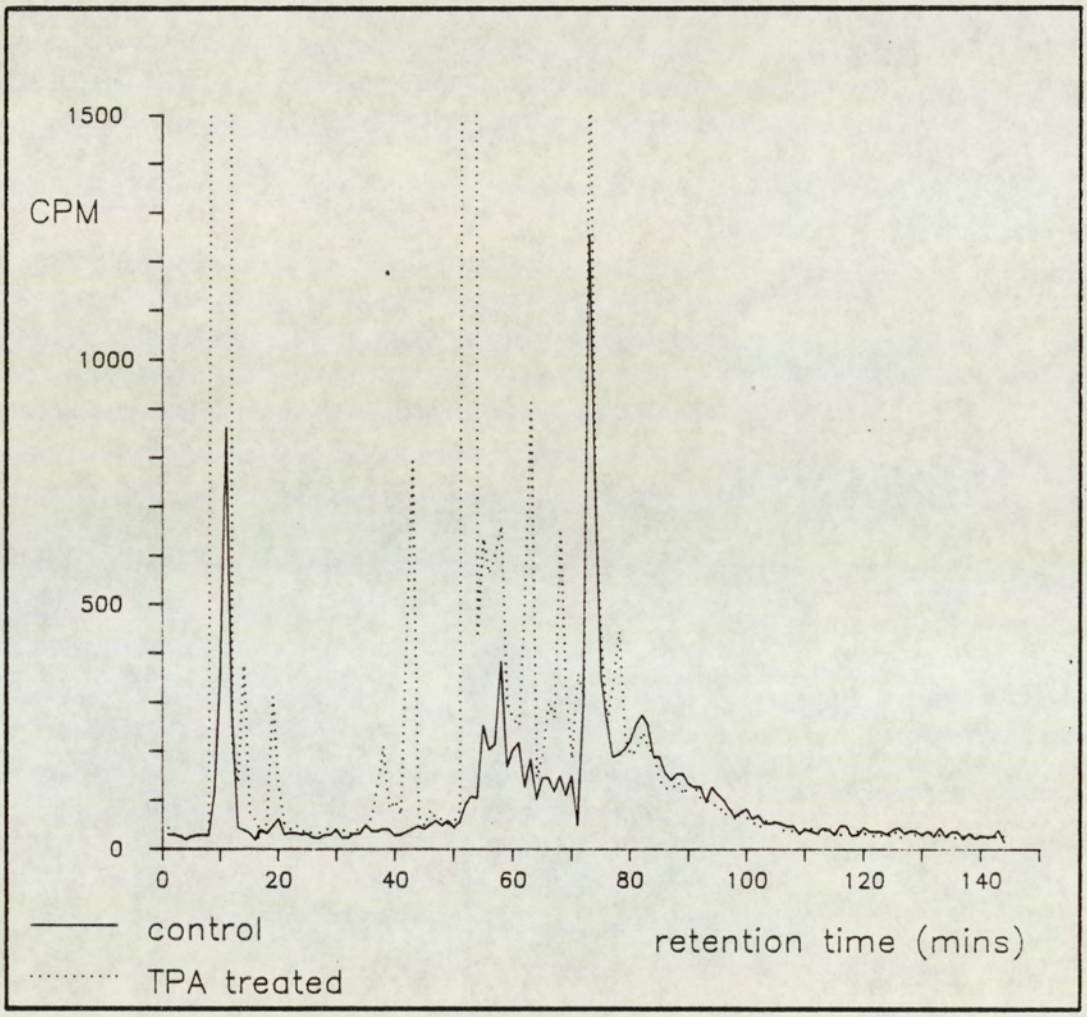

Figure.42 Phosphopeptide mapping of band 4.1 - effect of TPA
Human erythrocytes were metabolically labelled and incubated with or without of TPA (1µM). Membrane ghosts were isolated and band 4.1 was isolated by SDS-PAGE followed by blotting onto nitrocellulose. The protein was digested with trypsin and peptides were separated by HPLC.

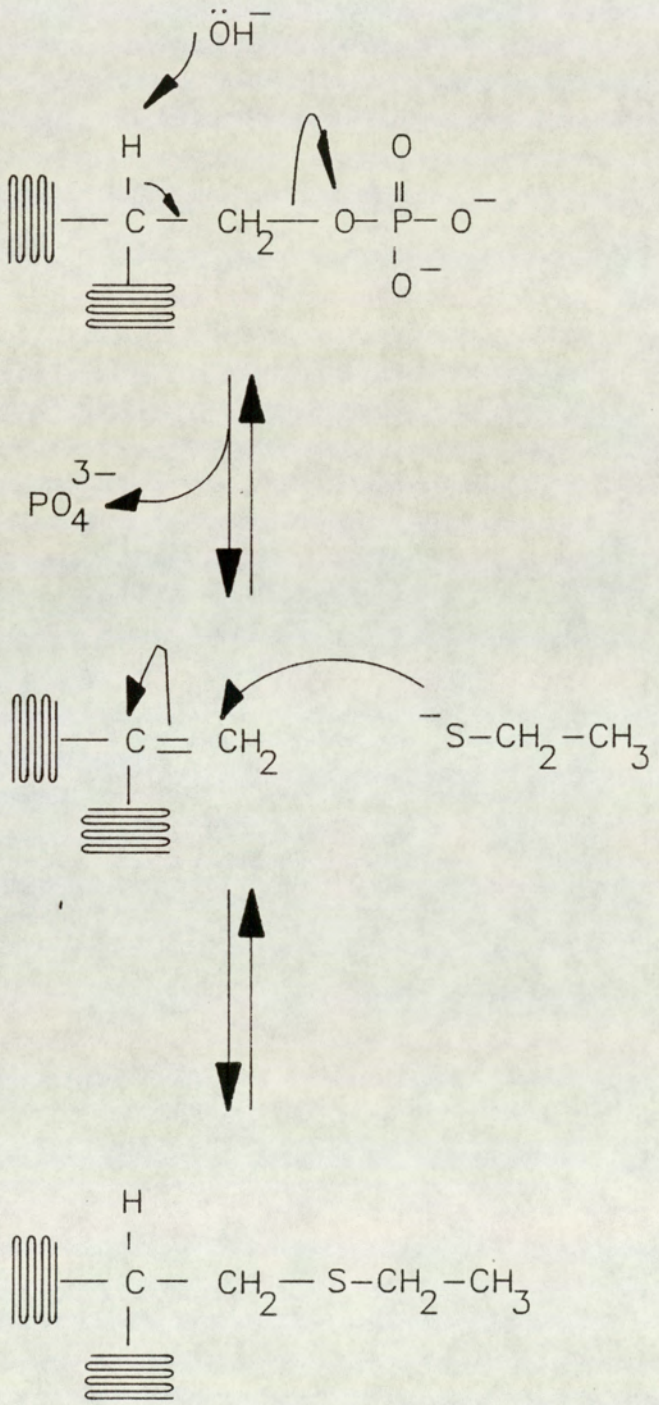

Figure.43. Chemical modification of phosphoserine residue by beta-elimination followed by ethanethio addition.

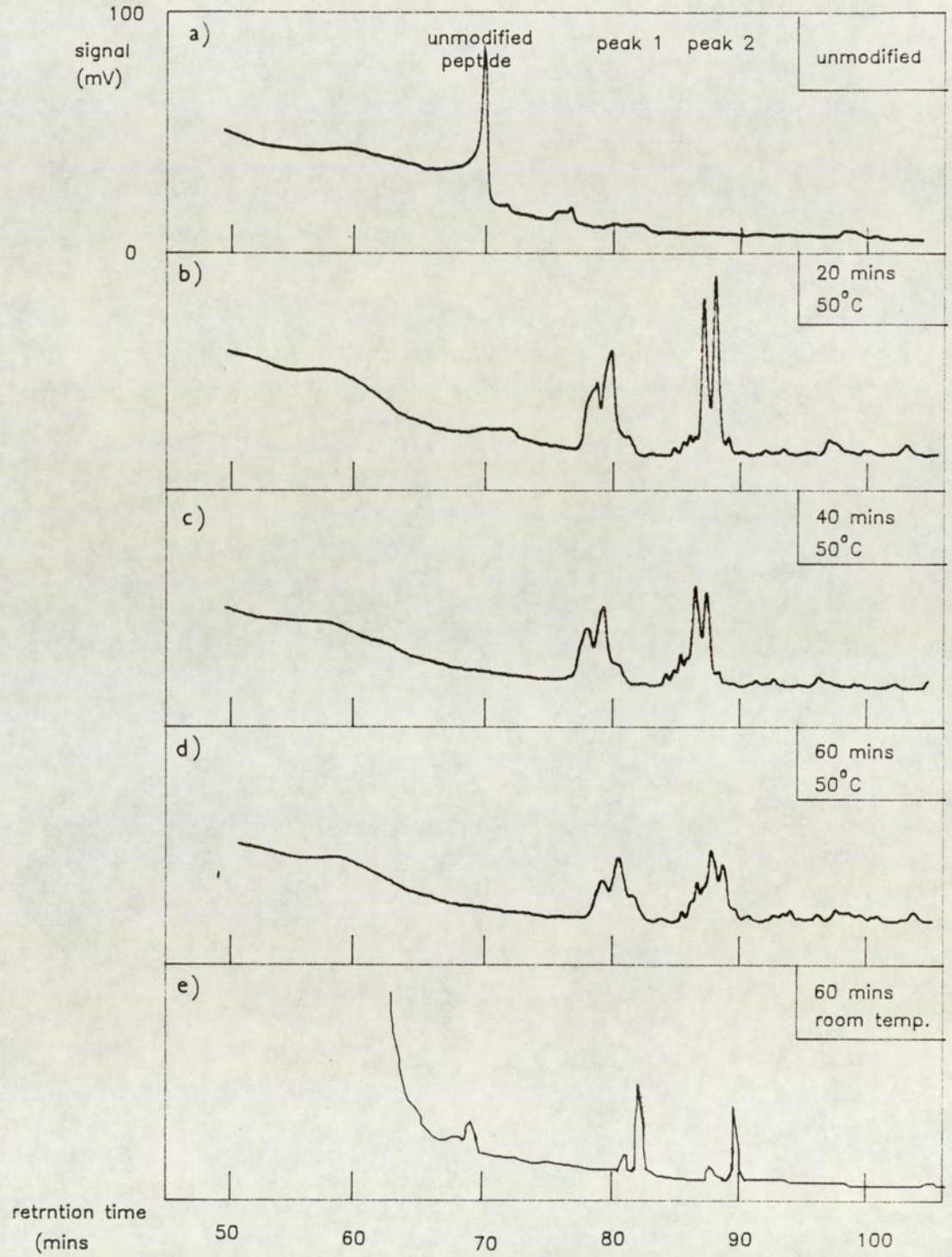

Figure.44 Chemical modification of synapsin peptide.

formation of 2-4 major products, which were further reacted to form other minor products. This caused a dramatic decrease in the yield after the reaction, the identification of the products was uncertain. It was found that the reaction would not proceed when NaOH was replaced by other weaker bases. In the end, the condition were modified such that the reaction gave rise to products separated as two doublets in HPLC analysis (figure 44e). Approximately 1-2 pmol of modified peptides were collected from the main peak in each doublet and subjected to mass spectroscopy. The results are shown in figure 45a-c. Figure 45a shows the molecular weight of the unmodified phosphopeptide had a mass of 1066, corresponding to the complete synapsin phosphopeptide. However, both samples obtained from PLC separation of the modified peptides had a mass of 1030, consistent with replacing a single phosphate with a single S-ethyl group. Thus the determination gave a mass fragment for the parent compounds only, with no cleavage, such that no information concerning structural differences could be obtained. More detailed studies have to be carried out to identify these products before the technique could be employed in identification and quantitation of phosphorylated residues in peptides during peptide sequencing.

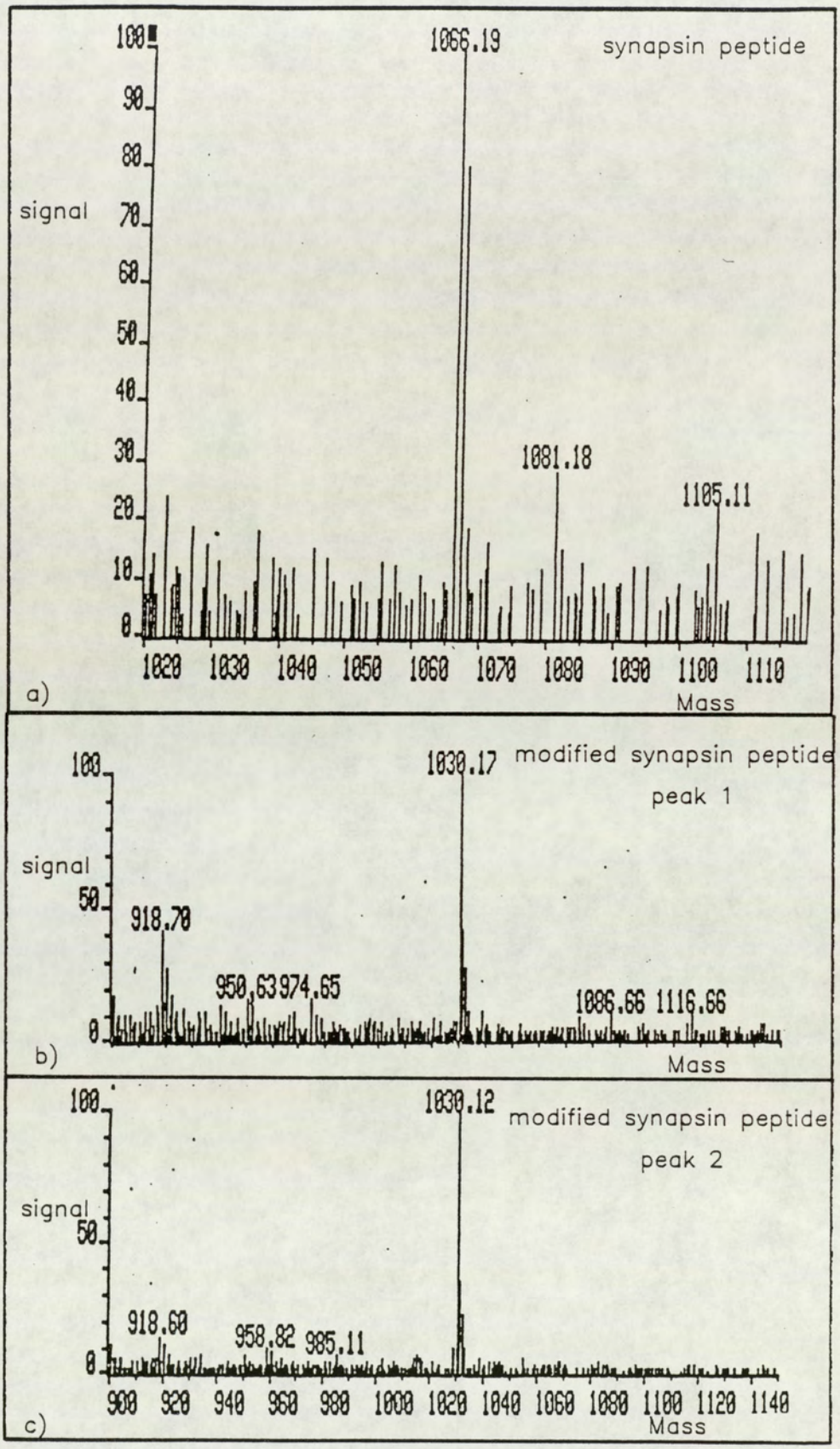

Figure.45 Mass spectrometry of synapsin peptides.
Synapsin peptides were chemically modified as described in methods and separated by reverse phase HPLC. Fractions containing modified peptides were processed for mass spectrometry.

CHAPTER FOUR

GENERAL DISCUSSION

In the previous chapters, the results obtained from the experiments detailed in this thesis were presented and discussed in relation to the underlying mechanisms of calcium-induced echinocytosis and the possible mode of action of Adriamycin. Briefly, it was shown that calcium-loading of human erythrocytes resulted in the controlled echinocytosis of erythrocytes and the proteolysis of band 2.1 and band 4.1, to a degree which was dependent upon the concentration of calcium used, and the time of exposure. Calcium-induced transglutamination reactions between cytoskeletal components and alterations in the phosphorylation level of cytoskeletal proteins were also observed. Pre-treatment of erythrocytes with 10μM Adriamycin resulted in the inhibition of band 2.1 (ankyrin) breakdown and a general decrease in the overall phosphorylation level of most cytoskeletal proteins, as judged by autoradiography. Although proteolysis of band 2.1 and an increase in phosphorylation level of cytoskeletal proteins both proceeded in a calcium concentration-dependent manner, and appeared to correlate well with the extent of the morphological change, there was no clear relationship between some of these biochemical changes and the effect of Adriamycin which would allow firm conclusions to be drawn regarding its mechanism of action.

The finding that Adriamycin inhibited ankyrin breakdown under conditions where it did not inhibit the change in morphology from the discocyte to echinocyte raises questions about the role of ankyrin in the control of cell morphology.

The findings do not support the suggestion that band 2.1 is a critical element involved in shape changes in human erythrocytes and for their deformability (Jinbu et.al. 1982, Jinbu et.al. 1984). Although these studies showed that proteolytic cleavage of ankyrin led to the weakening of cytoskeleton-membrane association as judged by electron microscopy, the use of trypsin as protease in the studies of Jinbu et al raises questions regarding the specificity of the cleavage site on ankyrin, and the extent of the cleavage under their conditions. Under the conditions used here, calcium loading of the cells (with > 200 μM) in the presence of Adriamycin, produced an irreversible change in morphology, despite the fact that ankyrin breakdown was inhibited.

Recently, it was proposed that human erythrocytes contain a second form of ankyrin, band 2.2 (M_w 186,000), which may be the activated form of ankyrin (Hall & Bennett 1987). It was shown that this form was capable of interacting with twice as many high affinity binding sites on ankyrin-depleted inside-out vesicles, as with native ankyrin molecules. They also reported that a proteolytic fragment (M_w 195,000), generated by the digestion of native ankyrin with calpain, also interacted with twice the number of high affinity sites in ankyrin-depleted vesicles, compared to native ankyrin, although the affinity was reduced 8-fold. However, these studies contradict those obtained from other investigators (eg. Allan & Thomas 1981a) and those of this thesis. These studies, including results from this thesis, have shown that treatment of erythrocytes with calcium and calcium ionophore

leads to the proteolytic cleavage of ankyrin into band 2.3 (M_w 174,000) as a result of calpain activation. However, Hall & Bennett (Hall & Bennett 1987) reported that exhaustive digestion of ankyrin with calpain resulted in the generation of a 195,000 Kd fragment, which was considerably larger than band 2.2 and band 2.3. Thus there is a possibility that the specificity of the cleavage site on ankyrin in vivo may be different to that observed in vitro, this was not verified by Hall & Bennett. It is still unclear whether band 2.2 is a proteolytic product of ankyrin (possibly after calpain digestion) or a product of differential RNA splicing during erythrocyte development. Unfortunately, since the state of band 2.2 during calcium-loading could not be observed in my studies (due to the use of the Laemmli gel system and a slightly higher acrylamide concentration, 7.5%), it was not clear whether band 2.2 was a proteolytic product of ankyrin, with band 2.3 being the end product of band 2.2 breakdown as the result of calcium loading. Nevertheless, these reports did show that proteolytic cleavage of ankyrin (band 2.2 and the 195,000 Kd fragment) resulted in enhanced interactions with both the transmembrane protein band 3 and the spectrin network, and there is therefore the possibility of enhanced interactions between the cytoskeleton and membrane as a result of band 2.2 or band 2.3 generation during calcium loading rather than a lowering of interactions, as suggested earlier (Jinbu et.al. 1982, Jinbu et.al. 1984).

The mechanism by which Adriamycin inhibited ankyrin proteolysis remains unknown; the drug had little effect on the

calcium-activated proteolytic enzyme calpain. It is possible, although unlikely, that different proteolytic enzymes were activated under conditions of calcium loading. Alternatively, it is possible that Adriamycin interacted with ankyrin in some way, so as to protect it from becoming a substrate for proteolysis. These questions await further experimentation.

Although the studies of the phosphorylation of the cytoskeleton discussed in this thesis (results section chapter six) were not able to show a specific or selective inhibition of protein phosphorylation by Adriamycin, as shown in figure 33 & 34, the phosphorylation level of each of the proteins identified correlated reasonably well with the morphological state of the erythrocytes. That is, Adriamycin reduced the overall phosphorylation levels observed, but when these rose above certain threshold levels, the cells changed their morphology. These "thresholds" were identified in the control cells treated with calcium, where parallel experiments allowed comparison of morphology with phosphorylation status. Thus it is feasible to postulate that the phosphorylation level of one or more cytoskeletal proteins may play an important role in the maintenance of erythrocyte morphology, and Adriamycin may inhibit echinocytosis by reducing the phosphorylation of some key component. Since changes in phosphorylation will inevitably cause a conformational change in the tertiary structure of a protein, the possibility exists that a threshold level of phosphorylation or phosphorylations at a specific site on a protein leads to the morphological transitions under conditions of calcium loading. Further

studies are required to clarify the role of phosphorylation changes in calcium-induced echinocytosis. This would include the study of changes in phosphorylation level of single proteins of the cytoskeleton at different phosphorylation sites. These would be facilitated by the recently developed peptide HPLC techniques reported in this thesis (see results section chapter seven).

Recently, it has been suggested that phosphorylation and dephosphorylation reactions in human erythrocytes may simply represent the remnants of reactions which took place during the course of erythrocyte maturation, in which phosphorylation may play an important role in the synthesis and assembly of the cytoskeletal elements (Backman 1988). However, no evidence is available to support this idea of the futile nature of the phosphorylation reactions of human erythrocytes. The hypothesis was based on the general failure of a decade or more of investigations which have sought to clarify the role of the phosphorylation of cytoskeletal proteins in erythrocytes. However, it is difficult to envisage that, during the course of evolution, erythrocytes would continue to utilise such a large proportion of intracellular ATP in order to phosphorylate such a large number of phosphoproteins. It is unreasonable to suggest this futile nature of the phosphorylation reactions of human erythrocyte, especially when most of the investigations on the erythrocyte cytoskeleton were actually performed under unfavourable conditions, as will be described below.

This thesis described (results section 3.3 & 3.5) the

development of a medium (medium B) so that human erythrocytes could be maintained in a metabolically active state, in discoidal morphology, with no cell lysis, for at least 6 hours. Using a similar medium for the isotopic labelling procedure with radiolabelled phosphate, it was revealed that there was an enhanced association of several phosphoproteins with the membrane ghosts, prepared as shown in figure 27 & 28. In contrast, when an isotonic medium (medium A), was used, which was similar to those commonly employed by previous investigators, significant cell lysis was observed after 3 hours of incubation and an echinocytic morphology was induced. Moreover, when erythrocytes which were isotopically labelled with medium B (in the presence of a physiological concentration of orthophosphate), were used to investigate the effects of the protein kinase C activator (TPA) on cytoskeletal phosphorylation, it was shown that phosphorylation was enhanced in many more proteins than had previously reported (Palfrey & Waseem 1985, Cohen & Foley 1986). In the present study, the dephosphorylation of cytoskeletal proteins was also observed after TPA treatment; this is a phenomenon which has not been reported previously. These results suggest that all previous investigations of the events associated with echinocytosis and other functions of various cytoskeletal proteins may have used experimental protocols and conditions such that the morphology and metabolism of the cells were not properly controlled. The erythrocyte suspensions used in these investigations might have suffered from premature echinocytosis, cell lysis, low

intracellular ATP levels and high intracellular calcium levels. In addition, incubation and treatment times were often prolonged unnecessarily such that early events, which may have primary importance in the processes under investigation, were missed, and were most certainly compromised by an on-going metabolic depletion.

This aspect of poor experimental design has not only been limited to investigations relating to human erythrocytes. In fact, many studies of phosphorylation reactions in other cell types have been performed under these conditions. Such experiments can be divided into two categories: firstly, preliminary studies of phosphorylation are often performed using partially isolated systems such as cell membranes or purified kinases; the complex interactions between various protein kinase systems and their counterbalance by various protein phosphatases, both present in the cytosol and cell membranes, has often been overlooked. It is possible that under non-physiological conditions, these interactions between proteins may be compromised.

Secondly, the explanations offered by many studies of phosphorylation which were performed on intact cells might be open to re-interpretation: these include the use of incubation media, including "phosphate free media", which cannot sustain normal cellular metabolism (eg. Palfrey & Waseem 1985, Cohen & Foley 1986). If cell density was too high during the labelling period, it is likely that cell viability was compromised during the early stage of the labelling period. In addition, the labelling time is often arbitrary, with

ranges from 15 minutes to 18 hours. In doing this, the state of isotopic equilibrium of both phosphoproteins and ATP is often neglected, resulting in the uneven labelling of proteins. Such investigations call for careful experimental design such that the protocols attempt to properly mimic physiological conditions.

Although the results obtained from this study do not allow conclusions to be drawn regarding the mechanism of the effects of Adriamycin which modulate red cell morphological change, the results obtained from both the studies of proteolysis and phosphorylation have provided the basis for further studies. In addition, other types of covalent modifications, such as protein methylation and fatty acid acylation of proteins, which were not investigated, could be usefully explored.

In the present study, morphological examinations were carried out in parallel with investigations of the biochemical changes which resulted from calcium loading. It was noted that, during calcium-induced echinocytosis at low calcium concentrations (10-200μM), morphological transitions gradually progressed from discocytes to stage I echinocytes, followed by stage II and stage III echinocytes to, finally, echinospherocytes. In the early stages of the transition process, particularly as stage I and stage II echinocytes were formed, it was noticed that the shape change was always initiated at the outer perimeter of the erythrocyte and then progressed inwards (see figure 46). It could be seen that the shape change was initially confined to a limited area, which

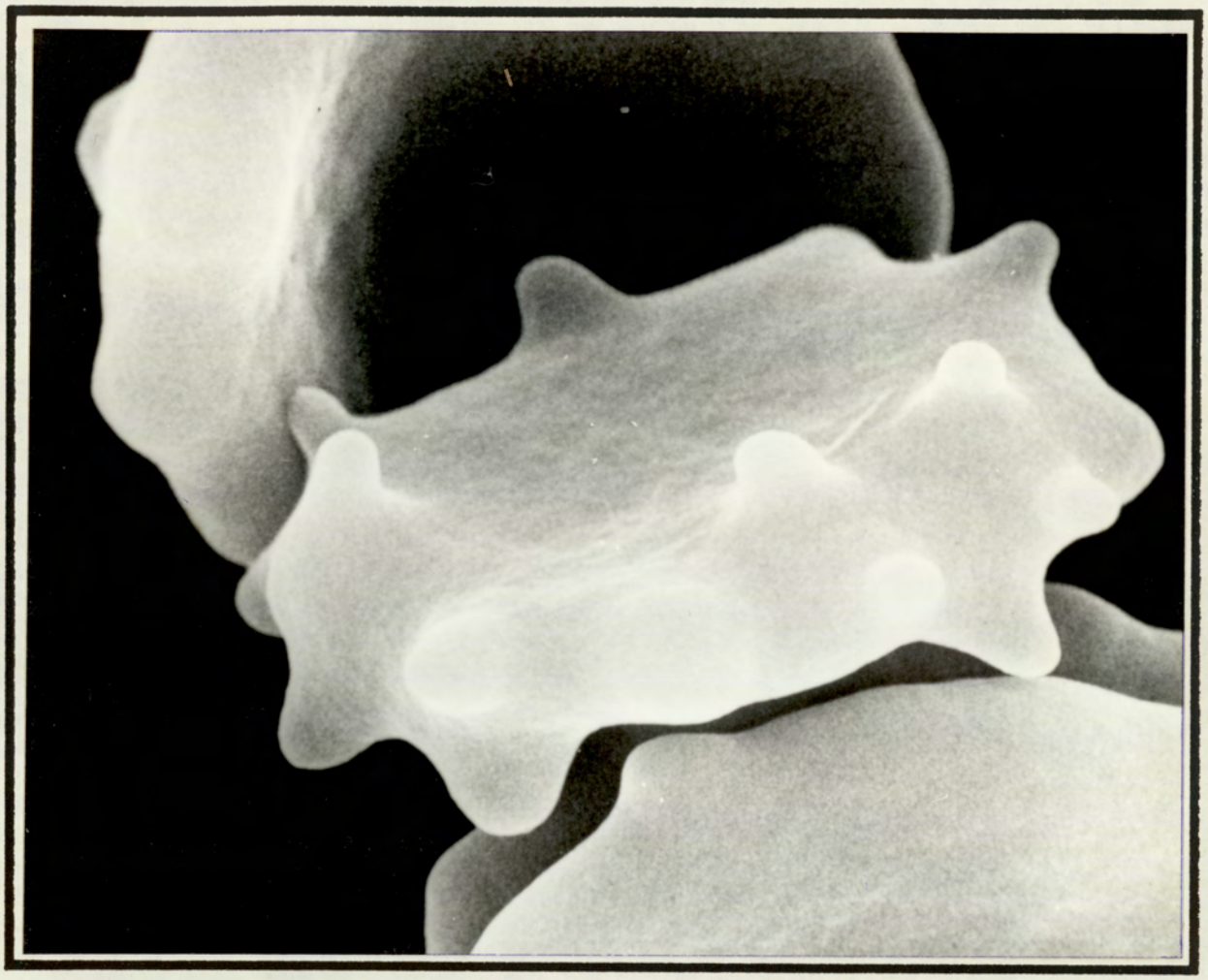

Figure.46 Illustration of the progression of echinocytosis.

progressed to form a spiky structure on the cell surface, followed by microvesiculation from these structures. It is difficult to explain this type of local effect, which suggests an element of structural polarity in the cell, simply by the invocation of the "bilayer couple" theory or by "mechanical" effects, as have been suggested by theoretical calculations which analyze the dynamic forces in the lipid bilayer (Brailsford et.al. 1976, Elgsaeter et.al. 1986). Such hypotheses do not appear to be able to accommodate the observed polarity of the transitions.

It has been discussed in the previous section that, under conditions described in this thesis, loading of human erythrocyte with calcium (<400μM) did not caused any significant changes in intracellular ATP level although extensive echinocytosis was observed (see result section). Although these results contradict reports from several investigators (eg. Weed et.al. 1969, Anderson & Lovrien 1981), several lines of evidence suggested the involvement of ATP in the underlying mechanism of the morphological transition process: firstly, isolated erythrocyte membrane ghosts, either treated with calcium or prepared from ATP-depleted cells, resulted in a dramatic decrease in deformability (Weed et.al. 1969), which could be reversed by treatment with ATP, EDTA or magnesium ions. Although the authors suggest these agents worked by reducing membrane associated calcium ions by chelation and mass action competition, the ineffectiveness of other nucleoside triphosphates suggested a specific effect of ATP.

It has been proposed that a contractile system is present in human erythrocytes (Patel & Fairbanks 1981, Fowler & Bennett 1984, Fowler et.al. 1985, Wong et.al. 1985, Patel & Fairbanks 1986, Matovcik et.al. 1986, Fowler 1987) since significant actin-activated ATPase activity has been demonstrated in human erythrocyte ghost preparations (Schrier et.al. 1981, Schrier et.al. 1986, Matovcik et.al. 1986). Recently, myosin has been identified as being associated with membrane ghosts prepared from human erythrocytes (Fowler et.al. 1985, Wong et.al. 1985, Matovcik et.al. 1986). Various other accessary proteins of a possible contractile system, including tropomyosin (Fowler & Bennett 1984) and a tropomyosin binding protein (Fowler 1987), have also been identified in human erythrocytes.

The above findings suggest that the human erythrocyte possesses a complete contractile system, which is operative in the vicinity of the membrane/cytoskeleton. It is therefore possible to propose that an actin-mediated contractile system may be a candidate for one of the mechanisms for the initiation of echinocytosis as a result of calcium-loading or ATP-depletion. As shown in figure 46, it could be envisaged that the localized change in the membrane structure, seen in the early stages of echinocytosis, could be brought about by a localized relaxation or contraction of a contractile system. It has been reported that human erythrocyte myosin was found associated with the membrane ghosts or membrane associated proteins but not with the cytoskeleton or a "triton shell" (Matovcik et.al. 1986). This may be compatible with a model

in which the myosin molecules, which are anchored or associated with the membrane, interact with the short actin filaments in the cytoskeleton, which are associated with tropomyosin, tropomyosin binding proteins, and band 4.9. Since the discoidal morphology allows the two separate area of plasma membrane to become adjacent to each other, near the periphery of the cell, then a limited 3-dimensional cytoskeletal structure, accommodating this contractile system, in these areas is possible. Contraction or relaxation of myosin and actin filaments would result in a change of the relative distance between the cytoskeleton and the plasma membrane. These changes might eventually bring about the observed morphological transitions. Such a contractile system would allow the imposition of intracellular controls, such as calcium-induced phosphorylation of myosin light chain, or of other accessary proteins as in other cell types. These activities may be able to accommodate the observations by Jinbu (Jinbu 1984a, Jinbu 1984b) that isolated "triton shells" exhibited MgATP-dependent expansion that reversed the shrinkage induced by calcium. Moreover, it was recently shown that the cytoskeletal ATPase, which could be released by extraction with ATP, was stimulated in the presence of spectrin and it was inhibited by Phalloidin and DNase 1, which inhibit actin (Sato et.al. 1986).

Actin purified from human erythrocyte has been shown to be distinct from the actin present in other cell types, since it possesses a greater capacity to activate an erythrocyte membrane actin-activated MgATPase than other actin isoforms

(Schrier et.al. 1981). Erythrocyte actin was found to arrange into regular oligomers, each consisting of 10-12 actin monomers, in contrast to the long F-actin filaments which were found in other cell types. Preliminary studies were performed by me to investigate possible alterations of the polymerization state of erythrocyte actin: human erythrocytes were loaded with various concentrations of calcium, such that morphology ranged from discocyte to echino-spherocyte were observed. The glutaraldehyde fixed cells were permeabilized and actin filaments were labelled with TRITC-phalloidin. This was followed by an attempt at quantitation using flow cytometry. The results of these preliminary experiments (not shown) indicated that there was no change in the degree of actin polymerization during the course of echinocytosis induced by calcium loading. However, there was also the possibility of actin filament rearrangements, which result from alterations in filament length. Indeed, it was recently reported that band 4.9, which is an actin bundling protein, reduced its actin bundling activity as a result of increased phosphorylation (Husain-Chishti et.al. 1988). The lowering of the actin-bundling activity resulted in the formation of actin oligomers containing 23 ± 5 actin monomers compared with 12 ± 1.5 monomers observed in normal cells. Since phosphorylation of band 4.9 was found in my studies to be increased as a result of calcium loading (figure 33b), it would be interesting to measure the polymeric state of the actin filaments in calcium loaded cells, and to observe the effects of Adriamycin on this process.

The loci at which Adriamycin exerts its action are, as yet, unidentified. It is shown in this study that Adriamycin was able to protect band 2.1 from calcium-induced proteolysis and to lower the basal phosphorylation level of erythrocyte cytoskeletal proteins, but there was no clear correlation between these effects and its ability in protect human erythrocyte against calcium-induced echinocytosis. Only the changes in overall protein phosphorylation appear to offer a clue to its mechanism. The proposal of a contractile system gives rise to another possible site for drug interaction. A recent report has shown that Adriamycin was able to interfere with actin polymerization by reducing the polymer size (Colombo & Milzani 1988). This observation may be compatible with the above proposal that if the size of actin filaments were increased as a consequence of band 4.9 phosphorylation during calcium-loading, this effect could be counteracted by pretreating the cells with Adriamycin, so as to prevent the onset of echinocytosis. This hypotheses remain speculative at this stage, and awaits further experimentation.

CHAPTER FIVE

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