THE ROLE OF TRANSGLUTAMINASE IN CELLULAR FUNCTION

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DECLARATION

This work has not been accepted in substance for any other degree, and is not concurrently being submitted in candidature for any other degree.

This is to certify that the work here was carried out by the candidate himself. Due acknowledgement has been made of all assistance received.

Signed .....R.A.Barnes.....

(Candidate)

Signed .....Martin Griffin.....

(Director of Studies)

TO MY WIFE

AND MY PARENTS

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ABSTRACT

The role of transglutaminase in cellular function

R.N. Barnes, 1980

Rat liver transglutaminase was purified (414-fold) and demonstrated to be a calcium - and sulphydryl - dependent, non-zymogenic enzyme with a molecular weight of 70000 ± 5000 and a K_m, app, for putrescine incorporation into N,N'dimethyl casein, of 5.4×10^{-4}M. This is the first literature report of a purification of this enzyme.

The enzyme sub-cellular localisation was demonstrated as a 3:2 distribution between the particulate-free supernatant and particulate (600g nuclear-membranous) fractions respectively. The plasma membrane was closely associated with the enzyme in terms of both locale and source of substrate protein. Actin was also shown to be a specific substrate during tissue slice experiments. The data indicated a role in the covalent incorporation of soluble substrates into, and consolidation of, protein aggregates.

The control of transglutaminase activity was shown to be mediated by a binding mechanism involving the particulate fraction, enzyme activation by a range of agents and the metabolic activity of the cell. Elution experiments demonstrated an enzyme reserve which was capable of translocation between the particulate (600g) and soluble cell fractions. This elution was achieved to varying degrees by the use of buffers involving electrostatic, hydrophobic, apolar and chaotropic interactions. Polyamines in conjunction with calcium completely reversed this finding resulting in loss of soluble enzyme and a reduced enzyme activity.

Studies involving model systems of foetal, hepatomegalic and regenerating liver demonstrate the enzyme to be repressed during proliferative growth. Restoration of full activity occurred during cell differentiation and especially maturation.

Platelet aggregation by various agonists was shown to induce insolubilisation of the transglutaminase activity, which may explain how this cell retains the enzyme during the release reaction.

Investigations of rat sarcomata indicate that transglutaminase may have a function in the metastasis of neoplastic tissues.

FIGURES AND TABLES

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1.0 INTRODUCTION

Functional protein units in many cases require the formation of covalent bonds subsequent to polypeptide chain assembly. The major role of these bonds, both within and between molecules, lie in maintaining gross structure and limiting extensibility. Many forms of covalent crosslink have been postulated in protein structure but, to date, direct evidence is available for only three types. These are the disulphide bridge (Friedman, 1973; Fluharty, 1974), the aldol and aldimide bonds (Feeney et al, 1975) and the $\varepsilon(\gamma$-glutamyl) lysine crosslink.

The transglutaminases, a group of transamidating enzymes, catalyse the formation of $\varepsilon(\gamma$-glutamyl) lysine dipeptide crosslinks resulting in the post-translational modification of some native proteins. The enzymes and their products have been the subject of a number of reviews (Lorand and Stenberg, 1976; Goldsmith, 1977; Folk and Finlayson, 1977).

Functional roles have been assigned to certain members of the transglutaminase family but in many cases, and one in particular, the ubiquitous "tissue" enzyme, the physiological role and mechanism of the control of its function have yet to be elucidated. At present the cellular aspects of the transglutaminases are poorly understood despite a wealth of biophysical data accumulated for a few extensively studied examples.

1.1.1 Reaction Mechanism

These enzymes may be described as thiol-mediated, Ca^{2+}-dependent acyl transferases (Folk and Chung, 1973). Only γ-carboxamide groups of peptide-bound glutamine residues act as acyl donors whereas a

variety of amine compounds may suffice as acceptors. When the ε-amino group of peptide-bound lysine is the acceptor the product is the monosubstituted γ-amide of peptide-bound glutamic acid. Other lysine analogues, and suitable primary amines, may be utilised as substrates, and in the absence of these, a hydrolytic reaction may take place to give the free γ-carboxy functions. These reactions are illustrated in Figure 1.1. The reaction has been proposed as a modified double displacement mechanism (Folk, 1969; Folk and Chung, 1973) in which three macromolecules (two protein substrates and the enzyme) come into contact in a highly ordered fashion. This situation is well illustrated by the action of various transglutaminases on fibrin and fibrinogen. Electrophoretic analysis of the products of catalysis of various transglutaminases, reveals different patterns of polypeptide crosslinking and the positions of ε(γ-glutamyl) lysine crosslinks, showing that enzyme specificity and protein conformation each play a part in the enzyme activity (Chung, 1972).

1.1.2 Nomenclature

The Enzyme Commission has recently assigned the transglutaminases the notation 2.3.2.13. The name, transglutaminase, was first coined by Waelsch and his co-workers (Mycek et al, 1959) in order to differentiate this group of enzymes from others performing catalysis of a similar kind upon free glutamine. Although descriptively inaccurate common usage has determined longevity. The accurate nomenclature, though less endearing, should read endo-γ-glutamine:ε-lysine transferase.

The enzyme originally described by Waelsch and co-workers was isolated from guinea pig liver (Clarke et al, 1959) and was often

referred to as "liver transglutaminase". However subsequent identification of similar enzymes from other tissues forced the term "tissue transglutaminase" to be used as the collective noun.

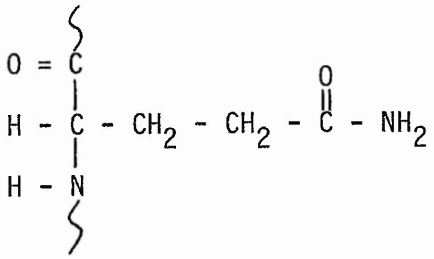
Zymogenic or protransglutaminases which may be found in various tissues and body fluids are collectively referred to as Factor XIII by workers in the blood coagulation field, the active enzyme being designated Factor XIIIa. The nomenclature was adopted by the International Committee on Blood Clotting Factors in 1963 (de Vreker, 1964) to clarify their role in blood coagulation.

1.1.3 Historical Significance

The recognition that proteins might be connected in a tertiary structure consisting of intermolecular linkages via glutamyl and lysyl residue was to take nearly fifty years. The concept of glutamic acid and lysine residues being interactive was initiated by Fischer (1906) but it was not until much later that Pauling and Niemann (1939) suggested amide linkages may contribute to conformational stability. Soon after, Mosiman and Singer (1964), using gelatin as a model, proposed that a "net structure" of intermolecularly linked polypeptides may be formed which conferred stability to an otherwise amorphous assembly.

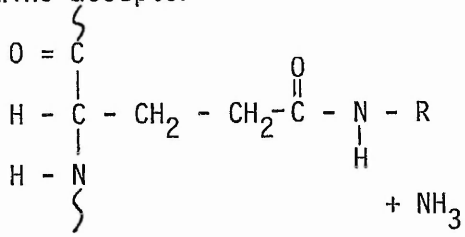
The observation that the uptake of labelled lysine into guinea pig liver homogenate was anomolously high in comparison to other amino acids was first reported by Borsook et al (1949). Since then linkages via the ε-lysine residues have been characterised in collagen (Mechanic and Loewy, 1959).

The subsequent identification of the crosslinks has posed a number of questions of technique and interpretation (see review Stevens et al,



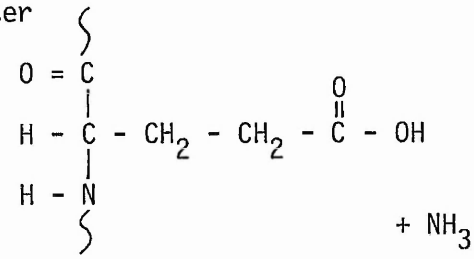
peptide bound glutamine (donor)

(i) + ε amino acceptor



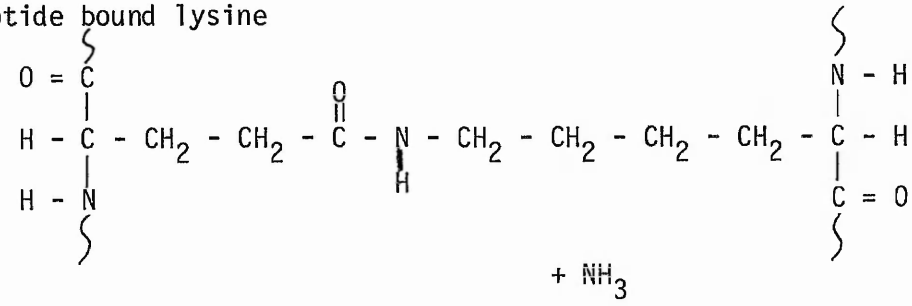
R = aliphatic or aromatic side chain

(ii) + water



peptide bound glutamic acid

(iii) + peptide bound lysine



ε(γ-glutamyl) lysine crosslink

(Wavy line denotes poly peptide backbone)

Figure 1.1 General Reaction Mechanism

1972). The demonstration of protein crosslinks involving lysine and glutamine residues evolved from the work of Schweet (1955,1956) on Ca^{2+}-dependent incorporation of [^{14}C-]lysine into guinea pig liver soluble fraction. The evidence for amide-group replacement on

Ca^{2+}-dependent incorporation of [^{14}C-]lysine into guinea pig liver glutamine residues by amine-containing moieties was strengthened when ammonia was detected as a reaction product (Clarke et al, 1957). Later, Neidle et al (1958) demonstrated that the γ-carboxamide groups of glutamine were the source of the ammonia, and furthermore, some of the lysyl side chains could be protected from nitrous acid deamination by initial incubation with liver transglutaminase.

Early work using guinea pig liver enzyme revealed that a variety of amines (Clarke et al, 1959; Clarke et al, 1957) could become incorporated into a variety of proteins (Mycek et al, 1959; Clarke et al, 1959).

The final confirmation of this crosslink was the isolation of ε(γ-glutamyl) lysine, which was obtained from crosslinked fibrin following exhaustive enzymic hydrolysis (Matacić and Loewy, 1968; Pisano et al, 1968) and has since been extended to other proteins.

1.1.4 Occurence of Transglutaminase Activity

The observation of catalytic activity in a particular system may rest on a number of different experimental approaches. The evidence may be produced by a simple transglutaminase enzyme assay, or more critically from a demonstration of protein crosslinking as in the analysis of fibrin after enzyme action. However, the most definitive proof remains the isolation of the ε(γ-glutamyl) lysine dipeptide.

Loewy et al (1971) have asserted that ε(γ-glutamyl) lysine linkage occurs universally in living systems. Whilst at present

this assertion may require some qualification, the presence of the crosslink has been described in an impressive array of eukaryotic and prokaryotic material.

Investigations based on crosslink analysis have demonstrated its presence in the plasmodium of Physarum polycephalum, in Paramecium, Escherichia coli and T_4 bacteriophage (Loewy et al, 1971). Various authors have also described crosslinking in fibrin in 45 species (7 classes) of vertebrate, in birds, reptiles, amphibians, bony fish, cartilaginous fish and even in the lamphrey (Schwartz et al, 1973b; Ciernewski et al, 1975; Murtaugh et al, 1973, 1974). Harding and Rogers (1971,1972b) have shown the crosslink to be present in the hair (or quills) of 10 different species encompassing monotremes, marsupials and placental mammals.

The occurence of the crosslink in tissues lies predominantly in body fluids and epithelia. Blood (as the source of plasma, platelet and erythrocyte transglutaminases) and seminal fluid have been extensively studied and these enzymes will be described later. Bovine colostrum (Klostermeyer et al, 1976) and microporulent sputum (Barton and Lourenco, 1971) have also been proposed as sites of crosslink localisation. Epithelial distribution has been demonstrated in wool (Asquith et al, 1970) hair, quill and inner root sheath of hair follicles (Harding and Rogers, 1971), hair-free cow snout epidermis (Goldsmith et al, 1974) and rabbit skin collagen (Stevens et al, 1972).

It has been proposed that the crosslinked proteins are more abundant in membranes and coverings. Examination of the subcellular fractions of cultured "L" cells (Birkbichler et al, 1973) for the presence of the crosslink have shown plasma membrane and endoplasmic reticulum to be the major sites of distribution. The crosslink has also

been identified from the capsule of whelk eggs (Price and Hunt, 1973). The presence of the crosslink in fibrous and membranous structures could conceivably exert a stabilising influence, however Birchbichler et al (1973) have observed that an extensive propogated system of crosslinking does not exist in membranes. They surmised that the linkages were intramolecular and connected closely related sites of polypeptides. In view of the lack of definitive evidence on the function of the crosslink in tissues much of the work done has been directed toward defining the function in individual proteins.

Difficulties may arise when interpreting transglutaminase function based on enzyme activity measurement alone. Chung (1972) in a survey involving 11 guinea pig organs discovered that activity in liver and spleen was over a thousand fold greater than in the muscle. However, to date, evidence of crosslink can only be found in muscle whereas the liver has no crosslink detectable by current techniques (Loewy et al, 1971). In the same investigation Chung (1972), using human tissues, found the distribution of enzyme was quite different to that of the guinea pig. In this case the liver and spleen contained very little activity and the highest levels were found in lung and uterus.

This survey of transglutaminase activity and its product, the $\varepsilon(\gamma$-glutamyl) lysine crosslink, has given an indication of the widespread occurence of the enzymes in organisms and their tissues. However, only limited information may be ascribed to the function of these enzymes at the cellular level. Extensive studies have been carried out on the family of transglutaminase enzymes resulting in a wealth of biochemical and biophysical data. Attempts to identify the substrate proteins of these enzymes (which will be dealt with in

detail in a later section) has permitted functional roles to be assigned in a few cases. However, the position in cellular physiology for the majority of the transglutaminases, and in particular the "tissue" enzyme, remains unclear.

1.2 Classification of Transglutaminase Enzymes

The transglutaminases have been subdivided into three distinct groups:

1.2.1 Protransglutaminases

1.2.1.1 Plasma Factor XIII

Plasma factor XIII (Folk and Chung, 1975) may be termed a pro-transglutaminase as it exists in plasma as an inactive zymogen. The enzyme has a molecular weight of approximately 300,000 and is composed of two identical 'a' chains (mol.wt. 75,000) containing the catalytic sites and two non-catalytic 'b' chains (mol.wt. 80,000) (Schwartz et al, 1971b, 1973a; Tagaki and Konishi, 1972; Chung, 1975). The sub-unit structure of the molecule may be designated a_2b_2.

Robbins (1944) first recorded the presence of the enzyme when he found that fibrinogen in the presence of thrombin, calcium and fresh plasma produced an insoluble clot when compared to a control system without plasma. Later, Laki and Lorand (1948) confirmed the presence of this plasma factor. Synonyms for this enzyme in common usage included fibrin-stabilising factor, fibrinoligase, fibrinase and Laki-Lorand factor. Evidence which has now accumulated, particularly from the isolation of the enzyme (Loewy et al, 1961) and from studies of factor XIII deficiency diseases (Duckert et al, 1960) led to its official recognition as the clotting factor XIII in 1968 (Duckert and Beck, 1968).

The activation of the zymogen initially involves the proteolytic cleavage of a 4000 mol.wt. peptide (39 amino acid) from the NH_2-terminal end of the 'a' chain (Schwartz, 1971b; Takagi and Doolittle, 1974). The structure is then designated $a_2'b_2$. Thrombin has been

shown to be the physiological activator of factor XIII (Buluk, 1961; Lorand and Konishi, 1964) although trypsin and papain have also been shown to activate this enzyme (Copec et al, 1969).

The presence of calcium ions in the plasma ensures the dissociation of the subunit structure into a_2' and b_2 dimers and the unmasking of the active site-SH group in the a' subunit (Lorand et al, 1974; Curtis et al, 1974a). Calcium ions have no effect on the original a_2b_2 conformation (Lorand et al, 1974) and may be replaced by other group 2a cations, though with much lower activity, in the order of effectiveness $Ca^{2+}>>Sr^{2+}>Ba^{2+}>Mg^{2+}$ (Curtis et al, 1974b). The function of the non-catalytic 'b' subunits at this stage has been the subject of a number of theories. Their possible role in promoting zymogen secretion (Schwartz, 1971b) and the prevention of catabolism of the active enzyme (Cooke et al, 1974) have proved popular. Also, evidence for a role in governing the rate of thrombin proteolysis (Chung, 1975) and in stabilising 'a' chains in plasma (Lee and Curtis, 1976) has gained acceptance.

The presence of a thiol residue at the active site was demonstrated after reports of enzyme inactivation by agents such as iodoacetamide, Ag^{2+} and p-mercuribenzoate (Loewy et al, 1961) in the presence of calcium ions. When labelled iodoacetamide was used all the incorporated radioactivity was present in the 'a' chains in the form of cysteine derivatives (Chung, 1975; Curtis et al, 1973; Holbrook et al, 1973).

The activation sequence is illustrated in Figure 1.2.

Mapping of the amino acid residues present at the active site of factor XIII was carried out by Holbrook et al (1973) and was shown to

be;

- Tyr - Gly - Gln - Cys - Trp -

This sequence was also shown to be identical to that found in the liver transglutaminase from guinea pigs (Folk and Cole, 1966a). A series of pseudo-substrates consisting of aliphatic amines were used to provide evidence for a common spatial arrangement about the glutamine binding site (Gross et al, 1973). However in general terms the pseudo-substrates have proved to be inferior to native proteins such as glucagon, acetylated B-chain of insulin (Folk and Chung, 1973), caseins and fibrin. Many amines have been investigated for their ability to inhibit fibrin crosslinking (Lorand et al, 1968b; Lorand and Ong, 1966). By varing R_1, R_2, R_3, X and n in the general formular;

$$R_1 - X - (CH_2)n - N \begin{matrix} R_2 \\ \diagdown \\ \diagup \\ R_3 \end{matrix}$$

Amines of the type Aryl-SO_2NH-$(CH_2)_5$ NH_2 were shown to be the most effective inhibitors of fibrin crosslinking by factor XIII (Nilsson et al, 1972). Primary amines and their derivatives are used routinely in a number of assays for factor XIII and other transglutaminases. Investigations of acceptor compounds have led to three classes being examined in depth, the amides, p-nitrophenylesters and the thiolesters. The data has been reviewed by Folk and Chung (1973) and the findings suggest the β-phenylpropionyl residue fits optimally into the structure of the enzyme.

The physiological role of factor XIIIa has been well documented particularly regarding its role in fibrin crosslinking (Losowsky and Miloszewski, 1977; Mandel, 1971; Bohn, 1978). Factor XIIIa catalyses the final stage in the blood clotting cascade (Figure 1.3) in which

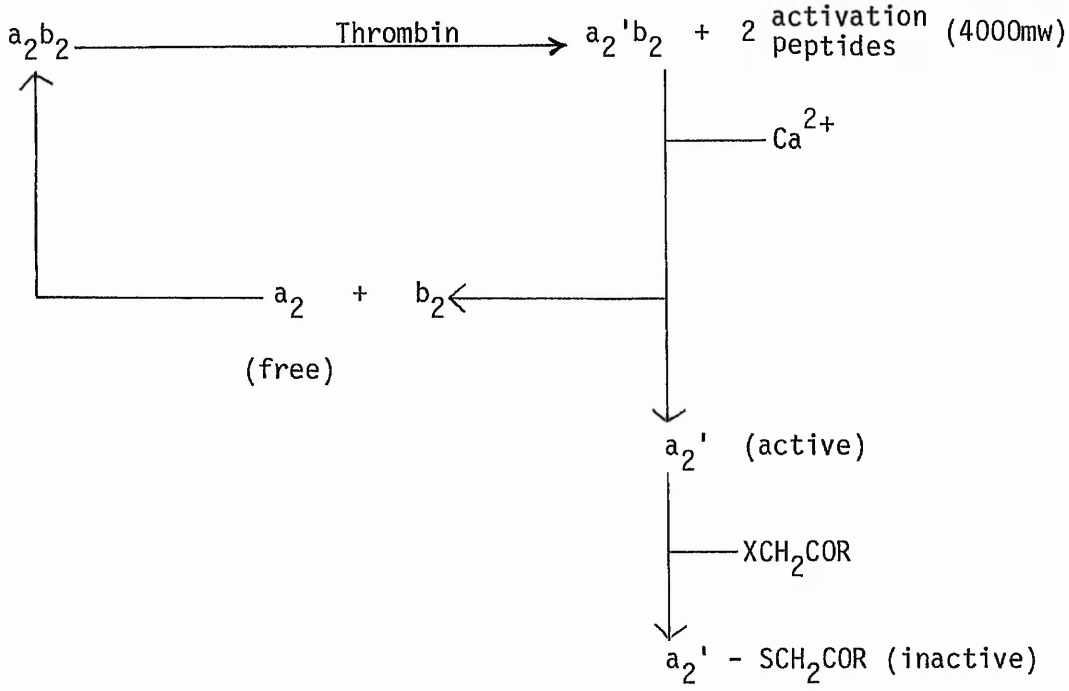


Figure 1.2 Schematic representation of plasma factor XIII activation and interactions

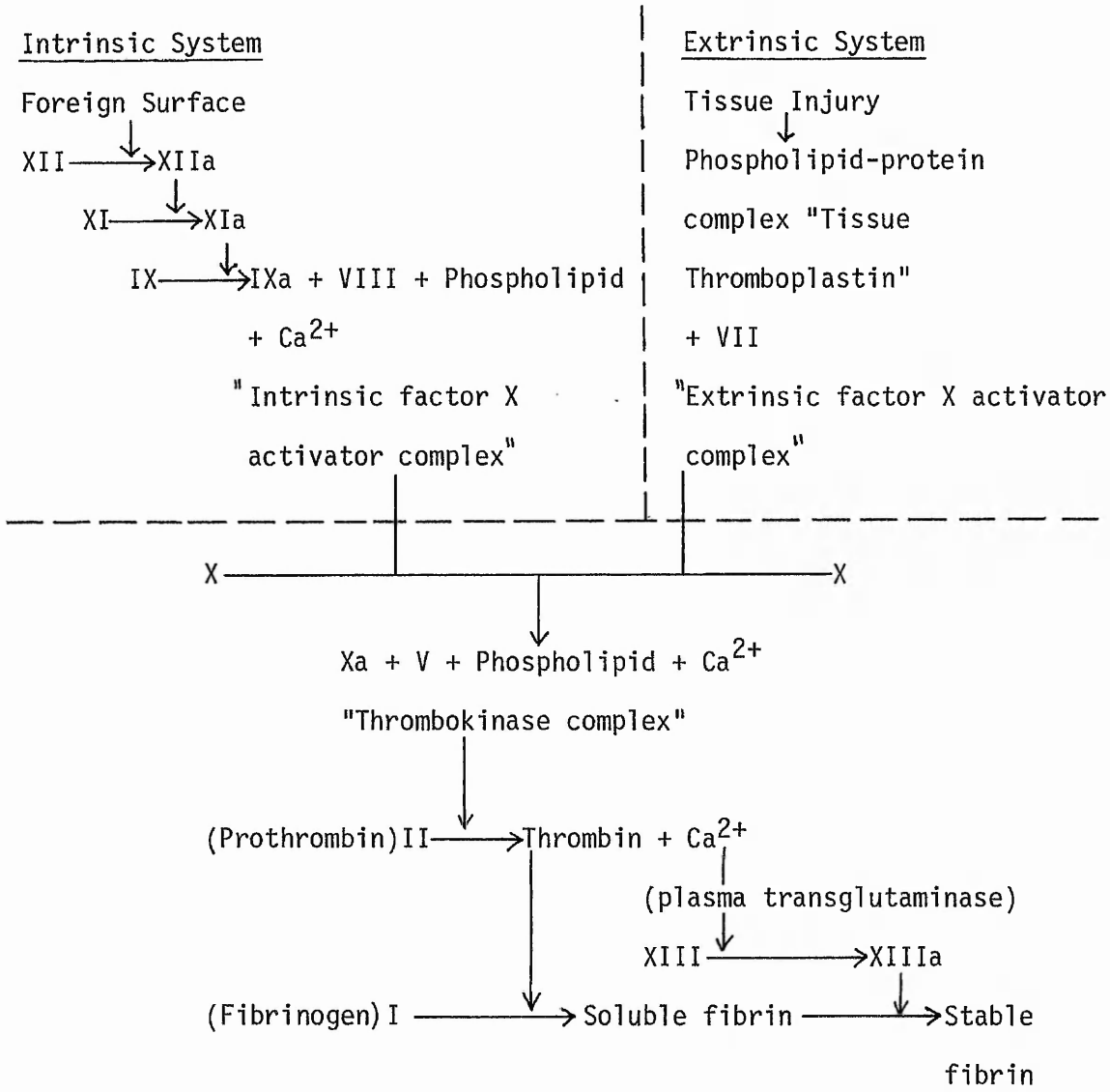


Figure 1.3 Scheme of coagulation system

Legend 'a' denotes the 'activated' form of the coagulation factor.

which soluble fibrin monomers, formed from fibrinogen by the action of thrombin, polymerise and are rendered stable by inter- and intra-molecular crosslinking. This process of fibrin stabilisation is essential for normal haemostasis as the resultant fibrin clots are mechanically stronger and more resistant to plasmin degradation. An indication of the potency of factor XIII in plasma may be illustrated using subjects with factor XIII deficiencies (Mandel, 1971). Normal haemostasis can still occur when the plasma concentration of factor XIII is reduced to 1% of its original concentration (2mg/100ml in man (Bohn et al, 1973)). When a congenital absence of factor XIII occurs (Greenberg et al, 1969) it is often as an autosomal recessive trait (Ratnoff and Steinberg, 1972) which manifests itself in the production of weak clots with a high susceptibility to proteolytic enzymes. The resultant poor haemostasis may be lethal but treatment by replacement techniques give excellent results (Losowsky and Miloszewski 1977).

Acquired abnormalities of factor XIII may occur following liver and renal diseases (Green, 1977), certain malignancies including leukemias and lymphomas (Nussbaum and Morse, 1964), disseminated intravascular coagulation (Miloszewski et al, 1974) and following major operations (Hosenfeld and Kaiser, 1969; Gerhake et al, 1970). These cases are normally without haemorrhagic manifestations, except when additional defects occur such as thrombocytopenia (Rasche et al, 1972). A small number of patients have been described with acquired inhibitors of factor XIII (Lewis, 1972; Lorand, 1972) which can, when present in great excess, produce severe symptoms comparable to the hereditary defect.

Observations of patients with factor XIII deficiency and the *in vitro* demonstration of collagen as a natural substrate for factor XIIIa (Nyman and Duckert, 1975) has led to speculation on the possible role of the enzyme in wound healing (Beck *et al*, 1961). This subject will be dealt with in greater depth in a later section.

1.2.1.2 Platelet Factor XIII

The presence of a clot stabilising substance in platelets has been recognised since 1955 (Buluk, 1955) and originally this activity was thought to be due to the plasma factor XIII. Subsequent studies during replacement therapy for plasma factor XIII showed that this enzyme did not penetrate the intact platelet membrane. Furthermore, it was found that during the platelet release reaction the transglutaminase activity remained within the platelet (Tsukada, 1977). These studies led Loewy (1972) to conclude that the platelet zymogen is a true platelet component which originates in the megakaryocyte (Kiesselbach and Wagner, 1972).

Determinations of molecular weight and subunit structure (Schwartz *et al*, 1971, 1973; Chung, 1975) have shown that the platelet zymogen consists of two 'a' subunits which have similar activation properties and reaction kinetics to the plasma enzyme.

The activity present within platelets represents about 50% of the total enzyme potential in blood (McDonagh *et al*, 1979). Furthermore it has been shown that the platelet enzyme a_2 subunits are capable of binding to free 'b' subunits, available from the plasma, with the consequent formation of a plasma-like zymogen (a_2b_2). It has been suggested that platelets may be the primary source of catalytic subunits for plasma factor XIII (Rider *et al*, 1976).

The role of platelets in haemostasis is complex. During the release reaction platelets secrete adenosine nucleotides (particularly ADP), 5-hydroxytryptamine (serotonin) and platelet factor 4 into the plasma in order to facilitate coagulation. The ADP and extracellular thrombin both stimulate aggregation of platelets (Hovig et al 1968) which then consolidate in the fibrin network and are later instrumental in clot retraction. Activation of platelets may also result from exposure to subendothelial basal laminae during vascular damage (Baumgartner and Haudenschild, 1972), collagen (Mason et al, 1976) and fibrin, particularly during the latters intermediate stages of its formation from fibrinogen (Niewiarowski et al, 1972). There are many other platelet agonists including platelet aggregation factor, the thromboxanes especially TxB_2, arachidonic acid etc but the previous protein examples are also transglutaminase substrates. This is particularly true in the case of fibrin where the platelet pseudopodia form a close association with fibrin strands. Clot reaction via the contractile proteins, particularly thrombosthenin, results in the consolidation of the haemostatic plug by the mechanical forces applied throughout the fibrin mesh (Sokal, 1960).

The presence of a further transglutaminase of the 'tissue' type was demonstrated by McDonagh et al (1972) and later confirmed by Tsukada (1977). The roles andpossible interrelation of activities of the platelet factor XIII and 'tissue' type enzyme require further investigation before a clear understanding of their functions may be reached.

1.2.1.3 Prostate Protransglutaminase

There exist in the prostate glands of humans and rodents (guinea pig, rat) two forms of transglutaminase, one present as a zymogen and

the other present in the active "tissue enzyme" form. The zymogenic enzyme is immunochemically indistinguishable from the platelet factor XIII a_2 conformation (Folk and Finlayson, 1977).

The role of the enzyme system in humans is poorly understood but in rodents the function has been more clearly demonstrated. The enzyme action on a seminal substrate produces a strongly stabilised post-copulation vaginal plug which prevents sperm loss and increases the likelihood of a successful fertilisation. The plug has been shown to possess the highest density of crosslinks per mole of any protein so far measured. At present it is not clear whether only one or both enzymes are active during the plug formation but it has been shown (Wing et al, 1974) that the tissue enzyme is definitely involved.

1.2.1.4 Placental Protransglutaminase

This zymogenic transglutaminase present in placenta and uterus is immunologically similar to the platelet factor XIII a_2 conformation (Bohn, 1970; Bohn and Schwick, 1971). The a_2 subunits will combine with free 'b' subunits present in plasma to form the a_2b_2 configuration (Schwartz et al, 1973) and molecular weight determinations of the 'a' polypeptides (76000 ± 4000 mol.wt.) show good agreement with the platelet enzyme (Chung and Folk, 1975). The function of the enzyme in this tissue is not understood.

1.2.2 Hair-Follicle and Epidermal Transglutaminases

1.2.2.1 Hair-Follicle Transglutaminase

Two forms of transglutaminase have been isolated from the hair-follicles of guinea pig (Chung and Folk, 1972b). The first is indistinguishable from the "tissue" enzyme and a second which is specific for this tissue. This unique hair-follicle transglutaminase

has been the subject of numerous reviews (Goldsmith, 1977; Folk and Chung, 1973).

The enzyme has been shown to have a molecular weight of 54000 consisting of two identical subunits each of molecular weight 27000. Studies by Harding and Rogers (1972a) using homogenates of hair-follicle from guinea pig, rat and sheep follicles have demonstrated the incorporation of glycine ethyl ester into casein. Isolation of $\varepsilon(\gamma$-glutamyl) lysine crosslink in sheep wool was reported by Goldsmith (1977). The substrate in wool was found to be a peptide of molecular weight 5-8000 which contained high levels of citrulline. The presence of the dipeptide was restricted primarily to the medullary regions and inner root sheath (Harding and Rogers, 1971, 1976) where approximately 1 in 13 lysyl residues were crosslinked.

The function of this enzyme unique to hair-follicles apparently lies in the stabilisation of certain hair and wool proteins thus conferring greater structural strength.

This enzyme is unusual amongst the transglutaminases for two reasons:

(i) The enzyme conformation, a dimeric structure consisting of 2 subunits each of molecular weight 27000, is unique to the transglutaminases so far characterised.

(ii) The enzyme is not inhibited by calcium chelating agents. As stated earlier the transglutaminase enzymes have an absolute requirement for divalent cations of which calcium is by far the most effective. Harding and Rodgers (1972a) reported that EDTA does not inhibit the enzyme and indicated that the removal of calcium produced a slight increase in activity.

These findings, anomalous in comparison to the other transglutaminases, have yet to be fully explained.

1.2.2.2 Epidermal Transglutaminase

Hair-follicle free epidermis of several vertebrate species including frog, turtle, rat, ox and man have been reported to contain transglutaminase activity (Goldsmith , 1976). The enzyme characteristic of this tissue has a molecular weight of about 55000 (Goldsmith et al, 1974; Goldsmith and Martin, 1975; Ogawa and Goldsmith, 1976). Investigations into substrate proteins have provided information suggesting that a soluble protein (150000 mol.wt.) may become crosslinked to insoluble structural proteins and that these macromolecules bear a structural simularity to fibrinogen which is a substrate for all transglutaminase enzymes (Buxman et al, 1976). Further studies have also suggested that pre-keratin subunits may be crosslinked to form fibrous proteins (Abernathy et al, 1977). Also transglutaminase substrates have been shown to be precursors of the cell envelope (Goldsmith et al, 1974) particularly in human epidermal keratinocytes (Rice and Green, 1978).

Histochemical investigations using frozen sections incubated with the fluorescent pseudosubstrate, monodansyl cadaverine, identified epidermal enzyme activity in the upper malphighian and granular cells of bovine snout epidermis, granular cells of human epidermis and cells of the keratinizing zone of the inner sheath of bovine vibrissae and hair follicles (Buxman and Wuepper, 1978). These observations suggest that the transglutaminase functions in the later phase differentiation of the epidermis and hair follicle inner root sheath.

Bures and Goldsmith (1978) have shown that insoluble "bound"

enzyme in chicken epidermis may be solubilised using chaotropic agents (eg potassium thiocyanate). Previously the majority of transglutaminases research has centred on the soluble forms of the enzyme and little or no mention has been made to the insoluble state. This report of a "bound" enzyme pool is therefore of particular interest.

A number of investigators (Goldsmith et al, 1974; Buxman and Wuepper, 1975) have pointed out the similarities between epidermal and hair-follicle transglutaminases. Immunological comparison of enzymes from glabrous and vibrissal tissues of the cow have indicated that there are two separate enzymes (Buxman and Wuepper, 1976). Later studies now indicate that these two transglutaminases may in fact be isoenzymes (Buxman and Wuepper, 1978).

1.2.3 Tissue Transglutaminases

This general term is used to describe a group of related transglutaminases that exist in a very wide spectrum of organs and tissues (Chung, 1972). They are non-zymogenic and are immunologically distinct from epidermal and hair-follicle transglutaminases.

Amongst the several well characterised tissue enzymes have been the liver enzyme from guinea pig and rabbit (Abe et al, 1977) and erythrocyte transglutaminase (Brenner and Wold, 1978). An investigation by Chung (1972, 1975) into the enzyme content of organs from guinea pig (using the soluble cell fraction) revealed the enzyme to be present in all organs studied. Large differences in total enzyme activity were found with the highest activities in liver and spleen and the lowest in muscle, including, in descending order of activity, kidney, adrenals, heart, lung, intestine, testis, brain and pancreas. A similar study carried out upon human tissue revealed an entirely different pattern with the lung and uterus containing the highest

levels of activity and liver and spleen the lowest. All the enzymes examined were found to be inhibited in the presence of antiserum to the liver transglutaminase demonstrating an immunological similarity existing throughout the tissue enzymes.

A study of bovine spleen transglutaminase, in a partially purified form (Zuch et al, 1974), described the presence of a pro-enzyme of splenic origin. This group of workers could not show a non-zymogenic form, a result which is in conflict with the work of Chung (1972) and is as yet unsubstantiated.

1.2.3.1 Liver Transglutaminase

The guinea pig liver enzyme has been extensively studied and the information so derived may, at present, be considered as representative of virtually all the tissue enzyme sources so far investigated. The experimental investigations carried out in order to accomplish this dissertation were mainly performed with the tissue transglutaminase derived from rat liver. This enzyme source has previously been poorly studied and the information gained from the guinea pig literature was used as a reference.

The physical and chemical properties of the guinea pig liver enzyme have been the subject of many reviews (Folk and Chung, 1973; Lorand, 1976; Finalyson, 1977). The enzyme consists of a single chain monomeric structure with a molecular weight in the range 70000-90000. The presence of disulphide bonds and carbohydrate have not been detected. The enzyme protein contains 16-18 sulphydryl groups, a number of which are susceptible to suphydryl reagents and one of which is necessary for catalytic activity. Calcium ions are essential activators of the enzyme by causing the induction of conformational changes without affecting the gross size and structure

of the molecule. These conformation changes, due to the formation of a metal-enzyme complex, have been demonstrated immunologically (Fesus and Laki, 1977).

The specificity of the enzyme for, and the binding of, substrates glutamine and lysine has provided many interesting details about the enzyme active centre.

(i) Glutamine binding

Gross and Folk (1973a,b) proposed that a polypeptide containing a α-glutamine residue attaches at the active site in such a way that the β and γ methylene groups of the glutamine side chain are bound to a hydrophobic pocket on enzyme surface, and its carboxamide group is directed toward the thiol group at the active centre. This binding pocket assumes overall dimensions of approximately 5Å by 5Å at some stage of catalysis as a consequence of a substrate-induced conformational change, and its specificity is directed towards the α-configuration of glutamine. The use of pseudosubstrates (Gross and Folk, 1973a,b; Gross et al, 1975) has demonstrated that the glutamine substrates bind to the enzyme with their carboxamide side chains in the fully extended conformation.

(ii) Lysine binding

Recent investigations by Gross et al (1977) and Schrode and Folk (1979) have presented evidence that the active sites of factor XIIIa and guinea pig liver transglutaminase possess the same stereochemistry. Based on observations from substrates and inhibitor studies they proposed that the amine

attachment site to the acyl intermediate was spatially restricted so that the uncharged amino group was positioned for reaction with the acyl-enzyme thiol-ester bond. The site, it was suggested, became more capacious as a function of distance from that region and appeared to be fashioned for the accommodation of the side chain of polypeptide bound α-lysine.

Lorand et al (1979) has reported in detail the three-dimensional aspects of the tissue enzyme active site. The active centre binding site was demonstrated to optimally accommodate an alkylamine side-chain of 5 methylene groups. In lysine the alkyl chain length of 7.5Å compares favourably with the 7.2Å measured for the commonly used pseudosubstrate, dansylcadaverine. Lorand et al (1976) have also shown a restriction with regard to cross-sectional dimensions that will allow a methylene group (2.8Å wide) but not a thiol residue (3.4Å) to enter the active site.

These results suggest that the amine binding site on the enzyme is a narrow crevice.

A second binding site has its significance in the hydrophobic locus where Lorand et al (1979) found that dansyl cadaverine had a 200-fold greater affinity for the enzyme than the non-hydrophobic n-butylamine. Many assays of transglutaminase rely on pseudo-substrates such as putrescine (1,4 diaminobutane, a low molecular weight aliphatic primary amine) but as previously described the enzyme has a very developed specificity toward peptide-bound lysine. In this way, since its natural substrates appear to be the best substrates, it can be said that the most efficient biological function

of the transglutaminases is the formation of the native ε(γ-glutamyl) lysine crosslinks.

Transglutaminase from rabbit liver has been investigated and has been found to be almost identical to the guinea pig liver enzyme in structure, amino acid composition and activity toward several substrates (Tyler and Laki, 1967; Abe et al, 1977). However, one instance exists where a difference in catalytic specificity has been shown i.e. the guinea pig liver enzyme will incorporate hydroxylamine into benzyloxycarbonyl-L-glutaminylglycine but this reaction does not occur with the rabbit enzyme (Abe et al, 1977).

1.2.3.2 Erythrocyte Transglutaminase

This intracellular enzyme identified in the red blood cells of humans and guinea pigs was first described by Divilansky (1970) and later by Chung and Folk (1975). More recent work by Brenner and Wold (1978) has fully characterised the enzyme.

The enzyme is a tissue transglutaminase requiring activation by calcium ions and is thiol-dependent. It has a molecular weight of 80000 ± 5000 and has catalytic properties essentially identical to the guinea pig liver transglutaminase. The enzyme is wholly intracellular and as such is not found free in plasma. When a tissue transglutaminase is experimentally introduced to the blood it is rapidly complexed to fibrinogen and is cleared from the circulation, to be later identifiable in the liver (Chung et al, 1971; Chung and Folk, 1975).

Lorand et al (1975) found that the intrinsic enzyme would catalyse the incorporation of monodansylcadaverine and putrescine into three major protein fractions, a high molecular weight protein possibly

spectrin, "band 3", a 88000 molecular weight polypeptide, and band 4.1. Fairbanks et al (1973) established the classification of erythrocyte membrane proteins. Lorand et al (1976) also reported that the erythrocyte enzyme was normally latent in the cell until a calcium concentration in excess of 0.5mM was reached. This regulatory activity corresponds to the calcium range which has been observed to precipitate physiologically significant alterations in erythrocyte shape, deformability and life span (Eaton et al, 1973; Palek, 1973).

Siefring et al (1978) have provided definitive proof of transglutaminase activity by the isolation of the γ-glutamyl-ε-lysine dipeptide from spectrin and band 3 proteins. They found about 10% of lysines were involved in dipeptide formation, and while this figure is less than the 30% given for seminal plasma (Willaims and Ashman, 1972) it is significantly higher than the 3% reported for fibrin (Matacic and Loewy, 1968). These results may go some way to explaining the phenomenon of Ca^{2+} related permanent alterations in red cell structure.

1.2.3.3 Tissue Transglutaminase as a Biochemical Tool

One practical adjunct to the academic approach to the study of these enzymes has been the realisation of the use of the tissue transglutaminases as a biochemical tool. Just as proteolytic enzymes have been widely employed as degradative structural probes (Mihalyi, 1972) the transglutaminases may be used for specific syntheses. Specific modification of substrate proteins have already yielded information on their structure and organisation.

The use of the transglutaminases for the covalent attachement of probes and/or labels has been described by Folk and Chung (1973)

and this approach has proved to have wide application. Brewer and Singer (1974) have labelled phage proteins whilst Dutton and Singer (1975) extended the work to membranes (mouse erythrocyte and muscle sarcoplasmic reticulum) using fluorescent probes. Fesus and Laki (1976) have labelled the surface proteins of malignant plasma cells with $[I^{125}]$-fibrinogen. Surface labelling of platelets by Okumura and Jamieson (1976) proved the method to be fifty times more efficient than the iodination (lactoperoxidase-catalysed) procedures previously employed. Dual labelling techniques incorporating probes both outside and within the cell have led to the identification of trans-membrane proteins by Evans and Fink (1977). Most recently Butler and Landon (1979) investigated the structure of cytochrome c and cytochrome c oxidase, with a view to the reactivity of the glutamine residues, using similar techniques.

One further example may be of use in the field of immobilised enzymes. The system of the immobilisation of enzymes onto inert supports at present requires chemical agents which are relatively severe in their effects on the enzymes in question. Although the results to date have proved disappointing Folk and Finlayson (1977) argue that this expanding area of study warrants particular attention.

A review of the use of transglutaminase in labelling experiments has recently been published (Iwanij, 1977).

1.3 Biological Significance

Folk and Finlayson (1977) have made the point that if crosslinking was to bestow a single feature to the interacting polymers then it would be "resistance". This term may be exemplified by:

(i) Resistance to deformation - as in the rigidity found in the haemostatic clot or copulation plug.

(ii) Resistance to breakage - conferred by high molecular weight aggregates.

(iii) Resistance to dissolution - where in the examples of hair and wool insolubility plays a direct functional role.

(iv) Resistance to chemical attack - particularly from proteolytic enzymes where Gormsen et al (1967) have shown that the degree of crosslinking of plasma clots is directly related to the resistance to fibrinolytic attack.

These properties may be related to more specific functions and pathological states in which their significance has been carefully evaluated.

1.3.1 Haemostasis

The definitive role of plasma factor XIII in haemostasis, although recognised as essential, has yet to be fully determined. The evidence from enzyme deficiency states has been previously discussed but the conclusions have been that although plasma factor XIII is critical, not all symptoms may be due to the lack of dipeptide formation. The most popular explanation is that factor XIII may be involved with the substrates in addition to fibrin.

Duckert (1972) proposed three roles for crosslinking in haemostasis (a) the production of a strong rigid clot; (b) a clot resistant to proteolysis; and (c) the support of tissue repair. At present the

conclusions which may be formulated are that the γ-crosslinking produces an insoluble matrix which forms a successful haemostatic plug (Ly, 1975). Whilst, more slowly, the α-polymerisation leads to the production of a stable long-term clot which is much more resistant to mechanical vascular forces and proteolytic degradation (Finlayson and Aronson, 1974). However this area is still subject to controversy over the possible roles of platelet factor XIII and erythrocyte transglutaminase and the uncertainties of the fibrin contribution to haemostatic control.

1.3.2 Fibrinogen Metabolism

Fibrinogen does not normally act as a substrate for factor XIII but evidence for a soluble fibrin-fibrinogen complex (cryoprofibrin) containing ε(γ-glutamyl) lysine dipeptide has been reported (Sasaki et al, 1966). Kanaide and Shainoff (1975) have shown that this mechanism may be useful in averting the formation of insoluble fibrin and hence its deposition which could lead to vascular damage and thrombus formation. The complexes are removed by the reticuloendothelial system (Shainoff and Sasaki, 1971) in which fibrinogen plays a major role.

1.3.3 Wound Healing

Duckert et al (1960) first reported poor wound healing and abnormal scarring in cases of factor XIII deficiency, thus linking the enzyme with tissue repair. In a series of experiments Beck et al (1961) suggested that a crosslinked fibrin support provided the basis for improved cell growth and collagen formation, as shown by fibroblast sub-cultures. Subsequent investigations have been lacking in conclusive proof, and the part played by dys- and afibrinogenemias in wound dehiscence (Menache, 1973) as a complicating feature. Work on animal models has provided substantial information. Biel et al (1971) treated

wounded guinea pigs with placental factor XIII and noted the diminished incidence of abnormal wound healing and a transient increase in the tensile strength of the healing tissue at the incision site. Most recently Knoche and Schmidt (1976) showed that a significant promotion of wound healing was evident, but at times in excess of 72hr post-operation. This recalls the work of Hosenfield and Kaiser (1969) who reported a post-surgical drop in factor XIII levels of about 40% between days 3 - 6 and suggested that this could be due to a factor XIII requirement in wound healing.

Again it must be considered that the substrates involved may not be restricted to fibrin. The action of factor XIII on collagen production and interactions with fibrin has been investigated (Soria et al, 1975; Nyman and Duckert, 1975) and may answer part of the question. The most promising aspect of this search lies with the possibility of fibronectin (cold-insoluble globulin) taking an active role. This protein, to be discussed in detail later, is a major cell surface protein which could serve as a link between neighbouring fibroblasts, as well as between developing fibroblasts and the organising fibrin network (Vaheri and Ruoslahti, 1975).

1.3.4 Tissue Integrity

The potential significance of the ε(γ-glutamyl) lysine dipeptide in maintaining integrity in tissues has been discussed at various points in this review. Although there is no dispute as to the importance of this function the rationale for its occurence may be different for different tissues.

The role of the ε(γ-glutamyl) lysine crosslink in the formation of hair, wool and quills may prove to be in stabilising the proteins during extrusion into the follicle, thus allowing alignment prior to

sulphydryl oxidation (Asquith et al, 1974). This postulate recalls that of Gerth et al (1974) which suggests "bundling" of fibrils at the α-chain crosslinking stage of fibrin formation. In the case of epidermal cells where keratinisation involves dipeptide formation Buxman and Wuepper (1975) noted that very soon afterwards the cells are sloughed into the environment. Folk and Finlayson (1977) suggest that rather than this crosslinking being a method of consolidation of the stratum corneum, the underlying cells may be stimulated by this cell-loss due to the improved system of waste disposal.

At the cellular level the significance of the dipeptide in membranes has been the subject of some controversy. Price and Hunt (1973) concluded that extensive crosslinking gave Whelk egg capsule its stability. Birckbichler et al (1973), using tissue culture L-cells, felt that no crosslinked network was present, rather that specific sets of proteins were crosslinked, possibly for insertion into the lipid bilayer. Here again the concept of bundling of proteins (Gerth et al, 1974) would appear to gain credibility.

When the density of crosslinking is low, as in the case of unicellular organisms, any functional role for the dipeptide becomes particularly elusive. Hydrolysates of both E. coli and Paramecium contain dipeptide, and both organisms are reported to contain trans-glutaminase (Loewy et al, 1966; Loewy, 1968). Identification of the major site(s) of dipeptide is difficult for such low levels but new methods of analysis may prove more accurate and sensitive. Paramecium contains, at its surface, the largest monomeric globular proteins known (Reisner et al, 1969) and these would bear close examination. E. coli cell wall has all the characteristics conferred by dipeptide formation but, as yet, no peptidoglycan derived dipeptide has been

measurable. Many characteristics of peptide crossbridges (Strominger and Ghuysen, 1967) found in bacterial membranes may be as a result of this dipeptide formation.

1.4 Substrate Proteins

The interaction of the transglutaminases with their substrate proteins has been considered to varying degrees throughout this review. Any investigation of the role of an enzyme in a tissue depends on a knowledge of the potential substrate and to this end a summary of those substrates may aid clarification. In some cases confirmation of $\varepsilon(\gamma$-glutamyl) lysine crosslinks have been made for a particular protein while other potential substrates have been included even though the presence of the dipeptide is still presumptive.

1.4.1 Fibrin

This subject has been covered in detail by Folk and Finlayson (1977) and earlier in this review. As this protein would not figure largely in an investigation of tissue transglutaminase only a brief summary is required.

The starting material, fibrinogen, consists of a polymeric structure of three polypeptide chains α, β and γ (approximate molecular weights 70000, 60000 and 50000 respectively) each of which occurs twice (Mossesson and Finlayson, 1976). Fibrin is formed by the action of thrombin which cleaves fibrinopeptides A and B from the NH_2 terminus of the appropriate chains, thus leaving the structure designated by $(\alpha\beta\gamma)_2$. The fibrin monomers assemble to form a gel which can, under the catalytic action of transglutaminase, become crosslinked. The extent of this series of reactions is determined by pH, solutes, Ca^{2+} ions, and the activation of the enzymes involved.

It has been shown that the γ-chains are crosslinked via the lysyl residue at position 6 (from COOH-terminus) and the glutaminyl residue at position 14 on another γ-chain. This situation results in an anti-parallel dimer configuration maintained by intermolecular

crosslinking (Doolittle, 1973).

The second form of intermolecular activity is shown by the catalytic polymerisation of the α-chains into high molecular weight associations of 5 or 6 molecules (McDonagh et al, 1971). This may be achieved through the two glutaminyl crosslinking sites per α-chain demonstrated for factor XIIIa by McDonagh et al (1976) and results in an anti-parallel arrangement. Full crosslinking corresponds to 6 crosslinks per molecule of fibrin as the β-chain plays no active part in polymerisation (Pisano et al, 1972).

The patten of fibrin polymerisation by factor XIIIa shows rapid γ-dimerisation followed by slower α-polymerisation. Tissue transglutaminase and hair-follicle enzyme show no such chain specificity but exhibit a greater reactivity toward the substrate, resulting in as many as 15 crosslinks per molecule (Chung, 1972; Harding and Rogers, 1972). Thus steric influences may govern the accessibility of the acceptor sites to the particular enzyme. This argument may be taken further in suggesting that crosslink density in vivo may be lower than determined in vitro because α-polymerisation may occlude the less accessible sites after first crosslinking the most readily available residues (Pisano, 1972).

Fibrin is probably the most extensive and widespread substrate utilised by the transglutaminases. The major source of fibrin is blood plasma and, via the action of factor XIII, is involved in haemostasis and wound healing. Other important sites of fibrin deposition are basement membranes where, particularly in uterus and lung, a definite functional role may be envisaged (Finlayson, 1974).

1.4.2 Fibrinogen

Fibrinogen, as previously described, is normally converted to fibrin before becoming an acceptable substrate for plasma factor XIIIa. This situation need not always apply since fibrinogen may act as a substrate though at a much reduced rate (Kanaide and Shainoff, 1975). There is one circumstance where fibrinogen is a normal substrate and that occurs in the haemostatic mechanism of the lobster (Fuller and Doolittle, 1971). Here a tissue transglutaminase (coagulin) acts directly on fibrinogen, needing no preliminary proteolytic activation by thrombin.

Factor XIIIa , liver transglutaminase (Farell and Laki, 1970) and hair-follicle enzyme (Chung and Folk, 1972) share the ability to gel fibrinogen. Liver transglutaminase brings about α-crosslinking prior to γ-dimerisation in fibrinogen as well as fibrin although neither are usual substrates. This order of crosslinking is the reverse of that exhibited by factor XIIIa on both substrates. One further anomaly is revealed by the report that liver transglutaminase may involve the Bβ chain of fibrinogen in the crosslinking action (Chung, 1972). This has been ascribed to the lower molecular weight of the tissue enzyme. This argument is strengthened by the observations of hair-follicle transglutaminase which catalyses the crosslinking of all three chains of fibrinogen at equal rates and has a molecular weight even lower that that of the liver enzyme (Chung and Folk, 1972).

The pathological implications of fibrinogen crosslinking may have considerable relevance in the future, as may the fibrinogen-fibrin crosslinking which also takes place. This latter complex has been demonstrated *in vitro* (Sasaki *et al*, 1966) and may occur *in vivo*

(Kierulf, 1974). One intriguing aspect of factor XIIIa catalysed crosslinking is that, on a molar basis, fibrinogen was found to be several thousand times more effective than the much used glycine methyl ester as a competitive inhibitor of fibrin crosslinking (Kanaide and Shainoff, 1975).

1.4.3 Hair, Quill, Wool and Epidermal Proteins

The hair-follicle, wool follicle and epidermal enzymes have been shown to catalyse fibrin crosslinking, and the $\varepsilon(\gamma$-glutamyl) lysine dipeptide has been isolated from their sites of action. However, unfortunately, the production of crosslink by the enzyme using native proteins has yet to be experimentally demonstrated. In this situation it is expedient to compare morphology to enzyme measurements in order to draw any conclusions.

Crosslink density is highest in the citrulline containing protein medulla of hair, but low in the adjacent keratin of the cortex. The inner root sheath, lying adjacent to the cortex has a crosslink density much lower than the medulla but three fold higher than in the afore mentioned cortex (Harding and Rogers, 1972b). The protein of the inner root sheath occurs in filaments (about 8nm in diameter) which like the medulla proteins are rich in citrulline (Steinart et al, 1971). It is interesting that the ratio of citrulline in guinea pig hair medulla and inner root sheath approximates to that of crosslink content (Harding and Rogers, 1971).

Merino sheep wool, which is not medullated, is reported as having a crosslink density intermediate between that of medulla and cortex in hair (Asquith et al, 1970). The enzyme isolated from wool-follicles has different catalytic properties to that isolated from follicles of guinea pig and rat where the hair contains large medullas

(Harding and Rogers, 1972a).

The outer root sheath of hair follicles is continuous with the epidermis, and therefore it may not be surprising that the enzymes isoalted from the two sources are similar in several respects. Immunological identification of the epidermal enzyme localises its activity to the malphigian and granular layers, where keratinisation takes place (Buxman and Wuepper, 1975). Goldsmith et al (1974), using amine incorporation as a measurement of enzyme activity, mapped the enzyme across the basal layer to the stratum corneum and found a steady increase in activity through the respective layers. Thus it would appear that the enzyme is more active in the mature and terminal cells undergoing keratinisation. Further evidence came from Rice and Green (1977, 1978) who, whilst not implicating keratin as such, showed that during terminal differention of keratinocytes an insoluble cornified envelope of crosslinked protein formed beneath the plasma membrane.

1.4.4 Fibronectin

This glycoprotein has been intensively investigated in recent years and has been the subject of a major conference, the proceedings of which have been published (Vaheri et al, 1978).

"Fibronectin" (from the Latin fibra (fibre) and nectere (connect, link)) refers to a series of antigenically and structurally similar glycoproteins found in vertebrate plasma, tissue fluids and tissues and cultures of adherent cells (Vaheri et al, 1976; Hynes, 1976; Mosher et al, 1977; Vaheri and Mosher, 1978). There are two major subdivisions of the proteins, the plasma (circulating) form isolated particularly from blood and the cellular form found associated with

cell surfaces and basement membranes. The two fibronectins, although immunologically and biochemically very similar, are not identical (Yamada and Kennedy, 1979).

(i) Plasma fibronectin

The circulating form of fibronectin was described 30 years ago as the major non-clottable protein of Cohn fraction 1, the fibrinogen containing fraction (Morrison et al, 1948). The impure protein precipitated in the cold and was first called cold-insoluble globulin (CIg) but subsequent purification (Mossesson and Umfleet, 1970) showed the pure form to be soluble. It has the electrophoretic mobility of a fast β-globulin, contains about 5% carbohydrate, is a major plasma protein (normal human value 0.33mg/ml approx) and appears as a globular disulphide bonded dimer of subunit molecular weight 200000-220000.

(ii) Cellular fibronectin

This major external protein has been studied under various synonyms includinglarge external transformation-sensitive (LETS) protein (Hynes, 1973), fibroblast surface antigen (Ruoslahti et al, 1973), cell surface protein (Yamada and Weston, 1974) and others. The protein is structurally and chemically very similar to CIg except that the subunit molecular weight is slightly increased (220000-250000). Many cultured cells, particularly fibroblasts, synthesise large amounts of fibronectin which is mainly shed into the medium, only 5-10% detectable in the cell layer, both intracellularly and externally (Vaheri et al; Mosher et al, 1977). Surface bound fibronectin exists mainly in the

form of disulphide bonded dimers but some multimers in excess of 1,200,000 molecular weight have been reported (Keski-Oja et al, 1977; McConnell et al, 1978). Disulphide bonds are also required for the binding of the protein at the cell surface (Ali and Hynes, 1978) and the orientation of the molecule is achieved by the presence of several stable domains connected by flexible polypeptide segments (Collonna et al, 1978; Alexander et al, 1979). This protein conformation plays an essential role in the interaction of extracellular macromolecules.

Mosher (1975) reported that plasma fibronectin was a substrate for plasma transglutaminase (factor XIIIa) and attached some physiological importance to the fact that the fibronectin became cross-linked to fibrin. Later studies by Jilek and Hörmann (1977) substantiated this claim by showing the incorporation of $[^{14}C]$-putrescine into plasma fibronectin with a single transamidation-sensitive site per subunit. Mossesson and Umfleet (1970), using clotting plasma, reported the serum concentration of plasma fibronectin to be 28% - 52% less than the plasma levels. Mosher (1976) reasons that in the presence of transglutaminase about 6% of a fibrin clot would consist of fibronectin crosslinked to the α-chain of fibrin and not to itself. This interchain crosslinking appears to be specific, in that in the absence of fibrin plasma fibronectin will polymerise up to the tetramer, but when fibrin is added the resulting multimers all form between fibrin and fibronectin or fibrin and fibrin (Mosher, 1975). The implication of these interactions in wound healing is clear, particularly with reference to the cell surface fibronectin which may also be produced in situ.

Cellular fibronectin also serves as a substrate for transglutaminases, both plasma and tissue, though covalent disulphide bonding may also play a role in the formation of large molecular weight multimers (Keski-Oja et al, 1976; Keski-Oja, 1976). These reports also show that most polymerisation occurs at low cell densities and when the cells are growing quickly. Mosher (1978) has concluded that transglutaminase-catalysed crosslinking of cell surface fibronectin may not always occur between fibronectin molecules, but between fibronectin and other constituents of the pericellular matrix. Birckbichler and Patterson (1978) have shown that a cellular transglutaminase with cultured cells produced fibronectin polymers containing the $\varepsilon(\gamma$-glutamyl) lysine dipeptide.

The implications of the role of cellular fibronectin and transglutaminase in physiology and function require a more detailed knowledge of interactions at the cell surface. Analysis of adherent cells grown from embryonic and adult human tissues indicates that the organisation of pericellular fibronectin is highly variable and depends on the tissue of cell origin (Mosher et al, 1977; Vaheri et al, 1976). Fibronectin may be in dense fibrillar (lung culture), discrete fibrillar (skin culture), punctate (some kidney cultures) or radial (astroglial cells) immunofluorescence patterns. Mosher et al (1977) concluded that there must be chemical differences between the fibronectins synthesised by different cell strains, or factors in the cell layer, which influence fibronectin binding and aggregation. Production and surface binding of fibronectin was found to be greatest in early passages of embryonic cells, whereas in established cell strains less of the protein was found. Fibronectin production was also found to be linked to cell cycle as the protein decreases at

mitosis but re-appears afterwards (Stenman et al, 1977). The action of transglutaminase on the pericellular fibronectin does not change the gross distribution observed using immunofluoresce methods (Keski-Oja et al, 1976).

The structures visualised at the cell surface may be supplemented by amorphous or fibrillar fibronectin which appears to mediate cell-to-cell and cell-to-substratum contacts (Hedman et al, 1978). This investigation also revealed a close association between fibronectin and the plasma membrane. In addition Engvall and Ruoslahti (1977) have shown that fibronectin binds strongly to, and co-distributes with, collagen at the cell surface and that collagen may be an intermediary in membrane binding.

Certain proteases, trypsin (Keski-Oja et al, 1976), plasmin (Blumberg and Robbins, 1975) and elastase (Zetter et al, 1976), in concentrations that are mitogenic to density-inhibited chick fibroblasts, remove surface-associated fibronectin. However it appears that the loss of the fibronectin is not the necessary condition required for proliferation as other factors are involved.

The effects of thrombin and other serum growth factors are interesting as hormonal rather than enzymic functions are integral in their actions. In cultures of embryonic and adult skin fibroblasts, thrombin and serum in mitogenic concentrations (Pohjanpelto, 1977) stimulate production of fibronectin into the medium (Mosher and Vaheri, 1978). Thrombin also denudes fibroblasts of pericellular fibronectin, without apparent changes in the protein, whereas the proteolytic release by trypsin appears to be due to cleavage of the molecule close to the disulphide bond; resulting in a reduction in molecular weight of the released fibronection of about 15000 (Keski-Oja et al, 1976). On cultures of fibroblasts small polypeptide

growth factors (epidermal growth factor, fibroblast growth factor, platelet factor) act jointly with proteases such as arginine esterases and thrombin in stimulating cell proliferation and production of matrix components (Chen LB et al, 1977; Lembach, 1976; Zetter et al, 1977). Glucocorticoid hormones, on the other hand, in concentrations inhibiting proliferation of fibroblasts, increase the amount of fibronectin in the cell layer (Vaheri and Kurkinen, 1978). Taken together these observations suggest that the release and production of matrix components may influence cellular proliferation. This would be achieved by provided the mechanisms by which cells may be stabilised during growth and released during mitosis. Furthermore, cytochalasin B in concentrations causing the disappearance of microfilament bundles ("actin cables") effect the rapid release of most external fibronectin (Kurkinen et al, 1977; Mautner and Hynes, 1977). Agents e.g. colchicine, vineblastine sulphate which cause the disruption of the microtubular system do not perpetrate similar fibronectin losses. Loss of fibronection from cell surfaces in cytochalasin-B treated cells, as well as mitotic, protease-treated and transformed cells is paralleled by altered cell morphology (rounding up or arborisation) and by loss of microfilament bundles. Thus, complex interactions may exist between fibronectin at the cell surface and the cytoskeleton organisation which may result in co-modulation in the cell.

The great interest shown in fibronectin in the last five years has been mainly due to the fact that the single most consistent difference between normal and neoplastic (transformed) cells is the loss of fibronectin-surface association. This phenomenon has been

found in viral transformation models including mutants (RSV, polyoma, SV40) temperature sensitive for transformation. Cells derived from naturally occurring human glioblastomas, fibrosarcomas, osteosarcomas and rhabdomyosarcomas produce fibronectin but fail to retain it at the cell surface. A decrease in external fibronectin has also been found when spontaneously and chemically transformed rodent cells are compared to parental normal cells. Furthermore, a close correlation has been shown between the decreased fibronectin at cell-cell interfaces and an increase in tumorigenicity in syngeneic rats and immunoincompetent (nude) mice (For reviews see Mosher and Vaheri, 1978; Yamada and Pouyssegur, 1978).

The information summarised above indicates that malignantly transformed cells have a defective cell-fibronectin matrix interaction, and this defect may contribute to the phenotype.

The tissue stroma is composed of several types of collagen, proteoglycans, fibronectin, elastin, microfibrillar protein and other unidentified components (Anderson, 1976; Kefalides, 1973) and although small differences exist between types of cells this matrix is generally known as the glycocalyx or cell coat (Mallucci et al, 1972). Transformation associated changes in synthesis of matrix components (collagen and proteoglycans) and overall chemical differences have been reviewed (Vaheri, 1978) and at least three of these alterations are known to be involved; fibronectin, sulphated glycosaminoglycans and collagen. Fibronectin has been discussed above, collagen will be discussed in a later section, and the glycosaminoglycan component (e.g. heparan sulphate (Chiarugi et al, 1974)) has been shown to become less surface-associated with transformation.

Fibronectin also possesses the ability, when isolated from normal

cells and added to transformed cells, of restoring morphological features characteristic of normal cells (Ali et al, 1977). These characteristics include defined actin cables (Ali et al, 1977) increased substratum adhesion (Yamada et al, 1976a) and less surface microvilli and membrane ruffles (Yamada et al, 1976b).

The reason for the effects of transformation on fibronectin are not clear at present. While transformed cells produce somewhat less fibronectin than their normal counterparts there is apparently no change in the molecule (Vaheri et al, 1976) nor do these cells lose their entire complement of the glycoprotein. Indirect evidence would suggest that proteolysis may be the cause of the increased shedding of fibronectin fibrin, collagen and sulphated proteoglycans from the cell surface. The release by cells of plasminogen activator and procollagenase has been reported (Reich, 1975; Sellers et al, 1977) but studies with inhibitors and mixed cultures contradict proteolysis as the basis of the release mechanism (Chen et al, 1977). There is a striking correspondence between fibronectin and the microfilament bundles (see Contractile Proteins 1.4.6) which is shown by their responses to transformation, mitosis, trypsin and cytochalasin-B. However no direct evidence is available on their interaction in the cell. Finally, fibronectin may require surface receptors which are missing in transformed states. Collagen, or collagenous proteins have been shown to form close associations with fibronectin and also with the plasma membrane (Lichtenstein et al, 1976) but results at present rely on methods which study pericellular structures and not structures which are actually membrane bound.

The malignant phenotype with respect to fibronectin and its properties may be used to explain some of the characteristics displayed

by these cells. The transformed cell, unlike its normal counterpart, would be free from the restraints of cell-cell and cell-substratum adherence and also to some extent free of internal cytoskeletal networks and thus have much greater freedom of membrane movement as suggested by the intermixing of surface antigens or "patching and capping" induced by divalent ligands (Nicolson, 1976). These cells freed of the glycocalyx and with abundant microvilli and surface ruffles (Ambros et al, 1975) would have a facilitated access to nutrients and use of glycosyltransferases and may be more agglutinable by lectins.

The affinity of fibronectin for collagen and fibrin and the crosslinking of fibronectin by transglutaminases may be of considerable physiological importance. In wound healing and tissue repair the incorporation of fibronectin into the fibrin clot and the involvement of fibroblasts and endothelial cells in producing and binding fibronectin (particularly under the proliferative stimulus of thrombin) provide the potential substrate for the transglutaminases. Fibronectin may also be involved in as yet less well researched areas of interest. Fibronectin has been identified in cerebrospinal fluid and shown to have drastically altered levels in response to various disease states such as multiple sclerosis and brain tumours (Kuusela et al, 1978). Amniotic fluid has been shown to contain fibronectin (Chen AB et al, 1976) produced by fetal cells in this location (Crouch et al, 1978) for use as a matrix protein and this may be of use in determining congenital diseases of connective tissue (Kuusela et al, 1978). Neutral proteases from polymorphonuclear leukocyte granules destroy the fibrillar networks of fibronectin showing a marked sensitivity of this biologic function to inflammatory conditions (McDonald et al,

1979). This work has been complemented by the report that raised levels of plasma fibronectin have been seen in systemic lupus erythromatosus and various states of rheumatoid arthritis (Fyrand et al, 1978). The identification of plasma fibronectin as the opsonic α_2SB (Surface Binding) glycoprotein has far-reaching implications with respect to the reticuloendothelial system and immunological recognition of cells in host defence following trauma, major surgery, burn injury and during neoplastic disease (Blumenstock et al, 1978). The deficiency in α_2SB glycoprotein may be due to its removal from the circulation in conjunction with fibrinogen-fibrin complexes under the catalytic action of plasma transglutaminse. This work is reinforced by the finding that fibrinogen and fibrin are bound to cultured fibroblasts, in close association with fibronectin, at the cell surface (Colvin et al, 1979) and that these compelxes are lost under the conditions already described for the shedding of fibronectin. Macrophages have also been shown to remove complexes from the blood through a fibronectin mediated process (Jilek and Hormann, 1978).

The action of transglutaminase on fibronectin has been shown to be of great importance in a number of pathological conditions particularly wound healing and tissue repair. However, further evidence of transglutaminase involvement in the wide range of fibronectin-associated physiological states is required in order to fulfill the potential of this area of study.

1.4.5 Collagen

The involvement of plasma transglutaminase in wound healing and the organisation of thrombi attached to vessel walls (Henry, 1965; Casley-Smith et al, 1976) have led to speculation regarding the role

of ε(γ-glutamyl) lysine crosslinks and collagen in these processes. There is little firm information, however Soria et al (1975) have shown that plasma transglutaminase acts on collagen and gives rise to a random rather than fibrillar polymer arrangement. Nyman and Duckert (1975) incubated radiolabelled fibrinogen with collagen in the presence of the enzyme, Ca^{2+} and thrombin and noted that the collagen fraction bound the radiolabel. Further work has shown that the γ-chains of fibrin were crosslinked to collagen during the fibrin clot formation in this experiment.

Cell biologists have used collagen as a substrate for cells for many years but only recently has the mechanisms for its supportive properties been demonstrated. It is concluded that fibronectin on cell surfaces binds to collagen particularly in the denatured form, gelatin (Grinnel and Minter, 1978; Balian et al, 1979; Hahn and Yamada, 1979). Ruoslahti and Hayman (1979) have shown that fibronectin has at least two distinct active sites, one for cell attachment and the other for binding to the αi(1) chain of collagen (Kleinman et al, 1978a) or in some cases fibrin-fibrinogen (Colvin et al, 1979). Although fibronectin will bind to all collagens there is some specificity displayed by various cells, Type IV (basement membrane collagen) is the most effective (Engvall et al, 1978; Kleinman et al, 1978b). Chondrocytes also require fibronectin for substratum attachment while growing and spreading until a point is reached where the secreted collagen matrix becomes able to support the growth on its own (Dessau et al, 1978). Vaheri et al (1978) have demonstrated that fibronectin co-distributes extensively with various collagens and procollagens in fibroblasts and trypsin removes both matrix proteins whereas collagenase only removes the procollagen

Transformed and tumour cell lines displayed little or no pericellular matrix collagen or fibronectin which may account for several phenotypic characteristics already described for transformed cells. Another example of the pathological implications of collagen-fibronectin interactions may result from work on the platelet in which Bensusan et al (1978) have demonstrated that fibronectin is the collagen receptor on platelet membrane. When the endothelial lining of a blood vessel is breached blood platelets come into contact with components of connective tissue and the collagen-platelet nidus provides a point around which aggregates form in the initial stage of haemostasis.

1.4.6 Contractile Proteins

Derrick and Laki (1966) using rabbit actin and tropomyosin demonstrated that muscle proteins would act as tissue transglutaminase substrates and form high molecular weight aggregates. Later, Loewy and co-workers (1968) isolated the crosslink dipeptide from muscle and proposed that covalent bond formation was involved in the contraction of skeletal muscle as displayed by an increase in dipeptide levels during contraction in a number of systems. Mui and Ganguly (1977) also demonstrated the substrate potential of actin in promoting and participating in the plasma transglutaminase dependent formation of aggregates with polymerising fibrin. This work was designed to explain the interaction of the platelet actomyosin-like protein, thrombosthenin (Bettex-Galland et al, 1961) with fibrin, and the subsequent clot retraction. Further evidence for this postulate was reported by Cohen et al (1979) when both platelet and muscle myosin were identified as substrates of plasma transglutaminase. The myosin heavy chains were particularly susceptible and formed

polymers in excess of heptamers. The crosslink dipeptide was isolated with an average of 19mol per mole of platelet myosin. These workers also concluded that the crosslinking of fibrin to actin and myosin plays an important role in the process of clot retraction, and hence effective thrombus formation.

Tissue transglutaminase was used in the study of chicken myofibril proteins (Gard and Lazarides, 1979) and specifically labelled proteins at or near the myofibril Z line. The Z line acts as the anchor point for the actin filament lattice in a sarcomere and is responsible for transmitting the forces of each sarcomere to those adjacent. The transglutaminase labelling implicated α-actinin, desmin, tropomyosin and most predominantly, actin. There were several other labelled proteins which could not be identified and also a substantial amount of extremely high molecular weight material possessing covalent linkages other than disulphide bonds. Exogenous guinea pig liver transglutaminase when added to this system became very tightly adsorbed strictly at the Z line, and whilst remaining active could not be removed by extensive and varied washing regimes. α-Actinin has also been localised in the tight junction (Zonula occludens) and/or the belt desmosome (Zonula adherens) of intestinal epithelial cells (Craig and Pardo, 1979).

There have been many investigations into the inter-relationship of the cytoskeleton with the surface of the cell in general and fibronectin in particular (Nicolson, 1976a,b). Ali and Hynes (1977)and Kurkinen et al (1978) have both reported that cytochalasin-B, which disassembles actin microfilaments (Mautner and Hynes, 1977) causes the release of fibronectin from the cell surface. The microtubular system does not appear to be involved as colchinine does not have the same effect. Mautner and Hynes (1977) and Hynes and Destree (1978)

provide evidence for a trans-membrane relationship between microfilament bundles and fibronectin due to a close proximity and co-distribution. Singer (1979) has demonstrated a dense submembranous plaque in which actin and fibronectin fibres congregate in a close one to one manner, joined either co-axially or by overlapping, and has named this junction the fibronexus. Here again a role for α-actinin is postulated in the stabilising of actin filaments in ordered arrays where these fibronexuses are found, for instance in the substrate attachment feet of cells (Heath and Donn, 1978) which bear a close resemblance to adherens-type junctions (Heaysman, 1973). Furthermore patching and capping by receptors in response to extracellular ligands involve actin and α-actininsub-caps on the cytoplasmic side of the membrane (Geiger and Singer, 1979). Thus α-actinin may prove to be an important adjunct to the stabilisation of actin at its sites of action. Transmembrane bridging has been the subject of intensive study, and the important bridging protein (protein X (Bourguignon and Singer, 1977)) that connects the cytoskeleton to the external face of the membrane has yet to be identified. However the interactions of actin with α-actinin resemble many of the properties attributed to the enigmatic protein X (Singer et al, 1978).

The patching and capping phenomenon has been examined with respect to transglutaminase activity (Davies et al, 1980). Transglutaminase has been shown to be essential in receptor-mediated endocytosis of α_2-macroglobulin. The enzyme makes the receptor aggregation process irreversible which results in the progressive accumulation of ligand-receptor complexes in the coated pit prior to endocytotic internalisation. The transglutaminase is thought to originate from the submembrane microfilament mat of the plasma membrane but the actual substrates involved have yet to be

determined.

The interactions of transglutaminase with the cytoskeleton is becoming of greater interest to research workers and is certainly one of the most promising fields of study.

1.4.7 Plasma Membrane

Birckbichler et al (1973) reported finding ε(γ-glutamyl) lysyl crosslinks in plasma membrane and endoplasmic reticulum fractions of L cells grown in suspension cultures. It seems improbable that there is any connection with fibronectin in this case. Although the fibronectin molecule may be present (Hunt and Brown, 1975) the work of Graham et al (1975) indicates that the ATPase-rich plasma membrane fraction analysed by Birckbichler et al (1973) should have been poor in this polypeptide. Furthermore the majority of the dipeptide containing membrane fraction was found to be sensitive to EDTA treatment suggesting the derivation to be the filamin molecules (Wang et al, 1975), on the cytoplasmic face of the plasma membrane.

Linnoila et al (1979) have also reported that membrane proteins are sensitive to rabbit hepatic transglutaminase activity by the formation of high molecular weight aggregates from smaller constituents. This was also found to be true of the cytosol constituents, where two proteins in particular (molecular weight >150,000) were markedly involved in polymeric assembly, with many other proteins capable of accepting radiolabelled amines.

This fraction of the cell along with the cytoskeleton have been investigated as possible sites of action of the intracellular tissue transglutaminases.

1.5 Pathology

Situations, such as those discussed, where a relatively few crosslinks can exert a strong influence may be adversely affected should there be any disruption of control mechanisms. The hazards to the organism of under or over- production of crosslink may be realised in the pathological conditions which can be evoked. Four major conditions are relevant to this argument and are active subjects of continued research.

1.5.1 Thrombosis

The clinical observation that thrombi become increasingly resistant to fibrinolytic therapy with the passage of time (Gormsen et al, 1967) causes concern for the potential hazards caused by the impendance of vascular flow. The resistance to lysis by the native system is accepted as being caused by the density of crosslinking and the failure of the lytic enzymes to penetrate the stabilised and retracted clot. However, the recent discoveries of the part played by collagen and fibronectin in haemostasis and wound healing may give a fresh approach to the problem of the dissolution of the clot. It is also recognised that in a sensitive coagulation system the potential triggering factors must be given due consideration. Fibrinogen and the transglutaminase mediated complex formation with fibrin and plasma fibronectin previously discussed, may form the stimulus necessary for coagulation to occur. It is already known that these complexes are formed during conditions such as Hodgkins Disease (Kainaide and Shainoff, 1975) and with renal allografts (Kanaide et al, 1973).

1.5.2 Hyaline Membrane Disease

This disordered respiratory syndrome of infants is related to thrombosis in terms of both chemistry of development and cause of

death. The intra-alveolar hyaline 'membranes' consists chiefly of fibrin and the inability to mount a fibrinolytic attack on this substrate appears to be the cause of the disease. Ambrus et al (1971) found that normal infants had half the levels of plasma transglutaminase found in infants with the disease. The high level of transglutaminase in the human lung (Chung, 1972) and the hypothesis of Barton and Lonrenco (1971) that chronic bronchitis may be aggravated by gelatinous, crosslinked sputum leads to the speculation of a role for the enzyme in other respiratory diseases. Furthermore, investigations into pulmonary fibrosis carried out in this laboratory (Griffin et al, 1979) also suggest a role for transglutaminase in fibrotic disorders.

1.5.3 Atherosclerosis

There are many theories concerning the pathogenesis of this disease and Laki et al (1972) have summarised some with particular reference to the role of transglutaminase. These investigations, using rabbits on a high-cholesterol diet, reported that the aortas showed twice the normal enzyme level in healthy areas and four times the control value in sclerotic regions. Also the intima of the sclerotic patches contained up to seven times the normal level of fibrinogen (Shainoff and Page, 1972) which appears to be in a partially cross-linked form. This suggests a role for tissue transglutaminase in vascular degenerative disease.

1.5.4 Cancer

The concept of neoplastic growth as a form of overhealing (Haddow, 1972) is both appealing and pertinent to a possible role for transglutaminase in this condition. Various scattered reports have been assembled during the past decade which have indicated a possible function for the enzyme in neoplastic growth and metastasis. Laki et al (1976) determined a direct relationship between the spread of breast

cancer and the transglutaminase activity of the organ in which secondary growth takes place. Furthermore, using transplantable tumours of high, medium and low enzyme activities in mice, the mean host survival time varied inversely with the transglutaminase level in the tumour. Injections of transglutaminase into YPC-1 tumour bearing mice promoted metastases to the lung and spleen, confirming an involvement in the proliferation of experimental tumours. Laki et al (1977) speculate on the role of the enzyme in tumour growth and invasion and propose three mechanisms (i) that a specific cellular protein e.g. actin is the transglutaminase substrate, (ii) that the enzyme may be released from the cell to interact with fibrinogen or fibrin in a situation analogous to wound healing or (iii) that transglutaminase action masks antigenic sites, rendering the immune surveillance system inoperative.

The involvement of fibrin in the spread of tumour metastases and the growth of the primary site is well documented. Dvorak et al (1979) have demonstrated that hepatocarcinomasbecome invested in a fibrin-gel within a few hours of implantation and Chew and Wallace (1976), using Walker 256 tumour cells, have shown that fibrin occurs very early in small amounts in association with tumour cell emboli. Defibrination of mice decreased the number and rate of growth of Lewis lung carcinoma (3LL) metastases in mice (Donati et al, 1978) showing that the spread of cancer cells is dependent on the fibrin(ogen) substrates. The growth of the primary site also requires a fibrin network for both the spreading of cells and the essential vascularisation of the tumour mass (Liotta et al, 1974; Folkman, 1976; Tanaka et al, 1977). The involvement of transglutaminase at this point may be seen by two experimental approaches. Fésüs and Laki (1976) have demonstrated that

tissue transglutaminse will crosslink fibrinogen to the surface of malignant murine plasma cells (about 10^6 molecules/cell) without impairing either viability or malignancy. Furthermore, Eipe et al (1977) have shown that plasmacytoma cells adsorb plasma transglutaminase, and may even endocytose the enzyme, providing an in situ source as and when required.

Birckbichler et al (1976, 1977, review 1978) have actively investigated cellular transglutaminase function in paired systems encompassing normal and transformed/neoplastic models. In rat liver, they have demonstrated that there is a substantial alteration in subcellular distribution and reduction in enzyme activity between normal and hepatoma cell types. Simple proliferation (as seen by regenerating and embryonic tissues) did not exhibit such changes in distribution, and throughout all these conditions the K_m and V_{max} for the isolated enzymes remained roughly similar. When paired cell culture systems were investigated the transformed states always had reduced enzyme activity (9-90 fold). The normal cells produced the highest enzyme activity when cell populations were in an essentially quiescent state which is in conflict with their earlier findings with proliferating tissue but which supports the findings for neoplastic proliferation. These studies have been reinforced by the finding that normal WI-38 (human lung) cultures contain 40-100 times more $\varepsilon(\gamma$-glutamyl) lysine bonds than their transformed WI-38VA13A counterparts, and that specific enzyme immunofluorescence shows an equivalent reduction in level. Birckbichler and his co-workers believe that these results may contribute to a better understanding of the altered cell surface in malignant disease. However, this observation of a reduction in enzyme production by transformed cells is in contradiction to Laki

et al's (1977) observation which suggests an integral role for the enzyme in growth and metastasis. This confused situation requires further investigation.

One further feature of this area is that some cancer chemotherapeutic agents have been shown to impede fibrin crosslinking (Komp et al, 1974). Furthermore, work in this laboratory (Griffin et al, 1978) has shown Bleomycin, an anti-tumour glycopeptide with a terminal amine side-chain, inhibits both tissue and plasma transglutaminases. Laki et al (1978) have also reported that the cancerostatic BCNU (1,3-Bis 2-chloroethyl-I-nitrosurea) caused a marked decrease in transglutaminase activity (close to 100%) in liver and spleen of mice. This area of investigation may, in future, help in elucidating some aspects of the interaction of chemotherapeutic agents with their target tissues.

In their review Folk and Finlayson (1977) regard the role of crosslinking processes in tumour growth and function to be firmly established and to be a valuable component of future research in this area.

1.6 Polyamines

Early investigators in this field established that a variety of primary amines, including diamines and spermine, could serve as acceptor substrates for guinea pig liver transglutaminase (Clarke et al, 1959). Expansion of the list of primary amines and enzymes involved have been the subject of more recent studies (Lorand and Ong, 1966; Gross et al, 1977). These substrates, or their analogues have been used in the assay, identification and inhibition of transglutaminase enzymes for many years. However, only recently have natural cellular polyamines been considered as possible mediators of the catalytic function of the enzyme. Schrode and Folk (1978) have demonstrated that tissue transglutaminase is capable of catalysing the production of crosslinks between peptide-bound glutamine residues by means of a transfer reaction involving both of the primary amino groups of a diamine or polyamine. Although no evidence is available from in vivo experiments the necessary spatial orientation of the contributing moieties would be much less than that required for the formation of peptide-bound $\varepsilon(\gamma$-glutamyl) lysine crosslinks. Thus, the reaction may be energetically favourable. The possible physiologic relationships of transglutaminases and polyamines are numerous and warrant further investigation.

Polyamines are found in highest concentration in those tissues with high rates of protein, ribonucleic acid and deoxyribonucleic acid synthesis and are formed from synthetic pathways starting with the decarboxylation of L-ornithine to give putrescine (Fig. 1.4). The study of polyamines has been the subject of a number of reviews (Bachrach, 1973; Russell and Durie, 1978). There is, as yet, no definitive function in cellular metabolism attributable to the

polyamines but they exert several biologic effects; (i) as polycations they have a high affinity for negatively charged compounds e.g. phospholipids of membranes and nucleic acids, where they stabilise structures and perform regulatory functions. (ii) Increased polyamine synthesis and accumulation are closely associated with growth processes. Some hormones enhancing synthetic mechanisms also strongly stimulate the synthesis of polyamines e.g. epidermal growth factor (DiPasquale et al, 1978, Stasny and Cohen, 1970). Rat liver following partial hepatectomy has often been used as a model system. (Russell and Snyder, 1968; Raina et al, 1970) for the study of the physiological effects of alterations in polyamine levels.

The polyamines have been shown to be involved with regulation of the cell cycle (Rupniak and Paul, 1978) and in the process of cytokinesis and cell division (Sunkara et al, 1979). Polyamine-induced actin orientation and polymerisation has also been recently observed in vitro (Oriol-Audit, 1978). Fluctuations in intra- and extra cellular polyamine levels have been reported in a number of disease states characterised particularly by increased cell loss or proliferation. Tumour cell proliferation in conjunction with polyamine levels have been intensively studied (Russell and Durie, 1978; Endo et al, 1978; Scalabrino et al, 1978) particularly to further the understanding of the growth processes and the possible use of serum or urinary polyamines as a marker for neoplasia. Other proliferative states have been investigated and these include cystic fibrosis, psoriasis, polycythomia vera, pregnancy and red blood cell pathologies (Russell and Durie, 1978).

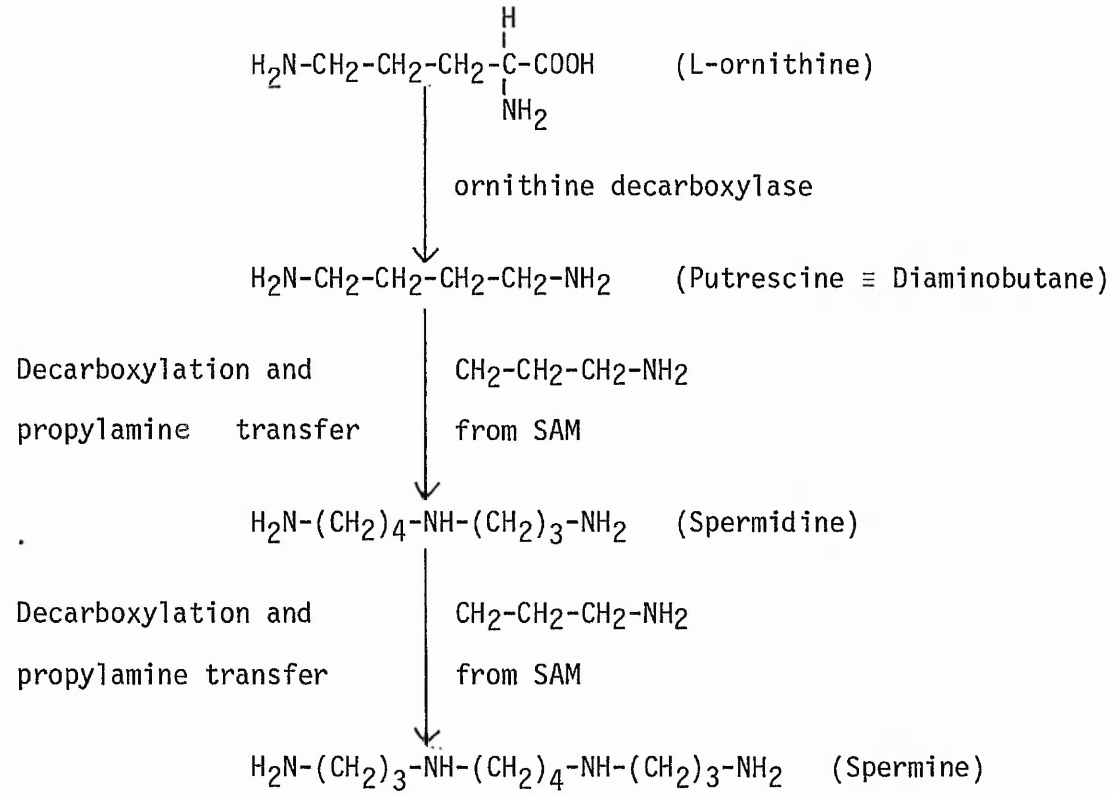


Figure 1.4 Polyamine biosynthesis in mammals

SAM ≡ S-Adenosyl-L-methionine

1.7 Purpose and Experimental Approach

The object of the research described in this thesis was to provide information on tissue transglutaminase with particular reference to its cellular role and function. A comprehensive knowledge of this enzyme and its place in the metabolism of the cell would facilitate the understanding of a number of pathological conditions related to aspects of the pericellular environment.

The most direct approach to obtaining this information encompassed three major areas of study:

(i) A comprehensive investigation of the rat liver transglutaminase is missing from the literature. This study using purified enzyme, would both enlarge the choice of animal model available for research and, importantly, give essential data on the application of the enzyme to cellular physiology.

(ii) Investigations were carried out to determine the subcellular localisation, mechanisms of control, and the identification of potential substrates for the enzyme.

(iii) Model systems, including embryonic, regenerating and hepatomegalic liver as well as platelet and tumour studies, were employed to observe the effects on the enzyme of an altered physiological balance of the cell.

The successful completion of this programme of research would, it is hoped, provide a significant contribution to the study of tissue transglutaminase.

2.0 MATERIALS AND METHODS

The majority of the work detailed here was carried out in the laboratories of the Department of Life Sciences, Trent Polytechnic. Studies using rat liver hepatomegaly, liver regeneration and platelets were carried out at I.C.I. (Pharmaceuticals) Ltd., Alderley Park.

All enzyme experimentation was carried out at 4°C unless otherwise stated.

All chemicals were of Analar or reagent grade and unless stated otherwise were obtained from Sigma Chemical Co. Ltd., Saint Louis, Missouri, U.S.A. Acids and solvents were obtained through Fisons Ltd., Loughborough, England.

Radiochemicals were obtained from The Radiochemical Centre, Amersham, England. The two major sources of radiolabel were as follows:

(i) 1,4(n)-[^{14}C]-Putrescine dihydrochloride:Specific activity 116mCi/mmol.

(ii) 1,4(n)-[^{3}H]-Putrescine dihydrochloride:Specific activity 19Ci/mmol.

pH measurements were made using a Pye-Unicam model 291 Mark II pH meter (Pye-Unicam Ltd.).

Centrifugation

The table overleaf shows the centrifuges and conditions used most often during the course of this work. All the equipment was manufactured by Medical Scientific Equipment Ltd. (M.S.E.).

Instrument	Rotor	Conditions
M.S.E. Magnum	r. max 31cm	600g x 10 min.
	r. max 31cm	410g x 10 min.
M.S.E. Superspeed 50	r.ave 5.84cm	15,000g x 3 min.
		40,000g x 6 min.
		71,000g x 40 min.
M.S.E. Bench		2,000g x 10 min.
M.S.E. Swing-out	r.ave 9.86cm	100,000g x 120 min.

Spectrophotometers

Instrument	Wavelength range (nm)
Pye-Unicam SP500	400-700
Pye-Unicam SP1800	200-800
Beckman Model 25	190-700

2.1 Animals

Three strains of rat (*Rattus rattus*) were used.

(1) The majority of work was carried out using Sprague-Dawley derived rats bred at the Department of Life Sciences, Trent Polytechnic.

(2) Liver regeneration and hepatomegaly studies used Wistar derived rats independently bred and based at I.C.I. (Pharmaceuticals) Ltd., Alderley Park, Macclesfield, Cheshire.

(3) Tumour experimentation used inbred AS/1 and /2 rats based at Charles Salt Research Institute, Agnes Jones Orthopaedic Hospital, Oswestry.

In all studies rats were killed by a common method. Animals were exposed to Fluothane (I.C.I. Chemicals Ltd.) anaesthetic until unconscious, rapidly followed by laparotomy, severing the diaphragm and exsanguination by aortic section at the renal bifurcation.

2.2 Enzyme Assays

2.2.1 Transglutaminase Assays (E.C.2.3.2.13)

2.2.1.1 1,4-[^{14}C]-putrescine incorporation into N,N'-dimethyl casein

Transglutaminase activity was measured by a modification of the "filter paper assay" of Lorand *et al* (1972). The reaction mixture contained in a final volume of 100µl:

(i) 5mM $CaCl_2$

(ii) 3.85mM Dithiothreitol

(iii) 500µg N,N'-dimethyl casein

(iv) 1.2mM putrescine containing 2.5µCi of 1,4-[^{14}C]-putrescine

(v) Enzyme sample (50-400µg protein)

All components were prepared in 50mM Tris pH 7.4.

The reaction vessels were incubated at 37°C in an aluminium heater block (Grant Ltd.) and the reactions were started by the addition of enzyme protein. Samples of 10µl were withdrawn from the sample cups (Sterilin) at appropriate time intervals using disposable pre-calibrated glass pipettes (Accupette) and dispensed onto 1cm squares of thick filter paper (Whatman 3MM).

The termination of the reaction and subsequent washing-out of unincorporated label was based on the methods of Bollum (1959), Thomas *et al* (1968), Martelo *et al* (1970) and Reinman *et al* (1971). The filter paper squares were placed in ice-cold, stirred 10% (w/v) trichloroacetic acid (TCA), for a minimum of 10 minutes each whilst maintaining a volume ratio of at least 5ml TCA per filter paper square. The samples were then transferred through a washing procedure that comprised:

(i) 3 x 5 min 5% (w/v) TCA (ice-cold)

(ii) 1 x 5 min Acetone-ethanol (50:50 (v/v))

(iii) 1 x 5 min Acetone

A volume:paper ratio in excess of 5ml/square was maintained throughout. The filter papers were then dried thoroughly in air before scintillation counting. A metal gauze retainer was found to be useful in accommodating the filter papers during the washing stages. A control sample was removed from each reaction vessel and placed on a filter paper square. The squares were omitted from the washing procedure in order to assertain a control value for the total 1,4-[^{14}C]-putrescine present in the individual vials.

Large numbers of graphite labelled sample filter-paper squares could be conveniently handled to produce rapid and accurate results by this method.

Unit enzyme activity is expressed as nmol putrescine incorporated /hour under the conditions of the assay.

2.2.1.2 Hydroxylamine Assay

This widely used assay measures the appearance of the peptide bound γ-glutamyl hydroxamate formed from the incubation of transglutaminase with the following components, as described by Folk *et al* (1966), but with modifications. The reaction mechanism is given in Fig. 2.1.

(A) 156.6 mg NH_2OH-Cl in 3ml H_2O.

The NH_2OH must be neutralised and the above must be prepared fresh by mixing equal volumes of 28% (w/v) NH_2OH-HCl and 14% (w/v) NaOH.

(B) 98.4 mg Benzyloxycarbonyl-(CBZ)-L-glutaminylglycine in 3ml H_2O plus 6ml 0.75m Tris-acetate, pH 6.

(C) 35.5 mg cysteine in 3ml H_2O.

(D) 1M $CaCl_2$.

(E) Equal volumes of (i) 5% (w/v) $FeCl_3$ in 0.1m HCl,
(ii) 15% (w/v) TCA,
(iii) 2.85M HCl.

After preincubation at 37°C for 5 min the enzyme reaction mixture contained 100 1µl, 300µl B, 100µl C and 5µl D in a final volume of 750µl. 200µl of test solution was added and the assay incubated for the required time (10-30 minutes). This was followed by the addition of 750µl of reagent E in order that the reaction would be terminated and the colour be developed. The reaction mixture was centrifuged (Beckman Microfuge) for 5 min to remove all suspended material and the absorbance measured ($A^{1\%}_{1cm525nm}$).

A standard curve was prepared using 0 → 5 µmol L-glutamic acid-γ-monohydroxamate. Controls were prepared by the ommission (i) enzyme, or (ii) CBZ-glutaminylglycine from the incubation mixture and a water blank was also included.

A unit of enzyme activity is expressed as µmol hydroxamate formed/min under the conditions of the assay.

2.2.1.3 Protransglutaminase Assay

Protransglutaminases (zymogens) of the Factor XIII type require limited proteolysis in order to express catalytic function (Lorand & Konishi, 1964). The proteolytic agent used was thrombin (Lorand *et al*, 1968) and preincubation of the test sample for 15 min at 37°C with approximately 50 NIH units/ml enzyme (Topical, Parke-Davies) was found

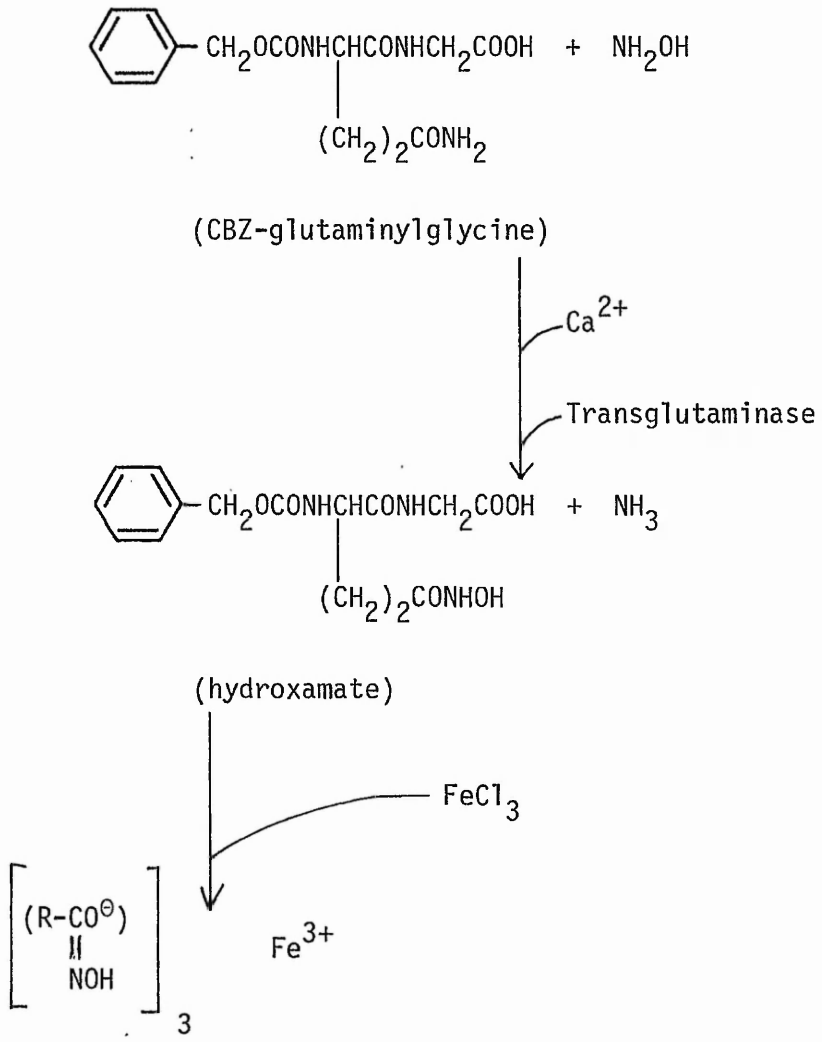


Figure 2.1 : Hydroxylamine assay reaction mechanism. The ferric hydroxamate complex is purple, absorbing at 525nm.

to ensure complete activation of the zymogen. The activated transglutaminase could then be assayed by the methods described above.

The preparation and assay of thrombin is described in Section 2.5.4.

2.2.3 Glucose-6-phosphatase (E.C.3.1.3.9)

Glucose-6-phosphatase was used as a marker enzyme for the microsomal fraction in tissue fractionation procedures. The assay measures the release of inorganic phosphate from glucose-6-phosphate by the enzyme.

Reagents used:

(A) 0.08M potassium glucose-6-phosphate adjusted to pH 6.5 with dilute HCl.

(B) 0.01M EDTA pH 6.5.

(C) 0.1M Histidine-HCl buffer pH 6.5.

(D) 8% (w/v) TCA.

Reaction mixtures contained, in a final volume of 1.0ml, 500µl reagent A, 100µl reagent B and 200µl reagent C. Reactions were started by the addition of 200µl of a suitably diluted enzyme sample and the volume made up with 100µl water. The assay was incubated at 37°C for 30 min and the reaction stopped by the addition of 5ml reagent D. Denatured protein was removed by centrifugation (1000g x 10 min) and determination of inorganic phosphate released was carried out on a sample of the supernatant. Inorganic phosphate was determined by the method described later (Section 2.3.3).

Water and reagent blanks were used as controls.

A unit of glucose-6-phosphatase activity is defined as the amount of enzyme which catalyses the hydrolysis of 1µmol of substrate per minute.

2.2.3 5'-Nucleotidase (E.C.3.1.3.5)

This enzyme has been commonly used as a plasma membrane marker in a number of methods for cell fractionation and membrane isolation (Aronson & Touster, 1974). The enzyme activity is measured by inorganic phosphate release from 5'-adenosine monophosphate (5'-AMP).

Reagents used:

(A) Na-AMP, 50mM, pH 7.0.

(B) Glycine-NaOH buffer, 0.5M, pH 9.1.

(C) $MgCl_2$, 0.1M.

(D) TCA 8% (w/v).

The reaction mixture containing 50µl reagent A, 100µl reagent B, 50µl reagent C and 250µl water was prepared and the composite was stable at -25°C, for at least a year. The reaction was started by the addition of plasma membrane (20-30 µg protein) via 50µl volume to give a final volume of 500µl. The assay mixture was incubated for 15-30 min at 37°C in a shaking water bath, and included controls of enzyme, reagent and water blanks.

The reaction was terminated by the addition of 2.5ml of reagent D and was centrifuged (2000g x 10 min) to remove denatured protein. Determination of inorganic phosphate was carried out upon a sample of the supernatant (Method 2.3.3).

A unit of enzyme activity is expressed as the µmol of phosphate released per minute under the condition of the assay.

2.2.4 Acid Phosphatase (E.C.3.1.3.2)

This enzyme provided a marker for lysosomal membranes (Appleman *et al*, 1955) by the rate of hydrolysis of β-glycerophosphate during a short incubation at 37°. Triton X-100 is added to the substrate

mixture in order to release the enzyme completely from the membrane. Reagents used:

(A) 0.5M Sodium β-glycerophosphate adjusted to pH 5 with dilute HCl.

(B) 1M Sodium acetate-acetic acid buffer, pH 5.

(C) 1M Sucrose.

(D) 8% (w/v) TCA.

(E) 2% (w/v) Triton X-100 (freshly prepared).

A total volume of 2ml contained 200µl A; 100µl B; 250µl C; 350µl water; 100µl E and 1ml enzyme solution in 0.25M Sucrose. The samples were incubated for up to 20 min at 37° and the reaction stopped by the addition of 10ml TCA. The denatured protein was removed by centrifugation (1000g x 10 min) and inorganic phosphate determined from a sample of the supernatant (Section 2.3.3). Reagent and water blanks were also prepared. Enzyme activity was determined as one unit corresponding to a rate of hydrolysis of β-glycerophosphate of 1µmole per min.

2.2.5 Adenosine Triphosphatase (E.C. 3.6.1.3)

ATPase was used as a plasma membrane marker enzyme and was assayed by the increase in rate of ATP hydrolysis when Na^+ and K^+ are present, over the rate for Na^+ alone (Hoezl-Wallach & Damat, 1955).

Reagents used:

(A) NaCl, 0.25M; $MgSO_4$, 0.00138M.

(B) NaCl, 0.25M; $MgSO_4$, 0.00138M; KCl 0.0125M.

(C) Na-ATP, 0.00125M, freshly prepared.

(D) Tris-HCl, 0.0625M; Na-EDTA 3.12×10^{-4}M, pH 8.6.

(E) Trichloroacetic acid (TCA) 30%.

Two series of measurements were undertaken:

(i) Series Na^+; in a final volume of 1ml, 0.4ml each of solutions (A) and (C) are mixed with 0.16ml of (D) and the mixture chilled membrane protein (5-50μg) was added in 0.04ml sample buffer.

(ii) Series Na^+-K^+; the conditions were identical except for the replacement of solution (A) by (B).

The samples were incubated for 30-60 min at 37° and terminated by the addition of 0.3ml ice-cold TCA and cooled. The samples were then centrifuged (3000g x 5 min) and 1ml samples of the supernatant assayed for the presence of inorganic phosphate (Methods 2.3.3). The phosphate determination was carried out at 4° to minimise ATP blanks.

Results were expressed as the increase in rate of ATP hydrolysis (μmoles/mg protein/hr) which occurs when K^+ is added to a system containing Na^+.

2.2.6 Phosphodiesterase I (E.C.3.1.4.1)

This enzyme provided another plasma membrane marker using an assay which does not rely on phosphate release. Phosphodiesterase activity is measured by the release of p-nitrophenol from the substrate p-nitrophenol-5'-thymidylate (Calbiochem. L.A. Calif.) (Touster *et al*, 1970).

Reagents used:

(A) Na-p-nitrophenol-5'-thymidylate, 5mM, stored frozen.

(B) Tris-HCl, 0.1M, pH 9.0.

(C) TCA, 8% (w/v).

(D) 0.133M glycine, 83mM Na_2CO_3, 67mM NaCl adjusted to pH 10.7 with NaOH.

Each assay contained 100µl A and B, 250µl water and about 10µg of protein in 50µl. The usual controls were performed plus a standard p-nitrophenol solution, 0.5ml of a 100µM solution, 1.5ml of 8% (w/v) TCA was used to terminate the reaction after 10-15 min at 37° in a shaking incubator. The reaction vessels were cooled and centrifuged and 1ml of the supernatant added into 3ml of buffer D and the resultant colour reaction determined at $A^{1\%}_{1cm400nm}$ against a water blank.

A unit of enzyme activity is expressed as the µmol p-nitrophenol released per minute under the assay conditions described.

2.2.7 Lactate:NAD:oxidoreductase (E.C.1.1.1.27)

This enzyme was used as a marker for the particle-free fraction of the cell fractionation scheme. Reactions were followed spectrophotometrically by monitoring a decrease in absorbance at 340nm when NADH was oxidised (Henry *et al*, 1960).

The reaction mixture contained, in a volume of 2.8ml, 100µl 10mM Na-pyruvate, 100µl 2mM NADH and 2.6 cm^3 0.03M phosphate buffer pH 7.4. The change in absorbance at 340nm was monitored continously using a Pye-Unicam SP1800 spectrophotometer.

The activity of the enzyme was expressed as the µmol NADH oxidised per minute under the conditions of the assay.

2.2.8 Cytochrome oxidase (E.C.1.9.3.2)

This enzyme was used as a marker for the mitochondrial fraction in a cell fractionation procedure. The enzyme activity was measured spectrophotometrically by observing the reduction in absorbance at 550nm due to the oxidation of reduced cytochrome-C (Applemans *et al*, 1955).

Reagents used:

(A) 41μM cytochrome-C (0.54mg/ml) in 1mM EDTA and 0.03M phosphate buffer, pH 7.4. This solution is reduced by adding small amounts of solid sodium dithionite until about 90% (w/v) of the cytochrome-c is reduced.

(B) Enzyme diluent (TVB) solution containing 0.1g/l Triton X-100, 1mM EDTA and 1mM $NaHCO_3$.

(C) Solid potassium ferricyanide.

The reaction mixture (3.1ml) contained 3ml reagent A and 100μl of enzyme diluted in reagent B. Absorbance was followed continously ($A^{1\%}_{1cm550nm}$) against a blank of completely oxidised (with added solid ferricyanide) substrate solution.

Unit activity of the enzyme is expressed as the amount of cytochrome oxidase which catalyses the oxidation of 90% of the reduced cytochrome present in 1 minute and in 100ml of solution. Thus $A^{1\%}_{1cm550nm}$ of the solution decreases by one unit per minute when the cytochrome oxidase concentration is 1 unit of enzyme per 100ml.

2.2.9 Succinate:Tetrazolium Reductase (E.1.3.99.1)

This enzyme provided a second marker for mitochondrial fractions. Activity was measured by the rate of reduction of the dye 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) (Pennington, 1961).

Reagents used:

(A) Sodium succinate, 0.25M, pH 7.5.

(B) INT, 0.5% (w/v) in 10mM EDTA.

(C) Phosphate buffer, 0.25M, pH 7.3 containing 5mg/ml BSA.

(D) Distilled water.

(E) Sample (or suitable dilution of).

Using 200µl of each of reagents B, C, D and E the reaction mixture was incubated for 1 min at 37°. Reagent A (200µl) was then added and the reaction incubated for 10-20 min at 37°. The reaction was terminated by the addition of 1.0ml of 10% (w/v) T.C.A. Formazan was extracted into 4ml ethyl acetate and the absorbance measured at $A^{1\%}_{1cm490nm}$. Enzyme activity was expressed at µmol formazan produced per min under the conditions of assay. Formazan has a molar extinction coefficient of 20.1 x 10^3 M^{-1} cm^{-1} at 490nm.

2.2.10 Arylesterase (E.C.3.1.1.2)

This enzyme is used as an alternative marker for the microsomal fraction, and is assayed spectrophotometrically by following the rate of formation of indoxyl from indoxylacetate.

Reagents used:

(A) Potassium phosphate, 0.25M, pH 6.8.

(B) Distilled water.

(C) Indoxylacetate, 25mM in 50% (v/v) Ethanol.

(D) Sample.

To the sample was added 1ml reagent A, 2.4ml reagent B and the mixture was preincubated for 2 min. The reaction was started by the addition of 100µl of reagent C and the change in absorbance at 386nm was recorded continuously.

Unit enzyme activity was expressed as µmol indoxyl formed per min under assay conditions. Molar extinction coefficient of indoxyl = 3 x 10^3 M^{-1} cm^{-1} at 386 nm.

2.3 Chemical Determinations

2.3.1 Protein Determinations

2.3.1.1 Method of Lowry

Protein estimation by the method of Lowry *et al* (1951) was performed on samples containing 25-500µg protein/ml.

A stock solution of final volume 1020ml was prepared by mixing 1000ml 2% (w/v) disodium carbonate in 0.1M NaOH, 10ml 1% (w/v) $CuSO_4.5H_2O$ in distilled water and, 10ml 2% (w/v) Na-tartrate in distilled water.

The protein was assayed by mixing 2ml of stock solution with 400µl of dilute protein solution and, after 10 min at room temperature, adding 200µl of Folin-Ciocalteau reagent with rapid stirring. After 30 min the absorbance of the solution was measured at 500nm for high protein concentrations and 750nm for low protein concentrations. Standards were prepared using bovine serum albumin.

2.3.1.2 Biuret Method

A modification of the Biuret method using the quantitate reagent of Gornall (1949) was used for protein concentrations in the range 2-20mg/ ml.

After precipitation in 1 ml 10% (w/v) TCA and centrifugation (1000 x g) the protein pellet was digested in 1 m 20% (w/v) NaOH and 2 ml Biuret reagent. Absorbance was measured, $A^{1\%}_{1cm55nm}$, and protein estimated against a standard curve prepared using bovine serum albumin.

Biuret reagent was prepared by dissolving 1.5g $CuSO_4.5H_2O$ (Analar) and 6g Potassium Sodium Tartrate (Rochelles Salt) in about 500ml distilled water. To this was added 300ml fresh, CO_2-free

10% (w/v) NaOH solution and the mixture was made up to 1000ml and stored in a plastic container.

2.3.1.3 Protein estimation by measurement at $A^{1\%}_{1cm260nm}$ and $A^{1\%}_{1cm280nm}$

The method of Warburg and Christian (1941) was used for rapid determinations of protein. It was particularly useful in monitoring the protein elution profiles of samples undergoing various forms of chromatography.

2.3.2 Determination of Deoxyribonucleic Acid

Deoxyribonucleic acid (DNA) determinations were made using the diphenylamine method of Giles and Myers (1965).

DNA was precipitated from test samples (0.5ml-1.0ml) by the addition of 2.6ml of ice-cold 10% (w/v) TCA. The resultant suspension was centrifuged at 2000g for 5 min using a bench centrifuge, and the procedure repeated on the pellet fraction. The pellet was resuspended in 5ml ice-cold 95% (v/v) ethanol and then centrifuged as above and the procedure repeated once more. The pellet was then drained and the precipitated DNA hydrolysed by the addition of 2.6ml 5% (w/v) TCA and incubation at 90°C for 20 min. After cooling and further centrifuging the supernatant was diluted 1 in 5 with 5% (w/v) TCA. A 1ml sample of the diluted supernatant was added to 2ml diphenylamine solution and the mixture incubated for 180 min at 60°C and the $A^{1\%}_{1cm600nm}$ was measured.

A standard solution of DNA (0.2mg/ml) in 5% (w/v) TCA was used to prepare a calibration graph and the DNA content expressed as μg DNA/ml of fraction.

2.3.3 Determination of Inorganic Phosphate

The procedure was that of Fiske and SubbaRow (1925) using the modifications shown:

Reagents used:

(A) 2.5% Ammonium molybdate in 2.5M H_2SO_4.

(B) 0.01M 1,2,4-aminonaptholsuphonic acid in 1.5M $NaHSO_3$ and 0.01M Na_2SO_4.

A sample of 2.5 ml was mixed with 0.5ml A and 0.2ml B and water was added to 5ml total volume. Absorbance at 660nm was measured after incubation at room temperature in excess of ten minutes.

A solution of 1mM KH_2PO_4 in 0.01 NH_2SO_4 was used as a standard.

2.3.4 Determination of radioactive compounds

Radioisotopes [^{14}C] and [^{3}H] were counted in a Packard Tri-Carb Liquid Scintillation counter Model 3300. The isotopes were present in a variety of environments and their treatment to facilitate the most efficient form for counting utilised a number of scintillation mixtures.

(i) Filter paper

The deposition of samples onto filter paper squares has previously been described (Section 2.1.1). These squares were placed in 8mls of Permablend 1 in a glass (20ml) Scintillation vial (Beckman Inc.). Permablend 1 consists of 5g 2,5 diphenyloxazole (PPO) and 0.5g dimethyl 1,4-di-2-)5-phenyloxazolyl)-benzene(POPOP) in 1000ml of toluene.

(ii) Aqueous samples

When samples were present in solution or as a finely dispersed

tissue homogenate (less than 10%) then dilution in 10ml of Instagel was normally sufficient to produce an accurate isotope determination.

(iii) Proteinaceous samples

When the radioactive samples were not readily soluble in aromatic hydrocarbon based scintillation solutions a special solubiliser was required to produce a homogenous system for reproducible measurement. Soluene-350 (Packard) was used in ratios not exceeding 100mg whole tissue to 1ml of Solubiliser. Samples of tissue slice that had been washed and eventually dried in acetone were rehydrated (e.g. 500mg protein plus 0.2ml H_2O) for 30 minutes prior to solubilisation. Samples were completely solubilised in 4 hrs at 60°C or overnight at 20°C.

To reduce the quenching created by the use of tissue solubilisers, the solubilised tissue (1 ml) was mixed with 10 ml Dimilume-30 (Packard Insts.) and counted after 30 min standing.

(iv) Polyacrylamide gels

Electrophoresis of radioactive proteins on polyacrylamide gels provided material which required special treatment. The gels were first cut to the required size (usually 1-10mm) and thoroughly dried. A solution (0.5ml to 5mm gel) of 19 parts H_2O_2 (30 vols):1 part ammonia solution (Sp.gr. 0.88) was added and incubated for 90 minutes at 37°C to dissolve the gel. The solution formed was taken

up in 10ml Instagel and counted for radioactivity. Quench correction was achieved by the use of internal standardisation using $[^{14}C-]$ or $[^{3}H-]$ n-hexadecane (Radiochemical Centre, Amersham).

2.4 Tissue Fractionation Procedures

2.4.1 Homogenisation

2.4.1.1 Potter-Elvehjem

This procedure (Potter & Elvehjem, 1936) has proved the method of choice in the literature (Fleischer & Kervina, 1971) for liver homogenisation.

Liver tissue was suspended in 3 volumes (w/v) homogenisation buffer (generally 0.25M sucrose/1mM Tris-Cl/1mM EDTA pH 7.4 at 4°C) and minced into 1mm cubes. Homogenisation was effected by three strokes of the teflon pestle (clearance 0.61mm) and a further three strokes of pestle clearance 0.29mm. The pestle was rotated at 1600-1700 rpm throughout and the tissues were maintained at 4°C in ice.

2.4.1.2 Ultrasonication

This method was used to disrupt platelets during sub-fractionation experiments (Barker *et al*, 1971).

Platelets, 3ml aliquots, suspended in Tangens buffer, were treated for 20 seconds in a M.S.E. sonicator at 4°C.

Preparation of Tangens buffer is described in Section 2.10.4.

2.4.1.3 Ultra-turrax

Mincing of tissue and the resuspension of post-centrifugation tissue pellets was facilitated by the use of a variable speed Ultra-turrax (Janke and Kundel, KG).

2.4.2 Differential Centrifugation

Cell fractionation following homogenisation was carried out using differential centrifugation.

The complete fractionation scheme was after the methods of DeDuve (1964, 1965) and is shown in Figure 2.2.

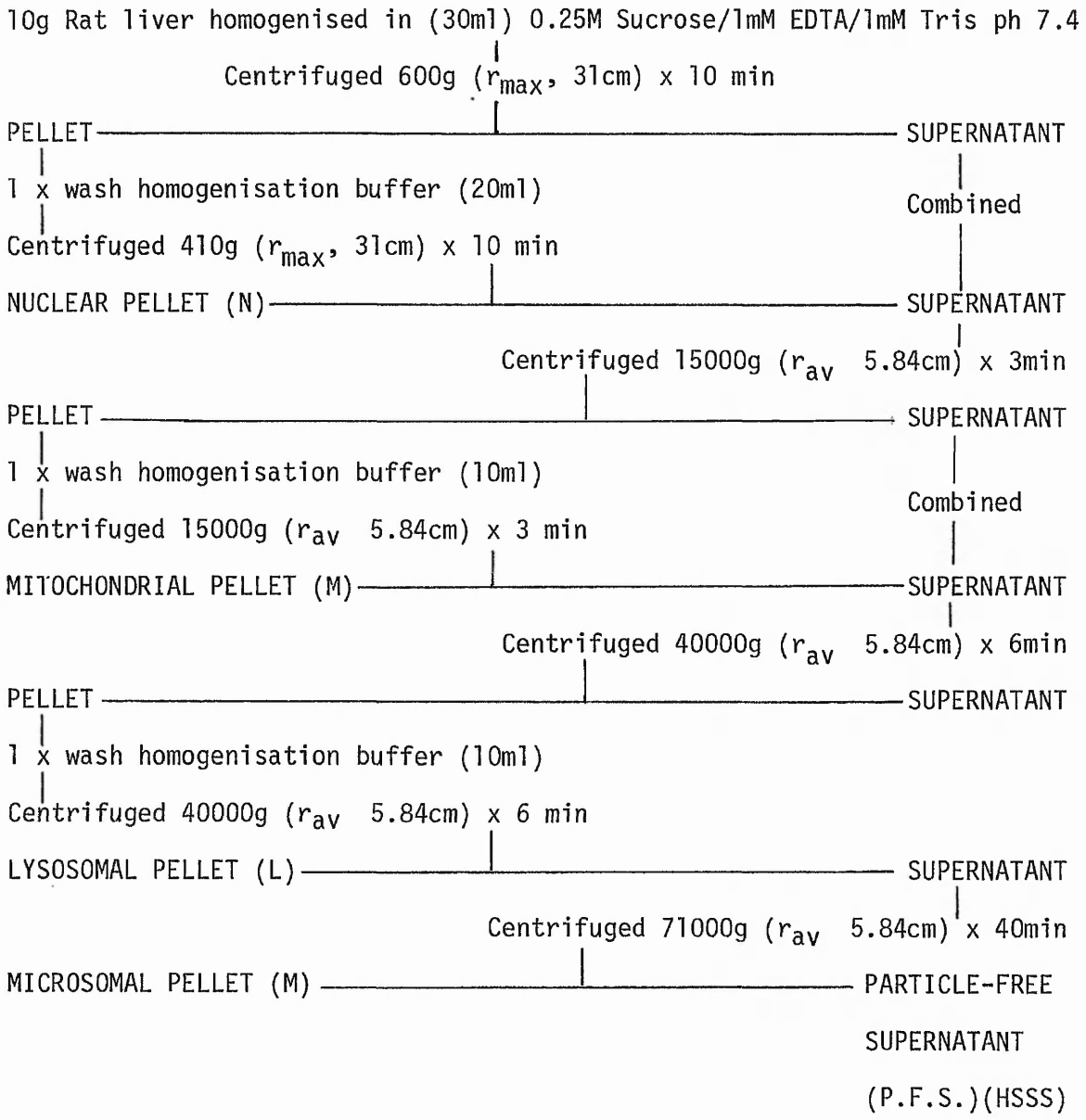


Figure 2.2 Fractionation Scheme

Many experiments required simple differentiation between the soluble and insoluble fractions of homogenised cells. For this, a shortened procedure was employed which allowed the following fractions to be produced:

(i) Homogenates were treated to produce the 'N' fraction and the resulting 'E' supernatant was centrifuged at 71,000g for 40 min to give the P.F.S. and a pellet containing the mitochondrial, lysosomal and microsomal fractions. These fractions were designated high speed spin supernatant (HSSS) and high speed spin pellet (HSSP or MLP).

(ii) When the samples available were small a further shortened procedure was employed in which a single centrifugation of of the homogenate at 71,000g for 40 min which produced the HSSS and HSSP fractions. In this instance the HSSP sample contained the nuclear fraction in addition to those detailed above.

2.4.3 Isolation of Plasma Membranes

The isolation procedure was a modification of the methods of Neville (1960). Plasma membranes were prepared from the 1000g nuclear pellet of rat liver homogenate previously dispersed in hypotonic media. Fractionation of this pellet on isopycnic density gradient centrifugation gave purified membranes at a density of 1.16 - 1.18 g/ml.

Rats were killed as described and the livers perfused with ice-cold 0.9% (w/v) NaCl containing 0.5mM $CaCl_2$. The livers were minced and passed through a loose fitting Dounce homogeniser (Kontes Glass Co.). Connective tissue was removed using a 0.8mm pore diameter sieve and the homogenate diluted to 1:100 (w/v) in 1mM $NaHCO_3$/

0.5mM $CaCl_2$/1mM Dithiothreitol (DTT), pH 7.4 and the filtrate was centrifuged at 1000g for 20 mins at 4°C (MSE Magnum).

The supernatant was discarded and the pellet resuspended in 1mM $NaHCO_3$/0.5mM $CaCl_2$/1mM DTT to half the original volume. This suspension was centrifuged at 1500g for 15 mins at 4°C (MSE Magnum). This latter procedure was repeated twice.

The final pellet was rehomogenised in 5ml of 56% (w/w) sucrose solution and placed at the bottom of a MSE 25ml polycarbonate centrifuge tube. This sample was then carefully overlaid with three 5ml layers of sucrose of densities 1.181 (43% w/w), 1.176 (39% w/w) and 1.153 (35% w/w) respectively. The rotor (3 x 25ml MSE Swing-out) was spun at 100,000g for 120 minutes. The compact white plasma membrane fraction was then collected at the interface of densities 1.176 and 1.153 using a hypodermic syringe with a needle bent to 90° about 1cm above the point.

The membrane fraction was washed twice by dilution and centrifugation and was finally suspended in the bicarbonate buffer described ready for assay.

2.4.4 Concentration of protein extracts

Three methods were employed:

(i) Ammonium Sulphate

Concentration of dilute protein solutions was achieved by complete precipitation at high concentrations of ammonium sulphate (approx. 70% (w/v)) followed by dissolution into much smaller volumes. Dialysis, where necessary, was used to remove the remaining ammonium sulphate.

(ii) Minicon B-15 (Amicon Ltd.)

These concentrators are disposable devices which provide an effective technique for the rapid enrichment of 5ml or less of dilute macrosolute. The B-15 model has a 15000 molecular weight cut-off and provides for a concentration factor of up to 100-fold from an original volume of 5ml

(iii) Centriflo-Membrane Cones (Amicon Ltd.)

The cone is formed from a Diaflo membrane (Amicon Ltd.) which fills a plastic support. This system allows the ultrafiltrate to flow into a collection tube during centrifugation. The CF25 model has a 25000 molecular weight cut-off and was used in the MSE Magnum centrifuge at 750g and 4°C.

2.4.5 Chromatography

2.4.5.1 Diethylaminoethyl-cellulose Chromatography

Anion exchange cellulose (Whatman DE52) (DEAE-cellulose) was supplied as a wet, fully swollen, microgranular slurry which was prepared, degassed, equilibrated, poured, packed and eluted according to the manufacturers instructions.

Elution of protein samples from the bed matrix involved the use of a linear salt gradient consisting 0 → 0.5M NaCl in 50mM Tris/ 1mM EDTA/1mM DTT pH 7.4 and was effected at 4°C.

2.4.5.2 Gel Filtration

Gel filtration using Sephadex beads (Pharmacia Fine Chemicals Ltd.) was used for both molecular weight determination and as a step in protein purification. The gel powder (Sephadex G-100 Superfine) was swollen, de-aerated and packed (using an extension column)

according to the manufacturers instructions. The column was allowed to stabilise in 50mM Tris/1mM EDTA pH 7.4 buffer at 4°C and was assembled in the ascending mode.

Calibration was achieved using a mixture of 0.1% (w/v) Blue Dextran (Pharmacia Ltd.) and 0.1% (w/v) potassium dichromate in 50mM Tris/1mM EDTA pH 7.4. Void (Vo) and elution (Ve) volumes were determined by this mixture. Further calibration of the column, to produce a standard graph of Ve versus log M.Wt. of protein, was carried out using a variety of proteins (see Results). Unknown samples were applied to the column under identical conditions.

2.4.5.3 Hydroxylapatite

Bio-Gel Hydroxylapatite (HTP) (Bio-Rad Laboratories) was supplied as a free-flowing powder and was prepared according to the manufacturers instructions. A syringe was used to accomodate a bed volume of 22ml and elution at 4°C of a protein sample was accomplished using a linear gradient of 200-600mM potassium phosphate in 1mM Tris/1mM DTT pH 7.4. The column was run at 8-10ml/hr using 27cm of head pressure and 3ml samples were collected over a total gradient volume of about 200ml.

2.4.5.4 Hydrophobic (Shaltiel) Affinity Chromatography

This method, first used by Shaltiel (1974) uses a homologous series of hydrocarbon coated agaroses.

A series of six columns, each of bed volume 1ml, (Miles-Yeda Laboratories) with alkyl chain lengths of 0, 2, 4, 6, 8 and 10 carbon atoms were used and elution at 4°C was by the method described by Goldsmith et al (1978). Initially each column was thoroughly washed in 25ml of starting buffer (50mM Tris/1mM EDTA/10mM Ca^{2+}/150mM NaCl).

Sample volumes of 1ml were collected during the elution procedure.

2.4.5.5 Affinity Chromatography

Deoxyribonuclease-I conjugated to Sepharose 4B (Pharmacia Fine Chemicals Ltd.) was used for the isolation of actin by the method of Lazarides and Lindberg (1974).

Cyanogen bromide activated Sepharose 4B was prepared according to the manufacturers instructions by washing 1g of swollen gel on a glass filter using 200ml of 1mM HCl. DNAse I (Sigma) (20mg) was dissolved in 2ml 0.1M $NaHCO_3$/0.1mM $CaCl_2$ and was gently mixed overnight at 4°C with the activated agarose. Unbound material was washed away with coupling buffer and the remaining active groups were reacted with 10ml 1M ethanolamine at pH 8 for 90 min. Non-covalently adsorbed protein was washed away using three cycles of:

(i) 0.1M acetate buffer containing 1M NaCl, pH 4.

(ii) 0.1M borate buffer containing 1M NaCl, pH 8.0.

The gel was then packed into a 5ml disposable syringe. A bed volume of 3ml was used in most procedures.

To standardise the column purified actin (0.5mg) was diluted in an equal volume of 0.5M sodium acetate/1mM $CaCl_2$/30% w/v glycerol pH 7.4 (Buffer A) and applied to the column. The protein was then eluted using 6 ml of each of 3 buffers applied in stepwise fashion:

(i) Buffer A

(ii) Buffer A + 0.75M guanidine-HCl pH 6.5

(iii) Buffer A + 3.0M guanidine-HCl pH 6.5

Fractions (1ml) were collected and assayed for protein spectrophotometrically at 280nm and 260nm.

2.4.6 Electrophoresis

Details of the procedures followed may be found as an appendix (Appendix 1).

2.4.6.1 Polyacrylamide gel electrophoresis (PAGE)

Conventional PAGE techniques (Ornstein and Davis, 1964) were employed in two procedures:

(i) Tris-glycine (pH 8.9)

(ii) TES (N-tris hydroxylmethyl methyl-2-aminoethane sulphonic acid) - TEA (Triethanolamine), pH 7-8.

Procedure (i) was used for the majority of experiments.

The methods followed involved rod or column gels consisting of approximately 6.7cm separating gel and 1.7cm stacking gel in a 10cm glass tube (5.5mm internal diameter, Bio-Rad Laboratories). Samples were investigated using a range of gel concentrations (5% - 10% (w/v) acrylamide) depending on the nature of the proteins included. At the end of a run the gels were placed in fixative, stained using Coomassie brilliant blue R-250 and subsequently destained. The resultant stained protein positions were recorded and the relative mobilities calculated. Molecular weight determinations were made by reference to a standard graph constructed from the relative mobilities of known proteins. Gels were also recorded using the SP1809 gel scanning attachment for the Pye-Unicam SP1800 spectrophotometer.

2.4.6.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis under denaturing conditions has been well documented since its introduction by Shapiro et al (1967) and

Weber and Osborn (1969).

The well known phosphate system (pH 7.1) was followed using LKB 2117 Multiphor equipment (LKB-Produkter AB, Bromma, Sweden) for horizontal flat-bed thin layer electrophoresis (Appendix 1). Molecular weight determinations were made by reference to a standard graph compiled from the relative mobilities of SDS-molecular weight markers (Sigma).

2.4.6.3 Isoelectric Focussing

Vesteberg and Svensson (1966, 1967) developed the technique of establishing pH gradients using carrier ampholytes (saturated aliphatics substituted to varying extents with carboxylic acid and amino groups). These compounds with molecular weights in the range 300-600 form a continuous spectrum of isoelectric points and are commercially available as Ampholine (LKB Produkter AB). pH Gradient stabilised in gels allow a rapid, small scale modification of the sucrose density medium used by the original method. Variations of the technique have also been published (for short discussion see Wrigley, 1972).

The method followed was to produce a pH gradient of approximately 3-10 in 5.5% (w/v) acrylamide rod gels and to focus the sample proteins within the gel at their isoelectric points. This was achieved by electrolysis using 0.2% (v/v) sulphuric acid at the anode and 0.4% (v/v) ethanolamine at the cathode.

The resultant gels were removed from the tubes and either processed through the staining procedure to establish the positions of the proteins in the sample, or cut up into small lengths and placed in a small volume of distilled water. This latter course of action

would, after allowing the water to assume the pH of the gel, lead to the construction of a standard graph from which the pH at any point on the gel could be derived. This information would provide the isoelectric points (pI) of the sample proteins.

Detailed information regarding the procedure followed may be found in Appendix 1.

2.4.7 Histochemical Studies

The use of monodansylcadaverine in its role as a fluorescent donor amine substrate for transglutaminase was utilised in order to ascertain the cellular distribution of the enzyme by fluorescence microscopy. The method of tissue slice staining has been used for demonstration of gross distribution of epidermal transglutaminase (Buxman and Wuepper, 1975) and monodansylcadaverine has been incorporated into enzyme assay procedures on a number of occasions (Lorand et al, 1971).

Rat liver tissue was rapidly excised and 2mm cubes were wrapped in Aluminium foil and plunged into liquid nitrogen. These tissue blocks were then fixed to the chuck of a freezing cryostat (Slee) using a polar spray. Sections (5μm) were prepared at -18°C and placed on prepared microscope slides.

The slice morphology was assessed after staining with Haemotoxylin and Eosin (Bancroft and Stevens, 1975). Stains for acid phosphatase (Gomori, 1950) and 5'-nucleotidase (Gomori, 1949) were used to assess enzyme activity after the freezing and thawing involved in the methodology.

The staining procedure for transglutaminase activity was as follows:

Stain used: 3mM Monodansylcadaverine

10mM $CaCl_2$

3.85mM Dithiothreitol or 5mM cysteine

Reagents all prepared in 50mM Tris/100mM NaCl pH 7.4.

Slices were incubated for 45 min at 37°C in the dark. Controls were prepared where 1mM EDTA replaced $CaCl_2$. After the incubation period the slices were washed using four changes of phosphate buffered saline over a period of 60 min.

Stained sections were viewed using a BG3 filter exciting wavelength 355nm, and a 530nm barrier filter (Leitz Ortholux II with incident ultraviolet illumination). Slides were stored at 4°C in the dark, maintaining a moist atmosphere.

2.4.8 Autoradiography

Autoradiography, at the light microscope level, was used to follow the distribution of $[^3H]$-putrescine in rat liver slices.

Rat liver slices were prepared and incubated according to the methods detailed in Section 2.8 with the exception that the amino substrate used was $[^3H]$-putrescine, and the incubation period was for 120 min at 37°C. The reaction was terminated by placing the slices in 1mM EDTA.

The slices were dehydrated through graded alcohols and prepared paraffin sections (5μm) were placed on gelatin-subbed slides and treated by the following procedure based on the work of Rogers (1979).

Ilford K2 gel was used in the following procedure. Two cylinders were marked, one at 30ml and another at 15ml and a third cylinder prepared containing 15ml water and 0.3ml glycerol. The safelight

(Ilford F904) was used at this stage when the emulsion was removed from the refridgerator and, using plastic forceps, added to the 30ml mark on the cylinder. The gel was placed at 43°C in a water bath until molten (about 10 min with gentle stirring) and using the second cyliner 15ml was removed and placed in the water bath. The 15ml molten gel and water/glycerol were combined and mixed and left to stand for 2 min whilst gently tapping the cylinder to remove air bubbles. When a test slice revealed an even coating of the gel on the slide the tissue sections were dipped, wiped on their free side, and placed on a cold plate (4°C) for 15 min. Finally the slides were left at room temperature for 20 min, dessicated over night and packed in light-tight boxes containing silica gel and stored at 4°C. A control slide was exposed to the light in order to assess latent image fading and all slides marked with a diamond pencil to aid identification by touch.

After a mathematically determined period based on the decay of the radioactive compound and the gel used, the slides were developed using Ilford Phenex. The following procedure was adopted:

(i) Developing, 2 min without agitation.

(ii) Stop bath (1% (v/v) acetic acid), 1 min.

(iii) Fixative, 30% (w/v) sodium thiosulphate, 10 min.

(iv) Wash, running tap water, 15 min.

Sections were stained in Harris' Haematoxylin for 2 min followed by washing in water for 15 min. Dehydration through 70% and 90% (1 min each) ethanol, then two changes of absolute alcohol and two changes xylene (2 min each) completed the treatment. Slides could be mounted in neutral balsam and following viewing were stored in boxes at room temperature.

2.5 PREPARATIONS

2.5.1 Actin

Actin was purified from an acetone powder by the method of Spudich and Watt (1971).

(i) Acetone Powder method (Morton, 1955).

Finely disintegrated rabbit leg muscle was suspended in 0.01M phosphate buffer, brought to pH 6.5 and cooled to 0°C. The suspension was very slowly added to 10 vol. of dry acetone at -15°C with rapid stirring, and held at this temperature for 10 min. The precipipate was then collected by filtration (Whatman No. 1) on a Büchner funnel at 0°C. The residue was washed twice by resuspension into 3 vol. dry acetone at -15°C and the acetone removed by drying under a stream of nitrogen and subsequently in vacuo over H_2SO_4. The dried material was stored in vacuo over $CaCl_2$ at -20°C, at which temperature it was stable for long periods.

(ii) Actin purification

Acetone powder (10g) was added to 200ml Buffer A (2mM Tris/0.2mM ATP/0.5mM β-mercaptoethanol/0.2mM $CaCl_2$ pH 8 at 25°C) and left at 0°C for 30 min. The suspension was filtered through a coarse scintered glass funnel and the residue washed with 100 vol. Buffer A. The filtrates were combined and centrifuged at 10000g for 60 min. About 400mg of protein was normally present. Potassium chloride (final concentration 50mM) and $MgCl_2$ (final concentration 2mM) were added and the actin allowed to polymerise for 2 hr. Potassium chloride was then added to a final concentration of 0.6M and the solution stirred for 90 min and then centrifuged at 80000g for 3 hr. The pellet was resuspended in 30ml Buffer A and dialysed with stirring

for 3 days against 100 vol. Buffer A (changed every 24 hr). The prepared G-actin was clarified by centrifugation (80000g/3 hr) and polymerised in 50mM KCl to yield about 200mg of protein. Actin was stored at 4°C and was used within 7 days of preparation.

2.5.2 N,N'dimethylcasein

N,N'dimethylcasein was prepared according to the method of Lin et al (1969).

Casein (1.5g) was dissolved in 150 ml 0.1M borate buffer pH 9.0 and then cooled to 0°C. Sodium borohydride (300mg) was added, with stirring, and 2 drops n-octanol prevented losses by foaming. Formaldehyde (3 ml) was added in 100µl aliquots over 30 min and the pH adjusted to pH 6 using 50% (v/v) acetic acid. This solution was dialysed against deionised water and finally freeze-dried and stored at -20°C as a fluffy white solid.

2.5.3 Thrombin

One vial of thrombin (Bovine Topical, Parke-Davies) was taken up in 3-5ml of 50mM Tris/100mM NaCl pH 7.4 and dialysed against the same buffer at 4°C for 120 min. A small volume of this thrombin was diluted to 1:10 and assayed in the following manner:

(i) 200µl thrombin solution

(ii) 200µl fibrinogen solution containing:

(a) 2ml 1.5% (w/v) fibrinogen

(b) 6ml 50mM Tris/100mM NaCl pH 7.4

(c) 2ml 0.3M KCl

(iii) 5µl 1.0M $CaCl_2$

The time taken for the fibrin clot to form at 37°C was measured. The dilution factor of the thrombin was adjusted so that the clotting

time was in excess of 10 sec. Activity was expressed as NIH units, one unit being the amount of thrombin required to produce a fibrin clot in 15 sec at 37°C.

Aliquots (500µl) of thrombin were stored at -20°C as a 1000 NIH units/ml solution.

2.5.4 Fibrinogen

A 2% (w/v) solution of human fibrinogen (Kabi, Sweden) was prepared in 50mM Tris/100mM NaCl, pH 7.4 and dialysed against 50 volumes of the same buffer containing 1mM EDTA for 120 min at 4°C. Aliquots (1ml) of this solution were stored at -20°C until required.

The fibrinogen prepared in this way was found to be contaminated with Factor XIII. Commercial sources claiming to be Factor XIII-free (Behrin Chemicals) were found to contain trace amounts. In order to produce fibrinogen which would not show stabilisation via ε(γ-glutamyl) lysine crosslinks in the presence of Ca^{2+} and thrombin, other than by the addition of tissue transglutaminase,the fibrinogen preparations were exposed to high urea concentrations (Schwartz et al, 1971) in order to irreversibly inactivate the endogenous Factor XIII.

Fibrin was prepared from fibrinogen by the methods detailed in Section 2.9.3.

2.6 Enzyme Solubilisation

A series of experiments were undertaken on membrane-bound rat liver transglutaminase in which the enzyme was subjected to conditions designed to remove it to the soluble state. These experiments entailed the addition of particular buffer systems to the 'nuclear pellet' cellular subfraction.

The systems used were based on the 0.25M Sucrose/1mM Tris/1mM EDTA, pH 7.4 buffer modified to give the following:

(i) 0.25M Sucrose/1mM Tris/1mM EDTA, pH 7.4

(ii) " " /2.5mM EDTA, pH 7.4

(iii) " " /5mM EDTA, pH 7.4

(iv) " " /1mM DTT/3mM Ca^{2+}, pH 7.4

(v) " " /1mM EDTA/1mM putrescine, pH 7.4

(vi) " " /3mM Ca^{2+}/1mM putrescine, pH 7.4

(vii) " " /3mM ATP/5mM Mg^{2+}, pH 7.4

(viii) 10mM Tris/0.1mM ATP/1mM DTT/0.1mM Ca^{2+}, pH 7.4
(Actin solubilisation buffer)

(ix) 0.9% (w/v) NaCl/1mM Tris/1mM EDTA, pH 7.4

(x) 0.25M Sucrose/1mM Tris/1mM EDTA/4% (v/v)n Butanol, pH 7.4

(xi) " " " /0.1% (v/v) Triton X-100
0.1% (v/v) Na taurocholate.

(xii) 0.25M Sucrose/1mM Tris/1mM EDTA/5mM phenylalanine, pH 7.4

(xiii) " " " /0→500mM KSCN

(xiv) " " " /0→50% (v/v) ethyleneglycol.

The total activity of transglutaminase was determined in each case and evaluated for release of the enzyme from the insoluble to the soluble fraction.

2.7 Tissue Slice Experimentation

Rat liver tissue was rapidly exised following the death of the animal and the chordate lobe trimmed to facilitate slicing. Slices (0.5mm) were cut using a tissue chopper (McIlwain, Mickle Lab. Eng. Co.) and immediately placed in 4°C buffer. The buffers used throughout were based on the modified Krebs-Ringer-Phosphate system with the exceptions that the $CaCl_2$ concentrations were altered and the phosphate was replaced by N-2-hydroxylthylpiperazine N'-2-ethane sulphonic acid (HEPES, Gibco-Biocult).

The incubation buffer consisted of:

130mM NaCl

5.2mM KCl

1.29mM $MgSO_4$

11mM glucose

20mM HEPES, pH 7.4.

Various additions to this buffer included 5mM and 1.9mM $CaCl_2$ and 1mM EDTA.

Incubation media were made up to include 1.2mM putrescine consisting of 0.3mM $[^{14}C]$-putrescine and 0.9mM 'cold' putrescine diluted in 50mM Tris-HCl, pH 7.4.

The protocol for the incubations was as follows:

Each rat liver slice was weighed (40-50mg wet weight approx.) and placed in a glass scintillation vial (Beckman). Approximately 100mg of tissue was used per millilitre of incubation mixture, to a maximum of 300mg/3 ml/vial. Vials were placed in a shaking water bath at 37°C and O_2/CO_2 (95:5(v/v)) was bubbled through the medium at a rate of 1-2 bubbles/sec. Incubation times varied but were generally of 60 minutes duration.

At the end of the incubation the slices were treated in different ways depending upon the analysis required.

(i) Samples were blotted on a clean glass surface and each placed in 1ml Soluene-350. These protein solutions were diluted into 10mls Dimilume and counted for radioactive decay. This result provided the total uptake of putrescine into cells and intracellular spaces.

(ii) Samples were homogenised (Potter-Elvejhem) in 5ml 10% (w/v) TCA to precipitate all proteins and then washed free of unbound putrescine. This was accomplished by rinsing the insoluble pellet in 2 x 5ml 5% (w/v) TCA, 1 x 5ml ethanol: acetone (50:50 (v/v)) and 1 x 5ml acetone each step requiring centrifugation (2000g for 5 min) to retain the pellet fraction. Samples were dried to remove the acetone, rehydrated for 30 min in 200µl water and, taken up in 1ml Soluene-350 and added to 10ml Dimilume and finally counted for radioactivity. This result gives the total covalent uptake of putrescine into the tissue proteins.

(iii) Samples for which the subcellular distribution of soluble/ insoluble protein uptake was required were placed in 1mM EDTA after the incubation. These samples were homogenised in 1mM EDTA buffer using a Potter-Elvehjem and centrifuged at 70000g for 40 min. The resulting fractions were suspended in 10% (w/v) TCA (about 5ml) and washed free of non-covalently bound putrescine as previously described.

(iv) A 10µl sample of incubation medium was placed in 10ml Instagel and counted as a measure of the amount of

$[^{14}C]$-putrescine present in the medium.

Appropriate controls were employed for each sample and quench correction was determined by internal standardization using $[^{14}C]$-n-hexadecane.

The viability of the tissue slices under the incubation procedure was assessed by measuring protein efflux from the slices. Slice to medium ratios of putrescine content were also monitored in each series of experiments.

2.8 Substrate Determinations

2.8.1 Self-Incorporation

(i) Samples of H, NP, E, HSSP and HSSS fractions were incubated with $[^{14}C]$-putrescine as in the casein incorporation experiment. However in this method the acceptor protein casein was ommitted and the activity of the enzyme towards native proteins determined. Casein was added to the system towards the end of the assay to verify that the enzyme had remained active.

(ii) SDS-PAGE of homogenised samples prepared as above was used to determine the molecular weights of the proteins into which $[^{14}C]$- putrescine had been incorporated. Samples in 2% (w/v) sodium dodecyl sulphate (SDS):2% (w/v) mercaptoethanol:6M urea were dialysed against 200 volumes 0.1% (w/v) SDS/0.1% (v/v) mercaptoethanol to remove non-covalently bound putrescine. The samples were then applied to 5% and 7.5% polyacrylamide gels and the electrophoresis was conducted as described in Section 2.4.6. The gels were stained using Coomassie Brilliant Blue G-250 and the relative mobilities of the proteins measured. The gels were then cut into 5mm sections dried, dissolved and prepared for counting of the incorporated radioactivity as described in Section 2.3.4. Standard proteins were electrophoresed simultaneously in order that the molecular weights of substrate proteins could be determined.

(iii) Samples derived from tissue slice incubations (Section 2.7) rather than from homogenate fractions were also used.

Liver tissue slices (300mg) were incubated as previously described for 120 min at 37°C. Total uptake and covalent incorporation was assessed using 200mg and the remaining 100mg was cooled to 4°C and homogenised (Potter-Elvehjem) in 1mM EDTA/50mM Tris, pH 7.4. The homogenate was centrifuged (71000g for 40 min) to give HSSP and HSSS fractions and these samples treated as were the homogenate samples above to yield the molecular weights of the substrate proteins.

2.8.2 Actin

Rat liver slices (300mg) were incubated as detailed in Section 2.7 for 60 min at 37°C. The slices were then cooled to 4°C and homogenised, using a Potter-Elvehjem, in 3ml 2mM Tris-Cl/0.2mM ATP/0.5mM mercaptoethanol/0.2mM $CaCl_2$, pH 8.0 (Actin Solubilisation Buffer). The resulting homogenate was centrifuged at 71000g for 40 min. The supernatant was passed through an affinity column as described in Section 2.4.5.5. Elution of actin was monitored by protein estimation ($A^{1\%}_{1cm280nm}$) for the 1 ml samples collected. Finally the samples were taken up in 10ml Instagel (Packard Instr.) and counted for radioactivity and quench correction (using [^{14}C]-n-hexadecane internal standardisation).

2.8.3 Fibrin and Fibrinogen Crosslinking

Crosslinking of fibrin and fibrinogen by transglutaminase was carried out using the following reaction medium (0.3 ml final volume):

(i) 0.8% (w/v) Factor XIII-free fibrinogen in 50mM Tris/100mM NaCl, pH 7.4.

(ii) 3.6mM Dithiothreitol

(iii) 20mM $CaCl_2$ or, 2mM EDTA

(iv) Enzyme sample

(v) 50mM Tris/100mM NaCl, pH 7.4.

Fibrin crosslinking was carried out following the addition of 5 NIH units of Thrombin to the incubation mixture. The addition of EDTA in place of Ca^{2+} was used as a control. Reactions were started by the addition of transglutaminase and incubated at 37°C. The addition of 300µl 3% (w/v) sodium dodecyl sulphate:3% (v/v) mercaptoethanol:9m (w/v) urea at time intervals 0, 10, 40, 120 and 240 min terminated the reactions. Samples were analysed using the procedure described in Section 2.4.6 for SDS-PAGE electrophoresis.

2.9 Model Systems

2.9.1 Hepatomegaly in rats

Four compounds of the chlorophenoxyisobutyrate (C.P.I.B.) family were used to induce hepatomegaly in adult rats (female). These compounds, of I.C.I. manufacture, produced a condition of work hypertrophy in the rat liver during the 14 days dietry administration prior to sacrifice. The following compounds and dosages were investigated:

(i) 112660 at 0.02% and 0.2% (w/w)

(ii) 65126 at 0.005% and 0.05% (w/w)

(iii) 55695 at 0.05% (w/w)

(iv) 79855 at 0.005% and 0.05% (w/w)

Livers were excised after 14 days and fractionated according to the shortened procedure. The fractions were assayed for marker enzymes glucose-6-phosphatase, 5'nucleotidase, protein, DNA and transglutaminase according to the methods previously described. Samples were also removed for tissue slice experiments (Methods 2.4.7) on I.C.I. 112660 (0.2%).

2.9.2 Rat Liver Regeneration

The surgical method followed was after Higgins and Anderson (1931) and resulted in the removal of the chordate and left lateral lobes close to the hilium (approx. 70% of liver). The portal cardiovascular system was ligatured using Merslik. Sham operated controls had their livers gently manipulated but no tissue was removed. Anaesthesia was maintained during the operations using Fluothane (I.C.I. Ltd.).

Samples of tissue were removed from the periphery of the remaining liver lobes at days 0, 3, 8, 13 and 20 post-operation. The tissue was taken up in 3 vol. 0.25M Sucrose/1mM Tris/1mM EDTA, pH 7.4, homogenised (Potter-Elvehjem) and centrifuged by methods described. Fractions H,

NP, HSSP and HSSS were assayed for protein, DNA, 5'Nucleotidase, glucose-6-phosphatase and transglutaminase.

2.9.3 Studies with Foetal Tissue

The pattern of transglutaminase distribution and activity was monitored during foetal maturation.

Day zero was taken as the day the copulation plug was discharged by the female rat. Sampling of liver tissue was carried out after 17, 18, 19, 20 and 21 days and 2 and 10 days after birth. Parturition occurred at day 21.

The tissue was homogenised (Potter-Elvehjem) in 0.25M Sucrose/ 1mM Tris/1mM EDTA, pH 7.4 and centrifuged (70000g for 40 min) to provide homogenate, particulate and soluble cell fractions. Samples were assayed for transglutaminase and protein and DNA.

2.9.4 Platelet Studies

Human blood (20ml) was removed into 3.8% (w/v) acid citrate dextrose (as 10% (v/v) of total volume).

Samples were centrifuged at 1200 rpm/15 min (MSE bench centrifuge) to prepare platelet-rich-plasma (PRP). Further centrifugation (2250rpm/ 15 min) of this PRP gave a supernatant of platelet-poor-plasma (PPP).

Platelet aggregation studies were carried out using a light-scattering aggregometer (Payton Co. Ltd.) and chart recorder (Kipp and Zonen BD8). The instrument was calibrated using 300µl of PRP and PPP to give 10% and 90% readings respectively. Aggregometry was carried out at 37° using a stirrer speed of 800 rpm and recording at 2mV and 20mm/min.

Washed platelets were prepared by the method of Tangen et al (1971). A Sepharose CL2B (Pharmacia Ltd.) column with a bed volume of 100ml was

prepared and 8ml PRP was layered on top. Elution (about 2ml/min) used Tangen's buffer containing:

100mg glucose

19.9mg $MgCl_2$

39.9mg KCl

147.2mg $CaCl_2$

Dissolved in 100ml 0.145M Tris-Cl, pH 7.6 and made up to 1000ml with 0.14M NaCl.

1.6% (w/v) Albumin was added prior to using the buffer.

Platelets were eluted at a volume of approximately 30ml and were identified by the opalescent quality they impart to the solution. The washed platelets were activated using a range of well characterised agonists. The required concentration of each was determined experimentally and subsequently used for further study. The agonists used included:

(i) Adenosine diphosphate to provide a basis for aggregation measurements.

(ii) Collagen (Sigma)

(iii) Arachidonic Acid (5, 8, 11, 14 Eicosatetraenoic acid from porcine liver as 10mg/ml in pre-gassed 10mm Na_2CO_3 stored in a N_2 atmosphere and in the dark).

(iv) Thrombin (Human; 2500 NIH units/ml).

Samples of washed platelets were activated and then treated to ultrasonic disintegration for 5 mins at 4^oC using 70% efficiency. The suspension was then centrifuged 48000g for 30 minutes. Samples of washed platelets, platelets + agonist, sonicated homogenates, and soluble and insoluble fractions of sonicated activated platelets were assayed. Assays included "active" transglutaminase (by the $[^{14}C]$-putrescine into casein assay), zymogenic transglutaminase (by a pre-

assay incubation of 15 minutes at 37°C with 5 units NIH Thrombin) and protein (Lowry).

2.9.5 Tumour Studies

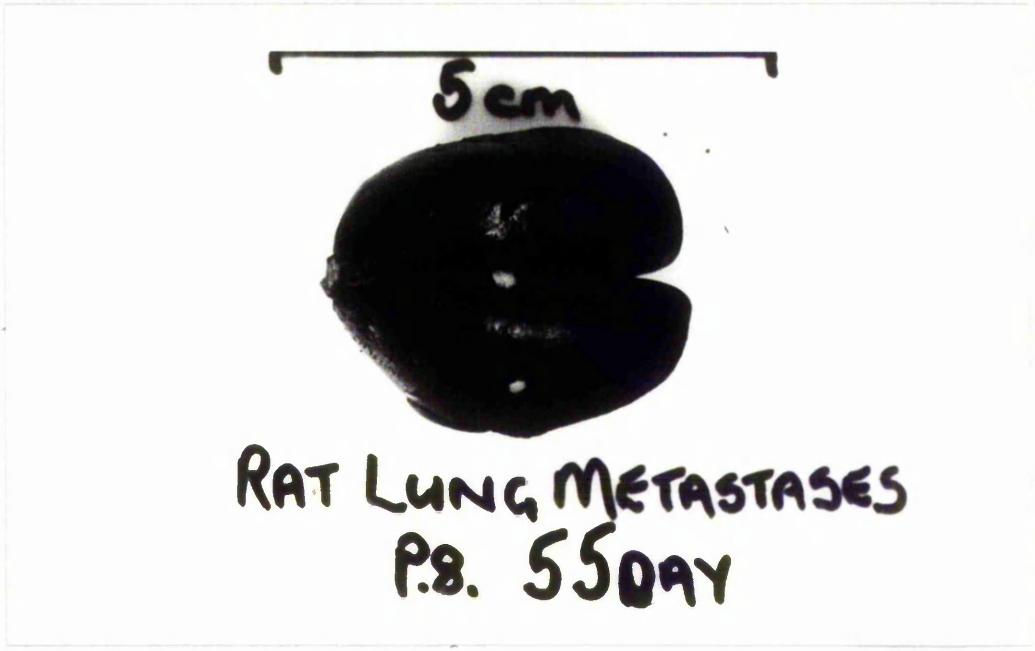
Studies with rat sarcomata

A series of tumours, of which 2 showed metastatic potential, were investigated in order that any changes in transglutaminase activity and distribution might be assessed. Tumours were sampled with particular reference to their pre- and post-metastatic states. All tumours were propogated in in-bred female AS rats and were kindly provided by the Charles Salt Research Institute, Agnes Hunt Orthopaedic Hospital, Oswestry.

The tumours were propogated by the implantation of fresh peripheral tumour tissue ($3mm^3$) into a subcutaneous pocket formed high on the shaved and sterilised flank of an ether-anaesthetised rat. The wound was closed using suture clips.

The life history of the rat sarcomata has been previously described (Moore, 1972). P8, a fibrosarcoma, was originally induced as an osteosarcoma by internal radiation with $[^{32}P]$. This tumour continously metastasises to the lungs and lymph nodes. P7, a sarcoma originally induced as an osteosarcoma by $[^{32}P]$, frequently metastasises to the lungs. MC3, a chemically induced sarcoma of soft tissue origin, does not metastasise. CC5, originally induced as in osteosarcoma by the carcinogen cupic chelated N-hydroxy-2-acetyl aminofluorene, does not metastasise.

Metastasis was detected by employing the method of Wexlar (1966) to determine the appearance of secondaries in lung tissue. The normal morphology of early tumour growth was difficult to assess as it was visually similar to lung tissue. The lungs were therefore filled with a contrast medium consisting of a 15% v/v solution of commercial Indian



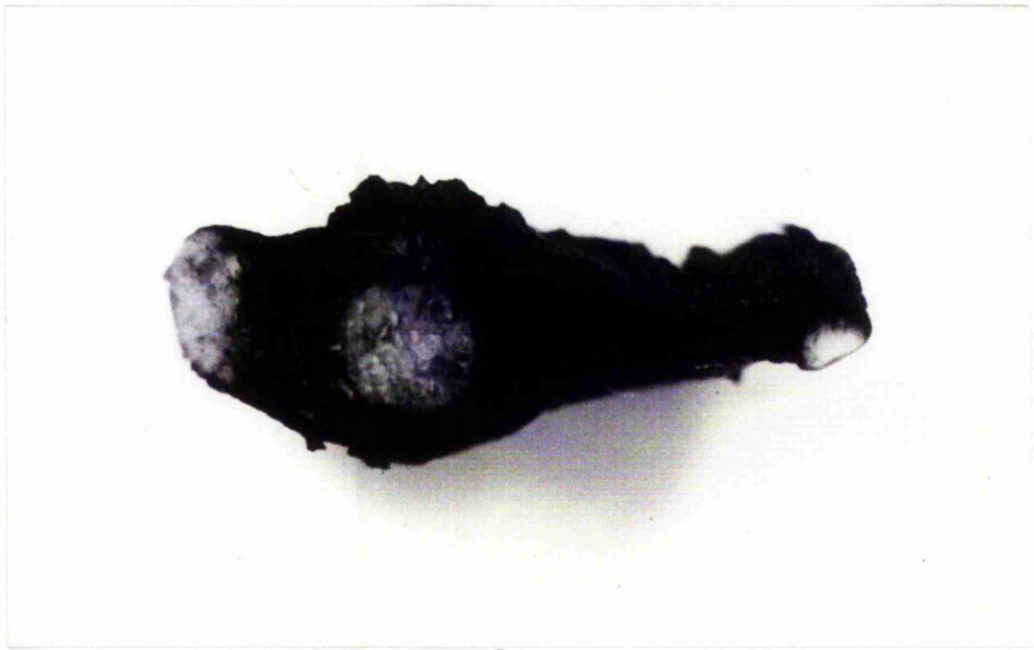


Figure 2.3 Lung Metastases of rat sarcomata P8
(tumour tissue contrasted white on black in whole and transverse section of rat lung)

ink in distilled water inclusive of a few drops of ammonia solution to aid dispersion.

This solution (7-10ml) was injected in to the lung using a broad, blunt needle fixed tightly at the transected end of the trachea. Massage of the lungs aided infiltration throughout the tissue. The lungs were then dissected en bloc from the thoracic cage and the trachea ligated. The tissue was rinsed in tap water and placed in Feketes solution for 24 hours. This fixative contained 100ml 70% Ethanol, 10ml formaldehyde, 5ml glacial acetic acid. The Indian ink stained the lung tissue black and was preserved indefinitely. The tumour metastases, where no ink had infiltrated, were contrasted white on black. Figure 2.3.1 shows stained rat lung with small white P8 metastases visible at the surface. Figure 2.3.2 shows a transverse section of a lobe containing large metastases.

Experiments were undertaken in order that detailed information about the tumour derived enzyme could be gained in addition to the activity and distribution of the tumour transglutaminase.

(a) A 600g pellet was derived from a P8 (39 day old) tumour equivalent to the NP fraction of liver. This pellet was then washed as described in Section 2.7 using two different buffer systems.

(b) A tissue slice incubation (Section 2.8) using the methods described was undertaken on a P8 (39 day old) tumour. Incubation media included 5mM and 1.9mM Ca^{2+} and 1mM EDTA as a control. Samples were assayed for total uptake, covalent incorporation and sub-cellular distribution of $[^{14}C]$-putrescine label.

3.0 RESULTS AND DISCUSSION

3.1 Purification and properties

3.1.1 Purification of Rat Liver Transglutaminase

The isolation methods of Folk (1969, 1971) were modified by Connellan et al (1971) to give a procedure which workers widely adopted. This method has been applied to guinea pig liver and, more recently, Abe et al (1977) have used a further modified procedure for the purification of the rabbit liver enzyme. The inclusion of a protamine sulphate extraction stage in both these methods was felt to be potentially deleterious to the enzyme and we have undertaken a further modification of the Connellan et al (1971) procedure. The results presented are derived from a total of four purifications.

Preparation of crude extract

Fresh rat livers (180g) were excised and placed in approximately 3 volumes of 50mM Tris/1mM EDTA pH 7.4. The tissue was roughly minced, then homogenised initially using an Ultra-turrax at low speed to disperse the tissue and further by using a Potter-Elvehjem with glass/teflon, "tight fitting", motor-drive apparatus (See Methods). This and all further operations were carried out at 2-4°C. The homogenate was centrifuged 23000g for 90 min (Medical, Scientific Equipment model 25 with 6 x 300ml rotor, r_{av} 9.5cm) and the supernatant fraction (400mls approx.) was collected.

Ammonium sulphate precipitation and extraction

The supernatant fluid was brought to 30% saturation of ammonium sulphate by the addition of a saturated solution made up in homogenisation buffer. This was accomplished by the gradual mixing of the two fluids over 15 min at 4°. The resultant suspension was centrifuged 71000g/40 min. This supernatant fluid was then adjusted to 65%

saturation by the gradual addition over 15 min/4° of solid ammonium sulphate. The suspension was again centrifuged (71000g/40 min) and the pellet fraction dissolved in approximately 100 mls homogenisation buffer. This fraction was dialysed overnight against 50 volumes 50mM Tris/1mM EDTA/1mM putrescine/1mM DTT, pH 7.4. Insoluble material was removed by centrifugation (71000g/20 min) prior to the next stage of purification. The highest quality (Aristar) ammonium sulphate was used throughout the experimentation.

1st DEAE-cellulose chromatography

The dialysed sample was applied to a column (150mm x 50mm) of DEAE-cellulose equilibrated with 50mM Tris/1mM EDTA/1mM DTT, pH 7.4. Elution was effected using 1500ml linear gradient of 0-1.5M NaCl in the same buffer (30ml/hr) and 15ml samples were collected. The fractions were assayed for transglutaminase activity by the incorporation of $\left[^{14}C\right]$ -putrescine into casein and the fractions showing high activity (volume 492-552ml) were pooled (Figure 3.1). The pooled eluate was concentrated by precipitation in 70% (w/v) ammonium sulphate, slowly added as solid, and centrifuged (71000g/20 min). The pellet obtained was dissolved in about 10mls homogenisation buffer. The linearity of the gradient used was by measuring the conductivity of the eluted fractions (Electrolytic Conductivity Measuring Set Model MC-1 Mark IV; Electronic Switchgear "London" Ltd.) and the NaCl concentration was derived from a nomogram.

Gel Filtration

The sample was applied to a column of Sephadex G-100 (superfine) (Pharmacia Fine Chemicals Ltd.) by passage through a 0.45μ filter (Millipore Ltd.). The column (25mm x 500mm) was pre-equilibrated in 50mM Tris/1mM EDTA and eluted with this buffer using the ascending mode, with a flow rate of about 12ml/hr. Fractions were assayed for

transglutaminase by $\left[^{14}C\right]$ - putrescine incorporation into casein and the peak fractions pooled (volumes 101-111ml) (Figure 3.3).

2nd DEAE-cellulose chromatography

The pooled samples (about 15mls) were applied to a second DEAE-cellulose column. This column (bed volume 22ml) was prepared in a plugged 20ml disposable syringe (65mm x 15mm). A linear gradient (0-0.6M NaCl in 50mM Tris/1mM EDTA pH 7.4) of total volume 200mls was applied to the column (20ml/hr) and the fractions obtained (3ml) assayed for transglutaminase. Peak activity fractions (volumes 100-126ml) were pooled and stored as 1ml aliquots at -25°C, where they exhibited negligible loss of activity for up to four weeks.

Table 1 summarises the purification and shows a mean 414-fold improvement in specific activity (best fraction ≡ 617) which compares favourably with published results. The yield of 4.7% is low in comparison to the guinea pig and rabbit liver yields (about 20%) but was sufficient to provide enzyme activity for the following experiments. The low yield is in some way explained by the rigorous collection of only the purest enzyme fractions after chromatographic steps and the number of sulphate precipitation steps required for the concentration of the enzyme.

Hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories Ltd.) was evaluated as an alternative to the final DEAE-cellulose chromatographic step (results not shown). This form of calcium phosphate was considered because the enzyme requires Ca^{2+} for activity and may have bound preferentially to this matrix. Elution, of the 22ml column, using a phosphate gradient of 0-200mM (as recommended by the manufacturers) failed to yield any enzyme protein. Increasing the gradient concentration finally yielded the enzyme in the range 250-350 mM phosphate, which

is close to the recommended concentration for the regeneration of the column. The enzyme protein was still active, though some reduction over what might have been reasonably expected was observed. This was probably due to the detrimental exposure of the active site of the enzyme during elution and despite the presence of dithiothreitol in the buffers. This method was therefore not adopted, as even though a good yield of purified enzyme protein was achieved, the specific activity was low.

No detailed literature reports of the purification of this particular enzyme have been described. The following investigation was undertaken in order to provide the necessary information for the design of further experiments.

Results - Figure 3.1

DEAE-cellulose chromatography (1)

The first DEAE-cellulose chromatographic step used a column (150mm x 50mm) equilibrated with 50mM Tris/1mM EDTA pH 7.4, and elution comprised 1500ml of a linear salt gradient, 0-1.5M NaCl, in the same buffer. Samples (15ml) were collected and assayed, as shown on the graph, for protein (by absorbance at 280nm (—————)), transglutaminase specific activity (——o——) and conductivity in milliMHO (—•——•—). Results as expressed are representative of four fractionation programmes.

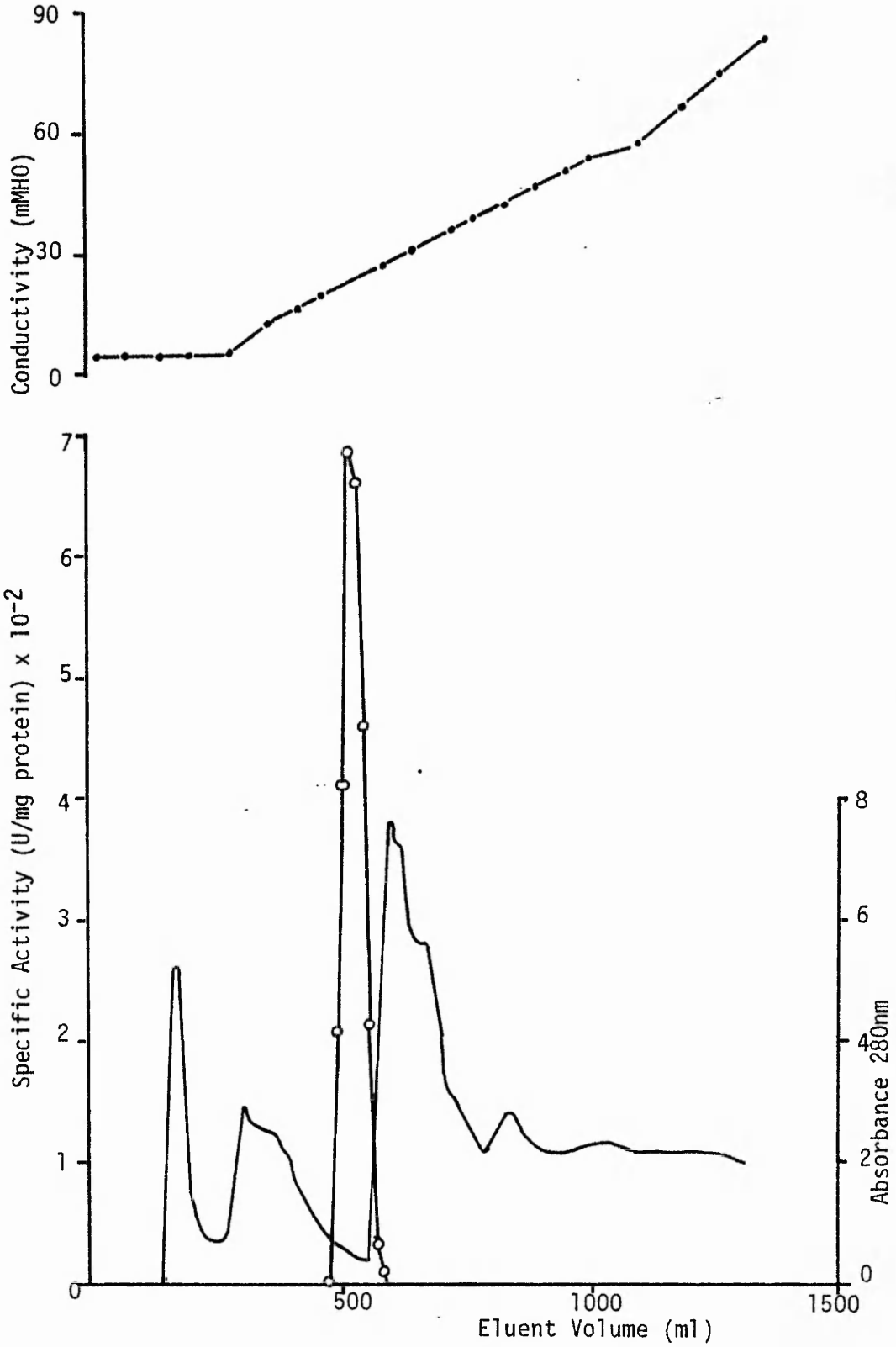

90
60
30
0
Conductivity (mMHO)
7
6
5
4
3
2
1
0
Specific Activity (U/mg protein) x 10^{-2}
8
6
4
2
0
Absorbance 280nm
500
1000
1500
Eluent Volume (ml)

Figure 3.1

Results - Figure 3.2

Standard Conductivity Curve

A standard curve was prepared for a 0-2M NaCl concentration range using a conductivity meter (Electronic Switchgear "London" Ltd.). "Aristar" grade NaCl was used to prepare solutions in 50mM Tris/ 1mM EDTA pH 7.4 which, itself was diluted using deionised water. The conductivity cell was rinsed thoroughly with the "test" salt solution before a reading was taken. The temperature was measured at 20°C throughout. Test solutions were prepared in triplicate and the results varied by less than 3%.

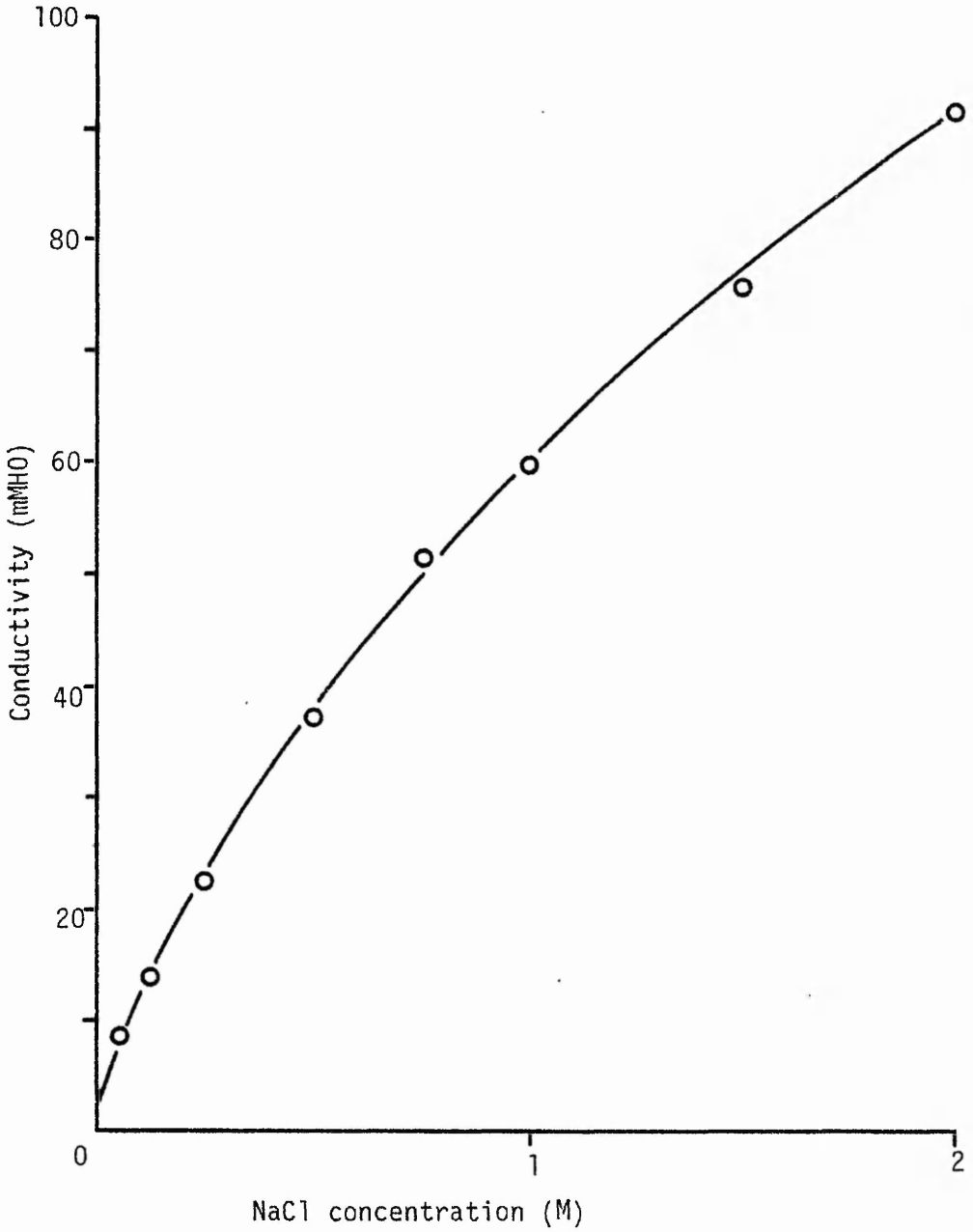


Figure 3.2

Results - Figure 3.3

Gel-filtration on Sephadex G-100

Gel-filtration (Sephadex G-100) was carried out using a 500mm x 25mm column in the ascending mode. The column was equilibrated in 50mM/1mM EDTA pH 7.4 and elution, using the same buffer at a flow rate of 12ml/hr was followed by assaying the fractions (7ml) for trans-glutaminase and protein. The sample was applied through a 0.45μ filter following protein concentration by ammonium sulphate of the DEAE-cellulose eluates. The elution profile was compiled from the results of four fractionation programmes.

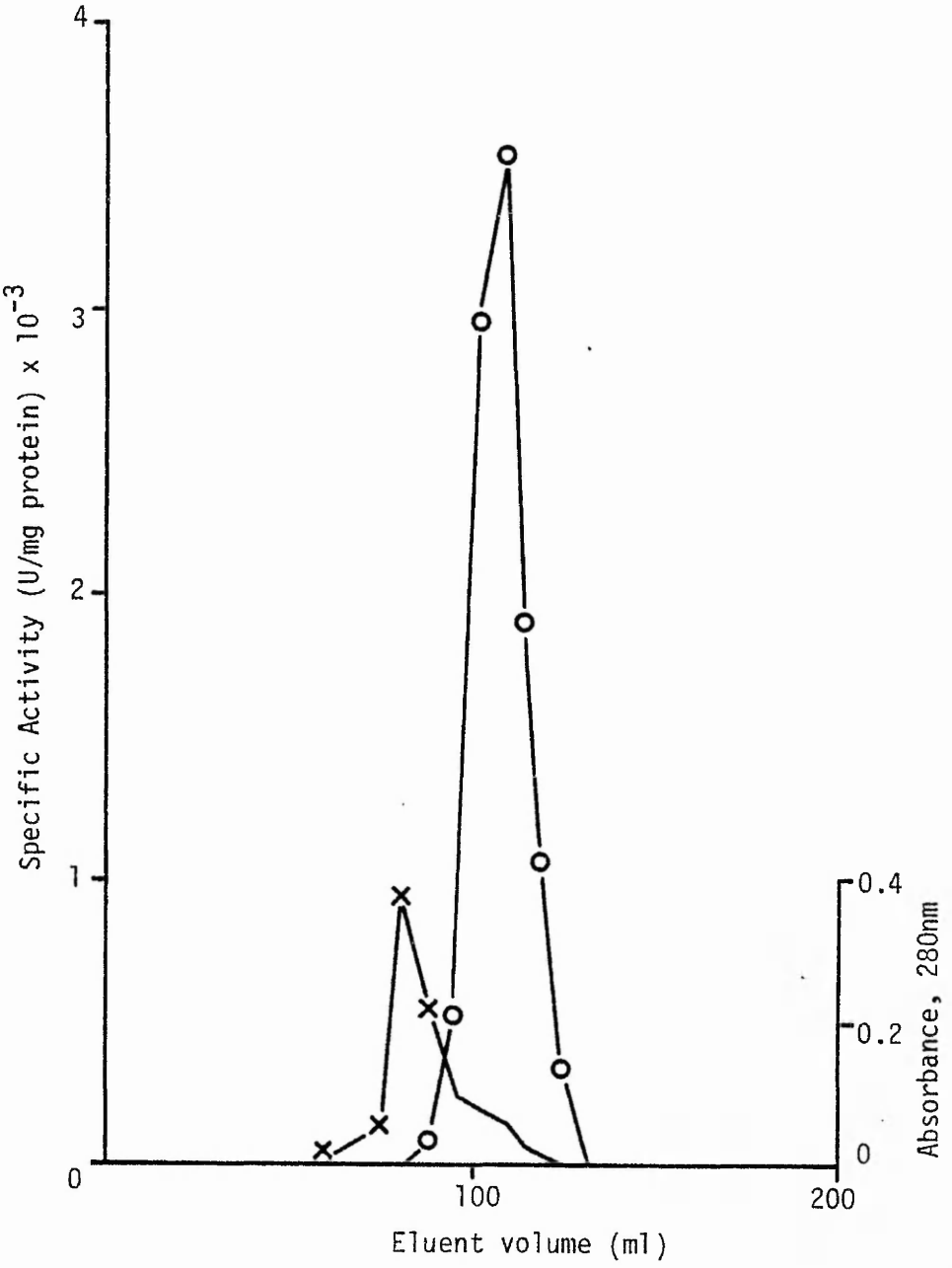

Specific Activity (U/mg protein) x 10^{-3}
4
3
2
1
0
100
200
Eluent volume (ml)
0.4
0.2
0
Absorbance, 280nm

Figure 3.3

Results - Figure 3.4

DEAE-cellulose chromatography(II)

The second DEAE-cellulose column (65mm x 15mm) was eluted by a 200ml linear salt gradient (0-0.6M NaCl) in 50mM Tris/1mM EDTA, at 20ml/hr with 3ml samples being collected. Fractions were monitored for transglutaminase activity and protein distribution and the peak fractions pooled. The results are compiled from four fractionation programmes where the elution profiles were identical.

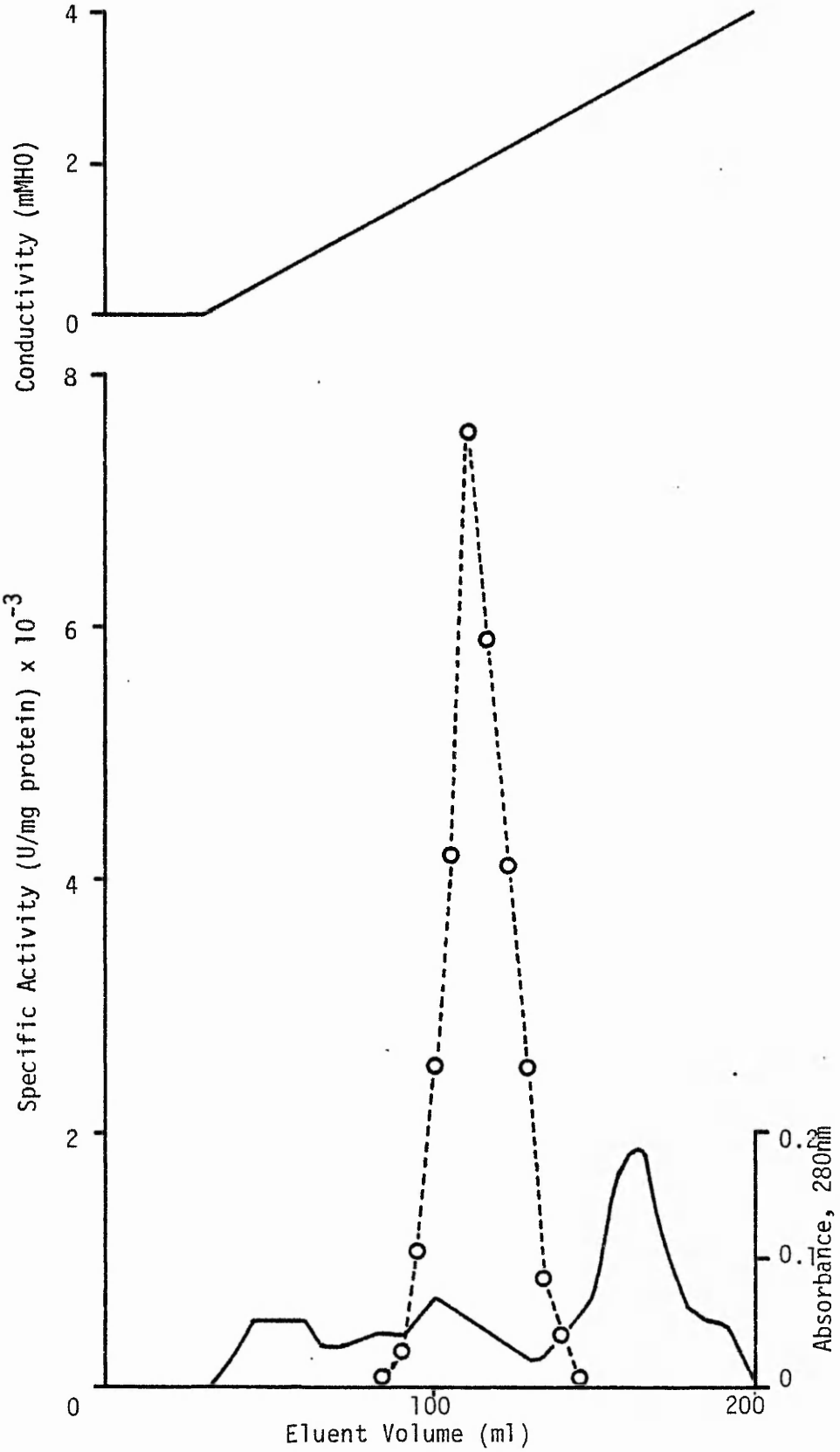

Conductivity (mMHO)
4
2
0
Specific Activity (U/mg protein) x 10^{-3}
8
6
4
2
0
0.2
0.1
0
Absorbance, 280nm
100
200
Eluent Volume (ml)

Figure 3.4

TABLE 1

Purification of Rat Liver Transglutaminase

Based on 100g of Liver

Purification Step	Total Protein (g)	Specific Activity units*/mg	Yield %
Homogenate	18.0 ± 1.0	12.2 ± 1.8	100
Supernatant fluid	5.7 ± 0.42	29.3 ± 2.2	76 ± 3.2
DEAE-cellulose chromatography I	0.11 ± 0.009	440.0 ± 37.0	22 ± 1.2
Gel filtration	0.004 ± 0.0001	2836.0 ± 78.0	4.8 ± 0.1
DEAE-cellulose chromatography II	0.002 ± 0.0001	5053.0 ± 56.0	4.7 ± 0.1
Best fraction		7527.0	

*One unit is defined as the amount of enzyme that catalyses incorporation of 1nmol of putrescine into casein per hour under the conditions given in the text (Methods 2.2.1.1).

The calculated values derive from four fractionation programmes and are expressed as the mean ± S.D.

3.1.2 Structural Properties

3.1.2.1 Gel-Filtration

Chromatography of the purified enzyme by elution from a molecular sieve (Sephadex G-100) provided single co-incident peaks, as followed by enzyme assay and protein measurement of absorbance at 280nm.

Determination of the molecular weight was achieved by the calibration of the column with various standard proteins of known molecular weight.

Standard	Molecular Weight
Ribonuclease I	13,683
α-Chymotrypsinogen	23,200
Ovalbumin	45,000
Serum Albumin (monomer)	65,000
Serum Albumin (dimer)	110,000

Source: Tanford (1961)

These results are plotted as a semi-log graph (Figure 3.5). Reference to the elution value of liver transglutaminase to this curve gave an apparent molecular weight of 69,000 ± 5,000.

The purified transglutaminases have been found to differ in their molecular weights depending on type and source of the enzyme (Lorand and Stenberg, 1976). In the main the tissue transglutaminases (non-zymogenic) fall in the range 70,000-85,000 with guinea pig liver at 85,000 (Connellan et al, 1971) and rabbit liver at 80,000 (Abé et al, 1977). The rat liver enzyme at 69,000 ± 5,000, whilst being somewhat lower in molecular weight than the other liver sources purified to date, still impinges on the lower end of the "normal" range.

Results - Figure 3.5

Estimation of the molecular weight of rat liver transglutaminase by gel filtration.

A Sephadex G-100 gel chromatography column was prepared as described in the text (Methods 2.4.5.2). The column (500mm x 25mm) was equilibrated and eluted (15ml/hr), using 50mM Tris/1mM EDTA pH 7.4, at 4°. Fractions (5ml) were collected and assayed for transglutaminase activity ($\left[^{14}C\right]$ -putrescine incorporation into casein) and protein concentration (absorbance at 280nm). The column was calibrated using 0.1% (w/v) Blue Dextran (Vo = 85ml), 0.1% (w/v) potassium dichromate ($Vi+V_o$=247ml) and a series of proteins of known molecular weight (see accompanying text). A semi-log plot was compiled in order to provide a standard graph for the determination of molecular weights. Results were derived from peak values and based on mean values of 2-3 determinations per point (variation less than 5%).

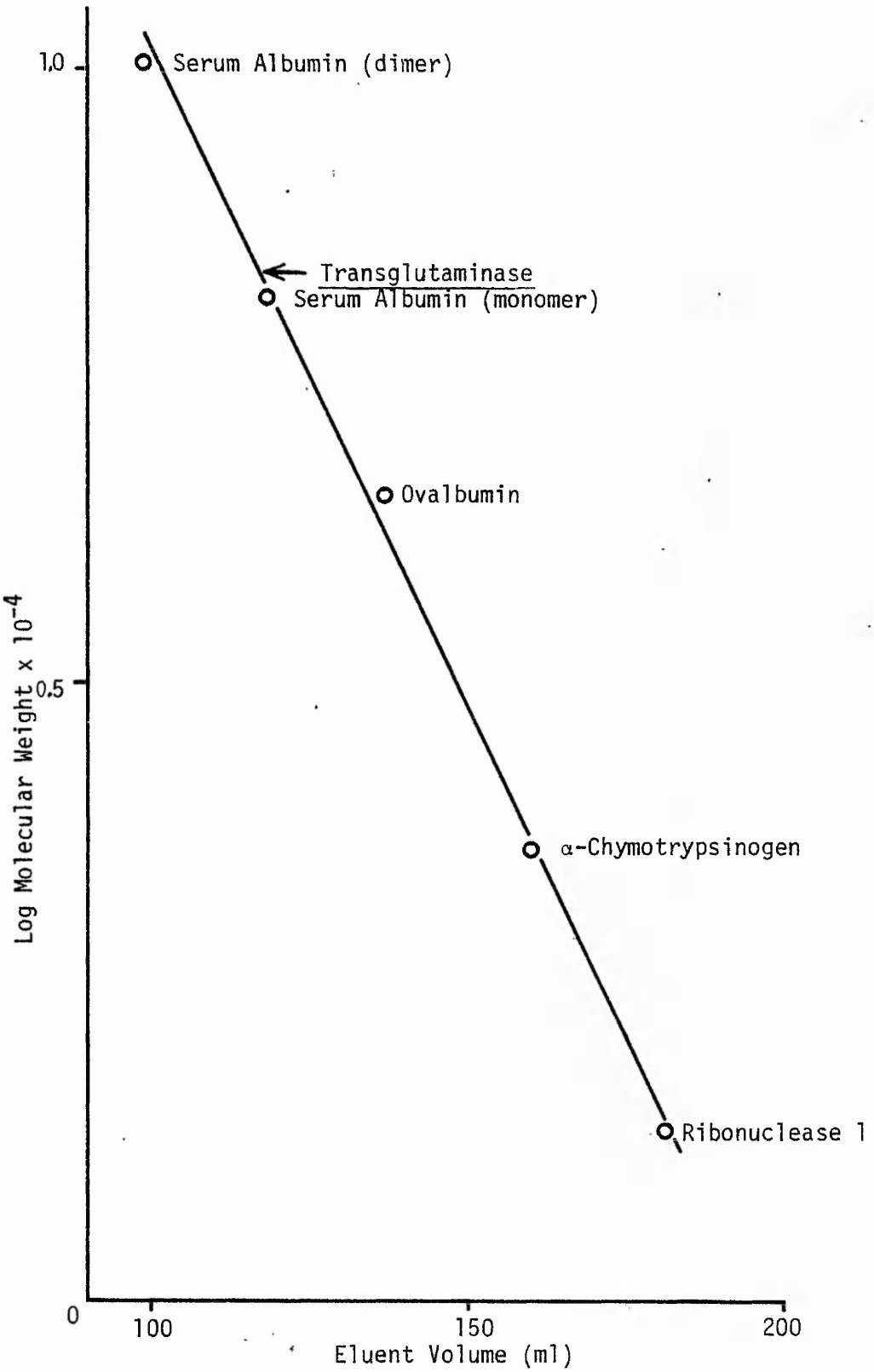

1.0
0.5
0
Log Molecular Weight x 10^{-4}
Serum Albumin (dimer)
Transglutaminase
Serum Albumin (monomer)
Ovalbumin
α-Chymotrypsinogen
Ribonuclease 1
100
150
200
Eluent Volume (ml)

Figure 3.5

The specific activity of the eluted enzyme was similar to that of the enzyme fraction prior to sample loading, and no contaminating protein peaks were observed in the elution profile.

3.1.2.2 Polyacrylamide gel electrophoresis

Electrophoresis of purified enzyme was carried out under both reducing and non-reducing conditions and a single band was identified in both cases.

The methods used have been detailed in Appendix 1 and are summarised below. The TES-TEA (pH 7-8) system using a separating gel of 7.5% acrylamide and a stacking gel of 2.5% acrylamide was loaded with approximately 250μg of protein and was electrophoresed until the marker (bromophenol blue) was within 1cm of the end of the gel. The gel was then removed and the protein fixed and stained. The resultant protein pattern was scanned using the gel-scanning attachment of a SP1800 spectrophotometer (Pye-Unicam Ltd.) and the reuslts may be seen as Figure 3.6.

Similar investigations of the purified enzyme using the Tris-glycine (pH 8.9) system described in Appendix 1 gave identical results (results not shown).

Electrophoresis under reducing conditions, using the sodium dodecylsulphate (SDS)-phosphate buffered system under conditions described in Appendix 1, also gave a single protein band. These 7.5% (u/v) acrylamide gels were further calibrated using SDS-gel molecular weight markers (MW SDS-70 and MWSDS-280 kits, Sigma Chemical Co. Ltd.) and a standard graph constructed (results not shown). The enzyme was found to be a single protein unit with a molecular weight of 70,000 ± 5,000 based on at least three determinations.

These results concur in that the enzyme was electrophoretically

pure, since no contaminant proteins could be observed even when the gel was moderately overloaded. Furthermore, under reducing conditions the molecule was seen as a single band, thus showing the absence of disulphide-bonded sub-units, and a monomeric single protein structure. The molecular weight determination (70,000 ± 5,000) was in good agreement with that described for the gel filtration of the purified enzyme (69,000 ± 5,000).

3.1.2.3 Isoelectric focussing

This experimental approach has been detailed in Appendix 1 and the results are summarised in Figure 7. The gels were subjected to one of three manipulations post-electrophoresis, and were therefore run in triplicate groups:

(i) The first gel having been fixed in 5% (w/v) TCA was stained to visualise the protein present. This gel was observed to have a single protein bond measured as 17-19mm from the anode (acid) extremity.

(ii) The second gel was sliced into 5mm sections and each was placed in 50mM Tris/1mM EDTA pH 7.4 (1ml) overnight to allow the enzyme protein to leach out. This gel was removed and the supernatant liquor assayed for transglutaminase activity by $[^{14}C]$-putrescine incorporation into casein. The enzyme activity was found to reside totally in the section 15mm-20mm from the anode end of the gel.

(iii) The third gel was also sectioned (5mm) and then each placed in distilled water (2ml) overnight. The pH of the resultant liquor was measured and a standard graph constructed to show the pH gradient throughout the length of the gel.

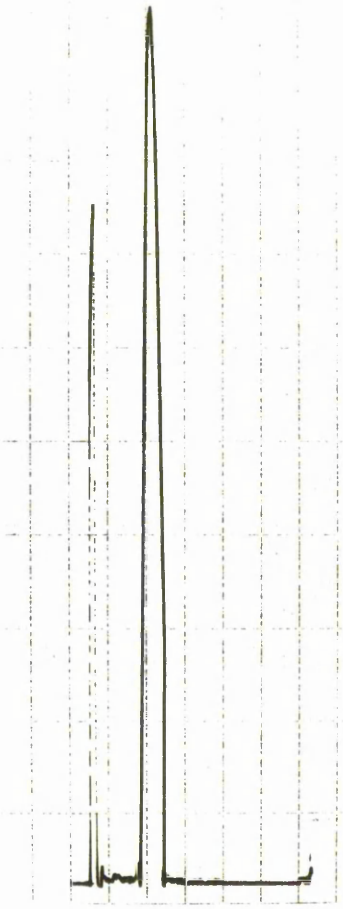
These results taken together indicate a single protein possessing

an isoelectric point (pI) in the range 4.5-4.7. The low value recorded for the enzyme activity can be explained by denaturation of the protein during the manipulations e.g. heating during electrophoresis. Also the formation of the gel, to include the enzyme within the gross structure, may have led to enzyme inhibition by the primary amines present with the acrylamides and the action of acrylamide as a sulphydryl agent.

Figure 3.6

Polyacrylamide gel electrophoresis of purified rat liver transglutaminase

The photographs show the gel resulting from the electrophoresis of a moderately overloaded sample of purified rat liver transglutaminase by the TES-TEA system (see text). The spectrophotometric analysis of the protein stain distribution within the gel confirms a single protein band with no detectable contaminating proteins.



Results - Figure 3.7

Isoelectric focussing of purified rat liver transglutaminase.

Acrylamide gels (5.5% (w/v) acrylamide) were formed (65mmin length) according to the methods described in Appendix 1. They contained a pH gradient of approximately 3.5-10 (LKB Ampholine, LKB Produkter Ltd.) and 75μg sample protein. The gels were run in triplicate in an Ethanolamine (cathode, pH 10.5) and H_2SO_4 (anode, pH 2.5) system for 180 min at 2mA/tube and treated according to the methods described in the text.

The figure shows the pH gradient obtained within the gel and the resultant position of the enzyme protein by (i) activity measured and (ii) a representation of the position of the single protein band as observed in the stained gel.

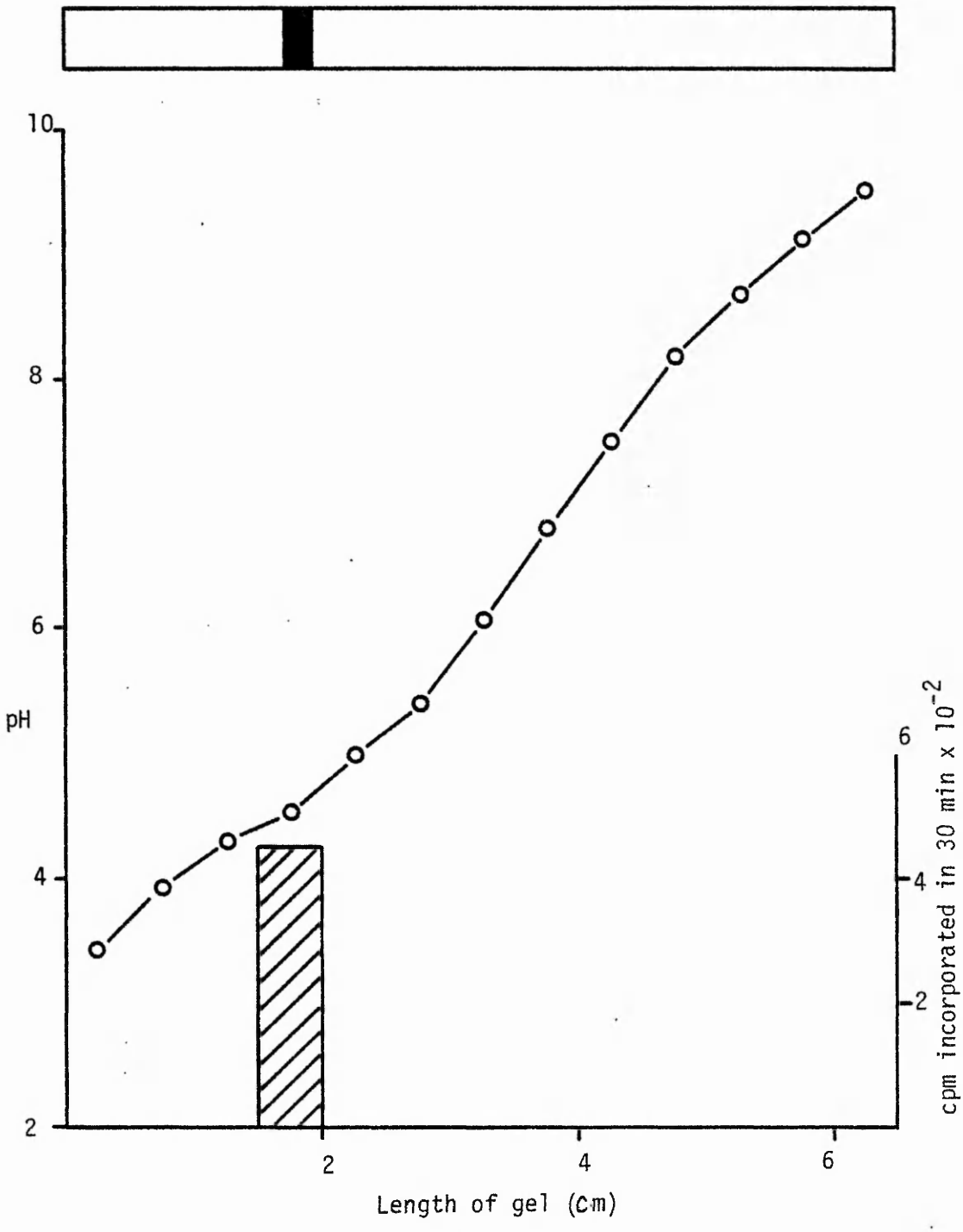

10
8
6
4
2
pH
2
4
6
Length of gel (Cm)
6
4
2
cpm incorporated in 30 min x 10^{-2}

Figure 3.7

3.1.3 Catalytic Properties of the Enzyme

3.1.3.1 Michaelis-Menton Kinetics

The evaluation of apparent Michaelis constant $K_{m,app}$ and V_{max} was undertaken using the incorporation of $[^{14}C]$-putrescine into casein as described in Methods 2.2.1.1. When the concentrations of the acceptor protein and enzyme were kept constant and that of the radioactive amine varied the changes in the initial velocity (Vo) of incorporation seemed to obey Michaelis-Menton kinetics. The results, expressed as the Lineweaver-Burk representation (Lineweaver and Burk, 1934) in Fig. 3.8, permitted the evaluation of apparent Michaelis constants for the isotopic amine substrate studied. Similar experiments using controls, where either no substrate was included or EDTA replaced Ca^{2+} in the system, showed a low background incorporation (non-covalent) as would have been expected to remain in washed filter paper squares. The linearity of the reaction with time was confirmed up to 30 min and subsequent experiments of this type used incubation periods not exceeding this figure.

The values obtained for the Michaelis contstants were as follows:

$K_{m,app} = 5.4 \times 10^{-4}M$ ($\pm 0.2 \times 10^{-4}$)

V_{max} = 28.2 pmol putrescine incorporated/min.

These data have no direct counterpart in the literature, however Lorand et al (1972) obtained a K_m value of $0.9 \times 10^{-4}M$ for guinea pig liver transglutaminase using $[^{14}C]$-putrescine incorporation into casein. Also, Birckbichler et al (1976) found that the K_m for rat liver enzyme was 1.2×10^{-4} when $[^{14}C]$-putrescine was used as the amine substrate and endogenous "particle-free supernatant" protein was used as the acceptor. Results from this laboratory, using rat lung tissue have indicated a K_m value of $2 \times 10^{-4}M$ for the soluble enzyme and

Results - Figure 3.8

Michaelis-Menton Kinetics

Lineweaver-Burk plot for incorporation of $[^{14}C]$-putrescine into casein (25mg/ml) by purified rat liver transglutaminase (13.2ng). The assay (Methods 2.2.1.1) included various concentrations of radioactive amine from 2.4mM to 0.075mM and reactions, incubated at 37^{o}, were stopped after 10 min. The results are expressed as the means of four determinations with standard deviations.

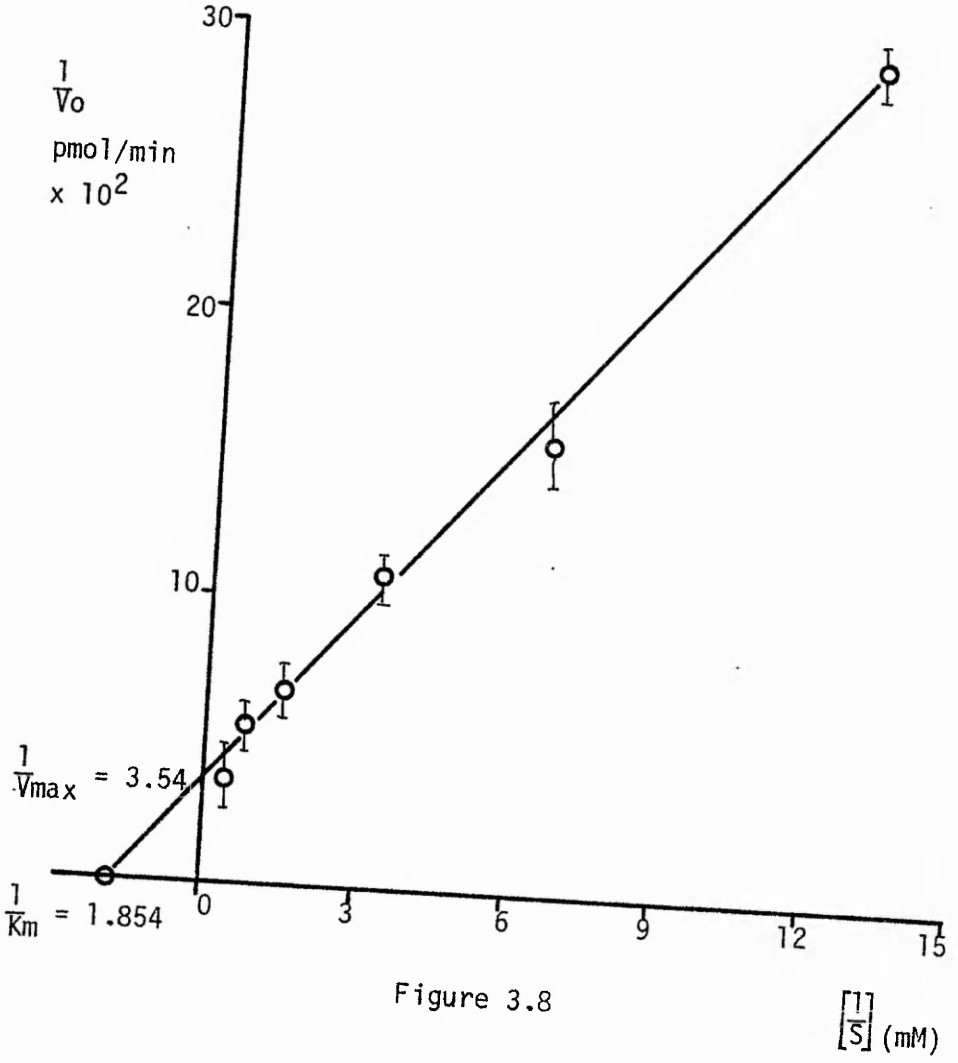


Figure 3.8

4.1 x 10^{-4}M for the "bound" enzyme in the particulate fraction of the cell (Griffin et al, 1978). Thus whilst no direct comparisons may be made the value quoted above indicates a certain agreement with the published figures.

3.1.3.2 Calcium Activation

This experiment was undertaken to investigate the calcium ion requirement of the purified enzyme.

Reaction mixtures were prepared which varied only in their Ca^{2+} concentations from those already detailed for the $[^{14}C]$-putrescine incorporation into casein assay. A series of ten Ca^{2+} dilutions (0-10mM final concentations) were utilised and the radioactive isotope uptake measured. The zero Ca^{2+} concentation samples were taken as those reaction mixtures containing 1mM EDTA, in conjunction with their more usual role as the enzyme blank controls.

The results, as shown in Fig. 3.9, display an absolute requirement for the Ca^{2+} ion. This feature of activity is characteristic of all but one of the family of transglutaminases so far studied. The exception, whilst remaining unsubstantiated, is the sheep hair follicle enzyme (Harding and Rogers, 1972) which was reported active even in the presence of EDTA. This apart, the general finding concurs with the published and accepted transglutaminase requirements.

The activity of the enzyme was also shown to be very sensitive to the available calcium. Up to 40% of the activity was present at the lowest calcium concentration used (40μm) and the majority of the enzyme was expressed at 0.1mM. There was little improvement on increasing the concentration above 0.5mM as the enzyme acceptor site(s) became saturated and the activity became nearly constant in the range

Results - Figure 3.9

Calcium Activation

The effect of Ca^{2+} concentration on purified rat liver transglutaminase activity was assessed by the variation of that parameter within the $[^{14}C]$-putrescine incorporation into casein assay (Methods 2.2.1.1). The reaction mixture (containing 6.6ng enzyme protein) included a range of Ca^{2+} concentrations from 10mM to zero which were then incubated for 5, 10 and 15 min at 37°. before the reaction was terminated. Zero values corresponded to reaction mixtures containing 1mM EDTA in place of Ca^{2+}, which also formed the enzyme blank control. The results were corrected for counting efficiency and the mean $[^{14}C]$-putrescine incorporation was plotted against Ca^{2+} concentration. Results are expressed as the means of three values (± S.D.).

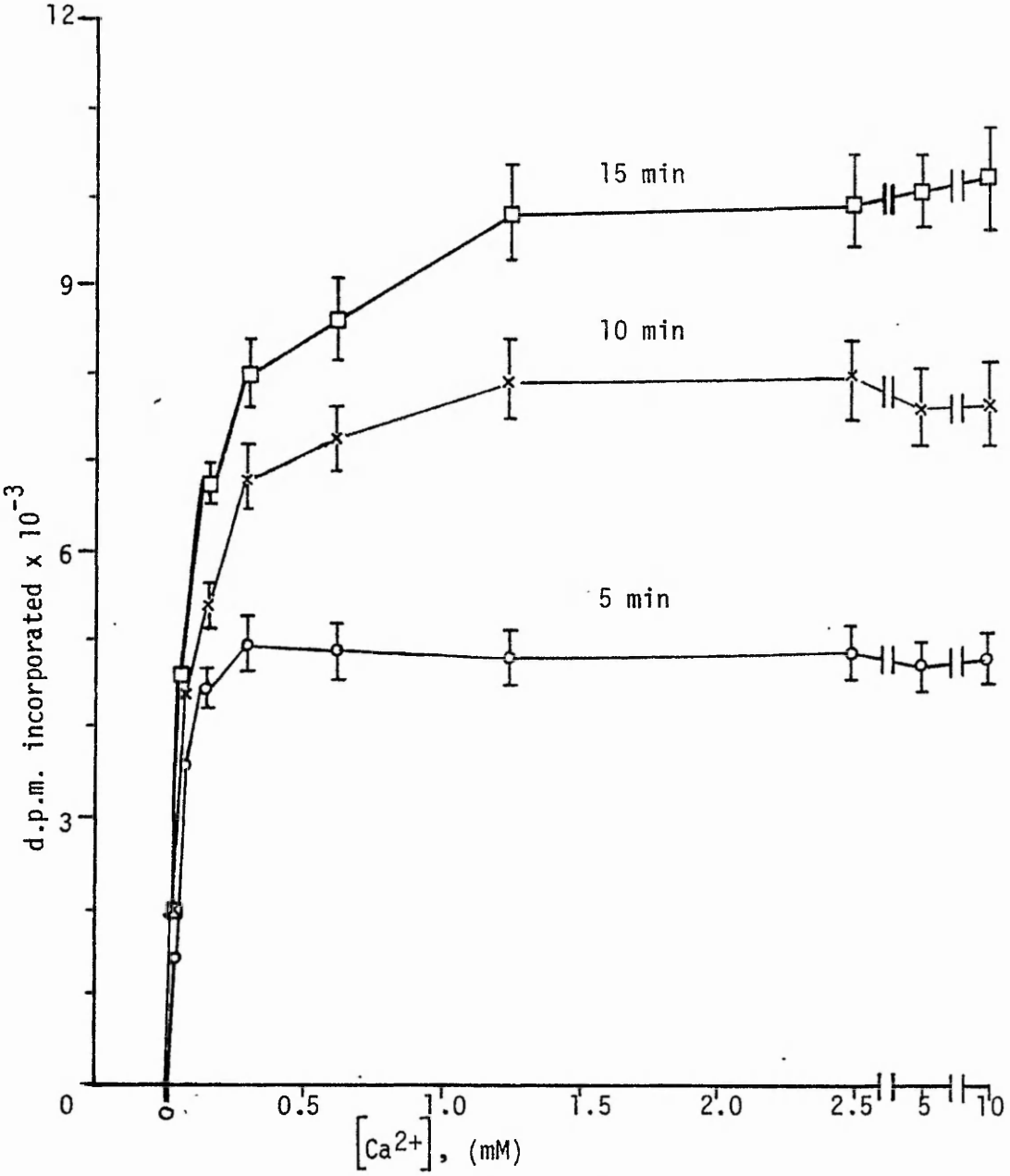

12
9
6
3
0
d.p.m. incorporated x 10^{-3}
15 min
10 min
5 min
0
0.5
1.0
1.5
2.0
2.5
5
10
$[Ca^{2+}]$, (mM)

Figure 3.9

0.5-10mM set by the experiment.

The Ca^{2+}-dependence in liver when compared to data from the erythrocyte transglutaminase exemplifies the sensitivity of this enzyme. The studies by Lorand et al (1976) and Seifring et al (1978) show that the erythrocyte transglutaminase only shows significant activity at calcium concentrations in excess of 0.3mM. This finding coincides with the calcium levels reported for old or abnormal red blood cells and is accompanied by losses of membrane deformability (Kirpatrick et al, 1975). This apparent method of control existing in erythrocytes would need to be far more sensitive in its application in hepatocytes. In liver the enzyme is significantly more active at much lower calcium concentrations and any control mechanism based on limiting the availability of Ca^{2+} would need to operate over a limited and extremely low concentration range.

3.1.3.3 Sulphydryl Dependence

Purified rat liver enzyme was tested for susceptibility to sulphydryl reagents since the activity of the guinea pig enzyme is known to be extremely sensitive to such reagents (Folk and Cole, 1966). Derivatives of iodoacetate (ICH_2COR) are known to form S-alkyl derivatives of cysteine (protein-S-CH_2COR) and iodoacetamide was chosen as it has been widely used for the inhibition of various transglutaminases. A second inhbitor , p-hydroxy-mercuribenzoate, an organomercuride compound which forms covalent mercaptide bonds (protein-S-HgAr) with cysteine, was also examined.

Using the $[^{14}C]$-putrescine incorporation assay for enzyme activity, the reaction mixtures were adjusted to remove the dithiothreitol component and replace it with the sulphydryl reagent. A serial dilution of inhibitor was prepared in the reaction mixture, resulting

Results - Figure 3.10

Sulphydryl Dependence

Stock solutions (100mM) of iodoacetamide (in 50mM Tris pH 7.4)(○) and p-hydroxymercuribenzoate (in 100mM glycylglycine buffer pH 8.0)(×) were used in a serial dilution scheme present as the replacement for dithiothreitol in the [^{14}C]-putrescine incorporation into casein assay reaction mixture (Methods 2.2.1.1). The enzyme (6.6ng) activity was monitored in the presence of the above reagents in the concentration range 10^{-3}M; 10^{-4}M; 10^{-5}M and 10^{-6}M. Normal controls were also prepared to provide the 100% values. Glycylglycine (100mM) buffer pH 8.0 was also tested as a component of the assay procedure and the enzyme activity was found to be identical to that observed using the Tris buffer system. The results (means ± S.D.; n=4) are expressed as the percentage of the normal initial velocity (Vo) achieved in the presence of the inhibitor compared to normal values obtained in the absence of the inhibitor.

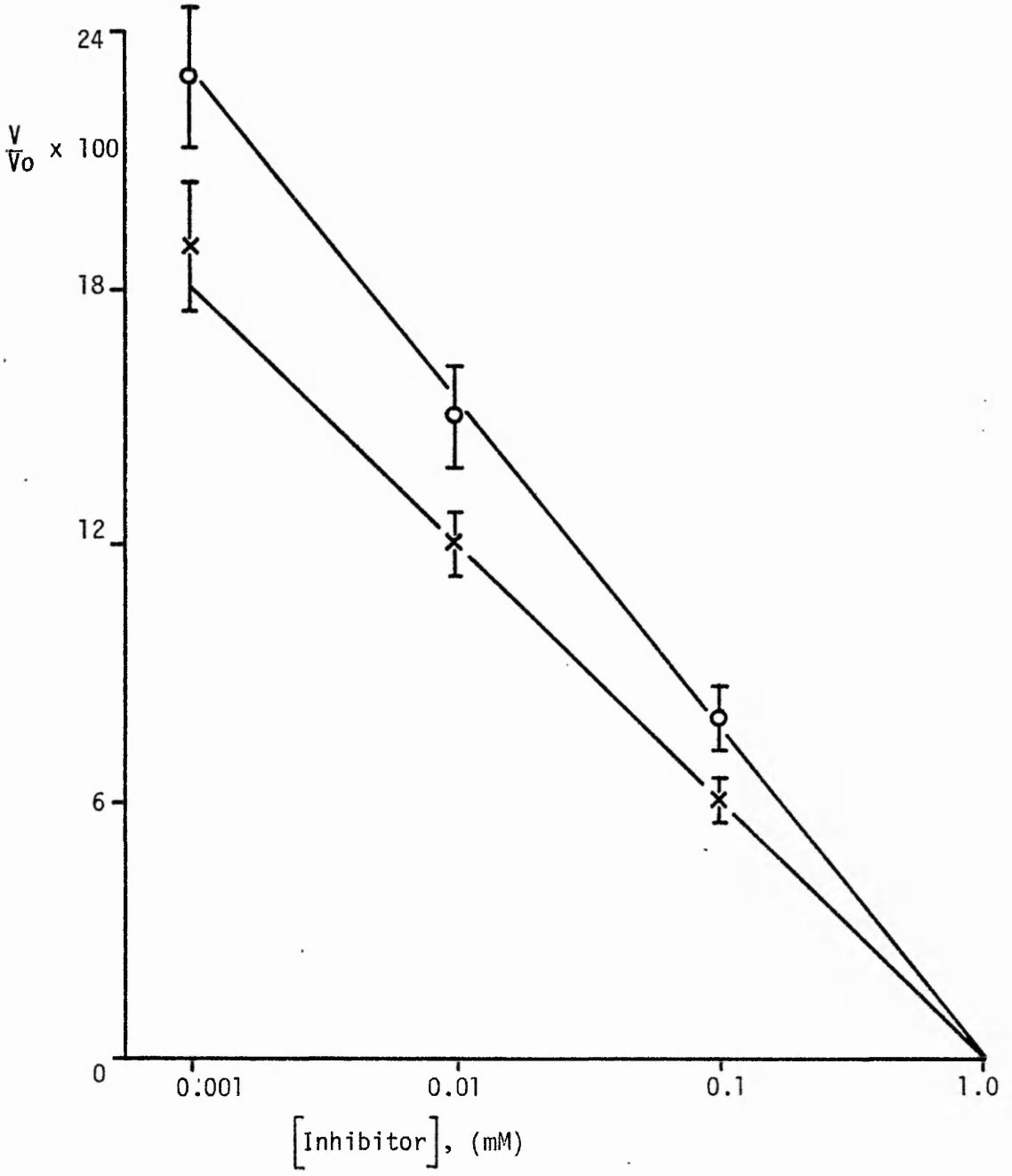


Figure 3.10

in a concentration range from 10^{-3}-10^{-6}M. The effect of the inhibitor was judged by the reduction in the initial velocity of the assay as compared to that of a control in the absence of inhibitor.

The inhibition of the transglutaminase activity by these reagents clearly demonstrates an active site sulphydryl dependence of this enzyme (Figure 3.10). The inhibitory effect of the two reagents monitored, iodoacetamide and p-OHmercuribenzoate were very similar and resulted in complete enzyme inhibition at a concentration of 1mM with only about 20% of activity at 1 μm. Thus the rat liver transglutaminase is very sensitive to the presence of sulphydryl reagents. Partial restoration of enzyme activity was observed by the addition of dithrothreitol (10mM) to the inhibited enzyme.

Tyler and Laki (1967) have reported complete inhibition of rabbit liver transglutaminase at 1mM concentrations of the same sulphydryl reagents measured by their influence on the fibrin cross-linking reaction. Erythrocyte transglutaminase is similarly affected by 1mM concentrations of the above reagents (Brenner and Wold, 1978) used in the fluorescent assay of Lorand et al (1971) with β-casein and dansyl cadaverine as substrates. Kinetic studies revealed the presence of one thiol-reagent sensitive site per enzyme molecule and are consistent with the postulated formation of a thiol-ester intermediate in transglutaminase catalysis (Folk and Cole, 1966b).

3.1.3.4 Pre-incubation with Thrombin

The proteolytic action of thrombin on plasma transglutaminase has been discussed above (see also Buluk et al, 1961; Lorand and Konishi, 1964) and is a pre-requisite of catalytic activity. It was of interest therefore to assess the influence that thrombin might have on the activity of the rat liver "tissue" transglutaminase.

Thrombin was prepared as described in Methods 2.5.3. The purified enzyme preparation (14.5ng in 100µl 50mM Tris/1mM EDTA pH 7.4) was pre-incubated for 20 min at 37° both in the presence and absence of approximately 100 NIH units of thrombin (1000 NIH units/ml in 50mM Tris/100mM NaCl, pH 7.4). A sample of this mixture was then removed and assayed by the catalysis of $[^{14}C]$-putrescine incorporation into casein (Methods 2.2.1.1) over 0.5; 5; 10 and 20 min time intervals.

The resultant radioactive incorporation from the test and control series were observed to be within 2%-3% of each other, indicating that treatment with thrombin had no effect on the catalytic activity of rat liver transglutaminase.

3.1.3.5 Hydroxylamine Assay

The reaction in which hydroxylamine may become incorporated into benzyloxycarbonyl-L-glutaminylglycine has been the basis of a simple and sensitive colourimetric assay for guinea pig liver transglutaminase (Folk and Cole, 1966a,b). The wide application of this assay to other transglutaminase systems, however, has been placed in doubt by recent evidence from studies with the rabbit liver enzyme which has been shown to have a particularly low activity against hydroxylamine incorporation (Abé et al, 1977).

The assay procedure (Methods 2.2.1.2) was followed and included 30-60ng purified rat liver transglutaminase in an incubation of up to 40 min at 37°. After the addition of the colour developer the absorbance was determined ($A^{1\%}_{1cm,525nm}$). A standard graph was also prepared using a range of 0-1.5µmol L-glutamic acid-γ-monohydroxamate.

There was no detectable hydroxamate production on incubation with the enzyme. The enzyme preparation was shown to be active by another assay method (Methods 2.2.1.1) and the reagent mixture was validated since the introduction of hydroxamate standard to the assay produced

the correct colourimetric response.

Rat liver transglutaminase was therefore shown to have very little or no activity towards the substrate of this assay. This finding concurs with that of Abe et al (1977) where the rabbit liver enzyme was found to have less than 2% of the maximum velocity displayed by the guinea pig liver enzyme in this reaction.

3.1.3.6 Fibrin-Fibrinogen Crosslinking

The action of transglutaminase on the sub-unit structure of fibrin and fibrinogen provides both physical evidence of crosslinking and some information as to the specificity of the enzyme involved (McKee et al, 1970; Chung, 1972).

The method by which the fibrin and fibrinogen substrates were incubated with rat liver transglutaminase has been described previously (Methods 2.8.3). The samples obtained were subject to polyacrylamide electrophoresis in sodium dodecyl sulphate (SDS) in the presence of a reducing agent(Methods 2.4.6 and Appendix 1) and the resultant gel stained for protein and photographed (Figure 3.11).

The electrophoretic pattern for fibrin reveals an early diminution of the α-monomer with the consequent production of covalently cross-linked α-polymers of higher molecular weight. Later in the incubation and to a lesser extent, the γ-monomer bond was seen to diminish with a low level of γ-γ dimer production. The β monomer and all bonds in the EDTA control remain unaltered throughout the incubation. The electrophoretic pattern of polymerisation of fibrinogen appeared almost identical to that for fibrin except that the β subunit was also involved in crosslinking.

This result is consistent with that of Chung (1972) who described an identical pattern of polymerisation using guinea pig liver

Figure 3.11

Fibrin and Fibrinogen Crosslinking

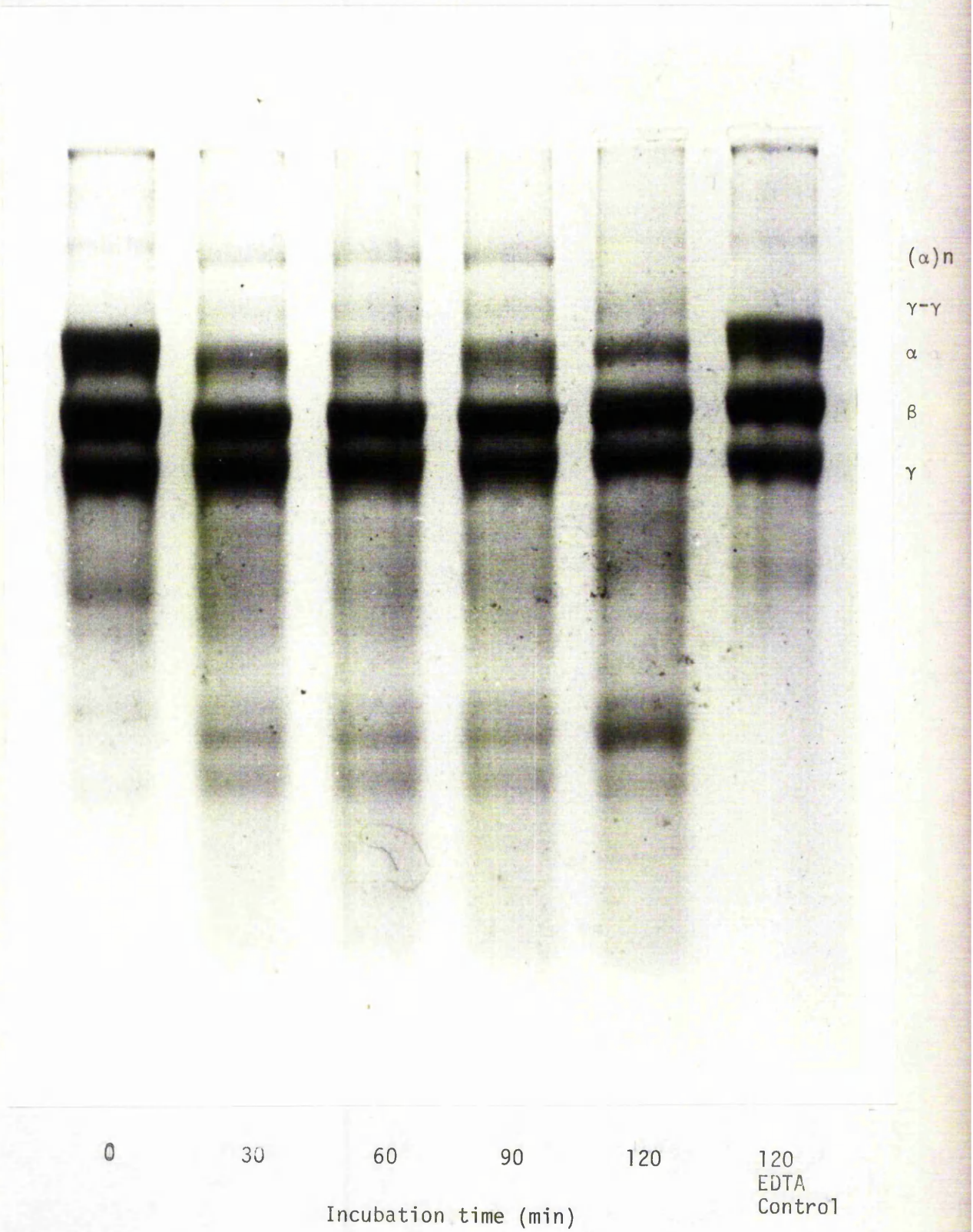
The photograph shows the crosslinking pattern of fibrin (and fibrinogen) due to the catalytic action of purified rat liver transglutaminase with respect to incubation time.

The early diminution of α-monomer and the appearance of α-polymers, and the slower dimerisation of the γ-bands and the lack of reactivity with the β-chains typifies a transglutaminase of the "tissue" type.

For methods see 2.4.6 and Appendix I.

Figure 3.11

Fibrin and Fibrinogen Crosslinking



transglutaminase. In contrast, the studies by Chung using plasma transglutaminase demonstrated γ- followed by α- subunit polymerisation and no reactivity with fibrinogen. Hair-follicle transglutaminase reacted with equal rates on the α and γ- subunits of fibrin but not at all on the β-subunit unless the substrate was fibrinogen, when all three subunits were involved.

The pattern of fibrin and fibrinogen polymerisation demonstrated by this method allows the identification of the purified enzyme as a characteristic tissue transglutaminase from its subunit specificity and protein crosslinking capabilities.

3.1.3.7 Hydrophobic Chromatography

Purifed rat liver transglutaminase was chromatographed on a series of alkyl agarose columns as shown in Fig. 3.12. Under the conditions described (see also Methods 2.4.5.4) the enzyme was retained primarily as a function of alkyl chain length, the longer the chain the more severe were the eluting conditions required to bring about its removal from the agarose. The elution programme involving a stepwise increase in NaCl concentration, with the addition of ethylene glycol in the final stage, demonstrated the varying hydrophobic interactions which took place in the columns. The C_0 column did not provide for any retention of protein and the activity recovered was identical to that applied. Similarly the C_2 column provided only minimal retention and the recovered activity was 95% of that applied. In the case of the C_4 column there was significant enzyme retention, in that, buffers with the higher NaCl concentrations were required to elute the enzyme protein. Furthermore the total enzyme activity recovered was calculated to be nearly 3-fold (283%) greater than a similar sample assayed prior to application. Thus the interaction of the enzyme with the column or a specific

constituent of the column appears to promote an enhancement of the activity of the enzyme. The subsequent columns used (C6, C8, and C10) all exhibited an ability to retain the enzyme protein, and a sequential relationship was apparent in that the protein was progressively more tightly bound to the matrix. These columns, therefore, were seen to release the protein in increasing levels as the buffers tended towards the highest NaCl concentrations and the addition of ethyleneglycol. These agarose columns also provided some enhancement of enzyme activity but as a decreasing function of alkyl chain length (C6, C8 and C10 showing 184%, 154% and 129% respectively).

Goldsmith et al (1978) found that in using this method they were able to demonstrate the hydrophobic interaction of epidermal transglutaminase with alkyl agaroses and also an enhancement of enzyme activity. Their results show that the binding of the enzyme to the column was such that only the C6-C10 retained the protein significantly and elution required the prescence of ethylene glycol at the final stage. There was little or no progressive release of protein with intermediate buffers. Also, with this enzyme, the stimulation of enzyme activity, whilst being of the same order as that shown for the rat liver enzyme, was biased towards C6-C10 alkyl chain lengths with a peak at C8.

These results demonstrate the prescence of a hydrophobic region on the liver enzyme molecule which responds to alkyl chain lengths, particularly butyl moieties, and which results in an *in vitro* stimulation of the enzyme. This finding corresponds to that of Lorand et al (1979) working with guinea pig liver and Factor XIII who determined the active sites of these enzymes to be receptive

to alkylamine chains of five methylene groups. The response to specific alkyl moieties may vary with the enzyme involved and may contribute to a method of control of transglutaminase activity.

Results - Figures 3.12.1 and 3.12.2

Chromatography of transglutaminase on alkyl agarose

Samples of purified rat liver transglutaminase (0.2mg in 0.2ml 10mM Tris-HCl, 1mM EDTA, 10mM Ca^{2+}, 150mM NaCl pH 7.4 were applied to a series of alkyl agarose columns, (12 x 7mm) equilibrated in the same buffer at 4°, consisting of agarose (Co), ethyl (C2), butyl (C4), hexyl (C6), octyl (C8) and decyl (C10) agarose. The columns were eluted with 6ml starting buffer (A) and 4ml each of starting buffer containing 0.5M NaCl (B), 2.0M NaCl (C) and 2.0M NaCl and 50% ethylene glycol. Fractions (1ml) were collected and assayed for protein (OD280) and transglutaminase (Methods 2.2.1.1) using the standard reaction mixture. Activity is represented as dpm incorporated during 30 min incubation. The total activity recovered (Fig.3.12.2) is expressed graphically as a function of alkyl chain length. The results are shown as the means (± S.D.) of values with a variation of less than 8%.

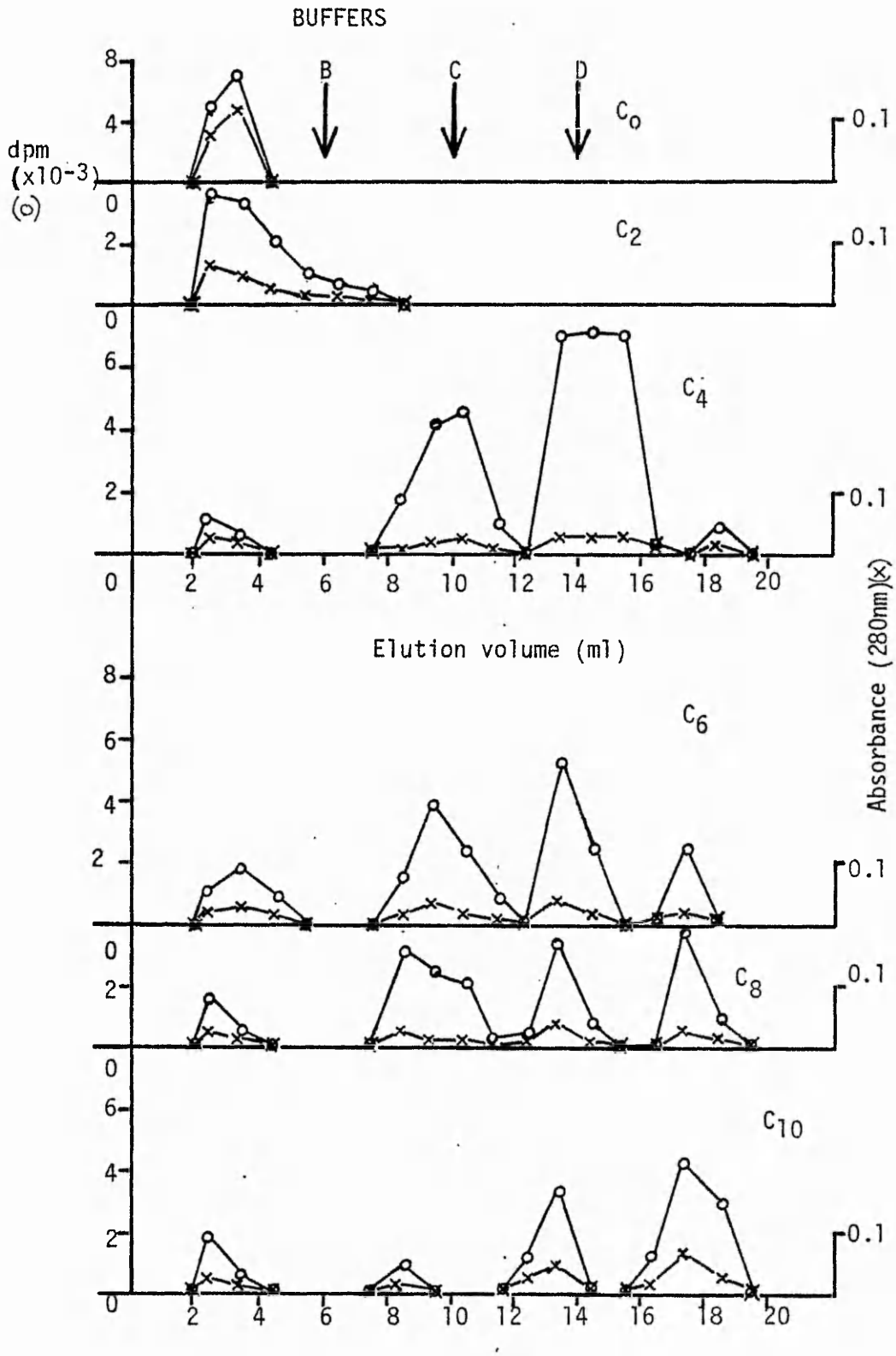

BUFFERS
B
C
D
dpm
(x10-3)
(o)
C_o
C_2
C_4
C_6
C_8
C_{10}
0.1
Absorbance (280nm)(x)
Elution volume (ml)
Elution volume (ml)

Figure 3.12.1

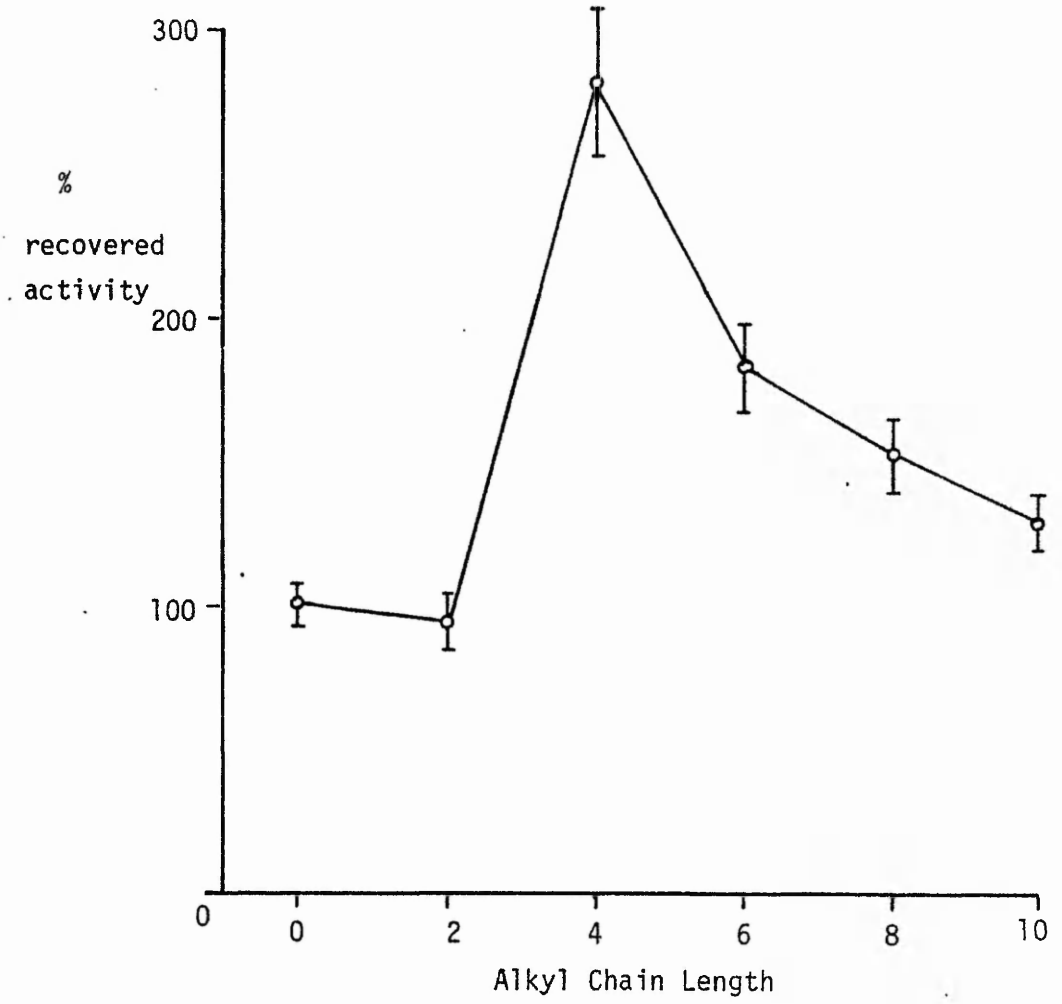

300
%
recovered
activity
200
100
0
0
2
4
6
8
10
Alkyl Chain Length

Figure 3.12.2

3.1.4 CONCLUSION

The procedure adopted for the isolation of rat liver transglutaminase gave a protein sample which was electrophoretically pure (purification factor of 414) and had a specific activity of 5053 units/mg protein. The yield, though disappointingly low, was adequate in providing sufficient protein for subsequent experimentation. The purification factor and specific activity were both well in excess of values quoted from isolation schemes in the literature. On the basis of the information summarised below the enzyme can be classified as (i) a transglutaminase and (ii) belonging to the tissue group. Furthermore, those studies involving the catalytic properties of the enzyme provide important information on the understanding of the physiologic controls.

(i) The molecular weight was determined to be 70000 ± 5000 by two independent methods. This corresponds with the low end of the "normal" range of the tissue group molecular weights.

(ii) The isoelectric point (ρI) was found to be 4.6 ± 0.1.

(iii) The Michaelis-Menton constants with respect to amine were as follows: $K_{m,app} = 5.4 \times 10^{-4}M$ ($\pm 0.2 \times 10^{-4}M$) and V_{max} = 28.2pmol putrescine incorporated per minute.

(iv) The enzyme was found to have an absolute requirement for Ca^{2+} ions. The activity was particularly sensitive to Ca^{2+} with approximately 40μM providing substantial enzyme function.

(v) The enzyme was found to have absolute requirement for a functional thiol moiety, most probably the cysteine-SH found in all transglutaminase active centres so far studied.

(vi) The enzyme was unaffected by the incubation with thrombin, showing that proteolysis by this enzyme is not required for activation and therefore the transglutaminase is not a zymogen.

(vii) The transglutaminase did not incorporate hydroxylamine into benzyloxycarbonyl-L-glutamin ylglycine. This result agrees with that described for rabbit liver enzyme (Abe et al, 1977). However guinea pig liver transglutaminase is reported to be active with this substrate (Folk and Cole, 1966a,b), thus displaying some heterogeneity between these otherwise similar enzymes.

(viii) The studies utilising fibrin and fibrinogen polymerisation confirm the identity of the transglutaminase by demonstrating the actual physical cross-linking of proteins, thus fulfilling "category 3" of Folk and Finlayson (1977) categories of evidence. Furthermore the order in which the subunits were reacted upon again suggest the enzyme is a member of the "tissue" group.

(ix) Hydrophobic chromatography of the enzyme demonstrates the presence of a hydrophobic region on the molecule (Shaltiel, 1974) which is sensitive to a range of alkyl chain lengths (C_4 - C_{10}) but in particular a C_4 or possibly a C_6 moiety. This result concurs well with the conformation of guinea pig liver and Factor XIII active sites (Lorand et al, 1979).

3.2 Subcellular localisation, substrates and control

Foreward

The standard assay method [^{14}C] putrescine incorporation into casein (Methods 2.2.1.1) was adapted from those procedures described by other workers. The concentration of putrescine used varies widely in the literature from 0.12mM with guinea pig liver (Lorand et al, 1972a) and rat liver enzyme (Birckbichler et al, 1976) through to 0.25mM (Seifring et al, 1978), 0.6mM (Ogawa and Goldsmith, 1976) and 4mM (Anderson et al, 1977) with epidermal and erythrocyte enzymes. In this study a putrescine concentration of 1.2mM was found to be saturating and, being 10-fold greater than the literature values used in the assay of liver preparations was adopted in all subsequent assays. A later experiment using purified enzyme (Results 3.1.3.1) demonstrated a K_m for putrescine of 0.54mM, which suggest that the assay concentration could be too low for accurate kinetics. However, experiments using a range of putrescine concentrations have shown that 1.2mM is saturating and throughout this study the putrescine consumed by incorporation into substrate has never exceeded 5% of that available in the assay mixture.

Two potential sources of tissue enzyme inhibition were considered, (i) the effect of the anaesthetic used to kill the animals and (ii) the presence of diamine oxidase (diamine O_2 oxidoreductase : deaminating , E.C.1.4.3.6) in the tissue preparation. Kinetic experiments using rat liver homogenate fractions from animals killed by different methods, and also fractions containing a diamine oxidase inhibitor, aminoguanidine, showed that neither caused significant inhibition of the enzyme. Furthermore, it has been reported in the literature (Tabor, 1964) that rat liver has no detectable diamine oxidase.

3.2.1 Rat Liver Fractionation

The methods used in the fractionation program may be found as follows; Potter-Elvehjem homogenisation and differential centrifugation in Methods 2.4, enzyme assays in Methods 2.2 and protein/DNA determinations in Methods 2.3.

The subcellular distribution of marker enzymes together with DNA and transglutaminase are shown in Figure 3.13. Cytochrome oxidase, acid phosphatase, glucose-6-phosphatase and lactate:NAD:oxidoreductase were found predominantly associated with the "mitochondrial", "lysosomal", "microsomal" and particle-free supernatant fractions respectively. There was a significant degree of separation between fractions and the enzyme recoveries were always in the range 87-96%.

Transglutaminase activity occurred in all sub-fractions though it was found to lie predominantly in the "nuclear" and soluble fractions. A more detailed breakdown of enzyme distribution suggests only negligible levels in the mitochondrial, lysosomal and microsomal fractions of 2.2%, 1.7% and 0.6% respectively, most probably due to contamination by the soluble fraction. The majority of the detectable enzyme was distributed as 38.2% in the nuclear and 57.3% in the soluble fractions. Electron microscopic examination of the nuclear fraction showed it to contain predominantly membrane components, nucleii and cell debris.

Previous investigations into various transglutaminases have rarely given any information as to their distribution within the cell-type. The majority of researchers have utilised the soluble fraction and made no reference to the presence or absence of further activity located in the particulate fraction. However Tyler (1972) has reported guinea pig liver transglutaminase to be an entirely cytoplasmic enzyme whereas work in this laboratory by Griffin *et al* (1978) have demonstrated rat lung transglutaminase to be almost entirely particulate bound.

Birckbichler et al (1976) have reported a distribution for rat liver in which greater than 90% of the enzyme activity was found in the 105,000g supernatant. This result, based on similar methodology, contrasts with our finding of approximately 60% soluble - associated activity in a series of three fractionations. There is no simple explanation for this discrepancy.

Figure 3.13

Subcellular distribution of transglutaminase, DNA and marker enzymes in rat liver.

The series of graphs demonstrate the subcellular distribution of transglutaminase, cytochrome oxidase, acid phosphatase, glucose-6-phosphatase and lactate:NAD:oxidoreductase on the basis of relative specific activity plotted against per cent protein in fraction. Enzyme activities were measured by the procedures described in the text. The distribution of DNA is represented by per cent DNA in fraction against per cent protein in fraction. "N" represents the nuclear fraction; "M" the mitochondrial fraction; "L" the lysosomal fraction; "P" the microsomal fraction and "S" the particle-free supernatant. The results expressed are the means of values from three fractionation programmes and with a variation determined to be below 9%.

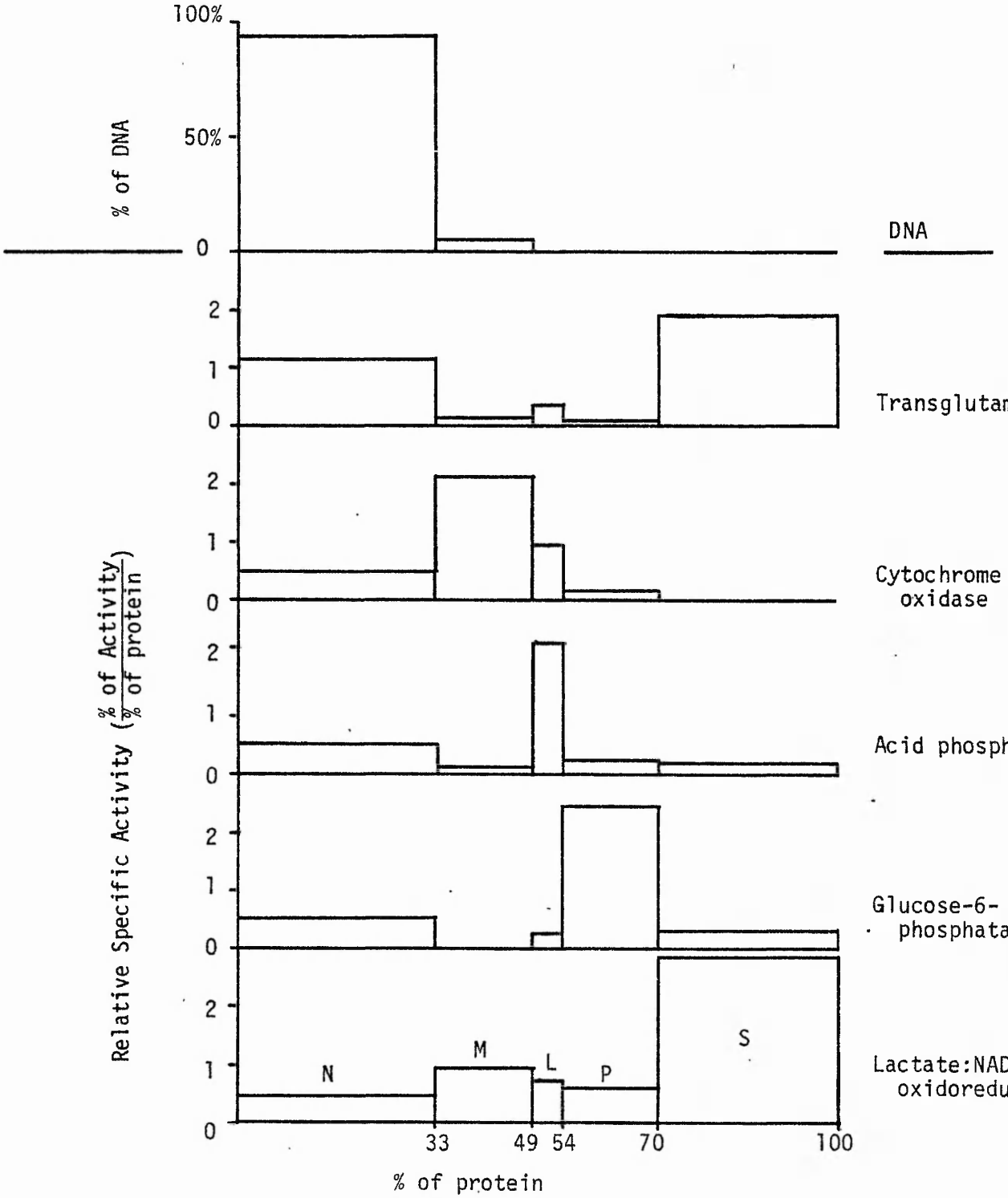

100%
50%
0
% of DNA
DNA
2
1
0
Transglutaminase
Cytochrome oxidase
Acid phosphatase
Glucose-6-phosphatase
Lactate:NAD: oxidoreductase
Relative Specific Activity (% of Activity / % of protein)
N
M
L
P
S
33
49
54
70
100
% of protein

Figure 3.13

3.2.2 Investigation of the site of action of transglutaminase

The potential sites of action of the enzyme were investigated by both histochemical and autoradiographical methods.

The incorporation of the fluorescent pseudosubstrate, monodansylcadaverine, into substrate proteins by a histochemical method using fresh frozen (5μ) rat liver slices has been described (Methods 2.4.7). This method, has been used previously (Buxman and Wuepper, 1975) to demonstrate gross transglutaminase distribution through a longitudinal section of cow snout epidermis, and it was hoped it would prove sensitive enough to allow the subcellular distribution of the label to be identified. Fluorescence microscopy of the resultant liver slices showed a disappointing lack of resolution of the internal distribution of label. The hepatocytes took up the label throughout their cytoplasm but there was no fluorescence detected within the nucleus. The cytoplasmic fluorescence appeared to be generally distributed but with concentrations of label at the plasma membrane, and as "fibrillar"-like structures which were both ubiquitous and of little apparent symmetry. These structures, whilst resembling endoplasmic reticulum or cytoskeleton in distribution, could not be fully identified. A reasonable photographic record was not obtainable because the specialist equipment required was not available during the limited half-life of the label. The controls prepared at the same time, using EDTA to prevent enzymic incorporation of the stain, showed a much less intense and much more diffuse cytoplasmic incorporation which was designated as "Background" fluorescence. Duplicate rat liver slices were also prepared and subjected to histochemical identification of acid phosphatase and 5'-Nucleotidase. The treatment of the slices during preparation could be shown to be without any deleterious effects on the restoration to

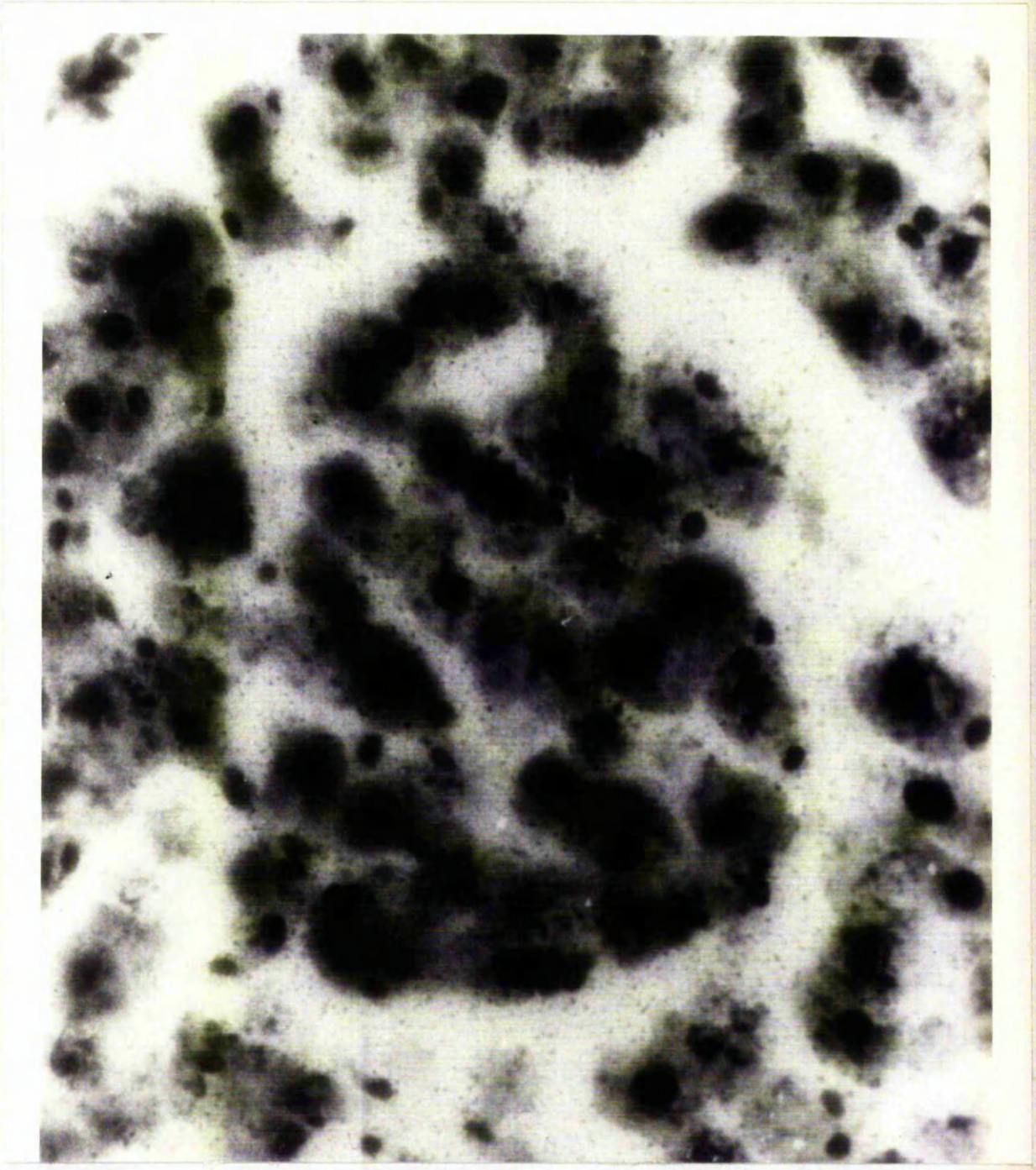
activity, from the frozen state, of these enzyme systems. Furthermore the cell integrity was assessed by Haemotoxylin-Eosin staining and was found to correspond to the standard hepatocyte structure. Thus, whilst the techniques employed in the isolation of viable rat liver slices was successful, the staining procedure for the transglutaminase was only partially so. This resulted in the visual confirmation of enzyme activity and an interesting but unresolved distribution of stain which may indicate some involvement with the plasma membrane and cytoskeletal components.

The autoradiographical studies, described in Methods 2.4.8, proved to give even poorer resolution at the light microscope level. The techniques involved in this method are of a specialist nature and ideally involve the use of equipment which was unavailable for these studies. An attempt was made to determine the subcellular sites of incorporation of $[^3H]$-putrescine, using a "tissue slice" incubation to achieve substrate uptake. The prepared slides (5μ slices) containing radiolabel were processed and the resultant developed slides examined by light microscopy. The cells, whilst appearing to be unaffected by the experimental procedure, did not display any significant pattern of substrate distribution. The high background levels of the emulsion may have masked any low level uptake of radiolabel. Alternatively the level of radioactive uptake may have been too low for detection however this is unlikely since duplicate slices were assessed for uptake, in order to calculate the exposure period, and found to contain significant levels of radiolabel. This indicates that the fault may lie in the experimental procedure. The results demonstrate that autoradiography at the electron microscopic level, which was anticipated as the next step in confirming the results obtained by the fluorescent studies, would have been entirely impracticable and for this reason was not attempted.

Figure 3.14

Autoradiographical investigation of the transglutaminase site of action.

(Cell nucleii stained with Eosin).



3.2.3 Plasma membrane studies

The determination of a possible subcellular niche for the enzyme had been prompted by two findings. Firstly, the fractionation of the liver cells had yielded a distribution that included a significant "particulate-associated" component (38% approx.) which when investigated showed the presence of plasma membrane, cell debris and nucleii. Secondly, the histochemical study had partially dismissed the nucleus as a possible site and also pointed to the plasma membrane as a candidate. These results prompted an investigation into the specific nature of a possible plasma membrane-enzyme interaction.

The methods followed for plasma membrane isolation have been described in Methods 2.4.3. Marker enzymes for plasma membrane (adenosine triphosphatase (ATPase) and 5'-nucleotidase (Methods 2.2)) and mitochondria (Succinate:tetrazoliom reductase (Methods 2.2)) as well as transglutaminase, DNA and protein (Methods 2.3) assays were determined.

The results, shown in Figures 3.15 and 3.16, demonstrate the distribution of plasma membrane components throughout the four fractions isolated but particularly to A and D. The sucrose density (discontinuous) gradient (Fig. 3.15) provided entire and clearly discernable samples which were virtually free from contamination. Assays for DNA and succinate: tetrozolium reductase activity (mitochondrial marker) showed that only 1.2% (± 0.8%) of these subfractions were present in the plasma membrane samples.

The distribution of transglutaminase in the samples (Fig. 3.16) is very similar to that of both of the plasma membrane marker enzymes assayed. This co-distribution accounted for 16% (± 1.4%) of the total transglutaminase activity in the liver homogenate and approximately 42% of the particulate-associated activity.

Previous investigations have been reviewed (see Introduction, Substrate proteins (vii)) and show the plasma membrane of rabbit liver to be both a source of substrate proteins and a site for polymeric aggregates (Linnoila et al, 1979). Also the presence of ε(γ-glutamyl) lysine dipeptide has been detected in the plasma membrane of "L" cells in culture (Birckbichler et al, 1973) and from the capsule of whelk eggs (Price and Hunt, 1973).

Self-incorporation studies which were carried out using the membrane sub-fractions and these experiments are described in the following section.

Figure 3.15

Sucrose density (discontinous) gradient

The photograph shows the distribution of the plasma membrane fractions isolated from the sucrose medium. The gradient comprised the sample (110-140 mg protein in 5ml 55% (w/w) sucrose) overlayed by successive 5ml sucrose layers of density 1.181 (43% (w/w)), 1.176 (39% (w/w)) and 1.153 (35% (w/w)). Centrifugation was carried out using conditions described in the text (Methods 2.4.3). Fractions concentrated at the three interfaces and the base of the centrifuge tube and were labelled A, B, C and D respectively from the least to the most dense with respect to the gradient.

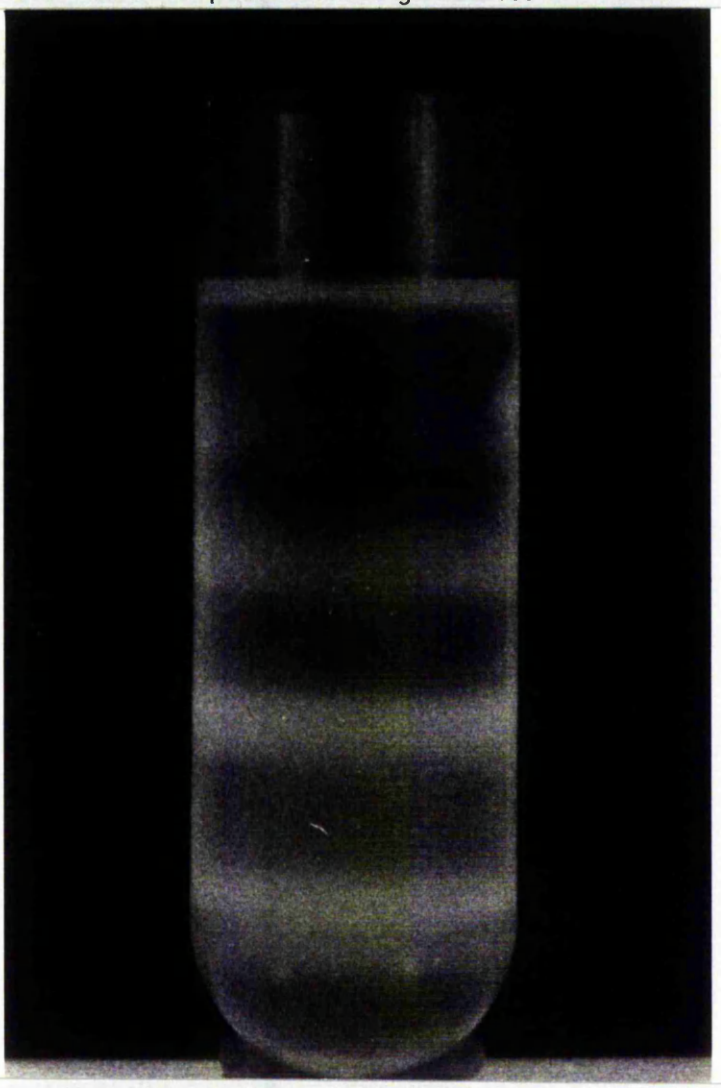


Figure 3.16

Distribution of transglutaminase and marker enzymes from the plasma membrane isolation

Plasma membrane fractions were prepared (Methods 2.4.3) by sucrose density gradient centrifugation and labelled A, B, C and D (Fig. 3.15) from the least to most dense samples respectively. The fractions were washed by dilution in 1mM $NaHCO_3$, 0.5mM $CaCl_2$, 1mM DTT, pH 7.4 and centrifugation (3000g/10 min) and the pellet fractions resuspended in 50mM Tris, 1mM EDTA, pH 7.4. The samples were assayed for plasma membrane marker enzymes adenosine triphosphatase (ATPase) and 5'-nucleotidase by methods previously described (Methods 2.2.5 and 2.2.3). Transglutaminase activity was assayed by the standard method (Methods 2.2.1.1). Histograms showing the co-distribution of transglutaminase with membrane marker enzyme are derived as the mean values of three experiments with a variation of less than 10%.

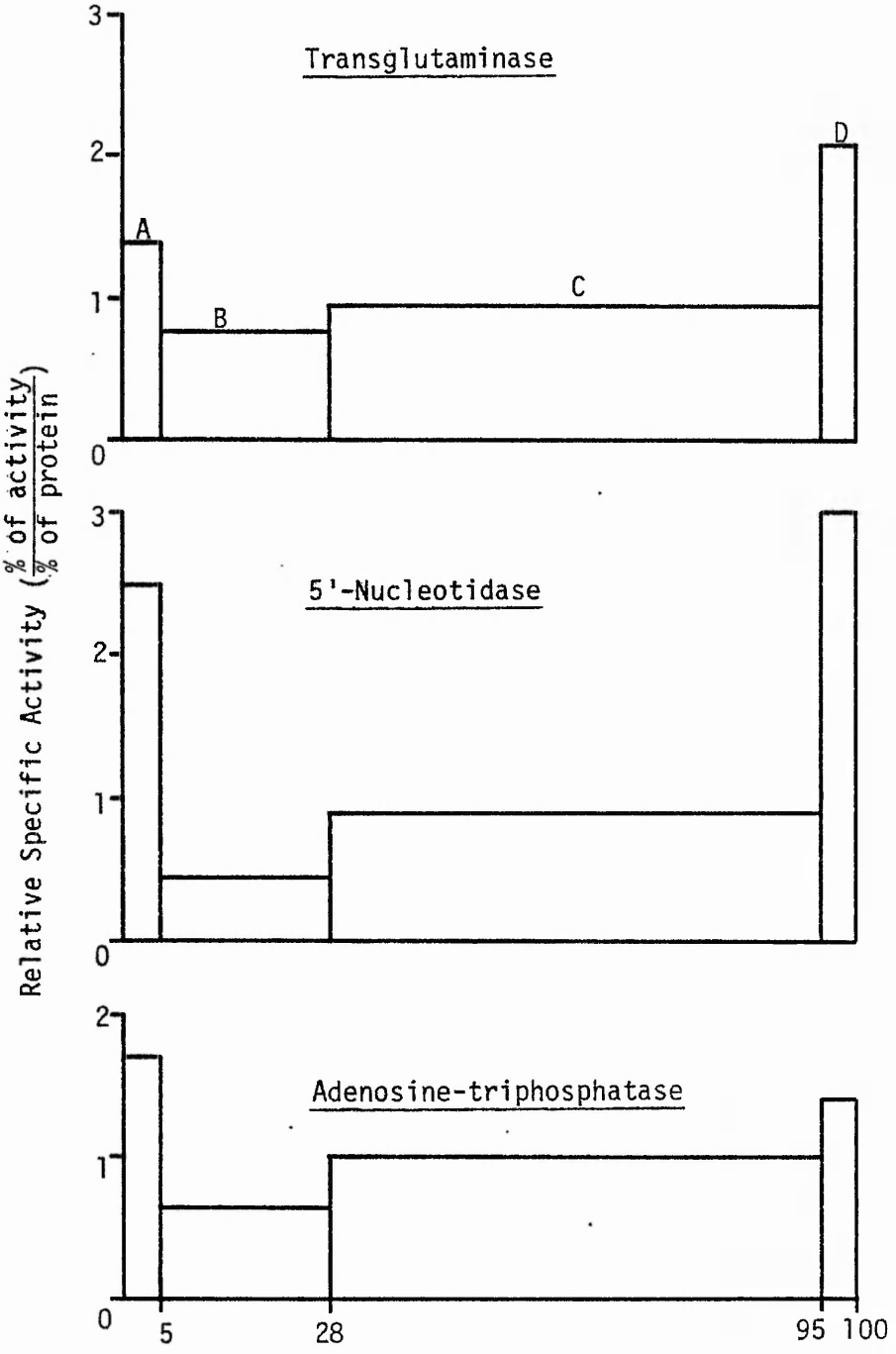

Transglutaminase
5'-Nucleotidase
Adenosine-triphosphatase
A
B
C
D
Relative Specific Activity (% of activity / % of protein)
0
1
2
3
5
28
95
100
% of Protein

Figure 3.16

3.2.4 Self-incorporation studies

The interaction of the enzyme with its endogenous cell protein was determined by means of adapting the standard assay method and has been described in Methods 2.8.1. The reaction mixture was modified by the replacement of the usual protein acceptor substrate, casein, by an equal volume of additional enzyme sample. Cell fractions were prepared from rat liver (Methods 2.4.2) in the usual way, except that the "M", "L" and "P" fractions were isolated as a single sample rather than separately. The enzyme activity, using endogenous cell protein as the acceptor substrate for the radiolabel, was determined as described above. Also, those plasma membrane samples isolated previously (Results 3.2.3) were similarly assayed to provide additional information as to their substrate potential.

The specific activity calculated for the various liver samples isolated showed a marked reduction in all fractions when endogenous protein, rather than casein, was the acceptor substrate (Fig. 3.17.1). The rate was seen to plateau after approximately 20 min of the 90 min over which the assay was made. At that time (90 min) a sample of casein was added to the assay to a final concentration of 8mg/ml. There was an immediate increase in reaction rate which when compared to a similar casein addition at time zero demonstrated that whilst the soluble enzyme retained only about 40% of its activity the particulate bound (N) enzyme had maintained in excess of 80% of its original activity over this lengthy incubation. Thus it would appear that the bound enzyme may be stabilised by its environment. Also this result demonstrates that the fall in reaction rate with endogenous protein was due to the saturation of acceptor sites rather than to any dramatic decrease in enzyme activity. The initial reaction rate was found to be approximately 2-4 fold greater in the presence of casein confirming its general use as an excellent

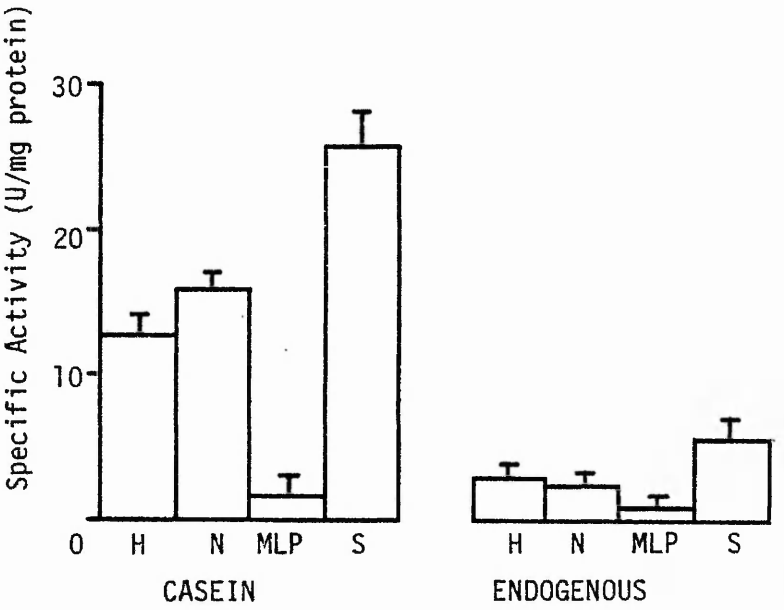
Figure 3.17

Self-incorporation activity towards rat liver whole tissue and plasma membrane sub-fractions

The following histograms demonstrate the specific activity (units*/mg protein) and the relative specific activity against per cent of protein for the various fractions. The legend "casein" beneath the histograms refers to the use of casein as the acceptor protein (standard assay-method 2.2.1.1) and "endogenous" to the use of endogenous protein for self-incorporation. The legend "H" refers to the homogenate fraction; "N", the nuclear fraction; "MLP", to the combined mitochondrial, lysosomal and microsomal fractions; "S", the soluble fraction; and A, B, C and D as the plasma membrane fractions isolated in Results 3.2.3.

*Unit activity is defined as that amount of enzyme which catalyses the incorporation of 1nmol of putrescine into casein per hour under conditions given in the text (Methods 2.2.1.1).

3.17.1



3.17.2

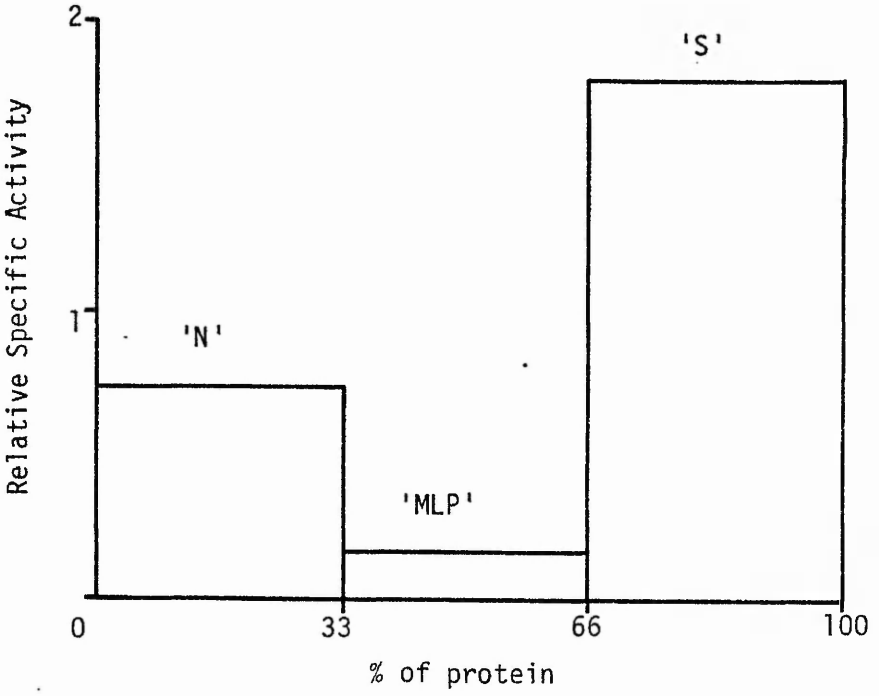
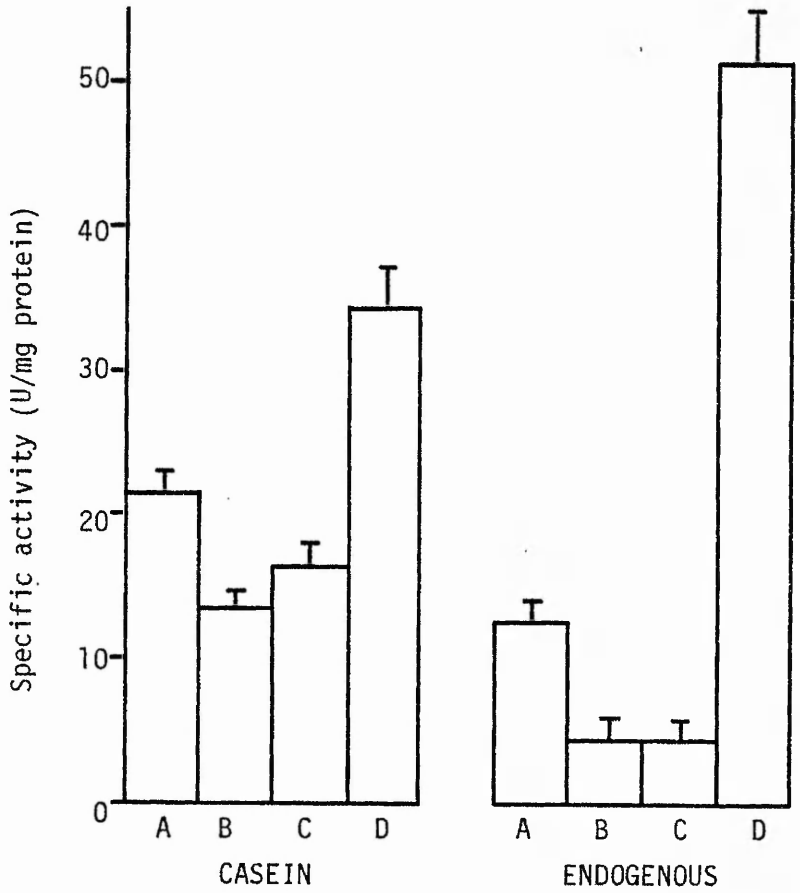
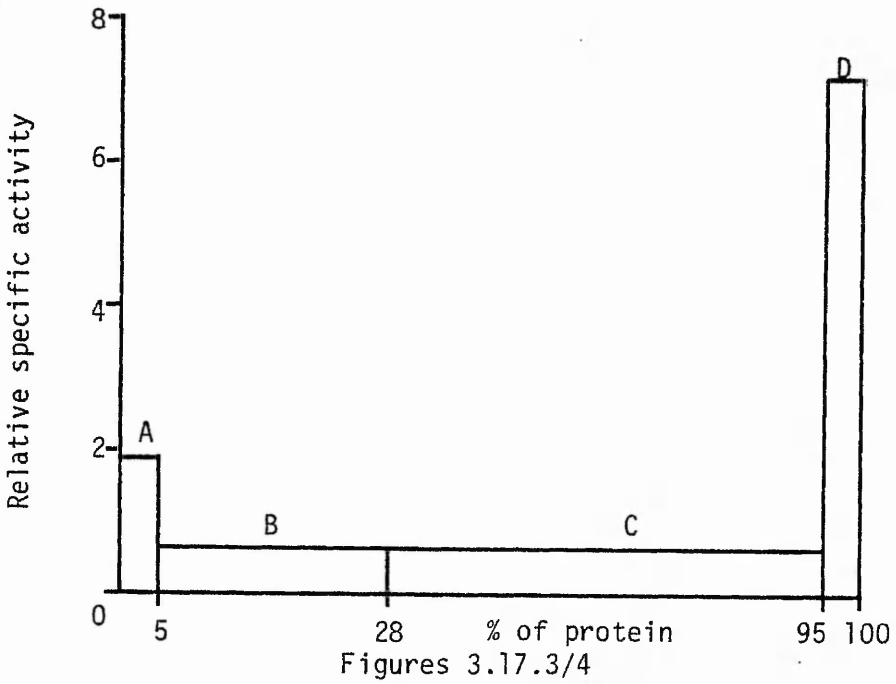


Figure 3.17.1/2

3.17.3



3.17.4



Figures 3.17.3/4

pseudosubstrate. The histogram (Fig. 3.17.2) of the relative specific activity with endogenous protein showed a very similar profile to that already derived with casein (Fig. 3.13).

An identical series of experiments were performed with the plasma membrane samples previously isolated. The specific activity of the fractions was only moderately altered with endogenous protein as the acceptor substrate. The adopted isolation method (Neville, 1960) designated sample A as the major sub-fractions of plasma membrane, though in addition, the results demonstrated a significant level of membrane marker enzymes present in sample D, along with the transglutaminase activity (Fig. 3.16). The specific activity of the enzyme with the endogenous protein from these fractions showed that sample D contained substrate(s) upon which the enzyme was particularly active, even more so than on casein (Fig. 3.17.3). Also sample A, the membrane sample, was seen to provide substrate(s) upon which the enzyme was significantly active. The relative specific activity histogram (Fig. 3.17.4) confirms this distribution of high levels of both enzyme and suitable acceptor substrate(s) in samples A and D, particularly the latter. Sample D was not subjected to complete analysis but had been found to contain the trace levels of DNA (but not the mitochondrial contamination which was found in Sample C) as well as the membrane marker enzymes. This information endorses the supposition that along with nucleii the other constitutents of this fraction might be large vesicles or polymer proteins and possibly aggregates of denatured protein.

Further analysis of endogenous protein used in self-incorporation experiments was performed in order to ascertain the molecular weights of substrate proteins. Two samples were prepared in which (i) homogenate proteins were incubated with radio-labelled putrescine and (ii)

tissue slices of rat liver were incubated with the same label. The resultant tissue was fractionated into low speed spin (1000g x 10 min) pellet (N) and supernatant (E) and later analysed by SDS-polyacrylamide electrophoresis. The resultant gels were measured for radioactivity and the position in the gel related to standard molecular weight markers.

The incubation of homogenate proteins with putrescine resulted in the label being incorporated in proteins throughout the gel (Fig. 3.18). The largest concentration of label was found in the first segment of low speed pellet (N) gel where 44% ± 3% of the incorporated putrescine was associated with very high molecular weight protein. The majority of this protein was excluded from passage through the 5% gel and would therefore have a molecular weight in excess of about > 400,000. There were two other significant concentrations of label, though at much lower levels. Approximately 15% of the label could be found in a gel segment pertaining to a molecular weight range 175,000-225,000, and, about 8% of the label was present in proteins in the range 45,000-57,000. The low speed spin supernatant (E), containing the "MLP" and "S" fractions described previously, showed very low levels of incorporation throughout the gel (approximately 9% of the "pellet" uptake). However the peak fractions were again found to be the highest molecular weight proteins (12% of total uptake) and the 45,000-57,000 molecular weight proteins (15% of total uptake).

The liver tissue slice incubation with putrescine gave a broadly similar pattern of label distribution. The majority of the label again went into the very high molecular weight protein of the "N" fraction (54% ± 4% approx.), with only one further small peak found in the range 45,000-57,000 (8% of the total uptake). The supernatant "E" uptake was very low (8% approx. of slice incorporation) and showed no discernable

concentrations of label after electrophoresis. The total putrescine incorporation by the slice method was 23% ± 2% of that achieved from homogenate protein incubation, but could more properly represent the availability of naturally occuring substrates to the enzyme in its normal physiological niche. EDTA in the incubation mixture instead of Ca^{2+} prevented amine incorporation into the cell proteins.

These investigations were furthered by the work of Linnoila et al (1979). Studies involving rabbit liver cytosol and plasma membranes identified two proteins in the molecular weight range 150,000-250,000 which were susceptible to the enzyme and were instrumental in the formation of very large molecular weight complexes. Furthermore, the coupling of amines to membrane proteins has been reported to be preferentially enhanced when those proteins are equal to or in excess of 150,000 daltons (Lorand et al, 1976; Okumura and Jamieson, 1976). The possible identity of the protein substrate(s) of molecular weight 45,000-57,000 was investigated in the following section.

Figure 3.18

Radio-labelled-protein analysis by SDS-polyacrylamide gel electrophoresis

Samples of tissue were incubated in the following manner (Methods 2.8.1) and analysed by SDS-polyacrylamide gel electrophoresis using the methods described in Appendix 1.

(i) Homogenised rat liver in 0.25M Sucrose/1mM Tris/1mM EDTA, pH 7.4 was added as a 650µl sample to 350µ of the standard transglutaminase assay mixture (Methods 2.2.1.1) but without casein. Following incubation (120min/37°) the reaction was stopped by the addition of EDTA (to 9mM) and the mixture centrifuged (1,000g x 10 min). The fractions were taken up in 2 parts 3% SDS:3% mercaptoethanol:9M urea and dialysed against 0.1% SDS/0.1% mercaptoethanol overnight. Samples (20µl containing 80-100µg protein) were electrophoresed (Appendix 1, using 5% gels) and the resultant gels either stained for protein or sectioned (5mm) and treated as Methods 2.3.4(iv). The molecular weight of the radio-labelled protein was determined by reference to a stand graph constructed using markers (SDS-MW 70, Sigma Chemical Co. Ltd.).

(ii) Rat liver tissue slices (300gm)(Methods 2.7) were incubated for 120 min at 37° and the reaction stopped by homogenisation in incubation buffer supplemented with EDTA to 10mM. The procedure was then identical to that described above.

The histograms compare the radio-labelled putrescine uptake by the various proteins identified by their relative mobilities. The following key has been observed: (1) refers to the low speed spin supernatant "E" profile of the homogenate derived sample; (2) to the "N" profile of the same sample; and (3) refers to the "N" fraction of the tissue slice-derived sample. The "E" fraction of the latter sample gave such low putrescine uptake as to tender little informative data. Results are expressed as the means ± S.D. for triplicate gels.

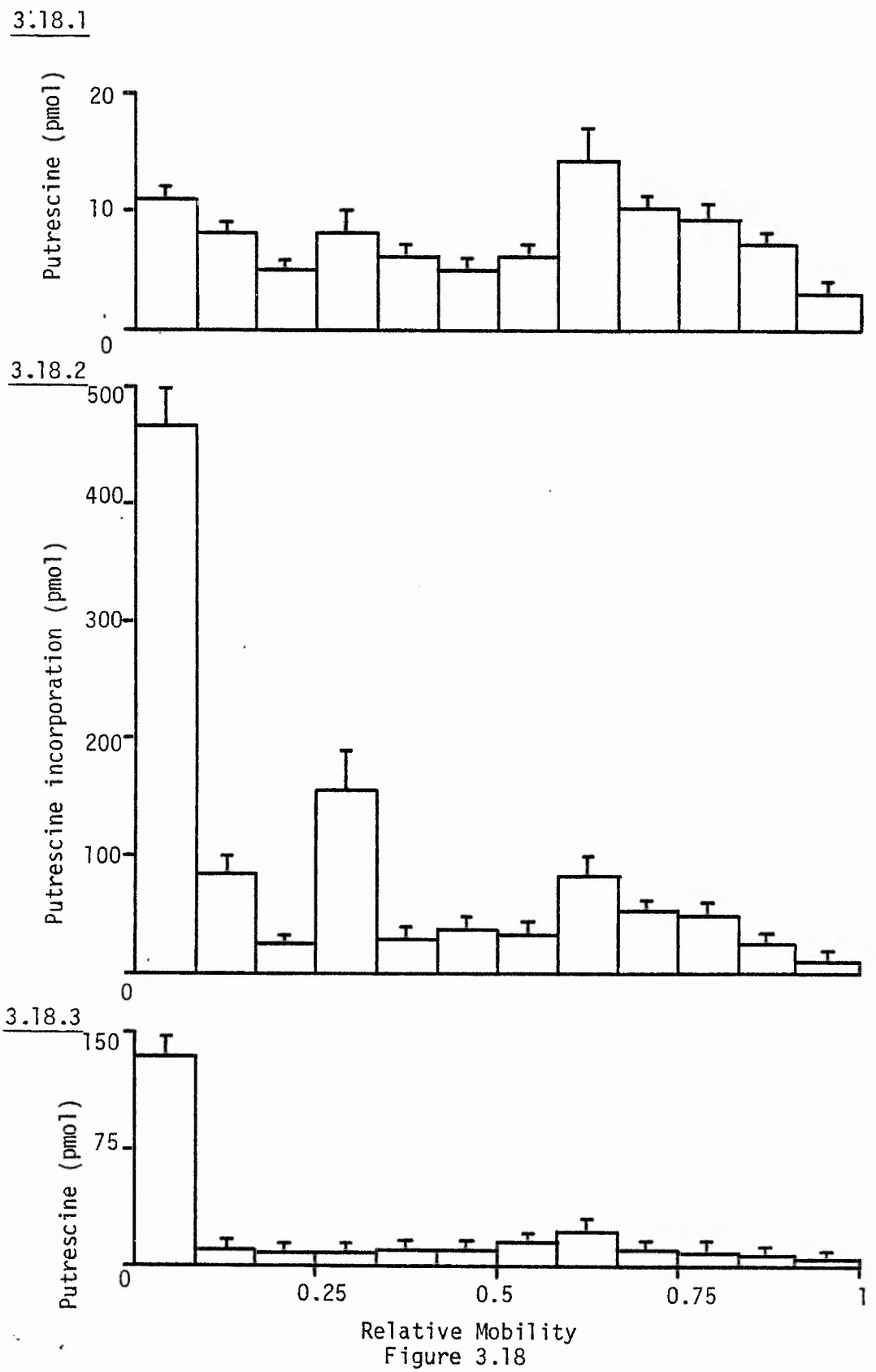

3.18.1
Putrescine (pmol)
20
10
0
3.18.2
Putrescine incorporation (pmol)
500
400
300
200
100
0
3.18.3
Putrescine (pmol)
150
75
0
0.25
0.5
0.75
1
Relative Mobility

Figure 3.18

3.2.5 Actin studies

Studies into the transglutaminase mediated incorporation of amine pseudosubstrate (the results of which have been described) have suggested that (i) the molecular weight range 45,000-57,000 included possible substrate protein and (ii) from histochemical labelling, a cytoskeletal protein may be involved. A survey of the relevant literature indicated that actin may fulfil both these criteria as it is the major element of the cytoskeleton and has a molecular weight of 48,000 (Spudich and Watt, 1971).

Purified actin was prepared (Methods 2.5.1) and passed through an affinity chromatography column (Sephanrose-4B) to confirm the procedure of Lazarides and Lindberg (1974) (Methods 2.4.5.5). Rat liver tissue slice incubations (Methods 2.8.2) with [^{14}C-] putrescine were fractionated and the particle-free supernatant fraction was passed through the affinity column (Fig. 3.19).

Purified actin was shown to bind tenaciously to the DNase-I affinity column and could be eluted with buffer containing 3M guanidine. The material passing through the column unabsorbed was due to excess ATP present in the actin preparation.

Samples of cytosol protein were found to contain actin into which was enzymically incorporated radioactive amine. The control experiments, in which EDTA replaced Ca^{2+} in the incubation medium, gave similar levels of actin in the cell but no detectable putrescine incorporation. This result suggests that actin may represent a natural substrate for the enzyme and, be present in the modified form in the soluble fraction of the cell.

Previous studies of the relationship between actin and the transglutaminase enzyme have always been carried out in vitro. Actin has been

demonstrated in high molecular weight aggregates with other contractile proteins (Derrick and Laki, 1966) and fibrin (Mui and Ganguly, 1977) but never in the isolated form from intact cells.

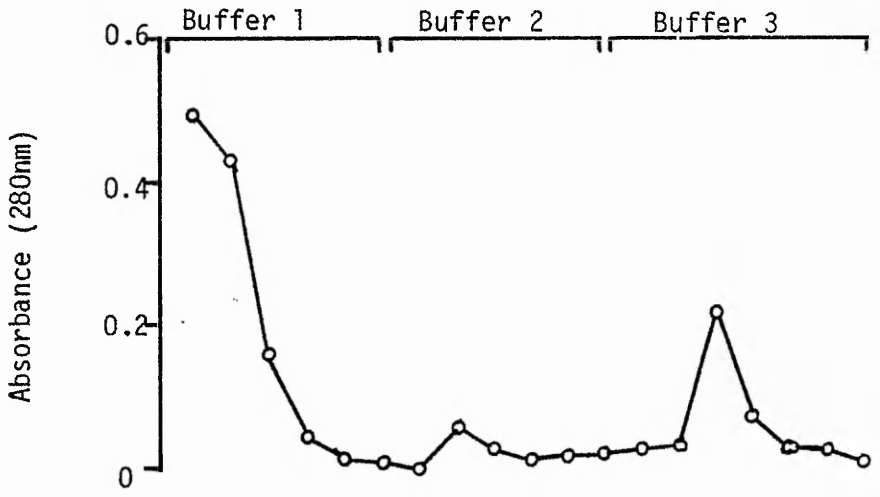
Figure 3.19

Affinity chromatography of actin

Rabbit muscle actin was purified (Methods 2.5.1) and passed through a DNase-I-Sepharase 4B affinity chromatography column as described in the text (Methods 2.4.5.5). A typical protein elution profile (Fig. 3.19.1) was determined by $A^{1\%}_{1cm280nm}$ from fractions (1ml) taken during elution by (1) 6ml Buffer A (0.5M sodium acetate; 1mM $CaCl_2$; 30% (w/v) glycerol, pH 7.4), (2) 6ml Buffer A plus 0.75M guanidine, pH 6.5 and (3) 6ml Buffer A plus 3M guanidine, pH 6.5.

A second experiment (Fig. 3.19.2) involved the elution of a sample of particle-free supernatant (4mg protein approx.), derived from a liver tissue slice incubation with $[^{14}C]$ putrescine (Methods 2.8.2), using a similar affinity column (3.5ml volume). The elution scheme was as above with the exception that unbound putrescine and non-actin protein was first eluted using 5ml 0.15M NaCl; 50mM Tris-HCl; 1mM Ca^{2+}, pH 7.4 and 6ml Buffer A. Fractions (1ml) were collected and assayed for protein ($A^{1\%}_{1cm280nm}$) and radioactive content (Methods 2.8.2 and 2.3.4).

3.19.1 Standard Actin



3.19.2 Tissue Slice Actin

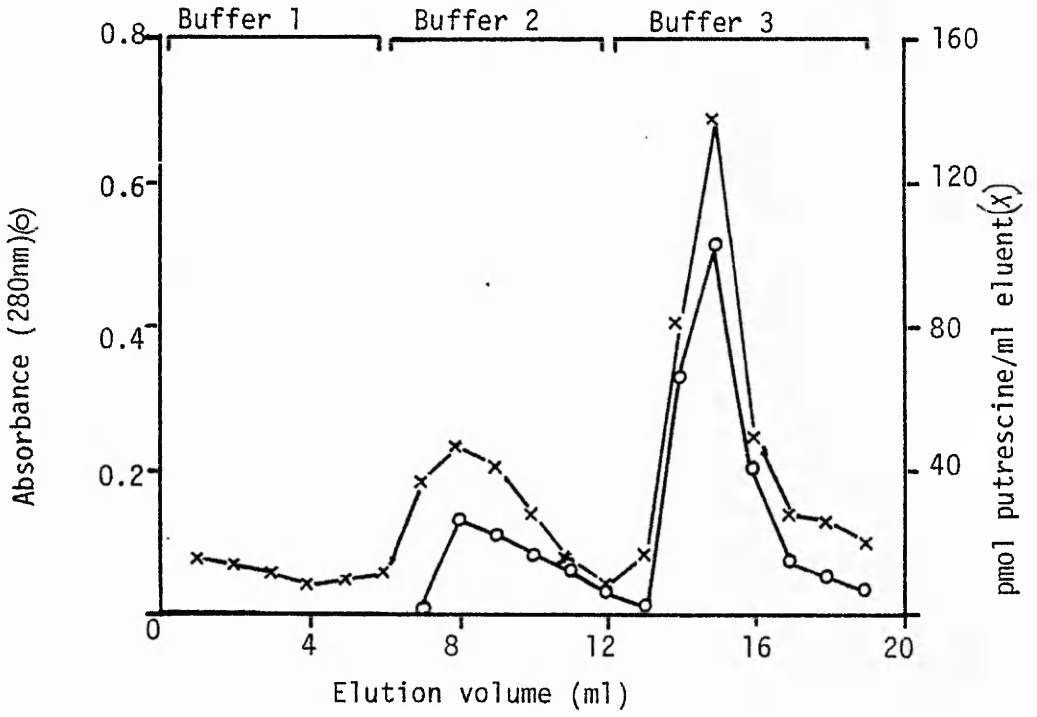


Figure 3.19

3.2.6 Enzyme Solubilisation

The subcellular "nuclear" fraction has been demonstrated to contain approximately 38% of the available transglutaminase activity (Results 3.2.1). Preliminary investigation of this association revealed a consistent and close relationship between the rat liver enzyme and the particulate fraction, and not one resulting from any non-specific binding during preparation. This relationship was examined during a series of experiments with the objective of discovering whether the cell control of the enzyme distribution may occur by a reversible binding mechanism.

Rat liver tissue was fractionated as previously described and the 600g "nuclear" fraction isolated. The "nuclear" fraction was then resuspended in the appropriate buffer system and "washed" twice before the pellet fraction was placed in homogenisation buffer. The relevant fractions were assayed for transglutaminase and the total activity of the enzyme determined. The accumulated data have been expressed in terms of (i) the amount of enzyme solubilised into the "washes" and (ii) the total measurable activity after solubilisation as compared to the enzyme level in the original "nuclear" fraction (Fig. 3.20.1).

The buffer systems were based on 0.25M Sucrose/1mM Tris-HCl, pH 7.4 and the homogenisation buffer included 1mM EDTA. This buffer was taken to be the standard as it has been used throughout the experimental programme.

The adoption of particular solubilisation buffers was directed at enzyme binding through active site properties though attention was also given to hydrophobic, electrostatic and other considerations.

The literature (and previous results) have shown enzyme activity to require Ca^{2+} ions, reducing conditions and substrate. It was anticipated

that these factors may exert some further control in this case over the distribution of the enzyme. The inclusion of 1mM concentrations of EDTA, Ca^{2+} and putrescine into separate buffers produced similar experimental data. All three systems eluted roughly the same levels of enzyme into the washes whilst the increase in the amount of enzyme due to the washing buffer ranged from 30% for putrescine to 51% and 61% for Ca^{2+} and EDTA respectively. Thus it would appear that the enzyme can be solubilised from its support and the overall enzyme level increased. This may be as a result of activation of existing enzyme or by the release of previously undetected enzyme from the "nuclear" fraction protein. Since the "washed" nuclear fraction activity was 70%-90% of that measured in the original fraction the activation of existing enzyme seems less plausible than the proposal of the release of enzyme from possible storage sites.

The effect of increasing the EDTA concentration was in lowering the enzyme eluted in the washes. The overall enzyme level measured was equal to that set by the standard 1mM EDTA buffer. This would infer that Ca^{2+} or some other chelated ion might mediate in the release of the enzyme and that 1mM EDTA was incapable of totally negating its effects.

An investigation of the effect of partition on enzyme distribution was undertaken by increasing the number of washes to five while using the standard buffer throughout. The amount of enzyme eluted was almost doubled over that achieved by the two-wash regime, whilst the "nuclear" fraction activity was reduced by only 20%. This partition effect produced a 109% increase in the total detectable enzyme level showing that a significant role may be played by the equilibrium balance of bound to soluble enzyme.

The use of dithiothreitol (DTT) to produce reducing conditions was examined in conjunction with a Ca^{2+} (1mM) containing buffer. Whereas Ca^{2+} alone gave a significant level of elution the addition of DTT caused very little enzyme to pass into the soluble phase. There was some enhancement of the total enzyme level, since some activity was eluted, but this was small in comparison to others (approximately 13% of standard buffer result). There would, on the basis of this result, seem little possibility of the enzyme binding being due to sulphydryl interaction.

The most prominent result in this series of experiments came from the combined use of Ca^{2+} and amine substrate. The previous discussion has shown each of these components, when examined separately, to cause significant elution and increased yield of enzyme. When Ca^{2+} and putrescine were combined the reverse situation was demonstrated where there was no detectable activity in the washes and a reduced (by approx. 10%) activity in the remaining nuclear fraction. This result was confirmed by the use of another polyamine, spermine, which, in conjunction with Ca^{2+} gave the same zero enzyme activity in the washes and further caused a substantial reduction (35%) in overall enzyme level. These data suggest that the enzyme may become covalently fixed to the "nuclear" fraction through the polyamine or that the binding of the substrate amine causes a substantial increase in the tenacity of the binding phenomenon. In both cases the greatly increased binding and overall reduction in activity in the presence of amine and Ca^{2+} activated enzyme suggests a clear role in the modulation of activity.

The possible interaction of hydrophobic moieties in the binding process was investigated using phenylalanine, butanol and a mixture of detergents. No clear pattern evolved but some interaction was demonstrated with specific molecules. Phenylalanine was used as a competitor for a

hydrophobic membrane binding site and was shown to cause a moderate level of elution from the nuclear fraction (50% of standard buffer) whilst causing a slight decrease in overall activity. Butanol (in the presence of DTT) gave a moderate level of solubilisation and produced a 30% increase in overall enzyme level. When the experiment was carried out in the absence of DTT (and therefore under denaturing conditions) the enzyme was inhibited. The detergents (Taurocholate and Triton X-100) have been successfully used to solubilise membrane bound β-glucosidase (Mueller and Rosenberg, 1977) and were tested to ascertain whether their properties extended to the transglutaminase phenomenon. The detergent mixture resulted in very low leaching of enzyme into the washes and a small decrease in overall activity. This suggests that this particular system promoted enzyme binding much more than did phenylalanine and in direct contrast to butanol. These results, whilst suggesting some hydrophobic interaction in the binding mechanism, are confusing since no pattern is involved. Thus a greater study of these interactions would be necessary to provide a clear picture, with particular emphasis on the removal of all traces of detergent prior to enzyme assay.

The involvement of electrostatic charges in binding was examined by the use of isotonic saline buffer. The data demonstrates that this buffer was only 55%-65% as efficient in elution and increasing overall enzyme levels as was the standard buffer.

Energy dependence was considered as another possible contributory factor in the binding mechanism. ATP and Mg^{2+} were used in a buffer system and caused a moderate level of elution which was inconclusive since it neither raised nor reduced to a minimum the solubilisation of the enzyme. However the overall level of enzyme was reduced (-14%)

due mainly to a large reduction in the activity of that enzyme remaining bound. This result suggests a lowering of available activity of bound enzyme when the energy level is high and a failure to stimulate enzyme stored in the pellet fraction.

Reference to the substrate potential of actin, as previously discussed, instigated an investigation into a buffer system which caused the solubilisation of this protein. A similar result to that above was encountered where there was a moderate elution of enzyme into the washes and a small decrease in the overall level of the enzyme. Thus it would appear that the removal of actin from the proteinaceous pellet to the soluble fraction may have also released some enzyme. However, since the overall enzyme level fell, the removal of actin caused a large decrease in the level of bound activity. This perhaps demonstrates a failure of this stimulus to activate the stored enzyme reserves, and so was acting independently of a general mechanism governing enzyme distribution.

A chaotropic salt, potassium thiocyanate (KSCN) was examined and a 125mM solution in buffer was used to wash a portion of "nuclear" fraction material. In addition, the effect of KSCN concentration on enzyme distribution was evaluated by preincubation of identical samples with a range of solutions (0-125mM). After incubation the samples were centrifuged and the particulate and soluble fractions assayed as described above. The washing of the "nuclear" fraction provided data for both elution and overall enzyme level which was similar to that described for the standard buffer solution. Therefore the decrease in strength of hydrophobic interaction brought about by the chaotropic effect of the salt caused the redistribution of the enzyme from its bound to soluble state. This was confirmed in the second experiment

(Fig. 3.20.2) where the redistribution was enhanced by increasing KCNS concentration until a plateau of effect was reached (approximately 50 -60mM KCNS) and further increases did not bring about any change.

Ethylene glycol, an organic solvent which produces a hydrophobic response by lowering the polarity of the eluent in which it is contained, was found to promote a change in distribution of the enzyme from the particulate to the soluble state. This experiment has been described in a later section (Results 3.2.7.2).

Figure 3.20.1

Enzyme solubilisation

Sample tissue (5-10g) was homogenised by the procedures previously described, in 3 vol. 0.25M Sucrose; 1mM Tris-HCl; 1mM EDTA, pH 7.4. The homogenate was centrifuged for 10 min. at 600g (rav 28cm) and the supernatant removed. The pellet was resuspended in 3 vol. sucrose buffer and recentrifuged as above. A sample of the pellet was resuspended in washing buffer (5 vol) for 15 min at 4° before centrifugation at 600g for 10 min (Wash 1). This procedure was repeated, but included an incubation of 100 min at 4° prior to centrifugation (Wash 2). The remaining pellet was resuspended in 3 vol. homogenisation buffer. All "wash" samples were dialysed against homogenisation buffer to remove those components which may influence enzyme activity. Fractions were assayed for transglutaminase by the standard method and protein (Lowry). All experiments were performed on 2-3 samples and a control using homogenisation buffer throughout was included. Results are expressed as means ± S.D. where appropriate. The following washing buffers were used and are shown as the additions to the basic buffer 0.25M Sucrose: 1mM Tris-HCl, pH 7.4 (except for buffer 0). The pH was constant at 7.4 all the buffers and all steps were carried out at 4°.

(a) 1mM EDTA (standard homogenisation buffer)

(b) 2.5mM EDTA

(c) 5mM EDTA

(d) 1mM EDTA (Partition experiment)

(e) 1mM Ca^{2+}

(f) 3mM Ca^{2+}:1mM Dithiothreitol (DTT)

(g) 1mM putrescine

(h) 1mM putrescine:1mM Ca^{2+}

(i) 1mM spermine:1mM Ca^{2+}

(j) 5mM phenylalanine

(k) 4% (v/v) butanol:1mM DTT

(l) 1% (w/v) sodium taurocholate:0.1% (v/v) Triton X-100

(m) 0.9% (w/v) NaCl

(n) 3mM Adenosine triphosphate (ATP):5mM Mg^{2+}

(o) 10mM Tris-HCl:0.1mM ATP:1mM DTT:0.1mM Ca^{2+}, (Actin solubilisation buffer)

(p) 125mM KSCN

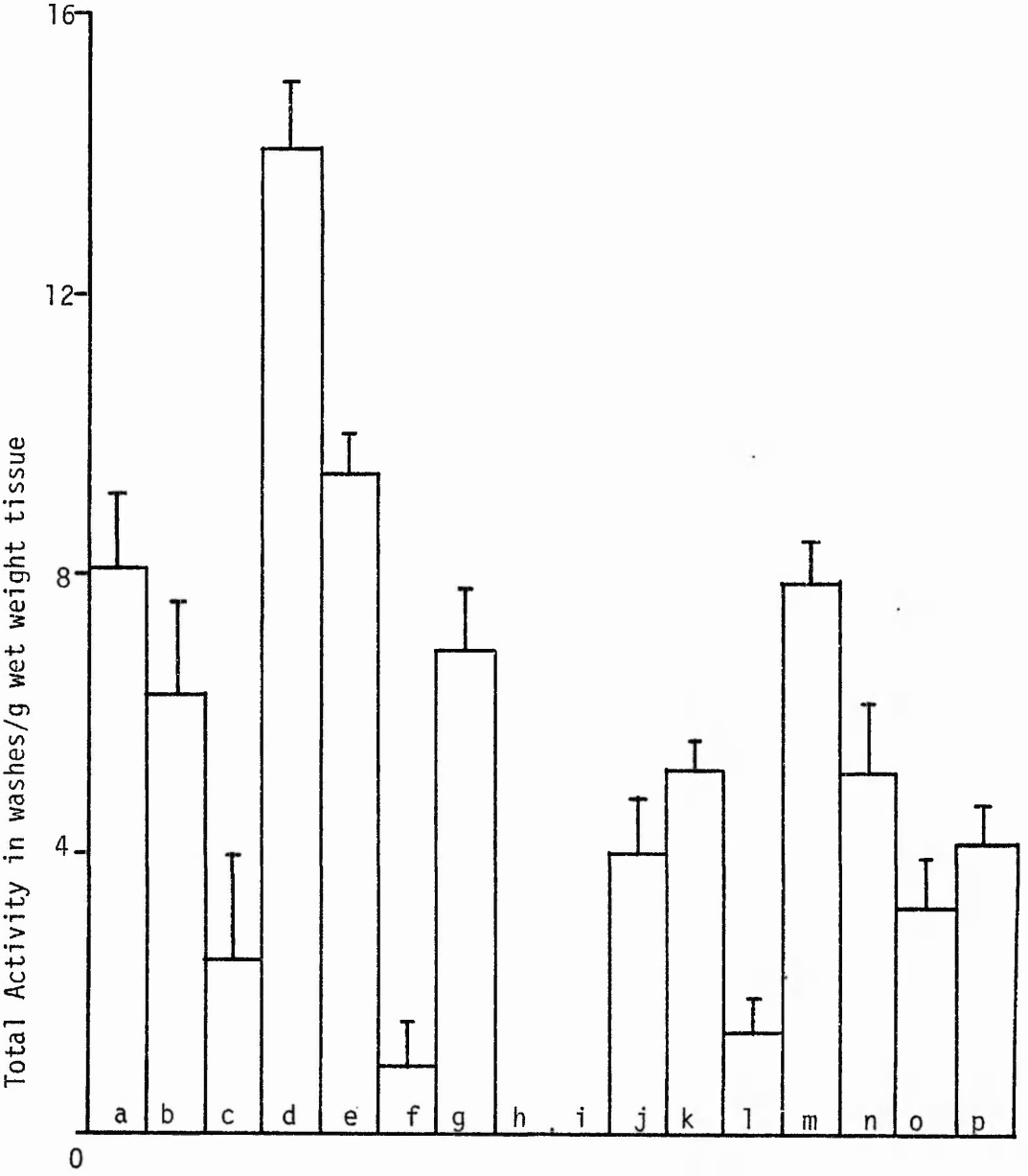


Figure 3.20.1.1

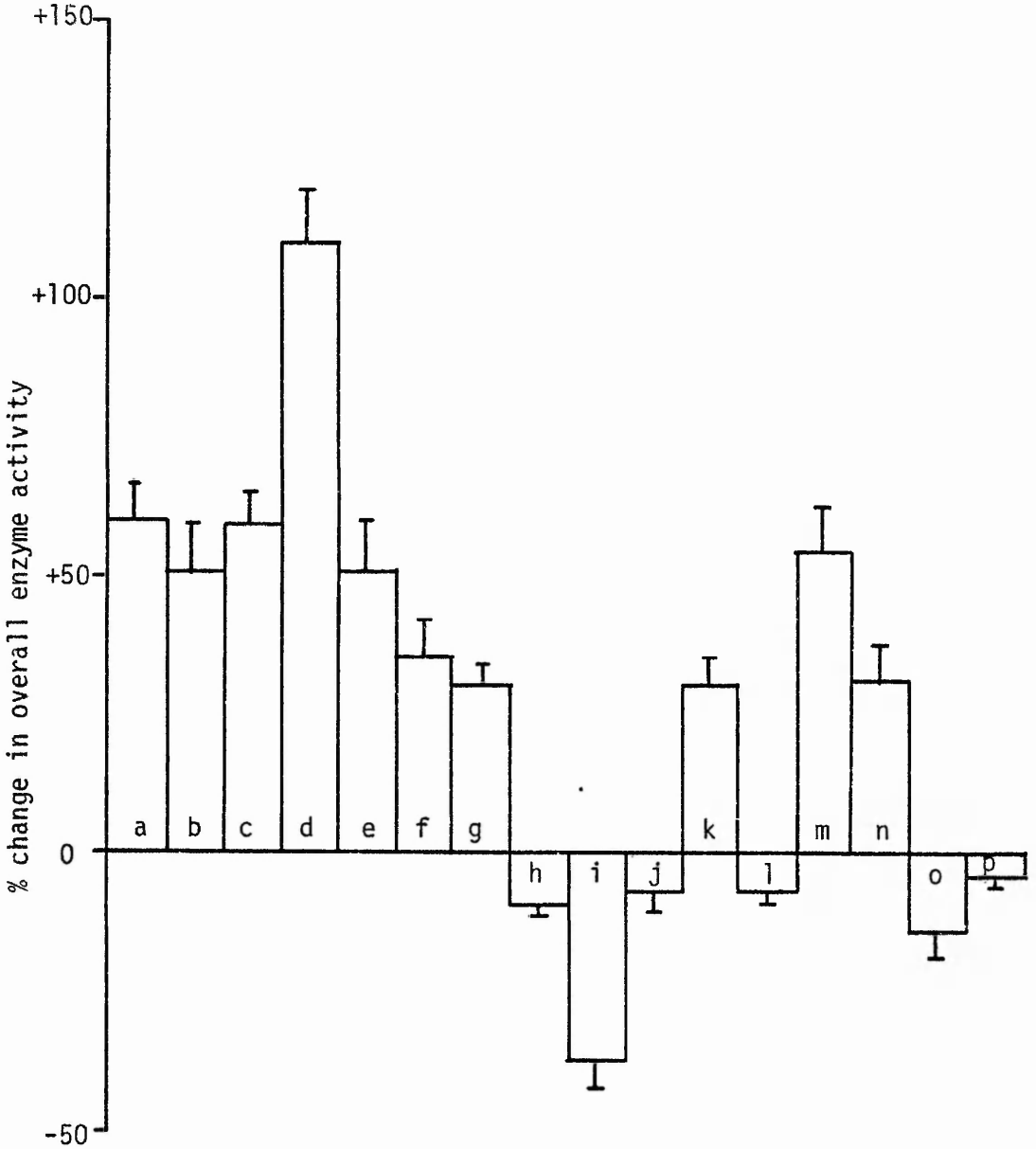


Figure 3.20.1.2

Figure 3.20.2

Effect of KCNS on enzyme distribution

Samples of "nuclear" fraction (see text) were incubated with 0; 32.25; 62.5; 125mM final concentrations of potassium thiocyanate (KCSN) for 30 min at 37°. The samples were then centrifuged (1,000g for 15 min) and the pellet fractions resuspended in homogenisation buffer. The samples were dialysed against homogenisation buffer (50 vol) for 120 min and assayed for transglutaminase and protein (Lowry). Experiments were carried out in triplicate and means ± S.D. are quoted.

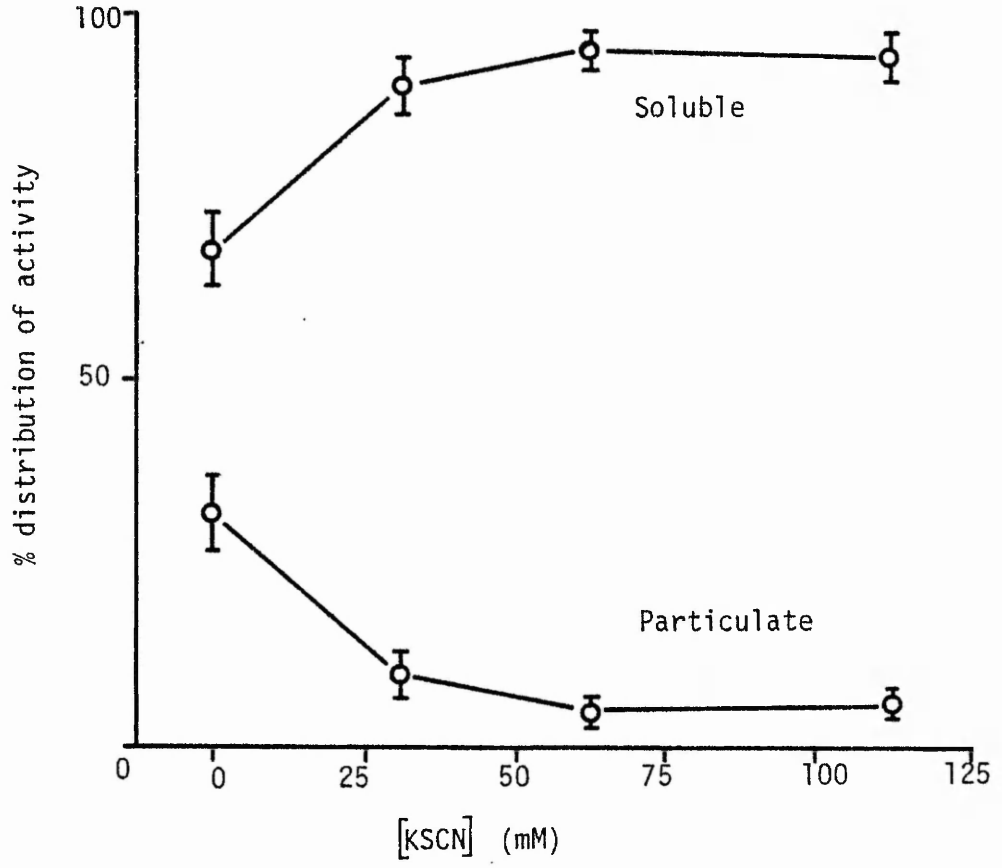

100
50
0
% distribution of activity
Soluble
Particulate
0
25
50
75
100
125
[KSCN] (mM)

Figure 3.20.2

3.2.7 Enzyme Activation

3.2.7.1 Purified enzyme studies

Ogawa and Goldsmith (1976) and Goldsmith et al (1978) have shown that human epidermal transglutaminase may be stimulated by treatment with various agents. A similar survey was undertaken using a small range of organic solvents, a chaotropic salt (KSCN) and trypsin to evaluate whether the observed effects may provide some insight into possible cellular control mechanisms.

Purified rat liver enzyme (Results 3.1.1) was pre-incubated for 20 min at 37^{o} with a range of concentrations of potassium thiocyanate (KSCN; a chaotropic salt), trypsin (a proteolytic enzyme), n-butanol (aliphatic alcohol) and dimethylsulphoxide (Me_2SO; an organic solvent). Pre-incubation was made in the presence of either EDTA or Ca^{2+} in order to assess the effect of the agent on the "activated" enzyme. The results, expressed in terms of percentage of control activity under varying concentrations of agonist, are compiled as Figure 3.21.1. Trypsin treatment produced a marked enhancement of enzyme activity at low concentrations (approximate range 20-100μg/ml) with a maximum 6-fold increase in activity at the lowest concentration measured (31.25μg/ml). Trypsin concentrations in excess of 150μg/ml, under the conditions used, caused a reduction in activity.

The data above relates to the activity determined for samples pre-incubated in the presence of Ca^{2+} ions. When EDTA was present the resultant activity showed only a moderate but nevertheless sustained increase (approximately 60%). This demonstrates a calcium-dependent factor in trypsin enhancement of the transglutaminase. The Ca^{2+} presence would also have stabilised the trypsin against autolysis. Note should also be taken of the results obtained for the pre-incubation

with thrombin (Results 3.1.3.4) which showed no such stimulation.

KSCN treatmentproduced a two-fold enhancement of activity at low concentration but this effect was reduced to a consistent 30-40% increase with higher concentrations. The results from the EDTA pre-incubation demonstrate a very similar pattern of activity but without the initial peak response. This suggests that though Ca^{2+} may influence activation at low concentrations, probably by active site protection, its effects are nullified at those higher KSCN concentrations which evoke a reduced response through the denaturing conditions. NaCl, used at the same concentrations, failed to produce any enhancement of enzyme activity.

DMSO produced a significant stimulation of activity which, as with previous reagents, showed as a peak response at the lowest concentration (4.5 fold increase at 5% DMSO) followed by a concentration-dependent decline in response to the higher concentrations. The EDTA pre-incubation samples produced a very similar response to that described but at a much reduced level.

n-Butanol stimulation in Ca^{2+} pre-incubated samples was only moderate at 5% (v/v) levels (66% increase in activity) and at higher concentrations failed to influence the enzyme activity.

EDTA pre-incubated samples showed a marked reduction in activity (approximately 20% of control values) showing that the Ca^{2+} ion played a role in protecting the enzyme from butanol inhibition. This result is diffcult to explain but may be due to the butanol-sensitive region of the enzyme becoming less accessible when the Ca^{2+} ion produces the conformational changes inherent in the activation sequence. More specifically butanol may have inhibited the enzyme through destabilisation of the active centre thiol residue which was less apparent at the lower concentrations and in the presence of Ca^{2+} ions.

The mechanism for the observed enhancement of activity may have been due to (i) the removal by proteolysis or solubilisation of an inhibitor complexed to the enzyme (ii) the dissolution of multimers or (iii) conformation changes of the enzyme brought about by the environment produced by the particular agent.

Examined in greater detail these proposed mechanisms may be evaluated as follows: The presence of a complex due to antizymes or phospholipids could have been observed on comparisons of data from gel-filtration and SDS-polyacrylamide electrophoresis. Under these conditions the molecular weight determined by these two procedures could be different whereas no such findings were made. Effects due to the solubility of the substrates or product may also be dismissed since it is the concentration of the agent in the pre-incubation which determined the degree of activation and not the concentration of the final assay. The theory of multimer formations may be doubtful since Ca^{2+} present in the pre-incubation would have caused these assemblies to be covalently crosslinked. These multimers would not have responded to disaggregation by the agents and would, moreover, have probably reduced rather than raised the enzyme activity. Furthermore previous experiments have shown the enzyme preparation to be free of any known transglutaminase zymogenic protein.

The enzyme collagenase has been demonstrated to react to KSCN and trypsin, in a model system, by increasing its activity without any apparent molecular weight change (Sellers et al, 1977; Stricklin et al, 1977).

The most likely explanation for the results must therefore be a conformational change, a conclusion also reached by Goldsmith et al (1978) using a similar system with epidermal transglutaminase.

Furthermore Neurath and Walsh (1976) have demonstrated activation by conformation transitions (which do not involve peptide bond cleavage) in plasminogen,complement factor C1 and plasma protransglutaminase (factor XIII).

The fact that the activity of this enzyme may be stimulated by up to 600% above control values must be of great importance in understanding by mechanism(s) by which the activity is controlled in the cell.

Birckbichler and Patterson (1978) have investigated the action of trypsin on cultured cells and report that concentrations in the range 1-5μg/ml promoted a 2-4 fold increase in transglutaminase activity. The increased activity was independent of protein synthesis and was accompanied by a marked re-distribution of the enzyme from bound to the cytoplasmic state. Their findings indicate that the cells have an extremely sensitive mechanism, triggered at the membrane level, which when activated results in the stimulation of existing transglutaminase molecules into much greater activity.

3.2.7.2 Ethylene glycol

Ethylene glycol is an organic solvent which possesses hydrophobic properties by lowering the polarity of system in which it is contained. This molecule, therefore was anticipated to produce the solubilisation of bound enzyme as has been described previously. Furthermore, Plisker et al (1978) have demonstrated that epidermal transglutaminase may be stimulated by ethylene glycol by an activation process. Should these effects be combined in an experiment the molecule may be shown to perform a dual role if applied to particulate-bound liver enzyme.

A range of concentrations (0-50% (v/v)) were added to the homogenisation medium and the resulting suspension centrifuged following a period of incubation. The fractions were assayed for transglutaminase

activity and the effects of ethylene glycol on distribution and activity evaluated (Fig. 3.21.2). A concentration of 20% (v/v) was found to stimulate enzyme activity with a 50% increase in the homogenate value over that of the control. Increasing the concentration to 33% and 50% (v/v) failed to stimulate enzyme activity further but resulted in a greater amount of activity being present in the soluble fraction.

Figure 3.21.1

An investigation into the activation of rat liver transglutaminase by various agents

Samples of purified rat liver transglutaminase (1mg/ml) in 50mM Tris-HCl, 1mM EDTA, pH 7.4, were pre-incubated for 20 min at 37° with the following concentration of agent (in 50mM Tris-HCl, pH 7.4): (i) KSCN: 0.25M; 0.5M; 1.0M; 2.0M, (ii) Trypsin: 31.25μg/ml; 62.5μg/ml; 125μg/ml; 250μg/ml, (iii) DMSO (v/v): 5%; 10%; 20%; 40%, (iv) n-Butanol (v/v):5%; 10%; 20%; 40%. Samples were assayed for transglutaminase activity by the standard method (Methods 2.2.1.1) over a 30 min incubation time incorporating all necessary controls. Samples were pre-incubated in two groups, the first containing 10mM Ca^{2+} + 0.5mM EDTA(⊗) and the second, 0.5mM EDTA(○) The enzyme activity was determined in each case and expressed as a percentage of the control activity for a minimum of 4 samples (± S.D.).

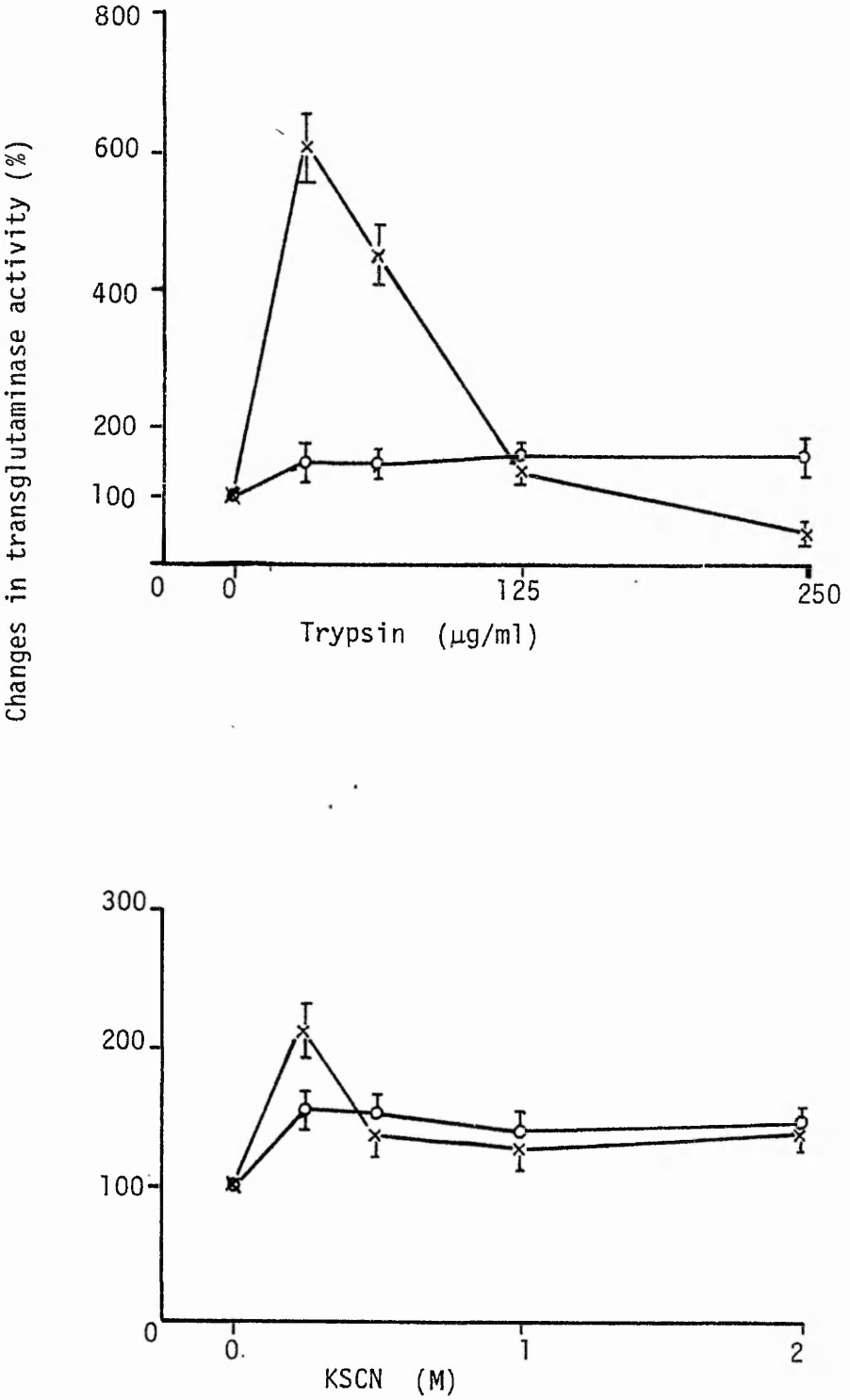

Changes in transglutaminase activity (%)
800
600
400
200
100
0
0
125
250
Trypsin (µg/ml)
300
200
100
0
0.
1
2
KSCN (M)

Figure 3.21.1

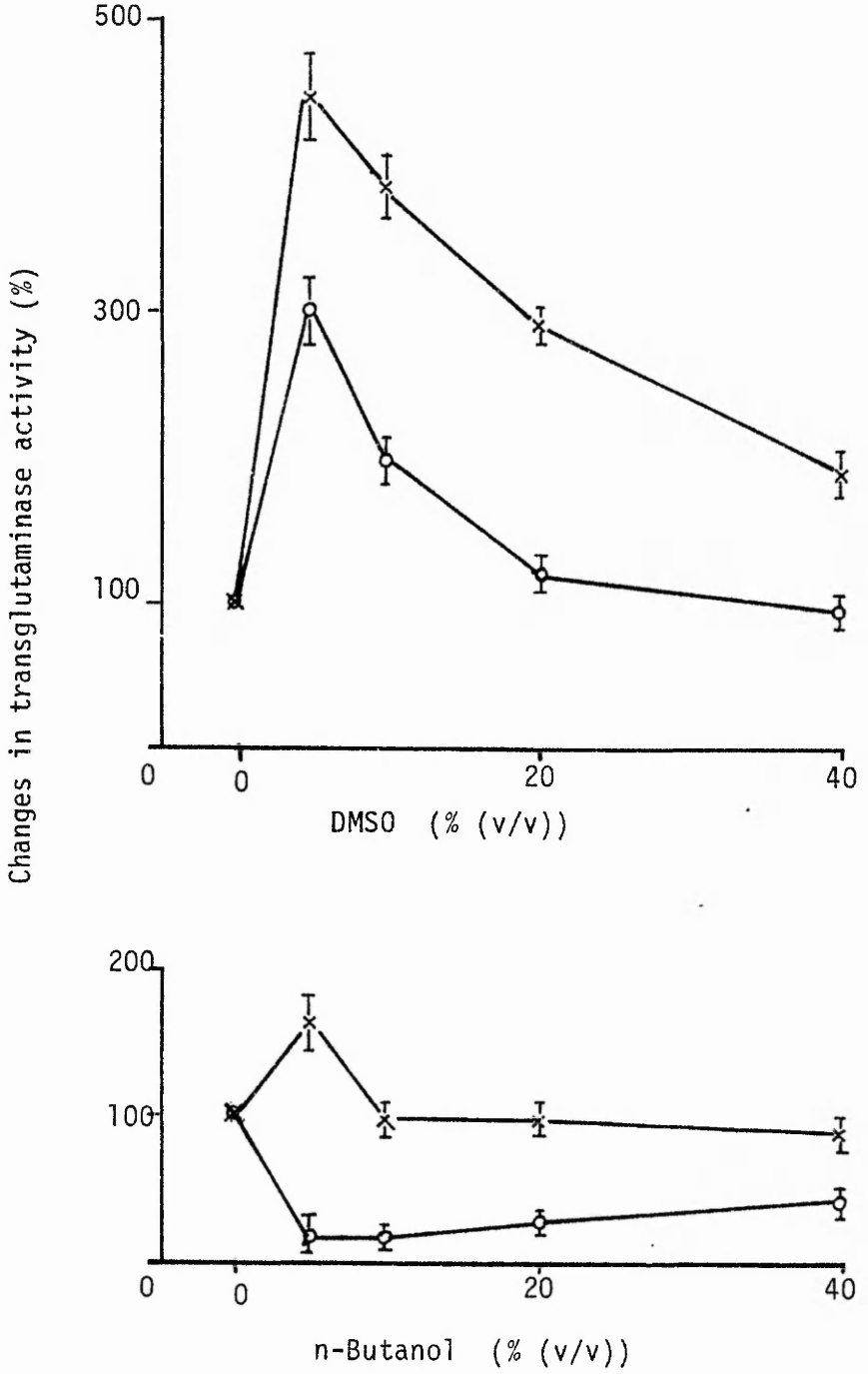

Changes in transglutaminase activity (%)
500
300
100
0
0
20
40
DMSO (% (v/v))
200
100
0
0
20
40
n-Butanol (% (v/v))

Figure 3.21.1

Figure 3.21.2

Changes in transglutaminase activity of liver fractions following the addition of ethylene glycol

Liver tissue (15g) was homogenised in 2 vol of 0.25M Sucrose: 1mM Tris-HCl:1mM EDTA, pH 7.4. Aliquots (5ml) were removed and ethylene glycol added to a final concentration of 20%, 33% and 50% (v/v). Samples were incubated at 4° for 20 min and then centrifuged 71,000g (rav 5.84cm) for 40 min. Each fraction was assayed for transglutaminase activity and protein. H is the original homogenate, P the 71,000g pellet and S the particle-free supernatant. Each value is the mean ± S.D. of 4 determinations.

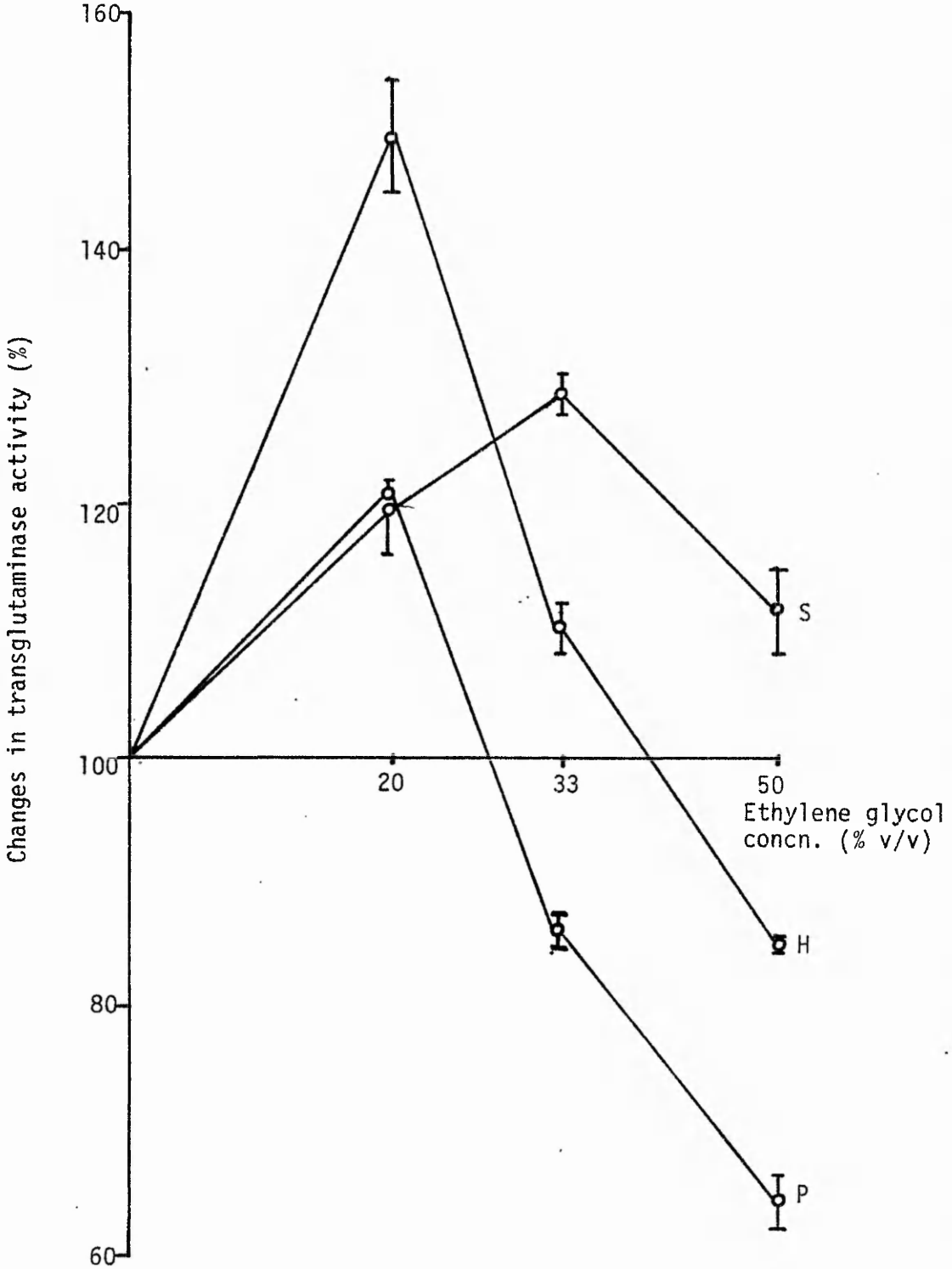

160
140
120
100
80
60
Changes in transglutaminase activity (%)
20
33
50
Ethylene glycol
concn. (% v/v)
S
H
P

Figure 3.21.2

3.2.8 Investigation of transglutaminase by tissue slice experimentation

This experimental approach was adopted in order that a closer approximation to the in vivo state could be examined. The methodology has been described previously (Methods 2.7) and consisted of the incubation of fresh, thin liver slices in a well oxygenated and defined medium containg radio-labelled substrate. The optimum conditions determined experimentally and were found to be very similar to a later published method (Lundgren and Hankins, 1978) for the same tissue. Cell integrity was determined by the measurement of protein efflux and also by following gluconeogenesis activity using lactate as the substrate . These findings confirmed that the tissue was viable for periods up to 180 min, the longest incubation time used (results not shown).

Putrescine accumulation was measured as total uptake by the cells in the slice and as the covalent incorporation of that putrescine into cell protein. Experiments (Figs. 3.22.1/2/3) demonstrated the time dependent increase in both uptake and covalent incorporation of the radiolabel. The uptakeof putrescine into the slice was initially rapid and a plateau was reached which was constant through 90-180 min. A measure of the uptake was used to confirm uniformity between the tissue slices where the radiolabel concentration in the slice was compared to that in the medium. This slice:medium ratio (s/m) gave an index of the putrescine accumulation by the cells and their interstitial spaces and was found to be constant for a given length of incubation. The maximum values of s/m were only slightly in excess of 1.0, indicating that the entry of putrescine was probably a passive process. This index rose rapidly with time to plateau at approximately 60 min were it remained constant to the end of the experiment.

Covalent incorporation of putrescine into acid-precipitatable protein was found to parallel the rate of uptake of the label into the cell. Approximately 9%-10% of the available label became covalently bound to cell protein and at 180 min there was still a slow increase in incorporation even when the rate of uptake had declined to a plateau level. On the basis of these results it was decided to use an incubation time of 60 min for the rest of the experiments since the s/m ratio was at its maximum and there was a significant level of covalent incorporation. EDTA added to the incubation medium, in place of Ca^{2+}, resulted in a >90% reduction in covalent incorporation. The values for total uptake and s/m ratio remained the same as for the Ca^{2+} incubations demonstrating that the presence of EDTA did not substantially alter the cellular metabolism. Ethylene glycol tetraacetic acid (EGTA), a more calcium specific chelating agent than EDTA, gave very similar results for the inhibition of covalent incorporation.

A series of experiments were carried out to investigate the role of calcium in the control of the enzyme and the possible metabolic fate of the putrescine (Fig. 3.22.4). The normal Ca^{2+} concentration used to supplement the incubation medium was 5mM and concentrations higher than this produced no further covalent incorporation of the label. This finding was confirmed when the Ca^{2+} ionophore X537A was used with 5mM Ca^{2+} and no significant increase was observed. Physiological buffers frequently contain Ca^{2+} at 1.9mM, the normal plasma levels, and incubation with this concentration produced over 65% of maximum incorporation. When the incubation medium was not supplemented with Ca^{2+}, and the cell was left to rely on its intracellular stores, the recorded activity was still 37% of the maximum.

The specificity of the reaction for Ca^{2+} was demonstrated when,

after incubation for 30 min in EGTA buffer, a sample of tissue was rinsed thoroughly and transferred to a buffer containing the similar divalent cation, Mg^{2+}. The resultant incorporation after the removal of Ca^{2+} and replacement by Mg^{2+} was similar to that of the control slice without Mg^{2+} or Ca^{2+}. Thus Mg^{2+} ions cannot replace Ca^{2+} in the activation sequence of the enzyme.

The site of incorporation of the putrescine was investigated on a macro-molecular scale (as described in Results 3.2.4) where the tissue slice method was used to determine the molecular weight of labelled proteins. This incorporation was Ca^{2+} dependent but it was felt that further proof was required that the putrescine had not entered the protein via any other metabolic route. Iproniazid, an amine oxidase inhibitor related to amino-guanidine, was incorporated into a Ca^{2+} containing incubation media and the resultant covalent incorporation of putrescine was found to be unaffected. Thus the amine oxidase metabolic route was ineffectual as the putrescine concentration always remained at saturating levels with respect to the enzyme within the slice. Furthermore it has been reported that rat liver does not contain detectable levels of diamine oxidase activity (Tabor, 1964). The metabolic fate of putrescine may vary according to the requirements of the cell and can lead to its incorporation in protein synthetic routes. It was though that the derivative, and therefore labelled, amino acids formed from the polyamine may have been incorporated into protein by the normal synthetic mechanisms and would give a false result for covalent incorporation by the transglutaminase. To this end protein synthesis inhibitors cycloheximide and puromycin were separately administered to the incubation media and did not significantly alter the overall incorporation of label. These experiments demonstrate that

the covalent incorporation reported here was entirely due to the transglutaminase activity and also that protein synthesis was not required for the expression of that activity.

The effect of cellular metabolic status on the transglutaminase activity was assessed by treatments which both raised and lowered the metabolic rate. When tissue slices were incubated under anaerobic conditions (incubation medium de-oxygenated and kept under nitrogen passed through 2% pyrogallol) to cause a low energy level, the enzyme activity was 70% of the maximum. A similar result was obtained when the incubation medium contained dibutyryl C-AMP, a molecule which readily passes through the cell membrane and acts as a secondary messenger raising the metabolic activity of the cell. These results are difficult to interpret as their effects encompasses many differing aspects of metabolism. Two factors emerge which may be taken as indications of the effect on the enzyme. The first concerns anaerobiosis and the fact that the reduction in transglutaminase activity was much less than might have been considered possible by such extreme conditions. This poses the possibility that perhaps the enzyme was either unaffected by the state of the cell or that it was in some way activated or given preferential treatment by the failing cell reserves. The second factor involves the increase of cellular activity and the previous finding (Results 3.2.6) that when buffer containing ATP was used for enzyme solubilisation, a significant decrease in overall enzyme level was found. These proposals, when taken together infer a cause and effect relationship existing between the metabolic state of the cell and the activity of the enzyme.

The stability of the incorporated $[^{14}C]$ putrescine was investigated by removing the labelled tissue and, after a thorough rinsing to remove unbound putrescine, placing the slices in a second incubation medium containing unlabelled putrescine or in a putrescine-free medium.

Only minor (± 7%) changes in the value for covalent incorporation were found. Thus the bound putrescine is not available for exchange or removal.

Tissue slices were incubated with mixtures of equal concentrations of [^{14}C-] putrescine and alternative polyamines in order to assess the effects on uptake and protein incorporation. The s/m ratio for putrescine remained unchanged despite the presence of other polyamines suggesting that there was either no competitive transport mechanism or that the transport available was sufficient to accommodate both polyamines simultaneously. The effect of the secondary polyamines was assessed by the reduction in covalently bound radiolabel putrescine. This was assumed to be due to replacement by the alternative substrate and the subsequent reduction in possible incorporation sites. The results (Fig. 3.22.5) demonstrate that all the polyamines produced an approximately 24% reduction in putrescine incorporation. This finding reveals that for every molecule of alternative polyamine incorporated 3 putrescine molecules were utilised thus displaying a preferential activity directed towards this polyamine.

The distribution of the covalently incorporated putrescine was established by homogenisation of the tissue slices in EDTA medium immediately following the incubation period. The homogenate was then centrifuged to give a 71,000g pellet (P) and particle-free supernatant (S). These fractions were analysed for distribution of label as described previously (Methods 2.7). The data (Table 2) shows that irrespective of the length of incubation (up to 120 min) the label is incorporated predominantly into the particulate cell fraction (87% approx.) with the remaining 13% associated with the soluble fraction. This result is of

particular interest since this distribution does not follow that of the enzyme where the ratio of particulate:soluble activity has been shown to be approximately 40:60 (Results 3.2.1).

Table 2

Distribution of covalently incorporated label

Medium	Incubation time (min)	Putrescine Accumulation (n=4)			
		Total Uptake nmol/g wet weight	Covalent Incorporation nmol/g wet weight	% distribution of covalent incorporation*	
				P	S
5mM Ca^{2+}	60	85.1 ± 5	6.20 ± 0.8	86.3 ± 8	13.7 ± 2
5mM Ca^{2+}	120	111.1 ± 7	9.33 ± 0.3	86.7 ± 7	13.3 ± 2
1mM EDTA	60	87.2 ± 5	0.57 ± 0.02	72.0 ± 6	28.0 ± 3

* "P" represents the 71,000g pellet fraction and "S" the particle-free supernatant. Data represents mean ± S.D. for at least 4 determinations.

Figure 3.22.1-5

The accumulation of [^{14}C-] putrescine in rat liver slices

The data presented here has been derived from experiments involving the incubation of 0.5mm fresh rat liver slices in an oxygenated and defined medium containing 1.2mM putrescine (final concentration). The incubation at 37^{o} and pH 7.4 was carried out over various time lengths (0-180 min) but 60 min was used for the majority of experiments. The experimental details have been described previously (Methods 2.7). The following data represents the mean ± S.D. for at least 4 determinations and includes:

Fig. 3.22.1 Total uptake of putrescine by the tissue slice (nmoles/g wet weight) against time (min).

Fig. 3.22.2 The index of putrescine concentration both within and outside the slice is expressed as the slice:medium ratio (s/m). This is derived from the disintegrations per minute (d.p.m.) of 1g of tissue slice and the d.p.m. of 1ml of medium.

Fig. 3.22.3 Total covalent incorporation of putrescine (nmole/g wet weight) into acid (TCA) precipitable protein against time (min).

Fig. 3.22.4 Total covalent incorporation of putrescine (nmole/g wet weight) into acid precipitable protein for a 60 min incubation. The legends describe the supplements made to the standard incubation buffer (see text for details).

Fig. 3.22.5 Percentage reduction in covalent incorporation of putrescine by the mixing in the incubation medium of equal concentrations (1.2mM) of [^{14}C-] putrescine and the individual amine given in the legend. GABA denotes γ-amino butyric acid. EDTA result is included as a basis for comparison of control level. Incubation was for 60 min at 37^{o} and pH 7.4.

Controls incorporating EDTA (1mM), iproniazid (5mM), cycloheximide (0.1mg/ml) and puromycin (0.09mM) were included at all stages of the experimentation.

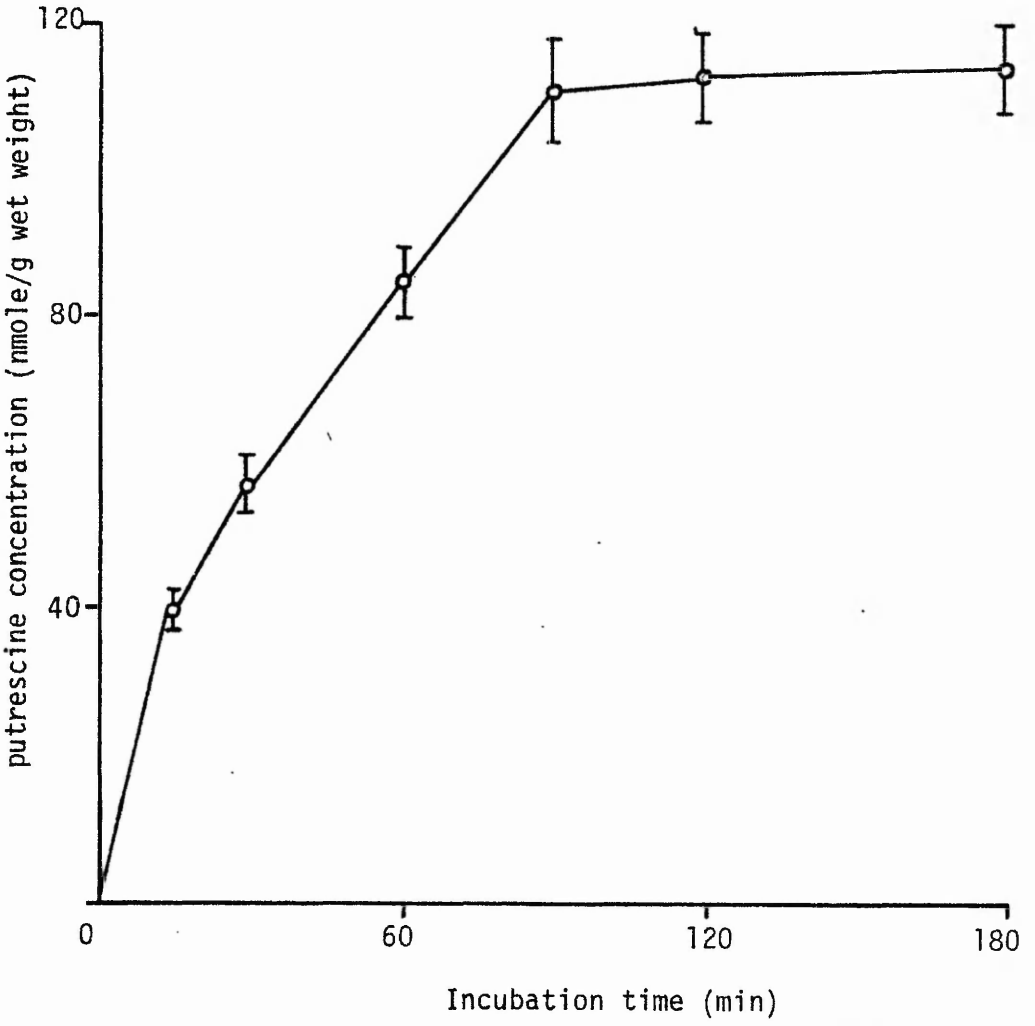

120
80
40
putrescine concentration (nmole/g wet weight)
0
60
120
180
Incubation time (min)

Figure 3.22.1

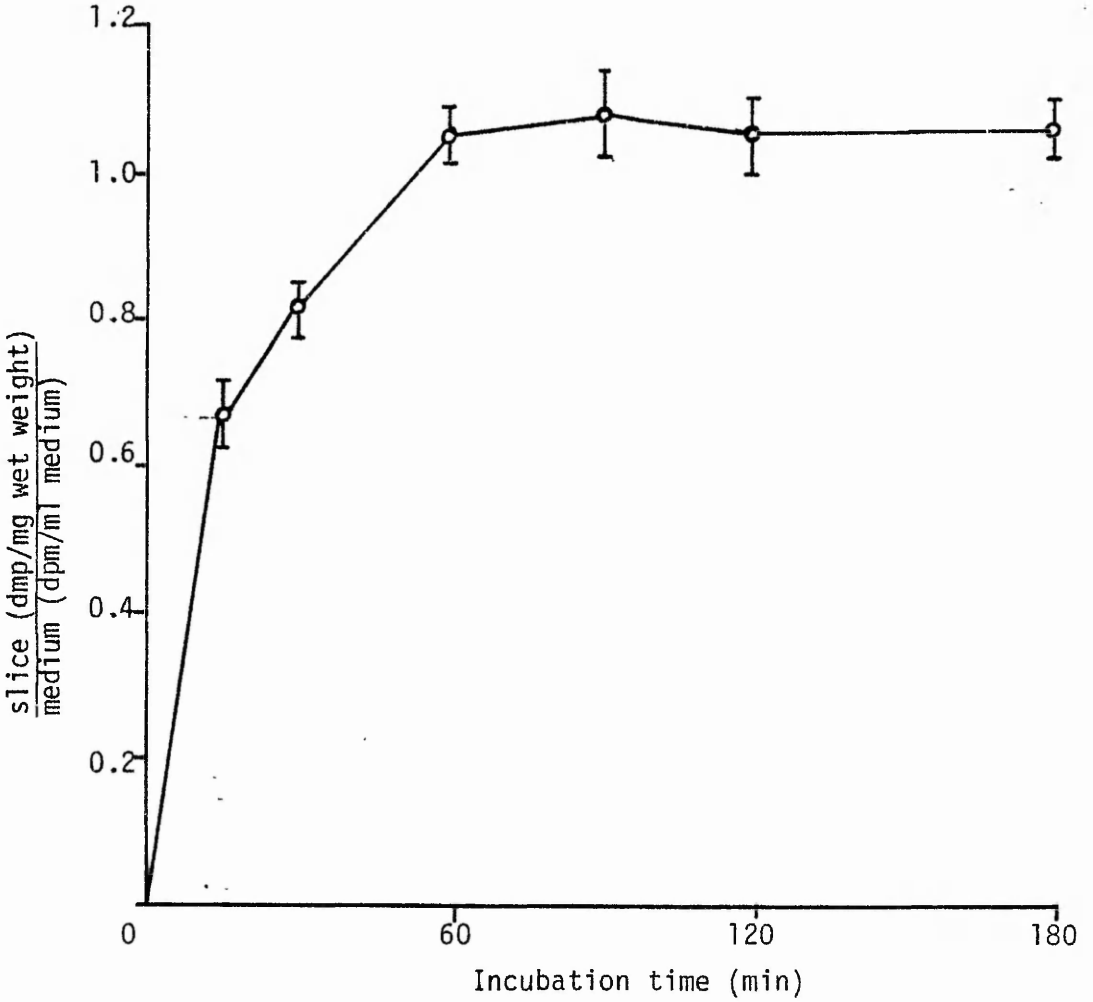

1.2
1.0
0.8
0.6
0.4
0.2
slice (dmp/mg wet weight) / medium (dpm/ml medium)
0
60
120
180
Incubation time (min)

Figure 3.22.2

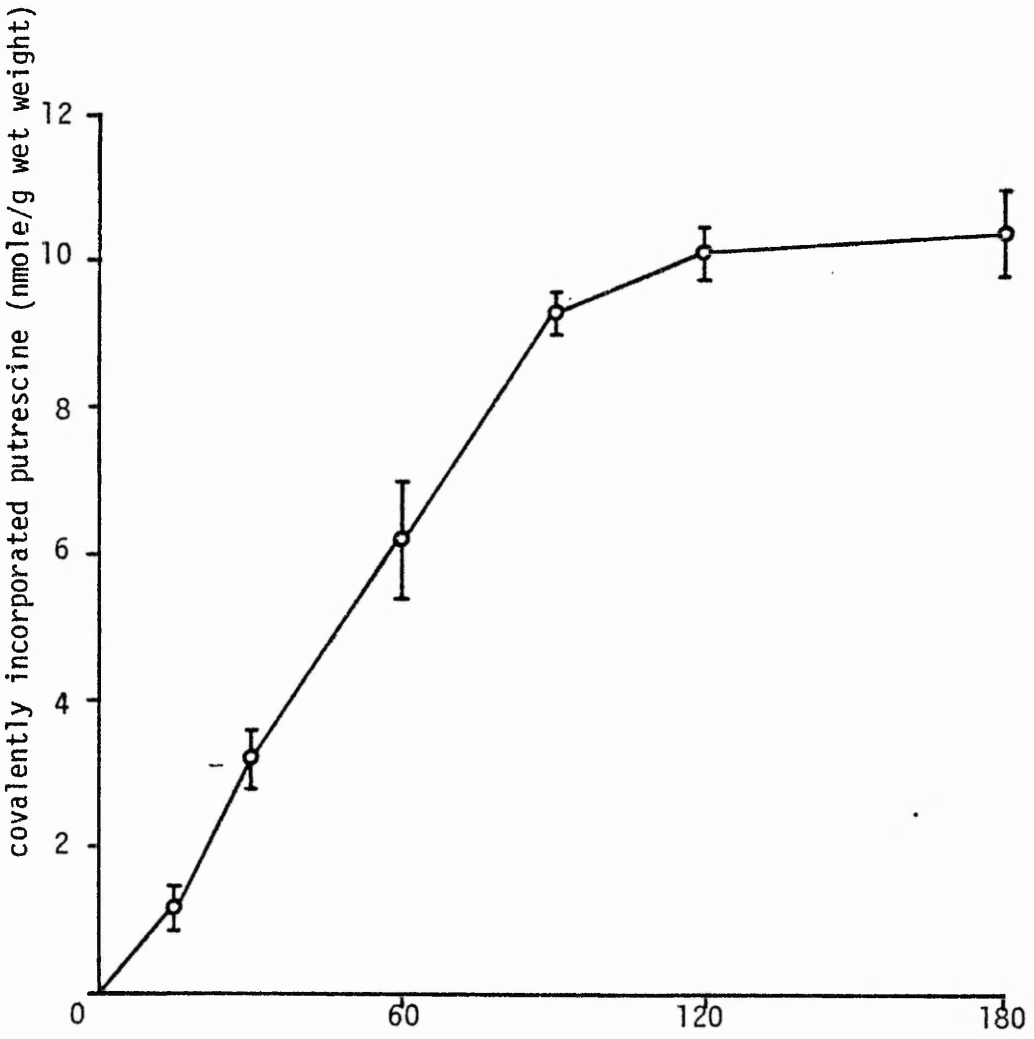

covalently incorporated putrescine (nmole/g wet weight)
12
10
8
6
4
2
0
60
120
180
Incubation time (min)

Figure 3.22.3

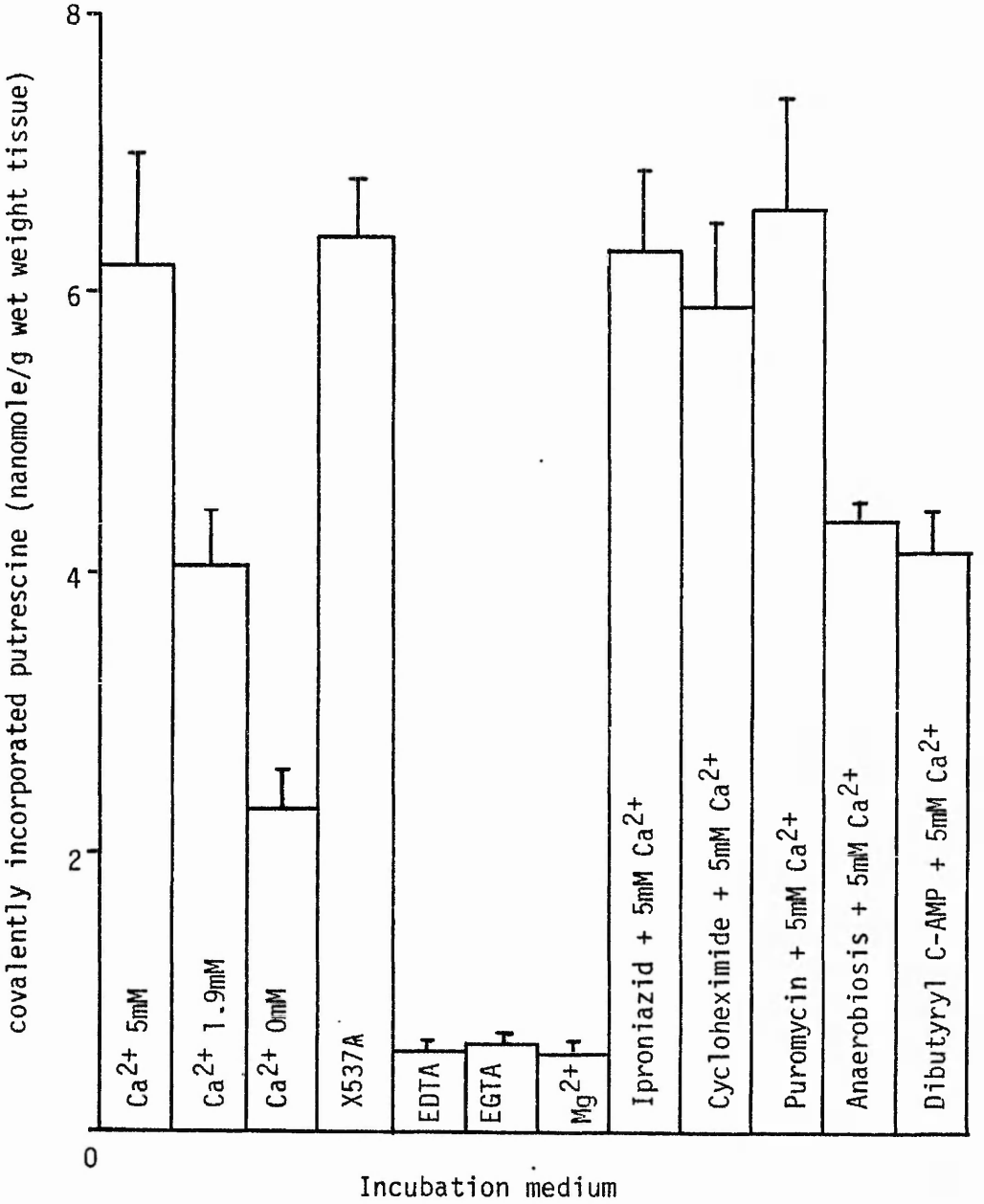

covalently incorporated putrescine (nanomole/g wet weight tissue)
8
6
4
2
0
Ca2+ 5mM
Ca2+ 1.9mM
Ca2+ 0mM
X537A
EDTA
EGTA
Mg2+
Iproniazid + 5mM Ca2+
Cyclohexeximide + 5mM Ca2+
Puromycin + 5mM Ca2+
Anaerobiosis + 5mM Ca2+
Dibutyryl C-AMP + 5mM Ca2+
Incubation medium

Figure 3.22.4

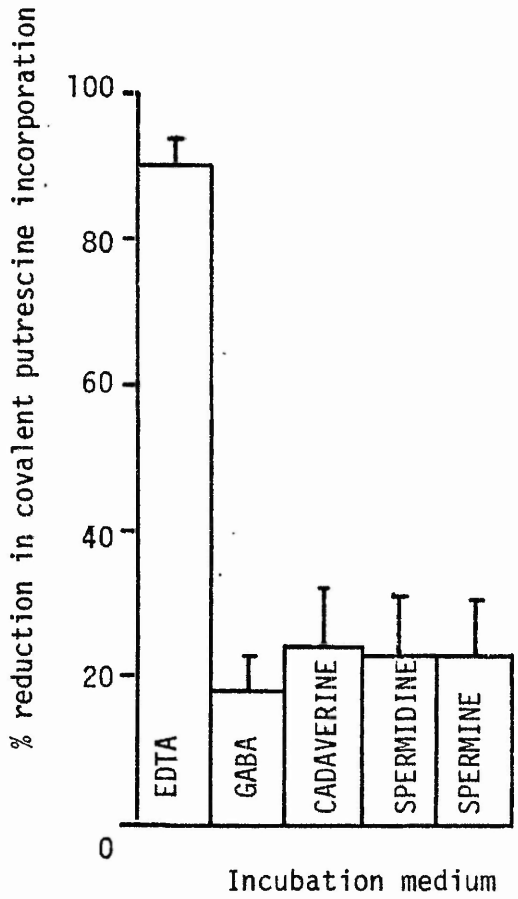

% reduction in covalent putrescine incorporation
100
80
60
40
20
0
EDTA
GABA
CADAVERINE
SPERMIDINE
SPERMINE
Incubation medium

Figure 3.22.5

3.2.9 DISCUSSION

The rat liver subcellular fractionation procedure provided good separation between fractions and high enzyme recoveries. The tissue transglutaminase was distributed in a ratio of approximately 3:2 between the soluble and particulate (600g "nuclear") fractions respectively. Examination of the 600g fraction by electron microscopy showed that it contained mainly membrane components, nucleii and cell debris.

(i) Enzyme Localisation

This bimodial distribution is unusual since literature reports to date have indicated either a totally soluble (Folk and Finlayson, 1977; Tyler, 1972) or totally particulate (Griffin et al, 1978; Bures and Goldsmith, 1978) localisation of the enzyme. However some reports by Birckbichler et al (1979) using paired cell culture lines indicate that the enzyme distribution may vary considerably depending on the cell type and its proliferative state. The difference in localisation between the rat liver enzyme as reported here and by Birckbichler et al (1976) has no simple explanation since the methods used were very similar. It should be noted that in all respects our fractionation procedure remained constant throughout the period of study.

A possible rationale which considers whether the enzyme is normally soluble and attaches itself to particulates under abnormal circumstances (e.g. tumourigenicity) or whether the reverse is the case, and also the possible effects of homogenisation on the release of the enzyme, is unresolved at present. The evidence to date, though incomplete, suggests the former case to be the more probable, and Birckbichler has postulated the presence of a separate third factor

which could mediate enzyme distribution particularly in the transformed state. However since the enzyme appears to be more stable in the bound form and utilises insoluble substrates there is a strong argument in favour of a significant particulate-bound enzyme component in intact cells. This bimodial enzyme distribution may prove to be a mechanism for compartmentalisation or control of activity *in vivo* and will be further discussed below.

The investigation of the cellular site of action of the enzyme using fluorescent markers and autoradiography was partially successful. The enzyme was observed to be widely distributed in the cytoplasm (but not in the nucleus) with concentrations at the cell periphery and along fibrillar strands. These features were missing in EDTA-treated control tissue. The possible involvement of the plasma membrane, endoplasmic reticulum and cytoskeleton inferred from these findings were further examined. There has been evidence for transglutaminase activity in the plasma membrane and endoplasmic reticulum (Birckbichler *et al*, 1973; Linnoila *et al*, 1979) and also cytoskeletal elements of muscle (Gard and Lazarides, 1979). A fibre-like enzyme distribution has also been reported by Birckbichler *et al* (1978) in WI-38 cell cultures, associated with either the cytoskeleton or extracellular fibronectin network.

During the isolation of plasma membranes there was a co-sedimentation of approximately 42% of the particulate-bound transglutaminase, which in turn comprises 16% of the total homogenate activity. These values may be lower than that which occurs *in vivo* since, during preparation, considerable leaching of the enzyme into the soluble fraction is thought to have taken place (Results 3.2.6). This result indicates a significant proportion of the enzyme localised in the 600g "nuclear" fraction, of which membranes were shown to form a substantial part.

(ii) Enzyme Substrates

Linnoila et al (1979) have reported that plasma membrane proteins are transglutaminase substrates, which, with the observation above infers a possible enzyme-membrane relationship. This was further examined by self-incorporation experiments in which endogenous transglutaminase was incubated with native protein. Plasma membrane protein was found to be a good substrate for tissue transglutaminase and, in particular, fraction D (Results 3.2.4) was shown to possess a greater number of available glutamine residues than the modified casein used for assays of the enzyme. The fraction D was thought to consist mainly of large polymers or aggregates of membrane protein, whilst fraction A corresponded to the purest source of plasma membrane.

Cell homogenates were also examined for self-incorporation by endogenous transglutaminase and, compared to the membrane fraction, were found to represent a poor substrate material. In these incubates the enzyme apparently rapidly saturated the available acceptor sites. However when casein was added to the incubation mixture, after putrescine incorporation had ceased, there was further incorporation indicating that substrate acceptor sites were rate-limiting. It was also found that during the lengthy incubation the soluble enzyme fraction declined in apparent activity at a much greater rate than that of the particulate enzyme.

Labelled protein derived from both homogenate and tissue slice self-incorporation studies were subjected to molecular weight analysis by SDS-PAGE. In both cases there was a large concentration of label (approx. 50% of incorporated putrescine) in the protein that failed to enter the gel i.e. extremely high molecular weight (>400,000MW). A second peak was observed in the range M.W. 45000-57000 which contained a further 10% of the incorporated label. The soluble cell fraction had only 10%-13% of the putrescine which had been incorporated

into the particulate material and the molecular weight distribution of label in the two fractions was similar. There was only one significant difference between the homogenate and tissue slice systems and that was in the identification of a 175000-225000 substrate in the homogenate proteins. This protein(s) would appear to be less available to the enzyme in intact cells and could be located at the cell exterior. A possible candidate could be fibronectin (Introduction - Substrate Proteins (iv)) in view of the molecular weight and location, but this possibility was not confirmed by further experiments.

These results indicate a close relationship between the enzyme and its major substrates which co-exist in the membrane fraction of the cell. The radiolabelling of proteins with large molecular weights indicates that the substrates which are preferentially utilised lead to the formation of polymers and aggregates which are subsequently incorporated into the plasma membrane and related structures. This possibility had been indicated by the work of Iwanij (1977) where membrane preparations from sarcoplasmic reticulum and rod outer segments were extensively labelled on incubation with transglutaminase. These findings noted the occurence of limited cell surface labelling using intact cells and viruses but, in contrast, demonstrated extensive labelling of the interior part of plasma membranes and intracellular membranes. Birckbichler et al (1973) isolated the $\varepsilon(\gamma$-glutamyl) lysine crosslink from plasma membrane and endoplasmic reticulum of tissue cultured L cells and reported its occurrence in various other membrane sub-fractions and in particular in the most insoluble residues. Thus, the demonstration of a large proportion of the cellular transglutaminase in the plasma membrane fraction, an excellent substrate material, agrees with the previous reports of crosslinks localised in these proteins and supports the hypothesis of a membrane directed role of the cellular enzyme.

The observation of putrescine incorporation into protein(s) in the molecular weight range 45000-57000 led directly to the identification of actin as a acceptor substrate in liver tissue slice preparations. This result may also explain the fibrillar distribution of fluorescent stain seen in the experiments on the site of action of the enzyme (see also Webster *et al*, 1978). Cytoskeletal elements actin and myosin have been shown to be substrates of the enzyme *in vitro* (Derrick and Laki, 1966; Cohen *et al*, 1979) and our observations with tissue slice provide an indication for this function of actin *in vivo*.

The involvement of membrane and cytoskeletal proteins *in vivo* may aid in interpreting the cellular role of the enzyme. Gard and Lazarides (1979) have demonstrated that substrate from the Z-line proteins of cultured muscle cells included both actin and myosin and also α-actinin, desmin and tropomyosin which further extends the possible field of influence of the enzyme. The cytoskeleton is intimately involved with the cell membrane in many processes (Singer *et al*, 1978; Lazarides and Revel, 1979). Transmembrane linkages, through α-actinin, to actin/myosin structures have been shown to be important in organising peripheral proteins on the external cell surface which include fibronectin (Hynes and Destree, 1978), collagen (Bornstein and Ash, 1977), fibrin (Mui and Ganguly, 1977) and other known transglutaminase-substrate matrix proteins. The most extensively studied system has been the erythrocyte where transglutaminase-mediated crosslinking has been shown to occur. Here, linkages joining red-cell actin, band 4.1, band 3 ankyrin and spectrin form a complete cytoskeleton to membrane system (Bennett and Stenbuck, 1979; Cohen and Branton, 1979) which could be extrapolated to other cell types. The control of the system in erythrocytes may be of physiological significance in the process of membrane stiffening in old and abnormal cells (Seifring

et al, 1978; Palek et al, 1978).

The close relationship which exists between the cytoskeleton and membrane components is thought to be of considerable importance in controlling cellular function. This may be exemplified by the "patching and capping" phenomenon which results in the apparent immobilisation of surface components and the subsequent internalisation by endocytosis. This has been reported to involve crosslinking of the proteins in the fluid membrane and the interaction of intracellular myosin and actin (Bourguignon and Singer, 1977). These authors suggest that the receptor aggregates, linked to actin and myosin, are collected into a "cap" by an analogue of the actin-myosin sliding filament mechanism of muscle contraction, a process already proven to involve the production of $\varepsilon(\gamma$-glutamyl)lysine crosslinks.

The endocytic process has been shown to involve protein crosslink formation, mediated by cellular transglutaminase, and can be inhibited by antagonists of transglutaminase action (Davies et al, 1980). The enzyme is thought to be located in the plasma membrane close to the inner aspect of the sub-membrane microfilament mat. The enzyme functions, in this respect, by the crosslinking of proteins in the ligand-receptor complexes. The endocytic vesicles, which form from the plasma membrane pass through the cytoplasm and are covered in a proteinaceous lattice work. This protein is called "coat protein" or calthrin and is composed of polypeptides of high molecular weight (Pearse, 1976). The calthrin from the vesicles has been shown to interact with contractile proteins, particularly actin and α-actinin (Schook et al, 1979) and may be another candidate substrate for transglutaminase.

(iii) Enzyme Control

The examination of cellular control of the enzyme encompassed translocation with respect to the bimodial distribution, activation and the effect of agonists. The major factors influencing enzyme activity are Ca^{2+} ion concentration, reduced sulphydryl moieties and specific substrates, preferably peptide-bound. The majority of investigations to date suggest that Ca^{2+} is the main enzyme modulator and propose that intracellular Ca^{2+} concentration, particularly in the erythrocyte, may be all that is required for expression of activity (Seifring et al, 1978; Linnoila et al, 1979). However, our studies show a much more complex pattern of interacting factors.

Enzyme redistribution from the particulate-bound to the soluble state was investigated using a variety of eluting buffers. A number of solutes, each containing either Ca^{2+}, EDTA or putrescine all produced an increase in overall enzyme levels of approximately 60% over that of the original sample. Despite this large increase in soluble enzyme the source i.e. the 600g pellet, was only slightly reduced in activity. This suggests the presence of undetected reserves which could provide the increased enzyme activity detected in the solution. A much greater elution of enzyme was accomplished by increasing the number of "washes" from two to five. The effect was to produce a 109% increase in detectable enzyme whilst the washed residue was only depleted by 20%. Since both bound and soluble enzymes possess similar activities the conclusion is that stored enzyme reserves are present in the particulate fraction and are capable of replacing or adding to the apparent cellular complement.

There was no evidence that this process involved the removal of inhibitory factors by dilution.

Inclusion of dithiothreitol in the buffer resulted in only minor levels of enzyme being eluted, indicating that disulphide bridging is unlikely to be the mechanism through which the enzyme is bound.

The tissue slice incubations were designed to investigate cellular enzyme control by monitoring the fate of radiolabelled substrate under various conditions of Ca^{2+} concentration, amine availability and cellular metabolic activity. The method adopted gave viable liver slices whose characteristics remained consistent within and between experiments providing reproducible results. The amine uptake was found to be a passive process and the fate of the putrescine taken up was not influenced by either protein synthesis inhibititors (cycloheximide and puromycin) or amine oxidase inhibitors (Iproniazid, an amino-guanidine). This observation also demonstrated that protein synthesis was not required for full expression of enzyme activity.

(a) Control through Ca^{2+} concentration

The role of Ca^{2+} concentration in enzyme activity was investigated as the enzyme is known to require Ca^{2+}-induced conformational changes which result in the active site becoming fully accessible (Fésüs and Laki, 1977). Erythrocyte transglutaminase has been shown to require Ca^{2+} concentrations in excess of 0.5mM for optimal enzyme activity but other tissue enzymes are active at lower Ca^{2+} concentrations. This has been demonstrated with purified liver enzyme (see Results 3.1.3.2) where significant activity has been recorded at 40μM Ca^{2+} and full activity at 0.1mM Ca^{2+}. These data show that the control of the enzyme within liver tissue is unlikely to be mediated by Ca^{2+} concentration alone.

Incubating liver tissue slices in medium containing 1mM EDTA or EGTA reduced enzyme activity by over 90%, demonstrating an absolute Ca^{2+} requirement for the putrescine incorporating activity. Full activity was observed at 5mM Ca^{2+} and additions of the Ca^{2+} ionophore, X537A, to the incubation medium did not increase the levels of putrescine incorporation. When the Ca^{2+} levels were reduced to those found in plasma (1.9mM) the enzyme was still very active. The removal of all Ca^{2+} from the medium resulted in 37% of maximum activity indicating that Ca^{2+} is available from intracellular pools. These reactions also appear to be Ca^{2+} specific as its replacement by Mg^{2+} resulted in the same levels of putrescine incorporation found for the EDTA/EGTA controls.

Calcium was also effective in promoting the release of enzyme during solubilisation experiments which suggest that either a conformational change or electrostatic charge are features of the enzyme binding. Increasing EDTA concentrations in the eluting buffer caused a reduction in the enzyme eluted, which confirms the finding above. This effect could be of particular physiological significance at the membrane and cytoskeleton where localised increases in Ca^{2+} concentration might potentiate enzyme activity. The mechanism for such fluctuation in Ca^{2+} levels may be mediated through the increasingly important calcium-binding proteins such as calmodulin, troponin C, parvalbumin and calsequestrin (for review and references see Cheung, 1980). An example of the localised action of the enzyme at the membrane level would be the recently described transglutaminase-mediated endocytosis of receptor complexes (Davies et al, 1980).

(b) Control through polyamines

The role of the polyamines, and putrescine in particular, in controlling enzyme activity in the cell may be envisaged as a much more generalised cellular phenomenon that the localised role suggested for calcium. The polyamines are involved in a number of biologic control processes, including the regulation of cell cycle and growth and have been reviewed in the Introduction. An early speculation on the role of transglutaminase was concerned with the fixation of histamine and other biogenic amines (Waelsch, 1962) although thishas never been proved. The metabolic fate of putrescine may be via pathways to other polyamines, to amino acids and proteins or through urea-cycle intermediates and depends on the metabolic activity of the cell. Incubation of tissue slices showed that during incubation (60 min) approximately 10% of the added putrescine was bound to acid-precipitable protein. Self-incorporation studies using native protein as the acceptor, demonstrated that the polyamine acceptor sites were rapidly saturated. This suggests that polyamines may control enzyme function by regulation of the available substrate acceptor sites. Evidence is available that synthetic amines can inhibit the formation of protein-protein bridges (Lorand et al, 1972) and prevent irreversible transformations in the visoelastic properties of erythrocyte membranes (Seifring et al, 1978; Lorand et al, 1976). Recently, Davies et al (1980) demonstrated that low concentrations of dansylcadaverine (0.1mM), methylamine and other alkylamines were able to block the transglutaminase mediated endocytosis of receptor complexes.

Experiments using liver tissue slices have demonstrated that of the covalently incorporated putrescine, 87% was found in the particulate fraction of the cell. Incubations using media containing various other

amines (spermine, spermidine, cadaverine and γ-amino butytic acid) showed that the uptake of amine was unchanged and the putrescine incorporation was only marginally reduced (18%-24%). These results suggest that whilst other amines may compete as enzyme substrates, of those tested, putrescine remained the most readily incorporated and the major sites of incorporation were concentrated in the particulate fraction.

Solubilisation experiments using putrescine or spermine in conjunction with Ca^{2+} resulted in no detectable enzyme activity in the washes and a reduced activity in the 600g pellet. This action of the polyamines and Ca^{2+} has been further examined using each independently and the results obtained indicated that both solubilisation and increases in apparent overall enzyme levels occurred. The above data indicate a substantial increase in the tenacity of binding of the enzyme, and possibly the covalent incorporation of the enzyme into a binding protein through the mediation of the polyamine in the presence of Ca^{2+}.

The proposal that the enzyme activity may be controlled through the intervention of polyamines, either by the restriction of glutamine acceptor sites and/or through particulate/soluble translocations prompts further consideration. Birckbichler et al (1979b) have shown that non-proliferating cells, when treated with polyamines which function as inhibitors of both enzyme and crosslinking, exhibit several properties of proliferating cells. This indicates a particular role for the enzyme in growth processes, especially in the polyamine-rich tissues such as neoplasms. Diamines and polyamines have also been shown to facilitate crosslinking by forming a "bridge" between adjacent peptide chains, both in vitro (Schrode and Folk, 1978) and in vivo

(Folk et al, 1980). These alternatives to the ε(γ-glutamyl)lysine crosslink might be expected to require less spatial orientation of the contributing glutamate residues and thus may be favourable in nature. These proposals allied to the data given for the control aspects of polyamines merit further investigation before a definite role for polyamines might emerge. Actin, which is a substrate for transglutaminase, has been shown polymerise from the monomeric state upon the addition of low concentrations of polyamines, and form fibrous thin filaments (Oriol-Audit, 1978). This phenomenon is also produced by divalent cations, particularly Ca^{2+} and Mn^{2+} (transglutaminase activators), (Strzelecka-Golaszeweska et al, 1978). The polyamine-induced polymerisation phenonemon has also been extended to another enzyme substrate, fibronectin (Vuento et al, 1980) where the resulting polymers resembled the pericellular structures isolated from fibroblast cultures.

The interactions of fibronectin and collagenous proteins has been reported to reside in specific cationic binding sites and the process as a whole to be mediated by polyamines (Vuento and Vaheri, 1978). Fibronectin and collagen co-distribute in vivo (Vaheri et al, 1978) and polyamines may form the bridge necessary to stabilise the complex. Finally Davies et al (1980) reported that transglutaminase-mediated endocytosis of receptor complexes could be inhibited by low concentrations of polyamine. This control of a specific process left the major enzyme fraction unaffected until the polyamine concentrations was increased to a point inhibiting the whole enzyme complement. This situation indicates that enzyme control via polyamines may involve a graded response to substrate/inhibitor availability and provides an interesting addition to the possible methods of regulations of activity.

(c) Control through cellular metabolic activity

The effect of the metabolic activity of the cell on the level of activity and distribution of the enzyme was examined by both the solubilisation and tissue slice experimental systems. Buffers containing ATP and Mg^{2+} caused less solubilisation of the enzyme than the standard buffers and greatly reduced the activity of the remaining bound enzyme. Similarly dibutyrylc-AMP caused a moderate reduction in the enzyme activity of liver tissue slices. This data suggests that a lowering of enzyme activity is accompanied by increased binding when the metabolic activity of the cell is raised. When the metabolic activity of the cell was reduced by incubation of tissue slices under anaerobic conditions, 70% of the normal enzyme activity was found. This result indicates that either the availability and level of enzyme activity remained almost unaffected by the condition of the tissue or that the enzyme may have a particular role in the processes involved in cell death. The latter postulate has been made previously by Rice and Green (1977) who report that the cornified envelope produced during terminal differentiation of epidermal keratinocytes requires $\varepsilon(\gamma$-glutamyl)lysine crosslinking. Furthermore, Seifring et al (1978) suggest that membrane stiffening in erythrocytes is a property of dying cells and that intracellular transglutaminases may play a general role in the completion of cell cycles. This postulate has been discussed in greater detailed in the following section of Results.

(d) Control through substrate protein

Actin having been found to be an enzyme substrate, was further investigated as a potential binding site for particulate-enzyme. The experiments showed that an "actin-solubilisation buffer" did not cause any significant translocation of enzyme activity.

(e) Control through hydrophobic interactions

Hydrophobic interactions were investigated as the basis for particulate binding since at least one functional hydrophobic binding site is known to be close to the enzyme active centre (see Introduction). Initial experiments using phenylalanine and a taurocholate/Triton X-100 detergent mixture were inconclusive as little solubilisation of the enzyme was observed. Butanol caused some solubilisation and an apparent 30% increase in enzyme levels, but was still less efficient than the Ca^{2+} or putrescine buffers. When butanol (5% v/v) was pre-incubated with purified enzyme and Ca^{2+} ions there was significant activation which may explain some of the observed increase in activity during the solubilisation experiment. Pre-incubation in the presence of EDTA caused a reduction in enzyme activity suggesting that the activation mechanism was Ca^{2+} dependent. Butanol may have produced denaturing conditions, especially at higher concentrations (10%-40% v/v), which were alleviated in the presence of Ca^{2+}. Hydrophobic compounds are known to cause destabilisation of thiol interactions and this may explain both the requirement for dithiothreitol in the buffers used in butanol experiments and the denaturing conditions.

Potassium thiocyanate was investigated in order to determine whether the chaotropic reduction in hydrophobic interactions would result in enzyme translocation. The observed effects during solubilisation experiments was similar to those of the standard buffers with substantial enzyme elution and an apparent increase in bound enzyme activity. Pre-incubation of purified enzyme with 250mM potassium thiocyanate brought about a two-fold increase in activity, and a 35% increase at higher salt concentrations. The activation process was slightly enhanced by Ca^{2+} ions but activation also occurred in the presence of EDTA.

Sodium chloride, a salt with no chaotropic properties, failed to produce any enzyme activation and gave less than 60% of the solubilisation achieved by potassium thiocyanate.

Ethylene glycol influences hydrophobic conditions by lowering the polarity of the medium in which it is contained. This agent caused substantial translocation of enzyme to the soluble state and a 50% stimulation of enzyme activity in homogenate fractions. This activation was further investigated using the solvent, dimethylsulphoxide, in pre-incubation with purified liver enzyme. This experiment resulted in a 450% increase in enzyme activity at low solvent concentrations and in the presence of Ca^{2+} ions, though EDTA evoked a similar though lesser response.

In summary, chaotropic and apolar effects have indicated an enzyme response, both in translocation to the soluble fraction and an increase in activity, which suggests that hydrophobic moieties may well be involved in the particulate-binding phenomenon. The use of hydrophobic chromatography (Results 3.1.3.7) provided evidence of interactions with an alkyl-agarose matrix, particularly C_4-C_6 substituents, and confirms that non-covalent binding via hydrophobic interactions may be a potential source of enzyme control.

(f) Trypsin activation of transglutaminase

Enzyme activation was also evident when treatment with low concentrations (20-100μg/ml) of the proteolytic enzyme trypsin produced a 600% increase in purified enzyme activity. The response was largely Ca^{2+} dependent and declined at higher trypsin concentrations, probably due to excessive proteolytic degradation. Birckbichler and Patterson (1978) reported a similar activation of transglutaminase in cultured cells. These authors also demonstrated a gross re-distribution of enzyme to the soluble fraction and indicated that the process may be mediated via a sensitive membrane site resulting in the stimulation

of existing transglutaminase activity.

3.3 Model Systems

The observation that transglutaminase activity could be influenced by polyamines and various "activating" moieties including the metabolic activity of the cell prompted a study of the enzyme in "model" cellular systems. Birckbichler *et al* (1978) have reported that the proliferative state of the cell may exert a controlling influence over the enzyme causing alterations in both distribution and activity. Polyamines are known to play a major role in cell growth and regulation of the cell cycle and may also function as transglutaminase-controlling factors *in vivo*.

The following investigation involved the measurement of transglutaminase activity and distribution throughout a sequence of model systems each representing a different aspect of cellular growth and control. Liver tissue was studied during foetal maturation, pharmocologically-induced hepatomegaly and regeneration following partial hepatectomy. Blood platelets were used to investigate whether well-characterised activation processes produced a concomitant effect on the transglutaminase enzyme. Finally, neoplastic tissues were studied with particular reference to alterations in the level and function of the enzyme which might be associated with metastatic growth.

3.3.1 Studies on Foetal Liver

Liver samples were obtained from foetal and newborn rats at specified times of gestation. The appearance of the copulation plug was regarded as day zero. Foetal liver was observed, with the aid of a dissection microscope at day 15/16, and was sampled at days 17, 18, 19, 20 and 21. Parturition occurred at day 21 and the newborn rats were allowed to remain in the maternal litter. Liver tissue was subsequently sampled at days 2 and 10 post-natal and also from adult animals to facilitate the comparison of data.

There was a rapid increase in foetal liver weight with an approximate 9-fold increase during the last 5 days of gestation (Fig. 3.23.1). Following parturition there was a diminution in the rate of gain in weight which persisted until at day 10 post-natal a further 2.7-fold increase was observed. At this time the newborn liver was approximately 8% of the weight of the normal adult rat liver (8.4g approx.).

The enzyme studies revealed interesting trends in both activity and distribution. In the period up to parturition there was a consistent and rapid increase in the specific activity of the enzyme in both the soluble and particulate fractions (Fig. 3.23.2). This position was changed after parturition when the enzyme specific activity quickly approached the levels of adult tissue. Birckbichler et al (1976) using liver from 18 day old foetii reported a similar enzyme activity to that of the control. In this tissue these authors demonstrated a 15% increase in specific activity and no alteration in distribution when compared to control (adult) tissue. In this study it can be seen that at 18 days there was a similar result to that cited, with specific activity raised by 20% and only a small alteration in distribution. However, the study by Birckbichler, using one isolated sample did not examine the situation in sufficient detail since substantial alterations in enzyme characteristics have been observed. The maximum specific activity observed, with values approximately 166% (soluble enzyme) and 454% (particulate-bound enzyme) above those found in adult tissue, coincides with the period approaching and including parturition. The enzyme results determined for cell homogenates follow a similar pattern, with respect to liver age, as those expressed for the cell fractions.

The investigation into the distribution of activity (Fig. 3.23.3) demonstrated the enzyme to change from a predominantly soluble distribution

(76% of total) at 17 days to a particulate-bound state (60% of total) following parturition. The distribution was later observed to correspond to adult levels of approximately 42%:52% "particulate" to "soluble" enzyme ratio. This alteration of distribution occurs over approximately the same time period as that described above for the increase in specific activity, but continues for a short time following parturition. The pattern of enzyme activity and distribution may be more clearly illustrated in Fig. 3.23.4 where the changes can be observed by reference to the "relative specific activity" of the isolated fractions.

The alterations in enzyme activity demonstrated in these experiments suggest a role for transglutaminase during foetal maturation and proliferative growth. The liver develops as a ventral diverticulum (endoderm) of the fore and midgut junction and extends anteriorly into the mesenchyme of the septum transversum. The proliferation of the endodermal cells gives rise to the cords and plates of hepatic cells. During rat fetal development and up to 3 days prior to birth the liver consists of 3 main cell types; parenchymal cells, haemopoietic cells and Kupffer cells (Greengard and Federman, 1970). Major changes in the relative cell mass occur in the final 3 days of gestation. There is an approximate doubling of the parenchymal cells, the virtual disappearance of the haemopoietic cells and a very slight increase in the Kupffer cells, though, in any case, the latter comprise only a minor component. At 2 days post-natal there is only the single major cell type present i.e. those of the parenchyme.

In summary, the results suggest that the parenchymal cells possess the majority, if not all, of the detectable transglutaminase activity. The disappearance of the haemopoietic cells might account for the increase in specific activity reported during the final stages of gestation if their transglutaminase activity was very low. Similarly, the activity

in parenchymal cells may be predominantly particulate-bound, and in the soluble phase in the haemopoietic cells, then this situation might produce an effect similar to the described alterations in enzyme distribution. However, until the cell types have been differentiated and their individual enzyme characteristics determined the true situation will remain unclear. Since the haemopoietic cells later become foetal blood cells there is reason to suppose that the tissue transglutaminase activity in these cells is small by comparison to the tissue enzyme derived from the hepatic cells. Thus, at present, it would appear that the enzyme characteristics observed during gestation may be due entirely to the cellular control mechanisms operating within, and upon, the parenchymal cells. The critical determination would be the measurement of the transglutaminase activity in a particular cell-type. This might be achieved by separation of the component cells followed by the measurement of either transglutaminase activity for each cell type i.e. by relating to DNA content or by the measurement of the $\varepsilon(\gamma$-glutamyl) lysine dipeptide in cross-linked proteins of the cell. The quantitation of DNA was followed during these experiments using whole tissue. The enzyme activity per cell was shown to increase during the latter stages of gestation. This may have reflected the disappearance of the haemopoietic cells and/or an increase in the parenchymal enzyme activity, but it is not clear which factor is the most significant.

Figures 3.23.1/2/3/4

Foetal Liver Studies

Liver tissue was completely removed from foetii at days 17, 18, 19, 20 and 21 after the appearance of the copulation plug and 2 and 10 days post-natal (Methods 2.9.3). Parturition occurred at day 21 and the samples therefore represent times of -4, -3, -2, -1, zero, +2 and +10 days. Adult tissue was also assayed in order to provide a basis for comparison. The samples were weighed, homogenised (Potter-Elvejhem) in 0.25M Sucrose/1mM Tris-Cl/1mM EDTA, pH 7.4 buffer, centrifuged at 71000g for 40 min and the fractions assayed for transglutaminase by the usual method.

The data represents the mean ± S.D. for 3 determinations each consisting of 6-8 pooled tissue samples. The following figures are included, Legends: soluble fraction, O; particulate fraction, X.

3.23.1 Mean liver weight with respect to tissue age

3.23.2 Specific activity of transglutaminase with respect to tissue age.

3.23.3 Percentage distribution of enzyme activity between the soluble and particulate fractions with respect to tissue age.

3.23.4 Relative specific activity with respect to percentage of protein. P and S legends refer to the 71,000g pellet and supernatant fractions.

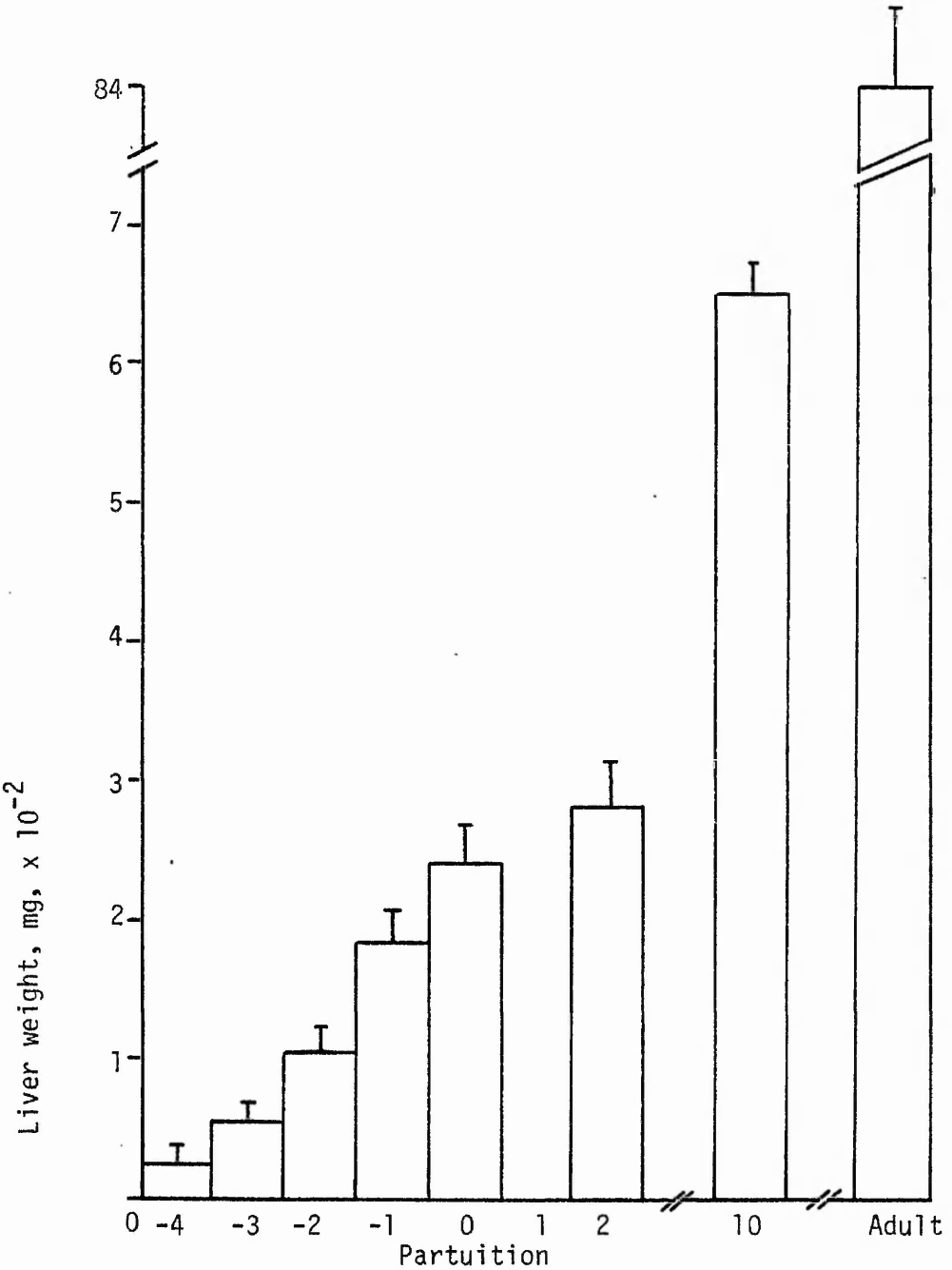

84
7
6
5
4
3
2
1
Liver weight, mg, x 10^{-2}
0 -4 -3 -2 -1 0 1 2 10 Adult
Partuition
Foetal Age (days)

Figure 3.23.1

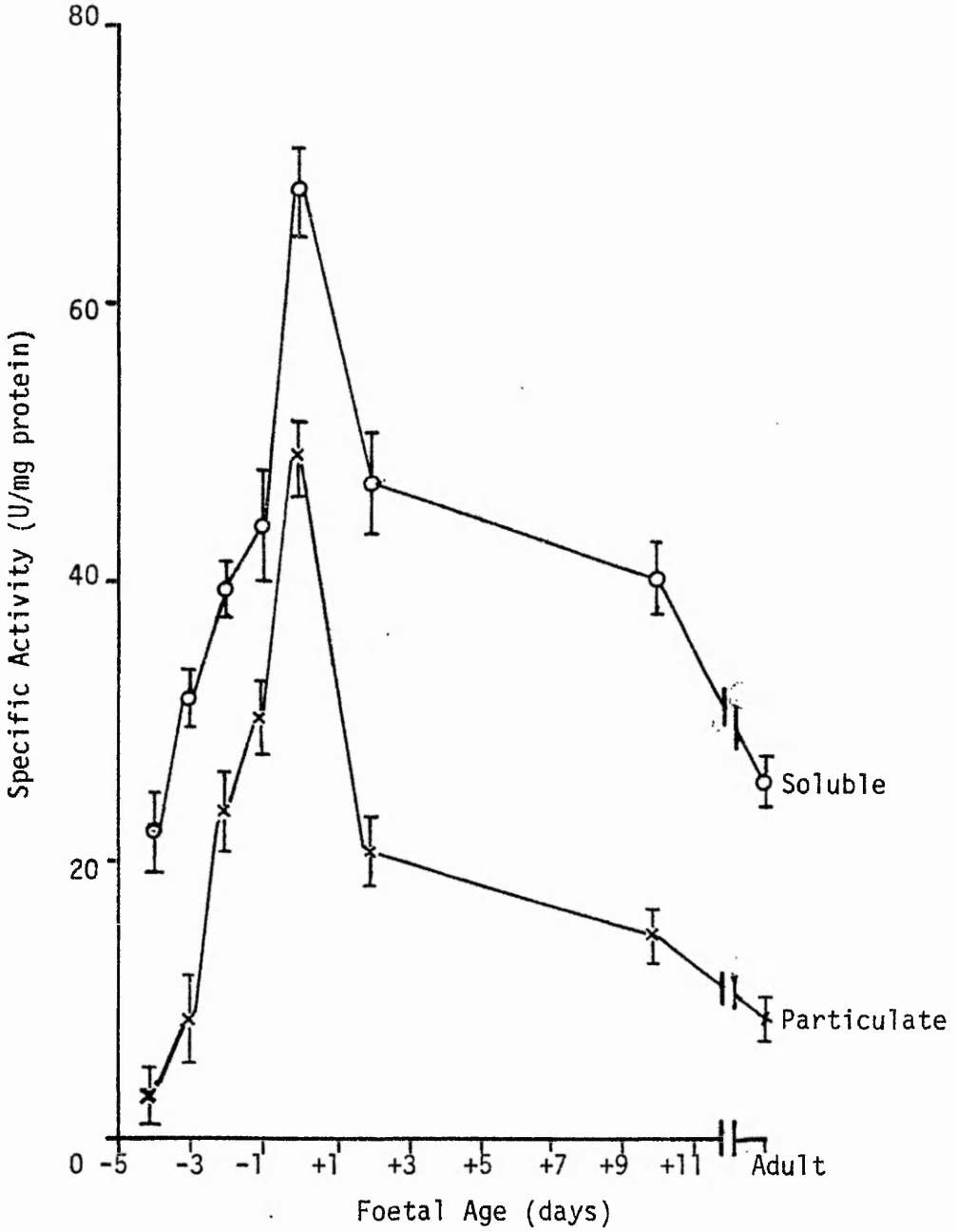

80
60
40
20
0
Specific Activity (U/mg protein)
-5
-3
-1
+1
+3
+5
+7
+9
+11
Adult
Foetal Age (days)
Soluble
Particulate

Figure 3.23.2

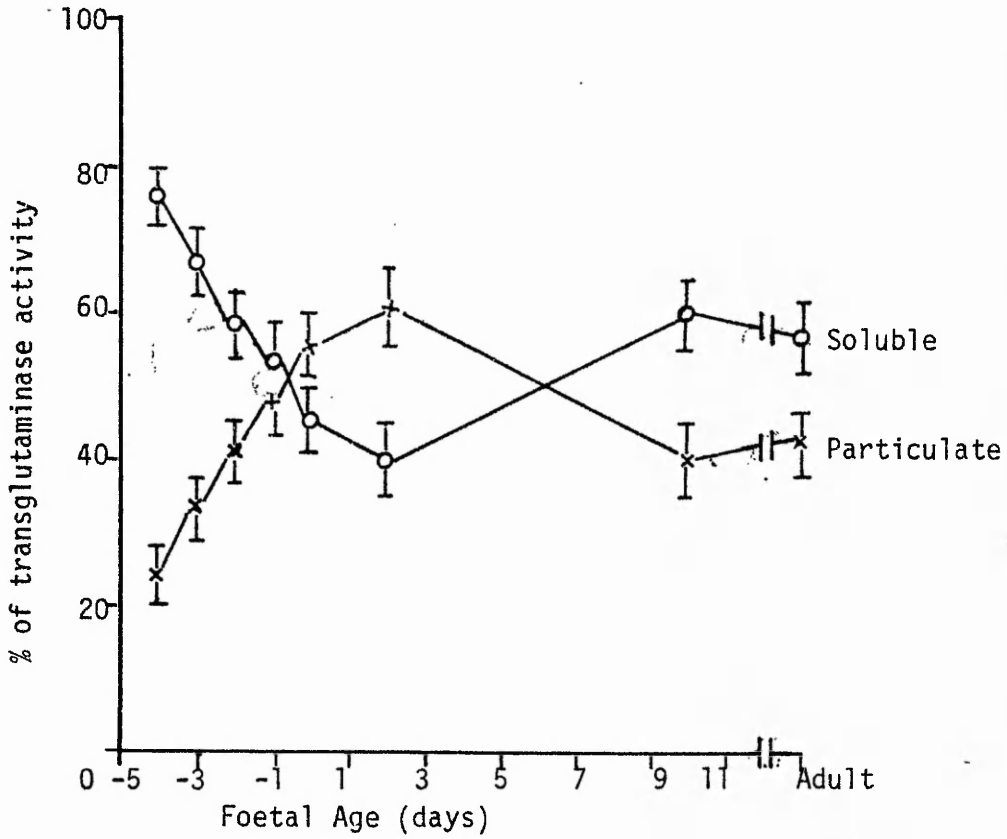

100
80
60
40
20
0
% of transglutaminase activity
-5
-3
-1
1
3
5
7
9
11
Adult
Foetal Age (days)
Soluble
Particulate

Figure 3.23.3

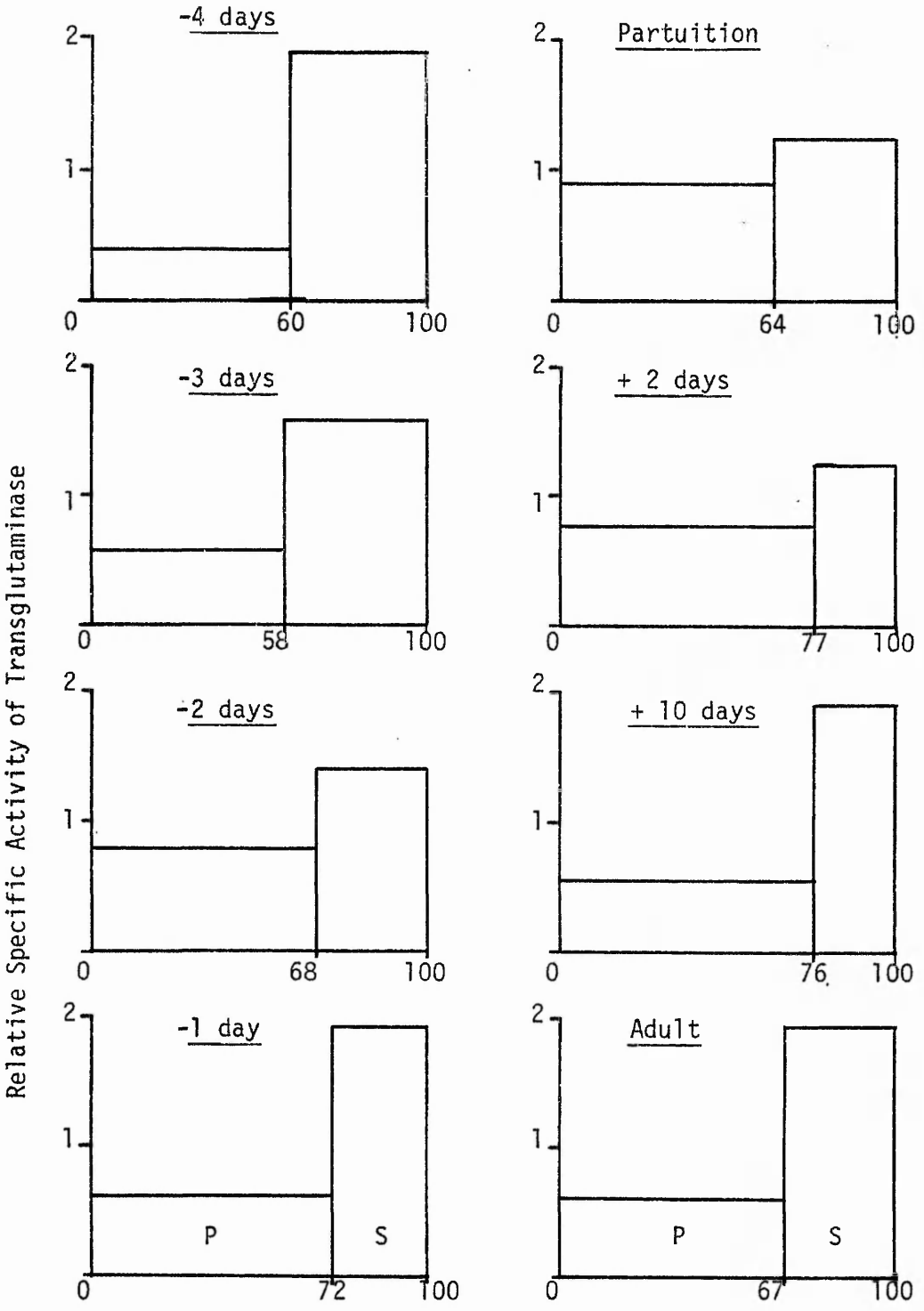

-4 days
Partuition
-3 days
+ 2 days
-2 days
+ 10 days
-1 day
Adult
2
1
0
60
64
58
77
68
76
72
67
100
P
S
Relative Specific Activity of Transglutaminase
% of Protein

Figure 3.23.4

3.3.2 Hepatomegaly

A series of hypolipaemic agents were employed to induce a condition of "work-hypertrophy" in rat livers resulting in hepatomegaly. The agents were drugs of the chlorophenoxyisobutyrate (C.P.I.B.) series normally used in anti-atherosclerotic treatment through lowering the plasma cholesterol and triglyceride concentrations (Thorp and Barrett, 1967). The cellular response includes a large increase in the peroxisome (microbody) fraction as oxidative phosphorylation is inhibited, and this can be seen in the accompanying electron micrographs.

The drugs (Methods 2.9.1) were administered in the feed over a period of 14 days at which time the livers were excised, weighed and fractionated according to the shortened procedure. Samples were also removed for tissue slice experimentation (Methods 2.7). The liver wet weights were greatly increased with the larger dose rates producing 50%-80% increases over the normal values. At the lower, therapeutic dosages the increases were less, being in the range 5%-25%.

Measurement of DNA concentration showed a marked reduction (22%-37%) with respect to normal values as the hepatocytes showed an increase in size rather than number. This was also clear from the electron microscope studies where a simple computation of numbers of nucleii per field of view gave a similar result.

Marker enzymes glucose-6-phosphatase (Fig.3.24.1) and 5'nucleotidase showed normal distribution in the control with a displacement of activity toward the 600g "N" fraction as the drug dosage was increased. The soluble fraction contained consistently small amounts of these enzymes. The activity of the 5'-nucleotidase was raised above the control values in all cases and, as this enzyme is involved in the synthesis of high-energy carriers, this reflects the condition of the cell.

Normal and hepatomegalic rat liver (magnification 40,000)

CONTROL

Nucleus

0.005% (w/w)
I.C.I. 65126
(hepatomegaly)
Note peroxisomes, nascent mitochondria and increased endoplasmic reticulum and ribosomes.

Glucose-6-phosphatase activity was reduced in the drug-treated livers confirming the increased energy production in the cells.

The total activity per microgram DNA determined for "homogenate" transglutaminase showed that the most physiologically active drug dosage evoked the greater response in the activity of the enzyme. The most active dose rate was normally the most concentrated (ten times therapeutic dose) except in the case of I.C.I. 79855 where the lower dose was the most active (P.L. Walton, personal communication) (Fig. 3.24.2). This measurement demonstrated that at these concentrations there was between two and three times the normal total activity present in the cell during this hepatomegalic condition. The specific activities recorded for the "homogenate" enzyme were reasonably consistent with the control (except for I.C.I. 112660 (0.2%)) showing only minor increases in response to the drug (Fig. 3.24.3). Thus with I.C.I. 112660 the total activity of the enzyme may be increased through enzyme concentration and/or activation, but with the other drugs the enzyme appears to be present in greater concentration only. Further experiments are needed to determine whether de novo synthesis or re-distribution of pre-existing, but inactive, enzyme reserves was the major contributor to this enhanced activity. The studies involving enzyme solubilisation (Results 3.2.6) have indicated that enzyme reserves may exist in the particulate cell fraction and this may prove to be the enzyme source.

Drug-induced hepatomegaly was found to affect the distribution of both protein and transglutaminase to a significant degree. The data (Fig. 3.24.4) demonstrates that all the drugs used produced a redistribution of protein towards the particulate fractions resulting in a reduction from approximately 28% to 13% for enzyme in the cytosol.

Coincidently the enzyme activity, which was distributed 43%:57% particulate to soluble respectively in the control, was found to be 25%:75% for the drug-treated tissue. The relative specific activity found in the soluble fraction was therefore shown to be very high compared to the control. The enzyme activity recorded for the "MLP" fraction was normally in the range 3%-5% of the total and was unaffected by drug or dosage.

Incubation of slices of 14 day hepatomegalic tissue induced by 0.2% I.C.I. 112660 in the presence of radio-labelled putrescine and 5mM Ca^{2+} gave data illustrating the changes in the enzyme activity discussed above (Table 3). The covalent incorporation of putrescine was increased by approximately 35% over the control values. The site of incorporation was also altered in that the soluble protein substrates were utilised to a greater degree than in normal tissue, which reflects the finding that the enzyme activity in this fraction was similarly increased. However, even with 80% of the enzyme activity in the soluble fraction, 67% of the label was still associated with the particulate protein.

The results from the isolated cell fractions would suggest that the cell response was directed toward raising the concentration of the enzyme (possibly through the release of bound-enzyme reserves) particularly in the soluble fraction. The tissue slice experiments indicate that whilst soluble protein substrates are present in increased concentration the particulate fraction remains the major site of enzyme function. The particulate fraction was also shown to be greatly increased with respect to protein distribution during hepatomegaly. The function of this response can only be the subject of speculation but from the present knowledge of transglutaminase a role is indicated in which soluble proteins are covalently immobilised into the particulate matrix.

Figures 3.24.1/2/3/4

Hepatomegaly

Liver samples (10g) were prepared by homogenisation (Potter-Elvehjem) in 0.25M Sucrose/1mM Tris-Cl/1mM EDTA, pH 7.4 buffer followed by centrifugation at 600g for 10 min to give the "N" fraction and then 71000g for 40 min to give "MLP" combined fractions and the "S" particle-free fraction. The fractions were assayed for glucose-6-phosphatase, 5'-nucleotidase, protein, DNA and transglutaminase according to the methods previously described (see Methods).

Hepatomegaly was produced by introducing compounds generously donated by Imperial Chemical Industries Ltd. These compounds were administered in the diet for a period of 14 days prior to sacrifice, and the dosages have been discussed above (Methods 2.9.1).

The data represents the mean ± S.D. for 4 determinations each derived from 3 pooled tissue samples. Control samples were derived from un-treated livers prepared in the same fashion.

The following figures are included:

3.24.1 Relative specific activity of glucose-6-phosphatase with respect to % of protein for the most active drug dosages. Enzyme activity was measured as μmoles phosphate released/min/ml fraction.

3.24.2 Total activity (nmoles putrescine incorporated/hr/fraction) of transglutaminase/μg DNA for all drugs and dosages tested.

3.24.3 Specific activity (nmoles putrescine incorporated/hr/mg casein protein) of transglutaminase for all drugs and dosages tested.

3.24.4 Relative specific activity of transglutaminase with respect to % of protein for the most active drug dosages.

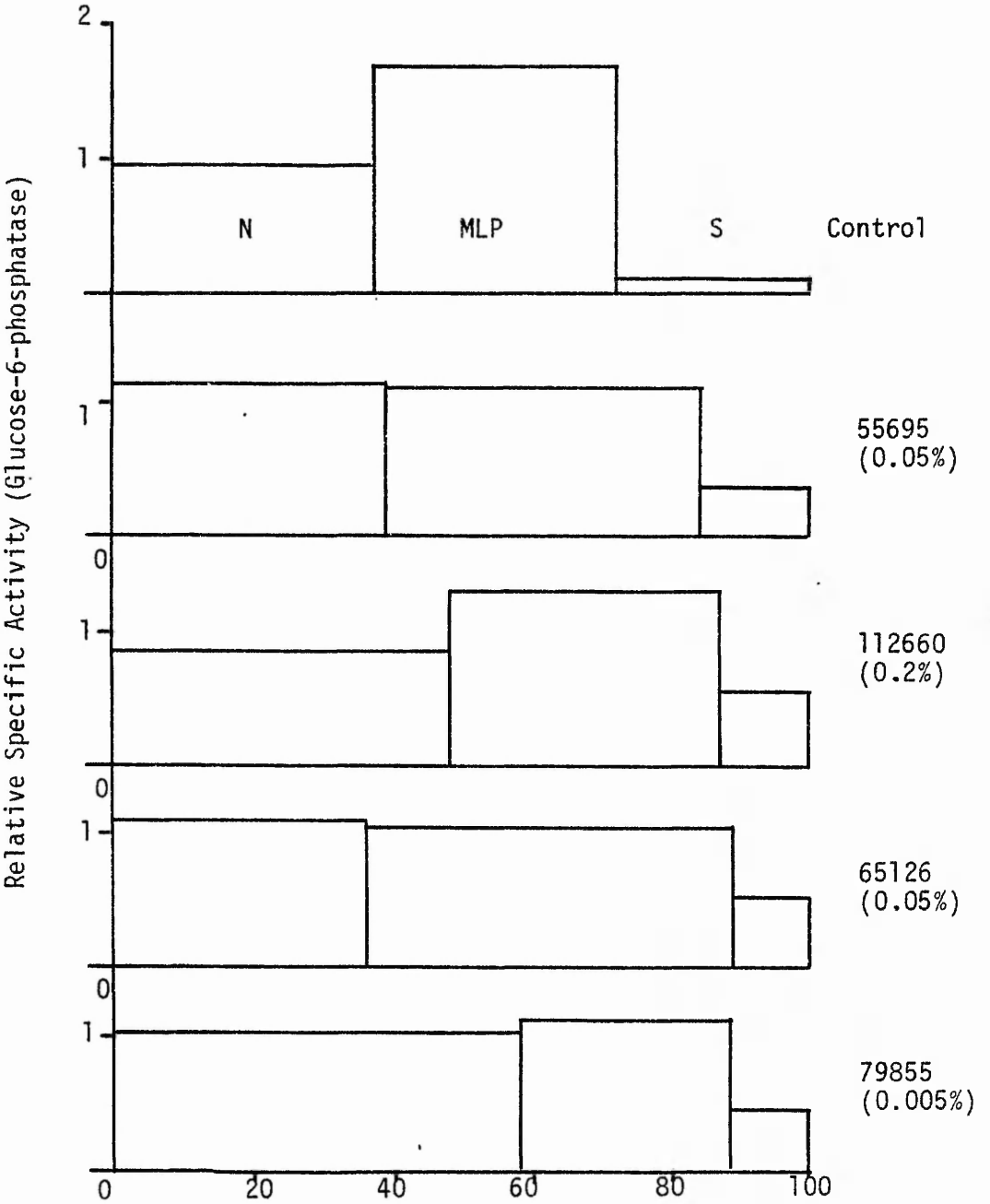
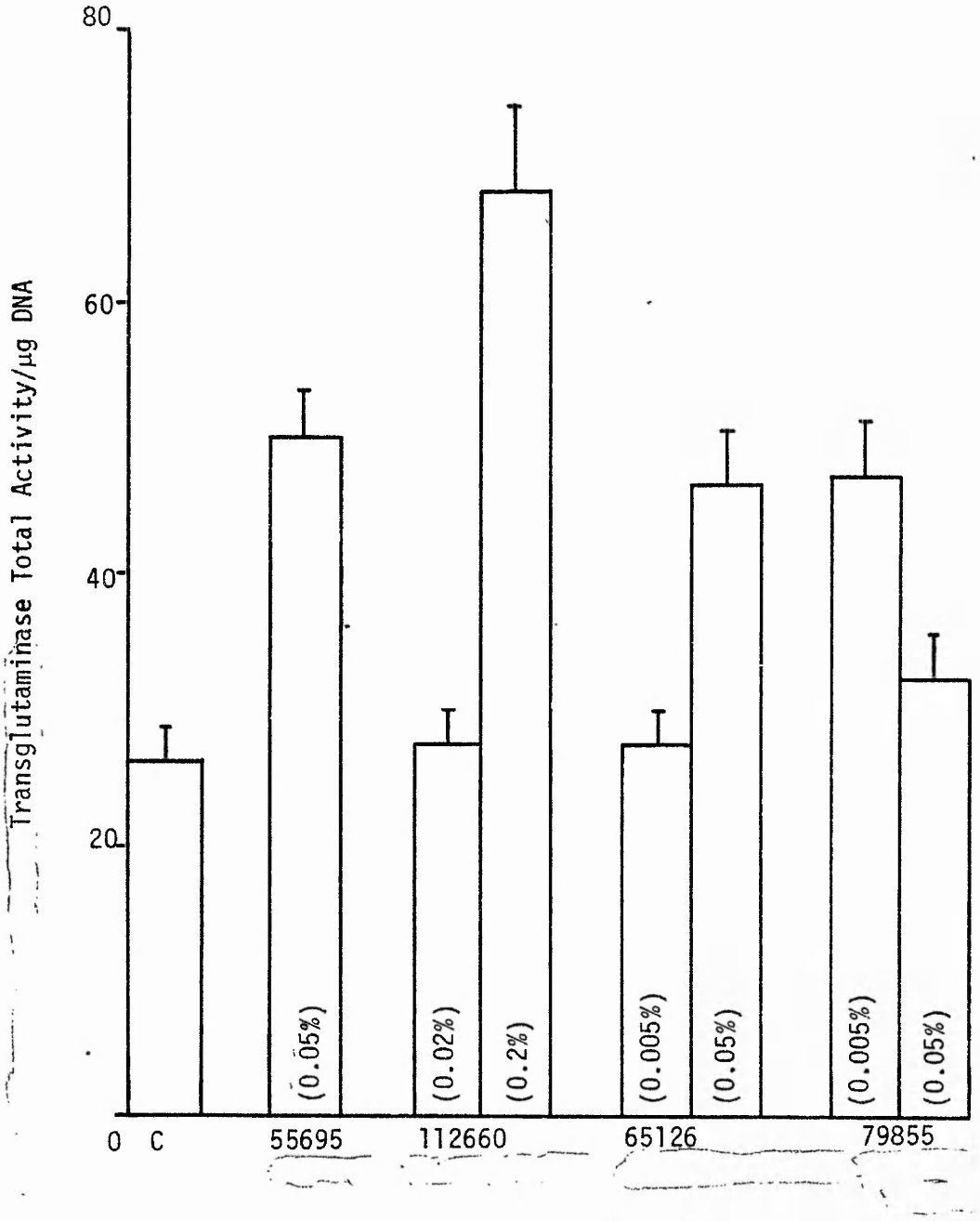
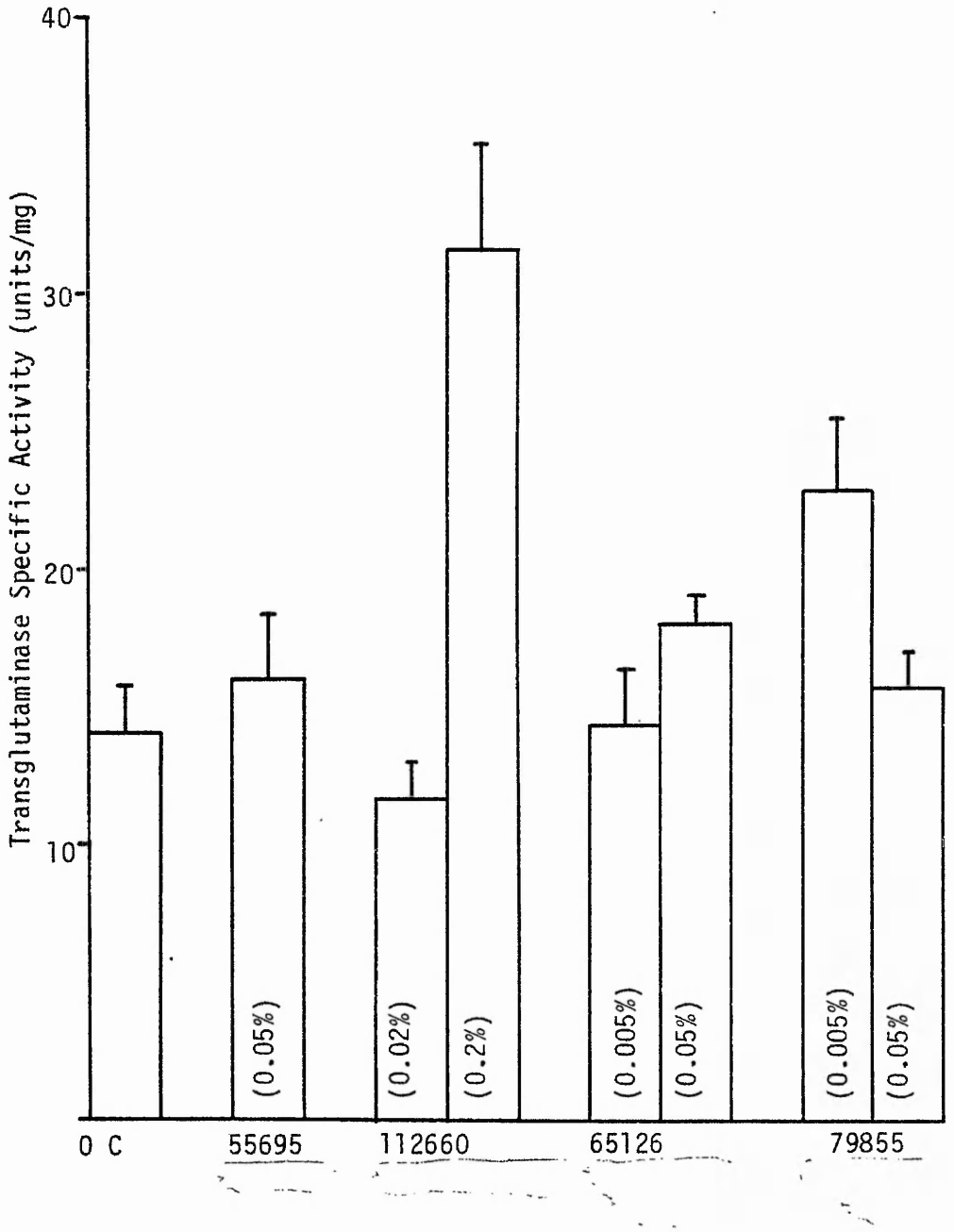

Relative Specific Activity (Glucose-6-phosphatase)
2
1
N
MLP
S
Control
1
55695
(0.05%)
0
1
112660
(0.2%)
0
1
65126
(0.05%)
0
1
79855
(0.005%)
0
20
40
60
80
100
% of protein

Figure 3.24.1



C.P.I.B. drug with dosage

Figure 3.24.2



C.P.I.B. drug with dosage

Figure 3.24.3

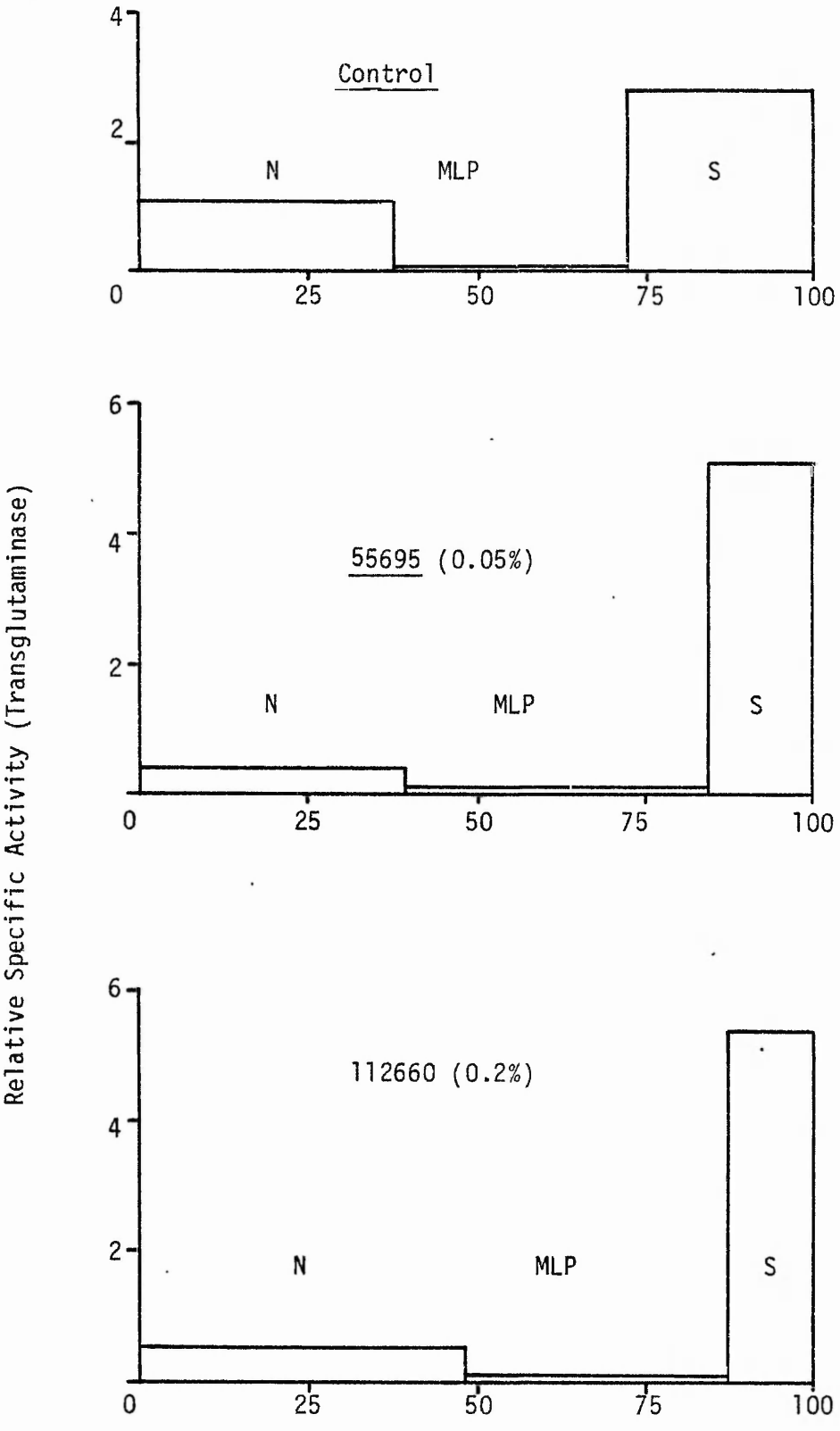

Control
N
MLP
S
55695 (0.05%)
112660 (0.2%)
Relative Specific Activity (Transglutaminase)
% of protein

Figure 3.24.4

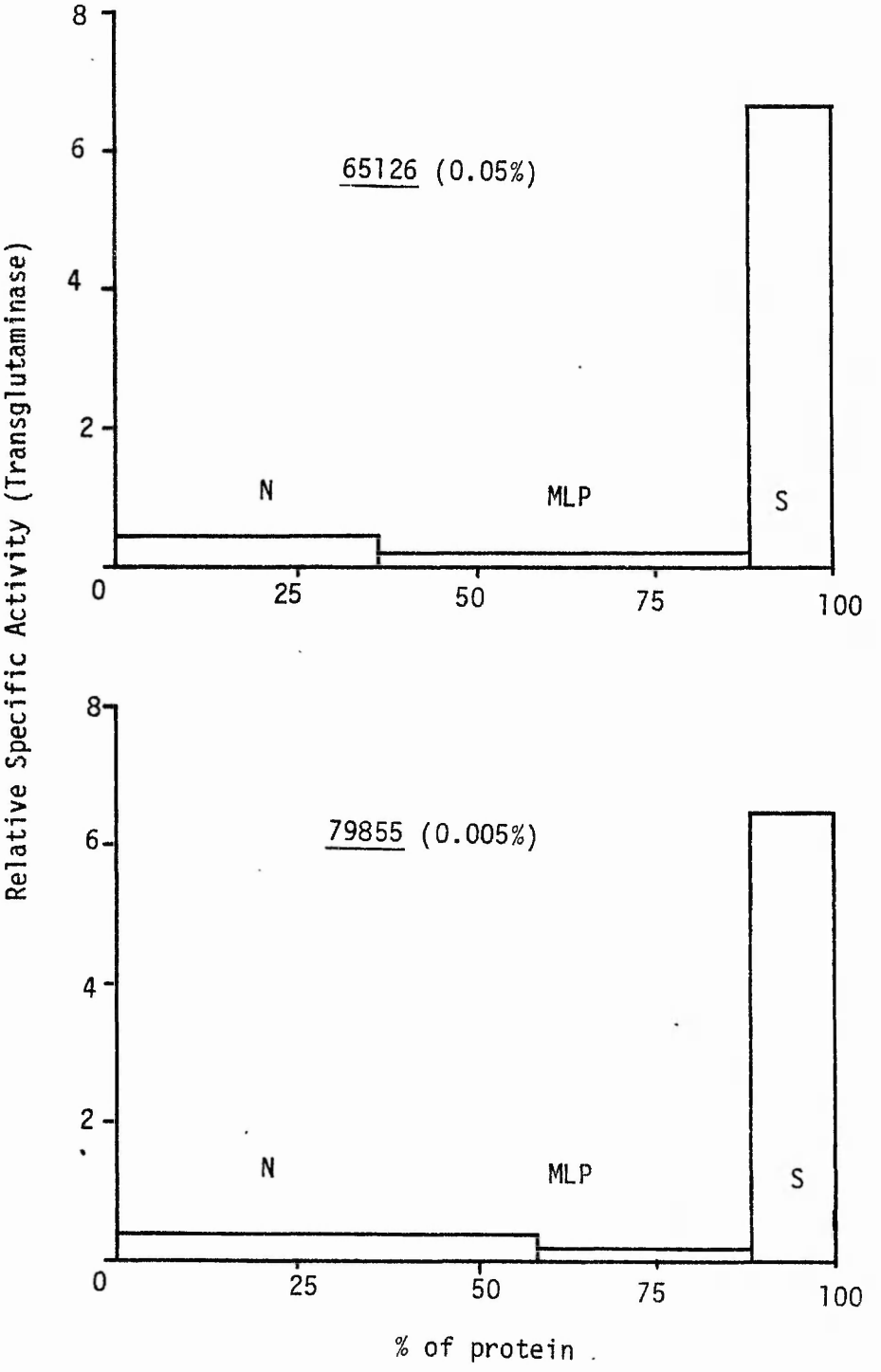


Figure 3.24.4 (cont'd.)

3.3.3 Liver Regeneration

The regenerating liver after partial hepatectomy is a useful model for studies of the regulatory mechanisms that control differentiation, proliferation and growth (Lewan et al, 1977). Partial hepatectomy is followed by an initial burst of rapid compensatory growth in the remaining liver lobes. After two or three cycles of cell division mitosis decreases and returns to normal (by 14 days after surgery). Restoration of the liver mass is then virtually complete and the liver returns to normal physiological function in approximately 20 days (Bresnick, 1971). Hepatic function is maintained at or above pre-surgery levels throughout regeneration and changes in soluble and plasma membrane enzymes have been well documented (Henderson and Kersten, 1970; Bucher and Malt, 1971; Wright, 1977).

Partial hepatectomy was carried out according to the method of Higgins and Anderson (1931) in which approximately 70% of the liver was removed (Methods 2.9.2). Samples of peripheral liver tissue (regenerated) were assayed at days 3, 8, 13 and 20 days post-operation as well as sham-operated control specimens. Samples were fractionated according to the shortened procedure to provide homogenate, 600g "N" fraction, 71000g "MLP" combined fraction and the particle free supernatant "S". These fractions were assayed for marker enzymes, DNA, protein and transglutaminase. Furthermore whole tissue (3 and 13 day) samples were also removed and incubated under the conditions described for tissue slice experiments (Methods 2.7).

Cell division was monitored by measuring the levels of injected [^{3}H-] thymidine incorporated into liver DNA during a period of 60 mins prior to sacrifice. Previous studies indicate maximal DNA synthesis approximately 36 hours after surgery which then slowly declines over the following 36 hours (Lewan et al, 1977), eventually returning to normal by 14 days. This was confirmed in this study where the rate of

DNA synthesis was approximately 5.5 fold higher than control levels at 3 days but only 2-fold higher at 8 days and normal at 13 days (Fig. 3.25.1).

The plasma membrane marker enzymes glucose-6-phosphatase (Fig. 3.25.2) and 5'-nucleotidase showed normal distribution in the control. The liver samples derived at various times throughout the period of regeneration showed a peak of activity at day 8 where coincidently the "MLP" protein fraction was also increased. The increases were of the order of 20% above control and at other sample times the marker enzymes were not significantly different from the normal liver values.

Protein distribution was found to vary between the "N" and "MLP" fractions. The "N" fraction was reduced during regeneration with a concomittant increase in the protein found in the "MLP" fraction. This alteration was found to be maximal at day 8 and involved approximately 10% of the total cell protein. The soluble fraction contained about 26% of the total protein throughout regeneration with only a small increase seen at day 3.

Transglutaminase activity was apparently reduced during the period of maximum cell production. The total activity/μg DNA and specific activity of the enzyme were reduced below control values and did not recover until later in the regenerative cycle. Total activity within the cell greatly increased at day 8 (Fig. 3.25.3) where it gradually reduced to control levels by days 13 and 20. Thus it would appear that transglutaminase activity is not expressed to any great degree during rapid proliferation but becomes more important as the cell matures (and perhaps during differentiation). This was shown more clearly as the specific activity of the enzyme increased rapidly from day 3, through day 8, and reached a peak around day 13 (Fig. 3.25.4). The activity in the soluble fraction was, at its maximum, more than double that of the control whilst that in the particulate fraction was increased by

approximately 40%. This situation apparently evolved from an increase in total enzyme around day 8 followed by an increase in specific activity a short time later. The relative specific activity of the transglutaminase (Fig. 3.25.5) can be seen to exhibit a pattern of redistribution of enzyme towards the soluble fraction during the period 8-13 days (approx.). As in the case of hepatomegaly it should be noted that an increase in the soluble fraction is closely followed by a cell protein redistribution into the "N" fraction. Both enzyme and protein distribution return to control values by day 20. The sham-operated control tissue gave data which was consistently within close statistical significance of normal liver.

Birckbichler et al (1976) have carried out a study of liver regeneration taking enzyme activity at 24 and 48 hours. Since, as in the case of hepatomegaly, they could find no significant alteration in activity they determined that no change occurred during regeneration. However, from the results of this study, it can be seen that whilst only a minor change takes place at 3 days the time length chosen by the above authors was much too restricted if they were to observe the effects of the full regenerative cycle.

Tissue slice incubations of liver removed at days 3 and 13 reflect the changes observed in the enzyme assays (Table 3). Compared to control tissue the covalent incorporation of radio-labelled putrescine in the presence of Ca^{2+} (5mM) was increased by 15% at 3 days and 47% at 13 days. Also the distribution of label was altered, in that, at 13 days there was far more incorporation into soluble substrate protein than was recorded for both the 3 day and control sample. This result reflects the previous finding that the enzyme activity in this fraction was greatly increased. However incorporation was still primarily into the particulate fraction where the majority of substrates were apparently

situated, or were placed by the enzyme catalysis. The uptake of putrescine by the liver tissue was only marginally different from the control value which demonstrated that the polyamine was fully available.

Sample	Covalent putrescine incorporation nmol/g wet weight	Slice: medium ratio	Distribution of label (%) of covalent incorporation*	
			P	S
3 day Regen.	7.80 ± 0.3	1.10 ± 0.04	81 ± 7	19 ± 2
13 day Regen.	9.92 ± 0.4	1.02 ± 0.06	67 ± 6	33 ± 2
14 day Hep.	9.10 ± 0.4	0.98 ± 0.01	63 ± 6	37 ± 2
Control	6.75 ± 0.3	1.02 ± 0.05	87 ± 5	13 ± 2
EDTA	0.54 ± 0.02	1.10 ± 0.03	72 ± 6	28 ± 3

TABLE 3 Distribution of label in tissues from hepatomegalic and and regenerating liver

"Regen" refers to regenerating tissue,

"Hep" to hepatomegalic tissue and

Control to mature liver.

* "P" represents the 71000g pellet fraction and "S" the particle-free supernatant.

Data received from 4 determinations (mean ± S.D.).

Slice:Medium ratio is a measure of concentration of radio-label in the two sites, designed to assess the mode of uptake and viability of the hepatocytes.

Figures 3.25.1/2/3/4/5

Liver Regeneration

Liver samples (3g) were prepared by homogenisation (Potter-Elvehjem) in 0.25M Sucrose/1mM Tris-Cl/1mM EDTA, pH 7.4 buffer followed by centrifugation at 600g for 10 min to give the "N" fraction and then 71000g for 40 min to give "MLP" combined fraction and particle-free supernatant "S". The fractions were assayed for glucose-6-phosphatase, 5'-nucleotidase, protein, DNA and transglutaminase according to the methods previously described (see Methods).

Regeneration was induced by surgical resection (see text) and samples were taken at 3, 8 13 and 20 days post-operation. Control and sham-operated tissue was also assayed.

[^{3}H]-thymidine (28 Ci/mMole) was injected at 15μCi/100g into rat tail vein 60 min prior to sacrifice and the DNA hydrolysate assayed for radioactive decay.

Data represents mean ± S.D. for 3 determinations each derived from 3 pooled tissue samples.

The following figures are included:

3.25.1 Histogram of [^{3}H-] thymidine uptake into DNA for regeneration of 3, 8 and 13 days in comparison with a mature liver control (0).

3.25.2 Relative specific activity of glucose-6-phosphatase with respect to % of protein through the regenerative period. Enzyme activity was measured as μmoles phosphate released/min/ml fraction.

3.25.3 Total activity of transglutaminase (nmoles putrescine incorporated/hr/fraction)/μg DNA during regeneration (days).

3.25.4 Specific activity of transglutaminase (nmole putrescine incorporated/hr/mg casein protein) during regeneration (days). The legend (0) refers to soluble enzyme and (X) to particulate bound "N" fraction enzyme.

3.25.5 Relative specific activity of transglutaminase with respect to % of protein during regeneration (days).

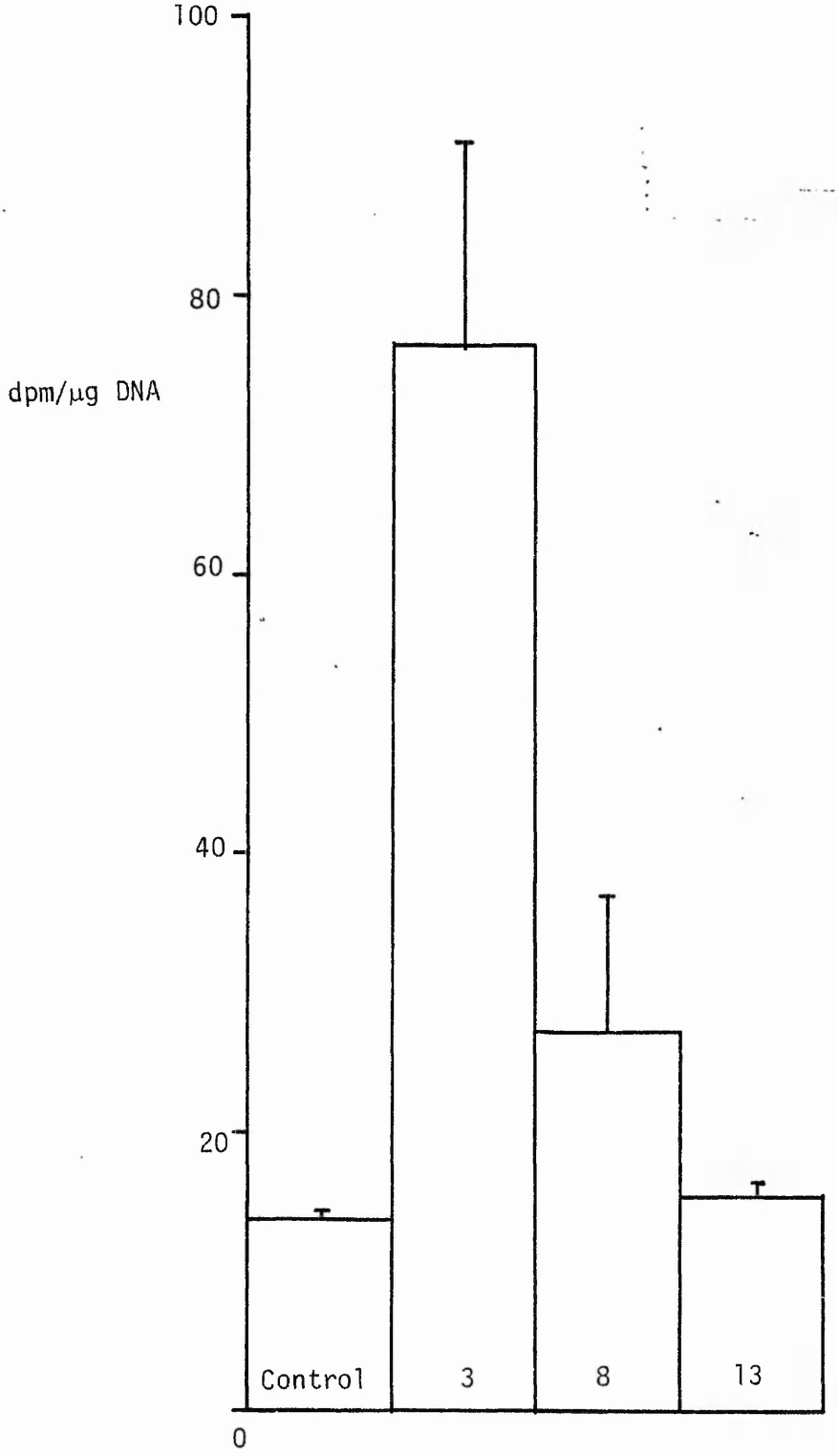


Figure 3.25.1

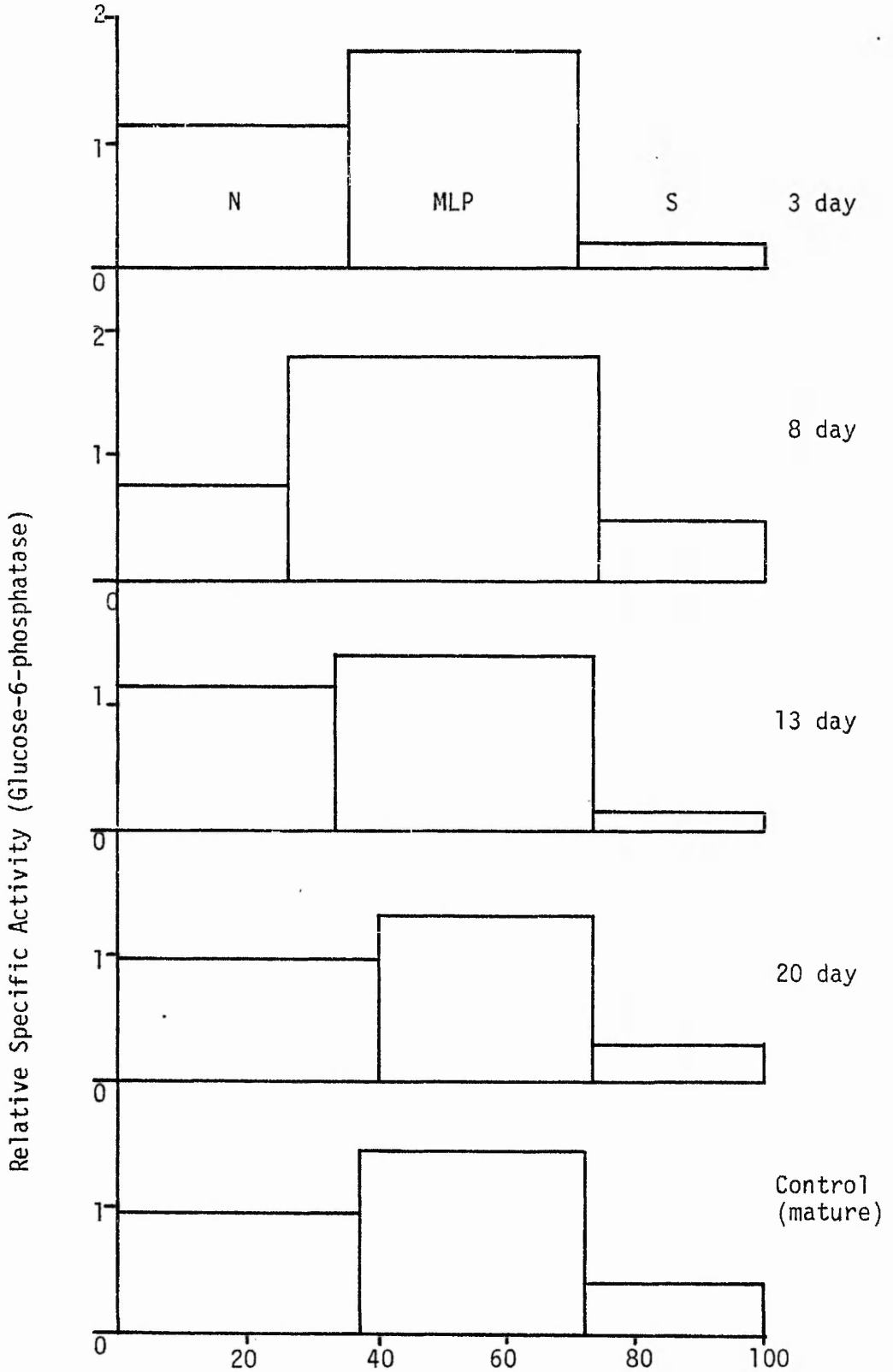

Relative Specific Activity (Glucose-6-phosphatase)
2
1
0
N
MLP
S
3 day
8 day
13 day
20 day
Control
(mature)
20
40
60
80
100
% of protein

Figure 3.25.2

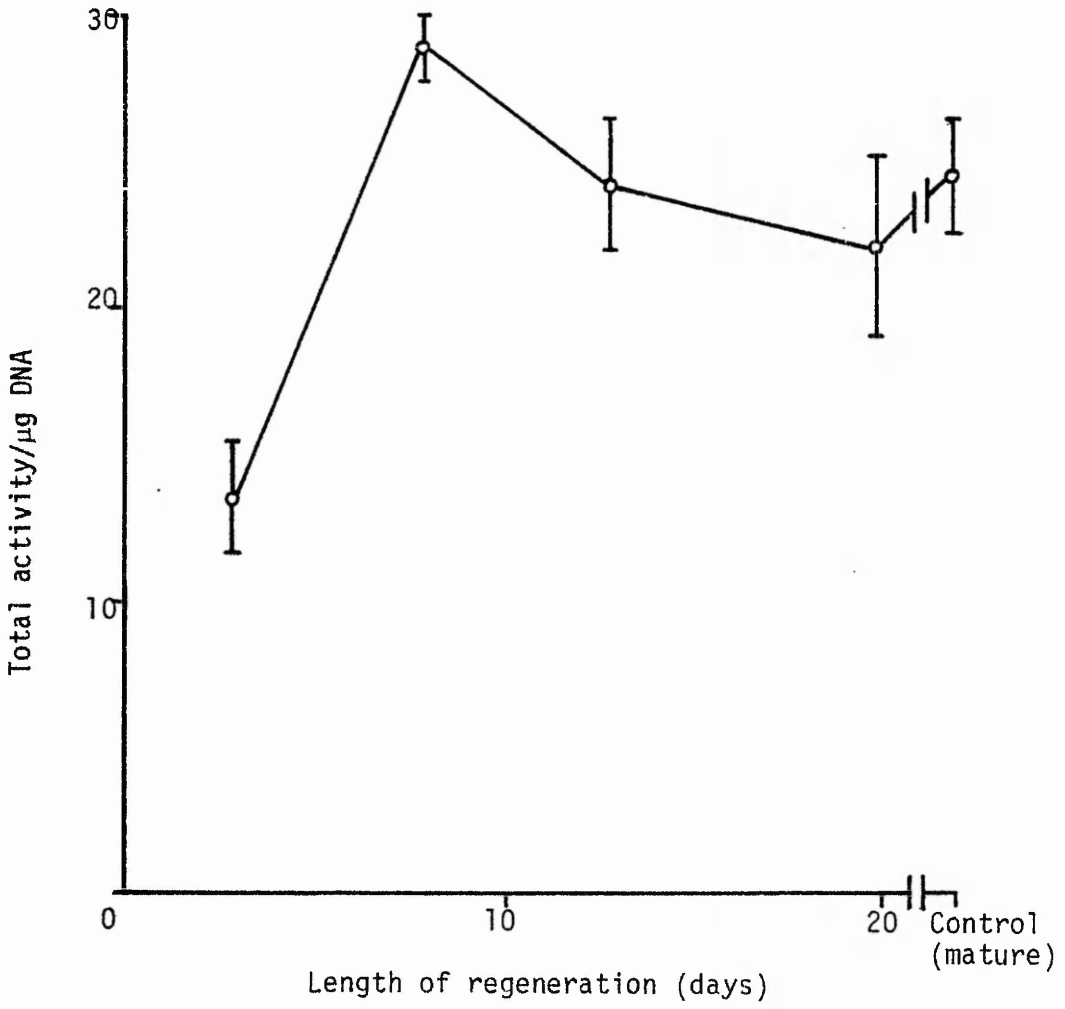

30
20
10
Total activity/µg DNA
0
10
20
Control
(mature)
Length of regeneration (days)

Figure 3.25.3

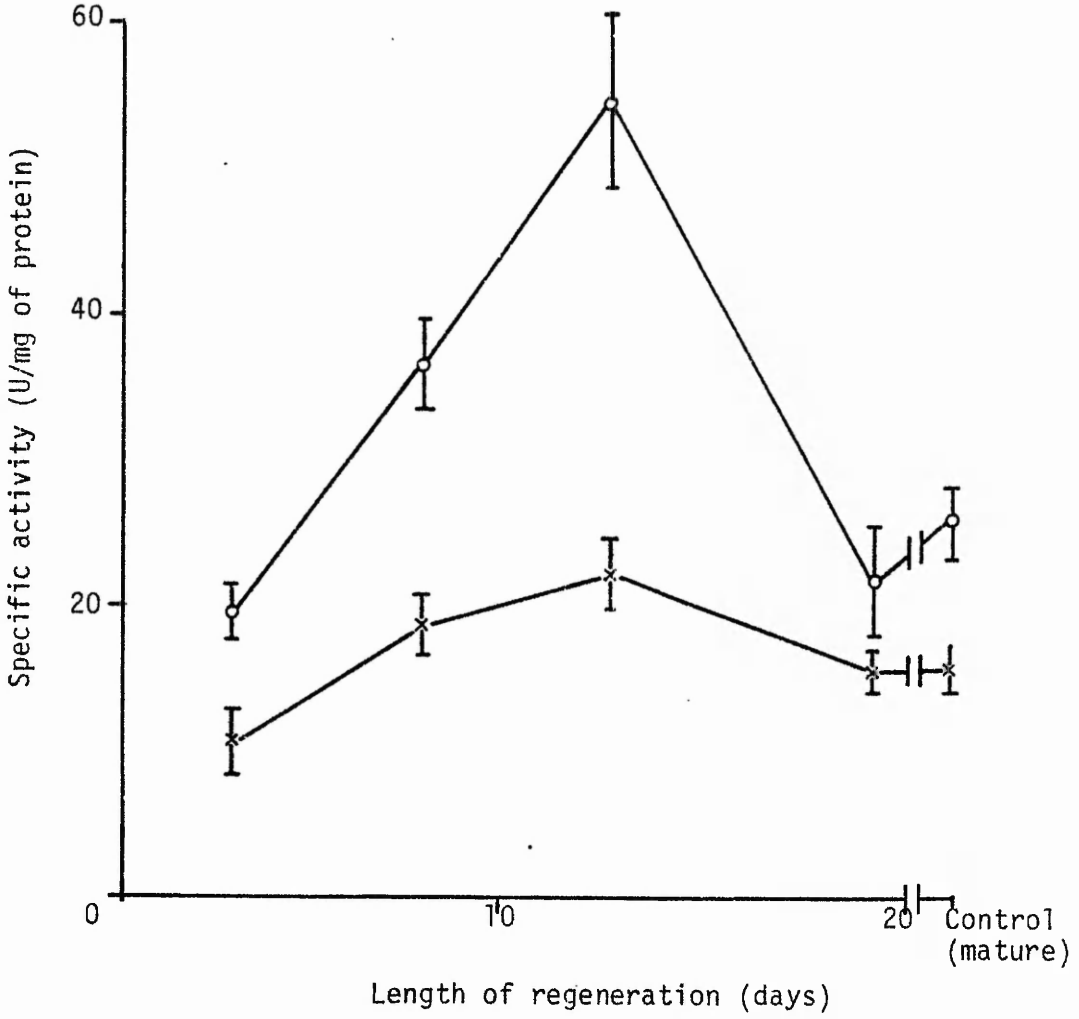

60
40
20
0
Specific activity (U/mg of protein)
10
20
Control
(mature)
Length of regeneration (days)

Figure 3.25.4

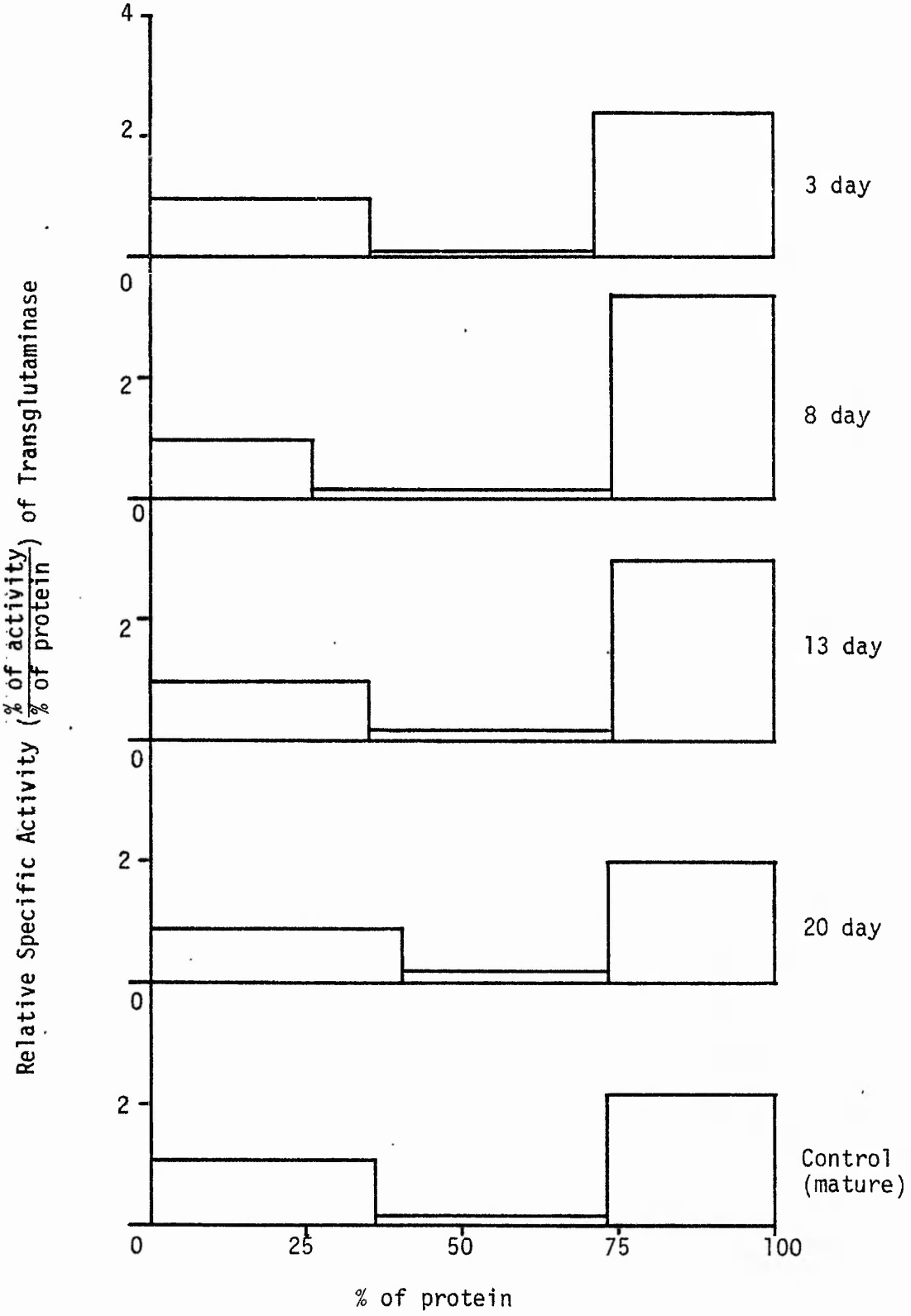

Relative Specific Activity (% of activity / % of protein) of Transglutaminase
4
2
0
3 day
8 day
13 day
20 day
Control (mature)
0
25
50
75
100
% of protein

Figure 3.25.5

3.3.4 Platelet Study

The hypothesis that endogenous transglutaminase may respond to extracellular stimuli has been examined in a series of preliminary experiments. Birckbichler et al (1977, 1978) have demonstrated that trypsin stimulated the level of transglutaminase in intact human lung cells and promoted redistribution of the enzyme into the soluble phase. Data given above (Results 3.2.7) indicate activation of the liver enzyme by a number of agents including trypsin, organic solvents and chaotropic salts, however, the mechanisms remains unclear.

This activation phenomenon may be of particular relevance to the platelet transglutaminase action since specific stimulation of this cell leads to aggregation and the release of various adjuncts to the clotting process (see Introduction). The normal platelet transglutaminase activity is made up of 80% tissue enzyme and 20% zymogenic platelet factor XIII, but it is claimed that neither of these are released during primary ADP-induced aggregation (Tsukada, 1977). The physiological function of the platelet tissue enzyme in relation to the platelet response to stimulation has not been detailed, however it may be relevant to platelet function and its role in haemostasis.

Platelets were isolated from human blood and purified according to the methods previously described (Methods 2.9.4). The yield of washed platelets was 79%, providing 2.53×10^9 cells/ml buffer. The platelets were washed thoroughly to remove from their surface any adsorbed clotting factors (including plasma factor XIII) and coagulants. These factors would have contributed to the activation of the zymogenic transglutaminase at a later stage of treatment.

Light-scattering platelet aggregometry was carried out using adenosine diphosphate as the standard. Collagen, arachidonic acid and thrombin were then investigated using the same procedure in order to

determine the concentration of each agent that would produce the same aggregation determined for ADP (Methods 2.9.4). The platelets were then sonicated and fractionated (by the shortened procedure) and the transglutaminase assayed for activity and distribution.

The control samples, which were treated in an identical manner to the aggregated platelets except for the addition of agonist, were found to contain a calcium-dependent transglutaminase which was non-zymogenic and existed almost entirely in the soluble fraction of the platelet cytoplasm. The platelet factor XIII was not activated during the experimental procedure since incubation with thrombin (after assaying for non-zymogenic enzyme) caused an increase in the observed activity in all relevant test systems. The finding that 98% of the tissue enzyme and 96% of the platelet factor XIII activity was observed in the soluble fraction was in good agreement with the 94% of total activity demonstrated by Lopaciuk et al (1976).

The results from the "activated" platelets demonstrated two distinct reactions by the enzyme. The first was observed as a result of activation by both collagen and arachidonic acid. In this case the tissue enzyme was observed to pass from the soluble to the particulate fraction producing an almost complete reversal of the normal distribution. The particulate fractions of collagen (82% of total activity) and arachidonic acid (69% of total) treated platelets compare with only trace levels determined in the control (Fig. 3.26.1). The specific activity of the enzyme under these conditions showed a substantial rise in the particulate fraction and a moderate fall in the soluble fraction (Fig. 3.26.2). The total activity of the enzyme did not alter substantially as a result of the redistribution.

Activation of the platelets by thrombin produced an entirely different enzyme response. This treatment with thrombin resulted in the concomittant activation of the zymogenic enzyme present but this

enzyme only forms approximately 20% of the total activity detected. Assuming that the zymogen is located entirely in the soluble fraction it was apparent that there was no large redistribution of activity. The soluble enzyme activity was reduced from approximately 98% in the control to 81% in the test (taking the factor XIII into account) or 61% (based on the tissue enzyme alone). The specific activity data recorded a similar increase in the particulate fraction to that of the other agonists. However a very great increase in the soluble activity (430% increase) was detected compared to the control which, in turn, is in direct contrast to the fall in activity recorded by both collagen and arachidonic acid. This increase would appear to be as a result of enzyme activation rather than by an significant increase in concentration. This activation was similar to that previously described for trypsin in purified enzyme (Results 3.2.7). The large increase in the soluble enzyme activity may also be responsible, in part at least, for the distribution figures quoted above. It may be argued that the level of enzyme activity in the soluble fraction does not fully represent the concentration of enzyme protein as it would with the other test agonists. Therefore, were the activation of the enzyme by thrombin to be set aside the distribution of enzyme protein of normal activity may be much more like that found for the other agonists.

In the absence of corroborating evidence it should be pointed out that the figure of 20% for the factor XIII fraction activity given by Tsukada (1977) may be low as the specificities of the enzymes for the assay substrates may be different. The factor XIII fraction may be substantially higher under different conditions (P.L. Walton, personal communication).

The speculation regarding thrombin activation of the enzyme from the soluble phase is in contrast to an earlier result in which thrombin failed to influence the purified liver enzyme (Results 3.1.3.4). The

reasons for this apparent contradiction are not fully understood. Two main alternatives exist which may be considered. The first is based on the differences which may exist between the platelet and liver "tissue" type enzymes with special regard to their susceptibility to proteolytic attack. Thus, thrombin, which fails to activate the liver enzyme, may produce the required changes in the platelet enzyme. Secondly, since the activation occurred using intact platelet cells and not purified enzyme, a necessary pre-requisite to thrombin action (e.g. co-factor) may have been missing during the earlier experiments. Only a more detailed investigation will resolve this apparent inconsistency.

The observation that the majority of the enzyme apparently becomes particulate bound during platelet aggregation by the agonists tested may explain a particular phenomenon of the release reaction. During this process, which involves many cytoplasmic constituents passing into the external millieu, the platelet transglutaminases remain within the cell. This may be accomplished by the enzyme (particularly the "tissue" type) becoming bound in the manner shown, thus preventing release. Similarly, the enzyme may have a function during and/or following haemostasis which requires it to become immobilised at the membrane. These translocations of the enzyme have been investigated in the liver cell and are discussed above (Results (3.2.6).

The rationale behind a function of the "tissue" enzyme within the platelet may be false since the enzyme has been in the cell since its formation from the megakaryocyte (Kiesselbach and Wagner, 1972). However, a function may become apparent since the platelet membrane is constantly interacting with transglutaminase protein substrates such as fibrin, collagen and fibronectin and the cell interior is concentrated with contractile proteins (Bensusan et al, 1978). A particularly

relevant role for the enzyme may be in clot retraction where covalent immobilisation of contracted cytoskeletal proteins such as actin (Results 3.2.5) would be of great value. Experiments might be performed with the initial purpose of using these well-characterised and eminently suitable cells to discover interactions at the membrane level. Dual-labelling by autoradiography using radiolabelled polyamines at the internal surface and fluorescent-conjugated antibodies to substrate proteins on the exterior may provide a link across the membrane. Subsequent studies may reveal a complete contractile system operating from the external fibrin meshwork of the stabilised clot to the contractile components within the platelet pseudopodia similar to that described for erythrocytes (Cohen and Branton, 1979).

Figures 3.26.1/2

Platelet Study

Human platelets were purified by centrifugation and gel-filtration using the method of Tangen (Methods 2.9.4). The platelets were subjected to identical aggregation by agonists as determined by aggregometry using adenosine diphosphate as the standard. The agonists used included collagen, arachidonic acid and human thrombin. The cells were sonicated for 5 min at 4^{o}C and centrifuged 48,000g for 30 min to provide the particulate (P) and soluble (S) cell fractions. Transglutaminase assay was carried out by the standard method and included the addition of thrombin (5NIH units) as a control assay to determine the platelet factor XIII activity. Control samples (without agonist) were also included. The results are expressed as mean ± S.D. for 3 determinations.

Figures include:

3.26.1 Relative specific activity of transglutaminase with respect to % of protein for the various agonists tested.

3.26.2 Specific activity of transglutaminase (nmol putrescine incorporated/hr/mg protein) with respect to the agonists tested.

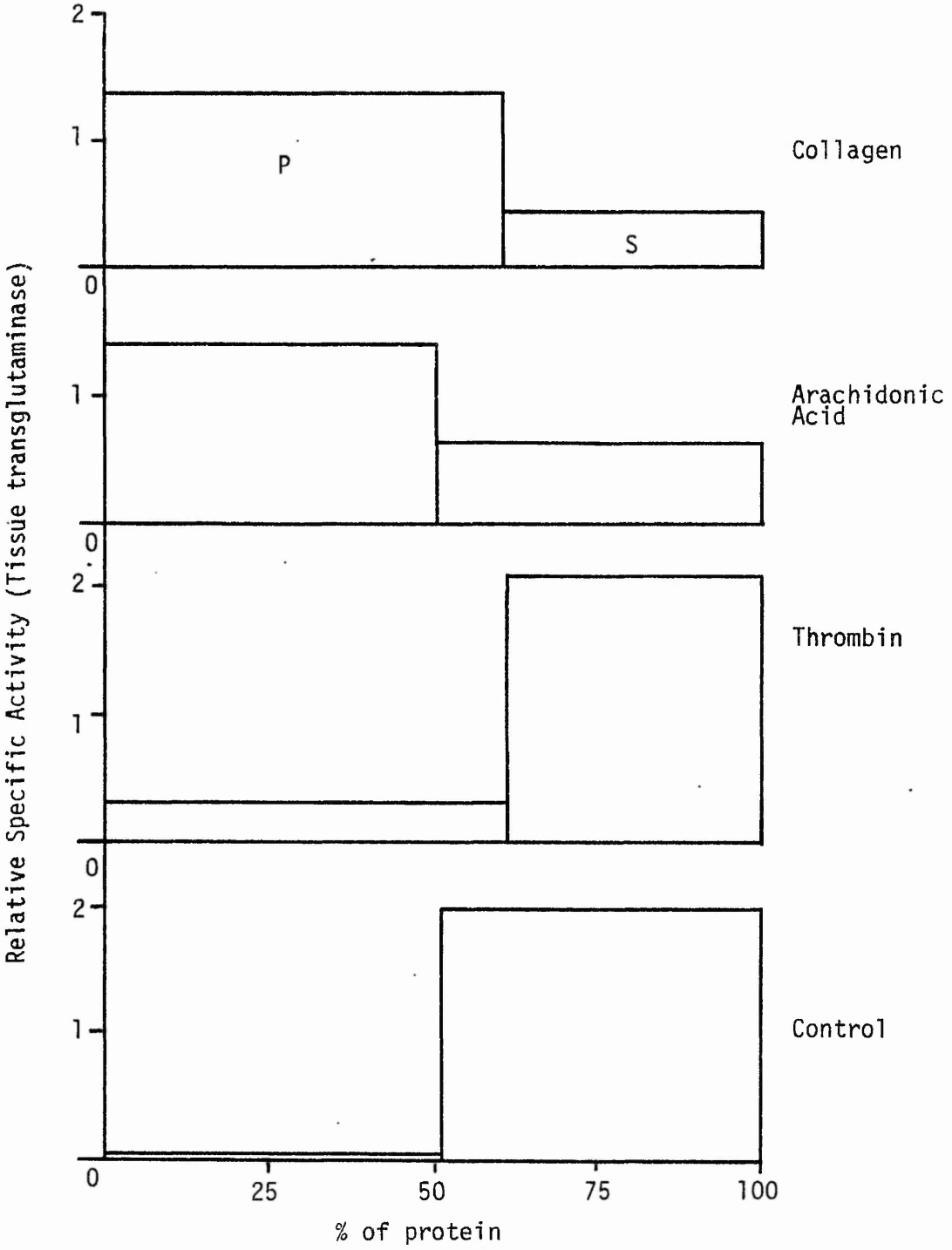

Relative Specific Activity (Tissue transglutaminase)
2
1
0
P
S
Collagen
Arachidonic Acid
Thrombin
Control
0
25
50
75
100
% of protein

Figure 3.26.1

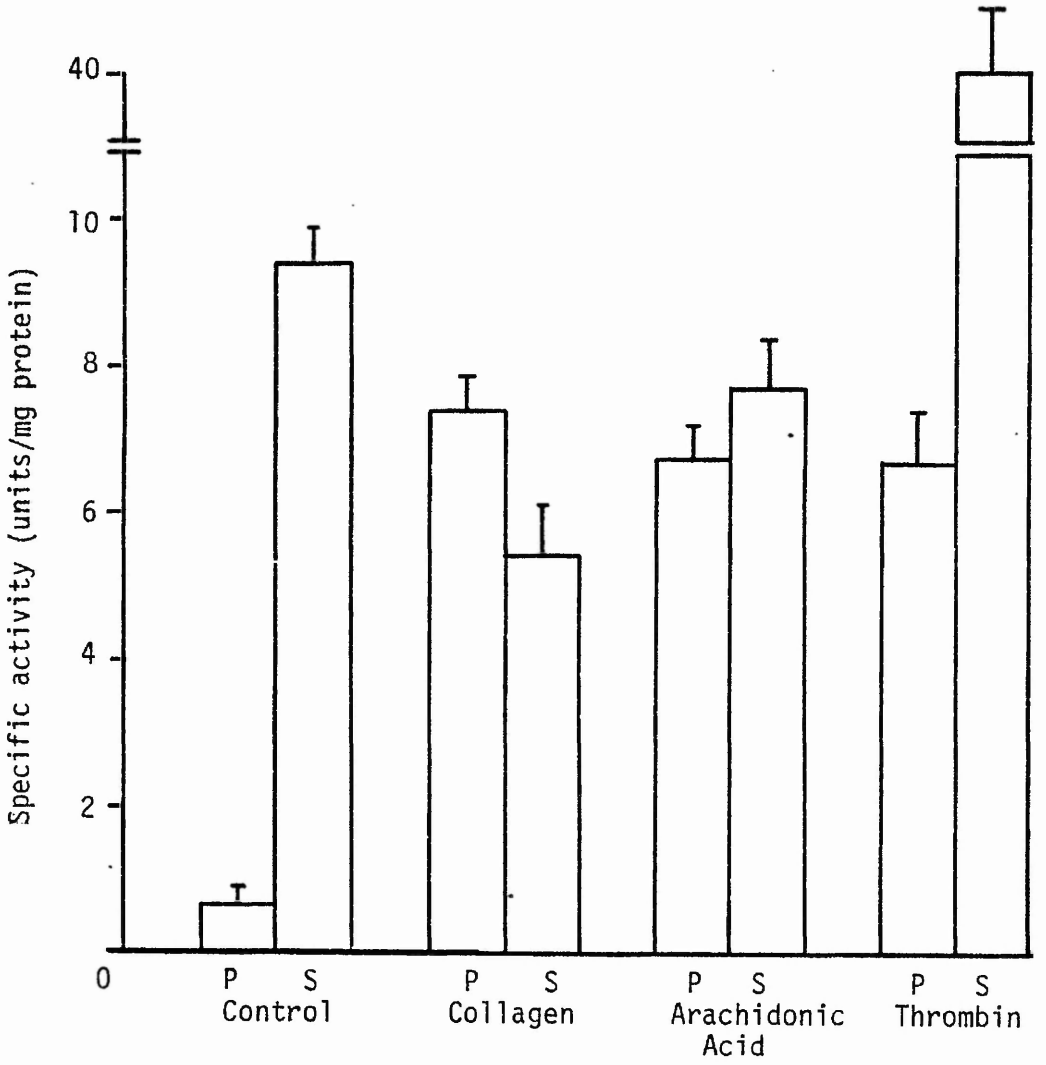

Specific activity (units/mg protein)
40
10
8
6
4
2
0
P
S
Control
P
S
Collagen
P
S
Arachidonic Acid
P
S
Thrombin

Figure 3.26.2

3.3.5 Tumour Study

Reports concerning the importance of transglutaminase enzymes in neoplasia have been contradictory. Birckbichler et al (1976, 1977) have demonstrated that reduced enzyme levels are a pre-requisite for proliferative growth whereas Laki et al (1977) propose that transglutaminase activity is required during a number of metastatic processes. This study was designed to assess the importance of the enzyme in a group of metastasising and non-metastasising rat sarcomata and to suggest possible methods by which the enzyme function in the cell may be controlled.

The experimental procedures have been given above (Methods 2.9.5) for the fractionation of the tissues and the subsequent assays, including solubilisation and tissue slice incubations.

Transglutaminase activity present in normal liver, lung and spleen was found in all cases to be substantially greater than that of the tumour lines (Table 4). Some variation of enzyme activity between tumours was apparent but no particular pattern was discovered that would distinguish the lines predisposed to metastasis.

The most striking result was the reduction in transglutaminase activity which occurred in the two tumour lines P7 and P8 during the period of metastasis. The length of time from implantation of the tissue to mestastasis (Wexlar, 1966) was determined to be approximately 40 days for P7 and 30 days for P8. Samples of tissue were derived from the tumours both before and after these times and the differences in enzyme activity were assessed. When expressed as activity per unit weight of DNA, an approximate measure of activity per cell, a reduction (after metastasis) of 73% was found for P7 and 86% for P8. The non-metastasising tumour line MC3 was also assayed over a similar time

TABLE 4 Transglutaminase activity in homogenates of normal rat tissues and rat sarcomata.

Tissue	Tumour Age(days)	Metastases Detected	Transglutaminase Activity *U/mg protein	*U/g tissue	*U/mg DNA
P7	21	-	2.56 ± 0.3	499 ± 32	297 ± 18
(n=4)	55	+	1.31 ± 0.12	219 ± 12	81 ± 4
P8	22	-	1.40 ± 0.15	148 ± 7	350 ± 26
(n=4)	39	+	0.176 ± 0.01	32 ± 2	50 ± 3
MC3					
(n=3)	27	-	1.88 ± 0.2	137 ± 8	136 ± 8
	43	-	1.14 ± 0.1	108 ± 6	115 ± 8
CC5	36	-	1.50 ± 0.18	188 ± 13	389 ± 26
(n=2)					
Liver		-	13.4 ± 2.6	2646 ± 230	4731 ± 327
(n=6)					
Lung		-	31.5 ± 4.2	3461 ± 282	2225 ± 525
(n=4)					
Spleen		-	20.3 ± 3.8	3094 ± 112	1426 ± 406
(n=4)					

* Unit of enzyme activity equals 1.0 nmol putrescine incorporated per hour under conditions of assay; Each value represents mean value ± S.D.

period and the enzyme activity was only slightly reduced (15%). The inclusion of Trasylol (Bayer Chemicals Ltd.), an inhibitor of a number of trypsin-like enzymes, into the homogenisation and enzyme assay media (1000 U/ml) did not alter the above findings. This diminishes the possibility that the reduction in activity was due to proteolysis by trypsin-like enzymes. The effect of the ageing process which may have contributed toward the reduced enzyme activity (as in MC3) was minimised by the removal of tumour tissue from the non-necrotic, peripheral regions.

The distribution of the enzyme activity in the tumours was found to be predominantly particulate (71000g pellet)(Fig. 3.26.1). However in the tumours P7 and P8 an apparent increase in the soluble enzyme fraction was observed following metastasis. This was brought about by the total activity of the particulate fraction being reduced to a much greater extent that that of the soluble fraction. This was demonstrated in P8 following a 15-fold reduction in particulate associated activity but only a 4-fold reduction in the soluble enzyme.

Further fractionation of the post-metastasis P8 tumour (Fig. 3.26.2) revealed that the majority of the particulate enzyme activity was associated with the 600g "N" fraction. This pellet is likely to contain cell debris, nucleii and cell membrane material.

The enzyme distribution in the normal tissues was also found to vary over a wide range. The liver tissue distribution has been described previously for the Sprague-Dawley rat (Results 3.2.1) and was found to be approximately 43%:57% for the particulate:soluble fractions respectively. The AS rat tissue analysed here gave a similar ratio of 35%:65% for liver tissue. The lung and splenic results demonstrate a contrasting distribution of 88%:12% and 84%:16% respectively. The latter examples

express a similar distribution to the tumour tissue with the majority of enzyme activity in the particulate (600g) fraction. A lung tissue fractionation for transglutaminase in Sprague-Dawley has been published from this laboratory (Griffin et al, 1978) and demonstrated the particulate fraction to contain in excess of 90% of the total activity.

The association of transglutaminase with the 600g "N" fraction was assessed by a series of "washings" using specified buffers as described previously (Results 3.2.6). Whereas washing this fraction in a buffer of 0.25M Sucrose/1mM Tris-Cl/1mM EDTA, pH 7.4 caused a substantial release of liver enzyme from the particulate to the soluble fraction, a similar buffer also containing polyamine and Ca^{2+} produced no such effect (Table 5). This result was true for both putrescine and spermine and only occurred in conjunction with Ca^{2+}. This requirement for the insolubilisation of the enzyme would infer that enzyme catalysis was involved in the binding mechanism. Washing of the 600g "N" fraction from a post-metastasis P8 tumour in the sucrose buffer also failed to solubilise any enzyme activity from the protein. This inability of the enzyme to leave its binding site, even in the absence of added polyamine and Ca^{2+}, indicated that this enzyme is already tightly associated, possibly through covalent bonding. The addition of polyamine and Ca^{2+} to the tumour washes did not affect this result. The solubilisation phenomenon has been investigated in greater detail in Results 3.2.6 and a number of factors have been demonstrated to alter the enzymes binding characteristics.

The accessibility of cellular protein substrates for transglutaminase was investigated by means of radio-labelled putrescine incorporation into tissue slices (Methods 2.7). Rat liver and post-metastasis P8 tumour were compared with regard to the activity of the tissue enzyme. The

TABLE 5 Leaching of transglutaminase activity from the 600 xg pellet of rat liver and p8 sarcoma.

Washing Medium	Sample	Specific Activity *U/mg protein	Total Activity/ of wet tissue
Sucrose medium	Liver	14.4 + 1.8	2391 ± 115
pH 7.4 plus	Homogenate	14.4 + 2.5	1054 ± 75
1mM EDTA	Original NP	25.2 + 0.8	628 ± 43
	Wash 1	17.0 + 4.2	186 ± 75
	Wash 2	18.4 + 1.3	927 ± 56
	Washed NP		
Sucrose medium	Homogenate	15.0 + 0.9	3740
plus 1mM Ca	Original NP	27.6 + 1.0	1821 ± 106
1mM putrescine	Wash 1	0	0
pH7.4	Wash 2	0	0
	Washed NP	14.8 + 2.5	960 ± 72
Sucrose medium	Homogenate	17.2 + 0.9	3891
plus 1mM Ca	Original NP	27.6 + 1.1	3014 ± 241
1mM Spermine	Wash 1	0	0
pH7.4	Wash 2	0	0
	Washed NP	14.5 + 1.7	688 ± 44
	p8		
Sucrose medium	Homogenate	0.173 + 0.01	31.0
plus 1mM Ca	Original NP	0.18 + 0.01	7.7 ± 0.4
1mM putrescine	Wash 1	0	0
pH7.4	Wash 2	0	0
	Washed NP	0.25 + 0.01	8.6 ± 0.6

*Unit of enzyme activty equals nmol putrescine incorporated per hour under conditions of assay.
Sample tissue (5-10g) was homogenised by the procedures previously described in 3 volumes of 0.25M sucrose/1mM EDTA/1mM Tris-chloride pH7.4. The homogenate was centrfuged for 10 min at 600 xg (rav 28cm) and the supernatant removed. The remaining pellet was resuspended in 3 volumes of sucrose medium and recentrifuged as before. The 600 xg pellet was divided and each component resuspended in the washing buffer (5 volumes) for 15 mins at 4° before centrifugation at 600 xg for 10 min (Wash 1). This procedure was repeated, but included an incubation of 100 min at 4° for the second wash prior to centrifugation. The washed 600 xg pellet was resuspended in 3 volumes of 0.25M sucrose/1mM EDTA/1mM Tris-chloride buffer and assayed for transglutaminase activity with the other buffer, pH7.4.

total uptake of radio-label into both tissues over the time periods assayed were similar and the slice:medium ratios of 1.1-1.2 indicate that entry of putrescine was probably a passive process (Table 6). Covalent incorporation of putrescine increased with the length of incubation. In liver, the incorporation expressed as a percentage of total accumulation at 120 min was 8.8% which contrast with the 0.9% calculated for the P8 tumour. This differential in polyamine incorporation was not as large as that between the homogenate enzyme activities but still demonstrated the obviously low transglutaminase activity in the tumour tissue.

Lowering the Ca^{2+} concentration in the incubation medium to that of plasma (1.9mm) did not affect the level of enzyme activity. This would indicate that the Ca^{2+} present normally within the cell is sufficient to allow optimum enzyme function.

The subcellular distribution of the incorporated putrescine also demonstrated a fundemental difference between the liver and tumour tissue (Table 6). Liver incorporated approximately 85% of the label into the particulate fraction compared to the extremely low value of 3% for the P8 tumour. Neither value followed the pattern of enzyme distribution (Fig. 3.26.1). This result is unusual as many normal transglutaminase substrates may be found in the membrane fraction (Results 3.2.4).

The experiments undertaken in this study support the claim that lowered levels of enzyme activity are required during proliferative growth (Birckbichler et al, 1976; Birckbichler and Patterson, 1978). Furthermore these authors also report the particulate nature of the tumour enzyme activity. This distribution, however, is not confined to neoplastic tissue since both lung and spleen exhibit a similar pattern.

TABLE 6 Incorporation of putrescine into tissue slices of normal rat liver and metastasising rat p8 sarcoma.

Tissue	Incubation Time (Min)	S/M	Putrescine accumulation (n=4)			
			Total Uptake nmol/g wet wt.	Covalent incorporation nmol/g wet wt.	Percentage distribution of covalent incorporation	
					P	S
P8	30	1.12	84.6 ± 7.6	0.17 ± 0.02	3.0 ± 2.1	97 ± 9.5
	60	1.10	82.0 ± 5.2	0.40 ± 0.03	2.8 ± 2.0	97.2 ± 4.2
	60	1.18	89.0 ± 6.1	0.38 ± 0.03		
	120	1.10	85.1 ± 5.2	0.76 ± 0.05	2.9 ± 2.3	97.1 ± 6.3
	120*	1.20	90.9 ± 6.5	0.06 ± 0.004	12 ± 4.6	88 ± 10.4
Liver	30	0.77	60.2 ± 4.2	3.20 ± 0.4		
	60	1.06	85.6 ± 4.9	6.02 ± 0.8	86.3 ± 8	13.7 ± 2
	60	1.10	87.2 ± 5.0	4.05 ± 0.2		
	120	1.35	105.7 ± 6.8	10.01 ± 0.4	86.7 ± 7	13.3 ± 2
	120**	1.10	87.8 ± 2.1	0.59 ± 0.06	72.0 ± 6	28.0 ± 3

S/M represents slice to medium ratio for putrescine accumulation calculated by dividing the total uptake of putrescine into 1g wet weight of tissue by the amount of putrescine in $1cm^3$ of medium at the time sampling.

P respresents the 71,000g pellet and S the particle free supernatant. Incubation mixtures contained 5mm $CaCl_2$ except for*, which contained 1.9mN $CaCl_2$ and **, which contained 1mM EDTA.

The insolubilisation of the enzyme in the presence of polyamine plus Ca^{2+} may be a result of catalysis since neither component acted independently. Birckbichler et al (1977) have also shown that transglutaminase will catalyse incorporation of polyamines into its own protein. This situation may well exist in the tumour where increased polyamine concentrations are known to occur (Williams-Ashman, 1972; Marton and Heby, 1974; Scalabrino et al, 1978). The influence of polyamines on transglutaminase activity may be manifold. The effect of immobilisation of enzyme may play a role in restricting activity in the cell. Alternatively these compounds may become incorporated into the available γ-glutamyl residues therefore limiting crosslinking of donor proteins. This may be a contributory factor in the data determined from the tissue slice experiments where the tumour acceptor protein was entirely soluble. This result, in direct contrast to that of normal liver tissue, may have been due to the insoluble substrates being either inaccessible to the enzyme, or perhaps, saturated with secondary substrates e.g. polyamines. The observation that liver protein substrates were mainly in the particulate fraction supports the proposal that cellular enzyme substrates are membrane-associated (Birckbichler et al, 1973; Davies et al, 1980).

The finding that the enzyme activity is reduced to exceedingly low levels during metastasis in P7 and P8 tumours correlates well with the functions which transglutaminase is thought to perform. Metastasis has been categorised into the sequential steps that lead to colonisation: (i) extension of the tumour mass into surrounding tissues, (ii) penetration of body cavities and vessels, (iii) release of tumour cells to other sites, (iv) re-invasion by tumour tissue at site of arrest and (v) manipulation of the new environment to promote tumour-cell survival,

vascularisation and growth (for review see Nicolson, 1979). Many properties that distinguish tumour cells from normal cells are expressed at the cell surface and may involve transglutaminase since alterations in membrane $\varepsilon(\gamma$-glutamyl) lysine crosslinking have been reported (Birckbichler and Patterson, 1978). The areas in which transglutaminase may be proved to exert some influence occur at all stages of tumour investment and metastasis.

One of the first manifestations of the early tumour mass is the formation of an encapsulating fibrin gel which provides both protection from host defence and a basis from which the cells might proliferate (Dvorak et al, 1979). This may involve the intervention of plasma factor XIII activity and relies on the tumour-secreted products. The thromboplastic activity of the cells is closely related to fibrin deposition, and the fibrinolytic activity present is a significant factor in the resolution of the fibrin mass especially at the advancing border of the tumour (Tanaka, 1977). A plasmacytoma in mice has been shown to substantially reduce plasma factor XIII levels by the selective absorption of the enzyme into the malignant cells (Eipe et al, 1977). The fibrin network is necessary for turnover growth since it supplies the cells with a surface over which they can proliferate, and also forms the framework for the endothelial cells of the stimulated vasculature. Fibronectin and collagen may also become involved at this stage as has been shown to occur in wound healing.

The small tumour emboli, which carry the tumour cells to their secondary sites, are formed from loosely associated cells at the tumour edge. The decreased association of malignant cells to matrix components is thought to be crucial to unregulated growth in that the cells are then free to divide and move (Vaheri and Mosher, 1978). The major matrix

proteins which are affected by transformation are fibronectin and collagen. The tumour cells lose their ability to hold fibronectin at the surface (Hynes et al, 1976) and this results in a significant reduction in cell-cell and cell-substratum adhesion (Yamada and Pouyssegur, 1978). Furthermore, collagen which extensively co-distributes with fibronectin, is also lost from transformed cell surfaces (Vaheri et al, 1978). Both these proteins have been shown to be substrates for transglutaminase and are known to be crosslinked by factor XIII (Mosher et al, 1980). The reduction in transglutaminase activity demonstrated during metastasis may be responsible for the failure of the cells to retain its matrix protein. This would require that fibronectin, in particular, is anchored to the membrane through the $\varepsilon(\gamma$-glutamyl) lysine crosslink. Birckbichler and Patterson (1978) have described a parallel distribution of the enzyme and fibronectin in paired cell lines (i.e. when one is low the other is low and vice versa) and suggest that the enzyme activity responds to the level of cell surface fibronectin. This phenomenon would take on an increased meaning should the normally intracellular enzyme be shown to be present in the extracellular environment.

The dispersed emboli are most successful in the next stage of metastasis if they accumulate fibrin and platelets on their surface (for review see de Gaetano and Garattini (editors), 1978). This greatly increased the liklihood of lodgement in the capillary bed of the target organ. Fibrin or fibrinogen is also thought to mask the tumour antigens from the host immunosurveillance systems therefore preventing cell destruction (Chew and Wallace, 1976; Fésüs and Laki, 1976). The fibrin also has the effect of disrupting the vascular endothelial lining thus facilitating the invasive stage (Kadish et al, 1979).

The invasion of tissue by the tumour cells calls for two major

attributes, deformability and motility. The low level of transglutaminase in these cells from their inception during proliferative growth may be shown to have decreased the crosslink density in their membranes. The resultant decrease in membrane stabilisation would aid deformability and promote growth. This phenomenon has been demonstrated in WI-38 human lung pair cell lines where the transformed culture contained 40-100 times fewer $\varepsilon(\gamma$-glutamyl) lysine crosslinks per cell than the normal counterpart. The second facet, i.e. that of motility, involves the cytoskeleton which contains many transglutaminase substrate proteins. Gabbiani (1979) describes the cytoplasmic contractile apparatus of cancer cells as more developed than that of normal cells, particularly at the cell periphery, and concludes this to be the explanation for the invasive quality of malignant cells. Actin bundles and fibronectin have been shown to co-distribute *in vivo* through some form of transmembrane association and to bind to one another *in vitro* (Keski-Oja *et al*, 1980). This association of cytoskeleton and matrix proteins may be transglutaminase-mediated in a similar way to that described for erythrocytes and others (see Results 3.2.9).

The tentative conclusions from this study indicate that while the tumour mass is growing there may be sufficient transglutaminase activity to allow the formation of the fibrin investment and the spreading of cells with reduced membrane stabilisation. Later when the tumour begins to metastasise the reduction in enzyme activity will promote dissemination of the emboli through decreased adhesions to other cells and the substratum. The residual activity may then be required, or obtained from platelets or circulating factor XIII, for further fibrin investment, platelet adherence and eventually tissue invasion. The motility of the cells during invasion may require transglutaminase-mediation of the cytoskeletal components and the newly-formed tumour secondary would

take on the characteristics of the initial primary site. Laki et al (1977) have demonstrated that the target tissue transglutaminase content is a determining factor. These authors suggest that a "low activity" tumour will metastasise to a "high activity" organ inferring that the tumour may be parasitic towards the target transglutaminase activity and utilise it for its own requirements.

The majority of the results expressed here have been submitted for publication to the International Journal of Cancer (see enclosure).

Figures 3.27.1/2

Tumour Studies

The subcellular distribution of tumour transglutaminase activity was determined following excision of a peripheral sample of tissue (4g), homogenisation (Potter-Elvehjem) and fractionation. Assays of normal tissues were also undertaken following the same procedure. The tumours were sampled at the times given in parenthesis. Fractionation of homogenates were carried out as follows:

3.27.1 Relative specific activity of transglutaminase activity with respect to % of protein for all tumours and normal tissues tested. Legend "P" represents the 71000g pellet and "S" the particle-free supernatant.

3.27.2 Relative specific activity of transglutaminase activity with respect to % of protein for normal liver and tumour P8 (pre- and post-metastasis). Legend "N" represents the 600g pellet, "P" the 71000g pellet and "S" the particle-free supernatant.

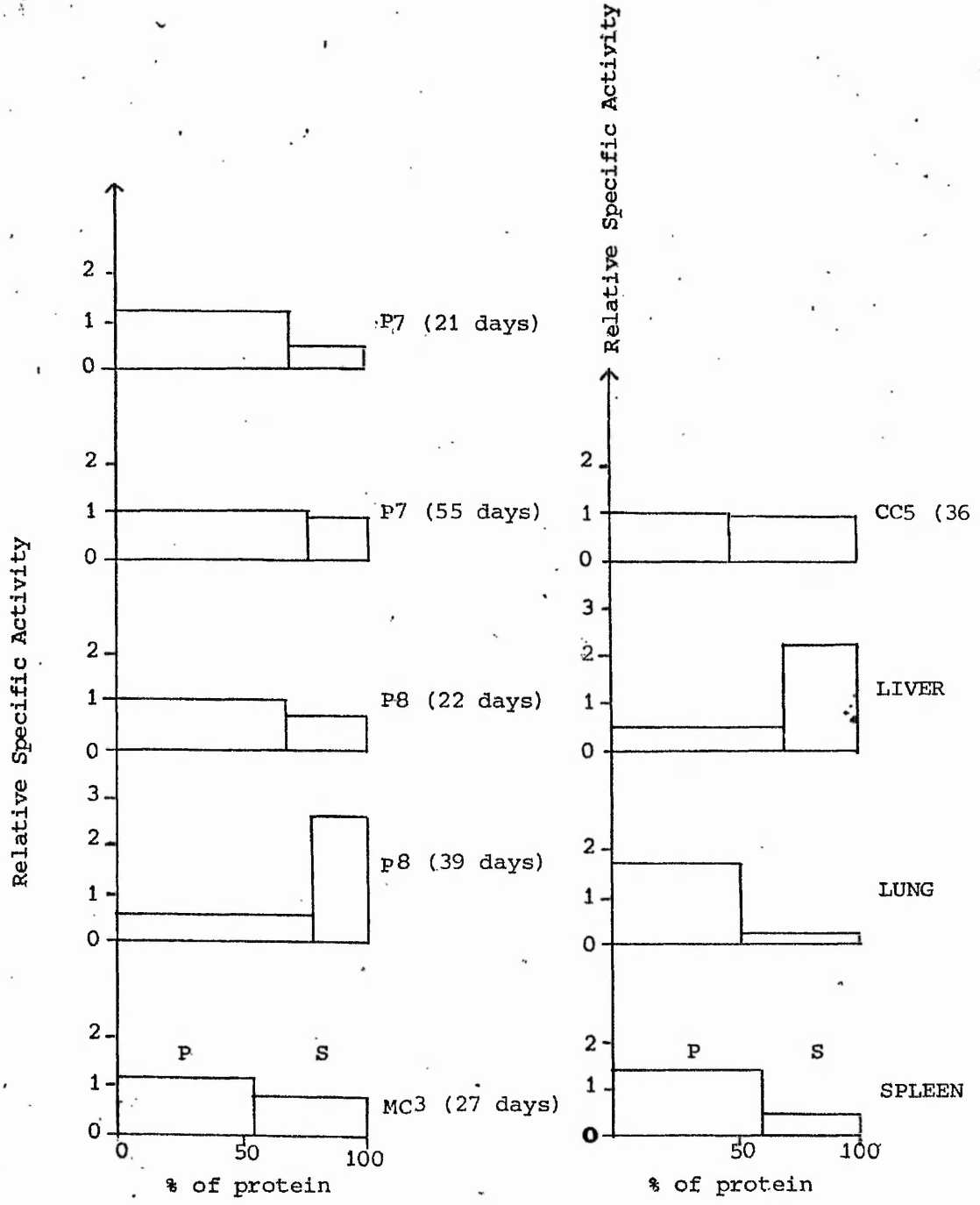

Relative Specific Activity
P7 (21 days)
P7 (55 days)
P8 (22 days)
p8 (39 days)
MC3 (27 days)
P
S
0
50
100
% of protein
Relative Specific Activity
CC5 (36 days)
LIVER
LUNG
SPLEEN
P
S
50
100
% of protein

Fig. 3.27.1

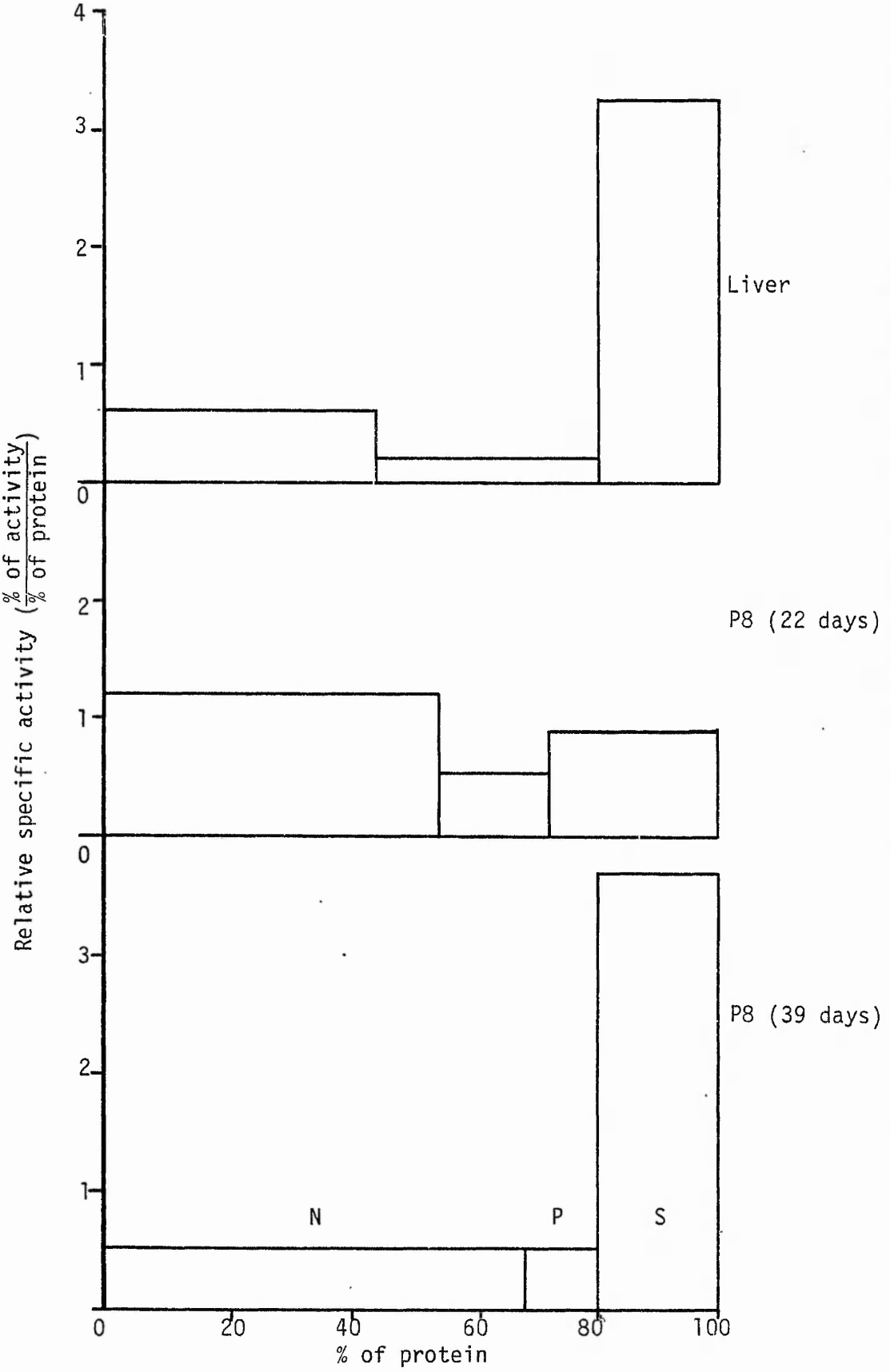

Relative specific activity (% of activity / % of protein)
4
3
2
1
0
Liver
2
1
0
P8 (22 days)
3
2
1
N
P
S
P8 (39 days)
0
20
40
60
80
100
% of protein

Figure 3.27.2

3.3.6 Discussion

The response of the transglutaminase enzyme system to the stimuli provided by each of the model systems may be seen to fall into three major categories.

(i) The studies of tumour, foetal liver and early regeneration of liver provided models of proliferative growth. In all cases the apparent enzyme activity was reduced and was associated with the particulate fraction to a greater extent than were the control systems. This situation was markedly altered following the decline of rapid proliferative growth. The level of polyamine present in cells during proliferation (Sunkara et al, 1979) may be a significant factor and this aspect of enzyme modulation will be the subject of future studies. It has, however, been shown that mitogens stimulate transglutaminase activity during lymphocyte blastogenesis (Novogrodsky et al, 1978) which illustrates that the situation is not straight-forward.

(ii) Hepatomegaly provided a model of tissue growth under work hypertrophy but without rapid cell division. The cellular response was to increase the total activity of the enzyme without affecting the specific activity. The enzyme and its potential substrate proteins were both found to be increased in the soluble cell fraction. However, the majority of acceptor sites remained in the membrane-containing particulate fraction. This indicates that the enzyme was possibly using soluble pre-curser proteins and immobilising them in the larger aggregates of protein in the membrane fraction. The induction of differentiation has also been shown, using glucocorticoids, to be associated with a concommittant increase in transglutaminase activity (Obinata and Endo, 1977). This induction, in epidermal cells, was followed by the synthesis of structural proteins and the accumulation of urea-mercaptoethanol insoluble

aggregates. Interestingly, glucocorticoid administration in other systems (Dexamethosone (Furcht et al, 1979) and epidermal growth factor (Chen et al, 1977) has resulted in increased fibronectin and collagen involvement in the cell glycocalyx or matrix proteins.

(iii) The later stages of liver regeneration are analagous to cell maturation. The enzyme in this situation responded by raising both the specific and total activity present whilst also following a redistribution to the soluble fraction. Here, again, there was an increase in the concentration of soluble substrates while the majority lay in the membrane fraction. This response to soluble substrates indicates a similarity with the epidermal cell model. As described, Obinata and Endo (1977) demonstrated the covalent fixation of soluble precursor proteins into large, insoluble aggregates. This phenomenon has been rationalised by Rice and Green (1979) who have identified a protein envelope beneath the plasma membrane which contains $\varepsilon(\gamma$-glutamyl) lysine crosslinks. This envelope is formed preceding cell death and is an act of terminal differentiation. Furthermore, Buxman et al (1979) report that in developing rat epidermis the transglutaminase is active only in cells approaching terminal keratinisation. Also, the erythrocyte ageing process involves transglutaminase-mediated membrane stiffening and is mainly relevant to dying cells (Seifring et al, 1978).

The conclusions from these studies would support an apparent role for cellular transglutaminase in the differentiation of growing tissue and the completion of cell cycles. The emphasis may differ between cell types and the enzyme may react more strongly in particular situations but the general role may remain consistent. The enzyme would appear to have little function during rapid proliferative growth and the major contribution of the enzyme-mediated crosslinking to cellular architecture is in a non-proliferative state.

4. SUMMARY

This investigation into the role of transglutaminase in cellular function encompassed a broad area of academic study and has related the data to specific model systems.

The purification (414-fold) of rat liver transglutaminase demonstrated the enzyme to be a calcium and sulphydryl-dependent, nonzymogenic protein with a K_m, app. for putrescine incorporation into N,N'dimethyl casein, of 5.4 x 10^{-4}M and V_{max} of 28.2 pmol putrescine $min.^{-1}$. The molecular weight was determined to be 70000 ± 5000 which impinges on the low end of the "normal" range for the tissue transglutaminases. The molecule was shown to have a hydrophobic region which was sensitive to a range of alkyl chain lengths (C_4-C_{10}) and in particular to the range C_4-C_6. The enzyme had no activity toward hydroxylamine incorporation into CBZ-glutaminylglycine. This latter result is consistent with reports of rabbit liver enzyme but differs from the guinea pig liver transglutaminase thus demonstrating the heterogeneity within these otherwise similar enzymes. This investigation provides the first literature report on purified rat liver transglutaminase.

Liver cell fractionation demonstrated 60% (approx.) of the enzyme activity in the soluble fraction with most of the remaining activity associated with the 600g particulate fraction (38%). Further investigation indicated that the latter enzyme activity was in close conjunction with the plasma membrane in terms of both locale and source of substrate. Actin was also shown to be an enzyme substrate using the tissue slice method of preserving cell function. Self-incorporation studies demonstrated the particulate fraction to be the major (87%) site of enzyme action during normal cell function, particularly the high-molecular

weight protein aggregates, though a range of substrates were observed.

The control of the enzyme was investigated by reference to translocation of activity and the effects of various agents on both the cell and the purified enzyme. The results demonstrated that the enzyme may be modulated by a variety of factors, each of which may become important as the environment of the enzyme changes.

The binding of the enzyme to proteins has been found to be both tenacious and possibly directed towards a particular site. Gard and Lazarides (1979) observed that the "Z" line proteins of myofibrils provided the enzyme with both substrate and binding sites. This mechanism would provide a local source of enzyme, as is thought to happen on the cellular scale in plasma membranes. The role of Ca^{2+} ions in this mechanism may be important where localised control of conditions could be used to elicit an enzyme response. Similarly, it might be envisaged that the cell may control the enzyme through a response to hydrophobic interactions brought about by a defined stimulus of a different kind. The metabolic activity of the cell would appear to dictate certain requirements as seen by the effects of raising and lowering the energy levels by the addition of c-AMP and anaerobiosis. Substrate proteins may also exert a controlling influence as Iwanij (1977) demonstrated that the degree of protein phosphorylation altered the reactivity of the enzyme. The finding that polyamines may be involved in protein crosslinking *in vivo* (Folk *et al*, 1980) may extend the range of enzyme substrates as bonds other than the $\varepsilon(\gamma$-glutamyl) lysine are discovered. The intervention of polyamines in crosslinking may result in either the saturation of acceptor sites or in providing aggregates of proteins with a reduced steric hindrance to enzyme action. Polyamines (in conjunction with Ca^{2+}) have been shown to exert a marked influence over

the distribution of the enzyme which may extend throughout the cell, rather than be confined to a localised phenomenon. The polyamines produce many responses in the cell, mainly concerned with growth and division, and, with related factors previously described, would appear to affect transglutaminase activity. Thus whilst Ca^{2+} remains the major factor the release of reserves with respect to particulate-binding may also play an important role in enzyme control.

The enzyme activation experiments demonstrate the extra activity available to the cell given the correct stimulus. The "activating" agents DMSO and KSCN also produced a marked solubilisation of the enzyme suggesting that it is the soluble enzyme which provides the major source of catalytic function. However, previously described studies have shown that the particulate fraction is the major site of enzyme action. This situation is not clearly understood but is consistent with soluble proteins being covalently incorporated to the insoluble matrix where further enzyme may provide both a source of localised activity and act as a reserve since protein synthesis is not required for full expression of enzyme activity. There is also the contrasting view in which bound enzyme may cycle through successive reactions with different amine incorporation sites without being released. This situation would be tantamount to an increased local concentration of acceptor sites in the protein substrate (Cohen et al, 1979).

The most striking enzyme activation resulted from treatment with trypsin. The possible explanations have been discussed and conclude that a beneficial conformational change may have been produced. Thrombin was unable to produce any similar response despite its own proteolytic activity. Birchbickler and Patterson (1978) reported the stimulation of cultured cell transglutaminase by trypsin in the culture medium and suggest a

membrane-level response transmitted to the enzyme. This effect, which is an intra-cellular response to an extra-cellular stimulus may be more widespread and constitutes a further possible factor in enzyme function and control. Hydrocortisone has been shown to induce epidermal transglutaminase in chick embryonic skin (Obinato and Endo, 1977) and interestingly, dexamethasone, another glucocorticoid, has been shown to promote the accumulation of fibronectin and collagen at the cell surface of transformed human cells (Furcht et al, 1979). One proposed mechanism for the latter observation was that polymerisation and adherance of the substrates had been affected. The recent studies of Davies et al (1980) and Maxfeld et al (1979), using internalisation of receptor complexes, led to the conclusion that transglutaminase activity may profoundly modify the way in which cells respond to hormones. These results extend the sphere of influence of the enzyme from the internal construction of cross-links within membranes and the cytoskeleton to a response to the status of the whole tissue. Therefore the control of the enzyme in the performance of its partially-characterised functions may be subject to many, as yet, unidentified factors but the basis for the cellular response is now much more clearly defined.

The study of model systems provided information about the enzyme during periods of physiological imbalance in various cell types. The conclusions from investigations of foetal, hepatomegalic and regenerative liver indicate that the enzyme is repressed during rapid proliferative growth. Restoration to full activity occurred during differentiation and, more particularly,maturation of the cell. The control of enzyme activity by increased binding to the particulate fraction was followed by release into the soluble fraction where the substrate proteins were present. The function of the enzyme was again consistent with the

covalent immobilisation of soluble substrates into the particulate matrix.

The effect on the enzyme of the activation of platelets was investigated and the result may aid in the understanding of a particular facet of the release reaction. Platelet activation resulted in a rapid translocation of the entirely soluble tissue transglutaminase activity to the particulate fraction. This may explain why the intracellular enzyme remains while the platelet is releasing many cytoplasmic constituents into the external environment. Among the agonists tested thrombin was the exception, in that, this proteolytic enzyme caused the non-zymogenic platelet transglutaminase to greatly increase in activity.

The investigation of various rat sarcomata demonstrated that these neoplastic tissues had a very much lower transglutaminase activity than normal tissues. Further more, upon metastasis the enzyme activity was observed to fall to trace levels. The results indicate that tissue dissemination is enhanced by extremely low transglutaminase activity but that the enzyme is required for tumour investment. These preliminary results require further investigation but may go toward elucidating the mechanism of metastasis, particularly with respect to matrix proteins and the possible contributory role played by transglutaminase in cell adhesion.

Among the questions still to be answered about the transglutaminases, perhaps the role played by polyamines is the most urgent. These compounds, recently identified as in vivo substrates for the enzyme (Folk et al, 1980) may extend the limits of transglutaminase activity set by the, up to now, only known catalytic product, the $\varepsilon(\gamma$-glutamyl) lysine cross-link. While extending the capability of the enzyme to stabilise proteins they may also perform the controlling function of limiting glutamyl acceptor sites. The resolution of these poorly understood interactions

will require an emphasis on the identification of an even wider range of possible substrates and their products.

The other major areas of interest involving the cellular enzyme function will probably lie in the interactions with the cytoskeleton and matrix proteins. If the present restricted knowledge of the interactions of actin, fibronectin and collagen in particular are extended to the in vivo situation it could have an important bearing on many pathological and other conditions. These conditions might include atherosclerosis, neoplasia, wound healing, embryogenesis, thrombosis and many others.

The bulk of the cellular transglutaminase activity is apparently directed toward the plasma membrane and its environs. Further work in this area may confirm transglutaminase involvement in transmembrane associations with the cytoskeleton (Singer, 1979), cell communication (Lloyd, 1976), immunological reactions and the reticuloendothelial system (Wajda, 1969) and endocytosis (Davies et al, 1980).

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APPENDIX

Gel Electrophoresis Procedures

(A) Conventional Polyacrylamide gel electrophoresis (PAGE)

(i) Tris-Glycine (pH 8.9)

The method followed was based on Ornstein and Davies (1964) with modifications.

As described the procedure yields 12 separating gels (6.7cm) that are 7.5% acrylamide and 0.2% methylenebisacrylamide, and 12 stacking gels (1.7cm) that are 2.5% acrylamide and 0.2% methylene bisacrylamide.

Solutions required:

Solutions	Component/100 ml aq. solution
Monomer solution I	30g acrylamide
	0.8g N,N'-methylenebisacrylamide
Monomer solution II	10g acrylamide
	0.8g N,N'-methylenebisacrylamide
Separating gel buffer	24 ml 1MHCl; 18.15g Tris
Stacking gel buffer	12.8 ml H_3PO_4; 2.848g Tris
Ammonium persulphate I	280mg
Ammonium persulphate II	80mg
Riboflavin	2mg
Lower tank buffer	0.374g glycine; 0.628g Tris
Upper tank buffer	5 ml 1MHCl; 0.757g Tris

Separating Gel:

6 ml monomer solution I

6 ml distilled water

6 ml separating gel buffer

6 ml ammonium sulphate solution I

Mix well and degas. Add 8µl TEMED, mix and pour gels (2ml). Layer with 1:3 aqueous dilution of stacking gel buffer and allow to polymerise for 30 min.

Stacking gel:

2 ml monomer solution II

2 ml stacking gel buffer

2 ml riboflavin solution

2 ml ammonium persulphate solution II

Mix and degas in dark; add 3µl TEMED; mix and pour gel (0.5ml); layer with upper tank buffer; photo polymerise for 30 min.

Sample should be adjusted to 5% (v/v) glycerol and 0.005% (w/v) bromophenol blue and added in 5-60µl aliquots to the gel surface once the upper and lower tank buffers are in place.

Gels are run at 6mA/gel until the tracking dye enters the separating gel and then 3mA/gel until the dye is within 1cm of the end of the gel. Running time is approximately 90 min.

Staining is accomplished in 0.25% (w/v) Coomassie brilliant blue R-250 in a 5:5:1 mixture of methanol, distilled water and glacial acetic acid respectively, for 60 min at 60°. Destaining used a 3:1:7 formulation of ethanol:acetic acid:distilled water.

The protein positions were then recorded and the gels scanned using a SP1809 scanning attachment to a Pye Unicam SP1800 spectrophotometer. Molecular weights were determined using a standard graph prepared from the relative mobilities of known proteins.

(ii) TES-TEA (pH 7-8)

This method is very similar to the Tris-glycine method described above, except that the buffers have been changed and the whole procedure is carried out at 4°. At this reduced temperature longer time

intervals are required for polymerisation (60 min with twice the TEMED included). The samples are then electrophoresed at 2mA/gel through the stacking gel and 5mA/gel through the separating gel, the running time for this system being approximately 2 hr.

Solutions required:

Solutions	Components/100ml aqueous solution
Separating gel buffer	2.4 ml 1MHCl;
	0.5 ml 20mM EDTA
	5.6 ml H_2O
	Adjust to pH 6.8 with TEA (4°) and make up to 100 ml.
Stacking gel buffer	2.4 ml 1MHCl
	0.5 ml 20mM EDTA
	5.1 ml H_2O
	Adjust to pH 5.8 with TEA (4°) and make up to 100 ml.
Upper tank buffer	0.879g TES
	0.037g EDTA
	80 ml H_2O
	Adjust to pH 6.3 with TEA (4°) and make up to 100 ml.
Lower tank buffer	0.33g 88% (v/v) H_3PO_4
	80 ml H_2O
	Adjust to pH 5.8 with TEA (4°)
	Add 0.037g EDTA
	Re-adjust to pH 5.8 with TEA (4°) and make up to 100 ml.

Equipment used consisted of a Shandon Ltd. electrophoresis unit incorporating a cooling jacket and Bio-Rad Laboratories glass precision tubing (11cm long, internal diameter 5.5mm). All chemicals used of electrophoresis grade, or the highest grade available (Sigma Chemical Co.) Abbreviations used include TES (N-Nis hydroxymethyl methyl-2-aminoethane sulphonic acid), TEA (triethanolamine), TEMED (N,N,N',N'-tetramethylethylenediamine), bromophenol blue (3,3',5,5'-tetra-bromophenolsulphonphthalein) and Coomassie brilliant blue (trisodium 4'-anilino-8-hydroxyl-1,1'-azonaphthalene-3,6,5'-trisulphonate).

(B) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The method described was used for horizontal flat-bed thin-layer electrophoresis under denaturing conditions, and are those prescribed by the manufacturer (LKB-Producter AB, Bromma, Sweden) for the LKB 2117 Multiphor.

Solutions used:

(i) Phosphate buffer; stock solution, 0.2M, pH 7.1, including 0.2% (w/v) sodium dodecyl sulphate (SDS).

(ii) Electrode buffer; 1 part stock buffer plus 1 part distilled water.

(iii) Phosphate sample buffer (0.01M, pH 7.5); 5 ml stock phosphate solution, 1 ml 2-mercaptoethanol, 1g SDS. Made up to 100 ml with distilled water.

(iv) Acrylamide solution; 22.2g acrylamide, 0.6g methylenebisacrylamide Made up to 100ml with distilled water, and stored in dark at 4°. Extreme care was taken throughout the handling of the toxic unpolymerised acrylamide.

(v) Ammonium persulphate solution; 150mg in 10 ml distilled water, prepared fresh.

(vi) Bromophenol blue solution (0.25% (w/v)); 25mg Bromophenol blue in 10 ml sample buffer.

(vii) Fixative solution; 57g Trichloroacetic acid (TCA)
17g Sulphosalicylic acid
150 ml Methanol
350 ml Distilled water

(viii) Staining solution; 1.25g Coomassie brilliant blue R-250
227 ml Methanol
227 ml Distilled water
46 ml glacial acetic acid
The solution was filtered (Whatman No. 1) and stored in the dark.

(ix) Destaining solution; 1500 ml Ethanol
500 ml Acetic acid
3000 ml Distilled water

The gel forming cassette had a nominal volume of 66 ml and was, in the case of a 7.5% acrylamide gel, formed from the following mixture of thoroughly mixed and deaerated solutions: 33 ml phosphate stock solution; 22.2 ml acrylamide; 3.2 ml ammonium persulphate; 100µl TEMED; and 7.5 ml distilled water. The gel was allowed to polymerise for approximately 60 min and stored overnight before use. Samples were normally dissolved in sample buffer but in some cases it was found to be advantageous to dissolve the protein using 3% (w/v) SDS: 3% (v/v) 2-mercaptoethanol: 9M urea in 5% (v/v) phsophate stock solution made up to 100 ml with distilled water. The final concentration of protein in the sample to be applied was in the range 0.2-2 mg/ml. Samples were

always heated for 3-10 minutes in a 100° water bath to ensure full solubilisation prior to sample application. Also 4% (v/v) of bromophenol blue dye and 2-mercaptoethanol were mixed with the sample prior to application.

The apparatus was assembled to include a cooling plate and the wicks were laid parallel to, and 1.5cm from, the edge of the gel. Pre-electrophoresis was performed using a constant current of 150mA for approximately 30 min. The samples were then applied to the pre-formed wells and electrophoresis was carried out at 20mA for the first 10 min and then this was increased to 190-200mA for the remainder of the run. The correct field strength was monitored at intervals using a LKB voltage probe and a D.C. voltage meter (5-6 Vcm^{-1}).

When the tracking dye had approached within 1cm of the wick (4-5 hr) the gel was removed and placed in fixative solution for 60 min. Staining for at least 120 min was followed frequent changes of destain until the background colour of the gel showed no residual stain.

Determination of molecular weights was achieved by the construction of a standard graph using SDS-molecular weight markers (Sigma Chemical Co.).

(C) Isoelectric focussing

The following preparation provided 8 5.5% (w/v) acrylamide gels each approximately 65mm in length using glass tubes of 5.5mm internal diameter (Bio-Rad Laboratories).

Solutions required:

Solutions	Component/100ml aq. solution
Acrylamide solution	30g acrylamide
	1g N,N'methylenebisacrylamide

Solutions (Cont.)	Component/100ml aq. solution (Cont.)
Ampholine (pH 3-10)	40% (w/v) solution as provided by manufacturer (LKB-Produkter AB).
Ammonium persulphate solution	1% (w/v) solution; prepared fresh.
Anode buffer	0.2% (v/v) H_2SO_4
Cathode buffer	0.4% (v/v) ethanolamine

Gels were prepared by mixing 0.6ml sample protein (approximately 1mg/ml) with 1.5ml acrylamide solution, 0.15ml ampholine, 0.3 ml ammonium persulphate solution and 3.6 ml distilled water. This preparation was thoroughly mixed and degassed prior to pouring the gels. Polymerisation was complete within 40 min.

Electrolysis was carried out with the anode and cathode buffers detailed above. A current of 2mA/gel was used for 20 min to establish the pH gradient and a further 180 min to allow the proteins to be focussed. The gels were then removed and treated in one of two ways.

(i) Gels were fixed, stained in Coomassie brilliant blue G-250 staining solution, destained and recorded using gel scanning equipment. This procedure was identical to that given for the treatment of conventional polyacrylamide gels.

(ii) Gels were cut into 5mm lengths and each was placed in 2 ml distilled water. These gel slices were left for approximately 18 hrs at which time the pH of the aqueous medium was measured. A standard graph was constructed in order that the pH at any point on the gel could be determined, and the isoelectric points (pI) of sample proteins established.

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