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THE IMPORTANCE OF TRANSGLUTAMINASE IN TUMOUR GROWTH AND METASTASIS

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DOCTOR OF PHILOSOPHY

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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 .....

(Candidate)

Signed .....

(Director of Studies)

DEDICATION

To my parents, my brother Paul and Neen.

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David Hand
'The importance of transglutaminase in tumour growth and metastasis'

ABSTRACT

Measurement of transglutaminase activity in chemically-induced hepatocellular carcinomas showed that it was significantly reduced. Subcellular fractionation studies revealed that this reduction occurred primarily in the cytosol fraction of the cell. Leaching studies with the particulate fraction of the cell indicated that the activity tightly associated with this fraction was not reduced in tumours. This was confirmed by separation of a cytosolic and a particulate form of transglutaminase activity using anion-exchange chromatography, which indicated that there was a specific reduction in the cytosolic enzyme in the tumours whilst the particulate enzyme was unaffected.

Comparison of the cytosolic transglutaminase existing freely in the cytosol with that loosely attached to the membrane fraction indicated that the association of this enzyme with the membrane fraction was not accompanied by any changes in molecular weight, Km for putrescine incorporation into N,N'-dimethylcasein and sensitivity to activation by Ca^{2+}. Similarly, the cytosolic enzyme from hepatocellular carcinomas was found to be identical to that from normal liver when the same characteristics were compared. This indicated that the reduction in cytosolic transglutaminase was most likely to be due to reduced expression of this enzyme by the tumour cells.

Normal liver and hepatocellular carcinomas were found to be equally capable of incorporating [^{14}C]-methylamine into tissue slice proteins in a Ca^{2+}-dependent manner. This was attributed to the action of the particulate enzyme since the radiolabel was mostly located in a plasma membrane rich fraction. SDS-polyacrylamide gel electrophoresis revealed that most of the radiolabel was located in a high molecular weight proteinaceous material ($Mr > 10^6$) and that the amount of incorporation into this material was equivalent in normal liver and tumours. Incorporation into smaller proteins was reduced in the tumour tissue and was attributable to the action of the cytosolic transglutaminase. This incorporation occurred mainly into proteins of Mr 43,600 and Mr 38,900. In the tumours a protein of Mr 35,900 was also found to be labelled.

Measurement of transglutaminase activity and polyamine levels in pre- and post- metastatic sarcoma tissue revealed that there was a 4-5 fold reduction in transglutaminase activity at the onset of metastases which was paralleled by a large increase in free putrescine levels (20 fold) during this same time period. A change in the levels of covalently-bound polyamine in pre- and post-metastatic P tissue was found to correlate with the changes seen in transglutaminase activity. These results indicate that transglutaminase may play an important role in mediating events associated with the metastatic process and that alterations in the levels of free and bound polyamines may also be of importance during this process.

PUBLICATIONS AND PRESENTATIONS

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Hand, D., Elliott, B.M. & Griffin, M. Polyamine levels in chemically induced rat liver tumours (1986) Biochem. Soc. Trans. 14 695-696

Hand, D., Elliott, B.M. & Griffin, M. Transglutaminase activity and its relation to the polyamine content of neoplastic tissue during the metastatic process (1987a) Noble Conf. Cell. Mol. Biol.; Transglutaminase & Protein Crosslinking Reactions, PB 12

Hand, D., Elliott, B.M. & Griffin, M. Correlation of changes in transglutaminase activity and polyamine content of neoplastic tissue during the metastatic process (1987b) Biochim. Biophys. Acta 930, 432-437

Abbreviations used in this thesis:

5'-AMP	- 5'-adenosine monophosphate
6-BT	- 6-p-dimethylaminophenylazobenzothiazole
c-DNA	- complementary DNA
DEN	- diethylnitrosamine
DMSO	- dimethylsulphoxide
DNA	- deoxyribonucleic acid
DNase 1	- deoxyribonuclease 1
DTT	- dithiothreitol
EDTA	- ethylenediaminetetraacetic acid
EGTA	- ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HPLC	- high performance liquid chromatography
NFT	- neurofibrillary tangles
OPA	- o-phthalaldehyde
PHF	- paired helical filaments
PMSF	- phenylmethylsulphonylfluoride
RCF	- relative centrifugal force
RNA	- ribonucleic acid
RNase 1	- ribonuclease 1
SDS	- sodium dodecyl sulphate
SE	- standard error of the mean
TCA	- trichloroacetic acid
TEMED	- N,N,N',N'- tetramethylethylenediamine
Tris	- Tris(hydroxymethyl)-aminomethane

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1.0 INTRODUCTION

1.1 INTRODUCTION TO THE TRANSGLUTAMINASES

1.1.1 Definition of Transglutaminases

Transglutaminases were first described thirty years ago by Sarkar et al. (1957). These workers had identified a calcium dependent transamidating activity in guinea pig liver which could be measured by the covalent incorporation of aliphatic amines (such as cadaverine or ethanolamine) into proteins. Since then a large amount of work has been published on these enzymes. They are also the subject of a number of reviews (Chung, 1972, 1975; Folk & Chung, 1973; Lorand & Stenberg, 1976; Folk & Finlayson, 1977; Folk, 1980; Williams-Ashman & Canellakis, 1980; Folk, 1983; Lorand & Conrad, 1984; Conrad, 1985).

Today, transglutaminases are defined as calcium dependent enzymes with an active site thiol which catalyse an acyl transfer reaction between peptide-bound glutamine residues and primary amine groups. The result of this reaction is the post-translational modification of proteins either by the specific incorporation of amines (such as putrescine) or, if the amine is the ϵ-amino group of protein bound lysine, the crosslinking of proteins via $\epsilon(\gamma$-glutamyl)lysine bridges. Clearly, the name transglutaminase is a misnomer since it is peptide bound glutamine which participates in the reaction and not free glutamine. The enzyme commission therefore recommends the systematic name R-glutaminyl-peptide:amine-γ-glutamyl transferase (E.C. 2.3.2.13).

1.1.2 Characterisation of Transglutaminases

Transglutaminases occur in a wide variety of tissues and are found both intracellularly and extracellularly (Folk, 1980; Conrad, 1985). Indeed it is generally thought that the occurrence of

transglutaminases will ultimately prove to be ubiquitous in animal cells and tissues. These enzymes are distinguished according to their localisation and their physical, chemical, immunochemical and catalytic properties (Chung, 1975). The following families of transglutaminase have so far been identified:

1) Factor XIII

This enzyme is best known for its involvement in the blood-clotting cascade. So far Factor XIII has been found in plasma (Lorand et al., 1968), placenta (Bohn & Schwick, 1971), platelets (Schwartz et al., 1973) as well as granulocytes, monocytes and macrophages (Berntorp et al., 1985).

2) Hair Follicle Transglutaminase

This enzyme is found in mammalian hair follicles (Harding & Rogers, 1972; Chung & Folk, 1972) and is involved in the crosslinking of hair fibre proteins. It is immunologically distinct from all the other forms of transglutaminase described here.

3) Epidermal (Type I) Transglutaminase

A transglutaminase has been identified in the epidermis of many mammals where it is thought to play a critical role in cornified envelope formation during the terminal differentiation of keratinocytes (Rothnagel & Rogers, 1984). This membrane located enzyme crossreacts immunologically with a similar enzyme found in rabbit tracheal epithelium (Jetten & Shirley, 1986) suggesting that it may not be restricted to epidermal tissues alone.

4) Tissue (Type II) Transglutaminase

This type of transglutaminase is found in the cytosol of many tissues, including: liver, muscle, kidney, lung, adrenal gland, brain, testis, pancreas, erythrocytes and the uterus (Chung, 1972;

Lorand and Stenberg, 1976; Conrad, 1985). It has also been reported that this enzyme coexists in epidermal tissue (Lichti et al., 1985) and hair follicles (Chung & Folk, 1972b) along with the type I enzyme and hair follicle enzyme respectively.

Nevertheless the classification of many types of transglutaminase remains unclear. For instance there is considerable controversy as to the nature of the transglutaminases found in the prostate gland of rodents (see section 1.2.4). Also of particular interest is the recent discovery that there is a particulate transglutaminase in rat liver (Chang & Chung, 1986) and lung (Cocuzzi & Chung, 1986) as well as the Type II liver transglutaminase. This novel enzyme has been shown to be immunologically distinct from Factor XIII and the tissue (Type II) liver enzyme although it was not tested for crossreactivity with the epidermal (Type I) family. Since both particulate and soluble forms of transglutaminase have been reported in several tissues (Liver, lung, spleen, epidermis, hair follicles and brain) it is tempting to suggest that both epidermal (Type I) and tissue (Type II) transglutaminases are constitutive in most if not all mammalian cells.

Recent work has revealed the presence of calmodulin dependent transglutaminases in human platelets and red blood cells and in chicken gizzard (Puszkin & Raghuraman, 1985; Puszkin & Billet, 1987). These enzymes are of great interest since not only do they afford an alternate mechanism for regulating polyamine incorporation and $\varepsilon(\gamma$-glutamyl)lysine formation in cells, it has also been shown that they are associated with cytoskeletal components (see section 1.3.3).

1.1.3 Reactions Catalysed by Transglutaminases

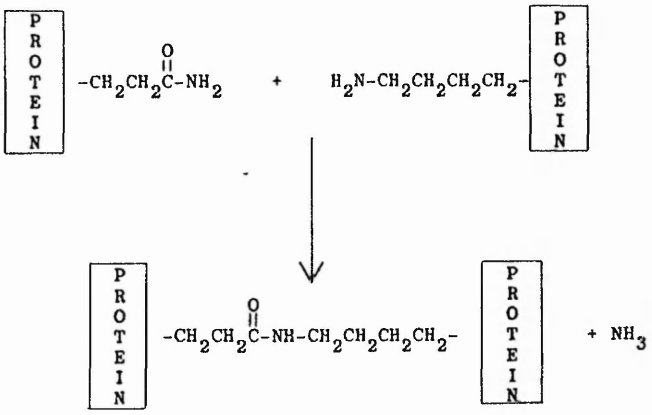
Transglutaminases can catalyse a wide variety of aminolytic and hydrolytic reactions. However only three of these reactions have been shown to occur *in vivo*. These are the crosslinking of proteins via ε(γ-glutamyl)lysine bridges, the incorporation of primary amines (such as putrescine) into proteins and the formation of N,N bis-γ-glutamyl polyamine derivatives, a reaction which also results in the crosslinking of proteins (Figure 1.1). The other reactions catalysed by transglutaminase involve the hydrolysis of an amide or ester (Table 1.1). However, there is no evidence to suggest that transglutaminase catalyses aminolytic or hydrolytic reactions *in vivo*.

The incorporation of primary amines into proteins (Figure 1.1b) forms the basis for the most widely used assay for all types of transglutaminase since it is one of the most sensitive assays available. This assay involves the incorporation of radiolabelled primary amines (such as putrescine, methylamine or histamine) into protein substrates which can then be estimated following precipitation of the protein in TCA (Lorand *et al.*, 1972). In recent years modification of the protein substrates by blocking the ε-amino groups of lysine (by succinylation, methylation or acetylation) has been preferred because this prevents crosslinking of the protein substrates (usually β-casein).

A less sensitive assay based on the production of hydroxamate from hydroxylamine and benzyloxycarbonyl-L-glutaminylglycine is also sometimes used. This reaction is equivalent to that depicted in reaction 3 in Table 1.1.

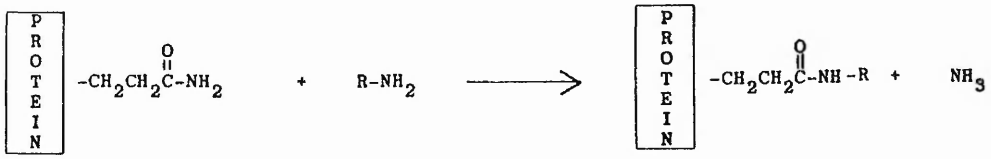
Figure 1.1 Reactions Known to be Catalysed by Transglutaminase In Vivo

A. Formation of ε(γ-glutamyl)lysine bonds : the crosslinking of proteins :

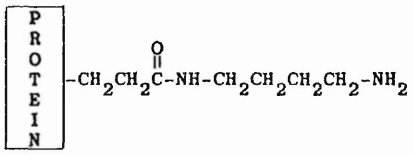


ε(γ-glutamyl)lysine crosslink

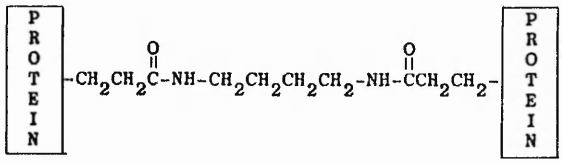
B. Incorporation of primary amines into proteins :

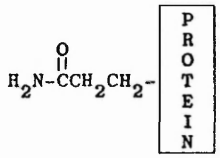


C. Formation of diamine crosslinks through the incorporation of polyamines (* reaction product from B with putrescine as the primary amine) :



N-(γ-glutamyl)putrescine

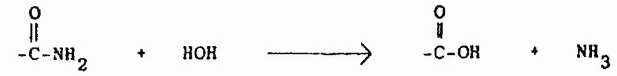




N,N-bis-(γ-glutamyl)putrescine crosslink

Table 1.1 Hydrolysis and Aminolysis Reactions catalysed By Transglutaminase

1. Hydrolysis of peptide-bound glutamine.

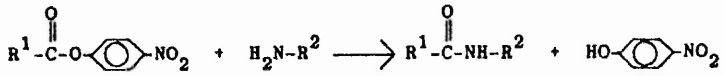


2. Hydrolysis of amides.

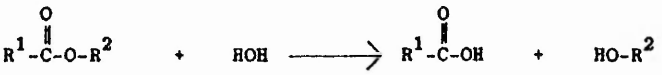
3. Aminolysis of amides.

4. Hydrolysis of p-nitrophenol esters.

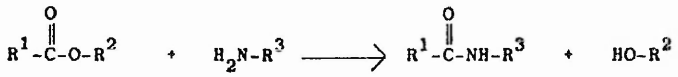
5. Aminolysis of p-nitrophenol esters.



6. Hydrolysis of aliphatic esters.



7. Aminolysis of aliphatic esters.



1.1.4 The Mechanism of Catalysis

The catalytic mechanism of transglutaminase was elucidated following isolation of a stable enzyme-substrate complex. It was found that the cysteine residue which was essential for catalysis formed a thioester intermediate with the peptide-bound γ-glutaminyl residue which could then undergo lysis by a nucleophile such as water or a primary amine. The kinetics of the reactions shown in Figure 1.1 and Table 1.1 were examined and found to be consistent with a modified double- displacement mechanism of this type (Figure 1.2). The full evidence for this is reviewed comprehensively by Folk (1983). Figure 1.2 shows the reaction mechanism as it would apply to reaction b in Figure 1.1, whereby peptide-bound glutamine first binds to the enzyme and forms an acyl-enzyme intermediate with the release of ammonia. This acyl-enzyme intermediate can then undergo nucleophilic attack either by water, forming peptide bound glutamic acid or by a primary amine such as putrescine, forming peptide-bound γ-glutamyl-putrescine.

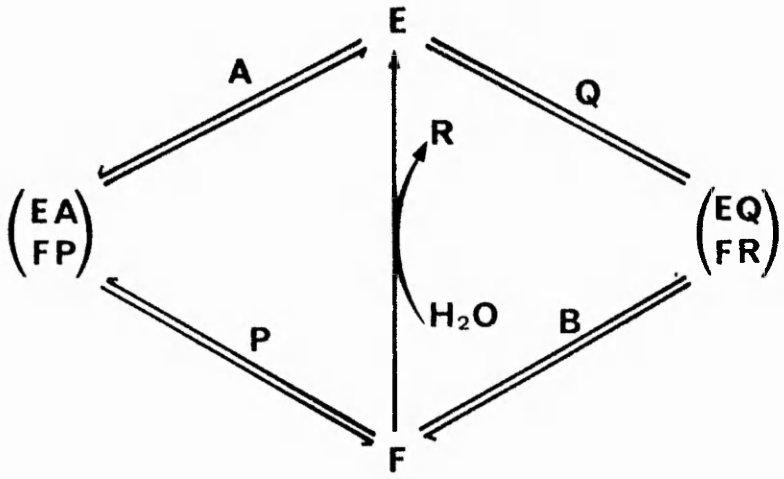
This reaction is dependent on calcium ions, although other divalent cations such as strontium or manganese can activate the enzyme to a lesser extent (Folk et al., 1967). Recently the sequencing of Factor XIII (Putnam et al., 1987; Ichinose & Davie, 1987) and the partial sequencing of a tissue (type II) transglutaminase (Stein et al., 1987) by c-DNA cloning techniques have shown that these enzymes have partial sequence homology with other calcium binding proteins such as calmodulin. Both of these transglutaminases have two such possible calcium binding domains although it is thought that only one of these will prove to be involved in enzyme activation. Furthermore, the activation of transglutaminases by calcium is thought to bring about small conformational changes in enzyme structure which expose the active site (Folk, 1983).

In the identification of transglutaminases two main criteria are used:

1) Calcium dependence as defined by the inhibition of enzyme activity by metal-chelating agents such as EDTA or EGTA.

2) Thiol dependence as defined by the inhibition of enzyme activity by thiol-reactive compounds such as iodoacetamide, p-chloromercurobenzoic acid and 5,5'-dithiobis(2-nitrobenzoic acid) (Folk & Cole, 1966; Connelan & Folk, 1969).

Figure 1.2 The Mechanism of Action of Transglutaminase



Key : Reaction 1.1b from Table 1.1 as an example

E = free enzyme

F = acyl-enzyme intermediate

A= peptide-bound glutamine

P = ammonia

R = peptide bound glutamic acid

B = primary amine

Q = peptide bound γ-glutamyl-amine

1.1.5 Energetic Considerations

In the reactions shown in Figure 1.1 there would be very little change in free energy ($\Delta G^{\circ} \sim 0$) since in all cases there is a γ-amide bond created for every γ-amide bond that is broken. These reactions should therefore be considered reversible although the production of NH_3 *in vivo* would promote the reaction to the right since this would be immediately protonated to form NH_4^+ (Lorand and Conrad, 1984; Conrad, 1985).

In actuality the demonstration of reversibility of protein crosslinking has proven difficult because clotting occurs and so far has only been reported for the Factor XIIIa catalysed crosslinking of $alpha_2$-plasmin inhibitor and fibrin (Ichinose & Aoki, 1982). However the reversibility of amine incorporation can more easily be demonstrated. Campbell-Wilkes (1973) was able to show the release of [^{14}C]-histamine from β-lactoglobulin following the covalent incorporation of this amine into β-lactoglobulin.

1.1.6 Substrate Specificity

So far substrate specificity studies have mainly been confined to the guinea-pig liver transglutaminase and Factor XIII from plasma. Whilst these two enzymes cannot be considered as representative of all types of transglutaminase, studies on their substrate specificity afford some valuable insights as to how these enzymes behave in biological systems. A comprehensive review of this work is given by Folk (1983), where substrate specificity is reviewed with respect to the following:

1) The Amide Site
2) The Extended Glutamine Binding Site
3) The Amine Site

1.1.6.1 The Amide Site

Studies by Chung et al.,(1970), Gross & Folk,(1973) and Chung, (1975) were undertaken in order to investigate the requirements for amides to act as transglutaminase substrates. The early work by Waelsch and co-workers (1962) where they determined that transglutaminase acts solely on glutamine residues and not asparagine residues was confirmed. Furthermore it was shown that transglutaminases exhibit stereospecificity for L-glutamine residues. Work with synthetic substrates revealed that only compounds blocked in the α-carbon NH_2 and COOH groups would act as substrates and that the enzyme has a preference for substrates with uncharged groups at or near to the glutamine residue (Folk & Cole, 1965, 1966).

1.1.6.2 The Extended Glutamine Binding Site

Substantial differences in the substrate specificity of Factor XIII and liver transglutaminase were recognised early on (Chung, et al., 1970). For instance the glutamine peptide derivative benzyloxycarbonyl-L-glutaminylglycine was found to act as a substrate for the liver enzyme but not for Factor XIII. Also, the patterns of fibrin crosslinking by the two enzymes were found to be different (Chung & Folk, 1972a). In this regard it was found that Factor XIII exhibits a far greater specificity than the liver enzyme.

Gross et al., (1975) examined the effect of varying the amino-acid sequence around the glutamine residue in synthetic substrates and found that the enzyme interacted with its substrates over a sequence of at least five amino-acid residues. Work with peptide fragments of β-casein revealed that varying the amino-acid composition of the fragments greatly affected the specificity of a substrate for Factor XIII and to a lesser extent for the liver enzyme

(Gorman & Folk, 1980, 1981). These interactions appeared to be significant for peptide fragments of up to 9 or 10 amino-acid residues and supported the idea that uncharged or hydrophobic amino-acids are important in substrate specificity.

1.1.6.3 The Amine Site

Once an acyl-enzyme intermediate has been formed the thioester linkage may undergo lysis by either water or a primary amine. The structure of primary amines was studied with regard to their specificity as transglutaminase substrates. It was found that for aminolysis to occur the amine must be in the uncharged form (Folk & Cole, 1966). Furthermore it should be noted that the nature of the acyl-enzyme intermediate may influence its specificity for amines (Chung et al., 1970).

A wide variety of primary amines were synthesised and tested for their ability to compete with [^{14}C]-putrescine for incorporation into N,N'dimethylcasein (Lorand et al.,1979a). It was found that the best substrates consisted of a large hydrophobic moiety attached to a 5 carbon alkylamine side chain. Similarly Gross et al.,(1975) showed that the enzyme expressed a preference for unbranched aliphatic amines unless the branch occurred at a distance of at least 5 carbons from the amine group. This close similarity with the side chain of lysine led to a series of studies with α-ω-diaminomonocarboxylic acid peptide derivatives which revealed the lysine derivatives to be the most effective substrates. In addition it was found that the enzyme showed some stereospecificity for L-lysine derivatives with a lesser ability to utilise D-lysine derivatives. Thus it can be concluded that these transglutaminases express a preference for the ε-amino group of L-lysine containing peptides as a second substrate.

Finally studies which examined the effect of altering amino-acid residues in synthetic lysine containing peptides revealed that only those amino-acids adjacent to the lysine residue appeared to affect the interaction with acyl-enzyme intermediates (Gross et al., 1977). In particular a single L-leucine residue on the amino side of the lysine residue enhanced the specificity of these synthetic peptides, thus providing further evidence for a hydrophobic domain on the transglutaminase molecule (Schrode & Folk, 1979).

1.1.6.4 Conclusion

The studies with guinea-pig liver transglutaminase and Factor XIII which have been discussed briefly show that these enzymes share a common specificity for the amide and amine sites but differ in their specificity for the extended glutamine site. The preference of these enzymes for peptide bound glutamine and lysine suggests that ε(γ-glutamyl)lysine formation is likely to be the main reaction in vivo. Indeed this is the case for Factor XIII and the transglutaminases from epidermal tissue and hair follicles (sections 1.2.1-3). However in the clotting of seminal plasma by a prostatic transglutaminase (see section 1.2.4) the incorporation of polyamines may also be involved (Williams-Ashman, 1984). The function of the tissue transglutaminase on the other hand remains an enigma, although ε(γ-glutamyl)lysine, γ-glutamyl-polyamines and polyamine crosslinks have been identified in tissues containing this enzyme (Birckbichler et al., 1973; Beninati et al., 1985). These findings would therefore appear to be in accordance with the wider specificity of this enzyme for γ-glutamine containing peptides and further, different acyl-enzyme intermediates can express different amine specificities once they have been formed (Chung et al., 1970; Folk, 1983).

1.2 KNOWN BIOLOGICAL ROLES OF TRANSGLUTAMINASES

The biological role of some transglutaminases has already been wholly or partially elucidated. It is these transglutaminases which will be described in this section. Following sections will deal with the putative roles of the tissue transglutaminase in cellular events.

1.2.1 Factor XIII

It has been known since 1944 that there is a factor present in blood which is responsible for the stabilisation of the fibrin clot during hemostasis (Robbins, 1944). However it was not until 1968 that this factor - 'Factor XIII' was proven to be a member of the transglutaminase family of enzymes (Matacic & Loewy, 1968).

The Factor XIII zymogen consists of two types of subunits (Siefring et al, 1978) of molecular weight 80,000 with a structure a_2b_2 (Mr 320,000). Activation of this zymogen is achieved by specific cleavage of a 4000 dalton activation peptide from the a subunits (Takagi & Doolittle, 1974), a reaction which is catalysed by the calcium dependent protease, thrombin. The modified zymogen a'_2b_2 can then be separated into a and b subunits by calcium (Lorand, 1974). This calcium dependent separation is enhanced by fibrinogen which interacts with the a'_2b_2 molecule and lowers the calcium requirement to levels found in the blood (Credo et al., 1978). Furthermore, fibrinogen also enhances the rate of cleavage of the activation peptide by thrombin (Janus et al., 1983). This is an important regulatory mechanism in vivo since Factor XIII can only be activated in the presence of its correct substrate (Greenberg & Schuman, 1982).

Fibrinogen is comprised of duplicate copies of three different polypeptide chains linked together by disulphide bridges; α,

β and & γ of molecular weights 70,000, 60,000 and 50,000 respectively (Mossesson & Finlayson, 1976). Fibrinogen undergoes a thrombin dependent cleavage which removes two polypeptides from the amino terminus of the fibrinogen polypeptides. The resulting structure (αβγ')2 then forms a gel which may be acted upon by Factor XIIIa. The initial crosslinking of the γ subunits occurs rapidly to form a hemostatic plug which is then stabilised by crosslinking the α subunits to form a stable clot (Finlayson & Aronson, 1974). The β subunits do not appear to play any part in the crosslinking process.

Factor XIII is also found in placenta, platelets, monocytes, macrophages and granulocytes (Conrad, 1985; Berntorp et al., 1985). However in these cells there is no evidence for the existence of the β subunit. Furthermore the granulocyte Factor XIII does not display thrombin dependence (Berntorp et al., 1985). The role of these sources of Factor XIII is unclear although their release into plasma results in association with β subunits whereupon they are indistinguishable from plasma Factor XIII (Folk and Finlayson, 1977). Thus it may be suggested that these sources of Factor XIII play specialised roles in aiding wound healing under different circumstances.

1.2.2 Hair Follicle Transglutaminase

Investigations into the insolubility of hair proteins led to speculation that a transglutaminase might play a role in crosslinking these proteins. In 1962 Rogers reported a citrulline rich protein which also had high glutamine content and few cysteine residues. This protein was considerably different to the keratins which were known to be crosslinked by disulphide bonds (Rogers, 1969). This protein was shown to be covalently linked and was resistant to extremes of pH, salt concentration, chaotropic agents, organic solvents and detergents.

ε(γ-Glutamyl)lysine was discovered in digests of wool, hair and quills (Harding & Rogers, 1970; Asquith, 1970) and subsequently it was shown that it was the citrulline rich proteins which were crosslinked (Harding & Rogers, 1976).

The transglutaminase activity responsible for these crosslinks was first identified by Rogers & Springell, (1959) in wool and was subsequently shown to exist in hair follicles (Harding & Rogers, 1972). This enzyme was then isolated and purified by Chung & Folk (1972b). It was in fact found that hair follicles contained two enzymes, one which was similar to the tissue transglutaminase from guinea-pig liver and another which was unique to this type of tissue. This hair follicle transglutaminase had a molecular weight of 54,000 and was comprised of two identical subunits, each of molecular weight 27,000. It was immunologically distinct from Factor XIII, epidermal transglutaminase and tissue transglutaminase (Buxman & Wuepper, 1976). It was also found to be different from Factor XIII and tissue transglutaminase since it possessed no esterase activity (reactions 4-7, Table 1.1). Whilst these studies are incomplete, the suggestion that hair follicle transglutaminase plays a role in hair fibre formation and stability is undisputed. For a review on this hair follicle enzyme see Peterson & Wuepper (1984).

1.2.3 Epidermal Transglutaminase

The presence of a transglutaminase in hair follicles led to a search for a similar activity in the epidermis itself. Goldsmith et al., (1974) found a transglutaminase activity in epidermis and this activity has now been isolated from several sources (Buxman & Wuepper, 1975; Ogawa & Goldsmith, 1976; Peterson & Buxman, 1981) and has a molecular

weight of approximately 54,000 from all sources. This enzyme is immunologically distinct from Factor XIII, tissue transglutaminase and hair follicle transglutaminase (Ogawa & Goldsmith, 1977). It does not however display cross-species immunoreactivity. The enzyme was found to be located in the granular region of the epidermis where keratinisation occurs (Buxman & Wuepper, 1975) suggesting that it may be involved in the manufacture of the cornified envelope during terminal differentiation of keratinocytes.

Hanigan & Goldsmith, (1978) demonstrated the presence of two high molecular weight substrates for this enzyme in human and new-born rat epidermis which contained large amounts of glutamine residues. In contrast Buxman et al., (1980) found two soluble substrates in bovine snout epidermis of molecular weights 150,000 and 36,000 as well as the high molecular weight substrates. The 36,000 protein is now named keratolinin and is known to be comprised of subunits of approximate molecular weight 8000 (Buxman et al., 1980). There is also evidence to suggest that polymerisation of keratolinin forms the 150,000 dalton protein which is then further polymerised to form the cornified envelope (Rothnagel & Rogers, 1984).

In cultured human keratinocytes a soluble cornified envelope precursor of molecular weight 92,000 has been found and named involucrin (Etoh et al., 1986). This protein is remarkable in that it contains 46 mole percent of glutamine residues and has also been reported as an excellent marker for squamous differentiation (Said et al., 1983). Kubilus & Baden (1984) also looked at bovine snout, new born rat and human epidermis and although there was some disagreement about the precise molecular weight of the soluble precursor proteins, their findings were essentially the same as the workers mentioned above.

It should however be noted that keratolinin and involucrin do not make up the whole cornified envelope in bovine and human keratinocytes respectively since their amino-acid composition is not the same as whole envelopes. Furthermore, the formation of the cornified envelope is known to occur only when all of the individual components are present in the keratinocyte, the stimulus apparently being calcium, although proteases such as cathpepsin B1 and cathpepsin D may participate in the activation of the epidermal enzyme (Negi, 1983).

1.2.4 Prostate Transglutaminase

The clotting of rodent seminal plasma has been recognised as a calcium dependent process for a long time (Gotterer et al., 1955). In guinea pigs the enzyme responsible for this clotting was identified as a transglutaminase of molecular weight 70,000 and was located in the prostate gland (Wing et al., 1974). However subsequent workers introduced some controversy into this area; Chung,(1977) reported two forms of transglutaminase from the prostate gland, one which cross-reacted immunologically with the guinea pig liver enzyme and another which was zymogenic and similar to Factor XIII. Wing,(1977) and Tong, (1980) also found two distinct enzymes but found them to be different to both Factor XIII and the liver enzyme on the basis of substrate specificity. Similarly Lorand et al.,(1979b) and Williams-Ashman et al., (1980) reported at least two novel forms of transglutaminase in the prostate gland of the rat and these were thought to be different to the guinea-pig enzymes. The precise nature of the transglutaminase activity responsible for the formation of the copulatory plug in rodents has yet to be clarified although the possibility of a Factor XIII enzyme being involved seems unlikely since this clotting process is not thrombin dependent.

Copulatory plugs from both guinea-pig and rat have been shown to contain ε(γ-glutamyl)lysine bridges (Williams-Ashman et al., 1972, In guinea-pig a basic protein of molecular weight 17,900 was shown to be involved in the formation of the plug (Notides & Williams-Ashman, 1967) whereas in the rat seminal vesicle protein II has been implicated in copulatory plug formation (Williams-Ashman et al., 1984). Polyamines are found in high concentrations in seminal fluid (Williams-Ashman, 1984) and γ-glutamyl polyamines and N,N-bis- γ-glutamyl-polyamine bridges have been found in copulatory plugs formed in vitro (Folk et al., 1980). Several reasons for this have been proposed; Williams-Ashman & Canellakis, (1980) suggested that it may act to attenuate clotting in the male urethra where it would be inappropriate. Mukherjee et al.,(1983) have suggested that polyamines in combination with uteroglobulin act to supress the antigenicity of spermatozoa in the female reproductive tract. This has been supported by the work of Paonessa et al., 1984) who found that transglutaminase can catalyse the covalent incorporation of spermidine onto the surface of the spermatozoa and can modify rat seminal vesicle protein IV so that it binds to the surface of the spermatozoa in vitro. It was postulated that these modifications could aid in the masking of the antigenicity of spermatozoa.

1.2.5 Conclusion

It can be seen that in plasma, epidermis, hair follicles and prostate there are distinct transglutaminases which catalyse ε(γ-glutamyl)lysine formation and thus confer mechanical strength and chemical resistance on the resultant structures. It must however be remembered that certain features of these enzymes' function and

regulation in vivo have yet to be elucidated. Perhaps more importantly the most commonly occurring form of transglutaminase has yet to have a biological role ascribed to it and in view of its apparent ubiquity this role may well be critical in the regulation and growth of cells in general. It is to this enzyme that this introduction will now focus its attention.

1.3 THE BIOLOGICAL ROLE OF TISSUE TRANSGLUTAMINASE

Tissue transglutaminases occur in a wide variety of animal tissues and cells (section 1.1.2) and it is generally thought that this enzyme will prove to be constitutive in animal cells. Indeed this widespread distribution implies that it must play a role in some process which is fundamental to the normal functioning of cells. Of particular interest is the calcium dependency of the enzyme since this divalent cation acts as a common trigger for biological events as diverse as muscle contraction, secretion and cell division. Thus calcium mediated post-translational modification of proteins by transglutaminase may play an important part in regulating protein function in cells. This need not be accomplished through crosslinking reactions alone since the incorporation of polyamines into cellular proteins may lead to large changes in behaviour of a protein.

In the last decade tissue transglutaminase has been implicated in membrane mediated events associated with receptor mediated endocytosis, the secretion of hormones and also in cell activation and differentiation. These processes are calcium dependent and therefore potentially mediated by transglutaminase.

1.3.1 Receptor Mediated Endocytosis

Competitive substrates of transglutaminases such as monodansyl cadaverine and methylamine have been shown to inhibit the receptor mediated endocytosis of hormones and proteins. The clustering and internalisation of epidermal growth factor and α_2-macroglobulin receptors was shown to be inhibited by primary amines in cultured fibroblasts, Chinese hamster ovary cells and normal kidney cells (Maxfield et al., 1979a, 1979b; Davies et al., 1980; Haigler et al., 1980; Levitzki et al., 1980). Davies et al (1980) were able to show a

strong correlation between the inhibition of endocytosis and the potency of the primary amines for transglutaminase. Similar experiments have also implicated transglutaminase in the internalisation of β-adrenergic receptors in frog erythrocytes (Chuang, 1981, 1984) 3,3',5-triiodo-L-thyronine by fibroblasts (Cheng et al., 1980) and the phagocytosis of Pseudomonas toxin (Fitzgerald et al., 1980) Shigella toxin (Keusch, 1981) and Diphtheria toxin (Dickson et al., 1981).

Some serious criticism of this work was made by King et al (1980) and Yarden et al., (1981) when they reported that internalisation of epidermal growth factor and α_2-macroglobulin was not inhibited by primary amines. They showed that it was the degradation of epidermal growth factor that was inhibited by primary amines which are known to be lysosomotropic (Okhuma & Poole, 1978). Thus the accumulation of high concentrations of basic amines in the lysosomes was thought to raise pH and therefore prevent degradation by lysosomal proteases (Seglen & Gordon, 1980). Similarly it was shown that the internalisation of chorionic gonadotropin by ovine luteal cells was not inhibited by primary amines but that its degradation was inhibited (Ahmed and Niswender, 1981).

However Van Leuven et al.,(1980) were able to dissociate the lysosomotropic effects of methylamine and monodansyl cadaverine from their effect on the internalisation of α_2-macroglobulin. They suggested that the decreased rate of uptake in the presence of these compounds was due to inhibition of receptor processing rather than a decrease in internalisation. This idea was supported by Kaplan & Keogh (1981) and Dickson et al.,(1981).

Work on the activation of macrophages has shown that accumulation of tissue transglutaminase in these cells is linked with an enhanced capacity for receptor-mediated endocytosis (Novogrodsky et al., 1978; Fesus et al., 1981; Leu et al., 1982; Teshigawara et al., 1985). In addition to showing a correlation between transglutaminase activity and phagocytic ability it was also demonstrated that primary amines could inhibit phagocytosis (Gunzler et al., 1982; Julian et al., 1983).

Evidence has also emerged for the involvement of transglutaminase in the internalisation of acetylcholine receptors (Higuchi et al., 1982; Hucho and Bandini, 1986) insulin receptors (Pastan & Willingham, 1981; Draznin & Trowbridge, 1981), somatostatin (Lewin & Reyl-Desmars, 1985), viruses (Leppla et al., 1980; Fitzgerald et al., 1980; Schlegel et al., 1980) and transport proteins such as low density lipoprotein and vitellogenin (Tucciarone & Lanclos, 1981; Pastan & Willingham, 1981; Kojima et al., 1987). For a review on this subject see Davies and Murtaugh (1984).

1.3.2 The Secretion of Insulin

As is the case in receptor mediated endocytosis a rise in the cytosolic concentration of free calcium ions plays a key role in the exocytotic release of hormones such as insulin (Wollheim & Sharp, 1981). In this laboratory glucose mediated insulin release in the pancreatic beta-cell was shown to be inhibited by primary amines (Bungay et al., 1982; Bungay et al., 1984a), a finding which was supported by the work of Gomis et al., (1983). The potency of primary amine substrates of transglutaminase was matched by their ability to inhibit insulin secretion (Bungay et al., 1984a). It was also suggested that polyamines may play a role in the secretory process (Bungay et al.,

1984b). However it became apparent that primary amines may act to inhibit insulin secretion by interfering with other targets than transglutaminase (Bungay et al., 1984a; Sener et al., 1984; Bungay et al., 1986), although it was shown that primary amines acted independently of either glucose oxidation or protein synthesis (Bungay et al., 1984a). These same workers also showed that the potent transglutaminase inhibitors monodansylcadaverine and N-(5-aminopentyl)-2 naphthalenesulphonamide inhibited insulin release whereas the corresponding N,N'-dimethylated analogues (which do not inhibit transglutaminase) were less effective (Bungay et al., 1986). They were also able to show that monodansylcadaverine did not inhibit lipid methylation, calmodulin stimulated phosphodiesterase or protein kinase C at the concentration of monodansylcadaverine used (50 μm). More recently the relationship between protein phosphorylation and transglutaminase mediated protein crosslinking has been investigated (Owen et al., 1987); this is important since phosphorylation is yet another means by which proteins involved in secretion may be modified and furthermore it provides a means of modulating transglutaminase activity through substrate availability. It was found that inhibitors of transglutaminase significantly inhibited the amount of ^{32}P associated with a membrane-associated high molecular weight phosphopolymer present in the glucose-stimulated intact islet. Since transglutaminase catalyses the formation of a similar high molecular weight polymer in calcium incubated islet homogenates (Bungay et al., 1986) it was suggested that protein crosslinking reactions and phosphorylation reactions are closely linked in the β-cell. A mechanism was proposed whereby activation of the islet transglutaminase would result in the stabilisation, through their crosslinking, of membrane or membrane-associated proteins. Stabilisation of predefined

membrane localities by the enzyme could then be important in promoting directional movement of membrane vesicles by providing defined anchorage points to the cytoskeletal network and/or preventing membrane intermixing during membrane recycling.

1.3.3 Tissue Transglutaminase and the Cytoskeleton

All eukaryotic cells contain a highly organised cytoskeletal network which is involved in cellular motile events and in specialised cases in muscle contraction and sperm motility. This cytoskeletal network is modulated by calcium and is therefore a potential target for transglutaminase mediated post-translational modification of proteins.

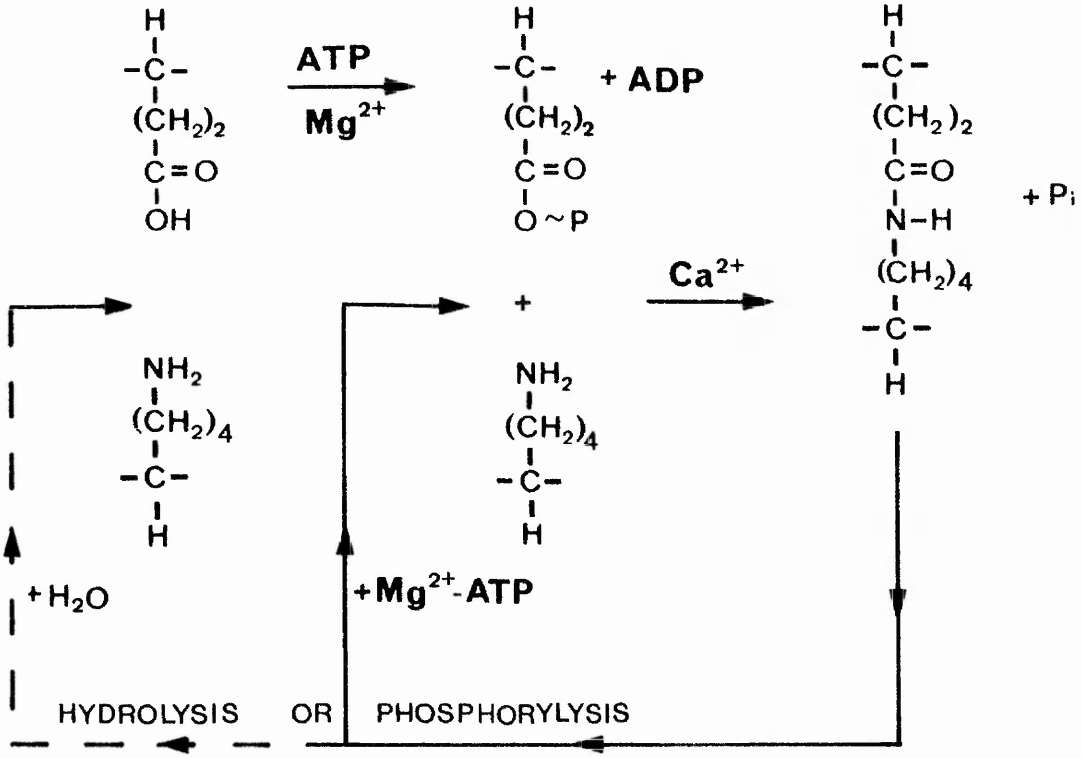
Transglutaminase-mediated crosslink formation was implicated in the modulation of the cytoskeletal network (Loewy & Matacic, 1981; Loewy *et al.*, 1981). They found that ε(γ-glutamyl) lysine in *Polycephalum Physarum* which was associated with the cytoskeleton was decreased in the presence of Mg^{2+}-ATP and increased by Mg^{2+}-ATP plus Ca^{2+}. They also demonstrated a similar decrease in crosslink formation on addition of Mg^{2+}-ATP in cultured chick skeletal myofibrils whereas in cultured embryonic chick heart myofibrils they were able to show an increase in ε(γ-glutamyl)lysine on addition of Mg^{2+}-ATP and Ca^{2+}. The model that was proposed is shown in Figure 1.3, whereby an acyl phosphate of a glutamic acid residue could be formed, which could then form an ε(γ-glutamyl)lysine bond and ultimately be cleaved by hydrolysis or phosphorolysis. It is interesting therefore that cytoskeletal proteins such as actin, α-actinin, desmin, tropomyosin and tubulin have been shown to act as transglutaminase substrates *in vitro* using polyamine incorporation techniques (Derrick & Laki, 1966; Iwanij, 1977; Gard & Lazarides, 1979; Cohen *et al.*, 1979, 1980; Conrad, 1985) although it was shown that intact microtubules were

better substrates than the individual microtubular proteins (Maccioni & Arechaga, 1986). The idea of transglutaminase mediated regulation of cytoskeletal function is supported indirectly by other workers; Denk et al., (1984) showed that griseofulvin (which blocks microtubule formation) caused tissue transglutaminase activity in mouse liver cells to increase. However it was also suggested that this increase may have been due to increased cell permeability to Ca^{2+} ions rather than microtubule antagonism. Alarcon et al., (1985) suggested that transglutaminase was involved in cellular motile events since primary amines were able to block proinsulin conversion to insulin, a process which requires the translocation of proinsulin to its site of conversion. Also, work on Arbacia Sperm motility has led to the proposal that transglutaminase may be involved in regulating the interaction of flagellar sliding fragments by the cycling of ε(γ-glutamyl)lysine bridges since the potent non-competitive inhibitor of transglutaminase, 2-(3-diallylaminopropionyl) benzothiophene caused an increase in the forward swimming speed of the spermatozoa and monodansylcadaverine (a competitive substrate with high affinity for transglutaminase) was also shown to have a similar but lesser effect (Cariello & Nelson, 1985; Gagnon & Lamirande, 1986). Finally recent work has demonstrated the presence of a calmodulin dependent transglutaminase with high affinity for cytoskeletal proteins in human platelets and red blood cells and in chicken gizzard (Puszkin and Raghuraman, 1985; Puszkin and Billet, 1987). This observation is of particular interest since this offers an alternate mechanism whereby transglutaminase-mediated reactions can be modulated in vivo.

This proposal of transglutaminase mediated cytoskeletal protein interactions is an attractive one since it offers an alternative explanation of this enzyme's involvement in endocytosis and

exocytosis. Receptor-mediated endocytosis is dependent on the translocation of internalised vesicles to the lysosomes and if the receptors are to be reprocessed, their return to the plasma membrane and similarly in secretion the movement of secretory granules to the plasma membrane and their ultimate release is dependent on the microtubule and microfilament system (Howell & Tyhurst, 1984). It is interesting to note that in a recent publication Matrisian et al.,(1987) reported that methylamine did not affect internalisation of epidermal growth factor but prevented its translocation within the cell.

Figure 1.3 A Hypothetical Cycle Accounting for the Making and Breaking of ε(γ-Glutamyl)Lysine Bonds



1.3.4 Tumour Growth and Metastasis

The involvement of transglutaminase in tumour growth was first suggested by Yancey & Laki (1972) who found that inhibitors of fibrin clot stabilisation increased host survival time in mice carrying the YPC-1 tumour and it was postulated that the formation of a stable fibrin clot by transglutaminase was essential for tumour survival. These same workers found a relationship between the transglutaminase content of a tumour and the transglutaminase content of the tissue to which that tumour metastasised (Laki et al., 1977). They also demonstrated that tumours with low transglutaminase content were more likely to survive than tumours with higher transglutaminase content, the idea being that transglutaminase modified cell surface proteins such as fibrin and fibronectin in a manner which favoured tumour viability (Fesus & Laki, 1976; Laki et al, 1977).

However in later years it became increasingly apparent that transglutaminase was essential to normal cells and the regulation of their growth and that reductions in transglutaminase activity were associated with neoplastic growth. Birckbichler et al.,(1976) showed that Novikoff hepatoma and primary hepatomas induced by 3'-methyl-4-dimethylaminoazobenzene contained reduced transglutaminase activity when compared to normal liver and that the subcellular distribution of transglutaminase in these tumours was more particulate than in normal liver. The same workers were also able to show that ε(γ-glutamyl) lysine crosslink formation in simian virus transformed cells was reduced in a comparable manner to transglutaminase activity (Birckbichler et al., 1977). It was therefore proposed that transglutaminase mediated ε(γ-glutamyl)lysine formation was important to the normal functioning of cells in the non-proliferative state (Birckbichler et al., 1978; Birckbichler & Patterson, 1980) where the

enzyme was thought to regulate membrane architecture. They then went on to show that there was an increase in proliferative markers following inhibition of transglutaminase mediated ε(γ-glutamyl)lysine formation by cystamine in proliferating WI-38 cells. Similarly Haughland et al (1982) were able to demonstrate that the inhibition of ε(γ -glutamyl)lysine formation in cultured mouse L-cells by dansylcadaverine and cystamine correlated with increased membrane fluidity. It has therefore been suggested that transglutaminase may be involved in regulating membrane rigidity during different proliferative states (Birckbichler et al, 1981) although recent work by Porta et al., (1986) with erythrocyte ghosts has contradicted this idea. Other workers have also demonstrated reduced transglutaminase activity in tumours; Vanella et al., (1983) observed reduced transglutaminase activity in Yoshida Ascites tumour cells and in Ascites tumour bearing liver compared to normal liver although they only measured the activity in the soluble fraction.

In this laboratory it was shown that transglutaminase activity was decreased in the sequence normal liver > tumour bearing liver > hepatocellular carcinoma using both diethylnitrosamine and 6-p-dimethylaminophenylazobenzothiazole as carcinogens (Barnes et al., 1984, 1985). In addition to this a redistribution of transglutaminase activity to the particulate fraction was also reported. In the same studies transplantable sarcomata were also investigated and found to contain very low levels of transglutaminase activity which was mostly particulate. The comparison of non-metastatic and pre-metastatic sarcoma tissue with sarcomas with established metastases showed that there was a significant (4-7 fold) reduction in transglutaminase activity in metastasising tumours. This interesting finding was recently supported by workers at Inserm in France who showed that in

cloned rat rhabdomyosarcoma cells transglutaminase activity decreased in the sequence non-metastatic > weakly metastatic > strongly metastatic (Delcros et al., 1986) and that in human colon tumours transglutaminase activity decreased in the sequence normal > benign > malignant (Roch et al., 1987) suggesting that transglutaminase measurements may be of prognostic value in the diagnosis of human cancer.

1.3.5 Regulation: Cell Activation and Differentiation

There is mounting evidence to suggest that transglutaminase is involved in cell activation and differentiation in a variety of systems. Of these the maturation of lymphocytes and monocytes has been most studied. The elevation in transglutaminase activity associated with stimulation of these cell types has led to the suggestion that this enzyme is involved in the phagocytic process (Fesus et al., 1981; Leu et al., 1982; Teshigawara et al., 1985) (see section 1.3.1). This has been supported by the observation that primary amines are able to inhibit phagocytosis (Gunzler et al., 1982; Julian et al., 1983) and that inflammatory macrophages contain higher transglutaminase activity than resident macrophages (Leu et al., 1982).

Workers were also able to show that in macrophages the level of transglutaminase was dependent on the cell type and its state of differentiation (Schroff et al., 1981) and that differentiation of myeloblastic cells to mature macrophages was accompanied by an increase in transglutaminase activity (Kannagi et al., 1982). Increases in transglutaminase were later shown to be due to rapid de novo synthesis of the enzyme following stimulation of macrophages by a heat labile serum component (Murtaugh et al., 1983). This component was identified as trans-retinoic acid (Moore et al., 1984) and its action was thought

to be dependent on serum retinol binding protein. Since then retinoic acid derivatives have been used to study the activation and differentiation of a number of normal and leukaemic monocytic cells and it has been shown that transglutaminase is synthesised rapidly in all these cell types following stimulation. It was also shown that the induction of transglutaminase was inhibited by actinomycin D and was therefore due to transglutaminase gene expression (Moore et al., 1984).

Subsequently it was shown that interferon could enhance the expression of transglutaminase in monocytes and that this induction was dependent on serum retinoids (Mehta et al., 1985). Using cultured monocytic leukaemia cells these same workers demonstrated that 12-0-tetradecanoyl-phorbol-13- acetate also induced transglutaminase synthesis and that dibutyryl cyclic AMP potentiated the retinoic acid dependent induction of tissue transglutaminase (Mehta and Lopez-Berestein, 1986). More recently Johnson and Davies (1986) have been able to show that the inhibition of retinoic acid induced expression of transglutaminase by pertussis toxin may be due to the ADP-ribosylation of a membrane protein of Mr 41,000 suggesting that the effect of retinoids may be mediated by a membrane receptor.

Transglutaminase has also been implicated in the activation and differentiation of a number of other cell types including platelets (Barnes et al., 1985) Chinese hamster ovary cells (Milhaud et al., 1980; Scott et al., 1982), neurones (Gilad & Varon, 1985; Maccioni & Seeds 1986), Friend erythroleukaemia cells (Hsu & Friedman, 1983) and embryonic cells (Cariello et al., 1984; Maccioni & Arechaga, 1986; Uhl & Schindler, 1987). In one instance it was suggested that transglutaminase was involved in cytoarchitectural changes in the development of murine embryos (Maccioni & Arechaga, 1986). This is of interest since the cytoskeleton is fundamental to the process of

differentiation and it has been suggested that transglutaminase is involved in microtubule stabilisation (Maccioni & Seeds, 1985), a finding which is in agreement with the ideas discussed in section 1.3.3.

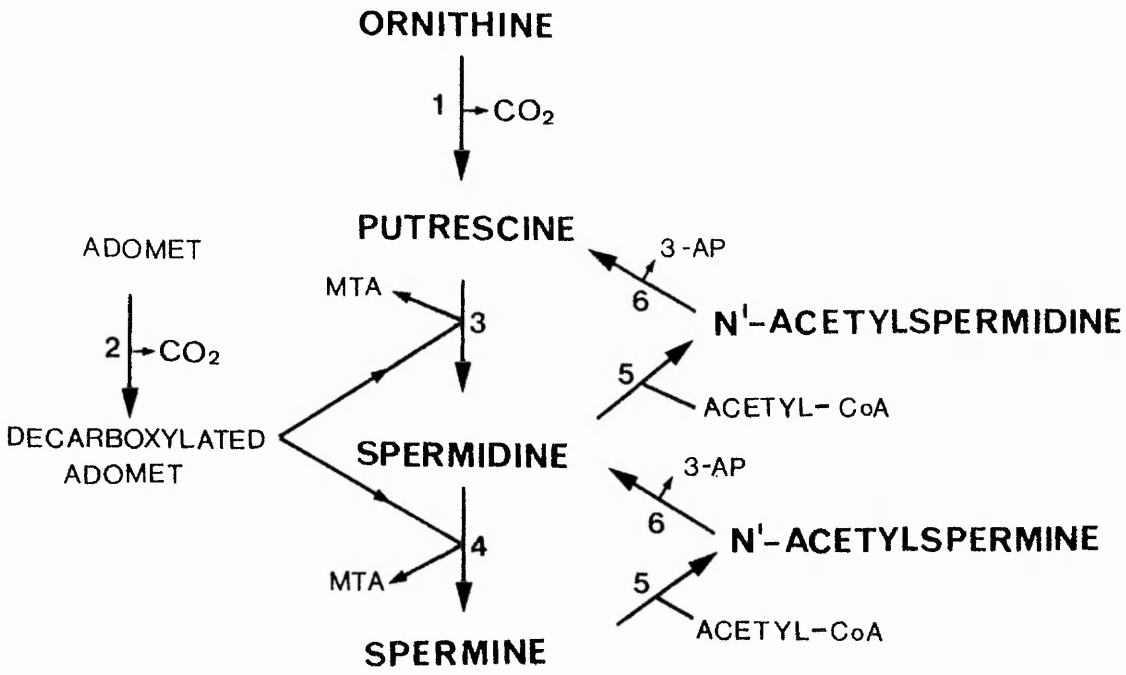
1.3.6 Transglutaminase and Polyamines in Normal and Neoplastic Growth

The polyamines putrescine, spermidine and spermine are potentially important physiological substrates for transglutaminase (Conrad, 1985). Covalently bound polyamines have been identified in acid-precipitable protein of normal tissues (Seale et al., 1979; Williams-Ashman & Canellakis, 1980; Chen et al., 1981; Haddox & Russell, 1981b) and in one instance bis-(γ -glutamyl) polyamine derivatives have been found in normal rat liver (Beninati et al., 1985).

Polyamines are thought to play an important role in cellular proliferation and differentiation (Heby, 1981; Pegg, 1986). The pathway for the biosynthesis and degradation of the common polyamines is shown in figure 1.4. Many studies have shown that the accumulation and biosynthesis of polyamines is a characteristic feature of proliferative stimulation by a variety of agents such as: partial hepatectomy, hormonal stimulation and treatment with drugs, carcinogens or tumour promotors (Janne et al., 1978; Matsui & Pegg, 1982; Scalabrino et al., 1978; Danzin et al., 1983). In support of these observations, recent work with inhibitors of polyamine biosynthesis has shown that polyamine depletion arrests cell growth and under extreme conditions can result in loss of cell viability (Pegg, 1986). Since alterations in transglutaminase activity have also been linked to cell growth and proliferation (Birckbichler et al., 1980; Russell, 1981) it is tempting to suggest that transglutaminase mediated polyamine

incorporation into proteins may be important in these growth states. Work on experimental tumours in animals has produced considerable evidence for the accumulation and enhanced synthesis of polyamines in tumours (Williams-Ashman and Canellakis, 1979; Noguchi et al., 1976; Kallio et al., 1977; Janne et al., 1978; Russell & Durie, 1978; Kaminski, 1983; Hand et al., 1985). As a consequence various workers have proposed that changes in polyamine levels may be of diagnostic value in cancer and in the evaluation of anti-cancer therapy (Nishioka & Romsdahl, 1974; Durie et al., 1977; Milano et al., 1981; Scalabrino & Ferioli, 1982, Janne et al., 1983; Kaminski, 1983).

Figure 1.4 Pathway for the Biosynthesis and Interconversion of Polyamines



The enzymes involved are : 1. ornithine decarboxylase (E.C. 4.1.1.17); 2. S-adenosylmethionine decarboxylase (E.C. 4.1.1.50); 3. spermidine synthase (E.C. 2.5.1.16); 4. spermine synthase (E.C. 2.5.1.22); 5. spermidine/spermine-N^1-acetyltransferase; 6. FAD-dependent polyamine oxidase.

Abbreviations : ADOMET, S-adenosylmethionine; 3-AP, 3-acetamido propanal; MTA, 5'-methylthioadenosine.

1.3.7 Pathological Conditions

All cells exist in a calcium rich environment necessitating the careful regulation of intracellular calcium levels since abnormal increases in cell calcium can result in toxicity and cell death. Since transglutaminase is extremely sensitive to calcium activation, (Hand et al., 1985) perturbations in the regulation of intracellular calcium may lead to the uncontrolled crosslinking of proteins in a variety of different biological situations (Lorand & Conrad, 1984). Indeed, highly insoluble protein polymers, which may be formed by transglutaminase are a characteristic feature of many disease states. Transglutaminase has therefore been implicated in the aetiology of several pathological conditions including erythrocyte ageing, lens cataract formation, lung fibrosis, Alzheimers disease, coeliac disease and cancer (see section 1.3.5).

1.3.7.1 Erythocyte Dysfunction

Erythrocyte transglutaminase (Brenner & Wold, 1978) has been implicated in the crosslinking of membrane proteins (Lorand et al., 1976; Anderson et al., 1977) to form heteropolymers containing spectrin, ankyrin, band 3, band 4.1 and haemoglobin (Bjerrum et al., 1981; Lorand & Conrad, 1984). This work was supported by the finding of ε-(γ-glutamyl)lysine bridges in polymers formed in Ca^{2+} loaded erythrocytes (Lorand et al., 1978; Seifring et al., 1978). More recently the use of dansyl-antibody labelling techniques revealed that band 3 was the main substrate in inside out vesicles of erythrocytes (Lorand et al., 1986) and the use of 2-(3-diallylaminopropionyl) benzothiophene (a potent non-competitive inhibitor of transglutaminase) has confirmed that spectrin, ankyrin, band 3 and band 4.1 are substrates for the enzyme (Lorand et al., 1987). Although the Ca^{2+}

loaded erythrocyte represents an artificial situation there is evidence to suggest that fluctuations in Ca^{2+} regulation due to the failure of the outward directed Ca^{2+} pump during ageing and in the irreversibly sickled erythrocyte may lead to stiffening of the cell membrane. The fact that non-erythroid cells contain similar membrane and cytoskeletal proteins to the erythrocyte (Burridge et al., 1982; Cohen et al., 1982) supports the idea that these proteins may be the in vivo substrates for transglutaminase in mammalian cells.

1.3.7.2 Lens Cataract Formation

The extreme insolubility and chemical stability of protein polymers in human lens cataracts led to the suggestion that transglutaminase-mediated crosslinking of proteins may be involved in cataract formation in the ageing lens. A Ca^{2+}-dependent transglutaminase was identified in the lenses of several mammalian species (Lorand et al., 1981). The presence of ε-(γ-glutamyl) lysine bridges in cataractous protein polymers was also demonstrated (Lorand et al., 1981; Hsu et al., 1981). In recent years the use of fluorescent and radio active primary amine probes to label γ-glutaminyl residues of proteins has led to the identification of the β-crystallin chains involved in cataract formation (Berbers et al., 1983; Lorand et al., 1981). In calf lens the γ-glutaminyl residues available for primary amine incorporation were found in the NH2-terminal regions of three different β-crystallin chains (Berbers et al., 1983). In rabbit lens two β-crystallin chains have been shown to act as substrates for transglutaminase-mediated amine incorporation (Lorand et al., 1981; Velasco & Lorand, 1987). Furthermore, antiserum raised to polymers formed from rabbit β-crystallin chains in vitro has been shown to crossreact with an Mr 50,000 protein polymer which is thought to

participate in cataract formation in calf and human lenses (Velasco & Lorand, 1987). This finding suggests that in situations such as ageing where the plasma membrane of the lens is damaged (Garner et al., 1981), activation of transglutaminase may lead to the formation of lens cataracts.

1.3.7.3 Pulmonary Fibrosis

Transglutaminase has also been implicated in the crosslinking of fibrous proteins during pulmonary fibrosis (Richards and Curtis, 1984). The major transglutaminase activity in lung tissue is a particulate enzyme (Griffin et al., 1978; Cocuzzi and Chung, 1986) which is associated with insoluble material in the lung and is thought to play a role in the formation of the extracellular matrix in lung tissue. Using a model of fibrosis induced by the herbicide, paraquat, it was shown that transglutaminase activity was increased in the fibrotic lung and it was therefore postulated that this enzyme was involved in the irreversible crosslinking of proteins during fibrosis (Griffin et al., 1979). Protein substrates for transglutaminase such as fibrin and fibronectin are present in lung tissue and may well be crosslinked together with collagen into insoluble matrices (Richards and Curtis, 1984).

1.3.7.4 Alzheimers Disease

The formation of neurofibrillary tangles (NFT) in patients with Alzheimers disease has been linked to transglutaminase mediated crosslinking of proteins (Selkoe et al., 1982a;b). It was suggested that transglutaminase may be involved in the formation of paired helical filaments (PHF), which are the major structural component of NFT. Because PHF are extremely insoluble it was suggested that

ε-(γ-glutamyl)lysine bridges might be involved in their formation (Selkoe et al., 1982a). Rat brain was shown to contain transglutaminase activity (Selkoe et al., 1982b; Gilad & Varon, 1985) and neurofilaments and microtubules from rat brain were shown to act as substrates for this enzyme (Selkoe et al., 1982b; Miller & Anderton, 1986). These protein substrates include all three neurofilament peptides, α and β-tubulin and MAPs 1 and 2. Since NFT contain all of these proteins it is possible that NFT formation is mediated by transglutaminase. However the in vitro incubation of neurofilaments and microtubules did not form PHF, thus indicating that other processes probably contribute to the formation of NFT in Alzheimers disease.

1.3.7.5 Coeliac Disease

It has been proposed that transglutaminase may be responsible for the binding of wheat gliadin and/or its peptide fragments to intestinal mucosa during the pathogenesis of coeliac disease (Bruce et al., 1985). The discovery of a Ca^{2+}-dependent transglutaminase activity in human jejunum (Bruce et al., 1985) and a variety of gastrointestinal tissues in the rat (Patel et al., 1985) lends credence to this idea. Bruce and coworkers (1985) were also able to show that in jejunal biopsies from patients with coeliac disease, transglutaminase activity was elevated up to three fold over control subjects. Furthermore it has been shown that gliadin is a substrate for transglutaminase since it contains 40 mole % of glutaminyl residues and that deamidated gliadin (which is non-toxic to patients with coeliac disease) is a poor substrate (Bruce et al., 1985). These findings strongly implicate transglutaminase in the binding of gliadin to mucosal surfaces and thus in the pathogenesis of coeliac disease.

1.3.8 Conclusion

Despite numerous studies on tissue transglutaminase using various model systems, the precise cellular role of this calcium dependent enzyme has yet to be elucidated. It is, nonetheless, becoming increasingly apparent that the ability of transglutaminase to modify membrane and/or cytoskeletal proteins is a common feature in many tissues. Studies on specific membrane proteins such as the HLA-A and -B antigens (Pober & Strominger, 1981), β_2-microglobulin (Fesus *et al.*, 1981) and membrane-mediated processes such as receptor-mediated endocytosis (Davies & Murtaugh, 1984) and the secretion of insulin (Bungay *et al.*, 1986) strongly implicate transglutaminase in the modification of membrane structure and function. Other reports suggest that transglutaminase may modify cytoskeletal proteins during embryogenesis and differentiation (see section 1.3.4) and that cytoskeletal proteins in erythrocytes (Lorand *et al.*, 1987) and other cells (Conrad, 1985) are substrates for the enzyme. Furthermore, in pathological situations where uncontrolled crosslinking occurs as a result of activation or stimulation of transglutaminase it is membrane and cytoskeletal proteins which act as substrates in polymer formation (section 1.3.7). Conversely, in tumour growth, transglutaminase activity is reduced (section 1.3.5) and may therefore by implication be linked to certain pleiotropic events associated with neoplasia such as: loss of cell shape; increased vesicular movement and reduced cellular adhesion (Winegardner, 1985; Pastan & Willingham, 1978).

The aim of this thesis is to further investigate the nature and cause of the reductions in transglutaminase activity that are seen during carcinogenesis and tumour progression (section 1.3.5). The hepatocellular carcinoma will be used as a model to study the role of transglutaminase in carcinogenesis and as a transglutaminase-negative

control to study the *in vivo* substrates of this enzyme in liver tissue. Metastasising and non-metastasising rat sarcomas will also be compared in an attempt to understand the role of transglutaminase in the metastatic process. In particular the relationship between transglutaminase and polyamine levels will be examined with a view to understanding how changes in polyamine metabolism may influence transglutaminase-mediated reactions and *vice versa*.

2.0 MATERIALS AND METHODS

2.1 MATERIALS

All of the work on fresh liver tumour tissues was carried out in the Central Toxicology Laboratories of ICI PLC, Alderley Park, Macclesfield, Cheshire. This included histology, fractionation, transglutaminase assay and tissue slice incubations; all samples were then frozen at -70°C. All other work on these tissues and on the transplantable rat sarcomata was carried out in the laboratories of the Department of Life Sciences, Trent Polytechnic, Nottingham.

Chemicals used for buffers and physiological media were of the highest grade available and were purchased from the following sources:

British Drug Houses (BDH) Limited, Poole, Dorset; Hipersolv grade: acetonitrile. Electran grade: acrylamide, ammonium persulphate, N,N'-methylenebisacrylamide, SDS, TEMED, PAGE blue 83. Analar grade: all other chemicals and solvents not listed below.

Amersham International PLC, Little Chalfont, Bucks.; [2-^{3}H]-5'AMP (19Ci/mmol), n-[1-^{14}C]-hexadecane (0.506mCi/g), [^{14}C]-methylamine hydrochloride (59mCi/mmol), [1,4-^{14}C]-putrescine dihydrochloride (109mCi/mmol), [1,4(n)-^{3}H]-putrescine dihydrochloride (22.5-24.6 Ci/mmol) and [^{14}C]-methylated protein standards (Mr 14,300-200,000).

Boehringer Corporation (London) Limited (BCL), Lewes, East Sussex; Standard DNA (from calf thymus).

Canberra Packard, Pangbourne, Berks.; Dimilume-30, Instagel, Soluene-350.

Clay-Adams, (A Division of the Becton Dickinson Company), Parsippany, New Jersey, USA; Autoclip 9mm (stainless steel wound clips).

The Deseret Company, Sandy, Utah, USA; Angiocaths, 16GA and 18GA.

Flow Laboratories, Rickmansworth, Herts; Williams' Medium E, foetal bovine serum.

Gibco Limited, Paisley, Scotland; Penicillin G, Streptomycin Sulphate USP.

Koch-Light Limited, Haverhill, Suffolk; methylamine hydrochloride.

ICI PLC, Pharmaceuticals Division, Macclesfield, Cheshire; Fluothane.

LKB Produktor AB, Bromma, Sweden; Ultrogel AcA 44.

Pharmacia Fine Chemicals Limited, Uppsala, Sweden; Aldolase (protein molecular weight standard), QAE Sephadex A-25.

Sigma Chemical Company, Poole, Dorset; Benzamidine, bovine serum albumin, Brij-35, collagenase type 1 (pfs), 1,7-diaminoheptane, diethylnitrosamine, diphenylamine, dithiothreitol, DNAse 1, gentamycin sulphate, HEPES, Lubrol-PX, 2-mercaptoethanol, phospholipase C type XI (from B.Cereus), putrescine dihydrochloride, PMSF, RNAse 1, spermidine trihydrochloride, spermine tetrahydrochloride, sucrose, Trizma base, octane sulphonic acid, o-phthalaldehyde and all protein molecular weight markers (except aldolase; Pharmacia).

Stuart Pharmaceuticals Limited, Cheadle, Cheshire; Dispray 1.

Whatman Labsales Limited, Maidstone, Kent; DE52 Ion-Exchange Media.

All other chemicals were purchased from BDH Limited and were of Analar grade.

Centrifuges MSE Scientific Instruments, Crawley, Sussex. (All centrifuge speeds in this thesis are quoted as g_{av}.)

	Centrifuge	Rotor	RCF (g_{av})
1)	MSE Microcentaur	-	7500
2)	MSE Centaur	8x10ml swingout (r_{av} = 12cm)	700/ 40
3)	MSE Major	24x30ml swingout (r_{av} = 23cm)	600/ 410

4) MSE Superspeed 50,	8x25 angle (r_{av} = 5.84 cm)	40,000/ 71,000
	or	
	8x25 angle fitted with 3ml adaptors (r_{av} = 6.22 cm)	40,000/ 71,000

Conductivity Meter Model 220. Corning Ltd., Essex.

Densitometer Model SP1809 (Accessory to a Unicam SP1800 ultraviolet spectrophotometer) Unicam, Cambridge.

Electrophoresis Apparatus Vertical slab-gel electrophoresis apparatus, model 2001. LKB Produktor AB, Bromma, Sweden.

Fluorimeter Model 1000. Perkin-Elmer, Beaconsfield, Bucks.

HPLC Apparatus All pumps and equipment supplied by Waters Associates, Milford, Mass., USA.

Integrator Model SP 4270. Spectra-Physics Inc., California, USA.

Liquid Scintillation Spectrophotometer Canberra Packard A 300 CD, Pangbourne, Berks.

pH Meter Model PW9409. Phillips (Pye-Unicam). All solutions were adjusted to the correct pH at 37°C unless otherwise stated.

Potter-Elvehjem Homogeniser MSE Homogeniser. MSE Scientific Instruments, Crawley, Sussex.

Sonicator Soniprep 150. MSE Scientific Instruments, Crawley, Sussex.

Spectrophotometers

(1) Pye-Unicam SP6-400. Pye-Unicam, Cambridge.

(2) Beckman DU-7. Beckman, California, USA.

Tissue Chopper McIlwain Tissue Chopper. Mickle Laboratory Engineering Company, Gomshall, Surrey.

Ultra-Turrax Janke and Kunkel KG. Copley Scientific Instruments and Equipment, Nottingham.

2.2 ANIMALS

Three strains of rat were used:

1) For the raising of hepatocellular carcinomas adult male Alderley Park (AP) rats (Wistar derived) were used. Further information on the dosing procedure is given in section 2.3.1. AP rats were also used for the majority of control experiments.

2) In addition some of the work on normal tissue was carried out using Sprague-Dawley rats of both sexes (200-300g), (sections 4.2.4.1, 4.2.4.2, 5.2.2 and 5.5.4).

3) For the propagation of the solid sarcoma lines P_7 and P_8, AS rats of both sexes (200-300g) were used.

2.3 TUMOURS

2.3.1 Administration of Carcinogens

Primary hepatocellular carcinomas were induced by oral administration of chemical carcinogens to male AP rats. The main carcinogen used was diethylnitrosamine (DEN), however one group of animals was dosed with 6-p-dimethylaminophenylazobenzothiazole (6BT). Rats (4-5 weeks old) were dosed by oral gavage 5 days per week. The dosing schedules are summarised in Table 2.1. The use of these carcinogens has been described previously (DEN- Argus & Hoch-Ligeti, 1961) (6BT- Elliott et al., 1983).

TABLE 2.1

GROUP NUMBER	CARCINOGEN	CONTROL	DOSE (mg/kg rat)	TERMINATION DATE
1	DEN	WATER	0.55 - 26 weeks	40 weeks
2	6-BT	CORN OIL	7.50 - 6 weeks	39-47 weeks
3	DEN	WATER	0.55 - 27 weeks	36 weeks
4	DEN	WATER	1.10 - 28 weeks	40 weeks

2.3.2 Histology of Chemically-induced Tumours

Animals were killed by fluothane intoxication and blood withdrawn from the heart (15-20ml) in order to minimise contamination of liver tissue by factor XIII. Livers were inspected for grossly visible nodules and polaroid photographs taken of the dorsal and ventral surfaces of the liver as a record of these. Sections were taken from all macroscopically abnormal areas and, in addition, three standard sections were taken from the left, right median and papillary

lobes. The tissues were fixed in buffered formalin, embedded in paraffin wax, cut into thin sections and stained with haematoxylin and eosin. Lungs were inflated with saline and inspected for metastases and any other abnormalities within the body cavity were recorded (for instance lymph node metastases).

2.3.3 Passage of Sarcoma Lines and Harvesting of Tissue

Two transplantable sarcomas whose origin and growth characteristics have been described previously (Moore, 1972) were used. These were originally induced by intraperitoneal injection of ^{32}P into AS rats; P_7 being an osteosarcoma and P_8 an intramedullary fibrosarcoma.

The sarcomas were propagated by subcutaneous implantation of freshly excised tissue into AS rats of both sexes. Rats were anaesthetised with ether, their flanks shaved and sterilised with Dispray 1 (Stuart Pharmaceuticals Ltd.). A small incision was then made in the upper flank and a small subcutaneous pocket (approx. 5mm x 3mm) formed using scissors. A piece of freshly excised tumour tissue ($2mm^3$) was inserted and the pocket sealed with a stainless steel wound clip (Clay-Adams) and sterilised with Dispray 1. The animals were allowed to recover and tissue harvested from animals when required.

For the harvesting of tissue, animals were sacrificed by cervical dislocation and tumour tissue from the outer edges of the mass taken for analysis. The central areas of the mass were discarded since this region usually contained necrotic tissue. P_8 sarcomas also contained bone inclusions which were discarded where possible. All tumour tissue was kept on ice until required. Tumour tissue was treated for histology as described in section 2.3.2.

2.3.4 Detection of Lung Metastases

The morphology of secondary tumour growth was visually similar to that of normal lung tissue, therefore a contrast medium was employed to detect secondaries in the lung according to the method of Wexler (1966). The lungs were inflated in situ with indian ink (15% (v/v) containing 0.5% (v/v) ammonia solution) via the trachea. The trachea was then ligated and the lungs removed from the thoracic cage and washed in distilled water before fixing in Feketes solution (100ml ethanol, 50ml distilled water, 15ml formaldehyde solution (40% (v/v)) and 7.5ml glacial acetic acid). Metastases appeared contrasted white on a black background.

2.4 FRACTIONATION PROCEDURES

2.4.1 Homogenisation of Tissues by Potter-Elvehjem

Excised tissue was weighed and washed in the appropriate buffered medium. The tissue was minced and then homogenised in four volumes of buffered medium (0.25M sucrose, 1mM EDTA, 5mM Tris-HCl pH 7.4) with five passes of a close-fitting (clearance 0.25-0.30mm) glass/teflon Potter-Elvehjem homogeniser unless otherwise stated. After homogenisation all homogenates were adjusted to 20% (w/v). Tissues were kept on ice before, during and after homogenisation.

2.4.2 Fractionation into 71,000g Pellet and Supernatant

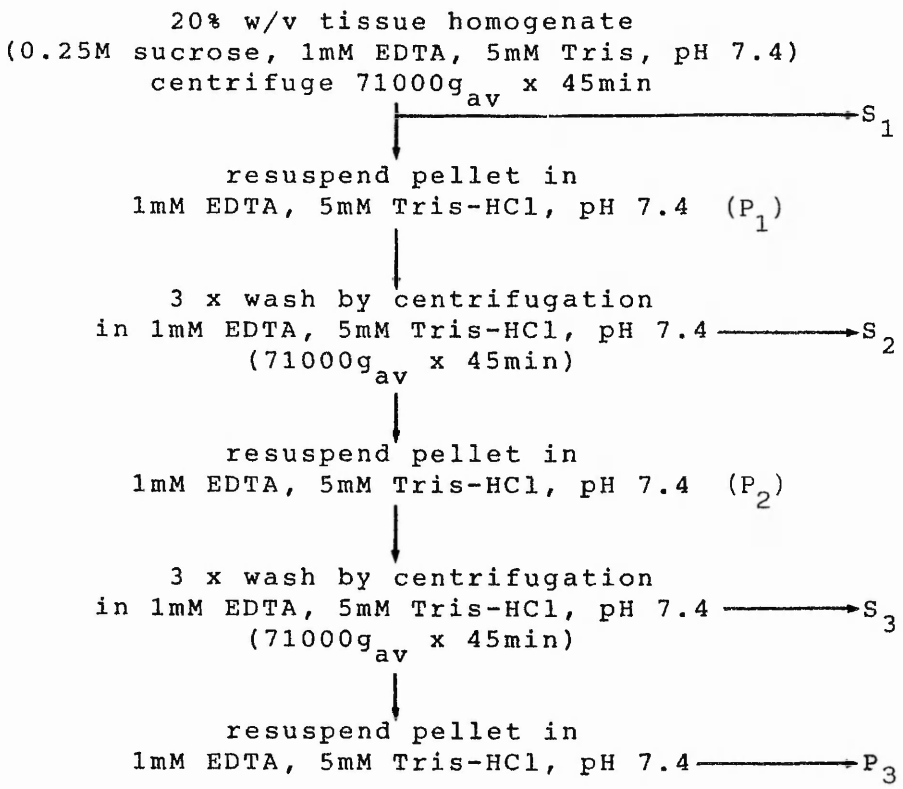
This procedure was used to determine the amounts of transglutaminase activity associated with the total membrane fraction and with the cytosol. Tissue was homogenised in 0.25M sucrose, 5mm Tris-HCl pH 7.4 containing 1mM EDTA as described in section 2.4.1. The homogenates were then centrifuged at 71,000g for 45 minutes. The supernatant was decanted and the pellet resuspended in a volume equal to that of the decanted supernatant by means of three passes of a hand held homogeniser. All procedures were carried out at $4^{o}C$.

2.4.3 Leaching of 71,000g Particulate Fraction

The leaching of transglutaminase and marker enzyme activities from a fraction containing all the cellular membranes and organelles was achieved using the schedule illustrated in figure 2.1. Rat liver and hepatocellular carcinoma tissues were homogenised in 0.25M sucrose, 5mM Tris-HCl pH 7.4 containing 1mM EDTA as described in section 2.4.1. All resuspensions were performed using three passes of a hand held homogeniser in 1mM EDTA, 5mM Tris-HCl pH 7.4 using a volume equal to that of the first supernatant, S_1. All procedures were carried out at $4^{o}C$ and fractions retained on ice.

Figure 2.1

Scheme for the Leaching of Transglutaminase from Tissues Using Detergent-Free Buffers

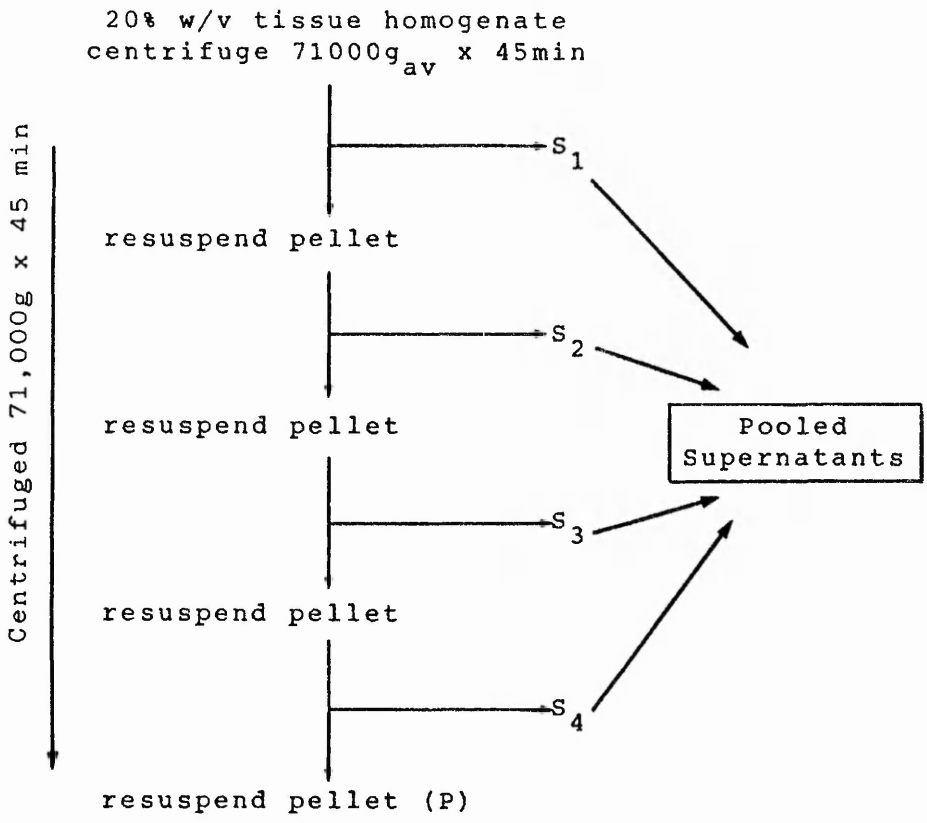


2.4.4 Transglutaminase Extraction Procedure

This methodology was used to prepare a pool of extracted transglutaminase activity for ion-exchange analysis (cf section 2.8.1) and is based on the methods of Chang and Chung (1986). The procedure is illustrated in figure 2.2. Tissue was homogenised by use of a variable speed Ultra-Turrax with three, 30 second bursts at low speed and allowed to stand for 30 minutes at $4^{o}C$ to reach equilibrium. One gram of tissue was homogenised in four volumes of the following buffered medium and adjusted to 20% (w/v) afterwards : 0.25M sucrose, 10mM benzamidine, 1mM PMSF, 1mM EDTA, 1% (v/v) Lubrol-PX, 5mM Tris-HCl pH 7.4. PMSF was dissolved in DMSO such that the final DMSO concentration in the medium was 1% (v/v). All resuspensions were carried out in the same way as the homogenisation and allowed to equilibrate for 30 minutes at $4^{o}C$. The Ultra-Turrax blade was precooled to $4^{o}C$ and tissues kept on ice at all times.

Figure 2.2

Scheme for the Extraction of Transglutaminase Activity from Tissues

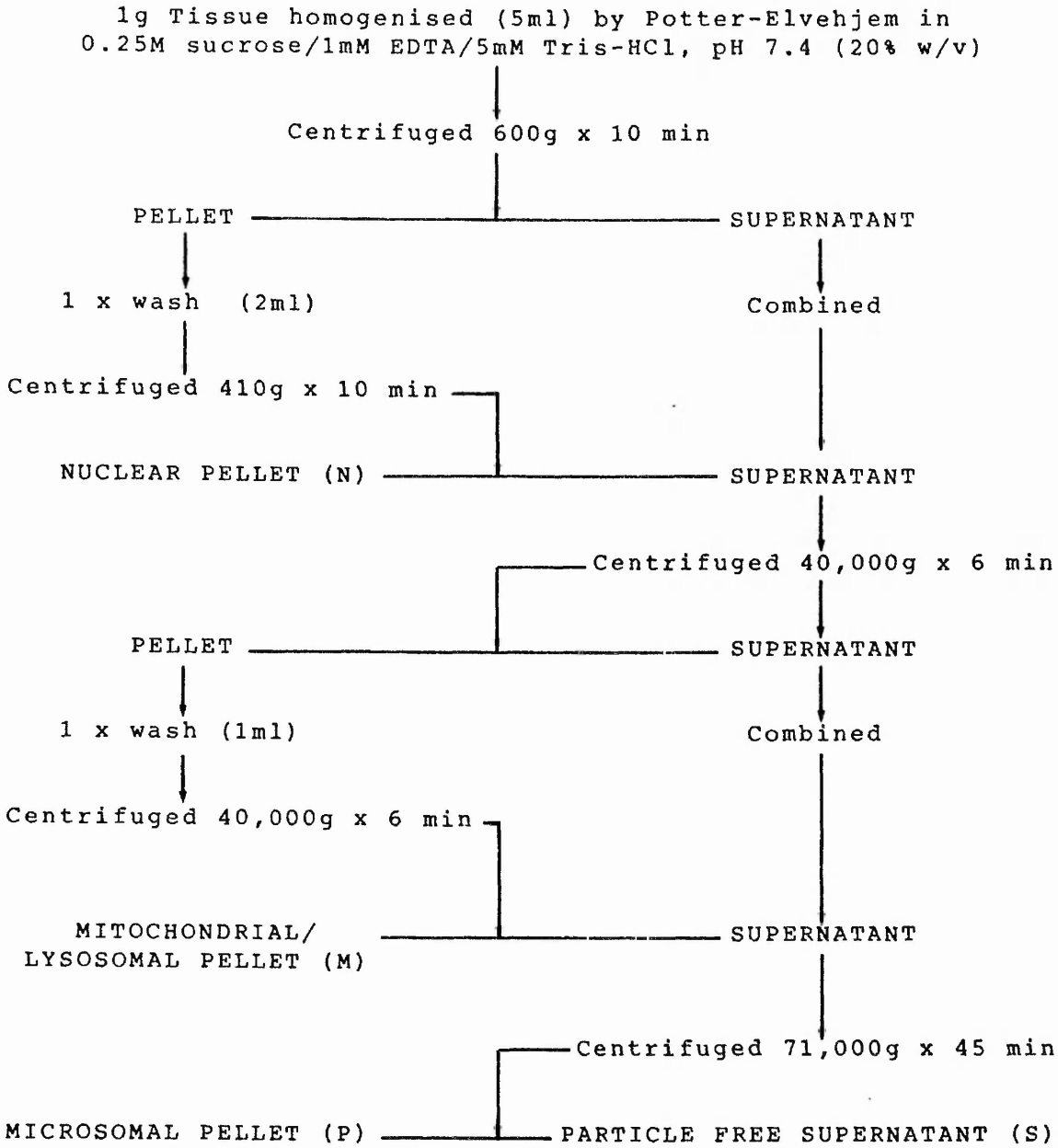


2.4.5 Differential Centrifugation

A variation of the methods of De Duve *et al* (1955) was utilised to fractionate tissue homogenates prepared as described in section 2.4.1. This procedure yielded a low-speed 600g pellet (N) containing plasma membranes and nuclei, a 40,000g pellet (M) enriched in mitochondria and lysosomes, a 71,000g pellet (P) consisting primarily of microsomes and a particle-free cytosol fraction (S). This fractionation is illustrated in figure 2.3. All resuspensions were achieved with three passes of a hand held homogeniser. All fractions were kept on ice and centrifugation was carried out at $4^{o}C$.

Figure 2.3

Scheme for the Subcellular Fractionation of Tissues



2.4.6 SDS Polyacrylamide Electrophoresis

The separation of proteins from homogenates and cellular fractions was achieved using a modification of the discontinuous system described by Laemmli (1970). An LKB 2001 vertical electrophoresis unit was used with 1.5mm thick slab gels consisting of a 2.5% stacking gel and a 10% resolving gel.

2.4.6.1 Solutions

Electrode Buffer	0.025M Tris-HCl pH 8.3
(5 litres)	0.192M glycine
	1% (w/v) SDS
Stacking Gel Buffer	0.25M Tris-HCl pH 6.8
	2% (w/v) SDS
Resolving Gel Buffer	0.75M Tris-HCl pH 8.8
	2% (w/v) SDS
Acrylamide Solution	30.0% (w/v) Acrylamide
	0.8% (w/v) Methylene bis Acrylamide
Sample Buffer	0.125M Tris-HCl pH 6.8
	40% (w/v) SDS
	20% (v/v) glycerol
	10% (v/v) 2-mercaptoethanol
	0.001% (w/v) Bromophenol Blue
Ammonium Persulphate	20 mg/ml

2.4.6.2 Sample Preparation

Samples of homogenates or cellular fractions containing 50-100µg of protein were mixed with sample buffer in a ratio of 3:1 (cf 2.4.6.1). Prior to electrophoresis, samples were boiled for five

minutes to dissolve proteins and then centrifuged at 700 g for 1 minute to remove undissolved material.

2.4.6.3 Electrophoretic Procedure

The gel mould was assembled according to the manufacturer's instructions using glass plates which had been thoroughly cleaned in detergent, distilled water and ethanol. The resolving gel was prepared by the combination of 9.9ml acrylamide solution, 15ml resolving gel buffer, 5.1ml distilled water and 20 µl TEMED. This mixture was degassed under vacuum for 5 minutes and then, immediately prior to use, 0.75ml of ammonium persulphate added. The mixture was swirled gently and gels poured to a height of 12cm and overlayed with butan-2-ol. This produced a flat surface to the gel and excluded oxygen. After one hour the butan-2-ol was removed and replaced with 50% (v/v) resolving gel buffer. The gels were then left to stand at room temperature overnight, thus ensuring that complete polymerisation had taken place.

The buffer was removed from the resolving gel surface and the top of the gel dried carefully. Stacking gel was prepared by mixing 2.5ml acrylamide solution, 10ml stacking gel buffer, 7.5ml distilled water and 10 µl TEMED and degassed as described above. On removal of the vacuum 0.5ml of ammonium persulphate was added, the mixture swirled gently and poured to within 1cm of the top of the glass plates. Sample wells were formed by inserting plastic combs and overlaying with butan-2-ol. After polymerisation had occurred (usually 1 hour) the combs were removed and the wells washed out with 50% (v/v) stacking gel buffer.

Samples were applied to the wells with a Hamilton syringe and overlaid with electrode buffer. The apparatus was assembled according to the manufacturer's instructions and electrophoresis performed at

15°C and a constant current of 30 milliamps per gel for 5-6 hours until the tracking dye was within 1cm of the edge of the gels.

Gels were calibrated using the MWSDS 200 kit supplied by Sigma Chemical Co, Poole, Dorset, which contained the following:

Myosin	Mr 205000
ß-Galactosidase	Mr 116000
Phosphorylase b	Mr 97400
Bovine Serum Albumin	Mr 66000
Egg Albumin	Mr 45000
Carbonic Anhydrase	Mr 29000

2.4.6.4 Fixing and Staining of Gels

Following the completion of electrophoresis, gels were freed from the glass plates, taking care to ensure that the stacking gel remained attached to the resolving gel in one piece. Gels were fixed for 20 minutes in 10% TCA, 3.4% 5-Sulphosalicylic acid and then placed in staining solution overnight. This staining solution consisted of 1.25g Page blue 83 dye dissolved in methanol, filtered and adjusted to 18% (v/v) methanol, 5% (v/v) glacial acetic acid in a total volume of 1 litre. The gels were destained using several changes of 18% (v/v) methanol, 5% (v/v) glacial acetic acid. When samples which were radiolabelled with amines were electrophoresed, 1% (w/v) methylamine was included in the destain in order to facilitate the dispersal of unbound radiolabelled amines.

The sectioning and autoradiography of gels is described in sections 2.5.4.3 and 2.5.4.4 respectively.

2.4.7 Isolation of Hepatocytes

Hepatocytes were isolated from male AP rat livers by the collagenase perfusion method of Rubin et al.,(1977). Rats were killed

by fluothane intoxication, the liver exposed *in situ* and the inferior *vena cava* ligated. The liver was cannulated through the hepatic portal vein (18 GA Angiocath, Deseret) and the superior *vena cava* (16GA Angiocath, Deseret). A waste line was attached to the superior *vena cava* and Buffer 1 (500ml) pumped through the liver (20ml/min) in order to remove any blood. The liver was perfused with buffer 2 (20ml/min) until buffer 1 had been replaced and the waste line was placed in the buffer 2 reservoir and the collagenase added (dissolved in a small volume of buffer 2; 10ml). This recirculating perfusion system was maintained until the reticular pattern of the liver began to disintegrate (10-15 minutes) and then stopped. The liver was placed in Williams medium and filtered through nylon bolting cloth (150 μm; Simons Ltd) into a centrifuge tube. The cell suspension was centrifuged at 40g for 2 minutes and the supernatant decanted (first supernatant). The pellet was washed twice in Merchants solution by centrifugation and finally resuspended in Merchants solution. This preparation consisted of 98-99% pure hepatocytes. The first supernatant from was re-centrifuged at 400g for 5 minutes and the pellet resuspended in Merchants solution. This non-parenchymal cell preparation consisted primarily of endothelial cells and Kupffer cells (R W Trueman - personal communication).

2.4.7.1 Solutions

Buffers 1 and 2 were gassed with 5% carbon dioxide in oxygen and pH adjusted at 37°C prior to use. Buffers were maintained at 37°C during perfusion.

Buffer 1:	10mM Hepes, 141.8mM NaCl, 6.6mM KCl,
(500ml)	2.5µg/ml Gentamycin Sulphate adjusted to
	pH 7.4 with 1M NaOH.
Buffer 2:	100mM Hepes, 66.7mM NaCl, 6.6mM KCl,
(500ml)	4.8mM $CaCl_2$, 0.8 g/ml Gentamycin Sulphate
	adjusted to pH 7.6 with 1M NaOH,
	plus 112 mg collagenase (Type 1) - see text.
Williams Medium:	500ml Williams Medium E (flow labs)
	10ml 200mM L-glutamine
	10ml Penicillin and Streptomycin (5000 Units/ml)
	25µl Gentamycin Sulphate (50 g/ml)
	50ml Foetal Bovine Serum
Merchants Solution:	0.14M NaCl, 2.7mM KCl, 0.53mM EDTA
	1.47mM KH_2PO_4/K_2HPO_4 pH 7.4

2.5 CHEMICAL DETERMINATIONS

2.5.1 Protein Assay (Lowry)

Protein was estimated by a modification of the method of Lowry et al., (1951). The determinations were carried out in the presence of SDS in order to ensure solubilisation of the samples. Two stock solutions were used:

Solution A : 0.4% (w/v) NaOH, 2.0% (w/v) Na_2CO_3, 0.02% (w/v) Na Tartarate.

Solution B : 0.5% (w/v) $CuSO_4$.

Samples were diluted approximately and 100 µl of sample mixed with 100µl of 2% (w/v) SDS. To this was added 1.0ml of Folins solution (49 parts Solution A plus 1 part Solution B). After 20 minutes 100µl of freshly prepared Folins-Ciocalteau reagent (1:1 dilution with distilled water) was added. Absorbance was read at 750nm after a further 20 min. Standard curves were constructed by the same procedure using bovine serum albumin in a range of dilutions from 100-600 µg/ml and distilled water as blank.

2.5.2 Protein Estimation by A280nM/A260nm Measurement

The method of Warburg and Christian (1941) was found to be useful for rapid estimation of the protein content of samples. This method was mainly used for monitoring protein elution from ion-exchange and gel filtration chromatography. The absorbance of samples was measured at A280nm and A260nm and the protein content calculated from the following formula:

Protein concentration (mg/ml) = A280nm x factor x 1/d

Where d is the light path in cm and the factor is obtained from a table (Dawson et al, 1974) using the ratio A280nm/A260nm.

2.5.3 DNA Assay (Burton)

DNA was estimated by the method of Burton (1956). Ice-cold 10% (w/v) TCA (2.6ml) was added to 0.4ml of a 20% (w/v) tissue homogenate and pelleted by centrifugation at 700g for 10 min. The resultant pellet was resuspended in 3ml 10% (w/v) TCA and recentrifuged. This pellet was washed twice in 95% (w/v) ethanol, centrifuged as above and then the final pellet drained thoroughly. DNA was hydrolysed by adding 3ml of 5% (w/v) TCA and incubating at 90°C for 10 minutes. The lysate was clarified by centrifugation and 1ml of lysate was added to 2ml of freshly prepared diphenylamine reagent (1.5g diphenylamine in 100ml Analar glacial acetic acid plus 1.5ml cH_2SO_4 and 0.5ml of 16mg/ml acetaldehyde) in glass tubes. Tubes were vortexed and left overnight in the dark at room temperature for colour to develop and absorbance was then read at 600nm.

A standard curve with calf thymus DNA in the range 10-100 µg/ml in 5% (w/v) TCA was used to estimate DNA concentrations in the samples.

2.5.4 Determination of Radioactivity

The radioisotopes ^{14}C and ^{3}H were detected by scintillation counting in a Packard A 300 CD liquid scintillation spectrophotometer. The channels used are shown in table 2.2.

TABLE 2.2

ISOTOPE	CHANNEL A (Kv)	CHANNEL B (Kv)
^{14}C	0-156	4-156
^{3}H	0- 19	2- 19

2.5.4.1 Filter Papers

For the transglutaminase assay (section 2.6) and in radiolabelled amine experiments (sections 2.7 and 5.5) samples for counting were precipitated onto filter paper (Whatman 3MM) and washed to remove unbound label. Samples precipitated onto 1 cm^3 Whatman 3MM filter paper squares were counted in 2ml of Optiphase Safe in plastic inserts. Filter paper discs of 2cm diameter were counted in 5ml of Optiphase Safe in glass scintillation vials.

2.5.4.2 Aqueous and Solid Tissue Samples

Whole tissue slices for the determination of total radiolabel uptake were solubilised in Soluene-350 at 45°C and then counted for radioactivity in 10ml Dimilume-30. Samples of media containing radioactive isotopes were counted in 10ml of Instagel. All samples were left at 4°C overnight in order to avoid interference from chemiluminescence.

2.5.4.3 Polyacrylamide Gel Sections

Polyacrylamide gels (1.5mm thick) consisted of a 2% (w/v) stacking gel (2cm) and a 10% (w/v) resolving gel (12cm), prepared as described in section 2.4.6. The stacking gel lanes were cut into an upper and a lower section, the upper section consisting of the first 2mm of the lane plus the teeth and the lower section being the remainder. The resolving gel lanes were cut into 1cm sections. Sections were placed into scintillation vials and dried in a vacuum oven at 60°C overnight. Samples were then dissolved in 0.5ml of a solution of hydrogen peroxide (30% (w/v))/ammonia solution (specific gravity 0.88) 19:1 (v/v) by incubation at 50°C for 1 hour. After cooling, 20ml Optiphase Safe was added and samples were counted for radioactivity.

2.5.4.4 Fluorography

Prior to autoradiography gels were impregnated with salicylate as a scintillator by the method of Chamberlain (1979). After destaining (section 2.4.6.4) the gels were immersed in 10% (v/v) glycerol overnight to remove the destain. Gels were then placed in a scintillator solution consisting of 1M sodium salicylate, 1M NaOH and 10% (v/v) glycerol. After one hour in this solution they were removed and placed on stiff card (Whatman Chroma 17) and covered with clingfilm. They were then dried for 6 hours under vacuum and at a constant temperature of 80°C.

The film (Fuji RX100 medical X-ray film) was hypersensitised by exposure to a flash of light. The intensity of the flash was adjusted to increase the absorbance of the developed film to 0.15 (A450nm) above that of unexposed film (Laskey and Mills, 1975). Dried gels were then placed in close contact with pre-flashed film in a light-tight cassette at -70°C for periods up to 21 days. Exposed film was developed according to the following procedure:

developer (D19 Kodak)	2 min.
stop bath (1% acetic acid)	10 sec.
fixative (Hypam, Ilford Ltd)	1 min.

Fluorograms were then washed copiously under running cold water and allowed to dry at room temperature.

The scanning of fluorograms was carried out on individual lanes using the scanning densitometer accessory of a Unicam SP 1800 spectrophotometer at 540nm coupled to a Spectro-Physics 4270 plotting integrator.

2.5.4.5 Determination of Counting Efficiency

The calculation of counting efficiency for filter paper assays was done by using known amounts of radioactive reaction mixture (see 2.8.1) which were spotted onto filter papers and counted in the same way as is described in 2.5.4.1. All squares within an assay were assumed to have equal counting efficiency. In addition, the Packard quench indication parameter and the channels ratio (B/A) were used to monitor for any unexpected quenching which occurred in the samples. Any results which were found to be affected in this way were repeated.

For dissolved aqueous and tissue samples internal standardisation was used with radiolabelled Hexadecane as internal standard.

In general 75-85% efficiency was found for all ^{14}C samples whatever the method of counting. For ^{3}H the counting efficiency was between 35-50%.

2.6 TRANSGLUTAMINASE ASSAYS

The quantitation of transglutaminase activity was performed by measuring the rate of incorporation of radiolabelled putrescine into N,N'-dimethylcasein using the filter paper assay of Lorand et al., (1972).

For most assays [1,4-^{14}C]-putrescine was used, however the increased sensitivity afforded by the use of [1,4(n)-^{3}H]-putrescine was occasionally required.

2.6.1 [^{14}C]-Putrescine Assay

Reactions were carried out in 0.4ml plastic microcentrifuge tubes incubated in a water bath at 37^{o}C. All solutions were at pH 7.4 (at 37^{o}C) and reaction vials contained the following in a total volume of 50µl.

Tris-HCl	27.50mM
Dithiothreitol	3.85mM
Calcium chloride	2.50mM
[1,4-^{14}C]-Putrescine (3.97mCi/mmol)	1.20mM
N,N'-dimethylcasein	5.0 mg/ml

Reactions were initiated by the addition of sample (25µl) to 25 µl of reaction mixture containing the above constituents at double the stated concentrations. At suitable time intervals (usually 2,5 and 10 minutes) 10 µl aliquots were removed and immediately spotted onto pencil marked filter paper squares (1cm^{2}) and placed in ice-cold 10% TCA. At the end of each assay 10 µl of reaction mixture from each vial was spotted onto a filter paper square and allowed to dry at room temperature and these were used as a measurement of the total counts in the assay in order to estimate the counting efficiency (cf section 2.5.4.5.).

The effective concentration of Ca^{2+} in the assay vials was 2.0mM since samples contained 1mM EDTA. Control incubations were carried out in which 10mM EDTA was substituted for $CaCl_2$. No rates were found for EDTA incubations in any of the samples measured; there was however a consistent background level of 60-100 cpm.

Once all filter paper squares from an assay had been placed in ice-cold 10% (w/v) TCA they were washed as follows:

1 x	10% (w/v) ice-cold TCA	- 10min
3 x	5% (w/v) ice-cold TCA	- 5min
1 x	1:1 Acetone:Ethanol	- 5min
1 x	Acetone	- 5min

The even washing of filter paper squares was facilitated by placing them in a perforated beaker which was suspended inside another beaker containing a magnetic flea. This apparatus was kept stirring and on ice during the washing procedure. At least 5ml of TCA per square per wash was maintained throughout. Filter papers were then allowed to dry at room temperature and counted as described in section 2.5.4.1.

When measuring the effects of other agents on transglutaminase activity (such as phospholipids or calmodulin) the sample volume was lowered to 20µl to accommodate the addition of these components.

When dealing with samples containing greatly reduced amounts of transglutaminase activity (eg P_8 tissue) the assay was scaled up. A total volume of 200µl was used and 50µl aliquots were taken at 5, 10, and 30 minutes and spotted onto 2cm diameter filter paper circles. (Total counts were estimated from 10µl of reaction mixture from each vial.) An extended wash procedure was also required allowing 10ml TCA per circle:

1 x 10% (w/v) ice-cold TCA - 20min

5 x 5% (w/v) ice-cold TCA - 20min

1 x 1:1 Acetone:Ethanol - 5min

1 x Acetone - 5min

EDTA background values ranged between 200-500 cpm, they were however consistent within an assay and did not vary with time.

Transglutaminase activity was expressed in units, one unit being the amount of putrescine in nanomoles that was incorporated into 5mg of N,N'-dimethylcasein per hour.

2.6.2 [^{3}H]-Putrescine Assay

The incubation conditions for this assay are as for the [^{14}C] assay detailed in the previous section. The specific activity of the [1,4(n)-^{3}H]-putrescine in the assay was 2.05 Ci/mmol. In addition filter papers were presoaked in 100mM EDTA and 1% methylamine and dried in an oven before use. This prevented anomalously high backgrounds caused by non-specific binding of radiolabelled putrescine to the filter papers. 1% methylamine was also included in the aqueous washes of the extended washing procedure. Background EDTA values were found to be in the range 100-200 cpm.

2.6.3 Assay for Chromatographic Profiles

For the detection of transglutaminase in ion-exchange and gel permeation chromatography fractions a simplified assay was found to be economical. A total reaction volume of 30µl was used, containing 15µl of sample. Two 10µl aliquots were removed after 30 minutes incubation, spotted onto filter paper and washed as described in section 2.6.1.

Where trace amounts of transglutaminase activity were expected the radiolabel used was [1,4(n)-^{3}H]-putrescine at a specific activity of 22.5-24.6 Ci/mmol in the assay and a concentration of 20μM. Filter papers were prepared and washed as described in section 2.6.2.

2.6.4 Estimation of the Sensitivity of Transglutaminase to Activation by Ca^{2+}

This assay involves the use of a $CaCl_2$/EGTA buffering system to produce free Ca^{2+} concentrations in the range 5-100μm. Great care was taken to acid wash all glassware with nitric acid prior to use and all solutions were made up in double-distilled deionised water. The N,N'-dimethylcasein used in other assays was found to contain phosphate groups (Hand et al.,1985) and it was thus necessary to dephosphorylate this substrate to remove the possibility of Ca^{2+}/phosphate interactions interfering with the assay (see section 4.2.4.3). Mg^{2+} was also included in the assay as an agent to swamp any remaining phosphate groups.

2.6.4.1 Dephosphorylation of N,N'-dimethylcasein

N,N'-dimethylcasein was dephosphorylated according to the method of Cooke and Holbrook (1974). 250mg of N,N'-dimethylcasein was dissolved in 3ml of 50mM Tris-HCl pH 7.4 containing 10μM $ZnSO_4$ and 10μl of a 20mg/ml solution of alkaline phosphatase was added. This mixture was incubated at room temperature for 2.5 hours. This material was then dialysed exhaustively against three changes of 500ml Tris-HCl pH 7.4 containing 10μM $ZnSO_4$ and then against 2 x 500ml of the same buffer without the $ZnSO_4$ to inactivate the alkaline phosphatase. The dialysed material was then adjusted to 10ml with 50mm Tris-HCl pH 7.4 and stored in 0.5ml aliquots at -20°C.

2.6.4.2 Calculation of Free Ca^{2+} Concentrations

Free Ca^{2+} concentrations were calculated by the methods of Schatzmann (1973) using an association constant of $10^{7.12}$ at pH 7.4 and 37°C. Samples (20 µl) containing 1mm EGTA were added to 30 µl of reaction mixture. The final concentration of EGTA in the reaction was therefore 0.4mM. The following Ca^{2+} concentrations were frequently used in assays and produced the listed free Ca^{2+} values:

TABLE 2.3

Total $CaCl_2$ Concentration	Free Ca^{2+} Concentration
0.395mM	3.50 µM
0.400mM	5.47 µM
0.410mM	12.43 µM
0.425mM	26.16 µM
0.450mM	50.60 µM
0.500mM	100.30 µM
2.500mM	2100.01 µM

2.6.4.3 Assay Conditions

Prior to assay all samples were dialysed exhaustively against 50mM Tris-HCl pH 7.4 containing 1mM EGTA and 0.5mM DTT in double distilled, deionised water. Each reaction vial contained the following:

[1,4-^{14}C]-putrescine (3.97 mCi/mnol)	- 1.20mM
dithiothreitol	- 4.05mM
magnesium chloride	- 2.00mM
dephosphorylated N,N'-dimethylcasein	- 5 mg/ml
EGTA (in sample)	- 0.40mM

Tris-HCl pH 7.4 at 37'C -50.0mM

calcium chloride (0.4mM-2.5mM, cf section 2.6.4.2)

The assay was otherwise performed as described in section 2.6.1, using 20 µl sample and a total reaction volume of 50 µl. The apparent Km for Ca^{2+} was then calculated from a Lineweaver-Burk Plot (Lineweaver and Burk, 1934).

2.7 TISSUE SLICE EXPERIMENTS

Fresh rat liver and hepatocellular carcinoma tissue was trimmed to facilitate slicing. In general the right lobe of normal liver was found to be ideal for this purpose, but hepatocellular carcinoma tissue required careful manipulation to produce adequate slices. The trimmed blocks of tissue were sectioned (5 μm) with a Mc Ilwain tissue chopper (Mickle Labs Eng Co) and immediately placed in medium at 4°C. The slices were blotted dry, weighed in scintillation vials (up to 300mg per vial) and medium containing either 1.9mM Ca^{2+} or 1mM EDTA added at a ratio of 900 μl per 100mg slice. The medium contained the following (these concentrations are at 10/9 the required concentration to allow for addition of radiolabel):

144.40mM sodium chloride

5.73mM potassium chloride

1.43mM magnesium sulphate

12.20mM glucose

22.20mM HEPES

The scintillation vials were capped with suba-seals and placed in a shaking water bath and attached to a gassing manifold which allowed a constant flow of 5% carbon dioxide in oxygen to be passed through the vials. After pre-incubation for 10 minutes radiolabelled 12mM methylamine (59mCi/mmol) was added (100 μl for every 900 μl medium). The vials were shaken gently for one hour and after this time slices were removed and treated in one of two ways:

1) For the Determination of Slice/Medium Ratio.

   Slices were quickly and thoroughly rinsed three times in medium containing 1.2mM 'cold' methylamine and 1mM EDTA, blotted dry and weighed. They were then placed in soluene-350 (1ml per 100mg slice) and left to dissolve in capped tubes at 45°C overnight.

Samples were then counted as described in section 2.5.4.2. Medium (10 µl) was also counted for radioactivity and slice/medium ratios calculated assuming a tissue density of 1.0.

2) For the Determination of Covalent Incorporation.

Slices were rinsed thoroughly in medium containing 1.2mM 'cold' methylamine and 10mM EDTA, blotted dry and weighed. They were then homogenised in 0.25M sucrose, 100µM PMSF, 1mM EDTA, 5mM Tris-HCl pH 7.4. Aliquots were spotted onto filter papers and washed and counted as described in sections 2.6.2 and 2.5.4.1. Homogenates were also fractionated as described in section 2.4.5 and fractions from this procedure counted for covalent incorporation in the same manner.

2.8 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

2.8.1 Ion-exchange HPLC of Proteins

The Waters Associates HPLC system consisted of two pumps (models 6000A and 510) controlled by an automated gradient controller (model 680). Protein elution was monitored with a model 440 absorbance detector at 280nm. The injection of samples was effected using a Rheodyne 7125 injector with a 2ml loop.

Two different columns were used: a Waters Associates DEAE 5PW Protein-Pak column and a Pharmacia Mono-Q column. Both columns gave similar separations. The columns were equilibrated overnight at 0.2 ml/min in 5mM Tris-HCl pH 7.4 containing 1mM EGTA. All buffers were filtered through 0.22µm Durapore filters (Waters Associates Ltd).

Samples (2ml) were predialysed against 5mm Tris-HCl pH 7.4 containing 1mM EGTA and 0.5mM DTT and then filtered through 0.22µm Durapore filters. They were then loaded and eluted at 0.5ml/min with a linear gradient from 0-0.5M sodium chloride in 5mM Tris-HCl pH 7.4, 1mM EGTA over 30 min.

2.8.2 Gel Permeation HPLC of Proteins

An LKB UltroPac TSK-G 3000 SW gel permeation column (7.5 x 600mm) with an UltroPac TSK-GSWP pre-column (7.5 x 75mm) was used to separate proteins according to their molecular weight. Elution was effected isocratically with an M-45 pump (Waters). The buffer used contained 0.1M sodium sulphate, 5% (w/v) glycerol, 1mM EGTA and 50mM HEPES pH 7.4. Samples (200µl) were loaded via a Rheodyne injector and eluted at 0.5 ml/min. Protein was monitored at 280nm using a Model 440 absorbance detector (Waters).

2.8.3 Polyamine Analysis

2.8.3.1 Extraction of Acid Soluble Polyamines

Tissue homogenates in 0.25M sucrose, 1mM EDTA, 5mM Tris-HCl pH 7.4 were spiked with internal standard (200 nmol/ml 1,7 diaminoheptane). They were then vigorously mixed and allowed to stand for 20 minutes at 4°C to equilibrate. Perchloric acid was added to a final concentration of 0.3M and extracts clarified by centrifugation at 700g for 10 min. The supernatants were filtered (0.22 µm Durapore filter), decanted and frozen at -20°C until required. It was found that samples extracted in this way could be kept for several months without significant loss of polyamine content.

2.8.3.2 Extraction of Covalently Bound Polyamines

The methodology for this extraction is essentially the same as that described by Haddox and Russell (1981b). Acid soluble polyamines were removed by a washing regime and the residue was then hydrolysed and following concentration, the hydrolysate analysed for polyamine content. The steps taken in this procedure are listed below:

1) Homogenates (1ml) in 0.25M sucrose, 1mM EDTA, 5mM Tris-HCl pH 7.4 were adjusted to 10% (w/v) TCA with a stock 50% (w/v) TCA solution and pelleted by centrifugation at 700g for 10 minutes.
2) The pellet was washed twice with 2ml 0.1M HCl and three times with 3ml of diethylether.
3) The pellet was then solubilised in 2M sodium hydroxide and salt saturated by the addition of 0.24g of a 7:1 mix of disodium sulphate and trisodium phosphate. This suspension

was then shaken with an equal volume of butan-1-ol for 30 minutes to extract ionically bound polyamines (Raina, 1963).

4) The aqueous fraction from above was made acid with 50% (w/v) TCA to a final concentration of 10% (w/v) TCA and pelleted by centrifugation at 700g for 10 minutes.

5) The pellet was suspended in 3ml 6M HCl (containing 20 nmol 1,7 diaminoheptane as internal standard) and sealed in Pyrex tubes under vacuum. Hydrolysis was carried out for 20 hours at 100^{o}C.

6) The hydrolysate was cooled to room temperature and then mixed vigorously with 3ml of butan-1-ol to extract the polyamines (Raina, 1963).

7) The butan-1-ol phase was dried by passing a continuous stream of nitrogen over the samples and the residue was taken up in 220µl of 0.3M perchloric acid, filtered (0.45µm Durapore filter) and used for polyamine analysis.

In order to check the efficiency of the extraction of polyamines 5 µCi of [1,4-^{14}C]-putrescine was added to 1ml samples of homogenate and following the butanol extraction, samples were solubilised in 500µl Soluene 350 and counted for radioactivity in 10ml Optiphase Safe. No appreciable amounts of radioactivity were found and the extraction was therefore assumed to remove 99%+ of all acid soluble polyamines.

2.8.3.3 HPLC of Polyamines

The HPLC of polyamines was performed using the methods of Seiler and Knodgen (1985). For routine analysis of acid-soluble polyamines the method described in his 1983 paper was used. However for the analysis of covalently bound polyamines a more complex gradient

was required owing to the large number of interfering peaks which occurred in samples extracted in this way (Seiler and Knodgen, 1985; 'Gradient 3').

Acid soluble polyamines were separated by paired-ion chromatography on a C-18 reverse phase column (Waters Associates) using a modification of the method of Seiler (1983). Polyamines were eluted with a linear acetonitrile gradient and detected post column with a fluorescent o-phthalaldehyde (OPA) reagent.

The Waters Associates solvent delivery system used to produce the gradient consisted of model 6000A and model 510 pumps controlled by a model 680 automated gradient programmer and a model M-45 pump to deliver the post-column derivatising reagent. Samples (usually 50 µl) were injected via a Rheodyne 7125 injector. Fluorescence was monitored with a Perkin-Elmer 1000 Fluorimeter (excitation wavelength = 340nm; emission wavelength = 455nm) coupled to a Spectra-Physics 4270 plotting integrator. A C-18 µ-bondapak reverse phase column (3.9 x 300mm - Waters Associates) which was jacketed and maintained at 35°C and protected by a guard column (3.9 x 75mm) containing Spherisorb General Purpose ODS (Phase Sep) was used for acid-soluble polyamine analysis. The buffers used are detailed below:

Buffer A	0.1M sodium acetate	pH 4.5	
	10mm octane sulphonic acid		
Buffer B	0.2M sodium acetate	pH 4.5)	10 parts
	13mm octane sulphonic acid	)	+ 3 parts acetonitrile

OPA Reagent:	component A:	50g boric acid
	(1 litre)	44g potassium hydroxide
		3ml Brij-35

component B 600mg OPA

7.5ml methanol

5.0ml 2-mercaptoethanol

The OPA reagent was prepared the day before use by mixing components A and B and was stored in a dark bottle overnight. All solutions were filtered through 0.22 µm Durapore filters and degassed before use. Elution of polyamines was effected at a constant flow rate of 1.5ml/min after equilibration of the column with buffer A for 10 minutes. The gradient programme used for elution is tabulated below:

TABLE 2.4

Gradient Programme for the Analysis of Acid-Soluble Polyamines

TIME (min)	% A	% B
0	100	0
25.0	0	100
30.0	0	100
35.0	100	0

The OPA reagent was mixed 1:1 with the column eluent and passed through a reaction coil which was incubated at 35^{o}C with a reaction time of 10 seconds. Fluorescence was then monitored as described above.

For the analysis of covalently-bound polyamines a more complex gradient was required due to the increased number of peaks detected. The method of Seiler and Knodgen (1985 - 'gradient 3') which is a modification of the above method was used. Buffers A and B were mixed with methanol 9:1 and the column was kept at room temperature. Polyamines were eluted at a flow rate of 1ml/min on a Zorbax ODS column (4.6mm x 25cm) (Dupont UK Ltd). The gradient is as described below:

TABLE 2.5

Gradient Programme for the Analysis of Covalently-Bound Polyamines

TIME (min)	% A	% B
0	100	0
12	100	0
16	60	40
36	60	40
48	0	100

All other conditions were as stated previously.

Polyamine levels were quantified by reference to the peak heights of known amounts of standard polyamines. The internal standard was used to correct for injection volume and for the efficiency of the extraction procedure. The response of the OPA reagent to known amounts of standard polyamines was linear in the range used (2.5-10nmol) (section 5.2.1). Examples of the traces obtained are shown in figures 5.1, 6.5 and 6.6.

2.9 MARKER ENZYME ASSAYS

Three marker enzyme activities were measured in tissue fractions obtained from differential centrifugation (section 2.4.5) and from leaching profiles (section 2.4.3). Lactate dehydrogenase was used as the cytosolic marker, 5'nucleotidase as the plasma membrane marker and cytochrome C oxidase as the mitochondrial marker.

2.9.1 Lactate Dehydrogenase

Lactate dehydrogenase activity was measured by following the decrease in absorbance at 340nm as pyruvate is converted to lactate. The reaction mixture contained 50mM potassium phosphate buffer pH 7.4, 1mM sodium pyruvate and 0.1mM NADH. Reactions were carried out at 37^{o}C in a DU-7 spectrophotometer (Beckman Instruments Inc). 50 µl of appropriately diluted sample was added to 1ml of reaction mixture and the rate of decrease of absorbance at 340nm followed for 3 minutes. Endogenous rates were measured by the substitution of phosphate buffer for pyruvate. Activity was calculated from a molar extinction coefficient of 3220 M^{-1} cm^{-1} after the subtraction of the endogenous rate. One unit of enzyme activity is equivalent to 1 millimole of NADH converted per minute.

2.9.2 5'Nucleotidase

5'Nucleotidase activity was measured by monitoring the conversion of [2-^{3}H]-5'AMP to adenosine using ion-exchange chromatography according to the method of Christie and Ashcroft (1985). All reactions were carried out at 30^{o}C and at pH 8.0 for one hour. A reaction cocktail which contained the following was prepared :3mM magnesium chloride, 2mM 'cold' 5'AMP, 25mM Tris-HCl, 0.1mg/ml bovine serum albumin and 0.1µCi [^{3}H] 5'AMP (specific activity - 19 Ci/mmol).

Reactions were initiated by the addition of 50µl of sample to 50µl of reaction mixture in 1ml plastic microcentrifuge tubes. Duplicates of each sample were run simultaneously with duplicate boiled blanks (samples boiled for 2 minutes to inactivate the enzyme). After one hour's incubation at 30°C reactions were terminated by boiling for 2 minutes and then 400 µl of 'cold' adenosine (0.1mM) was added. Each sample was passed down a separate ion-exchange column containing 0.75ml QAE Sephadex A25 (pre-equilibrated in 20mM ammonium formate) and washed with 2ml 20mM ammonium formate. The eluent from each column was collected in a scintillation vial and counted in 20ml Optiphase safe (cf section 2.5).

Known amounts of [^{3}H]-5'AMP were also counted in 2ml 20mM ammonium formate and 20ml Optiphase safe in order to calculate the counting efficiency. Following subtraction of boiled blank values (approx 2000cpm) activity was expressed in units. One unit of enzyme activity equals 1 nmol 5'AMP converted per hour.

2.9.3 Cytochrome C Oxidase

Cytochrome C oxidase activity was determined by following the oxidation of reduced cytochrome C at a wavelength of 550nm (Cooperstein and Lazarow, 1950). Cytochrome C reagent was prepared containing 41µM cytochrome C, 1mM EDTA in 30mM potassium phosphate buffer pH 7.4. This reagent was then reduced by the addition of solid sodium dithionite until the absorbance was approximately 0.8. Reactions were initiated by addition of 50µl sample to 600µl reagent and the rate of reduction in absorbance (550nm) at 37°C monitored in a DU-7 spectrophotometer. The rate of oxidation of cytochrome C was calculated from a molar extinction coefficient of 19.7×10^{-3} M-1 cm-1 and expressed in units. One unit of enzyme activity is equivalent to 1 millimole of cytochrome C oxidised per minute.

2.10 STATISTICAL ANALYSIS

1) Standard Error (SE)

Standard errors values were calculated from the formula

$$SE = \frac{\bar{x}}{\sqrt{n}}$$

where $\bar{x}$ is the sample mean and n the number of observations.

2) Students t-test

A two-sample t-test was used to estimate the difference between two means. This analysis was carried out in the Department of Computing Science, Trent Polytechnic, Nottingham, using the MINITAB System (MINITAB Inc, Enterprise Drive, State College, PA16801, USA). The test statistic, t , was calculated from the formula.

$$t = \frac{(\bar{x}_1 - \bar{x}_2)}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

Where t is the value from a t-table corresponding to 95% confidence and degrees of freedom (df) as defined below, $\bar{x}_1$ and $\bar{x}_2$ are the sample means, S_1 and S_2 the standard deviations and n_1 and n_2 the number of observations in each group. This test does not assume the two groups of data to have equal standard deviations.

$$d.f. = \frac{((s_1^2/n_1) + (s_2^2/n_2))^2}{\frac{(s_1^2/n_1)^2}{(n_1 - 1)} + \frac{(s_2^2/n_2)^2}{(n_2 - 1)}}$$

3.0 TRANSGLUTAMINASE ACTIVITY AND ITS SUBCELLULAR DISTRIBUTION FOLLOWING RAT LIVER CARCINOGENESIS

3.1 INTRODUCTION

The evidence for the potential involvement of transglutaminase in carcinogenesis and tumour growth has already been reviewed in the introductory chapter (section 1.3.5). To summarise, low levels of transglutaminase activity have been found in various neoplastic tissues when compared to normal tissue activity. Furthermore this reduction appears to be accompanied by a redistribution of enzyme activity to the membrane fraction. These changes have been reported in a wide range of aetiologically different neoplasms including chemically transformed cells, ^{32}P induced sarcomas (Barnes et al., 1984, 1985) and virus transformed cells (Birckbichler, 1978). In this study chemically induced liver cancer (hepatocellular carcinoma) was used as a model system since this allows direct comparison with a normal cell type, the hepatocyte.

3.2 IDENTIFICATION AND CLASSIFICATION OF TUMOURS INDUCED WITH DEN AND 6-BT

3.2.1 Histology of Tumours

Stained sections of normal liver, tumour bearing liver and abnormal tissue (see section 2.3.2) were examined under the microscope. Tumours were diagnosed on the basis of normal liver architecture and were classified according to their origin. In both DEN and 6-BT groups approximately 70% of the animals were available for analysis at termination.

In the DEN groups 60% of the animals were found to have developed hepatocellular carcinomas at termination. The morphology of these tumours was variable, the main type being well-differentiated trabecular hepatocellular carcinomas. Some tumours exhibited an acinar structure and others appeared as dense sheets of cells. One tumour was comprised of a trabecular hepatocellular carcinoma with a cholangioma-like lesion attached. In addition, 10% of the animals were found to have haemangiosarcomas. Lung metastases were found in all rats bearing haemangiosarcomas and in one animal with multiple hepatocellular carcinomas.

In the single 6-BT group that was set up, 36% of the animals had cholangiomas at termination and 21% of the animals were found to have hepatocellular carcinomas. All tumours in this study were of an extremely small size (100-500 mg), thus limiting the number of experiments which could be carried out using this tissue. None of the animals in this group had detectable lung metastases.

Examples of normal liver, trabecular hepatocellular carcinoma, haemangiosarcoma and cholangioma are shown in Figures 3.1 A - D respectively.

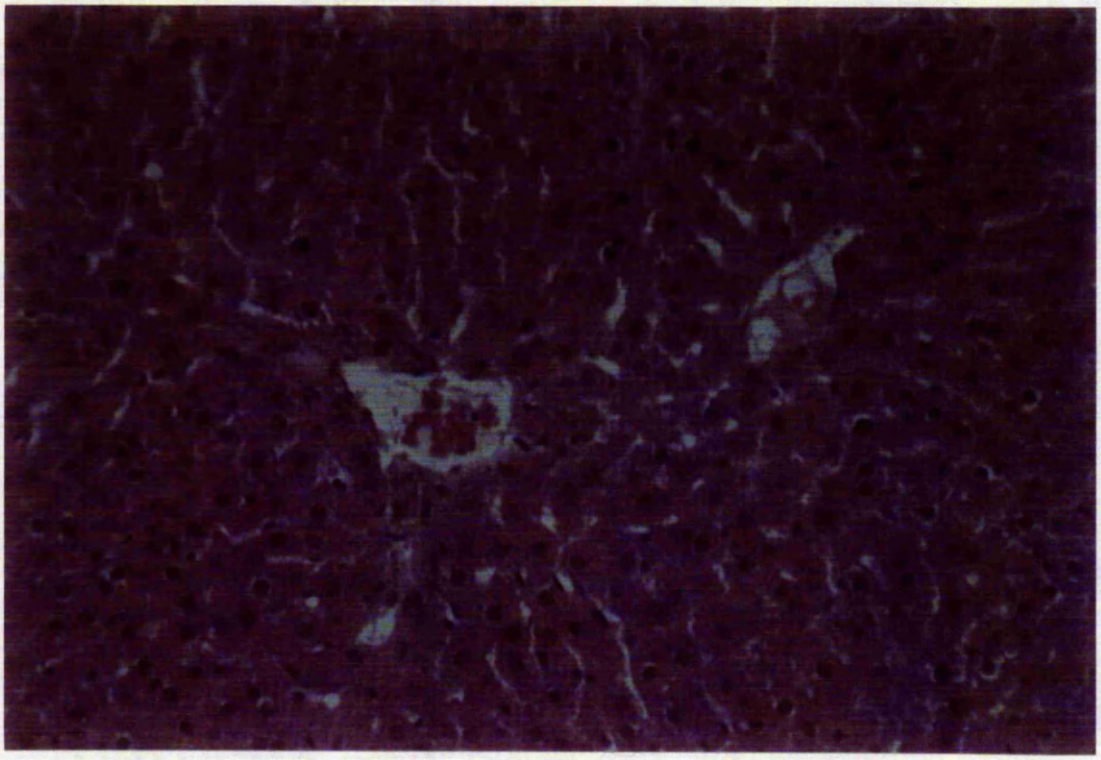
FIGURE 3.1

Histological Characterisation of Tumours

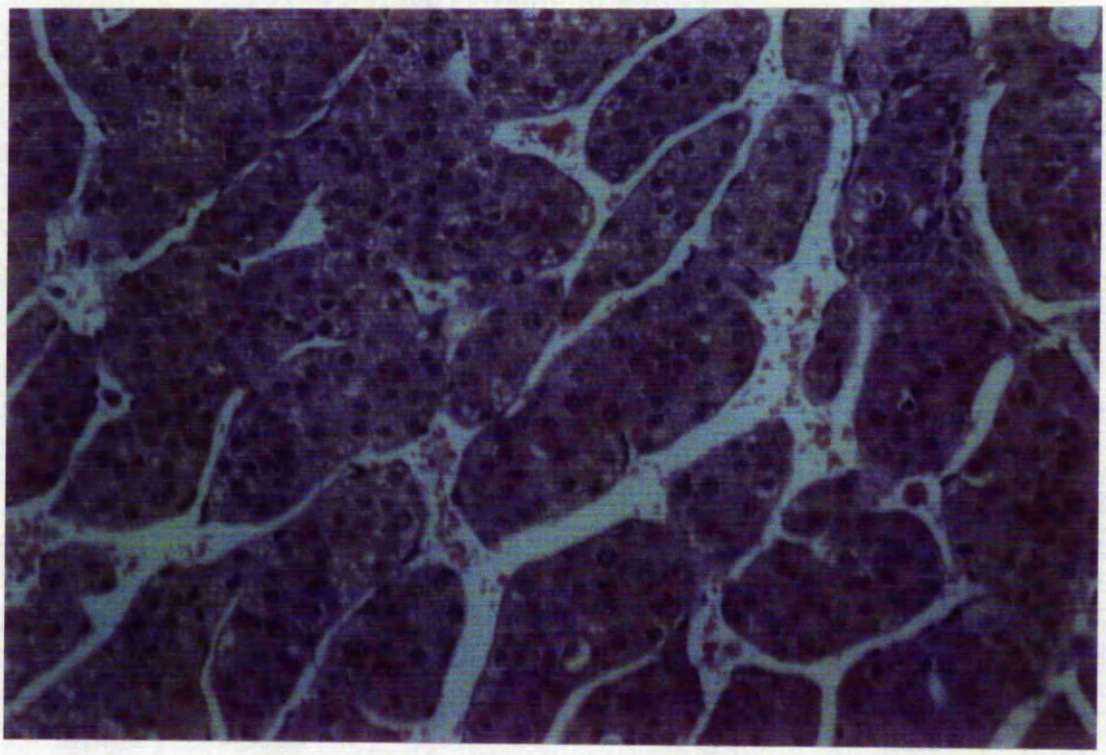
Induced by DEN in the Rat Liver

Tissues were stained with haematoxylin and eosin as described in section 2.3.2 and examined under the microscope: A normal liver, B hepatocellular carcinoma, C cholangioma, D haemangiosarcoma.

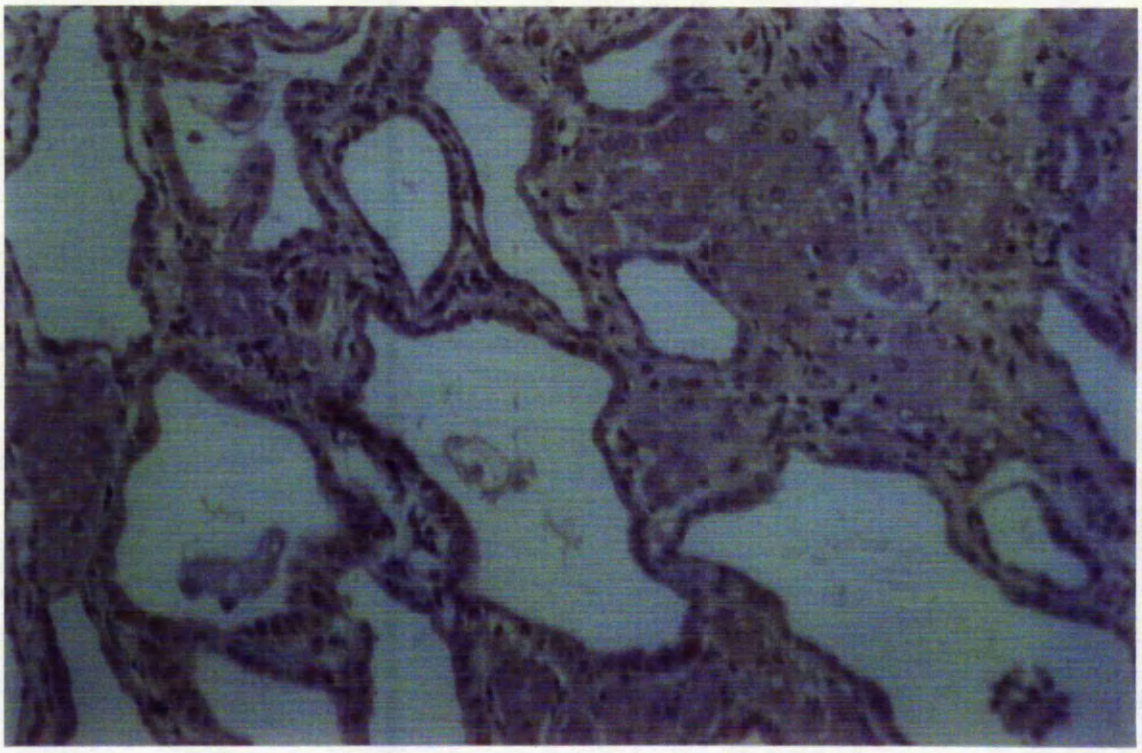
A Normal Liver (magnification x 2560)



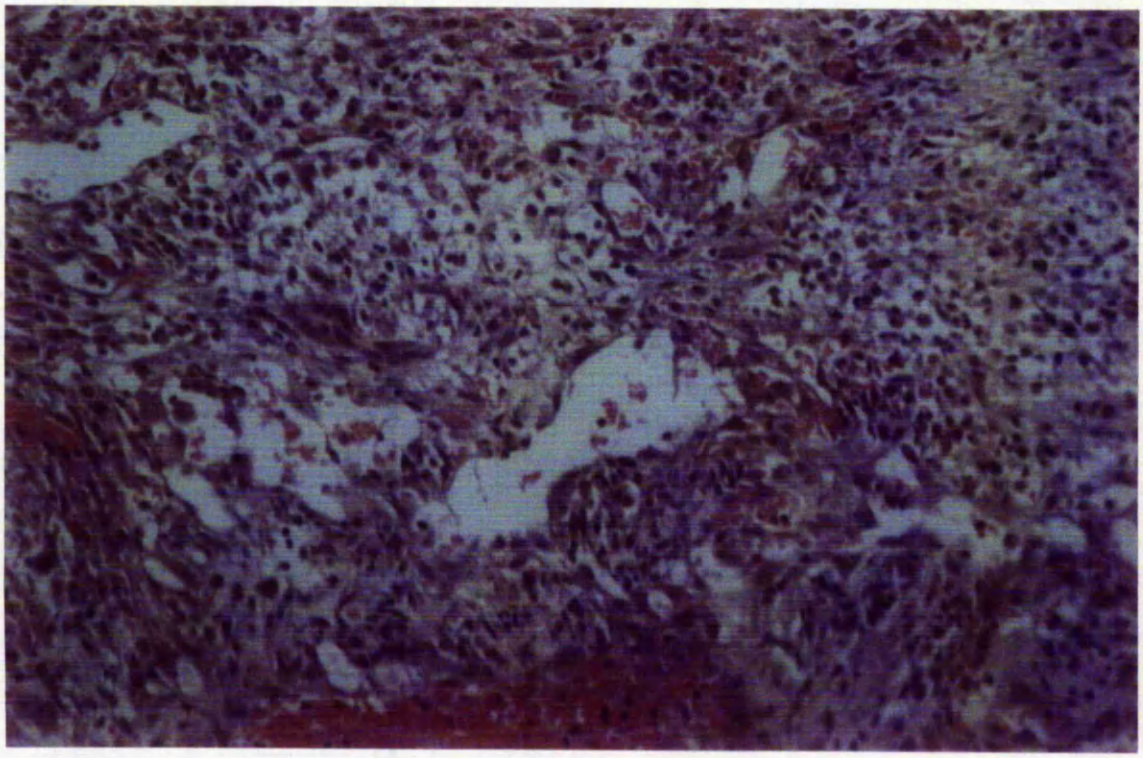
B.Hepatocellular Carcinoma (magnification x 2560)



C Cholangioma (magnification x 2560)



D Haemangiosarcoma (magnification x 2560)



The tumour bearing livers from both the DEN and 6-BT treated animals exhibited areas of altered vacuolated hepatocytes. This phenomenon was more pronounced in the 6-BT group where multiple cream coloured foci could be seen on the surface of all livers. In the DEN studies abnormal liver morphology could only be identified histologically.

3.2.2 Tumour Size and Mitotic Frequency

Tumour size (volume) and mitotic frequency were measured in some of the tumours in order to try to classify hepatocellular carcinomas according to their development or age. Protein and DNA content were also measured as references to correct for factors such as increased cell volume.

No correlation between tumour size and mitotic frequency was found in these tumours (Table 3.1). Protein and DNA content were relatively stable between individual tumours when expressed per gram weight of tissue and thus expression of the data as a function of these parameters could not account for the wide variation in either mitotic frequency or tumour size.

TABLE 3.1

Characterisation of Liver Tumours with respect to Tumour Type, Size, Mitotic Frequency, Protein and DNA Content

TUMOUR NO.	TUMOUR TYPE	SIZE (cm^3)	MITOTIC FREQUENCY	PROTEIN (mg/g)	DNA ($\mu g/g$)
10A	Hepatocellular Carcinoma	24.0	1.10	127.0	994.0
10B	Hepatocellular Carcinoma	0.8	2.90	167.0	1339.1
14A	Hepatocellular Carcinoma	7.1	1.10	155.3	1311.0
23A	Mixed Hepatocellular Carcinoma/Cholangioma	25.4	0.70	166.5	1051.4
23B	Hepatocellular Carcinoma	35-40	3.10	176.3	1148.0
23C	Hepatocellular Carcinoma	39.3	0.70	172.1	1198.0

Mitotic frequency was estimated by counting the number of cells undergoing mitosis for ten fields of view. Protein and DNA were measured by the methods of Lowry et al.,(1951) and Burton,(1956) respectively.

3.2.3 Discussion

DEN was found to be the most effective carcinogen under the experimental regime used. The tumours induced by DEN were mostly hepatocellular carcinomas and were of a size suitable for both histological and biochemical analysis. 6-BT, however, was found to yield much smaller tumour masses which in this study were mostly non-parenchymal in origin.

Attempts to further characterise the hepatocellular carcinomas into stages of development or growth were impractical. The large disparity between mitotic frequency values (a measure of the rate of growth) and tumour size showed that these tumours were a heterogenous group. Further attempts to correlate these factors with other measured parameters such as transglutaminase activity (see Table 3.4) and polyamine levels (see Table 5.3) also proved unsuccessful.

The development of large numbers of cholangiomas in the 6-BT group was unexpected. These tumours are derived from the biliary duct system of the liver and are not typically found in studies with this carcinogen (Elliott et al., 1983). The haemangiosarcomas on the other hand were of interest since these tumours are highly malignant and metastatic (Zimmerman, 1978). These tumours are derived from the vascular endothelium.

Finally the observation that 6-BT livers proved to have more frequent and sizeable areas of altered, vacuolated hepatocytes than those livers dosed with DEN was of interest, since later studies indicated some difference between these livers (see sections 3.2 and 3.3).

3.3 TRANSGLUTAMINASE ACTIVITY IN LIVER CARCINOGENESIS AND TUMOUR GROWTH

The comparison of biochemical parameters in normal tissue with those in tumours often depends on the use of whole organ homogenates as a control. In liver carcinogenesis it is generally accepted that whole rat liver is a good control for hepatocellular carcinomas. Hepatocytes represent 65% of the total liver cell population and owing to their large size (5000 μm^3), they represent 90-95% of the liver by weight (Weibel et al., 1969). However, since there have been no previous investigations into the distribution of transglutaminase activity within the liver cell population it was undertaken to verify that whole liver is a valid control for hepatocellular carcinomas by comparing transglutaminase activity in isolated hepatocytes and whole liver.

3.3.1 Measurement of Transglutaminase Activity in Whole Liver, Isolated Hepatocytes and Non-Parenchymal Cells

Transglutaminase activity was measured in whole liver, isolated hepatocytes and some non-parenchymal cells (Table 3.2). The method of isolation was that of Rubin et al., (1977) (section 2.4.7). Following perfusion with buffer 1 which freed the liver of blood, one lobe was clamped off as a sample of whole liver. The purified hepatocyte preparation was 98-99% pure and the non-parenchymal cell preparation consisted of approximately 40% endothelial cells and 60% Kupffer cells with some contamination by hepatocytes (R W Trueman - personal communication). Therefore any comparison of this non-parenchymal cell population with tumours derived from these cell types is only an approximation.

It was found that there was no difference between the transglutaminase activity present in whole liver, purified hepatocytes or the enriched sinusoidal cell preparation (Table 3.2). All three of these preparations having specific activities of approximately 30 Units/mg protein or 5 Units/µg DNA.

TABLE 3.2

Transglutaminase Activity in Whole Rat Liver, Isolated Hepatocytes and an Enriched Sinusoidal Cell Preparation

SAMPLE	Transglutaminase Activity	
	units/mg Protein	units/µg DNA
Whole Liver	30.4 ±1.5	5.5 ±0.3
Purified Hepatocytes	30.2 ±3.8	5.7 ±0.7
Enriched Sinusoidal Cell Preparation	30.6 ±0.6	5.2 ±0.1

Tissue was prepared as described previously (see sections 3.3.1 and 2.4.7). Whole perfused liver was homogenised by Potter-Elvehjem (section 2.4.1) in Merchants Solution (section 2.4.7.1). Purified Hepatocytes and the enriched sinusoidal cell preparation were homogenised in Merchants solution by sonication at an amplitude of 3µm using 3 x 30s bursts and cooling on ice for 1 minute between each burst. Transglutaminase activity was measured in all preparations using the method of Lorand et al., (1972) (see section 2.6.1) and protein determined by the method of Lowry et al., (1951) (section 2.5.1). Results are given as the mean values ±SE from three determinations.

3.3.2 Measurement of Transglutaminase Activity following DEN induced Carcinogenesis

Transglutaminase activity was measured in normal liver, hepatocellular carcinomas, haemangiosarcomas and tumour bearing livers (Table 3.3). The data shown are mean values expressed as a function of tissue weight, protein and DNA. In addition the activity in individual tumours and tumour bearing livers is shown in Table 3.4. This has been included primarily for comparison to other measured parameters (i.e. polyamine levels) which are presented later (section 5.2.3) but also for some comment here.

Hepatocellular carcinomas were found to contain reduced amounts of transglutaminase activity in comparison to normal liver (Table 3.3). When mean values were compared in a two sample t-test, this reduction was found to be highly significant ($P \leqslant 0.0001$) irrespective of how the data was expressed. It is however worthy of note that the mean protein content (mg/g tissue) of these tumours was reduced by 22% and that the mean DNA content (µg/g tissue) was increased by 25%. Thus the magnitude of the reduction in transglutaminase activity in hepatocellular carcinomas varied according to how it was expressed, being 56% when expressed per mg protein and 72% when expressed per µg DNA. Table 3.4 shows that all hepatocellular carcinomas had reduced transglutaminase activity when compared to the control mean activity. There was however considerable variation in transglutaminase activity within the tumour population; the smallest reduction occurring in tumour 10B (17% - units/mg protein) and the largest reduction occurring in tumour 65A (90% - units /µg DNA).

Comparison of the transglutaminase activity in hepatocellular carcinomas with that found in tumour bearing liver revealed that these tumours also contained reduced transglutaminase activity in comparison

to their own parent tissue. This reduction was statistically significant but the level of significance varied according to how the data was expressed; giving values of $P \leqslant 0.003$, $P \leqslant 0.047$, $P \leqslant 0.0013$ for units per gram tissue, mg protein and µg DNA respectively. Furthermore tumour bearing liver appeared to contain transglutaminase activity that was intermediate between that found in normal liver and hepatocellular carcinomas. The reduction (20-25%) was however not significant by a two sample t-test despite the fact that six out of a total of eleven tumour bearing livers had activity lower than the control mean activity (see Tables 3.3 and 3.4).

Finally, in the haemangiosarcomas it was interesting to note that the activity was even lower than in the hepatocellular carcinomas. This reduction was found to be statistically significant ($P \leqslant 0.0001$) when comparing the transglutaminase activity in haemangiosarcomas with that found in normal livers, hepatocellular carcinomas or tumour bearing livers, irrespective of how the enzyme activity was expressed. Since the reduction was much larger than in hepatocellular carcinomas, altered protein content (-27%) and altered DNA content (+12%) had less effect on the magnitude of this reduction which was 86% when expressed per mg protein and 90% when expressed per µg DNA or gram tissue.

TABLE 3.3

Transglutaminase Activity in DEN induced Tumours,

Tumour Bearing Liver and Normal Liver

TISSUE TYPE	Transglutaminase Activity		
	units/g tissue	units/mg protein	units/μg DNA
Normal Liver (8)	4160.0 ±198.0	23.3 ±1.3	4.3 ±0.3
Tumour Bearing Liver (11)	3329.1 ±477.0	18.2 ±3.4	3.2 ±0.4
Hepatocellular Carcinoma (17)	1446.7 ±201.7	10.2 ±1.3	1.2* ±0.2
Haemangiosarcoma(3)	432.9 ±94.4	3.3 ±0.5	0.4** ±0.1

Samples of tumour tissue, tumour bearing liver and normal liver were homogenised as described in section 2.4.1 and assayed for transglutaminase activity (section 2.6.1). Protein was determined by the method of Lowry et al., (1951) and DNA by the method of Burton, (1956). Results are shown as the mean values ±SE from several tissues. The number of tissues is given in parenthesis (*n=11, **n=12)

TABLE 3.4

Transglutaminase Activity in Individual Tumours and Tumour Bearing Livers

The methods for tissue preparation and the measurement of transglutaminase, protein and DNA are given in the legend to Table 3.3. All estimations were performed in duplicate and are presented as a mean value. They key to the tissue types is as follows:

HCC - hepatocellular carcinoma

CHOL - cholangioma

HAS - haemangiosarcoma

TBL - tumour bearing liver

In the column headed # numbers refer to animals and each letter represents a different tumour from that animal (i.e. 10A and 10B are two separate masses derived from the tumour bearing liver 10. (nd = not determined.)

TABLE 3.4

Transglutaminase Activity in Individual Tumours and Tumour Bearing Livers

#	TISSUE TYPE	TRANSGLUTAMINASE ACTIVITY		
		units/g Tissue	units/mg Protein	units/ g DNA
10A	HCC	2150.8	16.9	2.16
10B	HCC	3222.9	19.3	2.40
14A	HCC	2077.1	13.4	1.58
14B	HCC	1435.7	9.5	1.21
23B	HCC	811.7	4.6	0.71
23C	HCC	2515.0	14.6	2.10
61A	HCC	1574.0	11.4	1.27
65A	HCC	328.5	3.4	0.43
65B	HCC	2006.5	17.9	nd
65C	HCC	2010.9	16.0	nd
66A	HCC	559.2	4.6	0.50
79B	HCC	575.6	4.2	nd
80A	HCC	421.6	7.0	0.40
80B	HCC	1237.1	8.7	nd
84A	HCC	545.0	3.6	0.56
84B	HCC	1778.1	13.3	nd
87A	HCC	1343.4	5.2	nd
23A	HCC/CHOL	2029.8	12.2	1.93
61B	HAS	344.4	2.7	0.31
66C	HAS	332.8	2.8	nd
79A	HAS	621.5	4.4	0.51

/Table cont'd overleaf

TABLE 3.4 cont'd

#	TISSUE TYPE	TRANSGLUTAMINASE ACTIVITY		
		units/g Tissue	units/mg Protein	units/ g DNA
10	TBL	5790.0	40.9	5.01
14	TBL	3703.3	23.3	3.39
23	TBL	3398.3	20.5	2.97
61	TBL	2508.5	12.5	2.58
65	TBL	2269.6	11.1	2.21
66	TBL	2115.0	12.2	2.39
79	TBL	1821.8	9.1	1.66
80	TBL	3279.6	13.1	3.13
84	TBL	1446.0	6.0	1.61
87	TBL	3883.6	15.3	4.15

3.3.3 Measurement of Transglutaminase Activity Following 6-BT Induced Carcinogenesis

Transglutaminase activity was measured in normal liver, hepatocellular carcinomas, cholangiomas and tumour bearing livers (Table 3.5). The data is expressed as mean values per unit weight of tissue and protein but not DNA. The measurement of DNA content was not feasible for these tumours owing to their small size (see section 3.2.1).

In hepatocellular carcinomas transglutaminase activity was reduced (76% or 62%) when compared to normal liver if expressed per unit weight of tissue or protein respectively. This discrepancy could be accounted for by a 36% reduction in protein content of these tumours when compared to normal liver. The reduction in transglutaminase activity was significant in a two sample t-test ($P \leqslant 0.0007$ and $P \leqslant 00.032$ respectively).

Tumour bearing liver was also found to contain reduced transglutaminase activity when compared to normal liver. This reduction was less than that seen in hepatocellular carcinomas but was highly significant ($P \leqslant 0.0001$) irrespective of how the data was expressed. The transglutaminase activity in this tissue therefore appeared to be intermediate between normal liver and hepatocellular carcinomas. Statistical comparison of tumour bearing liver and hepatocellular carcinomas revealed that there was a significant difference when activity was expressed per gram tissue ($P \leqslant 0.026$) but not when activity was expressed per mg protein. A possible reason for this is the small sample number of hepatocellular carcinomas used (n=3) which is not suitable for this type of statistical analysis.

The cholangiomas also had significantly reduced levels of transglutaminase activity when compared to normal liver ($P \leqslant 0.0001$) irrespective of how the data was expressed. The activity in these tumours was similar to that found in hepatocellular carcinomas being 72% or 52% of control activity when expressed per unit weight of tissue or protein respectively. Similarly the activity in these tumours was lower than that found in tumour bearing liver. Two sample t-tests showed that the difference between these tissues (tumour bearing liver and cholangiomas) was significant ($P \leqslant 0.009$ when expressed per g tissue and $P \leqslant 0.022$ when expressed per mg protein). These differences in the magnitude and significance of the reduced transglutaminase activity are directly attributable to a reduction in the protein content of both cholangiomas (33%) and tumour bearing livers (12%).

TABLE 3.5

Transglutaminase Activity in 6-BT Induced Tumours, Tumour Bearing Liver, and Normal Liver

TISSUE TYPE	Transglutaminase Activity	
	units/g Tissue	units/mg Protein
Normal Liver (9)	6405.0 ±288.0	30.0 ±0.7
Tumour Bearing Liver (7)	3285.7 ±371.7	17.5 ±1.8
Hepatocellular Carcinomas (3)	1548.0 ±416.0	11.4 ±3.4
Cholangiomas (8)	1816.0 ±283.0	12.8 ±1.5

Samples of normal liver, tumour bearing liver and tumours were homogenised as previously described (section 2.4.1). The homogenates were then assayed for transglutaminase activity (see section 2.6.1). Protein was determined by the method of Lowry et al., (1951). Results are given as mean values ±SE with the number of tissues in parenthesis.

3.3.4 Discussion

The measurement of transglutaminase activity in whole liver and isolated hepatocytes confirmed that whole rat liver homogenates are a valid control tissue for comparisons with the hepatocellular carcinomas in these experiments (Table 3.2). The activity in whole liver and isolated hepatocytes was also found to be comparable to the normal liver activities in subsequent experiments (Tables 3.3 and 3.5).

In both DEN and 6-BT induced hepatocellular carcinomas, transglutaminase activity was shown to be substantially lower than in either normal liver or tumour bearing liver. The magnitude of this reduction varied according to how the data was expressed but was significant in all cases when assessed by a two sample t-test. The variation in the size of the reduction and thus in its level of significance could be accounted for by alterations in the protein and DNA content of the tumours or the tumour bearing liver. Unfortunately there was no single factor which could be reliably used as a reference for comparison of the tumours with normal tissues. Hence, all data were expressed as a function of tissue weight, protein and DNA. The first (units/g wet weight of tissue) would be subject to fluctuations in cell density and volume and the latter two would be subject to differences in protein and DNA synthetic activity in the tissues. Whilst these changes have not directly been demonstrated to occur in these experiments, they are certainly sufficiently well recorded to merit caution when assessing a potential marker of tumour growth such as transglutaminase. However, since transglutaminase activity was significantly and consistently reduced in hepatocellular carcinomas when compared to normal liver, irrespective of how the data was expressed, it appears that transglutaminase may be a useful marker for liver carcinogenesis and tumour growth.

Furthermore it was seen that transglutaminase activity in tumour bearing liver was intermediate between normal liver activity and tumour activity. However, since measurements of this kind are from a gross cell population it is unclear whether this represents a reduction in activity in altered 'pre-neoplastic' hepatocytes or is more directly related to toxic effects of the carcinogens. For instance alkylation of RNA can result in reduced protein synthetic activity (Zimmerman, 1978). The observation that this reduction was both consistent and statistically significant in 6-BT treated livers and was less apparent in DEN treated livers correlated with the observation that foci of altered hepatocytes were larger and more numerous in 6-BT treated livers than in DEN treated livers. The isolation of such foci would be necessary to confirm this and would be of interest in studying the development of these carcinomas although it was not attempted in this study.

The cholangiomas and haemangiosarcomas also provide some interesting circumstantial evidence for the involvement of transglutaminase in the carcinogenic process. The measurement of transglutaminase activity in the sinusoidal cell preparation (Table 3.2) affords a rough idea of the normal levels of transglutaminase in endothelial cells from which haemangiosarcomas are derived and thus the extremely low enzyme activity in this tumour type may possibly be attributed to carcinogenesis. Similar comparisons with cholangiomas were not possible due to the fact that the stromal matrix (which includes bile duct cells) was removed at an early stage in the hepatocyte isolation in an undigested form (see section 2.4.7). Nevertheless, the observation that the highly malignant and metastatic haemangiosarcomas contained less transglutaminase activity than either the hepatocellular carcinomas or the benign cholangiomas supports the

theory that transglutaminase may be involved, by virtue of such a reduction, in malignancy and the onset of metastasis (Barnes et al., 1984, 1985, Delcros et al., 1986, Hand et al., 1987, Roch et al., 1987).

It is also possible that the levels of transglutaminase activity in some tumours may be lower than reported here, due to the presence of blood which remained within the tumours. Despite care to remove clotted blood and the exsanguination of the livers via the heart, some blood remained within the tissue and therefore it is likely that some of the measured transglutaminase activity could be accounted for by the presence of Factor XIII or the red blood cell transglutaminase. This was particularly evident for haemangiosarcomas where large amounts of blood were associated with the tumour masses. Additionally, many tumours exhibited substantial mononuclear cell infiltration. This may also have contributed to the transglutaminase activity since cells of this type contain transglutaminase (i.e. monocytes, lymphocytes and granulocytes - Berntorp et al., 1985) and furthermore, monocytes and macrophages have been shown to contain very high levels of transglutaminase when stimulated (Moore et al., 1984, Mehta & Lopez-Berestein, 1986).

In conclusion, these data confirm the results of other workers (Barnes et al., 1985, Birckbichler, 1976), by showing transglutaminase activity to be lower in neoplastic tissue than it is in normal tissue. In addition, haemangiosarcomas were found to have lower transglutaminase activity than other less malignant tumours, a finding which is in agreement with the suggested importance of reduced transglutaminase activity in malignancy and metastasis (Barnes et al., 1984, 1985, Delcros et al., 1986, Hand et al., 1987, Roch et al., 1987). In tumour bearing liver it was found that transglutaminase activity was intermediate between normal and neoplastic tissue activities. However

in DEN treated animals this reduction was not found to be ubiquitous, whereas in 6-BT treated animals all tumour bearing livers had lower activity than normal liver. It is suggested that this may be due to differences in the number and size of pre-neoplastic foci of altered hepatocytes.

3.4 SUBCELLULAR DISTRIBUTION OF TRANSGLUTAMINASE IN LIVER CARCINOGENESIS AND TUMOUR GROWTH

The subcellular distribution of transglutaminase has been frequently reported as being bimodal (Barnes et al., 1985). In tumour tissue it has been found that in addition to the reduction in total enzyme activity there is an apparent redistribution of the enzyme activity to the membrane fraction (Barnes et al., 1985, Birckbichler, 1976). However, the recent discovery of a particulate cellular transglutaminase in mammalian liver (Chang and Chung, 1986, Slife et al, 1985) casts some doubt on the nature of this redistribution. The following experiments were therefore carried out in order to further investigate the nature of this apparent redistribution of transglutaminase activity. In these experiments tissue homogenates were fractionated into a particle free supernatant and a 71,000g pellet fraction and each fraction was assayed for transglutaminase activity and protein content. Since whole rat liver was used as a control in these experiments the distribution of transglutaminase in whole rat liver and isolated hepatocytes was also measured to confirm that the whole rat liver may be used as a valid control for comparisons with hepatocellular carcinomas.

3.4.1 Subcellular distribution of Transglutaminase Activity in Whole Liver and Isolated Hepatocytes

Homogenates of whole rat liver and isolated hepatocytes (98-99% pure) were fractionated into a particle free supernatant (S) and 71,000g pellet (P) and transglutaminase activity measured in each fraction (Table 3.6). It was found that whole liver contained a slightly higher percentage of activity in the pellet fraction than the isolated hepatocytes. Nevertheless, significant amounts of pellet associated activity were found in both preparations, thus indicating that both the cytosolic and the pellet associated activities occur in hepatocytes and that whole rat liver is a valid control for comparisons with hepatocellular carcinomas.

TABLE 3.6

Distribution of Transglutaminase Activity

in Whole Rat Liver and Isolated Hepatocytes

SAMPLE	TRANSGLUTAMINASE ACTIVITY			
	units/mg original protein		% Distribution	
	S	P	S	P
Whole Liver (3)	27.6 ±1.6	15.5 2.2	64.0 ±1.6	36.0 ±1.6
Purified Hepatocytes (3)	30.7 ±1.0	11.1 ±0.53	73.5 ±2.9	26.5 ±2.9

Homogenates of whole perfused liver and isolated hepatocytes were prepared and homogenised as described previously (see section 2.4.7 and the legend to Table 3.2). These homogenates were then fractionated into supernatant (S) and 71,000g pellet (P) fractions as described in section 2.4.2. Transglutaminase activity was measured by the method of Lorand et al., (1972) (section 2.6.1) and protein by the method of Lowry, (1951) (Section 2.5.1). Results are shown as the mean values ± S.E. from three determinations.

3.4.2 Subcellular Distribution of Transglutaminase Activity following DEN Induced Liver Carcinogenesis

Homogenates of normal livers, tumour bearing livers and hepatocellular carcinomas induced by DEN were fractionated by centrifugation into a particle free supernatant (S) and a 71,000g pellet fraction (P) (see section 2.4.2). Transglutaminase activity and protein were measured in these fractions and are presented as histograms of relative specific activity vs % protein distribution (figure 3.2). However since this representation does not take account of the total amount of enzyme activity in each fraction, these values are shown in Table 3.7.

From figures 3.2 A-C it can be seen that there is an apparent redistribution of enzyme activity from the supernatant to the particulate fraction in tumour bearing liver (12%) and hepatocellular carcinomas (44%) when compared to normal liver. In contrast the alterations in protein distribution were minor (hepatocellular carcinomas - 7%; tumour bearing liver - 3%) when compared to normal liver. However in hepatocellular carcinomas the apparent redistribution was clearly due to a large fall in the supernatant activity which was accompanied by a lesser reduction in the pellet activity (Table 3.7). In tumour bearing liver there was a smaller reduction in the supernatant activity which was mirrored by an increase in the pellet activity of similar amount.

It should, however, be emphasised that whilst normal livers and tumour bearing livers contained significantly higher amounts of activity than the hepatocellular carcinomas in both supernatant ($P \leqslant 0.0001$) and pellet fractions ($P \leqslant 0.05$), there were no significant differences between normal liver and tumour bearing liver. Furthermore, in the individual tumours it was found that whilst the supernatant

activity was always reduced when compared to normal liver, the pellet activity was not. Indeed the pellet activity was only reduced when the cytosolic activity was at the lowest levels (45-75 units/g wet weight) thus suggesting that the cytosolic activity may be reduced prior to a reduction in the pellet associated activity. This was supported by the observation that in tumour bearing liver the only reductions seen were in the cytosolic activity.

FIGURE 3.2

Distribution of Transglutaminase Activity in Normal Livers, Tumour Bearing Livers and Hepatocellular Carcinomas following DEN Induced Liver Carcinogenesis

Homogenates of normal livers, tumour bearing livers and hepatocellular carcinomas were prepared and fractionated according to the procedures given in sections 2.4.1 and 2.4.2. Transglutaminase activity was measured according to the method detailed in section 2.6.1 and protein content determined by the method of Lowry et al., (1951). Transglutaminase activity and protein were expressed as the percentage in each fraction and plotted as histograms of relative specific activity (% distribution of transglutaminase activity/% distribution of protein) vs % distribution of protein.

The number of tissue samples examined is given in parenthesis.

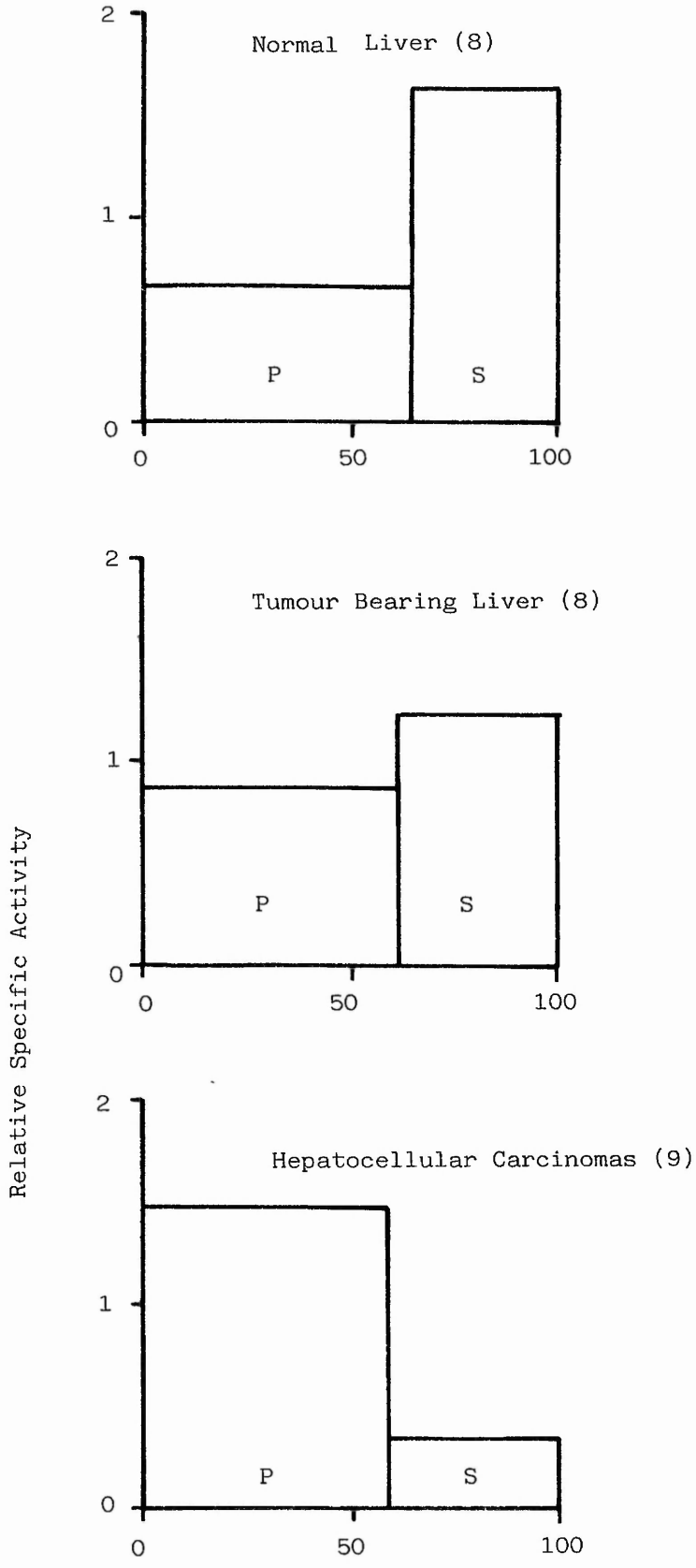

Normal Liver (8)
Tumour Bearing Liver (8)
Hepatocellular Carcinomas (9)
P
S
2
1
0
0
50
100
Relative Specific Activity
% Distribution of Protein

TABLE 3.7

Transglutaminase Distribution in Normal Livers, Tumour Bearing Liver and Hepatocellular Carcinomas following DEN Induced Carcinogenesis

TISSUE	Transglutaminase Activity (units/g original Tissue)	
	S	P
Normal Liver (8)	3095 ± 435	2170 ± 204
Tumour Bearing Liver (8)	2344 ± 333	2652 ± 344
Hepatocellular Carcinoma (9)	251 ± 110	1408 ± 269

See legend to figure 3.2 for methodology. In this case transglutaminase activity was expressed in units/g original tissue in each fraction. Results are given as the mean values ± SE with the number of tissue samples in parenthesis.

3.4.3 Subcellular Distribution of Transglutaminase following 6-BT Induced Liver Carcinogenesis

Homogenates of normal livers, tumour bearing livers and hepatocellular carcinomas were fractionated by centrifugation into a particle free supernatant (S) and a 71,000g pellet (see section 2.4.2). Transglutaminase activity and protein were measured in these fractions and are presented here as histograms of relative specific activity vs % distribution of protein (Figure 3.3) and also in units/gram original tissue (Table 3.8).

Figures 3.3 A-C revealed a similar pattern of distribution to that found in DEN carcinogenesis (see previous section). There was a significant redistribution of transglutaminase activity to the pellet fraction in both cholangiomas (46%) and tumour bearing liver (20%) when compared to normal liver, whereas the alterations in protein distribution were minor (cholangiomas - 4%; tumour bearing livers 2%). Examination of the total amounts of enzyme in each fraction (Table 3.8) showed that the apparent redistribution was caused by both a large fall in supernatant activity and a small reduction in the pellet activity. In tumour bearing livers the redistribution was caused by a substantial reduction in the supernatant activity and a small rise in the pellet activity (Table 3.8). The alterations in pellet activity were not statistically significant when normal livers were compared to tumour bearing livers. However tumour bearing livers and cholangiomas both had significantly lower amounts of supernatant activity than normal livers ($P \leqslant 0.0004$ and $P \leqslant 0.05$ respectively). Also, close examination of the distribution of activity observed in individual tumours and tumour bearing livers showed that the pellet activity was only reduced below the range of values found in normal liver when the supernatant activity was greatly reduced. Furthermore, in some tumours and all the tumour

bearing livers where the supernatant activity was only reduced partially, the pellet activity was always found to be within the normal liver range.

FIGURE 3.3

Distribution of Transglutaminase Activity in Normal Livers, Tumour Bearing Liver, and Cholangiomas following 6-BT Induced Liver Carcinogenesis

Homogenates of normal livers, tumour bearing livers and cholangiomas were prepared and fractionated according to the procedures given in sections 2.4.1 and 2.4.2. Transglutaminase activity was measured according to the method shown in section 2.6.1 and protein content determined by the method of Lowry et al., (1951). Transglutaminase activity and protein were expressed as the percentage in each fraction and plotted as histograms of relative specific activity (% distribution of transglutaminase activity /% distribution of protein) vs % distribution of protein.

The number of tissue samples examined is given in parenthesis.

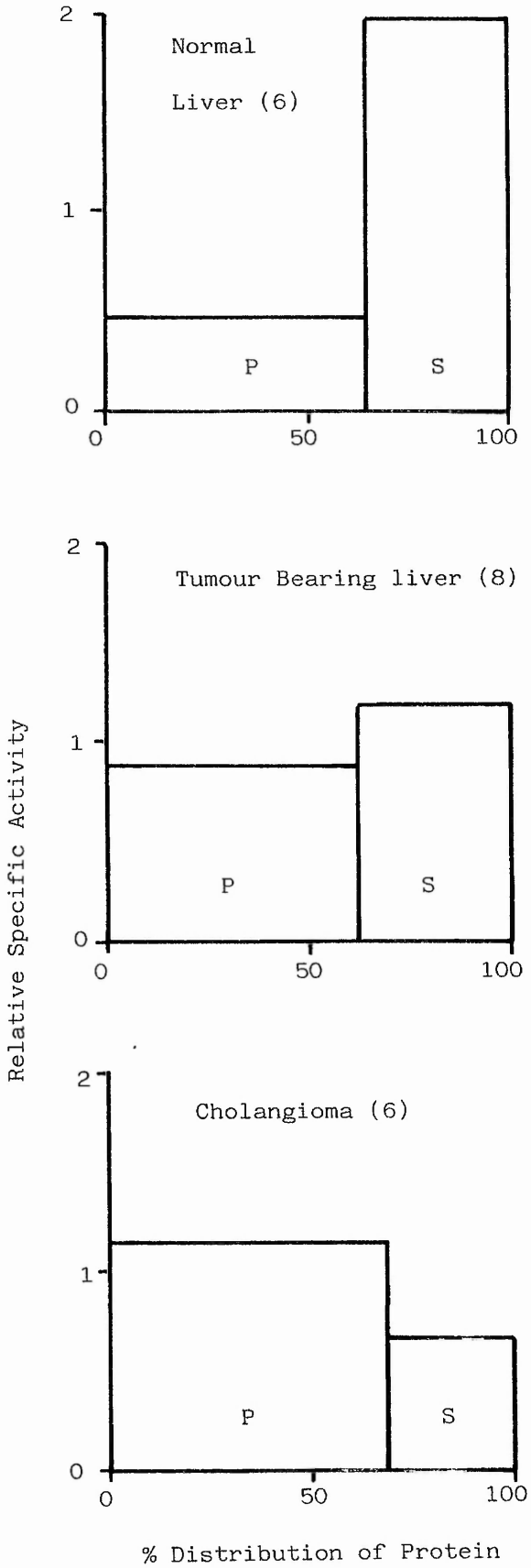

Normal
Liver (6)
P
S
Tumour Bearing liver (8)
P
S
Cholangioma (6)
P
S
2
1
0
0
50
100
Relative Specific Activity
% Distribution of Protein

TABLE 3.8

Transglutaminase Distribution in Normal Livers, Tumour Bearing Livers and Cholangiomas following 6-BT Induced Carcinogenesis

TISSUE	Transglutaminase Activity (units/g original Tissue)	
	S	P
Normal Liver (6)	5127 ± 521	2128 ± 206
Tumour Bearing Liver (8)	2461 ± 572	2420 ± 170
Cholangiomata (6)	491 ± 149	1478 ± 305

See legend to figure 3.3. for methodology. Results here are expressed in units/gram original tissue and are given as the mean values ± SE with the number of tissues in parenthesis.

3.4.4 Discussion

Comparison of whole liver with isolated hepatocytes indicated that the distribution of transglutaminase in these tissues was similar. The small difference that was found could be attributed to either the different homogenisation procedures used or a higher proportion of particulate activity in non-parenchymal cell types. Since non-parenchymal cell types were not examined in this context it is not possible to deduce the reason for the observed difference in distribution between whole liver and isolated hepatocytes, although different organs (and therefore different cell types) have been shown to contain widely different distributions of transglutaminase activity (Griffin et al., 1978, Barnes et al., 1984). Nevertheless, it would appear that whole rat liver is an appropriate control tissue that may be used as a comparison with hepatocellular carcinomas.

In hepatocellular carcinomas (DEN induced) and cholangiomas (6-BT induced) the transglutaminase activity was mostly found in the 71,000g particulate fraction. Histograms of relative specific activity vs % distribution of protein showed that there was a redistribution of activity from the supernatant to the pellet when comparing tumours to normal livers, however this was due to a large decrease in supernatant activity (90%) which was accompanied by a smaller decrease (30-35%) in pellet activity. These changes therefore give the appearance of a 'redistribution' of enzyme activity although it is clear that this is due to a specific reduction of enzyme activity in the cytosol which may be accompanied by a fall in enzyme activity associated with the particulate fraction when the supernatant activity is low enough.

In the tumour bearing liver of both DEN and 6-BT treated animals there was a redistribution of activity to the pellet fraction intermediate between that found in normal liver and tumour tissue. This redistribution was most apparent in 6-BT treated livers and could tentatively be attributed to a higher occurrence of pre-neoplastic foci of altered hepatocytes in 6-BT treated livers (see section 3.2.1).

Examination of the individual data for both tumours and tumour bearing livers revealed that the particulate associated activity only fell when there were minimal amounts of activity in the supernatant fraction (45-75 units/g original tissue). It may therefore be concluded that an initial fall in the activity in the cytosol is a prerequisite to the reduction in transglutaminase activity seen in the particulate fraction. Alternatively it is possible that the enzyme found in the cytosol becomes preferentially bound to the particulate fraction of the tumour cell (in a latent form). This particulate associated activity would only be reduced when there was insufficient enzyme present in the cytosol to saturate the particulate fraction.

An additional factor which became apparent during the course of these experiments is that the sum of the transglutaminase activity from the cytosol and particulate fractions was always greater (approx +20%) than the transglutaminase activity measured in the homogenates (compare Tables 3.3 with 3.7 and 3.5 with 3.8). A variety of factors may be responsible for this: the presence of inhibitors and physiological substrates for transglutaminase that are present in homogenates could interfere with the assay of enzyme activity (or with its ability to interact with the membrane fraction). These factors would become diluted out in fractionation experiments, thus resulting in less inhibition in the transglutaminase assay. Alternatively, this discrepancy may be due to the release of sequesterred enzyme from the

particulate fraction. The idea of a sequesterred or latent form of transglutaminase being favoured by some workers (Birckbichler et al., 1976). However, owing to recent reports of a novel particulate transglutaminase in liver (Chang and Chung, 1986) it is now clear that the 'particulate activity' described in this section may well be comprised of more than one type of transglutaminase.

The following experiments were therefore carried out in order to further investigate the association of transglutaminase with the particulate fraction and to attempt to distinguish between the soluble and particulate forms of transglutaminase in normal liver and hepatocellular carcinomas.

3.5 THE ASSOCIATION OF TRANSGLUTAMINASE WITH THE PARTICULATE FRACTION OF NORMAL LIVER AND HEPATOCELLULAR CARCINOMA TISSUE

In the previous section the results indicated that the observed reduction in transglutaminase activity in hepatocellular carcinomas and the apparent redistribution to the particulate fraction of the cell may be a result of sequestration of the enzyme in the particulate fraction of the cell resulting in an inactivation of enzyme activity.

In order to clarify whether the apparent redistribution of transglutaminase activity was in fact due to the altered binding characteristics of the tumour membranes for the enzyme or due to a selective reduction in the cytosolic activity the following experiments were carried out. A leaching procedure was employed for this evaluation (section 2.4.3). The methodology utilises an initial homogenisation in an isotonic sucrose buffer to gain an estimation of the free cytosolic activity; thereafter, non-isotonic conditions are used to remove as much transglutaminase activity as possible. The tumours used were DEN induced hepatocellular carcinomas.

3.5.1 The Leaching of Transglutaminase and Marker Enzymes from Normal Liver

Tissue was fractionated as detailed in section 2.4.3, and transglutaminase activity measured in all fractions. In addition a plasma membrane marker (5'nucleotidase), a cytosolic marker (lactate dehydrogenase) and protein (Table 3.9) were also measured in each of the fractions.

A large proportion of the transglutaminase activity was found to be leached out of the particulate fraction using this procedure.

However, a small but significant amount (5.2%) remained tightly associated with the particulate fraction. The use of an extraction buffer containing Lubrol-PX, a nonionic detergent did not release any additional enzyme activity, therefore confirming that this enzyme activity is tightly associated with the particulate fraction (see section 3.6). In the first (isotonic) wash (S_1) large amounts of transglutaminase (68.2%) and lactate dehydrogenase (91%) were released from the pellet whereas only 2.8% of the 5'nucleotidase was released. This suggests that a substantial proportion of transglutaminase activity is cytosolic but that the remaining 30% is either peripherally associated with the particulate fraction by adsorption or may truly be particulate in nature. The non-isotonic washes (S_2 + S_3) removed nearly all of the lactate dehydrogenase from the particulate fraction but left significant proportions of transglutaminase activity (5.2%) and 5'nucleotidase (32.5%) associated with the particulate fraction. It seems reasonable to assume that this remaining transglutaminase activity is particulate, especially since a large amount of 5'nucleotidase was also leached out of the membranes, a fact which demonstrates the harshness of these washing conditions. If the amount of 5'nucleotidase present in the washes was used as a marker of the amount of membrane-bound enzyme that would be freed from the membrane during this leaching procedure, then by extrapolation it was possible to calculate the amount of this enzyme that was present in normal liver.

The calculation used is shown below:

$$\frac{\text{Transglutaminase Activity in Final Pellet (units/g original tissue)}}{\text{\% of Total 5'nucleotidase Activity in Final Pellet}} \times 100$$

= Particulate Transglutaminase Activity in Homogenate (units/g tissue)

Using this assumption it was calculated that 16% of the total transglutaminase activity in normal liver was tightly associated with the particulate fraction of the cell.

TABLE 3.9

Percentage Distribution of Transglutaminase, Lactate Dehydrogenase, 5' Nucleotidase and Protein in Leached Fractions obtained from Normal Liver

Enzyme	Yield (% of total)			
	S_1	S_2	S_3	P
Transglutaminase (4)	68.2 ±3.6	20.7 ±2.3	5.9 ±1.5	5.2 ±1.4
Lactate (3) dehydrogenase	90.5 ±1.7	8.5 ±1.6	0.6 ±0.1	0.4 ±0.1
5'Nucleotidase (3)	2.8 ±0.3	62.6 ±2.2	2.1 ±0.1	32.5 ±1.9
Protein (3)	30.3 ±1.8	22.6 ±3.1	5.1 ±0.3	42.0 ±1.2

Normal liver was fractionated by the procedure detailed in Figure 2.1 (section 2.4.3). The following determinations were then carried out on each fraction: transglutaminase activity (section 2.6.1); lactate dehydrogenase activity (section 2.9.1); 5'nucleotidase activity (section 2.9.2) and protein (section 2.5.1). Results are shown as the mean percentage value ±SE in each fraction (100% = $S_1+S_2+S_3+P$). The number of tissues used for each determination are shown in parenthesis.

3.5.2 Comparison of the Association of Transglutaminase with the Particulate Fraction from Normal Liver and Hepatocellular Carcinomas

Normal liver and DEN induced hepatocellular carcinomas were fractionated as described in section 2.4.3. Transglutaminase activity was then measured in the resultant fractions (Table 3.10). The magnitude of the summed activities in the leached fractions and the final pellet was approximately 50% greater than the activity measured in the homogenate for both normal liver and hepatocellular carcinomas. The reduction in transglutaminase activity in hepatocellular carcinomas compared to normal liver was found to be the same (approximately 80%) irrespective of whether it was calculated from the original homogenate activity or the sum of the leached fractions and the final washed pellet. These results appear to suggest that transglutaminase does not bind more strongly to tumour membranes and that any interference with the measurement of enzyme activity either by soluble factors (such as inhibitors or endogenous substrates) or as a result of membrane adsorption does not affect the comparative measurement of this enzyme in homogenates of normal liver and hepatocellular carcinomas. Thus provided that normal liver and tumour tissue were treated in the same way and diluted to the same extent, the estimation of the reduction in transglutaminase activity in tumour tissue was unaffected.

It was also interesting to note that the amount of transglutaminase activity associated with the particulate fraction after leaching was comparable in tumours and control liver. This observation supports the idea that the particulate transglutaminase is not reduced in hepatocellular carcinomas, and that the major reduction of transglutaminase observed in hepatocellular carcinomas is due to a reduction in the cytosol enzyme.

TABLE 3.10

The Leaching of Transglutaminase Activity from the Particulate Fraction of Normal Liver and Hepatocellular Carcinomas

Tissue	Transglutaminase Activity (units/g Original Tissue)				
	Homogenate	S_1	S_2	S_3	P
Normal liver (4)	5931 ±978	5871 ±986	1776 ±59	507 ±26	448 ±130
Hepatocellular carcinoma (3)	1252 ±143	252 ±46	768 ±28	419 ±17	454 ±62

Normal livers and hepatocellular carcinomas were fractionated according to the regime shown in Figure 2.1 (see section 2.4.3). The resultant fractions: S_1, S_2, S_3 and P are described in Figure 2.1. Transglutaminase activity was measured in each fraction using the filter paper assay of Lorand et al., (1972) (section 2.6.1). Results are expressed as the mean values ± SE with the number of fractionations of each tissue given in parenthesis.

3.5.3 Discussion

The fact that only part of the total transglutaminase activity could be leached from the membrane fraction lends credence to the idea that there are two distinct types of cellular transglutaminase. Furthermore, studies with hepatocellular carcinomas indicated that only one of these, the cytosolic enzyme, is reduced in tumour growth. These results do not support the idea that altered associations between the membrane and transglutaminase are responsible for the reduction in transglutaminase activity observed in these tumours.

The homogenisation and fractionation of normal liver in an isotonic buffer resulted in the release of large amounts of both lactate dehydrogenase and transglutaminase, thus implying that this transglutaminase activity is cytosolic. The subsequent use of a harsher non-isotonic washing regime removed almost all of the remaining lactate dehydrogenase but left a significant amount of transglutaminase activity associated with the pellet. This tightly associated transglutaminase activity may be the particulate enzyme reported by Slife et al.,(1985) and Chang and Chung,(1986).

It was also clear from these studies that there was some underestimation of total transglutaminase activity when measured in tissue homogenates. However, since the magnitude of the reduction in transglutaminase activity in tumours was the same whether it was assessed from the homogenate activity or from the sum of the leached fractions and the final pellet, this underestimation appeared to have no effect on the comparison of normal liver with hepatocellular carcinomas. Also, although it is not possible to assess the ability of membranes to bind transglutaminase by these means it is unlikely that such binding contributes in any way to the reduction or apparent redistribution of transglutaminase activity in tumour tissue.

From these results it is clear that cytosolic transglutaminase activity is reduced in hepatocellular carcinomas of this type. However the evidence that particulate transglutaminase is unaffected in the same tumours is at best circumstantial. It was therefore decided to extract both transglutaminases from normal liver and hepatocellular carcinomas, separate and partially purify them and then to measure their relative amounts in these tissues.

3.6 CYTOSOLIC AND PARTICULATE TRANSGLUTAMINASE ACTIVITIES IN NORMAL LIVER AND DEN INDUCED HEPATOCELLULAR CARCINOMAS

The fractionation studies in sections previous to this have suggested that a significant amount of the total cellular transglutaminase activity measured in liver and hepatocellular carcinomas may be due to the particulate enzyme which was recently characterised by Chang and Chung,(1986). It was not possible, however, to accurately assess the relative proportions of cytosolic and particulate transglutaminase since marker enzyme studies indicated that particulate enzymes such as 5'nucleotidase may be leached into the supernatant fractions. It was therefore necessary to separate the two types of transglutaminase activity by other means. It was decided to extract both enzymes using a modification of the method of Chang and Chung,(1986) and then to separate them by ion-exchange chromatography. In this way it was possible to determine the relative amounts of the two enzymes in normal liver and tumour tissue.

3.6.1 Measurement of Cytosolic and Particulate Transglutaminase Activities in Normal Liver and DEN induced Hepatocellular Carcinomas

Transglutaminase activity was extracted from normal liver and hepatocellular carcinomas (DEN induced) as described in section 2.4.4. These extracts were then eluted on a Pharmacia Mono-Q anion-exchange HPLC column (Figure 3.4). The elution profiles for both normal liver and hepatocellular carcinomas showed two peaks, one eluting at 0.15-0.22M NaCl (P) and another at 0.28-0.45M NaCl (C). Typical examples of these elution profiles for normal liver and hepatocellular carcinomas are shown in Figures 3.4. Since the elution of an isotonic supernatant fraction on a Waters Associates DEAE 5-PW anion-exchange HPLC column revealed a single peak at 0.35-0.45M (see section 4.2.2) it seems likely that the second peak (C) is the cytosolic transglutaminase. This is corroborated by the work of Chang *et al*., (1986) who showed that the cytosolic transglutaminase eluted later than the particulate enzyme under similar conditions on a DE52 column. On comparing the hepatocellular carcinoma profiles with the normal liver profiles it was apparent that the cytosolic form eluting at 0.28-0.45M NaCl was greatly reduced and that the particulate form (0.15M-0.22M) was reduced to a much lesser extent. The active fractions for each peak were pooled and reassayed as shown in Table 3.11. In addition the activity in the pellet fraction (see section 2.4.4) was also assayed for transglutaminase activity.

Cytosolic transglutaminase activity (units/g original tissue) was markedly reduced in hepatocellular carcinoma extracts (92%) whereas the particulate activity was only reduced by 28.7% (Table 3.11). However if these activities were expressed per mg original protein then only the cytosolic form was reduced (89%).

Using the above methodology there is still a significant amount of activity unaccounted for, which is present in the particulate fraction. In view of the thorough extraction procedure used it seems likely that this is primarily particulate transglutaminase. On this assumption it is possible to calculate that in normal liver 15.5% of the total activity is particulate, a figure which is in close agreement with that calculated earlier (section 3.5.1) and also with that calculated by Slife et al.,(1985).

Finally it is perhaps pertinent to mention that comparison of the total activity (cytosolic + particulate + pellet) with the homogenate activity (data not shown) revealed that the total activity in both normal livers and hepatocellular carcinomas was some 40 - 50% higher than the homogenate activity. This underestimation of activity in tissue homogenates was of similar magnitude to that described in section 3.5 where non-detergent buffers were used.

Figure 3.4

Anion-Exchange Chromatography of Transglutaminase Extracted from Normal Liver and Hepatocellular Carcinomas with Lubrol-PX containing Buffers

Transglutaminase activity was extracted from normal liver and hepatocellular carcinomas with Lubrol-PX containing buffer as described in section 2.4.4. The extracted material was loaded via a Rheodyne injector (4ml) onto a Pharmacia Mono-Q column and eluted with a linear salt gradient from 0-0.5M NaCl over 30 min. Fractions (0.5ml) were collected and transglutaminase was then measured in these fractions using the assay detailed in section 2.6.3 with [^{3}H]-putrescine at a concentration of 20μM (Specific activity - 24.6 Ci/mmol). The elution profiles are representative separations of transglutaminase activity obtained from normal liver (o) and a hepatocellular carcinoma (●). The solid lines denote transglutaminase activity and the broken line shows the NaCl concentration in the fractions. C = cytosolic transglutaminase, P = particulate transglutaminase.

Transglutaminase Activity (CPM)
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
Time (min)
NaCl Concentration (M)
0.5
0.4
0.3
0.2
0.1
0
C
P

TABLE 3.11

Transglutaminase Activities in Normal Liver and Hepatocellular Carcinomas

Fraction	TRANSGLUTAMINASE ACTIVITY					
	U/g original Tissue		U/mg protein		% of Total Activity	
	NL	HCC	NL	HCC	NL	HCC
PEAK 2 (0.28-0.45M) (Cytosolic Transglutaminase)	5942 +766	475 ± 90	30.9 ±4.9	3.4 ±0.6	82.8 ±10.6	30.2 ±5.7
PEAK 1 (0.15-0.22M) (Particulate Transglutaminase)	454 ±72	324 ±51	2.4 ±0.3	2.4 ±0.4	6.3 ±1.0	20.6 ±3.2
Final Pellet Activity (Particulate Transglutaminase)	776 ±124	774 ±122	4.0 ±0.5	5.7 ±0.9	10.9 ±1.7	50.8 ±8.0

Data for cytosolic and particulate transglutaminase represent pooled fractions from a Pharmacia Mono-Q column (see Figure 3.4).

Transglutaminase Activity was measured by the filter paper assay of Lorand (Section 2.6.2).

Results represent the mean levels ± SE from three tissues of each type. NL = normal liver, HCC = hepatocellular carcinoma, Total Activity - peak 2 + peak 2 + final pellet activity.

3.6.2. Discussion

The presence of two different types of transglutaminase was demonstrated in both normal liver and hepatocellular carcinomas. These results therefore confirm those of Chang and Chung, (1986) whose investigations revealed the presence of a particulate enzyme in normal liver. In normal liver the cytosolic transglutaminase represents approximately 85% of the total transglutaminase activity, a figure which is in close agreement with the work of Barnes *et al.*, (1985) and Slife *et al.*, (1985) who also suggested that the particulate associated activity may be different to the cytosolic transglutaminase.

The reduction in transglutaminase activity seen in hepatocellular carcinomas was in the main found to be due to a reduction in the cytosolic transglutaminase. The smaller reduction in the particulate activity could be accounted for by the reduced protein content of these tumours. Such reductions in the protein content of DEN induced tumours have been reported previously (Magee, 1965, Zimmerman, 1978) and are thought to be due to methylation of RNA by DEN metabolites which results in reduced protein synthetic activity.

It is therefore proposed that in tumour growth of this type there is a sole reduction in the cytosolic enzyme and that the particulate enzyme is effectively unaltered. It also follows that the apparent redistribution of transglutaminase activity which has been reported (Barnes *et al.*, 1984, 1985; Birckbichler, 1976) is due to this same reduction and does not involve the increased storage or binding of transglutaminase at the plasma membrane.

Since the experiments in this chapter have identified a consistent and significant difference in the levels of cytosolic transglutaminase activity present in normal liver and liver tumours it

was decided to concentrate further studies on this enzyme. It is possible that the reduction of cytosolic transglutaminase observed in hepatocellular carcinomas is due to the production of a structurally altered and defective form of this enzyme rather than a lack of synthesis of enzyme molecules. With this idea in mind some basic molecular and enzymatic characteristics were measured for the cytosolic enzyme from both normal liver and hepatocellular carcinoma tissue. Furthermore, the association of this cytosolic enzyme with the particulate fraction of normal livers and hepatocellular carcinomas was investigated by comparison of the bound cytosolic enzyme with the free cytosolic enzyme in both tissues.

4.0 COMPARISON OF CYTOSOLIC TRANSGLUTAMINASE FROM NORMAL LIVER AND HEPATOCELLULAR CARCINOMAS AND ITS ASSOCIATION WITH THE PARTICULATE FRACTION OF THE CELL

4.1 INTRODUCTION

The experiments in this section were undertaken in order to address some important questions regarding the reduction in transglutaminase activity which was seen in hepatocellular carcinomas (see previous chapter). The first of these concerns the mechanism by which such a reduction may occur. Alterations in chromosomal DNA may lead to the production of a structurally abnormal enzyme molecule with reduced catalytic ability or alternatively, perturbations in the regulatory control of enzyme expression may have suppressed transglutaminase synthesis. It was therefore undertaken to characterise the cytosolic transglutaminase in hepatocellular carcinoma tissue and to compare it with the enzyme from normal liver. In the previous chapter the ion-exchange properties of these two enzyme sources were compared and found to be similar. This observation was confirmed using a different ion-exchange column which also served to semi-purify the enzyme activity for further analysis. The characteristics studied were molecular weight, elution on ion-exchange media, Michaelis-Menten constants for the incorporation of putrescine into N,N'-dimethylcasein and the sensitivity of the enzyme to activation by Ca^{2+}.

Additionally the nature of this enzyme's association with the particulate fraction of cells was investigated. It was unknown whether this activity was associated with the particulate fraction artefactually (by adsorbtion) or as a part of a physiological process involved in the regulation of enzyme activity. Indeed, since there is much evidence to suggest that the plasma membrane is the site of action of cytosolic transglutaminase (Barnes et al., 1985) it seems likely that interactions between cytosolic transglutaminase and the plasma membrane (or membrane proteins) play an important role in the regulation of

transglutaminase activity *in vivo*. It should, however, be emphasised that the enzyme under study in this chapter is the cytosolic transglutaminase and not the particulate enzyme described by Chang and Chung,(1986).

4.2 COMPARISON OF S_1 AND S_3 TRANSGLUTAMINASE FRACTIONS IN NORMAL LIVER AND HEPATOCELLULAR CARCINOMAS

Homogenates of normal liver and hepatocellular carcinomas were fractionated according to the procedure described in section 2.4.3. The supernatant fraction, S_1, was used as a source of free cytosolic activity and is referred to throughout this chapter as S_1 activity. The membrane-associated activity was obtained from the S_3 fraction and is referred to as S_3 activity. The presence of some particulate transglutaminase activity in these fractions necessitated the semi-purification of cytosolic activity prior to its use in kinetic studies. This semi-purification was achieved using ion-exchange chromatography as described in Section 4.2.2. However it is important to note that this particulate activity is a minor component of both S_1 and S_3 fractions and was not detected in chromatographic experiments due to the low sensitivity of the assay system used. It was therefore deduced that the majority of the particulate transglutaminase was in the S_2 fraction, a supposition which was supported by the leaching profile for 5'nucleotidase (Table 3.9).

4.2.1 Molecular Weight Analysis: Comparison of S_1 and S_3 Transglutaminase Fractions from Normal Liver and Hepatocellular Carcinomas

The comparison of S_1 and S_3 transglutaminase from both normal liver and hepatocellular carcinomas was achieved using high performance gel permeation chromatography according to the procedures detailed in section 2.8.2. Protein standards in the range of Mr 158,000 - 14,300 were used to calibrate the column (see Table 4.1a) and a plot of $\log_{10}$ molecular weight vs partition coefficient (Kav) constructed (Figure 4.1). This calibration plot was then used to estimate the

molecular weight of transglutaminase in each of the four fractions (see Table 4.1b). No significant differences were found between either S_1 and S_3 fractions or between normal liver and hepatocellular carcinoma. Indeed the molecular weight values were so close as to be well within the estimated experimental error for these experiments, which based on the fraction size of 250 µl was ±2000 daltons. These values are in agreement with the molecular weight for cytosolic transglutaminase reported by other workers (Conrad, 1985).

TABLE 4.1

Determination of the Molecular Weight of S_1 and S_3 Transglutaminase from Normal Liver and Hepatocellular Carcinomas

Calibration proteins (5 mg/ml) and S_1 and S_3 fractions of normal liver and hepatocellular carcinomas were chromatographed on a TSK G-3000 SW column as described in section 2.8.2. Elution volumes were calculated by peak triangulation and Kav calculated from the formula:

$$Kav = \frac{Ve-Vo}{Vt-Vo}$$

Where Ve is the elution volume, Vo the void volume (10.8ml) and Vt the total volume of the column (30ml), Vo was determined using blue dextran.

A plot of $\log_{10}$ Mr vs K_D is shown in Figure 4.1. Results in Table 4.1b are presented as the mean value ± SE from three tissues of each type.

TABLE 4.1

Determination of the Molecular Weight

of S_1 and S_3 Transglutaminase from

Normal Liver and Hepatocellular Carcinomas

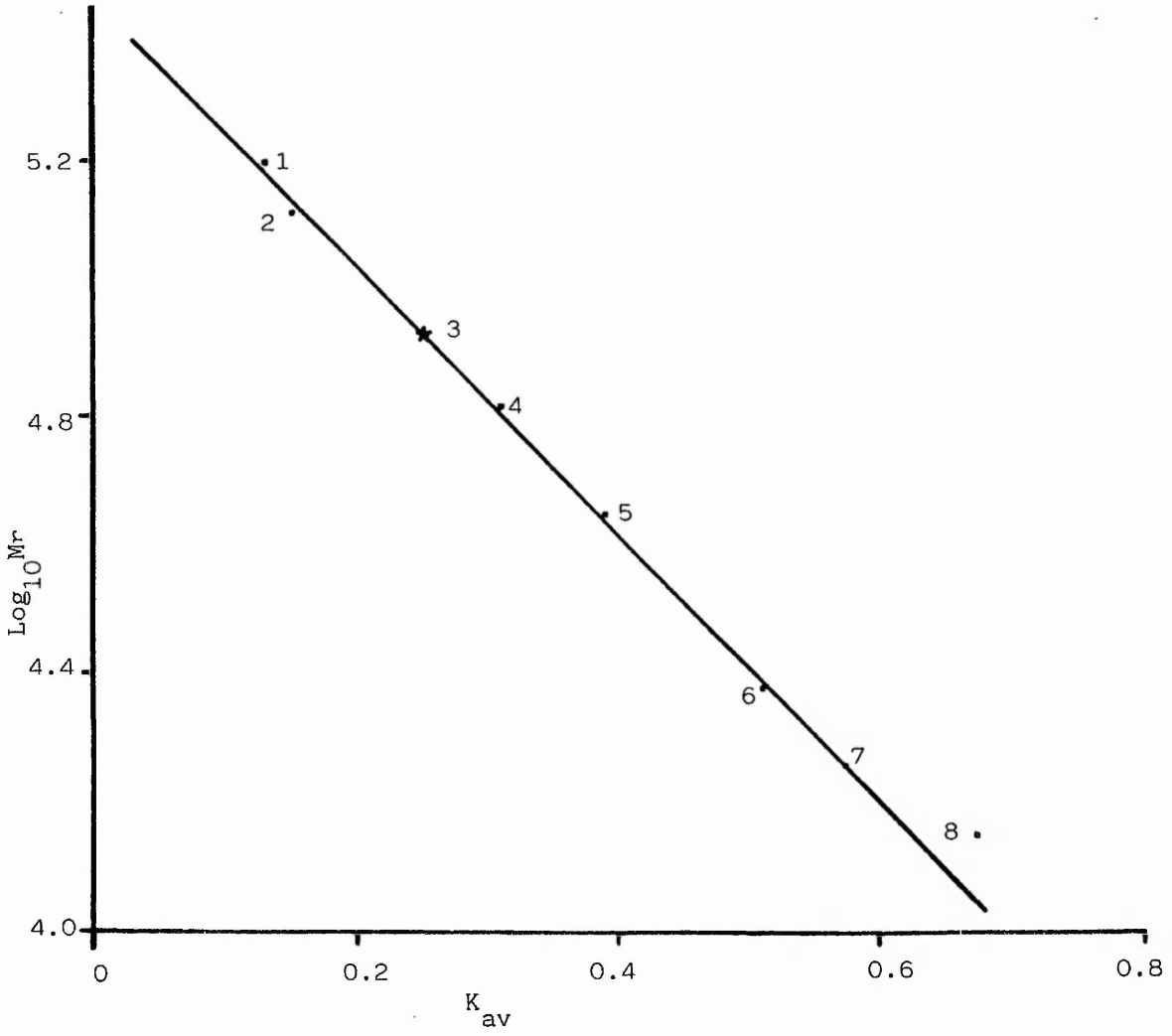
a) Standard Proteins

Protein	Molecular Weight (Mr)	Kav
1. Aldolase	158,000	0.127
2. Bovine Serum Albumin (Dimer)	132,000	0.151
4. Bovine Serum Albumin (Monomer)	66,000	0.308
5. Ovalbumin	45,000	0.388
6. Trypsinogen	24,000	0.510
7. β-Lactoglobulin	18,400	0.571
8. Lysozyme	14,300	0.672

b) Transglutaminase Fractions

Transglutaminase Source		Kav	Mr
Normal Liver:	S_1	0.255 ± 0.004	81900 ± 1500
	S_3	0.249 ± 0.001	83900 ± 400
Hepatocellular Carcinoma:	S_1	0.250 ± 0.002	83600 ± 500
	S_3	0.252 ± 0.004	83000 ± 1100

Figure 4.1 Molecular Weight Calibration of a TSK G-3000 SW column for the Estimation of the Molecular Weight of Transglutaminase



An Ultropac TSK G-3000 Sw column (LKB) was calibrated with the protein molecular weight standards shown in Table 4.1 according to the procedures described. Owing to the closeness of the elution positions of S_1 and S_3 transglutaminase fractions from normal liver and hepatocellular carcinoma, a single point (✱3) denotes the mean position of transglutaminase elution from all sources.

4.2.2 Ion-Exchange Chromatography: Semi-Purification and Comparison of S_1 and S_3 Transglutaminase Fractions from Normal Liver and Hepatocellular Carcinomas

Ion-exchange chromatography of fractions S_1 and S_3 from normal liver and hepatocellular carcinomas was undertaken using a high performance liquid chromatography system as described in section 2.8.1. This analysis served to compare the elution of transglutaminase in the four fractions and also to semi-purify the enzyme for further kinetic studies.

No significant differences in the elution of transglutaminase were seen in any of the samples run. A single peak of activity was found between 0.35 - 0.40 M NaCl. The absence of a second peak of activity in the range 0.15 - 0.22 M NaCl as seen in section 3.6 was attributed to the small amounts of particulate activity in these samples and the lower sensitivity of the assay used.

The most active fractions from each of these chromatographic separations were used as a semi-pure source of activity for kinetic studies (see sections 4.2.3 and 4.2.4.3). Table 4.2 shows the purification achieved in each case. For both normal liver and hepatocellular carcinomas, S_1 activity was purified approximately 50-fold with respect to the original S_1 fraction, whereas the S_3 fractions were purified by approximately 20-fold. In practice these fractions were found to be pure enough for kinetic assays since they gave apparent Michaelis-Menten constants for putrescine incorporation into N,N'-dimethylcasein and Ca^{2+} activation which were comparable to those found using purified rat liver cytosolic transglutaminase (Barnes,(1980); and section 4.2.4.2).

TABLE 4.2

Semi-purification of Transglutaminase

for Kinetic Studies

Fractions S_1 and S_3 (see section 2.4.3) from normal livers and hepatocellular carcinomas were chromatographed on a Waters DEAE-5PW Protein Pak column as described in section 2.8.1. Transglutaminase activity was measured in each original fraction (S_1 and S_3) and in the fractions collected from the ion-exchange chromatography using the assay procedure described in section 2.6.3. Protein was determined by the method of Lowry et al.,(1951). Finally, prior to use in kinetic studies the pooled fractions of highest activity were dialysed exhaustively against 50mM Tris, 1mM DTT, 1mMEGTA, pH 7.4 and centrifuged at 20,000g for 10 min to remove debris. These fractions were then ready for use in kinetic assays (see sections 4.2.3 and 4.2.4). Results are presented as the mean value ±SE from three fractionations of each tissue type.

(Sp. Act = Specific Activity; Purif. = Purification)

TABLE 4.2

Semi-purification of Transglutaminase

for Kinetic Studies

A. Control Liver

Sample	Total Enzyme Activity (Units)	Total Protein(mg)	Sp. Act. Units/mg Protein	Purif. Factor	Yield
S_1	3891.7 ± 905.1	77.7 ± 4.6	50.1 ± 11.6	-	100%
DEAE Pool	1437.3 ± 343.8	0.55 ± 0.1	2633.6 ± 584.2	52.6	37%
S_3	202.8 ± 10.6	4.6 ± 0.3	44.1 ± 2.3	-	100%
DEAE Pool	97.3 ± 9.4	0.11 ± 0.2	884.5 ± 93.2	20.0	48%

B. Hepatocellular Carcinoma

Sample	Total Enzyme Activity (Units)	Total Protein(mg)	Sp. Act. Units/mg Protein	Purif. Factor	Yield
S1	123.1 ± 20.7	47.2 ± 6.2	2.6 ± 0.5	-	100%
DEAE Pool	46.1 ± 4.3	0.32 ± 0.1	144.1 ± 1.8	55.4	37.4%
S_3	141.6 ± 21.3	4.2 ± 0.4	33.7 ± 4.9	-	100%
DEAE Pool	55.1 ± 12.4	0.07 ± 0.02	787.1 ± 52.1	23.3	38.9%

4.2.3 Michaelis-Menten Kinetics for Putrescine Incorporation into N,N'-Dimethylcasein: Comparison of S_1 and S_3 Transglutaminase Fractions from Normal Liver and Hepatocellular Carcinomas

The estimation of the apparent Michaelis-Menten constant (Km_{app}) for putrescine incorporation into N,N'-dimethylcasein by transglutaminase was undertaken using the methodology given in section 2.6.1. The samples were semi-purified prior to use (see section 4.2.2) and dialysed to prepare them for assay. This semi-purification served to remove any contaminating factors such as particulate transglutaminase, polyamine oxidases and competitive substrates.

Table 4.3 shows the Km_{app} values determined for S_1 and S_3 fractions of normal liver and hepatocellular carcinomas. Statistical analysis of the data indicated that there were no significant differences between the four groups of data. A typical double reciprocal plot for the determination of Km_{app} values is shown in Figure 4.2.

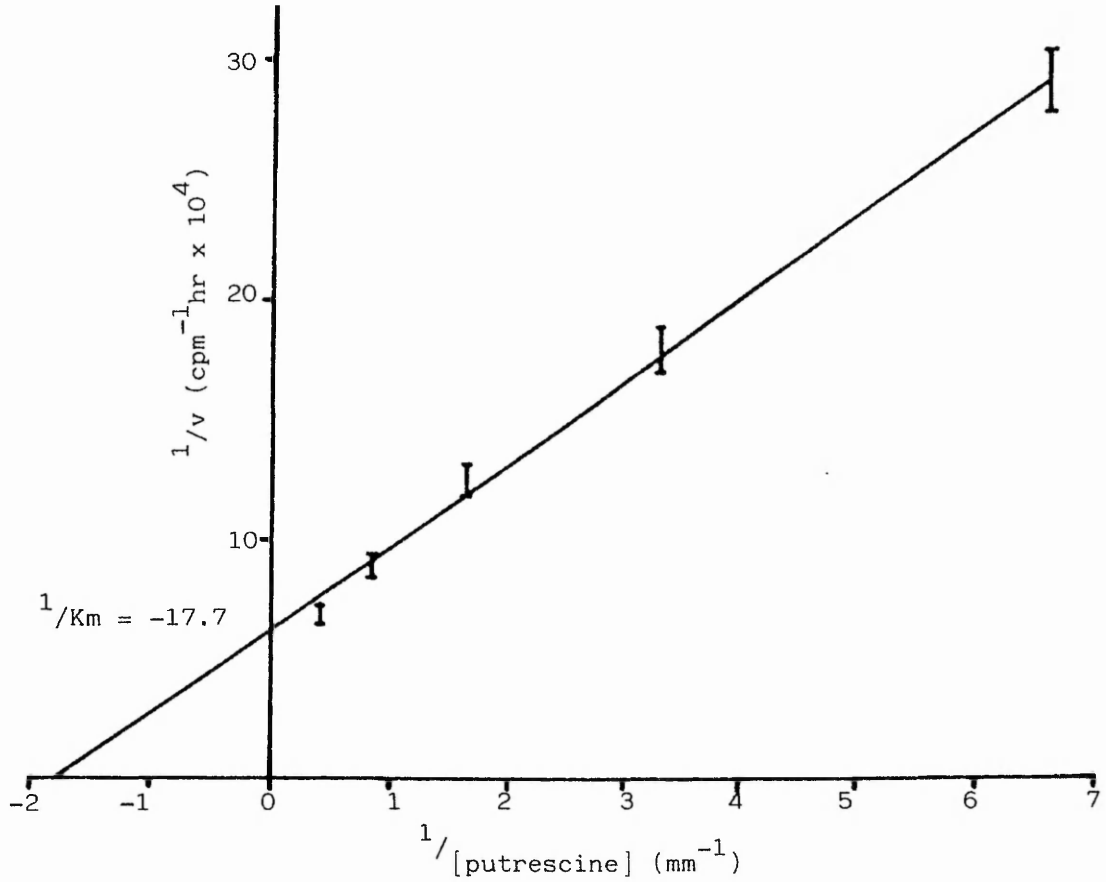
TABLE 4.3

Estimation of the Michaelis-Menten Constant for Putrescine Incorporation into N,N'-Dimethylcasein for S_1 and S_3 Transglutaminase from Normal Liver and Hepatocellular Carcinomas

SAMPLE	Km_{app} (mM)
Normal Liver S_1 (6)	0.50 ± 0.06
Normal Liver S_3 (4)	0.45 ± 0.03
Hepatocellular Carcinoma S_1 (3)	0.47 ± 0.08
Hepatocellular Carcinoma S_3 (3)	0.48 ± 0.05

The methodology for these assays is given in Figure 4.2. For each tissue type at least three experiments were carried out. Values are presented as mean ±SE with the number of fractionations in parenthesis.

Figure 4.2 Representative Double-Reciprocal Plot for the Incorporation of Putrescine into N,N'-Dimethylcasein by Rat Liver Cytosolic Transglutaminase



The apparent Km for the incorporation of putrescine into N,N'dimethyl casein was estimated by varying the putrescine concentration in the transglutaminase assay (section 2.6.1) in the range 0.15-2.40 mM with constant specific activity (3.97 mCi/mol). The above plot is a representative Lineweaver-Burk plot of $^1/v$ versus $^1/s$ for the control liver fraction S_1 . Km_{app} values were calculated from the x-axis intercept, which in this instance gave a Km value of 0.565 mM.

4.2.4 The Sensitivity of Transglutaminase to Activation by Ca^{2+}

In the course of these experiments it became apparent that the assay system commonly used for transglutaminase measurement was not suitable for determining the Ca^{2+} sensitivity of transglutaminase. A colleague in the laboratory, Dr P Bungay, found that the inclusion of Mg^{2+} at physiological concentrations (2mM - Veloso et al., 1973) in the assay system lowered the Ca^{2+} requirement of the enzyme and also that there was considerable batch to batch variation for the N,N'-dimethylcasein. This was thought to be due to competition by Mg^{2+} for Ca^{2+} binding sites on the N,N'-dimethylcasein. Since this protein is a phosphoprotein it was thought that these binding sites were likely to be phosphate groups and that batch to batch variation was probably due to the extent of phosphorylation. It was therefore decided to investigate the effect of dephosphorylation of N,N'-dimethylcasein on the activation of transglutaminase by Ca^{2+} and thus to develop a more accurate assay for determining the Ca^{2+} sensitivity of transglutaminase.

4.2.4.1 Purification of Cytosolic Transglutaminase from Rat Liver

Rat liver cytosolic (tissue) transglutaminase was purified from approximately 70g of liver from adult (200-250g) Sprague-Dawley rats. Prior to excision the livers were exsanguinated via the renal bifurcation to minimise contamination of the tissue with Factor XIII. The liver was homogenised in 4 volumes of ice-cold 0.25M sucrose, 50mM Tris-HCl, 2mM EDTA, 0.5mM dithiothreitol, 0.5mM putrescine, 200 µM PMSF, pH 7.4 using six passes of a variable speed Potter-Elvehjem homogeniser at $4^{o}C$. A particle-free supernatant was then prepared by centrifugation at 71,000 gav for 45 minutes at $4^{o}C$.

This particle-free supernatant was then concentrated by selective ammonium sulphate precipitation (30-65%) and the precipitate resuspended in 50mM Tris, 2mM EDTA, 0.5mM dithiothreitol, 2mM putrescine, pH 7.4 (60ml) and dialysed for 24 hrs against the same buffer (3 x 1 litre). The dialysed material was loaded onto a DE-52 anion exchange column (16cm x 5.5cm) which had been pre-equilibrated with 50mM Tris-HCl buffer, pH 7.4 containing 0.5mM dithiothreitol and 1mM EDTA. Protein was eluted with a linear salt gradient from 0-0.7 M NaCl at a flow rate of 30ml/hr overnight. The fractions with the highest specific activity of transglutaminase (0.3 - 0.45 M) were pooled and concentrated by the addition of ammonium sulphate to 60% saturation. The precipitate was taken up in the smallest feasible volume (5ml) and loaded onto a pre-equilibrated Ultrogel ACA44 gel filtration column and eluted with 50mM Tris-HCl buffer pH 7.4 containing 0.5mM dithiothreitol and 1mM EGTA. The fractions with highest activity were then pooled and dialysed overnight at $4^{o}C$ against 50mM Tris-HCl, 0.5mM dithiothreitol, 1mM EGTA pH 7.4.

Table 4.4 shows the purification data which indicate that the final preparations were approximately 800-fold pure with respect to the original homogenate and had an average specific activity of 10905 units/mg protein.

TABLE 4.4

Purification of Cytosolic Transglutaminase from Rat Liver

SAMPLE	Total Protein (mg)	S.Act (U/mg)	P.Factor	Yield (%)
HOMOGENATE	14220.0 ±2100.0	13.3 ±4.2	1.0	100.0
PFS	6028.0 ±1145.0	29.4 ±9.3	2.2	94.0
$(NH_4)_2SO_4$ Precipitation (30-65% cut)	2979.6 ±203.2	52.0 ±16.3	3.9	81.5
Pooled ion-exchange fractions	18.5 ±2.6	4416.0 ±932.0	332.0	43.0
Pooled Gel Filtration fractions	1.3 ±0.7	10905.0 ±317.0	820.0	7.6

Transglutaminase activity was measured by the method described in section 2.6.1. Protein was determined by the method of Lowry et al., (1951). Each value is the mean of three separate experiments ± SE.

4.2.4.2 The Sensitivity of Purified Cytosolic Liver Transglutaminase to Activation by Ca^{2+}

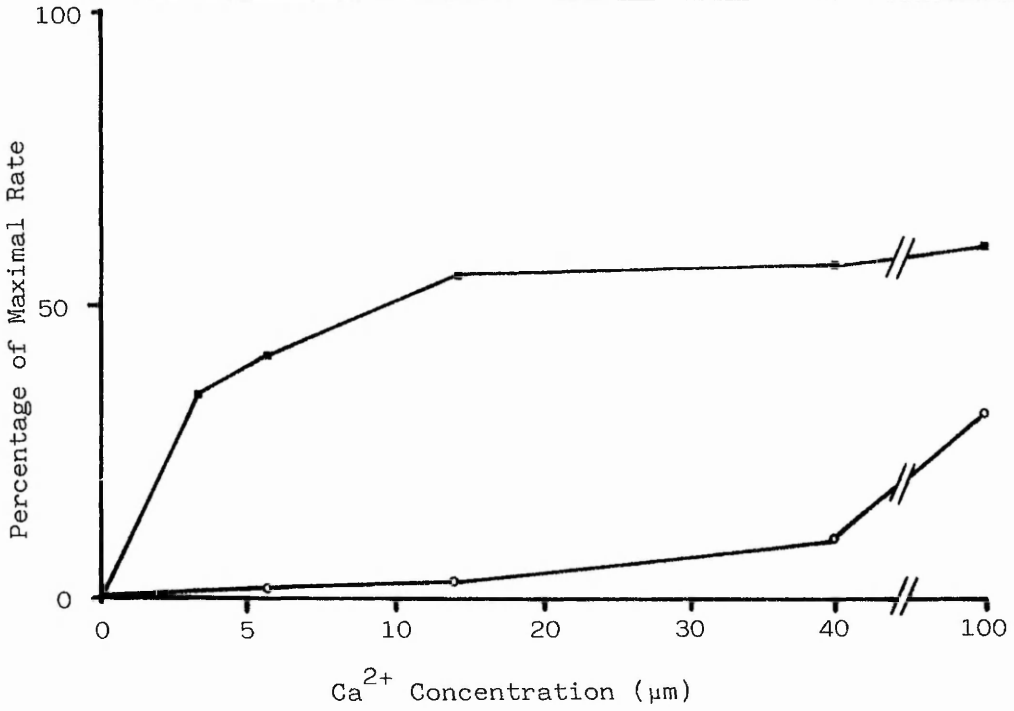
The idea that the estimation of the sensitivity of transglutaminase to Ca^{2+} might be affected by the degree of phosphorylation of the protein substrate N,N'-dimethylcasein was investigated using purified rat liver transglutaminase. Transglutaminase activity was measured at several Ca^{2+} concentrations in the range, 5-100 μM in the presence of either native N,N'-dimethylcasein or dephosphorylated N,N'-dimethylcasein (figure 4.3). It was found that the Ca^{2+} sensitivity of the enzyme was much greater if the N,N'-dimethylcasein was dephosphorylated. Treatment of the data in Figure 4.3 as double-reciprocal plots allowed the calculation of Km values for Ca^{2+}-activation of 38.5μM with native N,N'-dimethylcasein or 5.6μM with dephosphorylated N,N'-dimethylcasein. This modification was therefore used in all subsequent experiments investigating the sensitivity of transglutaminase to Ca^{2+}. It was also found that dephosphorylation reduced the maximal rate of incorporation into N,N'-dimethylcasein (see legend to Figure 4.3).

4.2.4.3 The Sensitivity of S_1 and S_3 Transglutaminase Fractions to Activation by Ca^{2+}: Comparison of Normal Liver and Hepatocellular Carcinomas

The Ca^{2+} sensitivity of S_1 and S_3 transglutaminase was estimated in both normal liver and hepatocellular carcinomas using the assay detailed in section 2.6.4. Three individual normal livers and three individual hepatocellular carcinomas were used to perform three determinations of the Km_{app} for Ca^{2+} of both S_1 and S_3 transglutaminase fractions using semi-purified material (section 4.2.2). Comparison of the four groups of data (Table 4.5) showed that there were no

significant differences either between S_1 and S_3 activity or between normal liver and hepatocellular carcinomas. The values obtained were similar to those found for purified rat liver transglutaminase, although they were slightly lower (1-2μM lower) (see Hand et al., 1985).

Figure 4.3 The Effect of the Dephosphorylation of N,N'Dimethylcasein on the Sensitivity of Transglutaminase to Activation by Ca^{2+}



The sensitivity of transglutaminase to activation by Ca^{2+} was measured (section 2.6.4) using either native N,N'dimethylcasein (○) or dephosphorylated N,N'dimethylcasein (■) as the amine acceptor substrate. The results are shown as a plot of the percentage of the maximal rate at 2.1mm Ca^{2+} versus the Ca^{2+} concentration (see section 2.6.4.2). The reaction rates for the substrate proteins at 2.1mm Ca^{2+} were as follows : native N,N' dimethylcasein, 3470 ± 1420 U/mg, dephosphorylated N,N'dimethylcasein, 2251 ± 919 U/mg.

TABLE 4.5

The Sensitivity of S_1 and S_3 Transglutaminase Fractions from Control Liver and Hepatocellular Carcinomas to Activation by Ca^{2+}

SAMPLE	Km_{app} (μM)
Normal liver S_1	3.36 ± 0.17 (3)
Normal liver S_3	2.82 ± 0.18 (3)
Hepatocellular Carcinoma S_1	3.50 ± 0.40 (3)
Hepatocellular Carcinoma S_3	3.64 ± 0.26 (3)

Transglutaminase activity was measured at various Ca^{2+} concentrations (5.7 - 100 μM) according to the procedure laid out in section 2.6.4. Km_{app} was estimated from double reciprocal plots by extrapolation. Values are given as the mean value ± S.E. with the number of determinations in parenthesis.

4.2.5 Discussion

Comparison of S_1 transglutaminase, semi-purified from normal liver and hepatocellular carcinomas indicated that there was no difference between the two sources of enzyme. This comparison was on the basis of molecular weight, elution on ion exchange media, Michaelis-Menten constant for putrescine incorporation into N,N'dimethylcasein and sensitivity to activation by Ca^{2+}. Furthermore comparison of the S_3 transglutaminase fractions with S_1 transglutaminase fractions in both normal liver and hepatocellular carcinomas also revealed that the enzyme in these fractions was identical. It is however important to emphasise that the S_3 transglutaminase is not identical with the particulate transglutaminase since the particulate enzyme was shown to elute at a different ionic-strength on a Pharmacia Mono-Q column (section 3.6). This confirms previous data (sections 3.3, 3.5 and 3.6) which indicated that the reduction in enzyme activity seen in hepatocellular carcinomas was principally due to a fall in cytosolic transglutaminase activity. Thus the reduction was due to the production of fewer enzyme molecules rather than the production of altered and functionally impaired enzyme molecules. The method by which such a reduction occurs is a matter for further consideration. It may occur as a result of mutations in the regulatory gene for transglutaminase synthesis or alternatively mutations in other genes may have resulted in co-ordinated regulatory events associated with neoplasia, which involve the suppression of transglutaminase synthesis. It is interesting to note that the regulation of transglutaminase occurs at the transcriptional level in a variety of cell types including macrophages and monocytes (see section 1.3.4).

Since S_1 and S_3 fractions were shown to contain an identical transglutaminase enzyme according to the criteria used in these studies it is likely that the association of this enzyme with the membrane fraction of the cell is not due to the presence of a latent, cryptic or even, activated sub-population of enzyme molecules in the cell. There is however a significant amount of cytosolic transglutaminase loosely associated with the membrane fraction in addition to the particulate transglutaminase reported by Chang and Chung,(1986).

The discovery that transglutaminase may be activated at Ca^{2+} levels found in stimulated cells (1-2μM, Beaven et al., 1984) is of great interest since previous measurements of this enzyme's sensitivity to Ca^{2+} activation have indicated that activation could only occur during cell ageing or death (Hand et al.,1985). Therefore this finding supports the idea that transglutaminase may play an important role in normal cellular function (see sections 1.3.1 - 1.3.5). The association of this enzyme with cell membranes further suggests that this association may be of importance in regulating the activity of the enzyme. For example membrane components such as phospholipids may affect transglutaminase activity (Fesus et al., 1983.) This has already been reported for Ca^{2+} and phospholipid dependent protein kinase C where diolein was found to enhance this enzyme's response to Ca^{2+} (Takai et al., 1980). An alternative mechanism whereby an enzyme's sensitivity to Ca^{2+} activation may be modified is via Ca^{2+}-binding proteins such as calmodulin. It was therefore undertaken to assess the effect of both phospholipids and calmodulin on the sensitivity of purified rat liver transglutaminase to Ca^{2+} activation.

4.3 EFFECT OF PHOSPHOLIPIDS AND CALMODULIN ON THE SENSITIVITY OF TRANSGLUTAMINASE TO ACTIVATION BY Ca^{2+}

The effect of various plasma membrane components on the activation of transglutaminase was tested at a Ca^{2+} concentration 5.7μM which gave approximately half maximal activation (Figure 4.4). Phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine were tested in the presence and absence of 1,2-diolein. Phosphatidyl choline was tested in the presence of cholesterol which was sonicated with the phosphatidyl choline to integrate it into the vesicles. Finally sphingomyelin and L-α-phosphatidic acid were also tested for their effect on transglutaminase activation. None of these preparations had any significant effect on the activation of transglutaminase as assessed in a two sample t-test. Diolein on its own also had no effect (Figure 4.4). Furthermore, in addition to the testing of phospholipids at a concentration of 40 μg/ml, phosphatidylcholine, phosphatidyl serine and phosphotidyl ethanolamine were also tested at 20μg/ml and 80μg/ml in the presence and absence of 1,2-diolein. These concentrations of phospholipids were also found to have no significant effect on the activation of transglutaminase by Ca^{2+}.

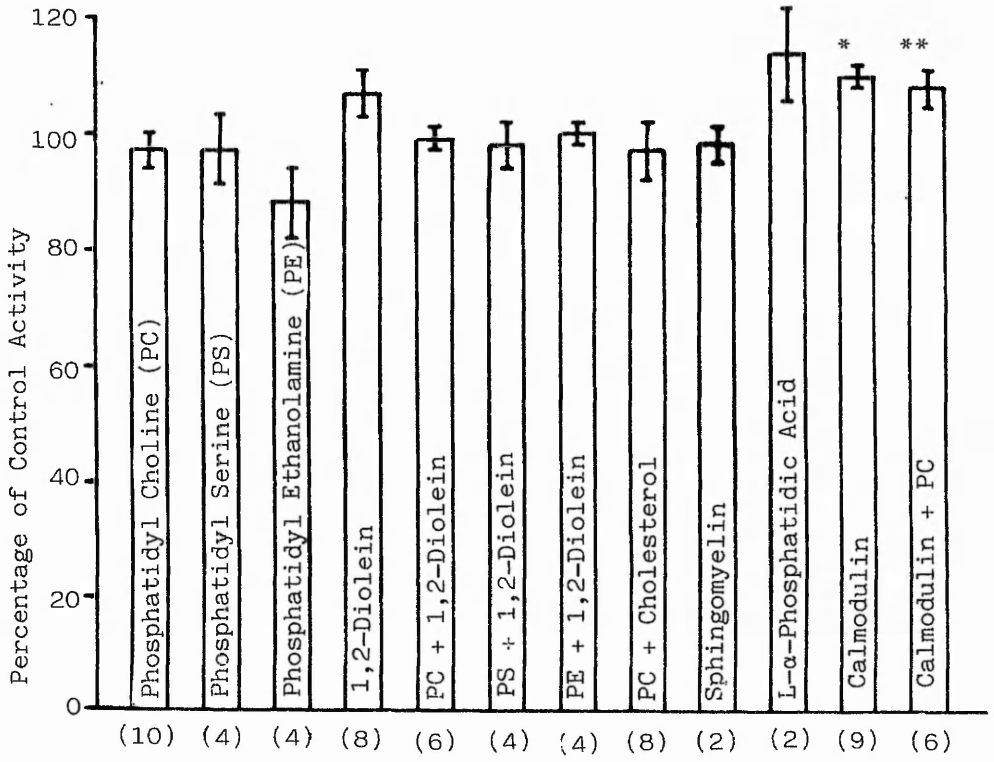
Since transglutaminase has now been shown to be activated in a Ca^{2+} concentration range in which the enzyme might be affected by calmodulin, a Ca^{2+} binding protein which increases the Ca^{2+} sensitivity of many cellular processes (Cheung, 1980), the effect of calmodulin on transglutaminase activity was also examined. In the presence of subsaturating Ca^{2+} (5.7 μM) transglutaminase activity was increased by 10%. Since calmodulin is thought to undergo small conformational changes on binding Ca^{2+} which result in the exposure of hydrophobic

domains (La Porte et al., 1980) the effect of calmodulin on enzyme activity was tested in the presence of phosphatidyl choline. Under these conditions a similar small increase (8%) in enzyme activity occurred. The increases in enzyme activity caused by the presence of calmodulin in the presence or absence of phospholipids were significant (see Figure 4.4).

FIGURE 4.4

The Effect of Phospholipid Vesicles, Diolein and Calmodulin on Transglutaminase Activation by Ca^{2+}

Transglutaminase activity was measured in the presence of various combinations of phospholipids, 1,2-diolein and calmodulin at a Ca^{2+} concentration which produced approximately half maximal activation (5.7μM). The methodology was identical to that given in section 2.6.4. Phospholipid vesicles were prepared by sonication in distilled water (3 x 30 second bursts) (Brocklehurst & Hutton, 1984). Diolein, cholesterol and calmodulin were sonicated with the phospholipids in the appropriate combinations (as shown in the Figure). The following assay concentrations were used; phosphatidyl choline (PC), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE), sphingomyelin (SM) and L-α phosphatidic acid (PA) - 40 μg/ml, diolein-2 μg/ml, cholesterol-20 μg/ml, calmodulin-5.5μg/ml. For each experimental treatment, control vials which did not contain added Ca^{2+} were included. Results (means ± SE) are expressed as % of control activity determined in the absence of test compound (2805 ± 267 units, n = 30) following subtraction of the appropriate 'no Ca^{2+}' treatment value (75-275 cpm). Figures in parentheses represent the number of determinations, *$P \leqslant 0.003$ (**$P \leqslant 0.026$) relative to the control as assessed by two sample t-tests.


Percentage of Control Activity
120
100
80
60
40
20
0
Phosphatidyl Choline (PC)
Phosphatidyl Serine (PS)
Phosphatidyl Ethanolamine (PE)
1,2-Diolein
PC + 1,2-Diolein
PS + 1,2-Diolein
PE + 1,2-Diolein
PC + Cholesterol
Sphingomyelin
L-α-Phosphatidic Acid
Calmodulin
Calmodulin + PC
*
**
(10)
(4)
(4)
(8)
(6)
(4)
(4)
(8)
(2)
(2)
(9)
(6)

4.3.1 Discussion

The plasma membrane components, phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, cholesterol sphingomyelin and L-α-phosphatidic acid were found to have no effect on the activation of transglutaminase in the presence of sub-saturating levels of Ca^{2+}. This finding is in contradiction to that of Fesus et al (1983) where a specific interaction between guinea pig liver transglutaminase and phospholipid vesicles of a small unilamellar size was reported. Whilst it is clear that in this study no particular attention was paid to vesicle size and structure it can be concluded that, in vitro, phospholipids per se did not affect the Ca^{2+} activation of transglutaminase. Furthermore the inclusion of 1,2-diolein in phospholipid vesicles also had no effect on transglutaminase activation indicating that transglutaminase is not activated in a similar way to Protein Kinase C (Takai et al., 1981), in which the Ca^{2+} activation requirement of this enzyme is lowered by diolein.

The Ca^{2+} binding protein, calmodulin was also found to have little effect on enzyme activation either in the presence or absence of phosphatidyl choline vesicles in comparison to its effect on other enzymes known to be activated by this Ca^{2+} binding protein (Klee et al., 1980). The activation that was observed (10%) in the presence of calmodulin alone was significant but was too small to be of any physiological significance. In view of the reports of calmodulin activated transglutaminases present in platelets, red blood cells and smooth muscle (Puszkin and Raghuraman, 1985, Puszkin and Billet, 1987) it should be pointed out that whilst purified rat liver transglutaminase (cytosolic) is not affected significantly by calmodulin it does not rule out the possibility of a novel calmodulin regulated transglutaminase being present in rat liver.

It can therefore be concluded that neither phospholipids nor calmodulin appear to be involved in the regulation of cytosolic transglutaminase by Ca^{2+} ions and that the cytosolic enzyme found in rat liver is activated by changes in the concentration of free Ca^{2+} in the cell.

The experiments in the following chapter were undertaken in order to assess the nature, quantity and location of transglutaminase substrates in normal liver and hepatocellular carcinomas.

5.0 POLYAMINES AND PROTEIN SUBSTRATES FOR TRANSGLUTAMINASE IN NORMAL LIVER AND TUMOURS; CHARACTERISATION AND SUBCELLULAR DISTRIBUTION

5.1 INTRODUCTION

The identification of the cellular substrates of transglutaminase is not only important to the understanding of this enzyme's role in normal cells, but also to the understanding of how reduced transglutaminase activity affects tumour cells. It has been shown that transglutaminase can act on membrane proteins (Birckbichler et al., 1973; Evans & Fink, 1977; Bjerrum et al., 1981) such as the HLA-A and -B Antigens (Pober & Stominger, 1981) and β_2-microglotulin (Fesus et al., 1981) as well as many of the cytoskeletal proteins (see section 1.3.4). Furthermore, since both $\varepsilon(\gamma$-glutamyl)lysine and γ-glutamyl polyamines have been isolated from normal tissues (Birckbichler et al., 1973; Conrad, 1985; Beninati, 1985) it is necessary to consider both ε-amino groups of lysine residues in proteins and free polyamines for their ability to react with available γ-glutaminyl residues of cellular proteins.

The hepatocellular carcinoma may be used as a convenient control for comparison with normal liver in order to identify the substrates of the cytosolic tissue transglutaminase since there is a specific reduction in this form of transglutaminase in these tumours (see chapter 3). Furthermore alterations in polyamine metabolism which are frequently associated with neoplastic growth (see section 1.3.6) may possibly influence the way in which transglutaminase acts on cellular proteins. The studies carried out in this chapter were therefore undertaken in order to evaluate the effect of polyamine levels on transglutaminase activity and to attempt to identify the cellular proteins acted upon by the cytosolic/tissue transglutaminase in intact cells.

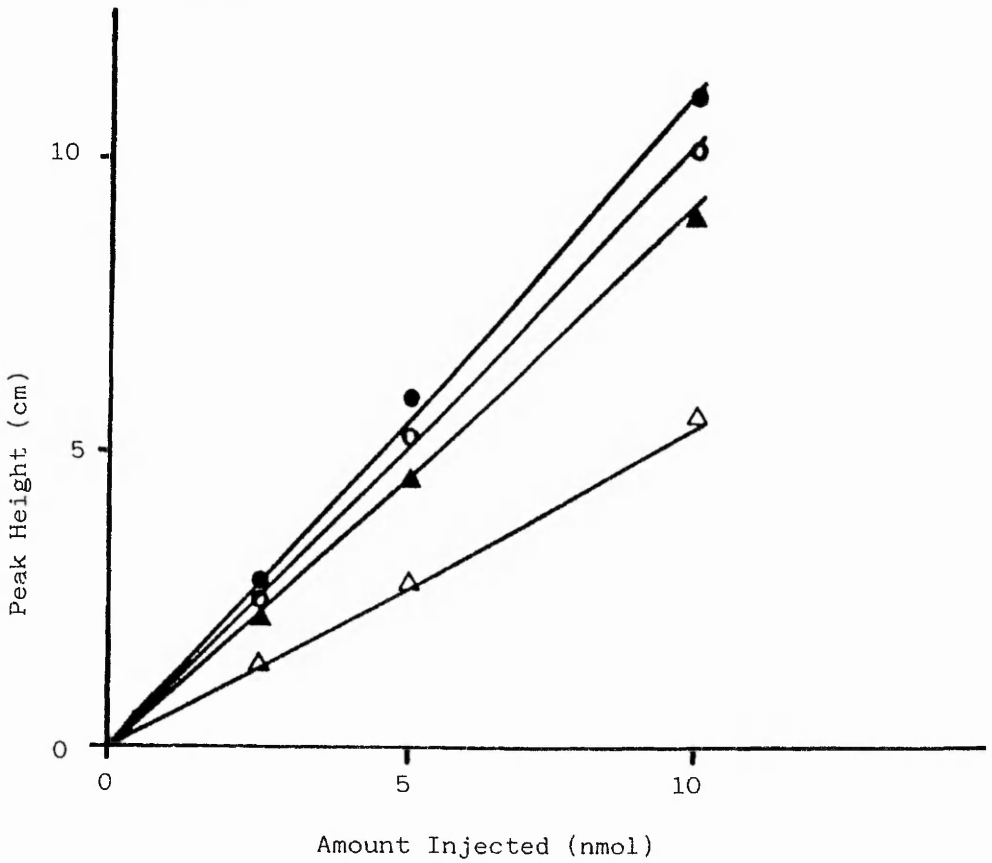
5.2 POLYAMINE LEVELS IN RELATION TO TRANSGLUTAMINASE ACTIVITY IN NORMAL AND NEOPLASTIC TISSUES

In the following studies the polyamine levels present in normal tissues, chemically-induced tumours and tumour bearing tissues were measured. This in turn was compared to the transglutaminase activity found in these tissues, so that any relationship existing between these parameters could be established.

5.2.1 Linearity of o-Phthalaldehyde Derivitisation

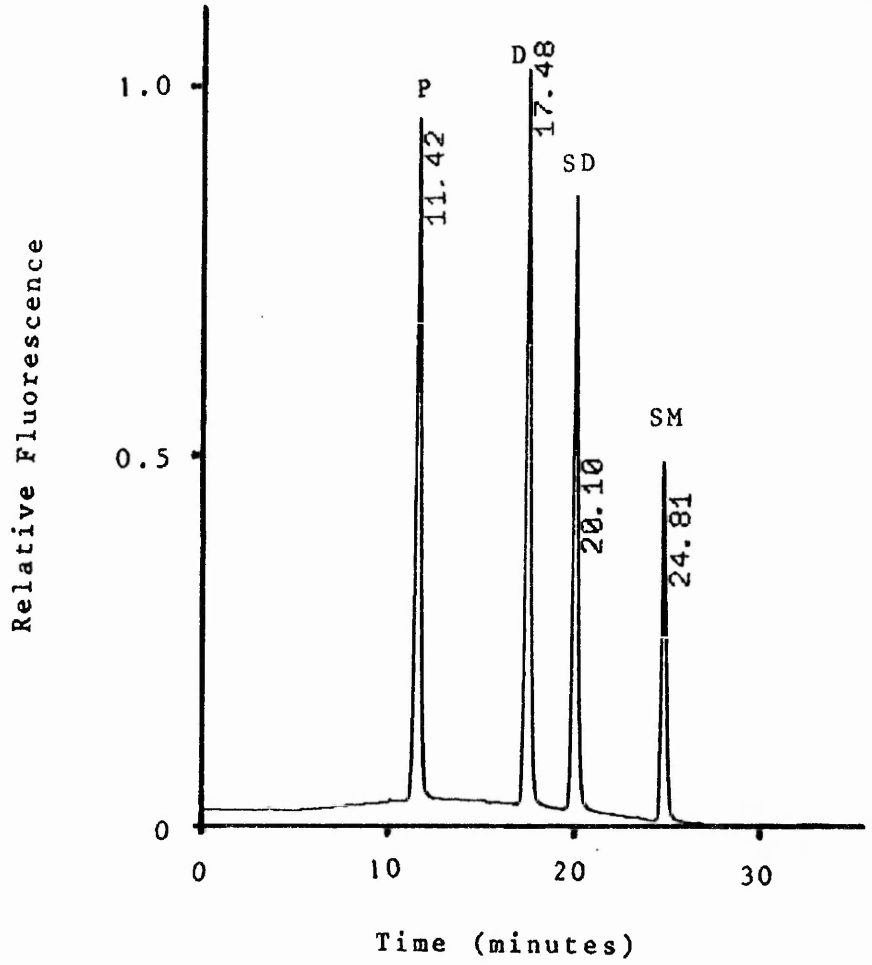
Since the method employed for polyamine analysis used a modified o-phthalaldehyde (OPA) reagent it was first necessary to confirm the linearity of OPA derivitisation in the appropriate concentration range (section 2.8.3). The derivitisation was found to be linear in the range 2.5 - 10 nMols per injection (Figure 5.1). Therefore for the accurate determination of polyamine levels in tissue samples, the amount loaded was adjusted to bring the levels of each polyamine loaded into this range of concentrations. A typical separation of the polyamines, putrescine, spermidine and spermine and the internal standard, 1,7-diaminoheptane is shown in Figure 5.2.

Figure 5.1 Linearity of Polyamine Derivitisation with o-Phthalaldehyde reagent.



Separation of polyamine standards was achieved using the reverse-phase ion-pair method of Seiler,(1983) (see section 2.8.3). Varying amounts of putrescine (●), 1,7-diaminoheptane (O), spermidine (▲) and spermine (△) were injected onto the column under identical conditions. Peak height was then plotted against the known amount of each polyamine loaded. A typical separation is shown in Figure 5.2.

Figure 5.2 Reverse-Phase Separation of Polyamine Standards



H.P.L.C. of polyamine standards was carried out as described in section 2.8.3. A typical separation of 10 nmol of each standard is shown above : putrescine (P), 1,7-diaminoheptane (D), spermidine (SD), and spermine (SM). Fluorescence was monitored with a Perkin-Elmer fluorimeter (excitation λ 340 nm; emission λ 455 nm; sensitivity x2) coupled to a Spectra-Physics 4270 plotting integrator (attenuation 16). The retention times for each standard are shown beside each peak.

5.2.2 Polyamine Levels in Relation to Transglutaminase Activity and Subcellular Distribution in Normal Rat Liver, Lung and Spleen

Since polyamines are substrates for transglutaminase (Folk et al, 1980) it was undertaken to compare polyamine levels in normal mammalian tissues with transglutaminase activity and its subcellular distribution in the same tissues. Rat liver, lung and spleen were chosen since these tissues contain high levels of transglutaminase activity (Barnes et al., 1984; 1985).

The levels of transglutaminase activity and its subcellular distribution were found to be similar to those reported previously (Barnes et al., 1984; 1985) (Table 5.1). Total activity was comparable in liver and spleen but slightly lower in lung tissue (70% of liver or spleen levels). The subcellular distribution of activity in liver was mostly soluble (76%) with some 24% associated with the particulate fraction. In contrast the majority of the activity in lung and spleen was associated with the particulate fraction (87% and 96% respectively). In liver this phenomenon has been shown to be due to the expression of two distinct forms of transglutaminase (Chang and Chung, 1986) whereas in lung and spleen the activity has yet to be fully characterised.

Polyamine levels were found to vary considerably between liver, lung and spleen (Table 5.2): spleen containing the highest total polyamine levels and lung the lowest. Further, the relative amounts of putrescine, spermidine and spermine in each tissue also varied considerably, indicating that the overall metabolic flux through the polyamine biosynthetic and degradative pathways was different in each tissue. This is clearly demonstrated by the spermidine/spermine ratio which increased in the sequence: liver (0.64); spleen (1.33); lung (2.05). Comparison of polyamine levels with transglutaminase

activity indicated that there was a rough correlation between spermine levels and total polyamine levels with total transglutaminase activity in these tissues but that there was no correlation between putrescine and/or spermidine levels and transglutaminase activity. However, since there is no known relationship between the particulate and soluble forms of transglutaminase in cells they should be considered individually rather than as a total activity value. Using the assumption that the subcellular distribution of activity corresponds roughly to the levels of each form of transglutaminase in tissues it was apparent that no true correlation between polyamine levels and either form of transglutaminase existed.

Finally protein and DNA were also measured in liver, lung and spleen as an indicator of any gross biochemical differences between these tissues (Table 5.1). It was found that lung contained less protein than liver or spleen and that DNA content of the tissues was substantially different: lung and spleen containing roughly 3 and 4.5 times as much DNA respectively as liver tissue. However, expression of polyamine levels and transglutaminase activity as a function of protein or DNA did not reveal any correlation between these parameters.

It may therefore be concluded that there is no obvious relationship between free polyamine levels in tissues and the levels or forms of transglutaminase present in a particular tissue type.

TABLE 5.1

Transglutaminase Activity and Subcellular Distribution, Protein and DNA Content in Normal Rat Liver, Lung and Spleen

Tissue	Transglutaminase Activity (units/g Wet Tissue)	% Distribution S / P	Protein (mg/g Wet Tissue	DNA µg/g Wet Tissue
Liver (3)	5759 ± 149	76/24	186 ± 23	1030
Lung (3)	3981 ± 589	13/87	107 ± 17	2907
Spleen(3)	5833 ± 561	4/96	215 ± 32	4549

Fresh Sprague-Dawley rat liver, lung and spleen tissue were homogenised by Potter-Elvehjem (section 2.4.1) in 0.25M sucrose, 1mM EGTA, 5mM Tris-HCl, pH 7.4 (20% w/v) and fractionated into a cytosol (S) and particulate fraction (P) by centrifugation (section 2.4.2). Transglutaminase activity, protein and DNA were determined according to the procedures described in sections 2.6.1, 2.5.1 and 2.5.3 respectively. Results are given as the mean value ± SE with the number of tissues examined in parenthesis.

TABLE 5.2

Polyamine Levels and Spermidine/Spermine Ratio in Normal Rat Liver, Lung and Spleen

Tissue	P	SD	SM	TOTAL	SD/SM RATIO
	Nanomoles/g Wet Tissue				
Liver (3)	20 ± 2	676 ± 79	1056 ± 65	1752	0.64
Lung (3)	57 ± 6	508 ± 32	248 ± 50	813	2.05
Spleen(3)	23 ± 6	1167 ± 75	879 ± 181	2069	1.33

Fresh Sprague-Dawley rat liver, lung and spleen tissue were homogenised by Potter-Elvehjem (section 2.4.1) in 0.25M sucrose, 1mM EGTA, 5mM Tris-HCl, pH 7.4 (20% w/v). Homogenates were spiked with internal standard (1,7-diaminoheptane) and acid-soluble polyamines extracted by the procedures detailed in section 2.8.3.1. The levels of putrescine, spermidine and spermine were then measured by reverse-phase HPLC as described in section 2.8.3.3. The internal standard was used to correct for injection volume and the efficiency of extraction procedure. Results are shown as the mean value ± SE with the number of tissues examined in parenthesis.

P = putrescine, SD = spermidine, SM = spermine.

5.2.3 Polyamine Levels in Relation to Transglutaminase Activity in Normal Liver, Tumour Bearing Liver and Hepatocellular Carcinomas

The levels of putrescine, spermidine and spermine found in five hepatocellular carcinomas, one mixed cholangioma/hepatocellular carcinoma, their respective parent livers and six normal livers are shown in Table 5.3. The wide variation in polyamine levels in DEN treated livers and the tumours derived from these livers necessitated the consideration of these tissues individually, whereas in normal liver tissue polyamine levels were relatively stable and are shown as mean values. The levels of transglutaminase activity, mitotic frequency, protein and DNA in these tissues are also shown here for direct comparison with polyamine levels (see also Tables 3.1 and 3.4).

Putrescine levels were found to be significantly elevated ($P \leqslant 0.05$) in the tumour group as a whole when the mean value was compared to the normal liver mean. However, only three tumours (23: A, B and C) contained significantly elevated levels of putrescine. Analysis of putrescine in tumour bearing livers revealed that there was a direct relationship between the putrescine levels in each liver and the putrescine levels in the tumours derived from that liver. Tumours with elevated putrescine levels were derived from tumour bearing livers with elevated putrescine levels and tumours with low putrescine levels were derived from tumour bearing livers with low putrescine levels.

Considerable variations in spermidine and spermine levels were found in all of the tumours examined, although these changes were not consistent within the tumour group and the mean levels of these two polyamines were in fact approximately equivalent to the amounts found in normal liver. In tumour bearing liver, two out of the three livers examined were found to contain elevated spermidine and spermine levels.

There was however no correlation between the levels of spermidine and spermine in tumour bearing livers and the levels of these polyamines in tumours derived from these livers.

The spermidine/spermine ratio, a measure of the overall metabolic flux through the polyamine biosynthetic and degradative pathways (Figure 1.4) was in general elevated in tumours when compared to normal liver. In tumour bearing liver only one such liver had an elevated spermidine/spermine ratio. Interestingly this was the same liver that contained elevated putrescine levels.

Attempts to correlate transglutaminase activity with polyamine levels in the same tissues were unsuccessful (Table 5.3). Similarly no apparent relationship between polyamine levels and mitotic frequency could be found, thus providing further evidence for the heterogeneity of these tumours and their growth states (see section 3.2.2). Finally, the measurement of total protein and DNA in these tumours showed some small variation of these parameters in the tissues examined. However it was found that these fluctuations could not account for any of the observed differences in polyamine content of the tissues.

The idea that elevated polyamine levels in tumours (especially putrescine) could be high enough to interfere with the transglutaminase assay (by diluting out the radiolabelled substrate) was checked by calculating the concentrations of each polyamine added to the reaction mixture in the tissue homogenates (Table 5.4). This was done for normal liver and tumour bearing liver 23 (the tissue with highest putrescine levels). From this calculation it can be seen that there is a difference in the amounts of tissue-derived polyamines added to the reaction mixture of approximately 200 μM. However, this difference was only seen in tumour bearing liver 23 and its tumours and

not in other tumours or their parent livers. Furthermore the levels of spermidine and spermine in these tissues are unlikely to affect the assay of transglutaminase since this enzyme expresses a higher affinity for putrescine (Clarke et al., 1959).

TABLE 5.3

Transglutaminase Activity, Polyamine Levels, Protein, DNA and Mitotic Frequency of DEN-Induced Tumours, Tumour Bearing Livers and Normal Livers

Tissues were prepared according to the procedures described in Tables 5.1 and 5.2. Mitotic frequency was estimated by counting the number of dividing cells for ten fields of view. Values are expressed as the mean from triplicate determinations on each individual tissue. P = Putrescine, SD = Spermidine, SM = Spermine, nd = not determined.

Tissue	P	SD	SM	SD/SM RATIO	Transglut-aminase Activity U/g Wet Tissue)	Mitotic Frequency	Protein (U/g Wet Tissue)	DNA (U/g Wet Tissue)
	nmol/g Wet Tissue							
a) Hepatocellular Carcinoma: 10A	52	712	647	1.10	2150.8	11.0	127.3	995.7
Hepatocellular Carcinoma: 10B	38	1340	1984	0.67	3222.9	29.0	167.0	1342.9
Hepatocellular Carcinoma: 14A	41	819	804	1.02	2077.1	11.0	155.0	1314.6
Hepatocellular Carcinoma: 23B	165	962	1137	0.85	811.7	31.0	176.4	1143.2
Hepatocellular Carcinoma: 23C	292	1702	1862	0.91	2515.0	7.0	172.3	1197.6
Mixed Cholangioma/ Hepatocellular Carcinoma: 23A	165	1220	1409	0.86	2029.8	7.0	166.4	1051.7
Mean Values ± SE for Tumours (6)	126 (±41)	1126 (±150)	1307 (±223)	0.90 (±0.06)	2134.5 (±321.1)	16.0 (±4.5)	160.7 (±7.3)	1174.3 (±56.7)
b) Tumour Bearing Liver: 10	38	1340	1990	0.67	5790.0	nd	141.6	1155.7
Tumour Bearing Liver: 14	20	907	1467	0.62	3703.3	nd	158.9	1092.4
Tumour Bearing Liver: 23	442	1986	2109	0.94	3398.3	nd	165.8	1144.2
Mean Values ± SE for Tumour Bearing Livers (3)	167 (±138)	1411 (±313)	1855 (±197)	0.75 (±0.1)	4297.2 (±751.5)	nd	155.4 (±7.2)	1130.8 (±19.5)
c) Mean Values ± SE for Normal Livers (6)	27 (±7)	1043 (±160)	1317 (±145)	0.78 (±0.08)	4160.0 (±198.0)	0	170.5 (±6.7)	966.1 (61.9)

TABLE 5.4

Levels of Polyamines derived from Tissue Homogenates in Transglutaminase Assays

Polyamine	Polyamine Concentration in Assay (μM)	
	Tumour Bearing Liver 23	Normal Liver (Mean)
Putrescine	44.2	2.7
Spermidine	198.6	104.3
Spermine	210.9	131.7
Total	453.7	238.7

The concentrations of putrescine, spermidine and spermine added to the transglutaminase assay (section 2.61) in tissue homogenates were calculated. The above examples were chosen since tumour bearing liver 23 contained the highest levels of polyamines and normal liver was a suitable comparison. Results were shown as the final concentration of tissue-derived polyamines in the transglutaminase assay. Assays also contained 1.2 mM [^{14}C]-putrescine (see section 2.6.1).

5.2.4. Discussion

The measurement of polyamine levels in normal rat liver, lung and spleen indicated that there were distinctive differences in polyamine metabolism in these tissues. Neither the levels of polyamines nor their ratios with respect to one another were comparable. Transglutaminase activity on the other hand was roughly equivalent in all three tissues, although its subcellular distribution was different. No correlation between transglutaminase activity/subcellular distribution and polyamine levels could be established in these tissues.

In experiments with DEN-induced tumours, putrescine levels were found to be elevated in some of the tumours, a finding which is in agreement with previous studies on chemically-induced tumours (Milano et al., 1981; Perin and Sesa, 1978; Scalabrino et al., 1978. However, contrary to the findings of Milano and co-workers (1981), spermidine and spermine levels did not increase in the majority of the tumours examined and the spermidine/spermine ratios were only slightly higher than in normal liver. Furthermore, elevated putrescine levels were not a ubiquitous feature of tumours in this study. The interesting relationship between polyamine levels in tumours and their tumour bearing livers merits some speculation as to its cause. It has been shown that DEN, the carcinogen used in this study, can cause putrescine levels to rise dramatically in liver tissue (Matsui and Pegg, 1982) both by increasing ornithine decarboxylase activity (Olson and Russell, 1978; Haddock and Russell, 1981a) and spermidine/spermine-N^1-acetyl transferase activity (Matsui and Pegg, 1982). However, since putrescine levels were not elevated in all of the tumours it is likely that any such elevation is a consequence rather than an obligatory

requirement of the carcinogenic process. It may therefore be concluded that considerable caution should be exercised when using polyamine levels as a marker for carcinogenesis and tumour growth.

Attempts to correlate polyamine levels with transglutaminase activity in tumours, tumour bearing liver and normal liver were unsuccessful. Further, the idea that enhanced polyamine levels (especially putrescine) in tumours could interfere with the assay of transglutaminase in tissues was discounted. This finding was supported by experiments in which purified transglutaminase was incubated with tumour homogenates (section 5.4.3) when it was found that the tumour homogenates did not affect the measurement of transglutaminase activity.

In conclusion it is clear that there is no correlation between free polyamine levels and transglutaminase in these tissues. This is perhaps not surprising in view of the wide variety of functions which polyamines have in cell growth and proliferation (Heby, 1981; Pegg, 1986).

Beninati and co-workers (1985) have shown that some 10-20% of the total polyamine content of liver tissue is covalently bound, thus demonstrating the significance of covalently bound polyamines. However, the precise function of these bound polyamines remains unclear since their site(s) of attachment have yet to be identified. The following experiments were therefore undertaken to try to identify the substrate proteins into which polyamines may be incorporated.

5.3 TISSUE SLICE EXPERIMENTS

Freshly prepared tissue slices of normal liver and hepatocellular carcinomas were incubated at 37°C in a well balanced salts medium under a continuous stream of O_2/CO_2 (95%/5%) in the presence of [^{14}C]-methylamine and either plasma levels of calcium (1.9 mM $CaCl_2$) or 1 mM EGTA. This procedure (see section 2.7) allows the measurement of radiolabelled methylamine uptake into cells and its subsequent incorporation into cellular proteins. Work in this laboratory (Barnes, 1980) has demonstrated that the uptake and incorporation of polyamines into tissue slices of liver is time-dependent and that slices prepared in this manner are viable under the conditions used (see also Lundgren and Hankins, 1978). The monoamine, methylamine was used rather than polyamines since its incorporation into proteins would not result in the formation of crosslinked bis-polyamine derivatives by transglutaminase, thus allowing an accurate measurement of the number of available γ-glutaminyl residues. Furthermore since there is no evidence to suggest that methylamine can enter the polyamine biosynthetic pathway, the artefactual incorporation of radiolabel into proteins caused by the conversion of polyamines to γ-aminobutyric acid (Lundgren and Hankins, 1978) or hypusine (Park et al., 1981; Conrad, 1985) can be ruled out. Also the myeloperoxidase catalysed formation of chloramine derivatives from primary amines, which can react with proteins (Thomas et al., 1982; Lorand and Conrad, 1984) can be distinguished from transglutaminase-mediated reactions because myeloperoxidase is not Ca^{2+}-dependent. Thus the use of [^{14}C]-methylamine as a primary amine and EGTA as a control for calcium incubations allows a valid assessment of the transglutaminase-mediated incorporation of methylamine into γ-glutaminyl residues of cellular proteins.

The following experiments were therefore carried out in order to compare the levels and subcellular distribution of methylamine incorporation into tissue slices of normal liver and hepatocellular carcinomas.

5.3.1. Incorporation of [^{14}C]-Methylamine into Cellular Proteins in Normal Liver and Tumours

The accumulation of methylamine in tissue slices was assessed by comparison of the concentration of radiolabel in the slice with that found in the external medium. This slice/medium ratio was used as an index of methylamine uptake by the tissue to confirm that any difference observed in radiolabel incorporation into protein was not due to differing abilities of the slices to accumulate this monoamine. The slice/medium ratios for normal liver, hepatocellular carcinomas and cholangiomas are shown in Table 5.5. In normal liver this ratio was calculated to be 1.49 ± 0.28, whereas for hepatocellular carcinomas and cholangiomas the ratios were 1.23 ± 0.2 and 1.20 ± 0.28 respectively. Although these differences were not statistically significant there was an underlying trend for tumour slices to take up less radiolabel than normal liver slices. However, since the Km for methylamine utilisation by transglutaminase has been reported to be 24 μM (Chang and Chung, 1986) it was clear that saturating levels of methylamine were present in all tissues. The replacement of calcium (1.9 mM $CaCl_2$) with 1 mM EGTA had no significant effect on the uptake of radiolabel by either normal liver or tumour tissue.

The level of radiolabel which was covalently incorporated into acid-precipitable protein was similar in tissue slices of normal liver and tumours (Table 5.5). This was true for both DEN-induced hepatocellular carcinomas and 6-BT induced cholangiomas, a finding

which was surprising in view of the reduced amount of transglutaminase in these tumours (see Chapter 3). Indeed, the highest levels of incorporation were found in the 6-BT induced cholangiomas. The covalent incorporation of radiolabel was Ca^{2+}-dependent since the substitution of EGTA for $CaCl_2$ in the incubation medium reduced the amount of radiolabel incorporated by 90-95%. Statistical comparison of the data indicated that there was no significant difference between the groups of data shown in Table 5.5 and that this was true whether the data were expressed as a function of tissue weight, protein or DNA.

TABLE 5.5

The Uptake of [^{14}C]-Methylamine into Tissue Slices of Normal Liver and Tumours and its Ca^{2+}-dependent Incorporation into Acid-Precipitable Protein

Tissue	Slice/Medium Ratio	[^{14}C]-Methylamine Incorporation		
		cpm/mg tissue	cpm/mg protein	cpm/ µg DNA
Normal Liver (13)	1.49±0.28*	1519.4±241.0	11966.3±1896.6	1561.3±251.5
Hepatocellular Carcinoma (16)	1.23±0.20**	1414.6±167.6	13213.3±1561.3	1184.1±136.2
Cholangioma (11)	1.20±0.28*	1802.3±167.6	16985.5±1582.2	-

Slice/Medium ratios and [^{14}C]-methylamine (specific activity 59 mCi/mMol) incorporation into tissue proteins were estimated by the methods described in section 2.7. [^{14}C]-methylamine incorporation is shown as the difference between the incorporation in the presence of 1.9 mM $CaCl_2$ and that found in the presence of 1 mM EGTA. The incorporation in the presence of EGTA was low and within a small range (50-120 cpm/mg slice). Protein and DNA were determined by the methods detailed in sections 2.5.1 and 2.5.3 respectively. Results are shown as the mean value ±SE with the number of tissues examined shown in parenthesis: except *n=8, **n=6. Counting efficiency in these experiments was determined to be 83% ± 1% (n=10).

5.3.2 Subcellular Distribution of Covalently Bound [^{14}C]-Methylamine followings its Ca^{2+}-Dependent Incorporation into Tissue Slices of Normal Liver and Hepatocellular Carcinomas

Tissue slices of normal liver and hepatocellular carcinomas were incubated with [^{14}C]-methylamine in the presence of $CaCl_2$ or EGTA as previously described. The slices were homogenised and fractionated by differential centrifugation into 600g(N), 40,000g(M), 71,000g(P) particulate fractions and a particle free cytosol fraction (S). As well as measuring the amount of radiolabel in each fraction, protein, DNA and the activities of lactate dehydrogenase, 5'-nucleotidase and cytochrome C oxidase were determined in each fraction (Figure 5.4).

The incorporation of radiolabel found in the presence of EGTA was only some 5-10% of that found in the presence of $CaCl_2$. However, the subcellular distribution of radiolabel was the same whether slices were incubated with EGTA or $CaCl_2$. The subcellular distribution of radiolabel is therefore shown as the percentage of the total Ca^{2+}-dependent incorporation following subtraction of EGTA control values for each fraction (Figure 5.4). In both normal liver and hepatocellular carcinomas the majority of the radiolabel (60-65%) was incorporated into the 600g(N) fraction. Smaller amounts were incorporated into the 40,000g(M), 71,000g(P) fractions and cytosol (S). Comparison of the subcellular distribution of radiolabel in normal liver and hepatocellular carcinomas with the marker enzymes revealed that it most closely resembled the subcellular distribution of 5'-nucleotidase. There was however a higher percentage of radiolabel in the cytosol (S) fraction than was seen for 5'-nucleotidase activity. Comparison of the distribution profiles for radiolabel in normal liver and hepatocellular carcinomas indicated that there was a higher

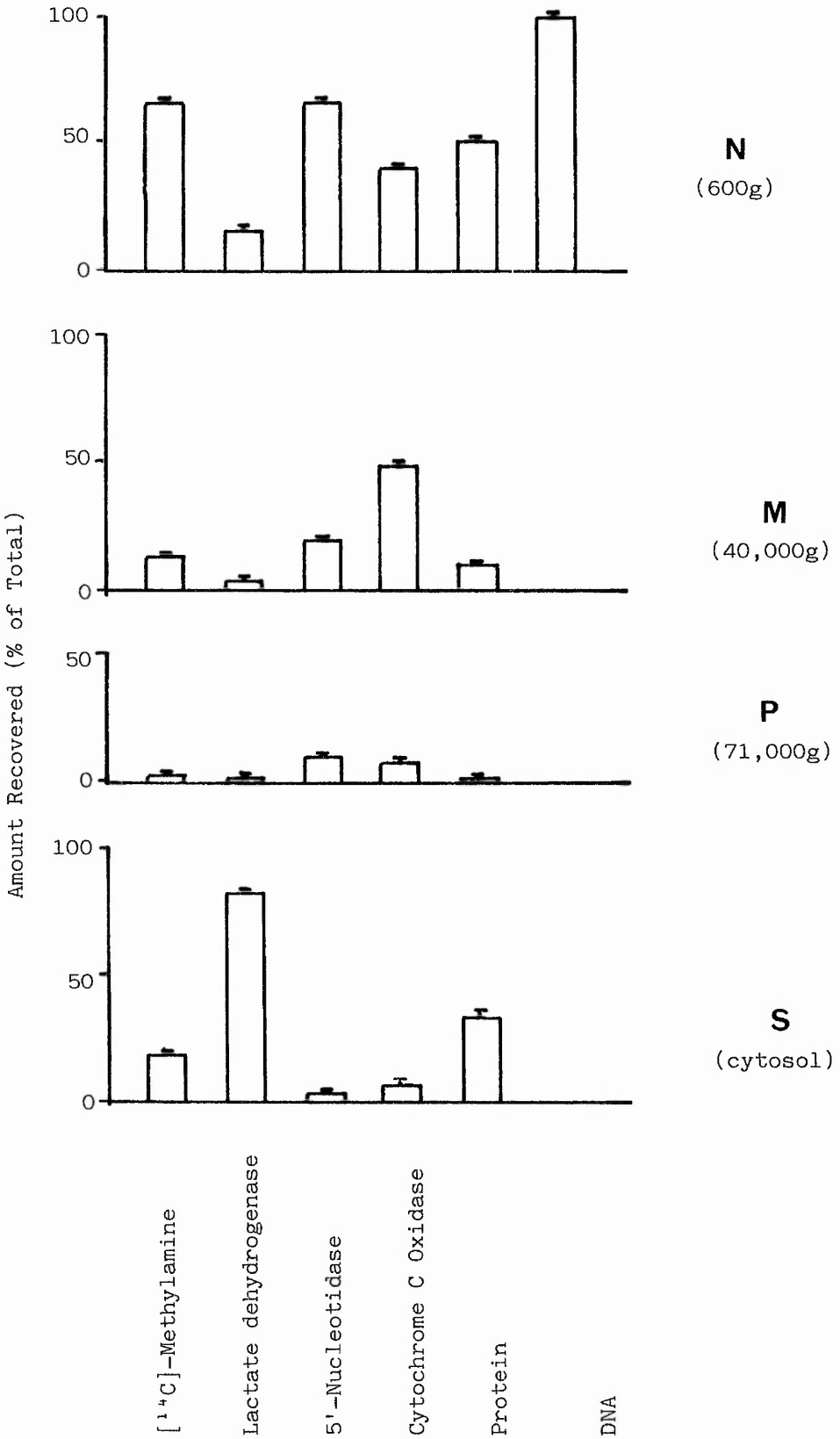
percentage of radiolabel associated with the 71,000g fraction (P) when the mean value for hepatocellular carcinomas (5.6%) was compared to that for normal liver (1.7%). Also in the cytosol fraction (S) there was a higher percentage of radiolabel associated with this fraction in normal liver (18%) than in hepatocellular carcinomas (11%). However, statistical analysis of this data indicated that these differences were not significant.

FIGURE 5.3

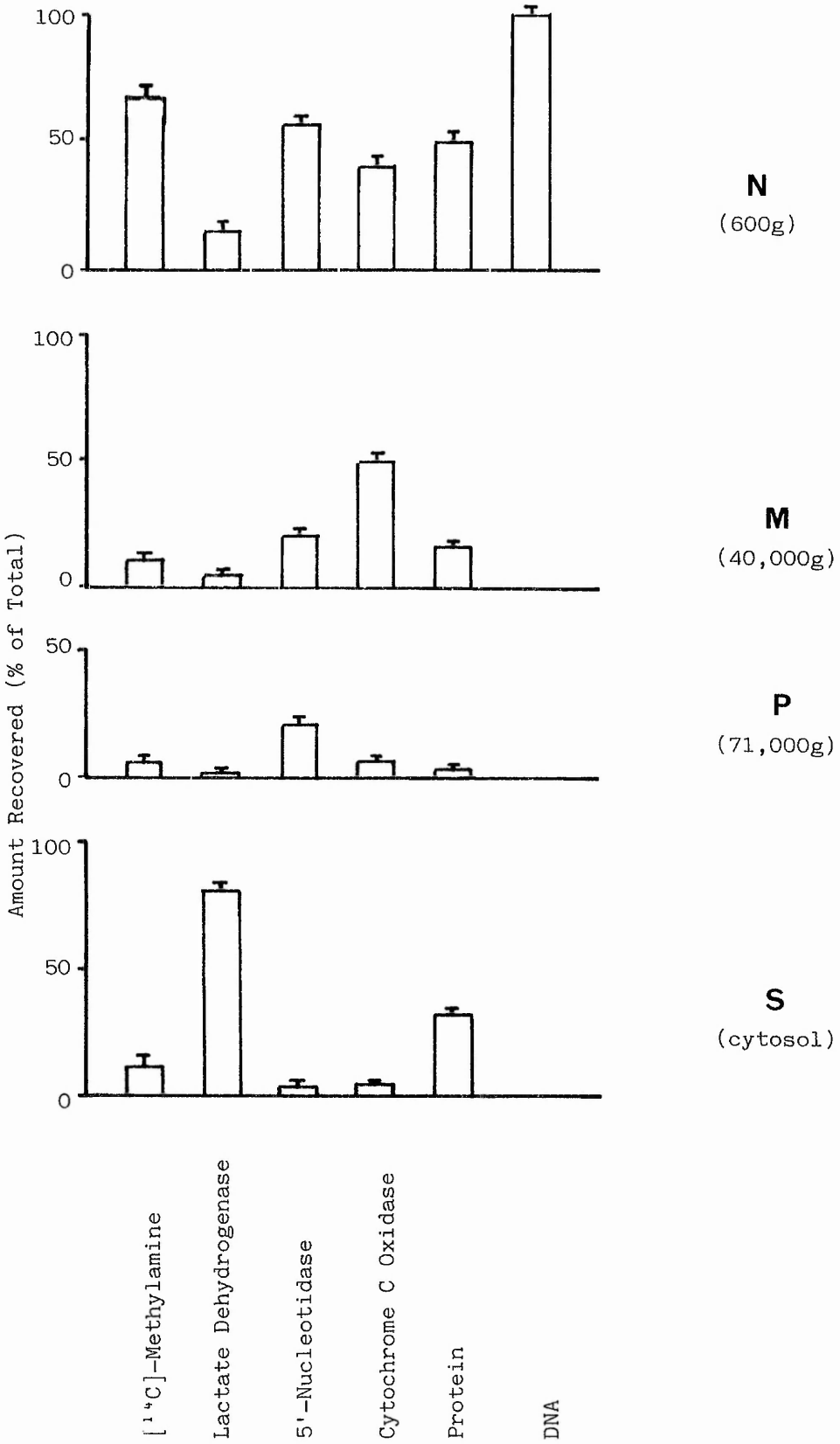
Subcellular Distribution of [^{14}C]-Methylamine following its Ca^{2+}-Dependent Incorporation into Cellular Proteins in Normal Liver and Hepatocellular Carcinomas: Comparison with Marker Enzymes, Protein and DNA

Tissue slices of normal liver and DEN-induced hepatocellular carcinomas were incubated with 1.2 mM [^{14}C]-methylamine (specific activity 59 mCi/mMol in the presence of 1.9 mM $CaCl_2$ or 1 mM EGTA as described in section 2.7. After incubation, slices were rinsed and homogenised in four volumes of 0.25 M sucrose, 1 mM EGTA, 100 µM PMSF, 5 mM Tris-HCl, pH 7.4 using a Potter-Elvehjem homogeniser (section 2.4.1). The homogenates were than fractionated by differential centrifugation into 600g(N), 40,000g(M), 71,000g(P) particulate fractions and a particle free cytosol fraction (S) (see section 2.4.5). The amount of radiolabel incorporated into each fraction was estimated on filter papers (section 2.5.4.1) and following subtraction of the appropriate EGTA controls, expressed as the percentage of the total Ca^{2+}-dependent incorporation of radiolabel (N+M+P+S) in each fraction, for: A, normal liver (2008.5 ± 355.2 cpm/mg tissue) and B, hepatocellular carcinoma (1630.8 ± 485.0 cpm/mg tissue). The incorporation seen in the presence of EGTA had the same subcellular distribution as that found in the presence of $CaCl_2$ and was some 3.8% of the levels found in the presence of $CaCl_2$. Marker enzyme activities, protein and DNA were determined in each fraction as described in sections 2.9, 2.5.1 and 2.5.3 respectively. All values are shown as the amount recovered (% of total) in each fraction from four normal livers and four hepatocellular carcinomas. Error bars are SE.

A. Normal Liver



B. Hepatocellular Carcinomas



5.3.3 Discussion

The measurement of methylamine uptake in tissue slices of normal liver and tumours showed that normal liver could accumulate this monoamine slightly better than tumour tissue. However it is unlikely that the small difference that was observed had any significant effect on these experiments since the methylamine concentration in the slices (1.2 mM) was above the saturation level for maximal transglutaminase activity (half maximal velocity is achieved with 24 μM methylamine - Chang and Chung, 1986). Furthermore the permeability of tissues to methylamine has been demonstrated in a number of other tissues (Okhuma and Poole, 1978; Seglen and Gordon, 1980; Bungay et al, 1984a; Gomis et al, 1984).

The incorporation of radiolabelled methylamine into cellular proteins in tissue slices was Ca^{2+}-dependent, the incorporation in the presence of EGTA being only 3-8% of that found in the presence of 1.9 mM $CaCl_2$. This is in accordance with a transglutaminase-mediated process, indeed there is no evidence to suggest that methylamine can be incorporated into proteins by any other Ca^{2+}-dependent process (see section 5.3.1).

The finding that tumour tissue and normal liver were equally capable of incorporating methylamine into cellular proteins was extremely surprising in view of the low activity of transglutaminase in hepatocellular carcinomas and cholangiomas (see Chapter 3). There are several possible explanations for this phenomenon: firstly, it is tempting to suggest that the particulate transglutaminase, which is not greatly reduced in tumours (see section 3.6), was responsible for this methylamine incorporation. This idea is supported by its subcellular localisation in the plasma membrane (Bruce et al., 1983; Barnes et al., 1985; Tyrell et al., 1986) where it may be readily activated by Ca^{2+}

influx/efflux in the hepatocyte, or alternatively it may be continuously active, owing to the high levels of Ca^{2+} stored at the membrane (Rasmussen and Barrett, 1984). Similar logic also leads to the suggestion that the cytosolic transglutaminase (which is reduced in tumours - Chapter 3) may be inactive during these experiments since Ca^{2+} levels in the cytosol are low under non-stimulatory conditions.

Studies on the subcellular distribution of covalently-bound [^{14}C]-methylamine indicated that the distribution was similar in normal liver and hepatocellular carcinomas. The majority of the incorporation of radiolabel (60-70%) was found in the 600g fraction (N) of both tissues, a finding which supports the suggestion that the particulate transglutaminase was responsible for this incorporation. It was found that tumour tissue had a significantly higher amount of radiolabel associated with the 71,000g fraction (P). However, since this increase was mirrored by similar increases in protein and 5'-nucleotidase activity in the same fraction, it was thought to be due to perturbations in the plasma membrane structure that can occur in tumours (Novikoff & Holtzman, 1976). Finally, it was found that normal liver had more radiolabel in the cytosol fraction (S) than was seen in tumours. This difference was thought to be due to reduced cytosolic transglutaminase activity in these tumours.

In order to gain a better understanding of the incorporation of [^{14}C]-methylamine into tissue slices of normal liver and hepatocellular carcinomas, the following experiments were undertaken using SDS-polyacrylamide gel electrophoresis and fluorography to identify the proteins which [^{14}C]-methylamine is incorporated into.

5.4 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF TISSUE SLICES

The experiments in this section were undertaken in order to identify the specific proteins which were radiolabelled with [^{14}C]-methylamine during tissue slice experiments. SDS-polyacrylamide gel electrophoresis was used to separate the proteins which were then examined by liquid scintillation counting and/or fluorography.

5.4.1 Examination of [^{14}C]-Methylamine-Labelled Tissue Slices of Normal Liver and Hepatocellular Carcinomas by SDS-Polyacrylamide Gel Electrophoresis and Fluorography

The incorporation of [^{14}C]-methylamine into proteins of tissue slices of normal liver and hepatocellular carcinomas was further investigated using SDS-polyacrylamide gel electrophoresis and fluorography. SDS-polyacrylamide gel electrophoresis of normal liver tissue slices incubated with [^{14}C]-methylamine and either $CaCl_2$ or EGTA revealed the formation of a high molecular weight material in tissue slices incubated with $CaCl_2$ but not in those incubated with EGTA (Figure 5.4). Since this polymeric material could not traverse a 2.5% (w/v) acrylamide gel, its molecular weight was in excess of Mr 10^6. However there were no apparent reductions in protein bands of lower molecular weight when comparing Ca^{2+}-incubated tissue slices with EGTA-incubated tissue slices. An identical situation was also found with tissue slices of hepatocellular carcinomas.

Fluorography of tissue slices incubated with [^{14}C]-methylamine and either $CaCl_2$ or EGTA (Figure 5.5) indicated that this high molecular weight material (SG_1 polymer) was the principal site of incorporation of radiolabel in both normal liver and hepatocellular carcinomas and that this incorporation was

Ca^{2+}-dependent. There was also some Ca^{2+}-dependent incorporation of radiolabel into smaller polymers in normal liver and tumours, which either entered the stacking gel (SG_2 polymers) or were blocked by the resolving gel (RG_1 polymer). For clarity, all polymers are referred to according to their location in the gel (see Table 5.7). In addition to these 'polymers', proteins in the resolving gel were also radiolabelled in the presence of $CaCl_2$ (Figure 5.5). Their molecular weights (Mr) were estimated using [^{14}C]-methylated protein standards (Table 5.6 and Figure 5.6). In normal liver two main radiolabelled proteins of Mr 43,600 and 38,900 were identified. There was also some Ca^{2+}-dependent radiolabelling of proteins of Mr 120,000; 87,000; 60,000, 49,000 and 29,000 in tissue slices of normal liver. In contrast, in tumour tissue only two proteins of Mr 38,900 and 35,900 were radiolabelled in the presence of $CaCl_2$. Interestingly the radiolabelling of the Mr 35,900 protein was unique to the hepatocellular carcinomas. Since many of these proteins were only just visible above the level of radioactivity found throughout the resolving gel they were not examined further. However, the proteins of Mr 43,600, 38,900 and 35,900 were radiolabelled sufficiently to allow further study and are referred to as bands 1, 2 and 3 respectively. Finally it should be noted that the incorporation of radiolabel seen in tissue slices incubated with EGTA was greatly reduced when compared to tissue slices incubated with $CaCl_2$. However, this incorporation appeared to follow the same pattern seen in incubations with $CaCl_2$ (Figure 5.5). Therefore for clarity, quantitation of the incorporation into these proteins and polymers was expressed as the Ca^{2+}-dependent incorporation following subtraction of EGTA controls.

Quantitation of the amount of [^{14}C]-methylamine incorporation into these proteins and polymers was carried out using two different techniques and results were expressed as the amount of radioactivity incorporated per mg total protein loaded. Firstly, gels were sectioned and counted for radioactivity (Table 5.7A). However, the use of this technique did not allow the detection of bands 1, 2 and 3 above the level of radioactivity found throughout the resolving gel. Gels were therefore developed as fluorograms and scanned densitometrically at 540 nM. Since X-ray film responds linearly to radioactive emissions under these conditions (Laskey and Mills, 1975) a plotting integrator was used to quantitate peak areas (Table 5.7B) from the fluorogram. A comparison of scans for normal liver and hepatocellular carcinomas is shown in Figure 5.7. It was found that both techniques gave similar results for the Ca^{2+}-dependent incorporation of radiolabel into all sections of the gel.

In normal liver the SG_1 polymer was found to represent some 40% of the total incorporation of radiolabel and SG_2 polymers and RG_1 polymers were found to represent approximately 5% and 15% respectively (Table 5.7A; 5.7B). The remaining incorporation was found to be distributed evenly throughout the resolving gel (35-40%) when liquid scintillation counting was used. However, the use of densitometric scanning revealed that protein bands 1 and 2 accounted for some 3% and 9% of the total incorporation of radiolabel respectively.

In tumour tissue it was found that the incorporation of radiolabel into SG_1 polymer was slightly elevated when compared to normal liver but that this difference was not statistically significant (Table 5.7). In contrast, there was a significant reduction in Ca^{2+}-dependent incorporation of radiolabel into SG_2 polymer, RG_1 polymer and the whole resolving gel ($P \leqslant 0.05$). Further, the

incorporation of radiolabel into protein band 2 was significantly reduced ($P \leqslant 0.05$) and band 1 was undetectable (Table 5.7B). Band 3, which was only found to be radiolabelled in tumour tissue, represented approximately 1% of the total Ca^{2+}-dependent incorporation of radiolabel into tumours.

Finally, in order to confirm that protein bands 1, 2 and 3 were not artefacts of the fluorographic process, it was undertaken to electrophorese excess amounts of normal liver and hepatocellular carcinoma protein (> 1mg) on 3 mM slab gels. Following electrophoresis, gels were sectioned and counted for radioactivity (Table 5.8). This technique gave similar results to those found using fluorography, although bands 1 and 2 were only detected in normal liver tissue. This is perhaps not surprising in view of the low level of incorporation into band 2 and band 3 in tumour tissue (Table 5.7B). Finally, it is interesting to note that as well as giving similar results for the incorporation of radiolabel into SG_1, SG_2 and RG_1 polymers, the incorporation into the whole resolving gel was comparable (compare Tables 5.7A and B with Table 5.8). This supports the idea that this radioactivity is due to covalent incorporation of radiolabel rather than non-specific binding of radiolabel throughout the whole resolving gel.

FIGURE 5.4

SDS-Polyacrylamide Gel Electrophoresis of Tissue Slice Homogenates of Normal Liver incubated with [^{14}C]-Methylamine and $CaCl_2$ or EGTA

Tissue slices of normal liver were incubated with [^{14}C]-methylamine (1.2 mM) in the presence of $CaCl_2$ (1.9 mM) or EGTA (1 mM) (see section 2.7). Following incubation, the slices were homogenised in 0.25M sucrose, 1 mM EGTA, 100 µM PMSF, 5 mM Tris pH 7.4 (section 2.4.1) and solubilised in sample buffer (section 2.4.6.2). Samples were then electrophoresed on discontinuous SDS-polyacrylamide gels which consisted of a 2.5% (w/v) acrylamide stacking gel and a 10% (w/v) acrylamide resolving gel (section 2.4.6.3). Following electrophoresis, the gels were fixed and stained with PAGE-blue 83 dye (section 2.4.6.4). Lane 1 - standard proteins; Lane 2 - incubation of normal liver slice with $CaCl_2$ (150 µg protein loaded); Lane 3 - incubation of normal liver slice with EGTA (150 µg protein loaded).

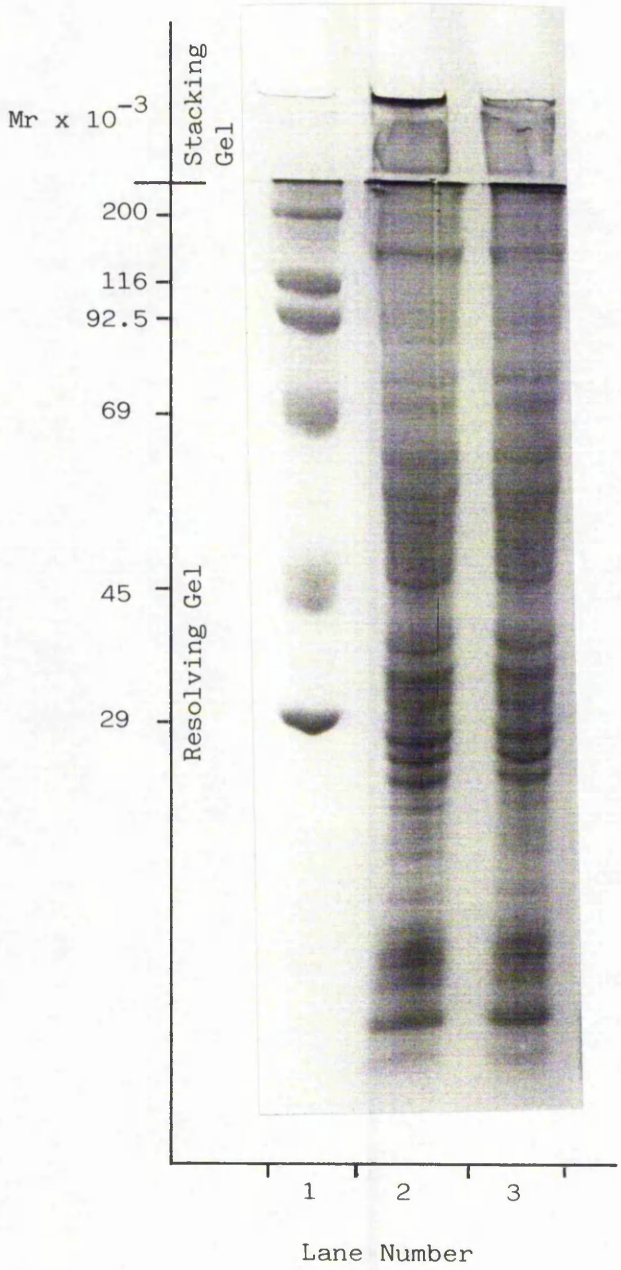

Mr x 10^{-3}
Stacking Gel
200
116
92.5
69
45
29
Resolving Gel
1
2
3
Lane Number

FIGURE 5.5

Examination of the Ca^{2+}-Dependent [^{14}C]-Methylamine Labelling of Tissue Slices of Normal Liver and Hepatocellular Carcinomas by SDS-Polyacrylamide Gel Electrophoresis and Fluorography

Tissue slices of normal liver and DEN-Induced hepatocellular carcinomas were incubated with [^{14}C]-methylamine in the presence of 1.9 mM $CaCl_2$ or 1 mM EGTA as described in section 2.7. Following incubation, slices were homogenised in 0.25 M sucrose; 1 mM EGTA; 100 µM PMSF, 5 mM Tris pH 7.4 (section 2.4.1) and solubilised in sample buffer (section 2.4.6.2). Samples were then electrophoresed on a discontinuous SDS-Polyacrylamide gel consisting of a 2.5% (w/v) acrylamide stacking gel and a 10% (w/v) acrylamide resolving gel (section 2.4.6.3). Fluorography was then performed as described in section 2.4.6.4 and fluorograms developed by exposure for 21 days at -70^{o}C. Lanes 1 and 10 contained [^{14}C]-methylated protein standards which were used to estimate the molecular weight of [^{14}C]-labelled proteins in samples (see Table 5.6). Lanes 2 and 9 contained 130 µg of normal liver protein and 110 µg of hepatocellular carcinoma protein respectively from homogenates of tissue slices labelled with [^{14}C]-methylamine in the presence of 1 mM EGTA. Lanes 3, 4 and 5 contained 130 µg of normal liver protein from homogenates of tissue slices labelled with [^{14}C]-methylamine in the presence of 1.9 mM $CaCl_2$. Lanes 6, 7 and 8 contained 110 µg of hepatocellular carcinoma protein from homogenates of tissue slices labelled with [^{14}C]-methylamine in the presence of 1.9 mM $CaCl_2$. The bands seen in Lane 9 are artefacts due to contamination from the standard mixture in Lane 10.

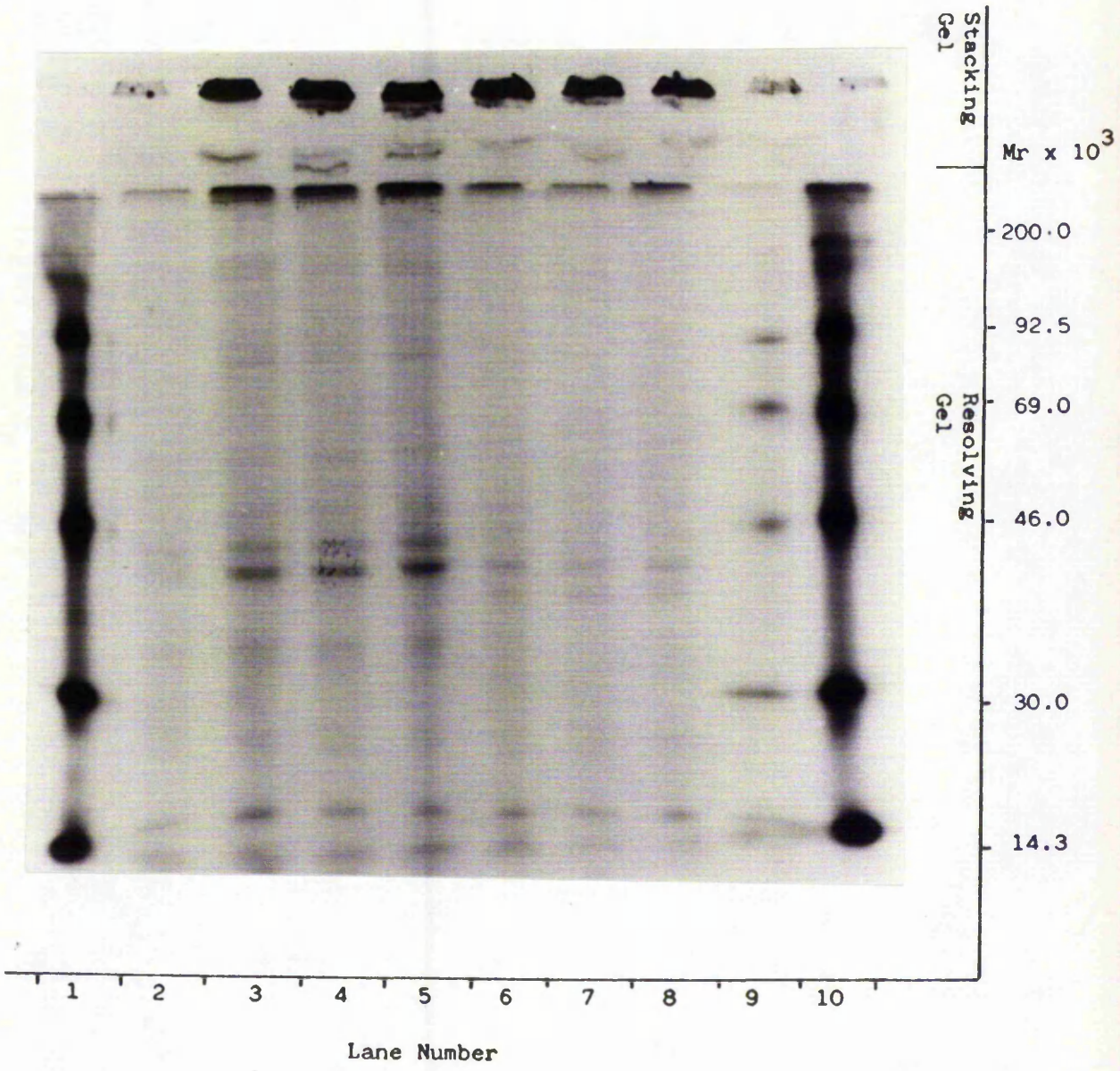

Stacking Gel
Resolving Gel
Mr x 10^3
200.0
92.5
69.0
46.0
30.0
14.3
1
2
3
4
5
6
7
8
9
10
Lane Number

TABLE 5.6

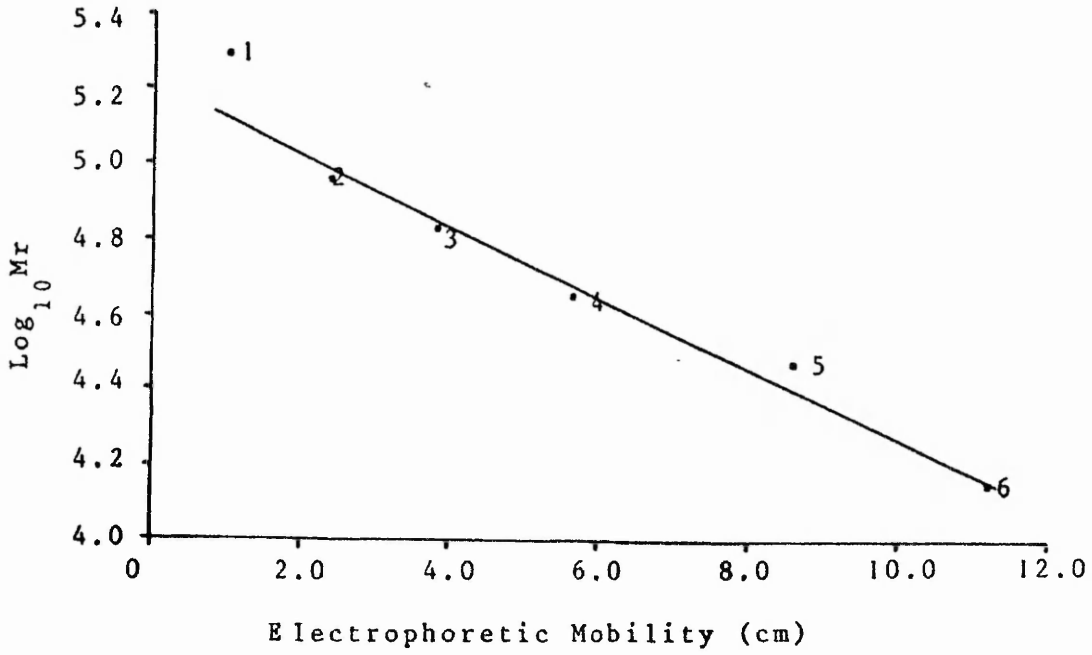
The Estimation of the Molecular Weight of [^{14}C]-Methylamine Labelled Proteins in Tissue Slices of Normal Liver and Hepatocellular Carcinomas

Protein	Mr($\times 10^{-3}$)	Electrophoretic Mobility* (mM)
1 Myosin	200.0	10.0
2 Phosphorylase B	92.5	24.0
3 Bovine Serum Albumin	69.0	38.5
4 Ovalbumin	46.0	56.5
5 Carbonic Anhydrase	30.0	86.0
6 Lysozyme	14.3	112.0
Band 1	43.6	61.5
Band 2	38.9	66.5
Band 3	35.9	71.0

* NB: These distances were taken from the original autoradiogram rather than the photograph in Figure 5.5.

The [^{14}C]-radiolabelled protein standards (Amersham International PLC) shown above were used to estimate the molecular weight of [^{14}C]-methylamine labelled proteins found in control liver and hepatocellular carcinoma slices (see Figure 5.5). A plot of $\log_{10}$ Molecular weight ($\log_{10}$ Mr) versus electrophoretic mobility was plotted and the Mr of [^{14}C]-methylamine labelled proteins calculated accordingly.

Figure 5.6 Calibration of SDS-Polyacrylamide Gels with [^{14}C]-Methylated Protein Standards



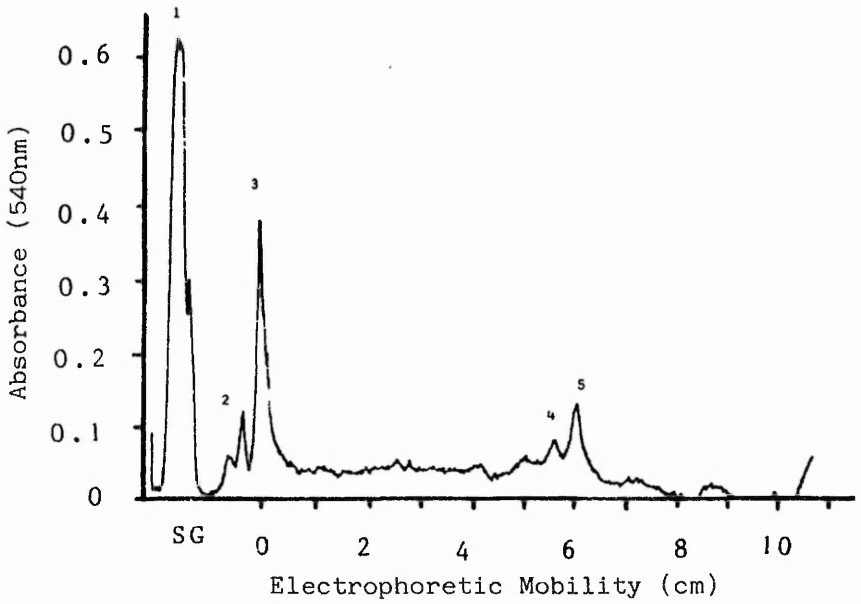
See legend to Table 5.6 for key to [^{14}C]-methylated protein standards (facing page).

FIGURE 5.7

Densitometric Scanning of Fluorograms of SDS-Polyacrylamide Gels of Tissue Slices incubated with [^{14}C]-Methylamine and $CaCl_2$: Comparison of Normal Liver and Hepatocellular Carcinomas

Fluorograms of SDS-polyacrylamide gels of normal liver and hepatocellular carcinoma tissue slices which had been incubated with [^{14}C]-methylamine and $CaCl_2$ (Figure 5.6) were scanned at A540 nm using the densitometer attachment of a Unicam SP1800 spectrophotometer (section 2.5.4.4). A540nm was recorded on a Spectra-Physics 4270 plotting integrator. Typical traces for scans of: A - normal liver homogenate (130 µg protein) and B - hepatocellular carcinoma homogenate (110 µg protein) are shown. The following peaks are labelled on the traces: 1 - SG_1, 2 - SG_2, 3 - RG_1, 4 - Band 1 (Mr 43,600), 5 - Band 2 (Mr 38,900), 6 - Band 3 (Mr 35,900).

A Normal Liver



B Hepatocellular Carcinoma

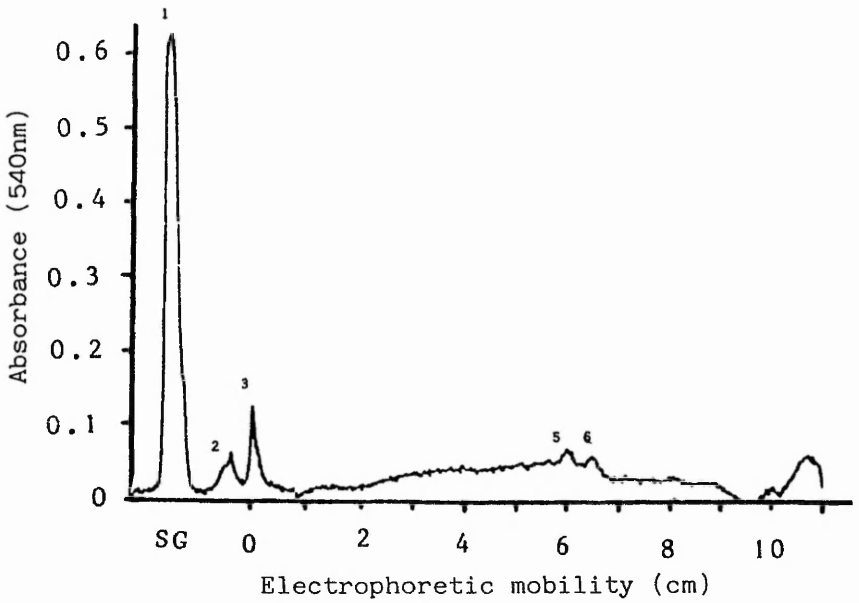


TABLE 5.7

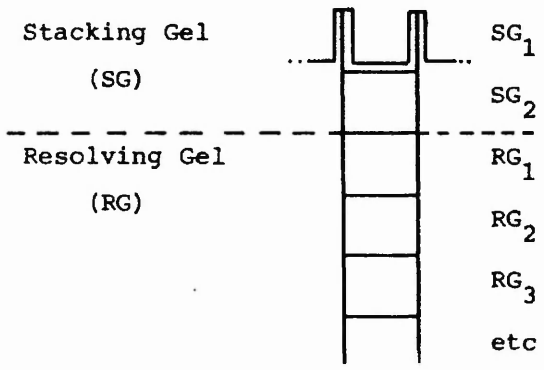
Pattern of Ca^{2+}-Dependent [^{14}C]-Methylamine Labelling in SDS-Polyacrylamide Gels of Tissue Slices of Normal Liver and Hepatocellular Carcinomas

The pattern of [^{14}C]-methylamine (59mCi/mmol) labelled proteins in tissue slices of normal liver and hepatocellular carcinomas was determined following their separation on discontinuous SDS-polyacrylamide gels (see figures 5.4 and 5.5) by the following methods:

A - Liquid Scintillation Counting of Sectioned gels (see section 2.5.4.3)

B - Determination of Peak Areas from Densitometric Scans of Fluorograms (see section 2.5.4.4).

The data are shown as mean values ± SE following subtraction of the appropriate EGTA controls. Typical values found in EGTA controls were: SG_1 polymer, 50-100 cpm or 1500 - 2000 peak area/mg protein loaded; all other sections, 200-500 cpm or 800-1500 peak area per mg protein loaded. The distribution (% of total) of radiolabel throughout the gel is also shown. The number of tissues examined is shown in parenthesis. A key to the sectioning of gels is shown below:



SG_1P Polymer in SG_1 fraction, SG_2P Polymers in SG_2 fraction, RG_1P Polymer at SG/RG interface, Bands 1-3 Proteins of Mr: 43,600, 38,900 and 35,900 (see Table 5.6). nd = not detectable.

A. Liquid Scintillation Counting of Sectioned Gels

Fraction	cpm/mg Protein loaded		Distribution (% of total)	
	Normal Liver (4)	Tumour (4)	Normal Liver (4)	Tumour (4)
SG_1	11034 ±2054	14114 ±1886	42.2 ±7.9	67.3 ±9.0
SG_2	1299 ±157	754 ±272	5.0 ±0.6	3.7 ±1.3
RG_1	3521 ±220	1939 ±461	13.5 ±0.8	9.2 ±2.2
RG_{2-12}	10258 ±2860	4149 ±1173	39.3 ±10.9	19.8 ±5.6
Total	26112	20956	100	100

B. Determination of Peak Areas from Densitometric Scans of Fluorograms

Fraction	Peak Area/mg Protein loaded		Distribution (% of Total)	
	Normal Liver (4)	Tumour (3)	Normal Liver (4)	Tumour (3)
SG_1P	20210 ± 80	22834 ± 340	43.6 ±0.2	63.6 ±1.0
SG_2P	2263 ± 260	1523 ± 160	4.9 ±0.6	4.2 '0.5
RG_1P	7130 ± 921	2644 ± 541	15.5 ±2.0	7.3 ±1.5
Band 1	1382 ± 20	nd	3.0 ±0.1	nd
Band 2	4086 ± 340	581 ± 140	8.8 ±0.7	1.6 ±0.4
Band 3	nd	300 ± 140	nd	0.8 ±0.4
RG(TOTAL)	16689 ±4922	8938 ±2081	36.0 ±10.6	24.9 ±5.8
Total	46292	35939	100	100

TABLE 5.8

Pattern of Ca^{2+}-Dependent [^{14}C]-Methylamine Labelling in 3mM SDS-Polyacrylamide Gels of Tissue Slices of Normal Liver and Hepatocellular Carcinomas

Tissue	cpm/mg protein loaded						
	SG_1	SG_2	RG_1	RG_{2-28}	RG_{13}	RG_{15}	Total
Control (n=2) Liver	9575	904	2305	5553	668	1270	20005
Hepatocellular(n=2) Carcinoma	11094	642	1139	2371	nd	nd	15246

The pattern of [^{14}C]-methylamine (59mCi/mmol) labelled proteins in tissue slices of normal liver and hepatocellular carcinomas was determined following their separation on discontinuous SDS-polyacrylamide gels. Conditions were identical to those described in Figures 5.4 and 5.5 except that gels of 3 mm thickness were used and 1.27 mg of normal liver protein and 1.10 mg of hepatocellular carcinoma protein were loaded. The resolving gel was cut into 28 sections (0.5 cm height) and counted for radioactivity (section 2.5.4.3). The results are shown as the mean Ca^{2+}-dependent incorporation (EGTA values have been subtracted) from two determinations on each tissue. See Table 5.7 for key to fractions: RG_{13} and RG_{15} are the fractions which correspond to band 1 (Mr 43,600) and band 2 (Mr 38,900) respectively. nd - not detectable above EGTA control values.

5.4.2 Subcellular Distribution of [^{14}C]-Methylamine Labelled Proteins in Tissue Slices of Normal Liver and Hepatocellular Carcinomas

These experiments were carried out in order to determine the subcellular location of the high molecular weight polymers and proteins which were radiolabelled during tissue slice experiments (see section 5.4.1). Homogenates of tissue slices were fractionated into 600g(N), 40,000g(M), 71,000g(P) particulate fractions and a particle-free cytosolic fraction (S). This fractionation procedure has already been characterised with marker enzymes (Figure 5.3). Protein from each subcellular fraction was separated by SDS-polyacrylamide gel electrophoresis and then treated in one of two ways: gels were either sectioned and counted for radioactivity (Table 5.9) or developed as fluorograms (Figure 5.8).

Fluorograms of normal liver fractionations (Figure 5.8A) and hepatocellular carcinoma fractionations (Figure 5.8B) showed a similar pattern of the Ca^{2+}-dependent radiolabelling of SG_1 and RG_1 polymers in both tissues. The SG_2 polymers which were found previously (see Figure 5.4) were not seen in these experiments. The Ca^{2+}-dependent radiolabelling of protein bands 1 and 2 was clearly visible in fluorograms of normal liver but in tumour tissue the radiolabelling of these bands was difficult to see.

Quantitation of the amount of each radiolabelled protein in the fractions was undertaken using liquid scintillation counting (Table 5.9) and densitometric scanning of fluorograms (Table 5.10). Liquid scintillation counting (Table 5.9) indicated that the SG_1 polymer was mainly in the 600 g fraction (N) (82%) with significant amounts in the 40,000g(M) and 71,000g(P) fractions, a distribution which is coincident

with that of the plasma membrane marker, 5'nucleotidase (see Figure 5.3). The RG_1 polymer was also mostly associated with the 600 g fraction (N) (65%), but as well as this, there was some radiolabel in the 40,000g(M) and 71,000g(P) fractions and also approximately 20% in cytosol fraction. Protein bands 1 and 2 were undetectable above the background level of radioactivity in the resolving gel. However, the Ca^{2+}-dependent radioactivity in this region was found to be bimodally distributed between the 600 g fraction (N) and the cytosol (S).

Densitometric scanning of fluorograms (Table 5.10) gave similar results for the subcellular distribution of the SG_1 and RG_1 polymers and also enabled the subcellular location of protein bands 1 and 2 to be determined. Band 1 was mostly located in the cytosol (S) fraction with smaller amounts associated with the 40,000g(M) and 71,000g(P) fractions. Band 2, however, was bimodally distributed between the 600 g fraction (N) (40%) and the cytosol (S) (47%) with smaller amounts associated with the 40,000g(M) and 71,000g(P) fractions. Attempts to characterise the subcellular location of band 3 in tumour tissue were unsuccessful owing to the low level of incorporation of radiolabel into this protein. However, visual examination of fluorograms indicated that it was mainly in the cytosol fraction (5).

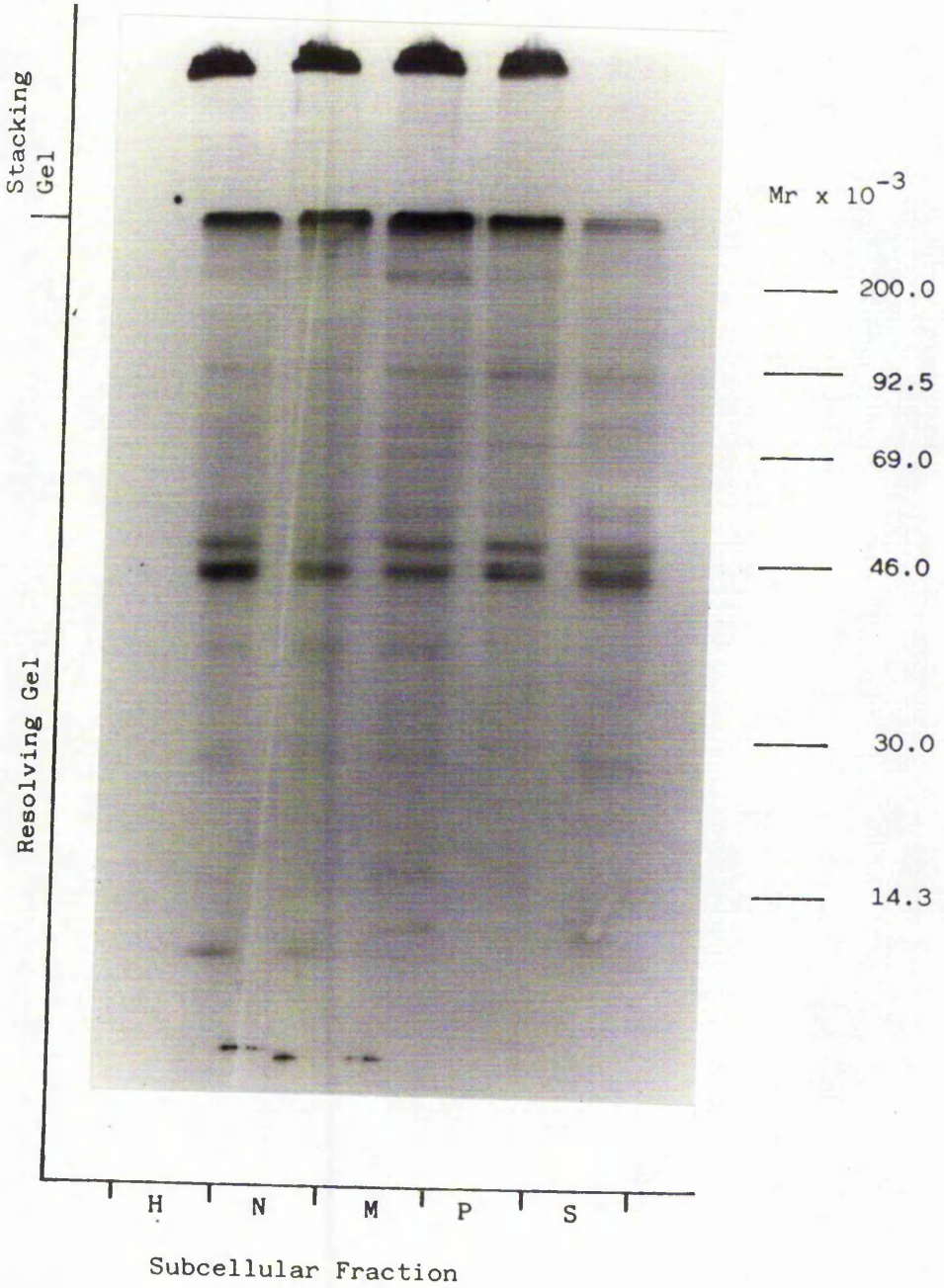
FIGURE 5.8

Examination of the Subcellular Distribution of the Ca^{2+}-Dependent [^{14}C]-Methylamine Labelling of Proteins in Tissue Slices of Normal Liver and Hepatocellular Carcinomas by SDS-Polyacrylamide Gel Electrophoresis and Fluorography

Tissue slices of normal liver and hepatocellular carcinomas were incubated with [^{14}C]-methylamine (1.2 mM) and $CaCl_2$ (1.9 mM) (Section 2.7). Following incubation, slices were homogenised in 0.25 M Sucrose, 1 mM EGTA, 100 μM PMSF, 5 mM Tris, pH 7.4 (see section 2.4.1) and fractionated into 600g(N), 40,000g(M), 71,000g(P) particulate fractions and a particle-free cytosol fraction (S) (see section 2.4.5). Fractions were then electrophoresed (section 2.4.6) on discontinuous SDS-polyacrylamide gels and developed as fluorograms (section 2.5.4.4): A, normal liver; B, hepatocellular carcinomas. The protein loadings for each fraction are shown below:

Lane	Subcellular Fraction	Protein Loading (μg)	
		A: Normal Liver	B: Hepatocellular Carcinoma
1	Homogenate(H)	130	110
2	600g(N)	125	75
3	40,000g(M)	295	285
4	71,000g(P)	70	75
5	cytosol(S)	190	135

A Normal Liver



B Hepatocellular Carcinoma

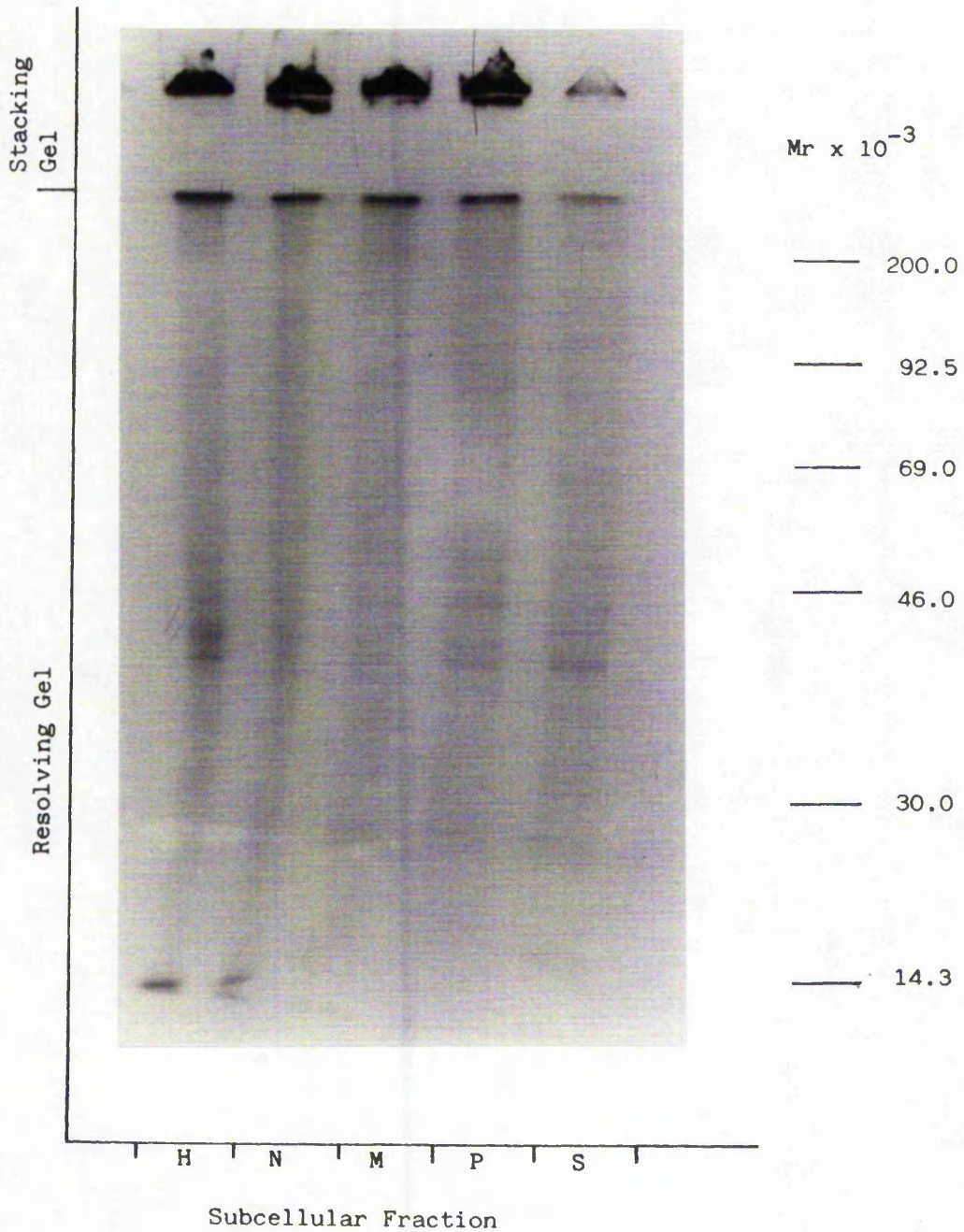


TABLE 5.9

Subcellular Distribution of Ca^{2+}-Dependent [^{14}C]-Methylamine Labelling of Proteins in Tissue Slices of Normal Liver and Hepatocellular Carcinomas: Liquid Scintillation Counting

Subcellular Fraction	% Distribution of Radiolabelled Proteins					
	Normal Liver			Hepatocellular Carcinomas		
	SG	RG_1	RG_{2-12}	SG	RG_1	RG_{2-12}
600g(N)	81.7±3.2	65.4±2.1	45.1±2.1	86.7±1.3	39.5±3.2	35.8±2.9
40,000g(M)	9.8±0.9	11.8±0.6	11.5±1.3	9.3±0.6	11.4±1.2	8.8±1.7
71,000g(P)	7.7±0.4	2.6±0.5	4.0±0.2	3.2±0.2	10.3±1.4	1.3±0.2
cytosol(S)	0.8±0.2	20.2±1.2	39.4±3.6	0.3±0.1	38.8±2.6	54.1±4.6
Total	100	100	100	100	100	100

Tissue slices of normal liver and hepatocellular carcinomas were incubated with 1.2 mM [^{14}C]-methylamine and either 1.9 mM $CaCl_2$ or 1 mM EGTA. Following incubations tissue slices were homogenised and fractionated as previously described (Figure 5.8). Subcellular fractions were electrophoresed (see Figure 5.8 for protein loadings) and following electrophoresis, gels were sectioned and counted for radioactivity (see section 2.5.4.3). Results are expressed as the mean percentage ± SE in each fraction from four fractionations of each tissue type (following subtraction of EGTA controls: 50-200 cpm/gel section).

SG = whole stacking gel, RG_1 = 1st cm of resolving gel, RG_{2-12} = remainder of resolving gel (see Table 5.7).

TABLE 5.10

Subcellular Distribution of the Ca^{2+}-Dependent [^{14}C]-Methylamine Labelling of Proteins in Tissue Slices of Normal Liver: Densitometric Scanning of Fluorograms

Subcellular Fraction	% Distribution of Radiolabelled Proteins			
	SG_1P	RG_1P	Band 1	Band 2
600g(N)	75	64	nd	40
40,000g(M)	12	9	8	5
71,000g(P)	13	12	10	8
Cytosol(S)	nd	15	82	47

Tissue slices were prepared, fractionated and electrophorised as described in Figure 5.8. Gels were then prepared for fluorography and exposed to X-ray film for 21 days at -70^{o}C (section 2.5.4.4). Individual lanes were scanned at 540 nM using a densitometer coupled to a plotting integrator (section 2.5.4.4). Peak areas were used to quantitate the incorporation into each section of the gel. Results are shown as the mean percentage in each subcellular fraction from duplicate determinations on a single fluorogram, SG_1P - polymer at the top of the stacking gel; RG_1P - polymer at the top of the resolving gel; Band 1 - protein of Mr 43,600; Band 2 - protein of Mr 38,900 (see Table 5.7); nd = not detectable.

5.4.3 Macromolecular Composition of the High Molecular Weight Polymers, SG_1 and RG_1, in Rat Liver Slices incubated with [^{14}C]-Methylamine and $CaCl_2$

The following experiments were carried out in order to determine the macromolecular composition of the high molecular weight polymers, SG_1 and RG_1, which were formed in tissue slices of normal liver when incubated with [^{14}C]-methylamine and $CaCl_2$ (section 5.4.1). Although it was shown that this Ca^{2+}-dependent incorporation of [^{14}C]-methylamine occurred in acid-precipitable material it was not known whether these high molecular weight aggregates were due to protein polymerisation or the association of proteins with other macromolecules such as DNA, RNA or membrane fragments which could not be dissociated by SDS. Therefore, prior to solubilisation for electrophoresis, radiolabelled tissue slices of normal liver were homogenised and subjected to a number of enzymic treatments, which included: deoxyribonuclease 1, ribonuclease 1, phospholipase C and trypsin. Following treatment, samples were subjected to SDS-polyacrylamide gel electrophoresis and the gels developed as fluorograms. These fluorograms were than scanned densitometrically at 540 nM and data were expressed as a percentage of an untreated control (Figure 5.9). Tissue slices which were incubated with EGTA instead of $CaCl_2$ are also included for comparison.

It was found that trypsin removed 80% of the radiolabel from the SG_1 position and 42% from the RG_1 position, thus demonstrating that the radiolabel was associated with protein. This radiolabel was found in the resolving gel as a diffuse staining below Mr 40,000, although the recovery was poor indicating that some radiolabel was freed as non-fixable protein fragments. Treatment with DNase 1, RNase 1 and phospholipase C, however, had little or no effect on the amount of

radiolabel found in the SG_1 or RG_1 positions. It can therefore be concluded that the high molecular weight aggregates SG_1 and RG_1 are comprised mainly of protein and that they may be covalently crosslinked.

FIGURE 5.9

Examination of the Ca^{2+}-Dependent [^{14}C]-Methylamine Labelling of SG_1 and RG_1 Polymers in Tissue Slices of Normal Liver by Treatment with Trypsin, DNase 1, RNase 1 and Phospholipase C

Tissue slices of normal liver were incubated with [^{14}C]-methylamine in the presence of 1.9 mM $CaCl_2$ or 1 mM EGTA (section 2.7). These slices were then homogenised in 0.25 M sucrose, 1 mM EGTA, 100 µM PMSF, 5 mM Tris-HCl, pH 7.4 (section 2.4.1). Aliquots of these homogenates were then treated as follows.

1) Ca^{2+} Incubation: no treatment.

2) EGTA Incubation: no treatment.

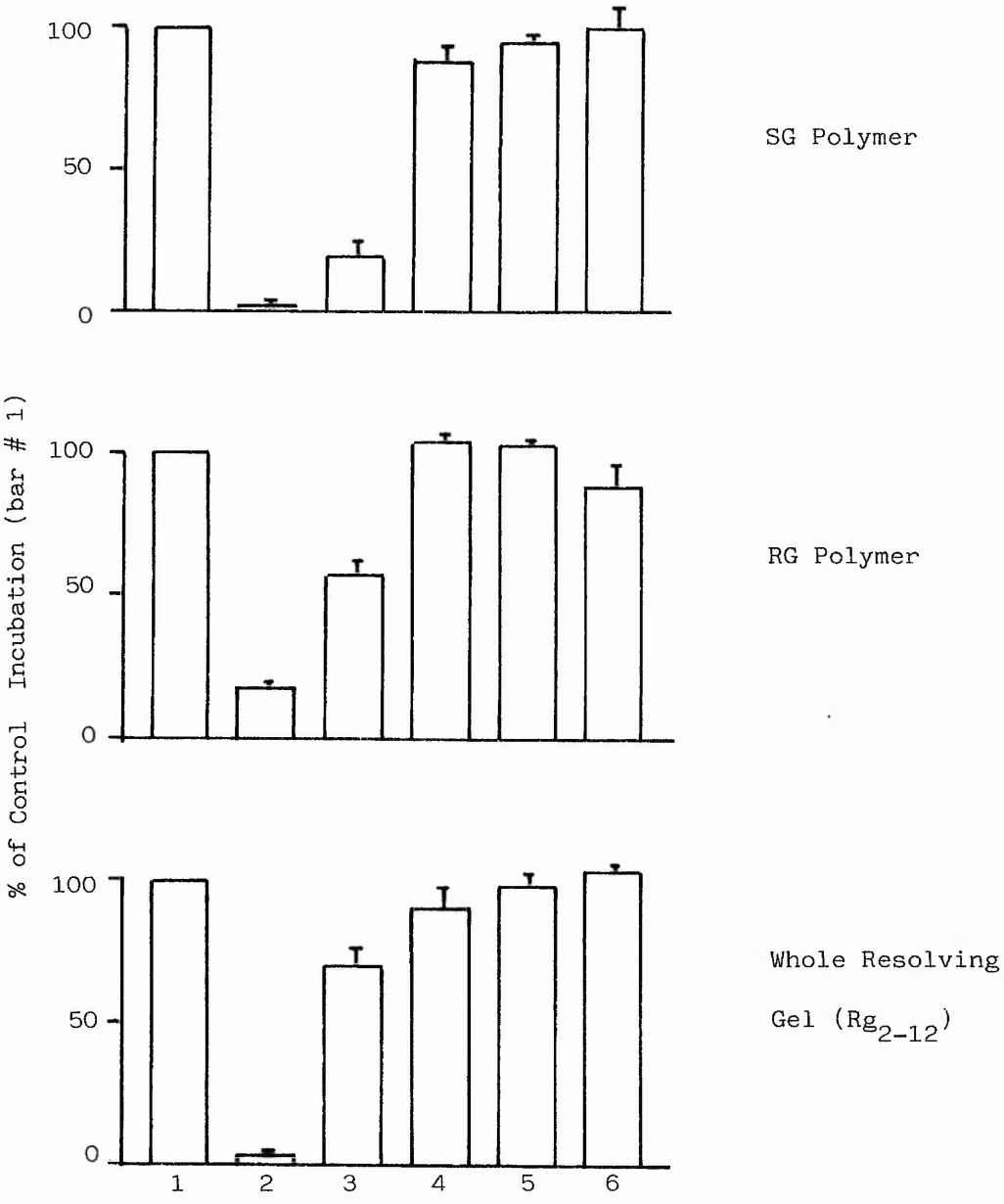
3) Ca^{2+} Incubation: 10 mg/ml trypsin at 37°C for 30 minutes; stopped by the addition of soybean trypsin inhibitor (5 mg/ml).

4) Ca^{2+} Incubation: 50 µg/ml DNase 1 with 10 mM $MgCl_2$.

5) Ca^{2+} Incubation: 50 µg/ml RNase 1.

6) Ca^{2+} Incubation: 200 µg/ml Phospholipase C (B Cereus EC 3.1.4.3) with 150 µM $ZnSO_4$ and 0.2% (w/v) deoxycholate.

Following treatment samples were solubilised in sample buffer (section 2.4.6.2) and electrophoresed on discontinuous SDS-polyacrylamide gels (section 2.4.6.3). Following electrophoresis, gels were developed as fluorograms at -70°C for 21 days (section 2.5.4.4). Fluorograms were scanned densitometrically at 540 nm and results expressed as the mean percentage incorporation into each section of the gel ± SE. Three separate experiments on one radiolabelled rat liver tissue slice (15,000 cpm/mg protein) were carried out.


% of Control Incubation (bar # 1)
100
50
0
SG Polymer
RG Polymer
Whole Resolving
Gel (Rg_{2-12})
1
2
3
4
5
6

5.4.4 Discussion

The proteins containing γ-glutaminyl residues available for transglutaminase-mediated [^{14}C]-methylamine incorporation in tissue slices of normal liver and hepatocellular carcinomas were investigated by SDS-polyacrylamide gel electrophoresis and fluorography. Staining of gels with the protein dye, PAGE blue 83, revealed the formation of a high molecular weight material (SG_1 polymer) in tissue slices which were incubated with [^{14}C]-methylamine and $CaCl_2$. This material could not enter a 2.5% ($^{w}/v$) acrylamide gel and was not found in tissue slices incubated with [^{14}C]-methylamine and EGTA, thus demonstrating that its formation was Ca^{2+}-dependent. Fluorography of gels indicated that the incorporation of [^{14}C]-methylamine into all sections of the gel was Ca^{2+}-dependent since tissue slices incubated with EGTA and [^{14}C]-methylamine had greatly reduced incorporation (90-95%) when compared to tissue slices incubated with $CaCl_2$ and [^{14}C]-methylamine. Therefore, because transglutaminase requires Ca^{2+} for activity, the results were expressed as the Ca^{2+}-dependent incorporation following subtraction of the appropriate EGTA controls.

It was found that the SG_1 polymer was a major site for the Ca^{2+}-dependent incorporation of [^{14}C]-methylamine in tissue slices of both normal liver and hepatocellular carcinomas. However, in addition to this, there was some Ca^{2+}-dependent incorporation of [^{14}C]-methylamine into smaller proteins, which were seen either in the stacking gel (SG_2 polymers), at the top of the resolving gel (RG_1 polymer) or in the resolving gel. This incorporation of [^{14}C]-methylamine in the resolving gel occurred mainly in proteins of Mr 43,600 (band 1) and Mr 38,900 (band 2) in normal liver and Mr 38,900 (band 2) and Mr 35,900 (band 3) in hepatocellular carcinomas. There was also some lesser incorporation into proteins of Mr 120,000-29,000 in normal liver but this was only just visible on the fluorograms.

Quantitation of the incorporation of [^{14}C]-methylamine by liquid scintillation counting and fluorography allowed a comparison of normal liver and hepatocellular carcinomas to be carried out. The incorporation of [^{14}C]-methylamine into the SG_1 polymer was similar in both tissues. Indeed in the tumours examined, there appeared to be more incorporation of [^{14}C]-methylamine (approximately 30%) into the SG_1 polymer than was found in normal liver. All other incorporation of [^{14}C]-methylamine into proteins of tumour tissue was reduced when compared to normal liver. The incorporation of [^{14}C]-methylamine into SG_2 polymers and RG_1 polymers being reduced by approximately 50% and for bands 1 and 2 by approximately 100% and 80% respectively. There was also a small amount of incorporation into a protein of Mr 35,900 (band 3) which was only radiolabelled in the tumour tissue. Finally it should be noted that the incorporation of radiolabel that was seen throughout the resolving gel was variable and that some of this radiolabel may be due to non-specific binding of [^{14}C]-methylamine to the gel. This is supported by the observation that the total incorporation of [^{14}C]-methylamine into normal liver protein and tumour protein was 25064 and 20957 cpm/mg respectively (Table 5.7) as compared to previous methods which employed more thorough acid-washing (11966 and 13213 cpm/mg respectively (section 5.3.1).

It was also undertaken to characterise these radiolabelled polymers and proteins with respect to their subcellular localisation. Differential centrifugation of tissue slice homogenates obtained from both normal liver and hepatocellular carcinomas showed that the distribution of the largest radiolabelled polymer, SG_1, was coincident with the plasma membrane marker, 5'nucleotidase, as seen in previous experiments with tissue slices (section 5.2.2). The smaller polymers were found in the 600 g fraction but were also present in significant

amounts in the cytosol fraction. The location of protein bands 1 and 2 was determined in normal liver by fluorography since tumour tissue contained insufficient amounts of these proteins for accurate determinations. Band 1 was found in the cytosol fraction with a distribution similar to that of lactate dehydrogenase (section 5.3.2), whereas band 2 was codistributed between the 600 g and cytosol fractions. Furthermore, the subcellular distribution of the total radiolabel associated with the resolving gel was also bimodal between the 600 g fraction and the cytosol fraction. This distribution is similar to that of total protein (section 5.3.2) and may either be explained by a low level incorporation of [^{14}C]-methylamine into many proteins or by the non-specific association of [^{14}C]-methylamine with the same proteins.

The macromolecular composition of the radiolabelled SG_1 and RG_1 polymers was further examined. Digestion with various enzymes indicated that the polymers were not caused by the association of proteins with either DNA (histones), RNA (ribosomal subunit proteins) or undigested membrane fragments (integral membrane proteins). The polymers were however shown to be comprised of trypsin digestible protein. Furthermore the approximate molecular weights of these polymers can be estimated from the known properties of acrylamide gels (Hames, 1979). The size of SG_1 polymer was therefore estimated to be in excess of Mr 1 x 10^6 and SG_2 and Rg_1 polymers were estimated to be of a size between Mr 1 x 10^6 and Mr 300,000. Similar high molecular weight substrates of transglutaminase have been reported in rabbit liver (Linniola et al., 1979), rat liver (Slife et al., 1986), cultured chick heart myofibrils (Loewy and Matacic, 1981) and human lung (Patterson et al., 1987). In all but one of these tissues (Linniola et al., 1979) the formation of these high molecular weight polymers has

been attributed to a particulate transglutaminase. Indeed, more recently a particulate transglutaminase and its high molecular weight substrate have shown to be located in a specific plasma membrane domain of rat liver which consists of gap junctional complexes and filamentous structures (Tyrell et al., 1986). It was also suggested that this high molecular weight substrate was similar to the adhesion factor previously found in chick hepatocytes (Slife et al., 1982).

It has been suggested that this high molecular weight substrate is either present in the native membrane or is formed rapidly following the addition of Ca^{2+} (Slife et al., 1986, Bungay et al., 1986). The results presented here indicate that the formation of this polymer is Ca^{2+}-dependent and it is therefore tempting to suggest that its formation is mediated by transglutaminase via ε(γ -glutamyl) lysine bridges. (Such ε(γ-glutamyl) lysine bridges have already been isolated from similar polymers in chick heart (Loewy et al., 1981), human erythrocytes (Lorand et al., 1976) and keratinocytes (Hanigan and Goldsmith, 1978).

In contrast there is scant information concerning momomeric protein substrates of transglutaminase in mammalian liver. Slife and co-workers (1986) briefly noted some radiolabelling of proteins which entered a 7% (w/v) acrylamide gel, when purified rat liver plasma membranes were incubated with[^{3}H]-putrescine, whereas Linniola et al., (1979) reported that two proteins of Mr 150,000 were involved in polymer formation in rabbit liver. However, the most plausible candidates as substrates for transglutaminase are the cytoskeletal proteins (Gard and Lazarides, 1979, Cohen et al., 1979, 1980), an hypothesis which ties in with the work on high molecular weight transglutaminase substrates by Loewy and Matacic (1981). Furthermore, work in other systems has implicated transglutaminase as a potential

modulator of cytoskeletal structure and function (see section 1.3). In our tissue slice experiments we have identified two principal monomeric substrates of transglutaminase, although it is unclear whether these play any role in polymer formation. Since these radiolabelled proteins were found in the cytosol it seems plausible that the cytosolic transglutaminase is the enzyme involved in the attachment of [^{14}C]-methylamine to these proteins. This idea is supported by the fact that there is a correlation between the reduced incorporation of [^{14}C]-methylamine into tumour proteins and the reduced cytosolic transglutaminase activity in these tumours (section 3.5). The labelling of a unique protein of Mr 35,900 in tumour tissue may be due to a variety of reasons. It may be due to proteolysis of one of the other radiolabelled proteins in the tumour tissue, since transformed cells can exhibit increased proteolytic activity with respect to their normal counterparts (Mahdavi and Hynes, 1979). Alternatively it may be due to the increased expression of a specific protein in the tumours or even the expression of an oncogenic product. Indeed a protein of this size has been shown to be a target for tyrosine phosphorylation by protein kinases and may be involved in transformation (Hunter, 1984). Furthermore, since phosphoproteins are thought to be substrates for transglutaminase (Iwanij, 1977, Owen et al., 1987), it is possible that there may be a link between the oncogenic phosphoproteins which are found in a variety of systems and transglutaminase. This possibility is particularly interesting since many of these proteins have been shown to be involved in the anchorage of the cytoskeletal network at the plasma membrane.

The co-distribution of the protein of Mr 38,900 and the RG_1 polymer between the 600 g fraction and the cytosol fraction may possibly be explained by the translocation of these proteins from the

cytosol to the plasma membrane or vice-versa or even their sequential incorporation into the plasma membrane as a part of a crosslinking process. This suggestion is pertinent since transglutaminase has been implicated in endocytosis and exocytosis, processes which are dependent upon translocation.

The following experiments were therefore carried out in order to further characterise the substrates of transglutaminase in vitro and to investigate the ability of cytosolic transglutaminase to act on membrane and cytosolic proteins.

5.5 IN VITRO PRIMARY AMINE INCORPORATION EXPERIMENTS

Investigations into the Ca^{2+}-dependent incorporation of [^{14}C]-methylamine into tissue slices have provided a preliminary picture of the nature and location of this incorporation into proteins of normal rat liver and hepatocellular carcinomas. The following investigations were therefore carried out using tissue homogenates and subcellular fractions in an attempt to further characterise and understand the Ca^{2+}-dependent incorporation of methylamine in tissues.

5.5.1 Measurement of Putrescine Incorporation into available γ-Glutaminyl Residues of Proteins in Normal Liver and Hepatocellular Carcinomas

The total number of peptide-bound γ-glutaminyl residues available for transglutaminase-mediated putrescine incorporation was determined in normal liver and hepatocellular carcinomas by the incorporation of [^{14}C]-putrescine into endogenous proteins. In order to ensure that the enzyme activity was equivalent in all determinations, an excess of purified rat liver cytosolic transglutaminase was added to the reaction system. Further, to confirn that saturation of γ-glutaminyl residues was achieved, exogenous protein was added to the reaction system after 35 min (Figure 5.10). The subsequent increase in incorporation of radiolabel demonstrated that the level of incorporation attained prior to the addition of exogenous protein was due to saturation of γ-glutaminyl residues of endogenous proteins rather than the exhaustion of enzyme activity. The mean values for putrescine incorporation into a number of normal livers, hepatocellular carcinomas and tumour bearing livers are shown in Table 5.11. It was found that the incorporation of [^{14}C]-putrescine

into endogenous protein was comparable in normal liver and tumour bearing liver. However these levels of [^{14}C]-putrescine incorporation were significantly higher than those found in tumour tissue ($P<0.05$) irrespective of whether the data were expressed as a function of tissue weight, protein or DNA.

FIGURE 5.10

Saturation of γ-Glutaminyl Residues of Proteins with [^{14}C]-Putrescine in Homogenates of Normal Liver and Hepatocellular Carcinomas using Purified Rat Liver Cytosolic Transglutaminase

Tissue homogenates (4-5 mg protein) were incubated with [^{14}C]-putrescine and purified rat liver cytosolic transglutaminase (7.3 μg protein, 9000 Units/mg protein - see section 4.2.4.1). Incubations were carried out at 37^{o}C in the presence of 1.8 mM Ca^{2+}, 3.85 mM dithiothreitol, 1.2 mM putrescine (3.97 mCi/mmol) in a total volume of 200 μl. Duplicate aliquots (10 μl) of reaction mixture were removed at 5, 10 and 30 min and spotted onto filter papers (1 cm^2) and placed in ice-cold 10% (w/v) TCA. At 35 min N,N'-dimethylcasein was added (final concentration 10 mg/ml) and further aliquots were removed at 40, 45 and 50 min and treated in the same way. All filter papers were then washed and counted as described in sections 2.6.1 and 2.5.4.1. Typical reaction plots for normal liver (●), hepatocellular carcinoma tissue (▲) and an incubation which contained only the purified enzyme (■) are shown.

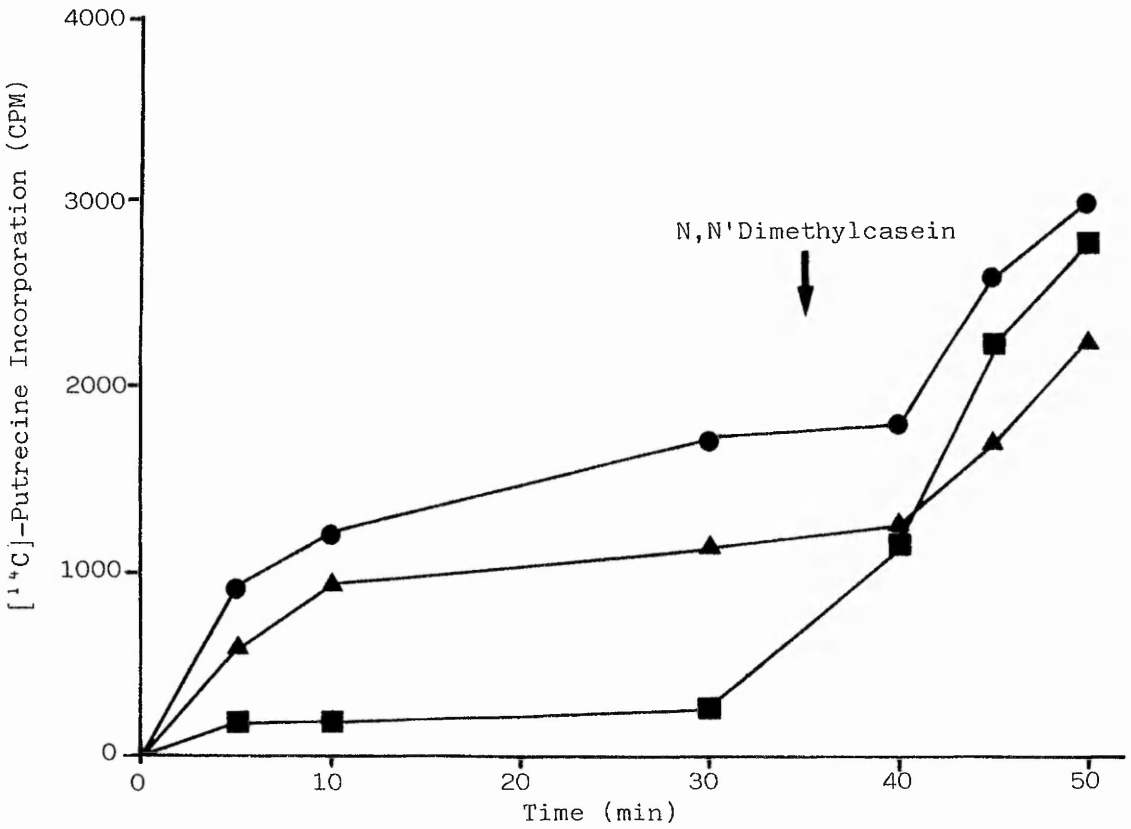

4000
3000
2000
1000
0
[14C]-Putrecine Incorporation (CPM)
N,N'Dimethylcasein
0
10
20
30
40
50
Time (min)

TABLE 5.11

Incorporation of [^{14}C]-Putrescine into γ-Glutaminyl Residues of Proteins in Homogenates of Normal Liver, Tumour Bearing Liver and Hepatocellular Carcinomas

TISSUE	[^{14}C]-Putrescine Incorporation		
	nmol/g tissue	pmol/mg protein	pmol/ g DNA
Normal Liver (7)	127.6 ± 16.1	781.4 ± 115.8	135.3 ± 19.3
Tumour Bearing Liver (4)	118.2 ± 6.4	735.7 ± 29.8	109.2 ± 8.7
Hepatocellular Carcinoma (6)	72.4 ± 11.4	457.0 ± 73.6	63.5 ± 10.9

Tissue homogenates were incubated with [^{14}C]-putrescine as previously described (Figure 5.10). The incorporation of radiolabel in the presence of EDTA (10 mM) was used as a control and was found to be constant throughout the time period of the assay (40-60 cpm). The Ca^{2+}-dependent incorporation of [^{14}C]-putrescine into tissues is shown as the mean value ± SE with the number of tissues examined in parenthesis. The incorporation of radiolabel into the purified enzyme preparation when incubated alone was 2747.0 pmol/mg protein but this represented only a small proportion of the total radioactivity incorporated into tissue samples under the conditions used (see Figure 5.10). Protein and DNA were estimated by the procedures described in sections 2.5.1 and 2.5.3.

5.5.2 Subcellular Distribution of γ-Glutaminyl Residues of Proteins in Normal Liver and Hepatocellular Carcinomas

The subcellular distribution of γ-glutaminyl residues of endogenous proteins in homogenates of normal liver and hepatocellular carcinomas was investigated by two methods. Firstly, normal liver and hepatocellular carcinomas were fractionated into a cytosol fraction and a 71,000 g particulate fraction. Excess purified rat liver cytosolic transglutaminase was then added to each fraction in order to determine the total number of available peptide-bound γ-glutaminyl residues in the fractions (see section 5.5.1). It was found that there was a consistent difference in the subcellular distribution of peptide-bound γ-glutaminyl residues when normal liver was compared to hepatocellular carcinomas (Table 5.12). In normal liver 27% of the total [^{14}C]-putrescine which was incorporated into proteins was found in the cytosol, whereas in tumour tissue some 43% was found in the same fraction. This could be seen to be due to a small increase in the number of incorporation sites in the cytosol and a larger decrease in the number of incorporation sites in the particulate fraction.

In the second method, whole normal rat liver homogenate was incubated with [^{14}C]-methylamine and either $CaCl_2$ or EGTA. Homogenates were then fractionated into 600g(N), 400,000g(M), 71,000g(P) particulate fractions and a particle-free cytosol fraction (S). The incorporation of radiolabel into each fraction was then expressed as the percentage of the total Ca^{2+}-dependent incorporation in each fraction (following subtraction of EGTA controls) (Figure 5.11A). The majority of the radiolabel (80%) was found in the 600g(N) fraction with small amounts in the 40,000g(M) and 71,000g(P) fractions and some 15% in the cytosol fraction (S). Homegenates were also submitted to SDS-polyacrylamide gel electrophoresis and gels were sectioned and counted

for radioactivity (Figure 5.11B). The majority of the radiolabel was found at the top of the stacking gel (78%) with the remainder spread throughout the resolving gel. The only significant peak of radioactivity in the resolving gel occurred in fraction R_8 which corresponded to a molecular weight range of Mr 25,000-30,000. Since this was clearly different to the labelling pattern seen in tissue slices (section 5.4.1) no attempt was made to study this further.

TABLE 5.12

Subcellular Distribution of γ-Glutaminyl Residues in Proteins from Homogenates of Normal Liver and Hepatocellular Carcinomas

TISSUE	[^{14}C]-Putrescine Incorporation			
	(pmol/mg protein)		% Distribution	
	S	P	S	P
Normal Liver (3)	444 ± 110	1170 ± 131	27.5 ± 8.2	72.5 ± 8.2
Hepatocellular Carcinomas (3)	594 ± 96	783 ± 59	43.1 ± 4.3	56.9 ± 4.3

Tissue homogenates (see section 2.4.1) were fractionated into a cytosol fraction (S) and a 71,000 g particulate fraction (P) (section 2.4.2). These fractions were incubated with [^{14}C]-putrescine (3.97 mCi/mmol) and purified rat liver cytosolic transglutaminase (section 4.2.4.1) as described previously (Figure 5.10). The incorporation of [^{14}C]-putrescine is shown as the mean value ± SE and as the mean % distribution ± SE with the number of tissues examined in parenthesis.

FIGURE 5.11

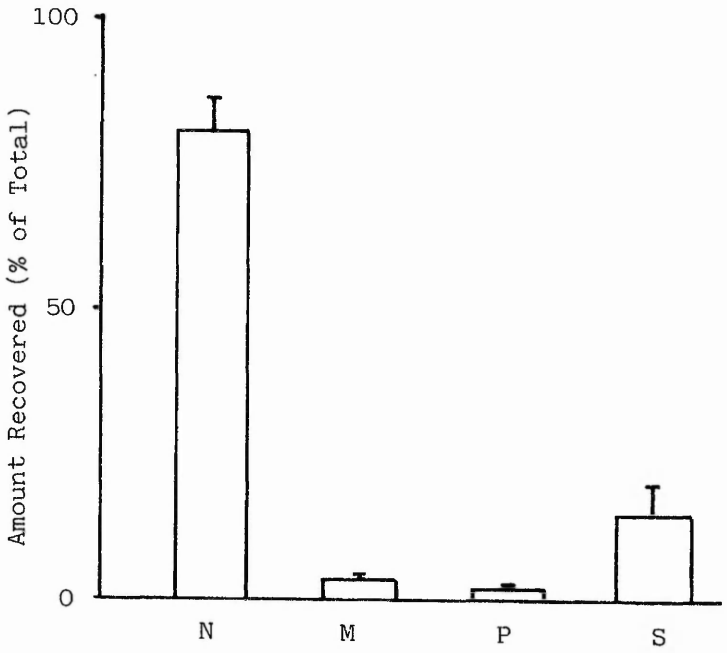
Examination of the Ca^{2+}-Dependent Incorporation of [^{14}C]-Methylamine into Normal Liver Homogenate by Differential Centrifugation and SDS-Polyacrylamide Gel Electrophoresis

Normal rat liver was homogenised in 0.25 M sucrose, 10 mM benzamidine, 1 mM PMSF, 1 mM EGTA, Tris-HCl, pH 7.4 (section 2.4.1). The homogenate was then incubated with [^{14}C]-methylamine (1.2 mM, 59mCi/mmol) in the presence of $CaCl_2$ (2.1 mM) or EGTA (1 mM) for 1 hour at 37^{o}C. The level of incorporation into these homogenates was as follows: Ca^{2+}-incubation - 19,000 ± 1350 cpm/mg protein, EGTA incubation - 640 ± 450 cpm/mg protein. These homogenates were then fractionated by the following procedures:

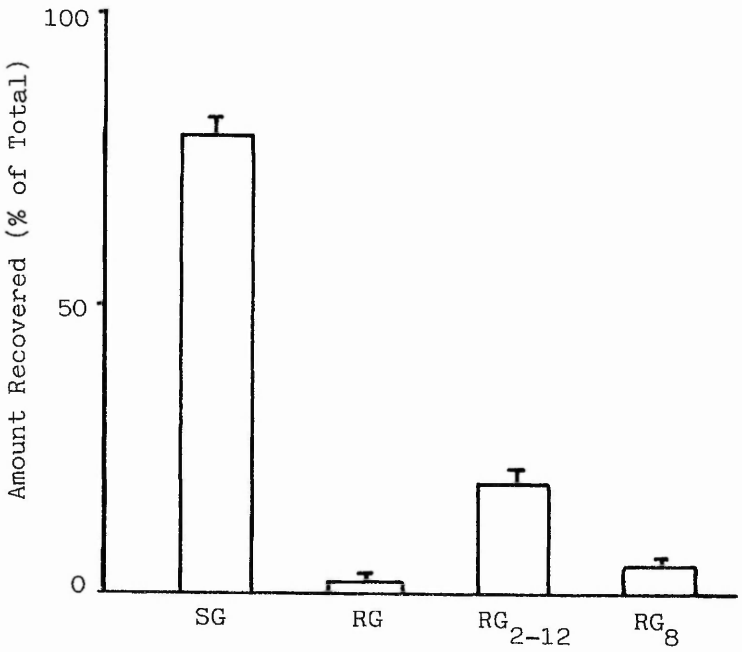
A Differential Centrifugation: The method described in section 2.4.5 was used to fractionate [^{14}C]-methylamine labelled homogenates. This procedure produced 600g(N), 40,000g(M), 71,000g(P) particulate fractions and a cytosol fraction (S). Results are shown as a histogram depicting the mean subcellular distribution of Ca^{2+}-dependent [^{14}C]-methylamine incorporation following subtraction of the appropriate EGTA controls. The standard error from duplicate determinations is shown as an error bar.

B SDS-Polyacrylamide Gel Electrophoresis: The method described in section 2.4.6 was used to fractionate proteins in [^{14}C]-methylamine labelled homogenates by SDS-polyacrylamide gel electrophoresis. Gels were then sectioned and counted for radioactivity (section 2.5.4.3). Results are expressed mean % of the total incorporation in each area of the gel following subtraction of EGTA controls (60-120 cpm/gel section). SG = stacking gel, RG_1 = 1st cm of resolving gel, RG_{2-12} = remainder of resolving gel (see Table 5.7). RG_8 corresponds to a molecular weight range of Mr 25,000-30,000.

A Differential Centrifugation



B SDS-Polyacrylamide Gel Electrophoresis



5.5.3 The Effect of Tumour Homogenates on the Activity of Purified Rat Liver Cytosolic Transglutaminase

Purified rat liver cytosolic transglutaminase (section 4.2.4.1) was used to investigate the effect of endogenous inhibitors and/or substrates of transglutaminase on the assay of enzyme activity in tumour homogenates. Transglutaminase activity was measured in the tumour homogenates, the purified enzyme preparation and in a mixture of both (Table 5.13). It was found that the activity in the mixture of tumour homogenate and purified enzyme was close to that expected from the assay of these samples alone. It would therefore appear that any endogenous substrates and/or inhibitors of transglutaminase that may be present in tumour samples (or other tissues) have little effect on the measurement of transglutaminase activity under the conditions used.

TABLE 5.13

Measurement of Purified Liver Cytosolic Transglutaminase Activity following its Incubation with Tumour Homogenate

Incubation	Transglutaminase Activity (Units)
1	63.3 ± 0.2
2	926.5 ± 47.0
3	954.1 ± 41.2

The following incubations were carried out at 4°C for 1 hour:

1) 60 µl tumour homogenate + 40 µl 50 mM Tris - HCl, pH 7.4

2) 40 µl purified transglutaminase + 60 µl 50 mM Tris - HCl, pH 7.4

3) 60 µl tumour homogenate + 40 µl purified transglutaminase

All samples were in 50 mM Tris-HCl, pH 7.4 containing 1 mM EDTA. Following incubation, transglutaminase activity was measured in each fraction (section 2.6.1). Results are expressed as the mean values ± SE from three determinations. Purified rat liver cytosolic transglutaminase was prepared by the procedure described in section 4.2.4.1. Tumour homogenate 23B was used for this assay (see Tables 3.1, 3.4 and 5.3).

5.5.4 In Vitro [^{14}C]-Methylamine Incorporation Experiments with Untreated and Iodoacetamide-Treated Rat Liver Membranes and Rat Liver Cytosol

These experiments were undertaken in order to study the ability of cytosolic liver transglutaminase to incorporate [^{14}C]-methylamine into cytosolic and/or membrane proteins. Fraction S_1 (see section 2.4.2) was used as a source of cytosolic transglutaminase since this fraction contains minimal amounts of particulate transglutaminase (section 4.2.2). In order to ensure that the incorporation of [^{14}C]-methylamine was due to the cytosolic enzyme, membranes were first incubated with iodoacetamide (5 mM) at 37°C for 1 hour, a treatment which completely inactivated the transglutaminase activity in these fractions as assessed by the incorporation of [^{14}C]-putrescine into N,N'-dimethylcasein. Both unwashed (P_1) and washed (P_2) membranes (see Figure 2.1) were used in order to discriminate between tightly and loosely associated peptide-bound γ-glutaminyl residues on the membranes. Following the removal of iodoacetamide by dialysis the treated membrane fractions (P_1I and P_2I) and untreated membrane fractions (P_1 and P_2) were incubated with or without the S_1 fraction (Figure 5.12).

The incubation of fractions S_1, P_1 and P_2 (bars 1-3) on their own with [^{14}C]-methylamine demonstrates that there are available γ-glutaminyl residues in proteins in all of these fractions. The reduced level of incorporation of radiolabel in P_2 fraction (when compared to P_1) is probably due to the removal of transglutaminase activity from the membranes by this washing procedure (see section 3.4.1). The incubation of iodoacetamide-treated membranes with the S_1 fraction showed that the level of incorporation of radiolabel was

similar for washed (P_2I) and unwashed (P_1I) membranes (bars 4 and 5), thus indicating that the membrane-bound γ-glutaminyl residues were not removed by the washing procedure. This supports the idea that the reduced incorporation into washed membranes (bar 3) when compared to unwashed membranes (bar 2) is due to reduced transglutaminase activity in the P_2 fraction. Further, since both incubations of iodoacetamide-treated membranes with the S_1 fraction (bars 4 and 5) resulted in higher levels of incorporation than expected from their incubation alone, it could be concluded that cytosolic transglutaminase was incorporating radiolabel into membrane proteins. (N.B. Incubations of P_1I or P_2I membranes on their own resulted in zero incorporation of radiolabel.) Finally, the incubation of membranes (P_2) which had not been treated with iodoacetamide with the S_1 fraction resulted in similar levels of incorporation to those seen with iodoacetamide treated membranes (bar 6). This demonstrates that the treatment of the membrane fraction with iodoacetamide did not alter the availability of γ-glutaminyl residues by altering protein structure or conformation.

Following incubation of P_2I membranes and the S_1 fraction (bar 5) they were centrifuged at 71,000 g for 45 min to generate a particle free supernatant (S*) and a 71,000 g pellet (P*) (bars 7 and 8). This revealed that 70% of the total incorporation was associated with the pellet (P*). This was surprising since the counts in the supernatant (S*) (bar 7) were lower than those found in incubations of the S_1 fraction alone (bar 1). Therefore S_1 fraction was incubated alone and then centrifuged at 71,000 g for 45 min. It was found that only 10% of the radiolabel was still in the supernatant (S_1*) after centrifugation (bar 9). It was therefore apparent that the incorporation of radiolabel into cytosolic proteins was associated with the formation of high molecular weight material, possibly similar to

that found in tissue slice experiments (section 5.4.1). Therefore several incubations of the type described above (Figure 5.12) were submitted to SDS-polyacrylamide gel electrophoresis (Figure 5.13). Fraction S_1 was found to contain a high molecular weight polymer similar to that found in tissue slice experiments (section 5.4.1) when incubated with $CaCl_2$ and [^{14}C]-methylamine. Centrifugation of this fraction removed the polymer from this fraction (S_1*), thus confirming the findings mentioned above. In addition P_1 and P_1I fractions were electrophoresed following their incubation with $CaCl_2$ and [^{14}C]-methylamine. It was found that polymer was only formed in the P_1 fraction, thus demonstrating that the formation of the polymer was thiol-dependent and therefore consistent with a transglutaminase mediated reaction. Finally the incubation of S_1 and P_1I fractions together with $CaCl_2$ and [^{14}C]-methylamine resulted in greatly increased polymer formation and therefore further implicated transglutaminase in the formation of this polymer.

Gels were also sectioned and counted for radioactivity (Table 5.14). The distribution of the radiolabel between the stacking gel (polymer) and the resolving gel was similar in all incubations except for that where S_1 fraction was first centrifuged at 71,000 g for 45 min (S_1*). In this S_1* fraction the radiolabel was mostly found in the resolving gel due to the loss of the radiolabelled polymer in the centrifugation. It should however be noted that the pattern of radiolabelling in these gels was different to that found in tissue slice experiments there being less incorporation at the stacking gel/resolving gel interface (RG_1 polymer) and somewhat higher incorporation in the molecular weight range Mr 48,000-37,000.

FIGURE 5.12

Experiments with Iodoacetamide Treated Membranes

Rat liver homogenates (20% w/v) were washed as described in section 2.4.3 as far as the generation of fraction P_2 (see Figure 2.1). The pellet fractions P_1 and P_2 were then incubated with 5 mM iodoacetamide for 1 hour at 37°C. These iodoacetamide-treated fractions, P_1I and P_2I, were then dialysed against 1 mM DTT, 1 mM EGTA, 50 mM Tris, pH 7.4 to remove the iodoacetamide. Samples of untreated S_1, P_1 and P_2 were also dialysed against the same medium in order to remove low molecular weight factors such as polyamines. These fractions were then incubated with [^{14}C]-methylamine on their own and in various combinations as shown below. The amount of radiolabel incorporated during each incubation is shown as the mean value ± SE from triplicate determinations on each incubation.

Bar	Samples in Incubation	Treatment after Incubation
1	S_1 + 50 mM Tris, pH 7.4	None
2	P_1 + 50 mM Tris, pH 7.4	None
3	P_2 + 50 mM Tris, pH 7.4	None
4	P_1I + S_1	None
5	P_2I + S_1	None
6	P2 + S_1	None
7	P_2I + S_1	Centrifugation (S*)
8	P_2I + S_1	Centrifugation (P*)
9	S_1 + 50 mM Tris, pH 7.4	Centrifugation (S_1*)

Each incubation contained 1.2 mM [^{14}C]-methylamine, 2.5 mM $CaCl_2$ and 3.85 mM DTT and 70 µl of each sample in a total volume of 200 µl. Bars 7 and 8 represent the supernatant (S*) and pellet (P*) fractions from centrifugation of the incubation shown in bar 5 at 71,000g for 45 min. Bar 9 represents the supernatant fraction (S_1*) from centrifugation of the incubation shown in bar 1 at 71,000 g for 45 min.

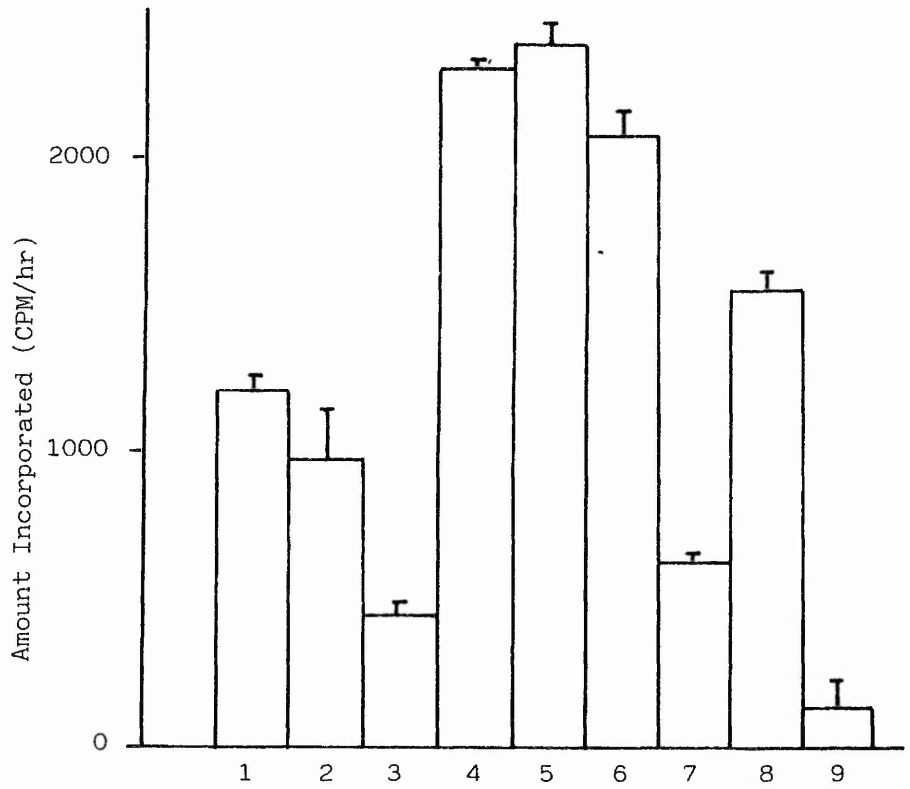

Amount Incorporated (CPM/hr)
2000
1000
0
1
2
3
4
5
6
7
8
9

FIGURE 5.13

SDS-Polyacrylamide Gel Electrophoresis of Untreated and Iodoacetamide-Treated Liver Cell Fractions following their Incubation with [^{14}C]-Methylamine and $CaCl_2$

Some of the incubations described in the Figure 5.12 were electrophoresed on discontinuous SDS-polyacrylamide gels (section 2.4.6). Following electrophoresis, gels were fixed and stained with PAGE blue 83 dye (section 2.4.6.4). The loadings of each incubation mixture are as follows:

Lane	Sample	Volume (µl)	Total Protein (µg)
1	Standards	-	-
2	-	-	-
3	S_1 (1)	25	120
4	S_1* (9)	25	120
5	P_1 (2)	25	200
6	P_1I -	25	200
7	$P_1I + S_1$ (4)	15	195

See Figure 5.12 for key to samples (Bar # is shown in parenthesis). N.B. There was no incorporation of radiolabel into P_1I fraction when incubated alone with [^{14}C]-methylamine.

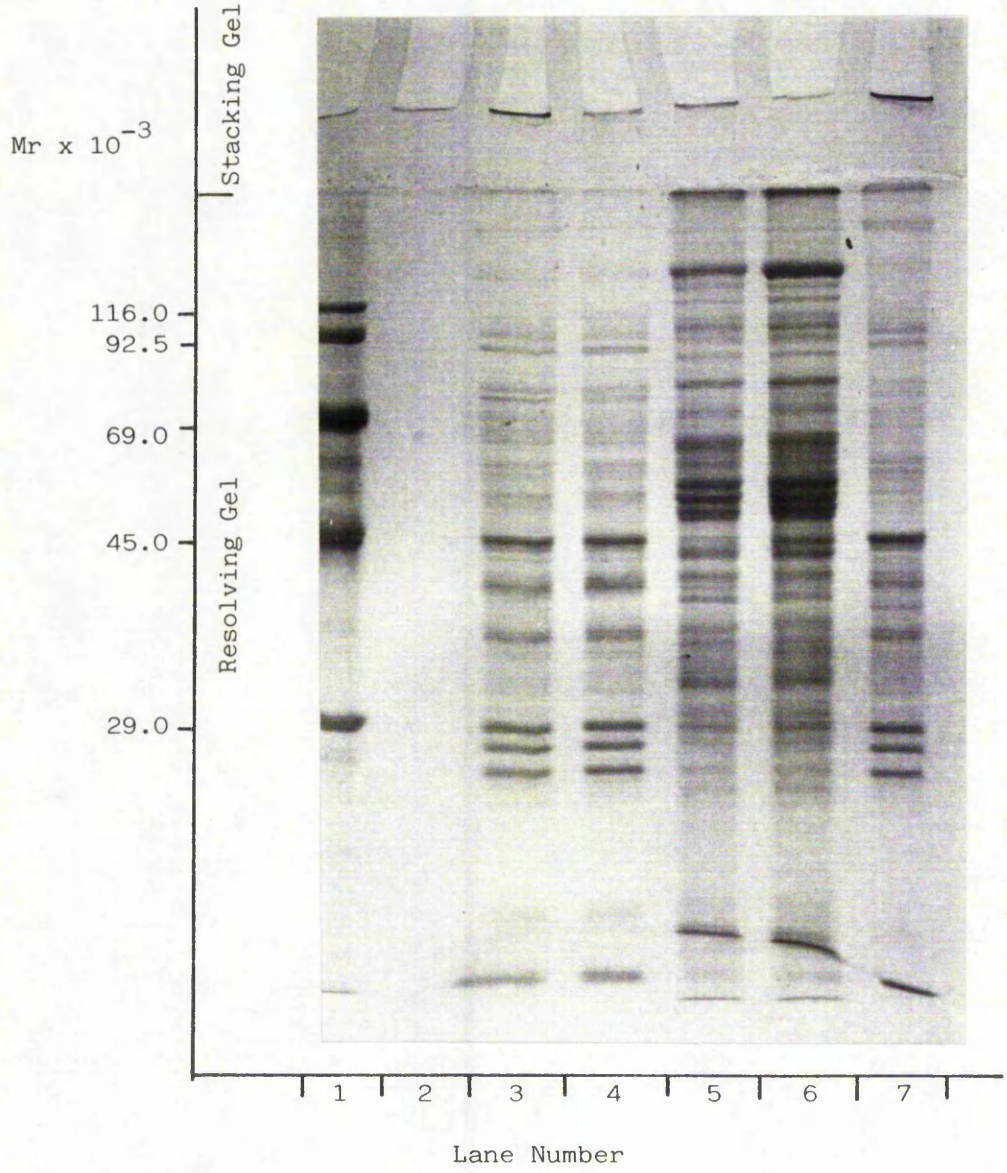

Mr x 10^{-3}
Stacking Gel
Resolving Gel
116.0
92.5
69.0
45.0
29.0
1
2
3
4
5
6
7
Lane Number

TABLE 5.14

Pattern of Ca^{2+}-Dependent [^{14}C]-Methylamine Incorporation in SDS-Polyacrylamide Gels of In Vitro Incubations of Iodoacetamide Treated Rat Liver Membranes and Rat Liver Cytosol

Incubation	Distribution of Radiolabel (% of Total)	
	Stacking Gel	Resolving Gel
S_1	33.0	67.0
S_1*	13.0	87.0
P_1	32.0	68.0
P_1I	-	-
P_1I+S_1	34.0	66.0

Samples were incubated and electrophoresed as described previously (Figures 5.12, 5.13). The gel shown in Figure 5.13 was sectioned and sections were counted for radioactivity (section 2.5.4.3). S_1 - rat liver cytosol, S_1* - S_1 centrifuged at 71,000 g for 45 min, P_1 - rat liver particulate fraction, P_1I - P_1 treated with iodoacetamide (see Figures 5.12). Results are expressed as the distribution (% of total) between the stacking and resolving gels from a single gel.

5.5.5 Discussion

The total number of peptide-bound γ-glutaminyl residues available for transglutaminase-mediated putrescine incorporation was measured in normal liver, tumour bearing liver and hepatocellular carcinoma tissue. Purified rat liver cytosolic transglutaminase was used to achieve complete saturation of available peptide-bound γ-glutaminyl residues. It was found that tumour tissue contained significantly reduced levels of available peptide-bound γ-glutaminyl residues when compared to normal liver or tumour bearing liver. This reduction (approx. 40%) was statistically significant ($P \leqslant 0.05$) whether the data were expressed as a function of tissue weight, protein or DNA. A number of factors may affect the total number of available peptide-bound γ-glutaminyl residues in tissues, including: changes in the expression of the proteins containing γ-glutaminyl residues, alterations in the expression or activation of transglutaminase(s) and the availability of polyamines. It has been shown that hepatocellular carcinomas contain reduced cytosolic transglutaminase activity (section 3.6) and it was therefore expected that the number of available peptide-bound γ-glutaminyl residues would be higher in normal tissue when compared to tumour tissue. Since the exact opposite was found, it was undertaken to examine the subcellular distribution of peptide-bound γ-glutaminyl residues. When the cytosol fractions of normal liver and tumours were compared, a higher number of peptide-bound γ-glutaminyl residues were found in the tumours, in agreement with the above hypothesis. However, measurement of the available peptide-bound γ-glutaminyl residues in the particulate fractions of these tissues revealed that there were significantly fewer present in tumour tissue. This may possibly be explained by reduced expression of specific membrane proteins in tumours as has been reported previously (Magee,

1965, Zimmerman, 1978), although other factors may have contributed to such a reduction. Furthermore, the total amount of primary amine incorporation into tissue homogenates was some 6-7 times higher than the incorporation found in tissue slices, thus indicating that in intact cells only a small proportion of the total peptide-bound γ-glutaminyl residues participate in transglutaminase-mediated reactions.

Purified cytosolic liver transglutaminase was also used to assess the effect of tissue factors such as polyamines (see section 5.2.3) on the assay of transglutaminase in tissue homogenates (see Chapter 3). It was found that tissue homogenates of tumours had little effect on the assay of transglutaminase activity. This indicates that the concentration of polyamines derived from the tissue homogenates is insufficient to interfere with the assay in the presence of 1.2 mM putrescine. Additionally it may also be noted that tumour tissue does not appear to contain any inhibitors of transglutaminase.

Incorporation of [^{14}C]-methylamine into rat liver homogenates (non-saturating) gave a similar labelling pattern to that found in tissue slice experiments with most of the radiolabel occurring in the 600 g pellet. However, analysis of these same homogenates by electrophoresis indicated that whilst high molecular weight polymers were the chief site of methylamine incorporation, the labelling of proteins which entered the resolving gel was different to that which occurred in tissue slices. There was more labelling overall and in particular there was significant radiolabel found in the range Mr 25,000-30,000. Therefore it was apparent that transglutaminase was either labelling proteins which it does not encounter in intact cells or that the cytosolic enzyme was not activated during tissue slice incubations. Furthermore, the formation of SG_1 polymer in these

experiments may be comprised of a different mixture of proteins to that found in tissue slices.

Experiments with iodoacetamide treated membranes provided some interesting insights into the action of cytosolic transglutaminase and into the nature and subcellular distribution of its potential substrates. It was clear that the cytosolic transglutaminase was capable of incorporating [^{14}C]-methylamine into both cytosolic and membrane located proteins. Furthermore, the incubation of rat liver cytosol on its own resulted in the formation of a high molecular weight polymer which was similar to that found in tissue slices when they were incubated under the same conditions (section 5.4.1). However, these protein substrates were tightly associated with the membrane since washing with a non-isotonic buffer did not remove them from the membrane fraction. This washing procedure did however remove significant amounts of transglutaminase activity from the membrane fraction (see section 3.5). Comparison of iodoacetamide treated membranes with untreated membranes revealed that the ability of these proteins to act as transglutaminase substrates was unaffected by this thiol-reactive agent. It can therefore be concluded that the availability of γ-glutaminyl residues on these proteins is not dependent on thiol groups. The labelling of protein(s) with [^{14}C]-methylamine in the molecular weight range Mr 48,000-27,000 is of particular interest since similar labelling was found in tissue slice experiments (see section 5.4.1).

Whether the cytosolic transglutaminase acts on membrane-bound proteins <u>in vivo</u> is a difficult question to answer since, in the intact cell, the particulate enzyme is also present. Also, the experiments in this section deal with only one aspect of transglutaminase catalysis since ε(γ-glutamyl)lysine bridges may also be formed by these enzymes.

Indeed, in light of the fact that polymeric material was formed in both tissue slice experiments and experiments with homogenates despite the presence of free methylamine it is possible that transglutaminase prefers the ε-amino group of protein-bound lysine residues as a second substrate rather than methylamine (see section 1.1.4). Furthermore, the labelling of these proteins with [^{14}C]-methylamine may suggest that they are rich in glutamine residues as has been shown for involucrin, a substrate of the epidermal transglutaminase (Etoh et al., 1986). The future development of methods for detecting the ε-lysine residues available for transglutamine-mediated crosslinking and of more sensitive techniques for the quantitation of ε(γ-glutamyl) lysine and γ-glutamyl-polyamine derivatives is undoubtably of crucial importance to the understanding of the role of transglutaminase(s) in cellular function and in pathological conditions such as carcinogenesis.

In the following chapter, transplantable rat sarcomata of different metastatic ability were used to investigate the role of transglutaminase and polyamines in the metastatic process.

6.0 THE P_7 AND P_8 SARCOMATA - A MODEL SYSTEM FOR THE STUDY OF THE INVOLVEMENT OF TRANSGLUTAMINASE IN THE METASTATIC PROCESS

6.1 INTRODUCTION

In contrast to the well established alterations in transglutaminase activity in tumour growth (see section 1.3.5), much less is known about the importance of this enzyme in tumour progression and metastasis. A further reduction in transglutaminase activity has been reported in connection with metastasis from this laboratory (Barnes et al., 1984, 1985) and workers at INSERM in France have demonstrated that transglutaminase is reduced in the sequence: non-metastasising > metastasising in rat rhabdomyosarcoma cells and in the sequence: normal > benign > malignant in human intestinal cancer (Delcros et al., 1986, 1987). The experiments detailed here are a continuation of the work of Barnes et al., (1985) on the transplantable rat sarcomata, P_7 and P_8. The aim of these experiments was to study the interaction of polyamines and transglutaminase during the metastatic process.

6.2 HISTOLOGY AND GROWTH CHARACTERISTICS OF THE P_7 AND P_8 SARCOMATA

The origins and growth characteristics of the sarcomata used in this study have been described previously (Moore, 1972). They were originally induced by intraperitoneal injection of ^{32}P into AS rats; P_7 is an osteosarcoma and P_8 an intramedullary fibrosarcoma.

6.2.1 Histology of P_7 and P_8 Sarcomata

Samples of tumour tissue were taken at appropriate intervals and prepared for microscopic examination (see section 2.3.4). P_7 tissue was found to consist of rapidly dividing cells in various stages of mitosis (Figures 6.1A and 6.1B). The cells appeared to be of a single type and there was little necrosis evident. P_8 tissue was sampled at 20 days (pre-metastasis) and 40 days (post-metastasis). In the pre-metastatic tissue the majority of cells were poorly differentiated although some areas of fibroblast-like cells were also present (Figure 6.1C). However in the post-metastatic tissue, the predominant cell type was the fibroblast-like cell (Figure 6.1D) with only small areas of undifferentiated cells being evident. In both pre-metastatic and post-metastatic P_8 tissue some areas of necrotic tissue were present, although the amounts appeared to be roughly equivalent in the two tissues. Figures 6.1E and 6.1F show the undifferentiated cells from pre-metastatic tissue and the fibroblast-like cells from post-metastatic tissue at high magnification.

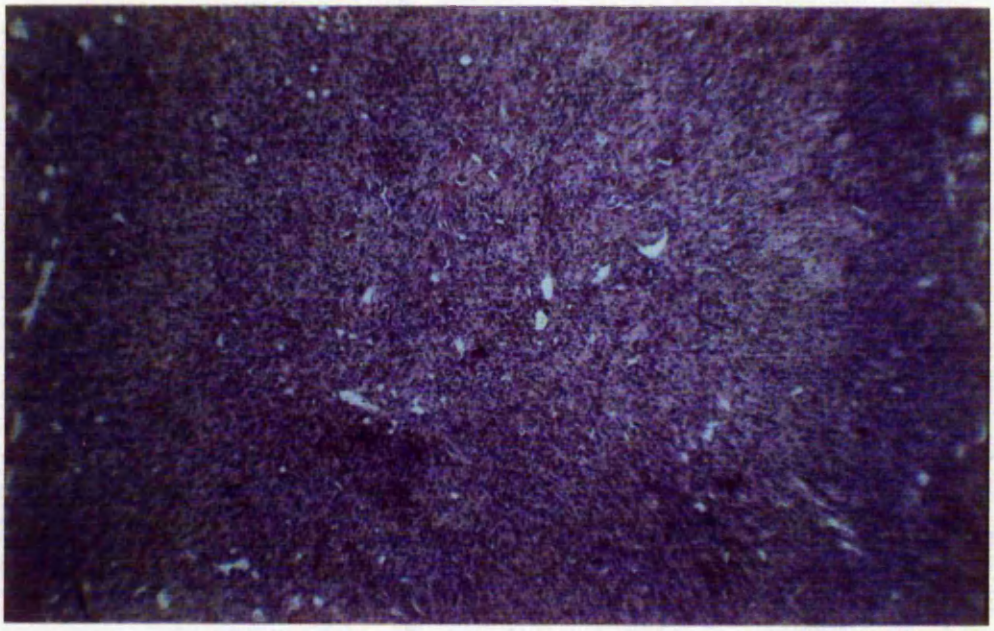
FIGURE 6.1

Histological Examination of P_7 and P_8 Sarcomata throughout their Growth

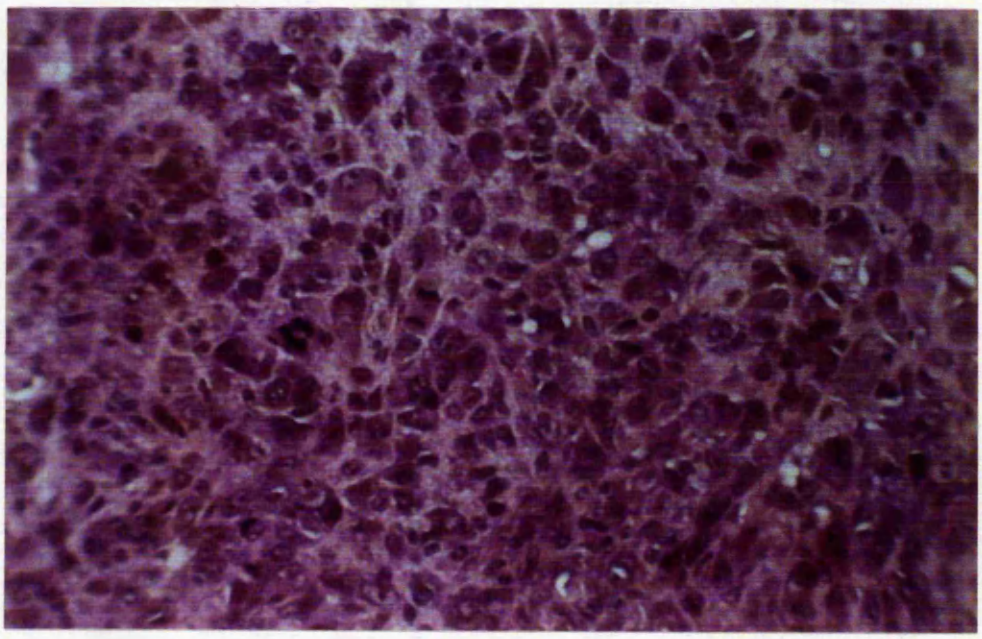
Tissues were stained with haematoxylin and eosin as described in section 2.3.2 and examined under the microscope. A,B - P_7 sarcoma (11 days), C,E - P_8 sarcoma (20 days), D,F - P_8 sarcoma (45 days).

Figure 6.1 Haematoxylin and Eosin Stained Sections of P_7 and P_8 Sarcomata

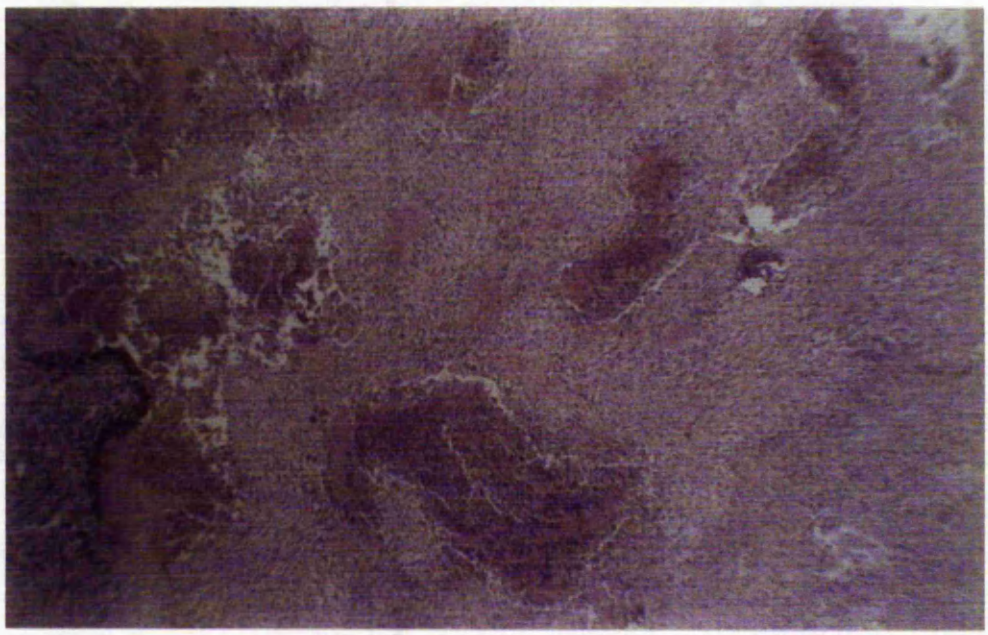
A P_7 Sarcoma (magnification x 154)



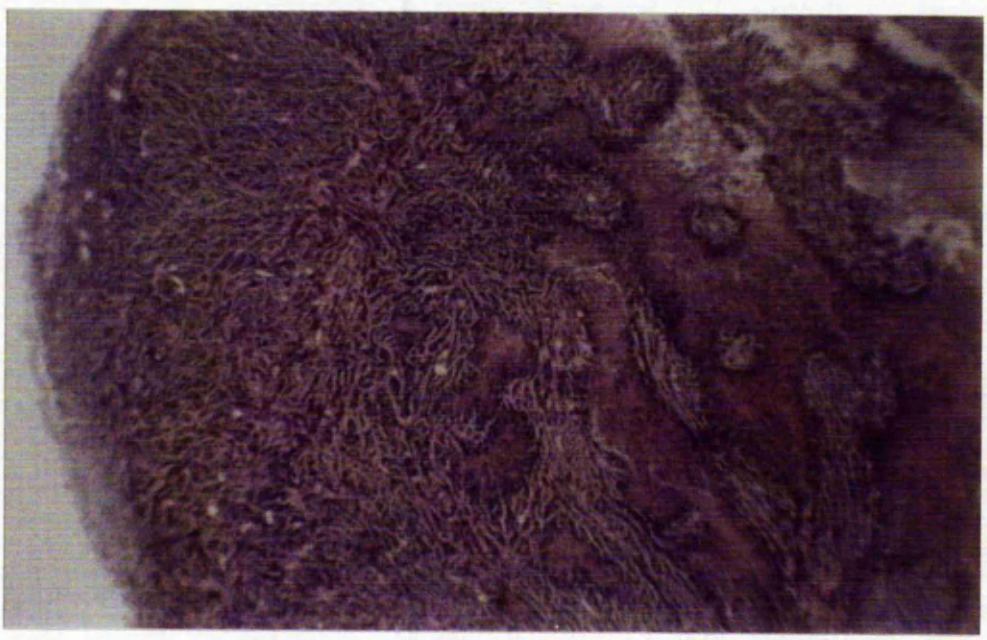
B P_7 Sarcoma (magnification x 960)



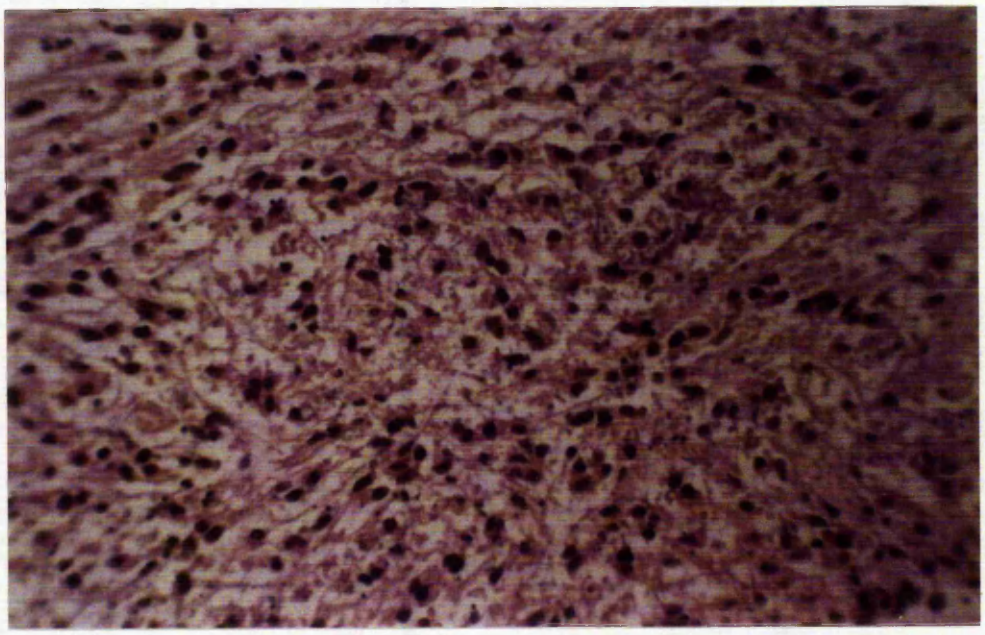
C P_8 Sarcoma (20 day) (magnification x 96)



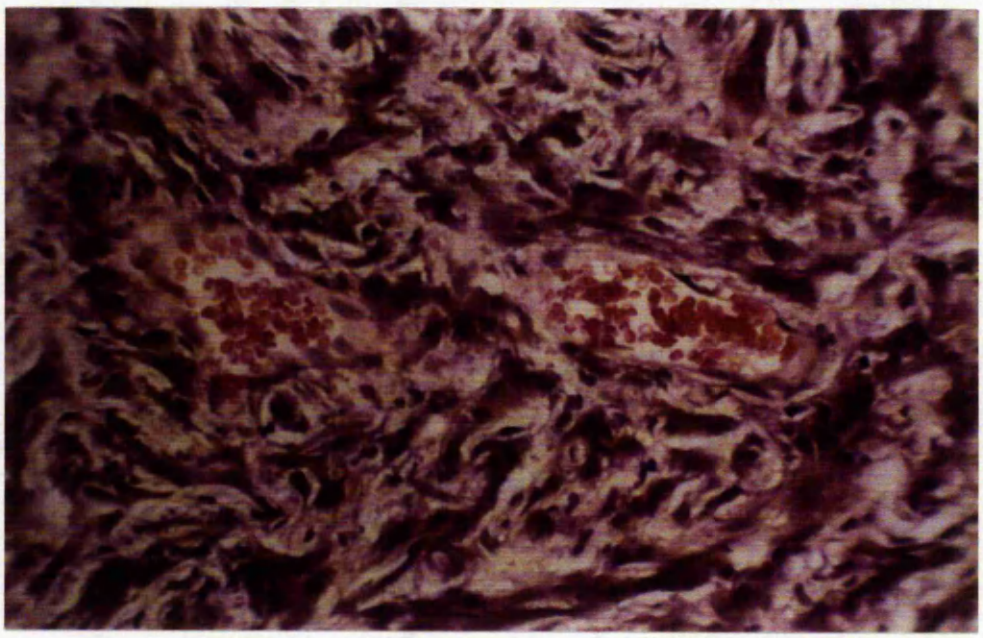
D P_8 Sarcoma (40 day) (magnification x 96)



E P_8 Sarcoma (20 day) magnification x 960)



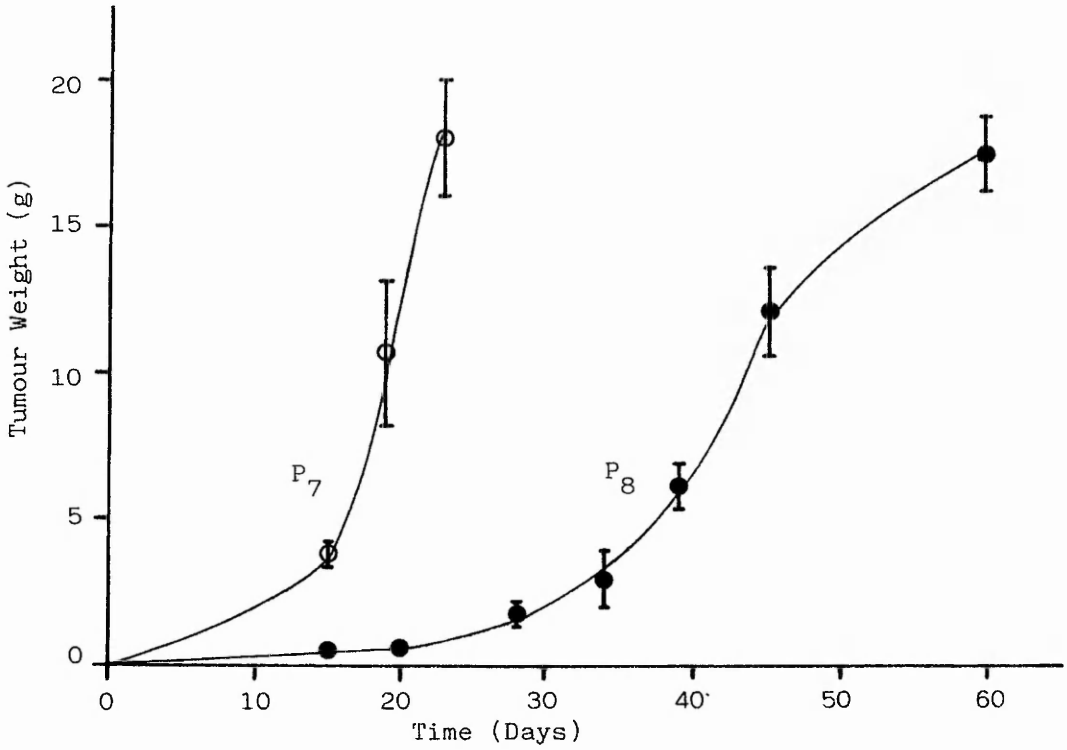
F P_8 Sarcoma (40 day) (magnification x 960)



6.2.2 Growth of P_7 and P_8 Sarcomata

The increase in Sarcoma weight with time was monitored in P_7 and P_8 sarcomata. Plots of the increase in sarcoma weight against time are shown in Figure 6.2. P_7 was clearly seen to grow much faster than P_8, reaching a maximal size at approximately 23 days. In contrast, P_8 took 60 days to reach a comparable size. When P_7 tumours reached maximal size (15-20 g) it was necessary to terminate the experiment since further tumour growth resulted in rupture of the flank on which the tumour was situated. In the P_8 tumours there was a considerable reduction in the body weight of tumour-bearing animals between 40-60 days, after which time animals became increasingly weak and ultimately died. For this reason 60 days was used as a maximal time for P_8 studies.

Figure 6.2 Growth Curves for P_7 and P_8 Sarcomata



Tumours were harvested at various times after implantation and weighed. Each point represents the mean weight ± SE from three tumours at each timepoint. P_7-O, P_8-●.

6.2.3 Detection of Lung Metastases in Sarcoma-Bearing Rats

Lung metastases were detected by the method of Wexler (1966) (cf section 2.3.4). In P_7 bearing animals no metastases were detected throughout these studies. In P_8 bearing animals metastases were consistently found in the lungs 30 days following implantation. One animal had detectable metastases at 28 days. Figures 6.3A and 6.3B show metastases detected in the lungs of a P_8 bearing rat at 39 days.

FIGURE 6.3

Detection of Lung Metastases in

P_8 Sarcoma-Bearing Rats

Lungs were inflated with an indian ink solution in order to highlight metastases (section 2.3.4). Metastases were contrasted white on a black background. Typical lungs from P_8 sarcoma-bearing rats are shown (39 days after implantation), as whole lung (A) and a transverse section of the left lobe (B).

A Whole rat lung (magnification x3.5)

B Transverse section of rat lung (left lobe, magnification x3.5)

6.2.4 Discussion

P_7 sarcomata were found to grow rapidly following their implantation into AS rats. This was demonstrated both by a rapid doubling time and by high mitotic figures seen in stained sections of cells. The morphology of these tumour cells was similar throughout the growth of the tumour with little necrosis evident.

P_8 on the other hand were slower growing than P_7 sarcomata, but they exhibited some considerable morphological changes during growth. In early or pre-metastatic tissue the main cell type appeared to be poorly differentiated with some small areas of 'fibroblast-like' cells present. Conversely, in older, post-metastatic tissue the majority of cells were 'fibroblast-like' with the numbers of undifferentiated cells having fallen considerably. It is impossible to determine whether this situation represents the progression of the tumour by differentiation of a single cell type on these data alone. However, the passage of P_8 sarcoma tissue from either young (20 day) or old (40 day) sarcomas results in the re-establishment of the same sequence of morphological changes. This would, therefore seem to suggest that the appearance of the 'fibroblast-like' cells is due to differentiation rather than the selective expression of a second cell type. It should also be noted that there was some necrosis evident in P_8 sarcomas. However the amount of necrosis did not appear to increase with age and was therefore unlikely to affect comparative measurements on pre- and post-metastatic P_8 tissue.

In agreement with previous work on these tumours (Moore, 1972; Barnes *et al.*, 1985), P_7 sarcomata, which metastasise infrequently, were not found to metastasise in these experiments. However in the studies with P_8 sarcomata metastases were consistently

found in the lungs of the sarcoma-bearing rats at about 30 days. In one instance metastases were detected at 28 days after implantation.

6.3 TRANSGLUTAMINASE ACTIVITY AND ACID-SOLUBLE POLYAMINE LEVELS IN P_7 AND P_8 SARCOMATA

Transglutaminase activity and the levels of the acid-soluble polyamines, putrescine, spermidine and spermine were measured in P_7 and P_8 sarcoma tissue. Tissue was sampled throughout the growth period of these sarcomata (see section 6.2.2) up until the time where tumour size (or metastases in the case of P_8) necessitated termination of the experiment. Protein and DNA content in these tissues was also measured; however, since neither of these parameters varied significantly with time, results are expressed per unit weight of DNA. Transglutaminase activity, protein and the levels of putrescine, spermidine and spermine are shown as a function of time for the P_7 sarcoma (Figure 6.4) and the P_8 sarcoma (Figures 6.5A and B). Where statistically significant differences are indicated in the following text, this was true whether data were expressed as a function of tissue weight, protein or DNA.

In the P_7 sarcoma none of the measured parameters varied significantly over the time period in which they were measured (11-23 days).

In the P_8 sarcoma transglutaminase activity fell dramatically between 28 and 34 days. The reduction in activity was approximately fourfold and coincided precisely with the detection of metastases in the lungs of the P_8 sarcoma bearing animals. When transglutaminase activity in pre-metastatic tissue (15, 20 and 28 days) was compared to post-metastatic tissue (34, 39 and 45 days) the reduction was statistically significant ($P \leqslant 0.05$). Transglutaminase activity in post-metastatic tissue at 60 days was significantly reduced with respect to

the activity found at 15 and 20 days ($P \leq 0.05$) but not that found at 28 days.

Putrescine levels in this same sarcoma (P_8) were also seen to undergo significant alterations during tumour progression. At 15 and 20 days putrescine levels were comparable to those found in P_7 tissue. At 28 days putrescine levels in two out of three tumours were also comparable to those found in P_7 tissue but the third tumour contained elevated levels (789.1 nMol/mg DNA) of putrescine. Interestingly this was the same tumour that had detectable metastases at this timepoint (see section 6.2.3). At 34 and 39 days putrescine levels were elevated in all sarcomas, reaching a maximum at 39 days. At this timepoint putrescine levels were 40 fold higher than at 15 or 20 days. This elevation was highly significant ($P \leq 0.02$) and was effectively maintained until 45 days by which putrescine levels had only fallen minimally. However by 60 days putrescine levels had fallen back to pre-metastatic levels, being only 1.8 times higher than at 15 or 20 days. The initial rise (34 days) of putrescine in these sarcomata coincided with the onset of metastasis and the fall in transglutaminase levels. However whilst putrescine levels were seen to return to levels approaching those in pre-metastatic tissue, transglutaminase activity remained low throughout the growth of the P_8 sarcomata once metastases had been detected. The massive rise in putrescine levels seen in post-metastatic P_8 tissue is highlighted in figure 6.6 which shows typical HPLC traces from perchloric acid extracts of P_8 tissue.

Spermidine and spermine levels were also seen to fluctuate in the P_8 sarcoma. Between 15 and 28 days the levels of both of these polyamines doubled, then fell back to the same levels as at 15 days by 39 days. By 60 days they were on the increase again. Comparison of the levels of spermidine and spermine in each group of tumours at each

timepoint using a two sample t test indicated that at 28 days the levels of both spermidine and spermine were higher than at 15, 20 or 39 days ($P \leq 0.05$). All other comparisons were not significant.

In P_7 tissue spermidine/spermine ratios were in the range 1.9 - 2.7 and in P_8 tissue in the range 4.0 - 6.9. Indeed in P_8 tissue fluctuations in spermidine/spermine ratios mirrored the changes in spermidine and spermine levels, thus indicating that spermidine levels fluctuated more than spermine levels in this sarcoma. This relationship was not found in the P_7 sarcoma.

The idea that elevated polyamine levels in tumours (especially putrescine) could be high enough to interfere with the transglutaminase assay (by diluting out the radiolabelled substrate - see section 2.6.1) was checked by calculating the concentrations of each polyamine added to the reaction mixture in homogenates of P_7 and P_8 sarcomata. This was done for P_7 sarcomata at 11 days (a representative timepoint - see Figure 6.4) and P_8 sarcomata at 15 and 45 days (minimal and maximal timepoints for putrescine levels - Figure 6.5). Taking into consideration that transglutaminase has a lower affinity for spermidine and spermine (Clarke et al., 1959) it is clear that these polyamines would have little effect in the presence of 1.2mM putrescine. Similarly, the putrescine levels in 11 day old P_7 tissue and in 15 day old (pre-metastatic) P_8 tissue were too low to have any appreciable effect. However, in 45 day old (post-metastatic) P_8 tissue the putrescine levels were high enough to dilute the specific activity of the radiolabel from 3.97 mCi/mmol (see section 2.6.1) to 2.98 mCi/mmol. Nevertheless, this dilution factor (25%) does not account for the large drop (fourfold) in transglutaminase activity that was seen in these sarcomata and furthermore, transglutaminase activity was not found to have risen significantly by 60 days, a timepoint at which polyamine levels were at premetastatic values (see Figure 6.5).

FIGURE 6.4

Levels of Transglutaminase, Polyamines and Protein in P_7 Sarcoma Tissue During Growth

Samples of tissue were taken at various times during tumour growth and homogenised (section 2.4.1). Homogenates were assayed for transglutaminase ▲ (section 2.6.1), protein □ (section 2.5.1), DNA (section 2.5.3), or extracted for Polyamine analysis (section 2.8.3), putrescine ○, spermidine △, spermine ●. Results are expressed as the mean value from three individual sarcomas ± SE.

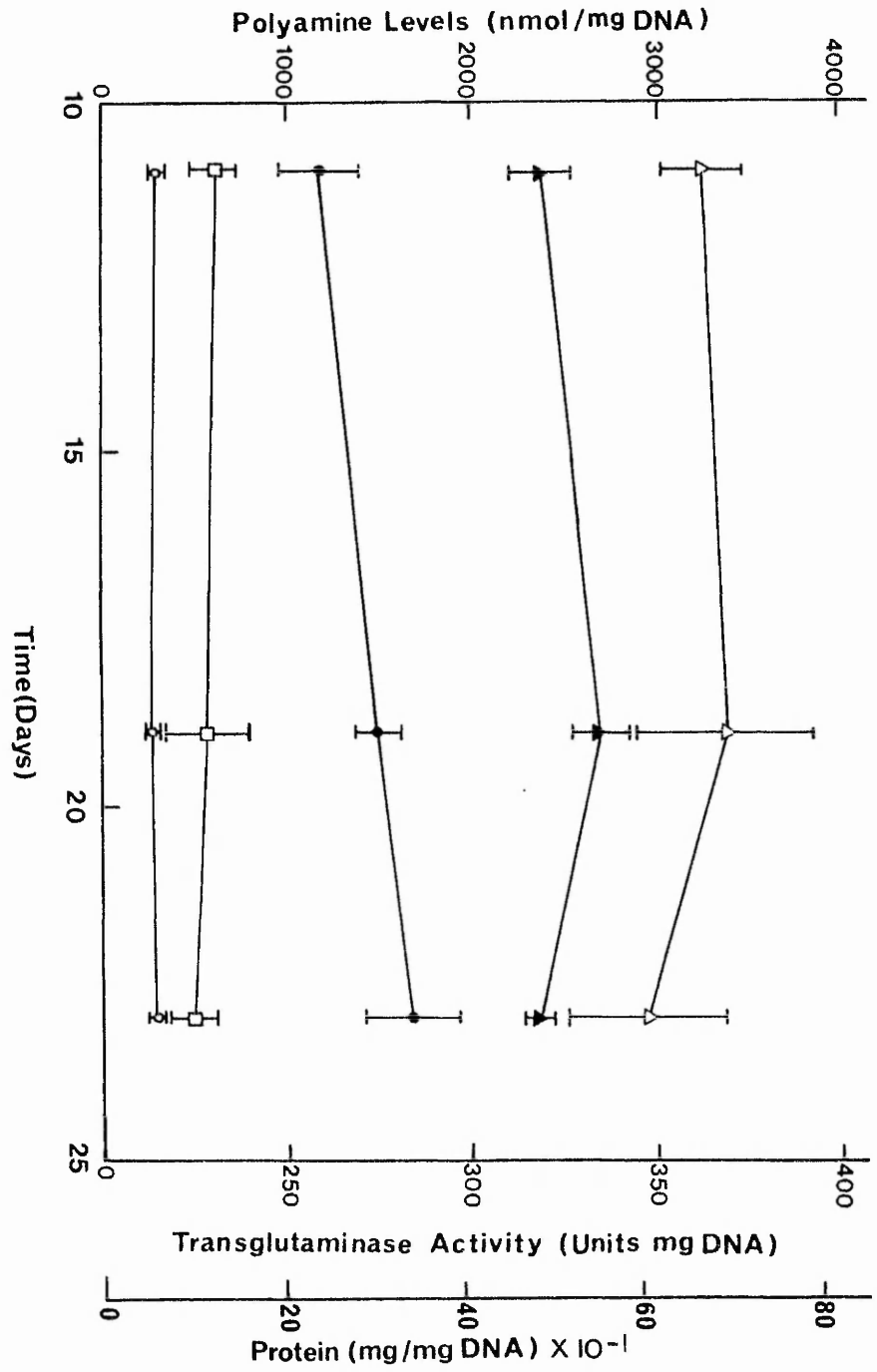
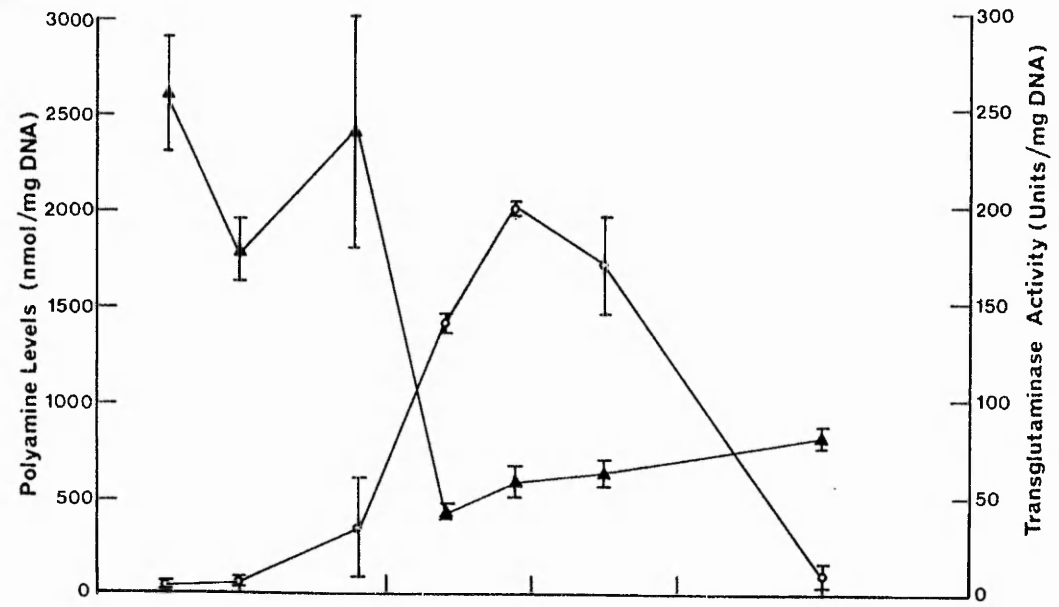

Polyamine Levels (nmol/mg DNA)
0
1000
2000
3000
4000
Time(Days)
10
15
20
25
0
250
300
350
400
Transglutaminase Activity (Units mg DNA)
0
20
40
60
80
Protein (mg/mg DNA) X 10⁻¹

FIGURE 6.5

Levels of Transglutaminase, Polyamines and Protein in P_8 Sarcoma Tissue during Growth

Samples of tissue were taken at various times during tumour growth and homogenised (section 2.4.1). Homogenates were assayed for transglutaminase ▲ (section 2.6.1), protein □ (section 2.5.1), DNA (section 2.5.3), or extracted for polyamine analysis (section 2.8.3), putrescine ○ , spermidine △ and spermine ● . Results are expressed as the mean from three individual sarcomas ± SE. A: transglutaminase activity and putrescine, B: spermidine, spermine and protein.

A


Polyamine Levels (nmol/mg DNA)
Transglutaminase Activity (Units/mg DNA)
3000
2500
2000
1500
1000
500
0
300
250
200
150
100
50
0

B

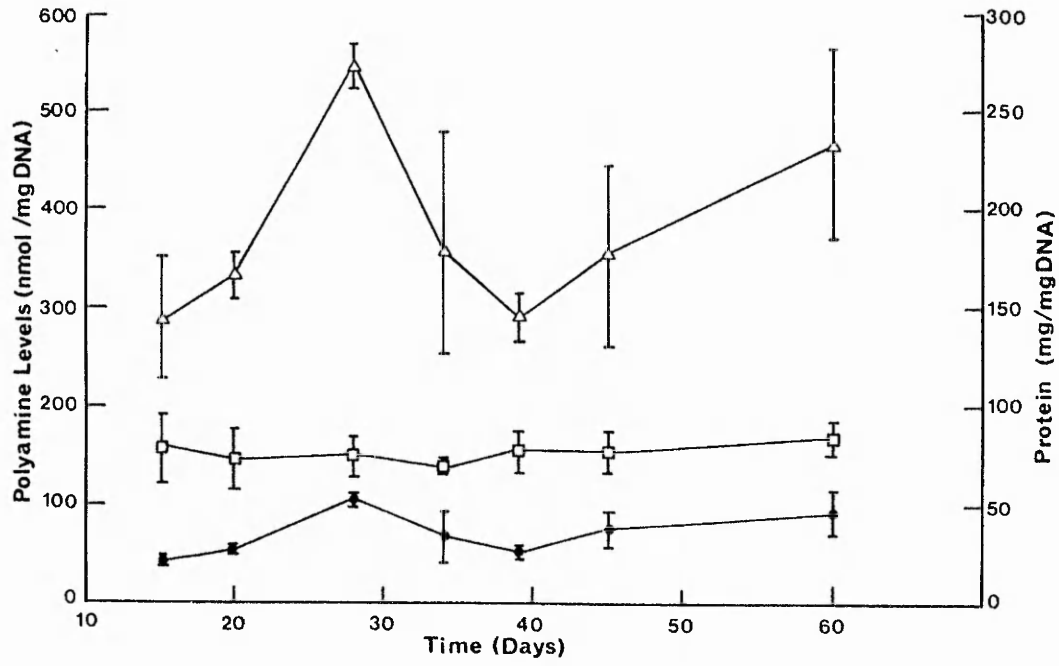

Polyamine Levels (nmol /mg DNA)
Protein (mg/mg DNA)
Time (Days)
600
500
400
300
200
100
0
300
250
200
150
100
50
0
10
20
30
40
50
60

TABLE 6.1

Levels of Polyamines derived from Homogenates of P_7 and P_8 Sarcomata in the Transglutaminase Assay

Polyamine	Polyamine Concentration in Assay (μM)		
	P_7 (11)	P_8 (15)	P_8 (45)
putrescine	20.3	8.5	379.9
spermidine	215.8	53.8	76.8
spermine	119.6	7.8	16.8
total	355.7	70.1	473.5

The concentrations of putrescine, spermidine and spermine which were added to the transglutaminase assay (section 2.6.1) in homogenates of sarcoma tissue were calculated. Results are shown as the mean concentration of tissue-derived polyamines in the transglutaminase assay from three sarcomata. The age of the sarcomata is given in parenthesis.

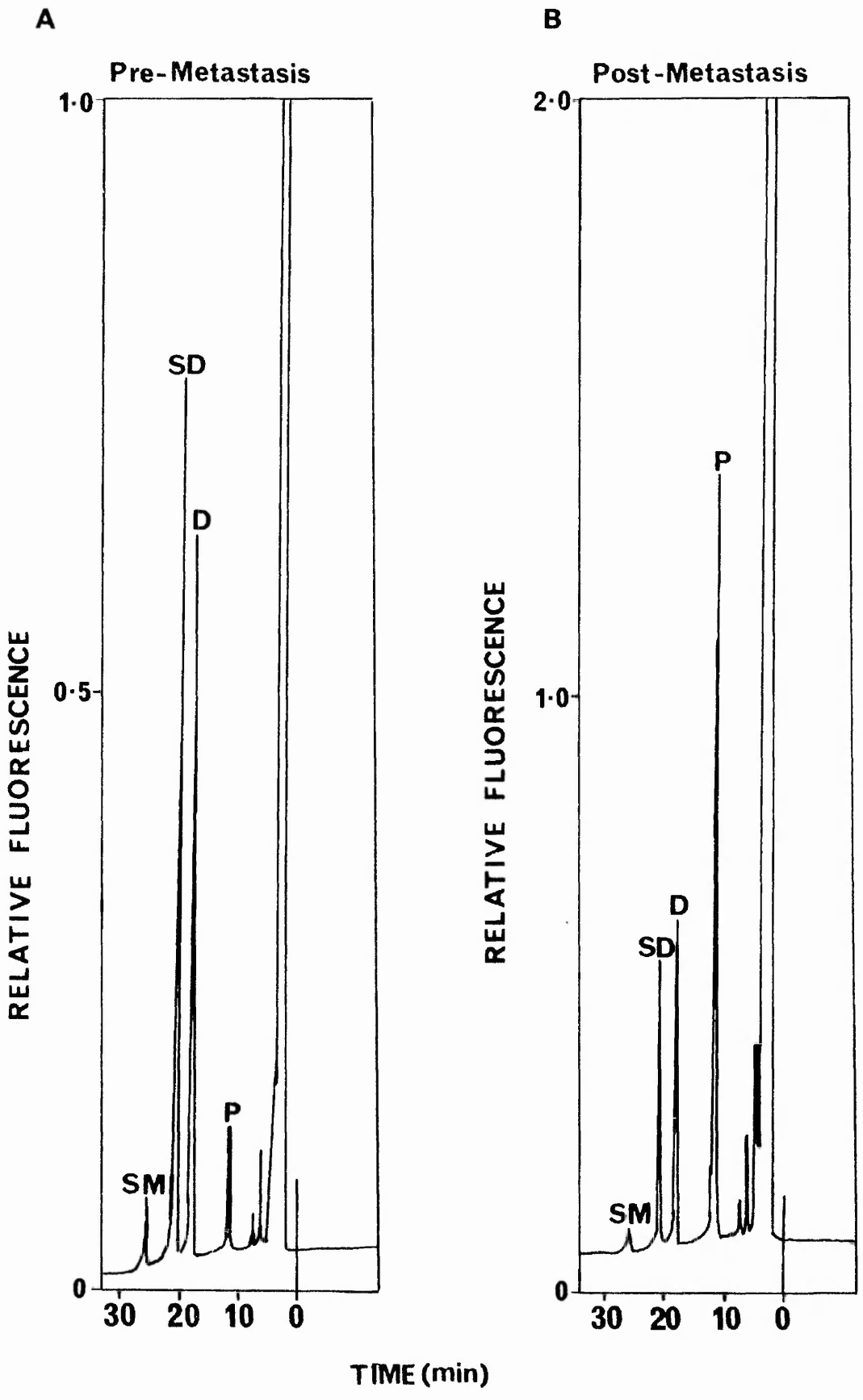
N.B. The transglutaminase assay contained 1.2mM [^{14}C]-putrescine (3.97 mCi/mmol) (section 2.6.1).

FIGURE 6.6

HPLC Analysis of Pre- and Post-Metastatic

P_8 Sarcoma Tissue

Homogenates of P_8 sarcoma tissue were spiked with internal standard (1,7-diaminoheptane, D) and extracted with perchloric acid (section 2.8.3.1). Clarified acid extracts were then subjected to polyamine analysis according to the method of Seiler (1983). Fluorescence was monitored with a Perkin-Elmer fluorimeter (excitation 340 nM, emission 455 nM; sensitivity x 2) coupled to a Spectra-Physics 4270 plotting integrator (attenuation: A,16, B,32). Representative traces for pre-metastatic (A) and post-metastatic (B) P_8 sarcoma tissue are shown. The peaks labelled are: putrescine (P), 1,7-diaminoheptane (D), spermidine (SD) and spermine (SM).


A
Pre-Metastasis
1·0
0·5
0
SD
D
P
SM
30
20
10
0
B
Post-Metastasis
2·0
1·0
0
P
D
SD
SM
30
20
10
0
RELATIVE FLUORESCENCE
RELATIVE FLUORESCENCE
TIME (min)

6.4 COVALENTLY BOUND POLYAMINES IN NON-METASTASISING AND METASTASISING P_8 SARCOMA TISSUE

The levels of polyamines which could only be released from tissue by acid-hydrolysis were also measured. Because great care was taken to remove soluble and ionically conjugated polyamines from the tissue prior to acid hydrolysis it was assured that the polyamines released by acid hydrolysis were present as γ-glutamyl derivatives, the formation of which is likely to be transglutaminase mediated. This analysis was carried out on pre-metastatic and post-metastatic P_8 tissue (Table 6.2). Since the methodology used for this analysis was somewhat different to previous polyamine measurements a typical trace is shown in Figure 6.7.

It was found that the main polyamine present was putrescine, the magnitude of incorporation of each polyamine decreasing in the sequence putrescine $>$ spermidine $>$ spermine. When comparing the two groups of data the bound polyamine content of post-metastatic tissue was consistently lower than that found in pre-metastatic tissue. The amount of reduction in the post-metastatic tissue varied from polyamine to polyamine and according to how the data was expressed but was in the region of 77 - 87%. Statistical analysis of the same data indicated that only the reduction in putrescine was significant ($P<0.05$).

TABLE 6.2

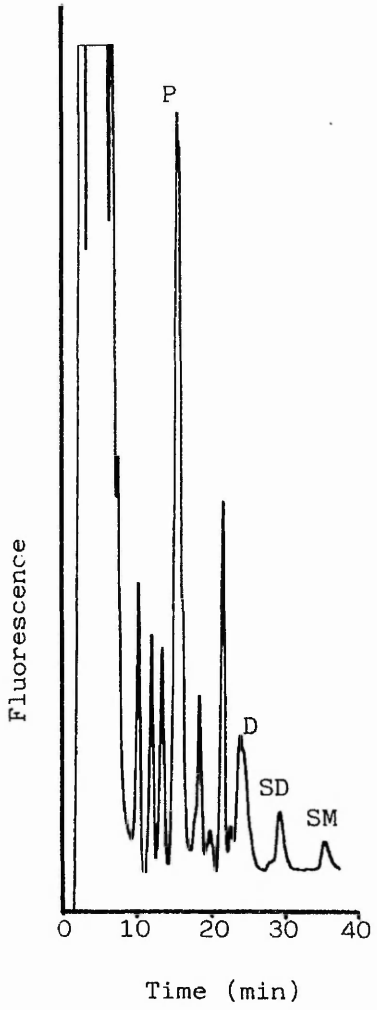
Covalently Bound Polyamine Levels

in P_8 Sarcoma Tissue

Units	Polyamine Content		
1. nmol/mg DNA	No metastases detected	Metastases detected	
PUTRESCINE	69.0 ± 15.5 (4)	16.2 ± 4.3 (4)	$P \leqslant 0.05$
SPERMIDINE	10.8 ± 4.2 (4)	1.7 ± 0.1 (3)	N.S.
SPERMINE	6.7 ± 0.5 (3)	1.3 ± 0.2 (3)	N.S.
2. pmol/mg protein			
PUTRESCINE	1008.7 ± 239.8 (4)	210.5 ± 29.4 (4)	$P \leqslant 0.05$
SPERMIDINE	166.5 ± 65.4 (4)	21.1 ± 3.6 (3)	N.S.
SPERMINE	84.3 ± 64.3 (3)	13.3 ± 2.6 (3)	N.S.
3. nmol/g wet weight			
PUTRESCINE	135.9 ± 34.9 (4)	34.8 ± 6.3 (4)	$P \leqslant 0.1$
SPERMIDINE	20.0 ± 6.9 (4)	3.55 ± 0.8 (3)	N.S.
SPERMINE	11.3 ± 8.4 (3)	2.63 ± 0.2 (3)	N.S.

P_8 sarcoma tissue was sampled before and after the detection of metastases to the lung and immediately homogenised (section 2.4.1). Acid soluble polyamines were extracted and the residue then acid hydrolysed (see section 2.8.3.2). The polyamines released were then extracted and concentrated according to the method of Raina (1963) (see section 2.8.3.2). Analysis of polyamines was carried out by the method of Seiler & Knodgen, (1985) (section 2.8.3.3). The results are expressed as the mean levels ± SE with the number of determinations in parenthesis. Where n = 3, quantitative analysis of the remaining peak could not be performed owing to limitations in the separation and detection procedure (N.S. $P > 0.1$).

Figure 6.7 Measurement of Covalently-Bound Polyamines in P_8 Sarcoma Tissue by Reverse Phase HPLC



Following extraction of acid-soluble polyamines (section 2.8.3.2), the residual material was hydrolysed with 6M HCl under vacuum for 20hr at 100°C. The polyamines were then extracted from this hydrolysate using butanol (Raina, 1963) and concentrated by evaporation. The polyamines were then reconstituted in 0.3M perchloric acid (55 µl per 100mg original tissue) and filtered (0.22 µm Durapore filter, Waters Associates). HPLC was performed according to the procedure described in section 2.8.3.3. A representative trace for the separation of putrescine (P), 1,7-diamino heptane (D), spermidine (SD) and spermine (SM) is shown.

6.5 THE SUBCELLULAR DISTRIBUTION OF TRANSGLUTAMINASE ACTIVITY IN P_7 AND P_8 SARCOMATA

The gross distribution of transglutaminase activity between the cytosol and the particulate fraction was measured (Table 6.3). As was the case for other tumours (section 3.3), the enzyme activity in P_7 and P_8 sarcomata was mainly associated with the particulate fraction. Upon metastasis, the reduction in transglutaminase activity in P_8 sarcomata (see section 6.3) was greater in the cytosol fraction (72%) than in the particulate fraction (57%) when compared to pre-metastatic P_8 tissue.

TABLE 6.3

Distribution of Transglutaminase Activity

in the Rat Sarcomata P_7 and P_8

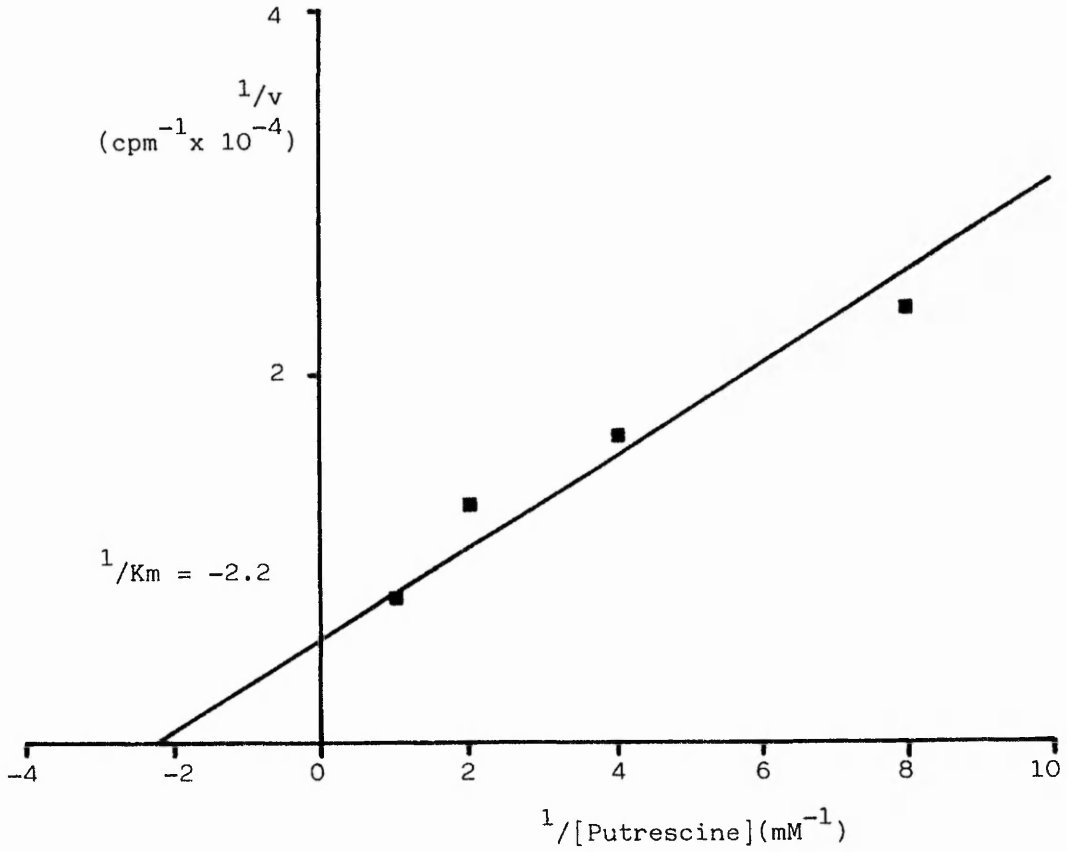
Tissue	Transglutaminase Activity			
	Units/g original tissue		% Distribution	
	S	P	S	P
P_7 (8)	46.2 ±2.8	404.0 ±28.4	10.7 ±1.7	89.3 ±1.7
P_8 (3) (Pre-Metastasis)	101.9 ±7.6	327.6 ±14.2	23.7 ±1.6	76.3 ±1.6
P_8 (6) (Post-Metastasis)	29.1 ±3.7	143.7 ±8.0	16.8 ±1.3	83.2 ±1.3

Tissue was homogenised in four volumes of 0.25 M sucrose, 1 MM EGTA, 5 mM Tris pH 7.4 by Potter-Elvehjem (section 2.4.1) and then fractionated into a 71,000g particulate fraction (P) and a cytosol fraction (S) (see section 2.4.2). Transglutaminase activity was measured in each fraction according to the method given in section 2.6.1.

6.6 PARTIAL CHARACTERISATION OF THE TRANSGLUTAMINASE ACTIVITY IN P_8 SARCOMA TISSUE

The transglutaminase activity in P_8 sarcoma tissue was partially characterised with respect to several properties common to transglutaminases. These were Ca^{2+} and thiol dependence and Michaelis-Menten kinetics for the incorporation of putrescine into N,N'-dimethylcasein. However, much of this work was hindered by the instability of the enzyme and by its low activity in this tissue. Transglutaminase has been reported to be unstable in other systems (Croall and De Martino, 1986), although such problems were not found to significantly affect studies with rat liver (Chapter 3 and 4). It was therefore not possible to accurately estimate the sensitivity of this transglutaminase enzyme to activation by Ca^{2+} as described previously (section 4.2.4). However, using the incorporation of [^{14}C]-putrescine into N,N'-dimethylcasein to measure transglutaminase activity, the enzyme in P_8 sarcoma tissue was found to have maximal activity in the presence of 2.5 mM $CaCl_2$. The substitution of EGTA (1 mM) for $CaCl_2$ completely abolished the incorporation of putrescine into N,N'-dimethylcasein. The dependence of this transglutaminase enzyme on reduced thiol groups was demonstrated by the inclusion of iodoacetamide (5 mM) in the assay, which prevented the incorporation of putrescine into N,N'-dimethylcasein in the presence of $CaCl_2$ (2.5 mM). The kinetics of putrescine incorporation into N,N'-dimethylcasein were also investigated (Figure 6.8) by varying the putrescine concentration in the assay and keeping the concentration of N,N'-dimethylcasein constant. The use of double-reciprocal plots indicated that the Km for the incorporation of putrescine into N,N'-dimethylcasein was 0.45 mM.

Figure 6.8 Determination of the Michaelis-Menten Constant for the Incorporation of Putrescine into N,N'-Dimethylcasein by P_8 Sarcoma Transglutaminase



Transglutaminase activity was extracted from P_8 sarcoma tissue (20 day) using the procedure described in section 2.4.4 and dialysed over night against 50mM Tris, 1mM EGTA, 0.5mM DTT, pH 7.4. The activity of this preparation was then assayed at various concentrations of [^{3}H]-putrescine (0.15 - 2.4mM) in the presence of $CaCl_2$ (2.1mM) or EGTA (10.4mM) with a total reaction volume of 200 µl (section 2.6.1). Triplicate incubations were carried out at each putrescine concentration. v = rate of putrescine incorporation per minute in 50 µl of reaction mixture. The above plot gave a Km value of 0.45mM putrescine.

6.7 DISCUSSION

The transplantable rat sarcomata, P_7 and P_8, were used to investigate changes in transglutaminase activity and polyamine levels during the metastatic process. The P_8 sarcoma was found to metastasise to the lungs at about 30 days after implantation. However, the P_7 sarcoma did not metastasise during the course of these experiments and was therefore used as a non-metastatic 'control' tissue for comparison with the P_8 sarcoma. Histological examination of these tumours revealed that there was only one cell type evident in P_7 sarcomata but that there was more than one cell type in P_8 sarcomata. In early or 'pre-metastatic' P_8 sarcomata most of the cells were poorly differentiated, although there were also some areas of fibroblast-like cells. In later or 'post-metastatic' P_8 sarcomata the fibroblast-like cells were predominant with the differentiated cells only present in small areas. However since this change was gradual it was difficult to envisage how these morphological changes were related to metastasis, a process which appeared to occur suddenly in the P_8 sarcoma. Furthermore, it was tempting to suggest that these changes were due to the development of a single cell type, especially since the transplantation of P_8 sarcoma tissue from all stages of growth resulted in the same sequence of morphological changes.

It should also be noted that whilst there was very little necrosis in the fast growing P_7 sarcomata there was some necrosis evident in P_8 sarcomata. However since this did not appear to increase during the course of these experiments it was possible to carry out valid comparisons of pre- and post-metastatic P_8 sarcomata.

Whilst transglutaminase activity and polyamine levels were fairly constant throughout the growth of P_7 sarcomata, substantial

alterations were found in P_8 sarcomata when tissue was sampled before and after the detection of metastases to the lungs. In P_8 sarcomata transglutaminase activity underwent a fourfold reduction at about 30 days, a finding which correlated with the onset of metastasis and was in agreement with previous work from this laboratory (Barnes et al, 1984, 1985). The complete characterisation of this enzyme was not possible due to its extremely low activity in this tissue and its instability. However, it was demonstrated that the incorporation of putrescine into N,N'-dimethylcasein was Ca^{2+}- and thiol-dependent which is consistent with a transglutaminase mediated reaction. Measurement of the Km_{app} for putrescine incorporation into N,N'-dimethylcasein indicated that it was similar to that of the cytosolic liver enzyme with a value of 0.45 mM. This demonstrates that the low levels of transglutaminase activity in the P_8 sarcoma when compared to other tissues such as normal liver (see Chapter 3) are genuine and are not due to the expression of a transglutaminase enzyme with different substrate specificity. However, examination of the subcellular distribution of transglutaminase activity in P_7 and P_8 sarcomata indicated that the activity was mostly associated with the particulate fraction. Comparison of pre- and post-metastatic P_8 sarcomata revealed a small apparent redistribution of activity to the particulate fraction in post-metastatic sarcomata. It was however unclear whether this represented an analogous situation to that found in hepatocellular carcinomas where the redistribution was due to a specific reduction in one form of transglutaminase, the cytosolic transglutaminase (see section 3.6).

There was close correlation between the onset of metastasis in P_8 bearing animals and elevations in the putrescine content of these tumours. However, these elevated levels were not maintained throughout

the growth of these sarcomata, putrescine levels returning to approximately pre-metastatic levels by 60 days. Despite this massive (40 fold) increase in acid-soluble putrescine levels between 34 and 45 days, spermidine and spermine levels in these tumours were virtually unaffected. The only significant alteration in spermidine and spermine levels occurred at 28 days (a timepoint immediately prior to the detection of metastases for most tumours) when these levels were twice those found at 15 days. Whether these changes in polyamine levels have any direct effect on the metastatic process is unclear, although a rise in putrescine levels certainly seems to be indicative of the onset of metastasis in this particular sarcoma.

The suggestion that the reduction in transglutaminase activity seen in these sarcomata at the onset of metastasis is due to interference of factors such as polyamines with the transglutaminase assay may be ruled out on several grounds: 1) the concentration of polyamines found in these tumours would only dilute the radiolabelled substrate in the assay by approximately 25%, a figure which does not match the large fall in activity, 2) transglutaminase activity was not seen to rise at 60 days when putrescine levels were lower than at other post-metastatic time points. Furthermore, the measurement of bound polyamine levels in these same tumours revealed a strong correlation between the fall in transglutaminase activity and a reduction in bound polyamine levels, suggesting that there was an actual reduction in transglutaminase levels in P_8 sarcomata during metastasis. It is interesting to note that this fall in bound polyamine levels occurs despite a massive increase in acid-soluble putrescine levels, thus demonstrating that enzyme availability is a key factor in determining the levels of bound polyamines. Hougaard and Larsson, (1986) have shown that polyamines are present at specific sites

in cells and thus they may be at saturating concentrations regardless of large fluctuations in polyamine levels which occur under different circumstances (Pegg, 1986). Recent work in this laboratory has also shown that the levels of ε(γ-glutamyl)lysine bridges in these P_8 tumours decrease dramatically at the onset of metastasis. A 4-5 fold decrease in the levels of ε(γ-glutamyl)lysine has been demonstrated in post metastatic P_8 tissue when compared to pre-metastatic P_8 tissue (C R L Graves, personal communication) using a modification of the method of Griffin et al., (1982).

Since metastasis is a multistep process (Nicolson, 1979) it is interesting to speculate as to how alterations in transglutaminase activity may affect a tumour's propensity to metastasise. Recently Roch et al.,(1987) have shown that transglutaminase activity decreases in the sequences: normal > benign > malignant and low metastatic> high metastatic (see section 1.3.5), the latter being supported by the work presented here. These findings suggest that reductions in transglutaminase activity may aid the invasion of normal tissue by malignant tumours, thus promoting the spread of metastases. The discovery of a particulate liver enzyme which is associated with a specific plasma membrane domain (Tyrell et al., 1986) and contains gap junctional complexes has led to speculation on this enzyme's role in cell-cell contact. Indeed there is already some evidence to suggest that it may be involved in the formation of extracellular matrix (Cocuzzi and Chung, 1986) and a cellular adhesion factor (Slife et al., 1982). Thus, reductions in transglutaminase activity may favour the invasion of normal tissue by tumour cells and the subsequent shedding of cells into the circulatory system. Furthermore, since reductions in transglutaminase activity led to reduced crosslinking of proteins and/or reduced incorporation of polyamines into proteins in the P_8

sarcoma (Hand et al., 1987) and in other tumours (Birckbichler et al., 1978, 1980), this may lead to alterations in the cell surface structure and antigenicity of tumour cells and thus aid its spread throughout the circulatory system.

It has been proposed that transglutaminase may play a role in regulating polyamine metabolism by modifying the rate-limiting polyamine biosynthetic enzyme, ornithine decarboxylase, via the incorporation of putrescine (Russell, 1981). The parallel increase of bound polyamines and transglutaminase in rat liver nuclei during liver regeneration (Haddox and Russell, 1981b) and the presence of γ-glutamyl-polyamine conjugates in rat liver (Beninati, 1985) suggest that this reaction may occur in vivo. Furthermore, stimulation of both mouse melanoma cells and Chinese hamster ovary cells with retinoids resulted in the stimulation of transglutaminase activity and a reduction in ornithine decarboxylase (Scott et al., 1982). Conversely, the administration of DEN to rat liver has been reported to result in both a decrease in transglutaminase activity and an increase in ornithine decarboxylase (Olson and Russell, 1979). However, whilst it is tempting to suggest that the alterations in free and bound putrescine levels and transglutaminase activity in the P_8 sarcoma support this model, the lack of significant and concomitant increases in spermidine and/or spermine levels suggests that elevated putrescine levels are not simply caused by perturbations in the regulation/inactivation of ornithine decarboxylase by transglutaminase. Reduced diamine oxidase activity or alternatively reduced activity of the spermidine and spermine synthases and/or S-adenosylmethionine decarboxylase or even increased putrescine uptake could all, equally, be responsible for the observed increases in free putrescine levels (see Figure 1.4). Indeed, evidence is now accumulating to suggest that

transglutaminase is not involved in the regulation of ornithine decarboxylase activity since both enzymes are stimulated under the following conditions: partial hepatectomy (Remington & Russell, 1982), administration of tumour promotors to keratinocytes (Yuspa et al., 1983), insulin administration to chicken liver (Grillo et al., 1982), and in bipuvacaine-induced muscle regeneration (Sadeh et al., 1983). Also, the induction of transglutaminase and rise in bound polyamine levels in regenerating rat liver occurs in the nuclei (Haddock and Russell, 1981b) and not in the cytosol where ornithine decarboxylase is found. Furthermore, Tyagi et al., (1982) have demonstrated that the inactivation of ornithine decarboxylase in yeast is not caused by the covalent attachment of putrescine to this enzyme.

In conclusion it seems unlikely that transglutaminase plays any direct role in regulating polyamine metabolism in eukaryotic cells. It was shown that a significant 4-5 fold reduction in transglutaminase activity was associated with the onset of metastases in the transplantable rat sarcoma, P_8. This fall in activity was accompanied by a reduction in the levels of covalently incorporated polyamines (and of $\varepsilon(\gamma$-glutamyl)lysine bridges) which may have profound effects on the structure and function of cytoskeletal and/or membrane protein structure. Such alterations may in turn be related to the metastatic process. Furthermore, in the P_8 sarcoma a large rise in putrescine levels appears to be a specific marker for the onset of metastasis.

Future work should include the characterisation of the transglutaminase enzyme(s) present in these sarcomata and its/their protein substrate(s) in order to gain a deeper understanding of how the reduction in transglutaminase activity affects the propensity of a tumour cell to metastasise.

7.0 GENERAL DISCUSSION

Since transglutaminase activity has been shown to be reduced in tumour tissue (see section 1.3.6) initial studies were undertaken using chemically-induced tumours of liver as one model system to investigate the role of this enzyme in normal liver and its importance in tumour growth. Studies with hepatocytes demonstrated that whole rat liver was a suitable control tissue for comparisons with the hepatocellular carcinoma in the rat. This was impor tant since any study involving carcinogenesis should refer to an appropriate control. The measurement of transglutaminase activity in chemically-induced tumours confirmed previous reports of reduced levels of this enzyme in these tissues (Birckbichler et al.,1976, Barnes et al.,1985). In addition, the subcellular distribution of the enzyme activity in normal and neoplastic tissues indicated that the reduction occurred primarily in the cytosol. It has previously been suggested that this is due to a redistribution of enzyme activity to the particulate fraction (Birkbichler et al., 1976, Barnes et al., 1985). However, it has recently been reported that there are two distinct forms of transglutaminase in rat liver (Chang and Chung, 1986), a cytosolic enzyme and a particulate enzyme. It was therefore thought that this redistribution of enzyme activity could be due to a selective reduction in the cytosolic enzyme. Indeed, leaching studies with non-isotonic detergent-free buffers revealed that whilst the total transglutaminase activity in tumours was reduced, the

amount of activity which remained in the particulate fraction of tumours after extensive leaching was comparable to that found in normal liver. The extraction of normal liver and tumour tissue with detergent (Lubrol-PX) enabled the separation of the two forms of transglutaminase by anion-exchange chromatography. This confirmed that there was a significant reduction in the activity of the cytosolic enzyme in these tumours (~ 90%) but not of the particulate enzyme.

However, subcellular distribution studies also indicated that the particulate fraction contained both the cytosolic and particulate enzymes in significant amounts. Therefore the free cytosolic transglutaminase and the cytosolic transglutaminase which was associated with the particulate fraction were compared in order to establish whether any biochemical or biophysical changes were responsible for the association of this enzyme with the membrane fraction. It was found that these enzyme fractions were indistinguishable according to molecular weight, Km_{app} for the incorporation of putrescine into N,N'dimethylcasein and sensitivity to activation by Ca^{2+}, thus suggesting that the association of this enzyme with the particulate fraction was brought about by any modification of the enzyme within the cell. Furthermore, since chemical carcinogenesis may lead to the expression of abnormal proteins (Hunter, 1984), it was also important to establish that the cytosolic enzyme from hepatocellular carcinomas was the same as that found in normal liver. It was found

that these enzymes were identical with regard to the characteristics outlined above and thus the reduced transglutaminase activity in these tumours was most likely to be due to the reduced expression of the cytosolic enzyme rather than the expression of an abnormal enzyme by the tumour cell.

Interestingly, during these studies, it was discovered that the sensitivity of the cytosolic form of transglutaminase to activation by Ca^{2+} was much higher than previously reported. It was calculated that the enzyme could operate at half-maximal velocity at concentrations of Ca^{2+} in the range 3-4 μM (Hand et al., 1985) as compared to previous reports of values of between 40-500 μM (Gomis et al., 1983, Bungay et al., 1984a, Lorand and Conrad, 1984). This was interesting since it demonstrates that transglutaminase can be activated by levels of Ca^{2+} found in the stimulated cell (1-2 μM -Beaven et al., 1984) and is therefore likely to play a role in normal cellular function. Since molecules such as calmodulin and phospholipids have also been reported to affect the Ca^{2+} sensitivity of enzymes (Cheung, 1980, Takai et al., 1981), their effect on transglutaminase was also investigated. However it was found that these molecules had little effect on the sensitivity of transglutaminase to activation by Ca^{2+}. This indicates that the cytosolic enzyme can be directly activated by changes in the concentration of free Ca^{2+} in the cell and that the association of the cytosolic enzyme with the cell membrane is unlikely to affect its

sensitivity to activation by Ca^{2+}.

The measurement of free polyamine levels in hepatocellular carcinomas and in normal liver revealed that these divalent cations were unlikely to significantly affect the assay of transglutaminase activity in these tissues. Furthermore, whilst elevated putrescine levels were found in some of the tumours examined it was clear that polyamines were not an ubiquitous marker for this type of tumour and that caution should be exercised when attempting to use them as such (Hand et al., 1986).

With the intention of clarifying the role of the cytosolic transglutaminase in the cell, the ability of tissue slices to incorporate [^{14}C]-methylamine into cellular proteins in a Ca^{2+}-dependent manner was investigated. In this way the hepatocellular carcinoma could be used as a cytosolic transglutaminase negative' control for comparison with normal liver in order to discriminate between protein modifications catalysed by the particulate enzyme and those catalysed by the cytosolic enzyme. However, the level of Ca^{2+}-dependent incorporation of [^{14}C]-methylamine into acid-precipitable protein in these tissues was found to be approximately equivalent. This was surprising in view of the known reduction in cytosolic transglutaminase in these tumours. However, since the subcellular distribution of this radiolabel was shown to coincide with the plasma membrane fraction it was thought that this might be due to the activation of the particulate transglutaminase alone. It was therefore

thought that the cytosolic enzyme was not significantly activated under these conditions. In retrospect this is perhaps not surprising since free Ca^{2+} levels in the resting cell are probably too low to activate the cytosolic transglutaminase (0.1-0.3 μM, Beaven et al., 1984). However, the particulate form of transglutaminase may have readier access to free Ca^{2+} by virtue of its location at the plasma membrane. Further study of this Ca^{2+}-dependent process was undertaken in order to determine the nature of the proteins labelled with [^{14}C]-methylamine using SDS polyacrylamide gel electrophoresis. It was found that the main site of [^{14}C]-methylamine labelling was a high molecular weight proteinaceous material of Mr > 1 x 10^6. Smaller polymers of molecular weight Mr 300,000 - 1 x 10^6 were also radiolabelled. However, whilst the amount of incorporation of [^{14}C]-methylamine into the high molecular weight material was roughly equivalent in normal liver and tumours, the incorporation into smaller polymers (Mr 300,000 - 1 x 10^6) was reduced in the tumours. In addition there was some labelling of normal liver proteins in the molecular weight range 200,000-30,000, in particular into proteins of Mr 43,600 and 38,900. The incorporation of [^{14}C]-methylamine into these proteins was reduced in the tumours, although there was some low-level incorporation into a protein of Mr 35,900 which was unique to the tumour tissue.

Subcellular distribution studies revealed that the high molecular weight material was associated with a plasma

membrane rich fraction, a finding which is in agreement with that of other workers (Slife et al., 1986, Tyrell et al., 1986) who also postulated that it was a substrate of the particulate transglutaminase. However some of the incorporation of [^{14}C]-methylamine in normal liver was found to occur in cytosolic proteins. In particular the protein of Mr 43,600 was found to be mostly located in the cytosol fraction and the protein of Mr 38,900 was codistributed between the cytosol and the plasma membrane rich fraction. Since the incorporation of [^{14}C]-methylamine into these proteins was reduced in tumours it was thought that they may be substrates of the cytosolic form of transglutaminase. Further studies were undertaken using tissue homogenates in order to determine whether there were any gross differences in the number and distribution of γ-glutaminyl residues available for transglutaminase mediated polyamine incorporation in normal and neoplastic tissues and to determine whether cytosolic transglutaminase could act on membrane proteins as well as soluble proteins. It was found that there were significantly fewer available γ-glutaminyl residues in the tumours when they were compared to normal liver or tumour bearing liver and that this apparent reduction occurred primarily in the particulate fraction of the cell. However, whilst the subcellular distribution of radiolabel was not dissimilar to that found in proteins from tissue slices, the electrophoretic labelling pattern was different. Furthermore the actual level of incorporation was much higher (6-7 fold) than in

tissue slices, therefore indicating that the enzyme was labelling proteins that it either did not encounter in the intact cell or that the cytosolic enzyme was not activated under the conditions used. Experiments with membranes which had been treated with iodoacetamide to inactivate the particulate enzyme did however indicate that not only could the cytosolic transglutaminase act on membrane proteins but that it could form high molecular weight polymeric material from either cytosolic or membrane associated proteins. This probably represents uncontrolled crosslinking of proteins into insoluble aggregates by transglutaminase and highlights the importance of considering the formation of ε(γ-glutamyl)lysine formation in conjunction with the attachment of polyamines to proteins, particularly since this high molecular weight material appears to be the main site of polyamine incorporation. Also, the high molecular weight material formed in homogenates may bear little resemblance to the polymer formed in the intact cell since it may contain proteins that are not present in the polymer formed in the intact cell.

Using a different model system, a non-metastasising sarcoma, P_7, was compared with a metastasising sarcoma, P_8. In P_7 it was found that transglutaminase activity remained constant throughout the growth of the sarcoma. However, in P_8, transglutaminase activity dropped significantly at the same time that metastases were detected in the lungs of sarcoma-bearing rats. This is in agreement with previous work from this

laboratory (Barnes et al, 1984, 1985) and others (Delcros et al, 1986, Roch et al., 1987). The reduction in enzyme activity (4-5 fold) was accompanied by a parallel decrease in the levels of bound polyamines and of ε(γ-glutamyl) lysine crosslink in this tissue, thus strongly implicating changes in transglutaminase mediated reactions as being important to the metastatic process. In addition, a dramatic increase in the levels of acid-soluble putrescine was found to coincide with the onset of metastasis in the P_8 sarcoma. This massive increase (20 fold) lasted until at least 45 days after which the levels of putrescine fell again. In view of these findings, the suggestion that transglutaminase may regulate ornithine decarboxylase via the incorporation of putrescine into enzyme molecules, a theory first put forward by D.H. Russell (1981), remains an intriguing one. However, this idea is still controversial and much evidence has now accumulated against it (see section 6.7) The observation that cytosolic transglutaminase is reduced in hepatocellular carcinomas highlights the potential importance of this enzyme in carcinogenesis. Whether this occurs as a direct result of changes in the regulatory gene for tissue transglutaminase or as a result of co-ordinate events associated with neoplasia is as yet unknown, although it has been demonstrated that in macrophages the tissue enzyme is under transcriptional control (Moore et al., 1984) and that its synthesis can be stimulated by retinoic acid (Murtaugh et al., 1983). Furthermore, neoplastic cells have also been

shown to synthesise the cytosolic enzyme when challenged with agents which have the ability to cause tumour regression, such as sodium butyrate (Lee et al., 1987, Byrd and Lichti, 1987) and retinoids (Mehta and Lopez-Berestein, 1986) which further supports the proposal that reduced transglutaminase activity accompanies and may therefore be associated with neoplasia. There is now considerable evidence to suggest that the function of the tissue transglutaminase may be related to calcium-mediated cytoskeletal events important to membrane function and cellular architecture (Bjerrum et al., 1981, Loewy and Matacic, 1981, Loewy et al., 1981, Bersten et al., 1983, Cariello et al., 1984, Maccioni and Arechaga, 1986, Maccioni and Seeds, 1986, Uhl and Schindler, 1987). Reduced expression of tissue transglutaminase may therefore be directly related to a number of pleiotropic events which occur in neoplastic cells following transformation; such examples may include cytoskeletal disorganisation leading to loss of cell shape and polarity, increased vesicular movement and impaired cell communication, all of which are characteristic features of the neoplastic cell (Weinberg, 1983).

With regard to the particulate form of transglutaminase it is now clear that the intracellular modification of proteins by transglutaminases requires careful identification of the enzyme(s) responsible. Indeed it has now been shown that a variety of cell types contain more than one form of transglutaminase, including:

epidermis (Lichti et al., 1985), epithelial cells (Jetten and Shirley, 1983) and pheochromocytoma cells (Byrd and Lichti, 1987) and that a variety of tissues other than liver contain bimodal activity distributions (Griffin et al., 1978, Barnes et al., 1984, 1985, Gilad and Varon, 1985, Cocuzzi and Chung, 1986). It was therefore interesting that the particulate enzyme was not found to be reduced in the hepatocellular carcinoma since a number of studies have implicated the involvement of the particulate enzyme from liver in cellular adhesion (Slife et al., 1982, Tyrell et al., 1986) a process which is impaired in neoplastic cells. However a reduction in a particulate transglutaminase enzyme (which has yet to be fully characterised) does occur in the metatasising rat sarcoma, P_8. It is therefore tempting to suggest that the reduction which occurs in the metastasising P_8 sarcoma leads to an increased ability of this tumour to invade surrounding tissues by impairing cell-cell contact between tumour cells. Alternatively altered transglutaminase expression may lead to perturbations in cell surface antigenicity which could favour the tumour cells propensity to metastasise. Similarly, since putrescine is a known immunosuppressant (Williams-Ashman & Lockwood, 1970, Byrd et al., 1977) the secretion of large amounts of putrescine by the tumour tissue may also favour the spread of metastases.

Future studies should address themselves to the identification of the intracellular substrates of transglutaminase since this will allow a role for the

enzyme to be clearly prescribed. The use of stimulated and non-stimulated model systems should enable workers to discriminate between the actions of the different forms of transglutaminase and possibly to establish the relationship between these different forms. Furthermore, the measurement of transglutaminase activity in biopsies of human cancers may potentially lead to the use of this enzyme as a marker in the prognosis of cancer.

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Polyamine levels in chemically induced rat liver tu

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The polyamines, putrescine, spermidine and spermine, are biological cations thought to play an important role in cell proliferation and differentiation (Williams-Ashman & Canellakis, 1979, Heby, 1981). Studies have indicated that in these different states there is an increase in synthesis and an accumulation of polyamines (Jänne *et al.*, 1978; Raina *et al.*, 1980). As a consequence, various workers have proposed that changes in polyamine levels in physiological fluids may have potential clinical use as tumour markers and have attempted to demonstrate their use as early indicators of chemotherapeutic efficiency (Durie *et al.*, 1977; Milano *et al.*, 1980). However, although considerable evidence has been gained for the occurrence and enhanced synthesis of polyamines in experimental tumours in animals (Noguchi *et al.*, 1976; Kallio *et al.*, 1977; Williams-Ashman *et al.*, 1972), this work has generally been limited to studies on well-established cell lines or transplantable tumours. Relatively few studies have been undertaken with chemically induced primary tumours where direct comparisons of polyamine levels in tumours of similar type, tumour-bearing tissue and normal parent tissue can be made.

In the following study we have measured polyamine levels in a series of histologically characterized rat hepatocellular carcinomas induced by the chemical carcinogen, diethylnitrosamine. Hepatocellular carcinomas were induced by previously published methods (Barnes *et al.*, 1985). Animals were killed by controlled exposure to fluothane (ICI PLC), after which the livers were removed. Tissue was taken for histology and the remaining tissue homogenized in 3 vol. of ice-cold 0.25 M-sucrose/1 mM-Tris/HCl, pH 7.4, containing 1 mM-EDTA. Polyamine levels were measured by h.p.l.c. according to the method of Seiler (1983). DNA was measured by the method of Burton (1956). Mitotic frequency was estimated by counting the number of cells undergoing mitosis under high powe

The levels of putrescin hepatocellular carcinom shown in Table 1. Sinc comparable in all tissues nmol/g of tissue. The l present in the tumours from the levels found in ation was found when i but no apparent trend parison of the mean tumour group with that significant differences b apparent between indivi

The only polyamine ($P < 0.05$) in the tumo mean value approximat control livers. However, 23C) demonstrated cons tumours (10A, 10B and levels of putrescine. In putrescine levels in the in bearing livers was also bearing tumours 23A, 23 levels, while livers 10 and 14A, had putrescine leve control livers.

Our results are ther studies relating to chem elevated levels of putre Sesa, 1978; Scalabrino However, in our studies significant alterations in mine, or any changes (Milano *et al.*, 1981) co Measurement of the n tumours indicated a co rate but this could not levels or the spermidin relationship shown bet tumour and its tumou

Table 1. *Polyamine levels in rat hepatocellular carcinomas, tumour-bearing livers a*

	Tissue	Polyamine levels (nmol/g of tissue)* Putrescine	Spermidine	Spermine	Spe
(a)	Hepatocellular carcinoma: 10A	52	712	647	
	Hepatocellular carcinoma: 10B	38	1340	1984	
	Hepatocellular carcinoma: 14A	41	819	804	
	Hepatocellular carcinoma: 23B	165	962	1137	
	Hepatocellular carcinoma: 23C	292	1702	1862	
	Mixed cholangioma/ hepatocellular carcinoma 23A	165	1220	1409	
	Mean values (±S.E.M.) for tumours	126 (±41)	1126 (±150)	1307 (±223)	
(b)	Tumour-bearing liver: 10	38	1340	1990	
	Tumour-bearing liver: 14	20	907	1467	
	Tumour-bearing liver: 23	442	1986	2109	
	Mean values (±S.E.M.) for tumour-bearing livers	167 (±138)	1411 (±313)	1855 (±197)	
(c)	Mean values (±S.E.M.) for control livers (n = 6)	27 (±7)	1043 (±160)	1317 (±145)	

*Polyamine values are the mean of three determinations.

that any occurrence of elevated putrescine levels is likely to be a consequence rather than an obligatory requirement of the carcinogenic process.

In conclusion our data indicate that considerable caution should be exercised when using polyamine levels as a marker for carcinogenesis and tumour growth.

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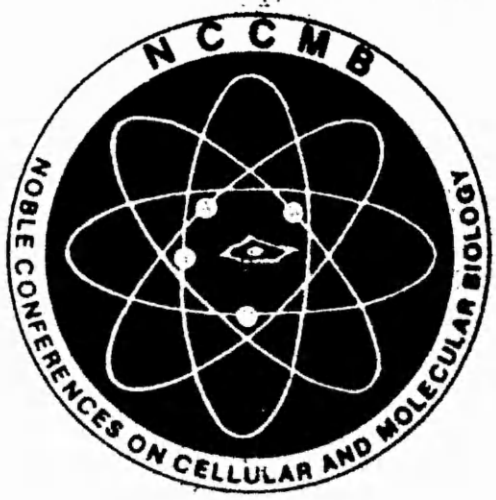
TRANSGLUTAMINASE & PROTEIN CROSSLINKING REACTIONS



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Poster Boar

Transglutaminase A
Relation to the Polyamine Co
Tissue during the Metastatic

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Transglutaminase activi
of putrescine spermidine and
measured in two transplantab
one, P8 which metastasises c
lung and the other P7 which
infrequently. In P7 no meta
detected following implantat
significant changes occurred
transglutaminase, putrescine
spermine. In the P8 tumour
30 days after implantation t
decrease in transglutaminase
mirrored exactly by a 20-fol
level of acid soluble putres
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tumour indicated a correspon
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compatible with the decrease
transglutaminase activity.
changes coincided with the t
P8 sarcoma was shown to have
the lung and suggests that t
these biochemical parameters
to the metastatic process.

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Correlation of changes in transglutaminase activity and polyamine content of neoplastic tissue during the metastatic process

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Key words: Transglutaminase; Polyamine; Tumor growth; Metastasis

Transglutaminase activity and the levels of the polyamines putrescine, spermidine and spermine were measured in two transplantable rat sarcomata: P_8 which metastasises consistently to the lung, and P_7 which metastasises infrequently. With the P_7 sarcoma no metastases were detected following implantation; similarly, no significant changes occurred in the levels of transglutaminase activity, putrescine, spermidine or spermine during tumour growth. However, with the P_8 sarcoma at approx. 30 days after implantation there was a marked decrease in transglutaminase activity, mirrored exactly by a 20-fold increase in the levels of acid-soluble putrescine. Measurement of covalently-bound polyamines in the P_8 sarcoma indicated a significant and corresponding decrease in the levels of bound putrescine. The timing of these changes coincided with the time at which the P_8 sarcoma was shown to have metastasised, and suggests that the changes observed may be related to this phenomenon.

Introduction

Transglutaminase (EC 2.3.2.13) catalyses an acyl transfer reaction between peptide bound glutamine residues and primary amine groups. The result of this reaction is the post-translational modification of proteins, either by the incorporation of amines or, if the amine is peptide bound lysine, by the crosslinking of proteins through ε(γ-glutamyl)lysine bridges [1].

The most common of these enzymes, the tissue transglutaminase, has yet to have a function ascribed to it although it may play a role in membrane-mediated events such as receptor mediated endocytosis and membrane recycling [2,3,4]. Changes in transglutaminase levels may also be an important feature in tumour growth and metastasis as there have been several reports of reductions in transglutaminase levels in neoplastic tissue [5,6,7,8]. Previous data from this laboratory have indicated that up to a 7-fold reduction in transglutaminase activity can occur in the primary tumour of rat sarcomata following detection of metastases to the lungs [8]. More recently, studies with cloned cell lines also demonstrated an inverse relationship between transglutaminase levels and metastatic potential [9]. There are as yet, however, insufficient data to determine whether these changes observed with transglutaminase are involved in a causal manner in the development of the neoplastic phenotype, or whether they are parallel phenomenon.

In common with the changes observed for transglutaminase in the carcinogenic process, studies with proliferating tissue have indicated that changes may also occur in the levels of polyamines

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found in neoplastic tissue [10,11]. This is particularly interesting, since the high levels of polyamines found in cells coupled with their ability to act as substrates for transglutaminase when present at these concentrations [12] suggests that the function of polyamines and transglutaminase may in some way be interrelated. Indeed, polyamine conjugates whose synthesis is thought to be transglutaminase mediated have been found in proteins of normal tissue [13,14,15]. It has also been suggested that ornithine decarboxylase, the major enzyme responsible for the synthesis of putrescine, may be down-regulated by transamidation reactions involving the covalent incorporation of putrescine into the enzyme [16].

In view of this close relationship between transglutaminase and polyamines in cellular processes, and its potential importance in neoplastic events, the present study was undertaken in order to investigate any correlation between transglutaminase activity and polyamine levels in a model system which allows discrimination of the metastatic process.

Materials and Methods

Chemicals. [1,4-^{14}C]Putrescine (118 mCi/mmol) was purchased from Amersham International, Amersham, Bucks, U.K. Brij-35, dithiothreitol, 2-mercaptoethanol, 1-octanesulphonic acid, *o*-phthalaldehyde, Tris-base, putrescine, 1,7-diaminoheptane, spermidine and spermine were purchased from Sigma Chemical Co., Poole, Dorset, U.K. All other reagents were obtained from BDH Chemicals Ltd., Dagenham, Essex, U.K., and were Analar grade with the exception of acetonitrile which was Hipersolv grade.

Tissue propagation and homogenisation. The propagation and characteristics of the transplantable rat sarcomata, P_7 and P_8 have been described previously [17]. Tumour metastases to the lung were detected by the method of Wexler [18] in which tumour secondaries appear contrasted white on a black background following staining of lung tissue with 15% (v/v) Indian ink and fixation in Feketes solution (60% ethanol/4% glacialacetic acid, 9% formaldehyde). Tissue was homogenised in 0.25 M sucrose/5 mM Tris/1 mM EDTA (pH 7.4) at 4° C using a Potter Elvejem homogeniser.

Assay of transglutaminase activity. Tra
glutaminase was assayed by a modification of
method of Lorand et al. [19] using the calcium-
pendent incorporation of [1,4-^{14}C]putrescine i
trichloroacetic acid-precipitable protein. A t
reaction vol. of 200 μl was used comprising
mM dithiothreitol/2.5 mM calcium chloride c
mM EDTA/5 mg/ml *N N'* dimethylcasein/
mM [1,4-^{14}C]putrescine (0.397 mCi/mmol),
mM Tris-HCl (pH 7.4)/100 μl of sample (2–3
protein). Samples (50 μl) were removed at vari
time points (5, 10 and 15 min) from both C
incubated and EDTA controls blotted onto fi
discs (2-cm diameter) and immersed in ice-c
10% (w/v) trichloroacetic acid. Filter discs w
then washed three times in ice-cold 5% (w
trichloroacetic acid, once in a mixture of eq
vols. of ice-cold ethanol/acetone and finally
acetone. Filter discs were then dried and coun
in 10 ml scintillation fluid (Optiphase S
Packard Ltd.).

Extraction of polyamines from tissue. For
extraction of free or acid-solubilised polyamir
tissue homogenates (normally 1 ml) contain
internal standard (1,7-diaminoheptane,
μmol/ml) were mixed with the appropriate vol
12.2 M perchloric acid to bring the final c
centration of perchlorate to 0.3 M. The mixt
was clarified by centrifugation at 1000 × *g* fo
min, after which the supernatant was remov
filtered through a 0.22-μm durapore filter (Wa
Associates, Ltd.) and used directly for polyam
analysis.

For the analysis of covalently conjuga
polyamines, acid-soluble polyamines and any
ther electrostatically complexed polyamines w
first removed from tissue homogenates using
method of Haddox and Haddock-Russell [20].
efficiency of this extraction procedure was chec
by addition of 5 μCi of [1,4-^{14}C]putrescine
tissue homogenates (1 ml) which revealed
greater than 99% of the radiolabel was removed
the washings. This is in agreement with the res
of Haddox and Haddock-Russell [20], who
demonstrated that this washing procedure
effective in the removal of radiolabelled sper
dine and spermine from tissue fractions.

The washed precipitate obtained from 2 m
original tissue homogenate was then suspended

3 ml of 6 M HCl (containing 10 μmol of 1,7-diaminoheptane as internal standard) and hydrolysed under vacuum for 20 h at 100 °C. The polyamines were then extracted from this hydrolysate using butanol [21], concentrated by evaporation and finally reconstituted in 220 μl of 0.3 M perchloric acid before analysis.

Analysis of polyamines by HPLC. Polyamine levels in tissue extracts were analysed by the HPLC method of Seiler [22] with slight modifications where required. For the estimation of recoveries and quantitation of sample injection, 1,7-diaminoheptane was used as an internal standard. For analysis of acid-soluble polyamines, a linear gradient from 0–100% (v/v) solvent B [22] over a time period of 25 min was used. For the analysis of covalently conjugated polyamines, gradient III described by Seiler [22] was required to achieve separation of the large number of peaks present. A Zorbax ODS column (4.6 mM × 25 cm) (Dupont (U.K.) Ltd, Stevenage, Herts) was used for this second gradient instead of the Waters C18 μ-bondapak column.

Chemical determinations. Protein and DNA were determined by the methods of Lowry et al. [23] and Burton et al. [24], respectively.

Expression of data. For clarity, transglutaminase activity, polyamine levels and protein are expressed as a function of tissue DNA content unless otherwise stated. Statistical tests for significance were performed using Student's *t*-test (two-tailed) and values of $P \leqslant 0.05$ are taken as significant.

Results

Levels of acid-soluble polyamines and transglutaminase in P_7 and P_8 sarcomata

Transglutaminase activity together with the levels of the acid-soluble polyamines putrescine, spermidine and spermine were measured in both P_7 and P_8 sarcomata (Figs. 1 and 2). The measurements were made at intervals over the whole period of time prior to the tumour size (or metastases in the case of P_8) necessitating the termination of the experiment. In animals implanted with P_8, metastases were detected in the lungs of all animals when tumour samples were taken at 30 days or later. Before this time period, only one animal (sampled at 28 days) was found to have detectable metastases in the lungs. In animals implanted with P_7 no metastases were detected during the course of these experiments.

Analysis of the polyamines putrescine, spermidine and spermine in the P_7 tumour samples indicated that the amounts of these three polyamines varied very little over the time course used (Fig. 1), whether the levels were expressed per wet weight of tissue, per mg protein or per mg DNA. In the P_8 sarcoma tissue, the levels of the polyamines spermidine and spermine were, in general, found to fluctuate more than the levels in P_7 tissue. This was especially true for the level of spermidine, which at 28 days in P_8 tissue was found to be approximately double ($P \leq 0.05$) that found in P_7 tissue sampled at 15 and 20 days; however, this elevation in spermidine levels did not appear to correlate with any other measured parameters. The spermidine/spermine ratio measured in P_8 tissue was found to be twice that found in P_7 tissue. However, the actual levels of spermidine and spermine found in the P_7 tumour were approx. 10-times greater than those found in the P_8 tumour.

The levels of putrescine found in the P_8 tumour at 15 and 20 days, when no metastases were detected appeared to be comparable to those found in P_7. However, in the case of P_8 tumours sampled at 34, 39 and 45 days, time points at which metastases were detected, putrescine levels were found to be approx. 20-times greater than those measured in tumour tissue sampled at 15 or 20 days, whether the data were expressed as a function of DNA (Fig. 2), wet weight of tissue or protein (data not shown). Only at 60 days, where the main part of the tumour is necrotic, did putrescine levels return to pre-metastatic levels. Only one P_8 tumour sample prior to 30 days (28 days) was found to have associated metastases in the lungs. This tissue also contained elevated levels of putrescine (789.1 mmol/mg DNA), though not as high as tissue sampled at 34, 39 or 45 days.

Transglutaminase activity was measured in homogenates of tumour tissue in an attempt to correlate any changes in enzyme activity with tumour progression and with the observed changes in polyamine levels reported above. In the P_7 sarcoma, no significant differences in trans-

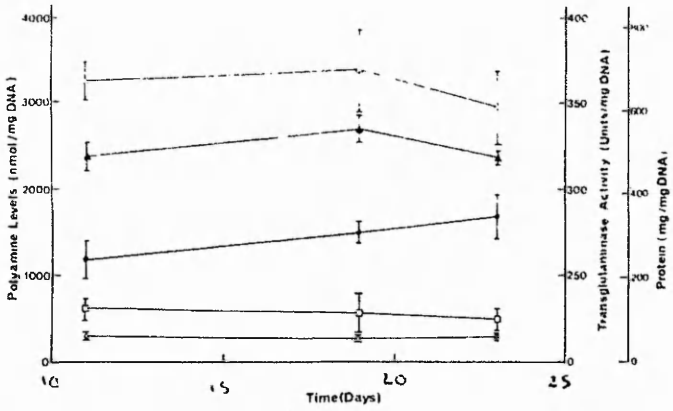

Fig. 1. Levels of transglutaminase activity, polyamines and protein in P_7 sarcoma tissue during growth. Samples of tissue were taken at various times shown during tumour growth and assayed for transglutaminase, ▲; putrescine, ○; spermidine, △; spermine, ● and protein, □. Results are expressed as the mean value ± S.E. ($n = 3$). 1 unit of transglutaminase activity equals 1 nmol putrescine incorporated per h.

glutaminase activity were found at the different sampling times whether expressed as a function of DNA (Fig. 1) or as a function of wet tissue weight or protein (data not shown). In contrast, the levels of transglutaminase activity found in the P_8 sarcoma (Fig. 2), although relatively constant in pre-metastatic tissue, were significantly reduced (4–5-fold) in post metastatic tissue ($P \leq 0.05$). This reduction in transglutaminase activity was consistent whether expressed as a function of DNA (Fig. 2) or as a function of wet tissue weight or protein (data not shown) and was found to correlate with the onset of metastasis and the rise in putrescine levels. However, although putrescine levels were seen to fall back to normal levels by 60 days, transglutaminase activity did not significantly rise at this time period.

Levels of covalently bound putrescine, spermidine and spermine in P_8 sarcoma

Covalently bound polyamines which could only be released by acid hydrolysis were also measured in four of the P_8 sarcomas at days 15, 20 and 28 prior to the detection of metastases, and in four tumours at days 34 and 45 which had detectable metastases.

The tumours which had detectable metastases were compared as a group to those with no detectable metastases. Statistical analysis of these two

groups revealed significant reductions for cov
lently bound putrescine in the tumour group wi
detectable metastases. The level of significan
was greatest ($P \leq 0.05$) when the levels were e
pressed as a function of protein or DNA (Table
This 4–5-fold reduction in bound putrescine co
pares well with the corresponding reduction in t
levels of transglutaminase activity.

Comparison of the levels of covalently bou
spermidine and spermine in the two groups
tumours indicated an increased amount of vari
tion in the levels of these polyamines compared
that found for bound putrescine. Although
apparent reduction in both polyamines was e
dent when the two groups were compared,

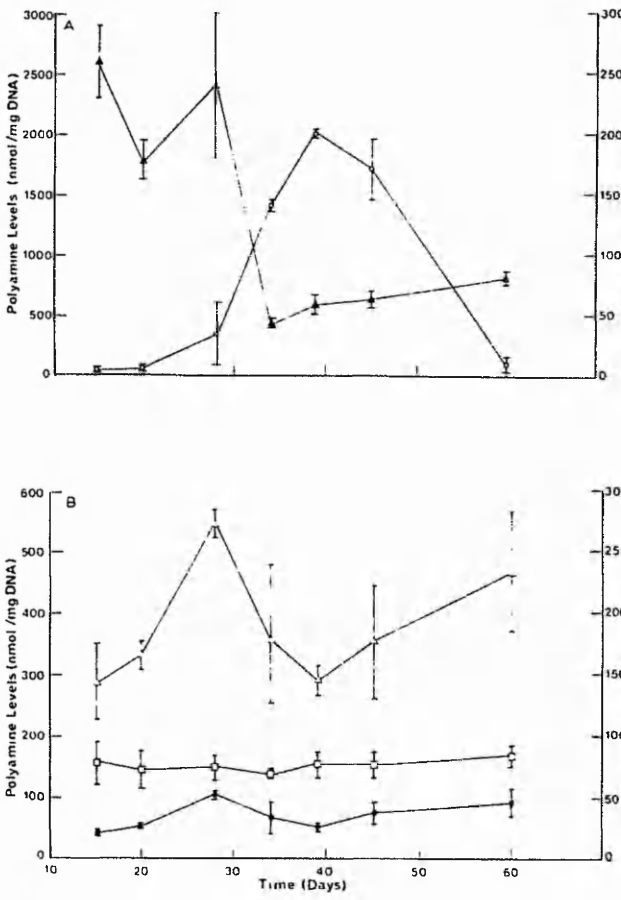

Fig. 2. Levels of transglutaminase, polyamines and protein
P_8 sarcoma tissue during growth. Samples of tissue were tak
at various times during tumour growth and assayed for (
transglutaminase (▲) and putrescine (○) and (B) spermidi
(△), spermine (●) and protein (□). Results are expressed as t
mean value ± S.E. ($n = 3$). 1 unit of transglutaminase activ
equals 1 nmol of putrescine incorporated per h.

TABLE I

COVALENTLY CONJUGATED LEVELS OF POLYAMINES IN P_8 SARCOMA TISSUE

P_8 sarcoma tissue was sampled before and after detection of metastases to the lung. Acid-soluble polyamines were first extracted from tumour tissue and the residue acid was hydrolysed. The polyamines released were then extracted and concentrated. Analysis of polyamines was carried out by the method of Seiler [21]. The results are expressed as the mean levels ± S.E. with the number of determinations in parenthesis. Where $n = 3$, quantitative analysis of the remaining sample could not be performed owing to limitations in the separation and detection procedure. n.s., $P > 0.1$.

Units	Polyamine content		
	no metastases detected	metastases detected	
nmol/mg DNA			
Putrescine	69.1 ± 15.5 (4)	16.2 ± 4.3 (4)	$P < 0.05$
Spermidine	10.8 ± 4.2 (4)	1.7 ± 0.05 (3)	n.s.
Spermine	6.7 ± 0.5 (3)	1.3 ± 0.2 (3)	n.s.
pmol/mg protein			
Putrescine	1008.7 ± 239.8 (4)	210.5 ± 29.4 (4)	$P < 0.05$
Spermidine	166.5 ± 65.4 (4)	21.1 ± 3.6 (3)	n.s.
Spermine	84.8 ± 64.3 (3)	13.3 ± 2.6 (3)	n.s.
nmol/g wet weight			
Putrescine	135.9 ± 34.9 (4)	34.8 ± 6.3 (4)	$P < 0.1$
Spermidine	20.0 ± 6.9 (4)	3.5 ± 0.8 (3)	n.s.
Spermine	11.3 ± 8.4 (3)	2.6 ± 0.2 (3)	n.s.

significant difference was found between tumours with or without detectable metastases.

Discussion

Two rat sarcomata, P_7 and P_8, were used in this study. The P_7 sarcoma metastasises infrequently, whereas the P_8 sarcoma consistently metastasises to the lungs at about 1 month after implantation. These two tumours, therefore, enable an investigation of any correlation between transglutaminase activity and polyamine levels in tumour progression, since they allow discrimination of the metastatic process.

The only significant changes observed occurred in P_8 tissue, where at 28 days, a period immediately prior to when this tumour is known to metastasise, a 2-fold increase in acid soluble spermidine was observed. Further, at approx. 30 days after implantation, there was a marked decrease (approx. 4–5-fold) in transglutaminase a tivity mirrored almost exactly by a large increas (approx. 20-fold) in the levels of acid-solub putrescine. The timing of these changes coincid with the time at which the P_8 sarcoma was show to have metastasised to the lung and suggests th the changes in the parameters measured are some way related to this phenomenon. The lack any changes in either transglutaminase or pol amine levels in the P_7 sarcoma during its rapi growth leading to termination, indicates that th changes observed in the metastasising P_8 sarcom are unlikely to be nonspecific changes which sim ply accompany tumour growth.

There is now increasing evidence to sugge that polyamines might have an important role i mammalian tissues through covalent linkage wit proteins through their primary amine group Post-translational modification of proteins throug incorporation of polyamines is a reaction whic transglutaminase is capable of effecting [1]. Tran glutaminase-mediated reactions of this kind hav been implicated in the regulation of nuclear even [20], the 'down regulation' of ornithine d carboxylase [16] and in the stabilisation of mem brane processes [12,25]. It therefore seemed pert nent in this study to compare the levels of cov lently bound polyamines which could be release by acid hydrolysis with the levels of tran glutaminase activity, and in turn relate these t the levels of acid-soluble polyamines and the ons of the metastatic event. Our data indicate th there is a significant reduction in the levels covalently bound putrescine in the post-metastat P_8 sarcoma. The magnitude and time of this chang parallels that seen for transglutaminase activit and it is tempting to suggest that the two a interrelated. However, further work including iso lation of γ-glutamyl putrescine and perhaps γ glutamyl spermidine conjugates would be neede to fully confirm this.

It is not possible at this stage to conclud whether the observed increase in acid-solubl putrescine is in any way directly related to th consistent reduction in transglutaminase activit and associated reduction in covalently boun putrescine. The lack of significant and concom itant increases in the levels of acid solubl spermidine and spermine in the metastasising

sarcoma would suggest that the increase in acid-soluble putrescine levels is not simply due to a transglutaminase-mediated perturbation in the regulation of ornithine decarboxylase activity [20]. Reduced diamine oxidase activity or alternatively reduced activity of either spermine and spermine synthase and/or *S*-adenosylmethionine decarboxylase could equally be responsible for the observed increases in acid-soluble putrescine levels.

In conclusion, the overall changes in the biochemical parameters that we have measured show an excellent correlation with the onset of the metastatic process, although our data do not allow us to determine whether the changes observed occur uniformly within the cell population, or to a greater or lesser extent within sub-populations. It is tempting to suggest that the result of the biochemical changes observed, whether exerted at the level of the nucleus [20], the cell membrane [2,3,4] or both, may be fundamental to the expression of the metastatic phenotype.

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