THE CELLULAR ROLE OF TRANSGLUTAMINASE

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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 P.J. Bunyan .....

(candidate)

Signed Martin Griffin .....

(Director of Studies)

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ABSTRACT

The Cellular Role of Transglutaminase

P.J. BUNGAY, 1984.

Rat pancreatic islets of Langerhans were found to contain a Ca^{2+}- and thiol-dependent transglutaminase enzyme. The apparent Km of the enzyme for Ca^{2+} was found to be approximately 40μM. Primary amines inhibited islet transglutaminase activity with a potency which decreased in the order monodansylcadaverine > ethylamine > methylamine > propylamine.

Primary amine inhibitors of islet transglutaminase activity also inhibited glucose-stimulated insulin release from intact islets with a potency which matched their relative potency as transglutaminase inhibitors. Although at high concentrations methylamine, ethylamine and propylamine displayed non-specific toxic effects, lower concentrations were found which gave rise to inhibition of insulin release in the absence of perturbing non-specific effects. These observations suggest a role for islet transglutaminase in the mechanism of glucose-stimulated insulin release.

Although putrescine was found to be a good substrate of islet transglutaminase (Km= 0.59mM), its effect on glucose-stimulated insulin release was modest by comparison. Whilst uptake studies suggested that this anomaly may be explained by the limited ability of putrescine to enter islet cells, the possible involvement of this polyamine in the mechanism of insulin release could not be discounted as an explanation.

Evidence was obtained which suggested that rapid synthesis of the polyamines putrescine, spermidine and spermine was not required for stimulus-secretion coupling in the pancreatic β-cell. The high concentrations of these compounds in islets implied a possible role as substrates for islet transglutaminase.

Homogenates of islets contained proteins possessing γ-glutamyl residues available for amine incorporation catalysed by endogenous transglutaminase. Initial studies suggested that the substrate specificity of islet transglutaminase may be limited to a small number of these proteins.

Studies on passaged tumour lines and primary hepatocellular carcinomata suggested that transglutaminase activity was in general lower in these tissues than in normal tissues. A predominantly particulate distribution of transglutaminase activity appeared to be a marker of tumour tissue and may therefore be important in tumour growth.

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Abbreviations used in this thesis.

6-BT	6-p-dimethylaminophenylazobenzothiazole
p-CMB	p-chloromercuribenzoic acid
DEN	diethylnitrosamine
DFMO	α-difluoromethylornithine
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
HBSS	Hanks' Balanced Salt Solution
Hepes	N-2-hydroxylthylpiperazine N'-2-ethane sulphonic acid
HPLC	High-pressure liquid chromatography
KRB	Krebs Ringer Bicarbonate medium
ODC	ornithine decarboxylase (E.C. 4.1.1.17.)
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane

1. INTRODUCTION

1.1. INTRODUCTION TO THE TRANSGLUTAMINASES.

1.1.1. The Definition of Transglutaminase.

In 1959, an enzyme activity from guinea-pig liver was described which catalysed the Ca^{2+}-activated covalent incorporation of primary amines into proteins (Clarke et al., 1959). During its subsequent characterisation, this enzyme activity was named transglutaminase in order to describe the reaction catalysed, namely, the exchange of amide groups of peptide-bound glutamine with primary amine groups to yield new γ-amide bonds and ammonia (Mycek et al., 1959). Transglutaminases have since been identified in and purified from a large number of other animal tissues and body fluids and have been the subject of a number of reviews (Chung, 1972; 1975; Folk & Chung, 1973; Folk & Finlayson, 1977; Folk, 1980; Folk, 1983; Lorand & Stenberg, 1976; Lorand & Conrad,1984; Williams-Ashman & Canellakis, 1980). They are now defined as enzymes which catalyse the Ca^{2+}-dependent acyl transfer reaction between peptide-bound glutamine and primary amine groups, resulting in the incorporation of amine into protein or the formation of ε-(γ-glutamyl)lysine cross-links between proteins if the primary amine is peptide-bound lysine. Due to the ability of transglutaminase to catalyse the formation of ε-(γ-glutamyl)lysine crosslinks, these enzymes are often known as γ-glutamyl-ε-lysine transferases. They have been systematically named as R-glutaminyl-peptide:-amine-γ-glutamyl-yl-transferases (E.C. 2.3.2.13), although the alternative nomenclature glutaminyl-peptide:-amine- γ-glutamyl transferase has now been recommended (Eur. J. Biochem. 116(3), 1981).

1.1.2. The occurrence of transglutaminases: classification.

Transglutaminases are very widely distributed enzymes and are found intracellularly and extracellularly (Folk & Finlayson, 1978; Folk, 1980). Indeed, the occurrence of transglutaminases is so widespread that it is generally thought that these enzymes are ubiquitous in mammalian tissues and cells. However, transglutaminases differ in their structure and function according to their source. Therefore, the classification of these enzymes was required and was based upon their location. Thus, the following families of transglutaminases have been identified:

- Plasma Factor XIII
- Platelet Factor XIII *
- Placental protransglutaminase
- Prostate transglutaminase
- Epidermal transglutaminase *
- Hair follicle transglutaminase *
- Erythrocyte transglutaminase *
- Tissue transglutaminase *

Together with this; the above have been grouped further into three major types comprising protransglutaminases (zymogens), the hair follicle and epidermal transglutaminases and the tissue transglutaminases, as indicated above. These distinctions are based upon physical, chemical, immunological and catalytic properties of the enzymes (Chung, 1977). Those types which perform their function intracellularly are indicated by * above.

A few of these types of transglutaminase have been studied sufficiently for it to be possible to assign to them a biological function: these enzymes are the plasma Factor XIII, rodent prostate

transglutaminase, epidermal transglutaminase and hair follicle transglutaminase. The biological role of the most widely distributed transglutaminase, the tissue transglutaminase, is not known although many studies have been undertaken in an effort to understand its structure and function.

1.1.3. The subject of the thesis.

The tissue transglutaminase enzyme will form the subject of this thesis whose aim is to improve our understanding of the biological role of this ubiquitous intracellular enzyme.

1.2. GENERAL CATALYTIC FEATURES OF TRANSGLUTAMINASES.

1.2.1. The reactions catalysed by transglutaminases.

Table 1.1 describes the reactions catalysed by the transglutaminase enzymes. The most important of these are the first two, the incorporation of amines into protein and the crosslinking of proteins, for these reactions represent the covalent post-translational modification of proteins and as such have great potential for the regulation of biological processes. Indeed, they are the only transglutaminase-catalysed reactions which have been observed *in vivo* and which form the basis of the physiological role of the transglutaminases whose function has been elucidated. These reactions are illustrated in fig. 1.1..

Several other reactions have been shown to be catalysed by transglutaminases *in vitro*, but in common with reactions 1 and 2 involve the hydrolysis or aminolysis of an amide or ester. None of these reactions (reactions 3-9 in table 1.) have yet been shown to have any physiological significance.

It is clear that the transglutaminse-catalysed incorporation of an amine into protein will be subject to competition by other primary amines leading to an inhibition of incorporation of the first amine which can be viewed as an inhibition of enzyme activity with respect to that amine. Likewise, cross-linking reactions will be inhibited by primary amines which will be incorporated into the protein substrate containing γ-glutamyl residues.

The incorporation of amines into protein (reaction 1.) forms the basis of the most commonly used and most sensitive assay for

transglutaminase enzymes of all types. Thus, the incorporation of radiolabelled amine substrates (e.g. 1,4-[^{14}C]-putrescine, [^{14}C]-methylamine and 2,5-[^{3}H]-histamine) into protein substrates (e.g. N,N'-dimethylcasein and acetylated B-chain of insulin) may be conveniently measured by precipitation of the substrate protein with trichloroacetic acid and counting of the bound radiolabel (Lorand et al., 1972).

A less sensitive and less preferred assay procedure is based upon the measurement of hydroxamate formation from hydroxylamine and carbobenzoxy-L-glutaminyl glycine catalysed by the enzyme (Folk & Cole, 1966b). This reaction is equivalent to that depicted in reaction 5., in Table 1.1..

Table 1.1. The reactions catalysed by transglutaminases

1. Aminolysis of peptide-bound glutamine: incorporation of amines into protein.

2. Aminolysis of peptide-bound glutamine: ε(γ-glutamyl)lysine cross-link formation.

3. Hydrolysis of peptide-bound glutamine.

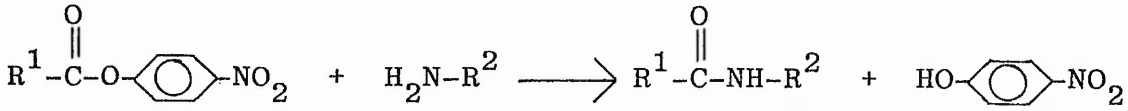
4. Hydrolysis of amides.

5. Aminolysis of amides.

6. Hydrolysis of ρ-nitrophenyl esters.

Table 1.1. continued

7. Aminolysis of ρ-nitrophenyl esters.



8. Hydrolysis of aliphatic esters.

9. Aminolysis of aliphatic esters.

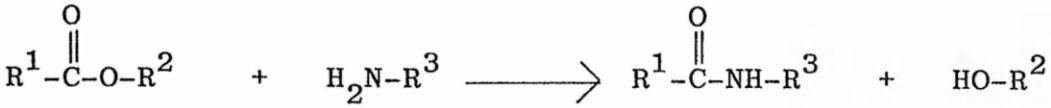
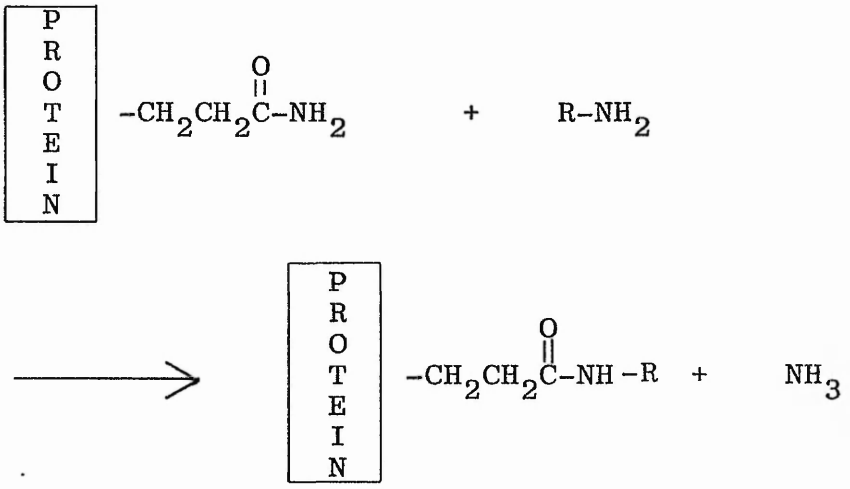
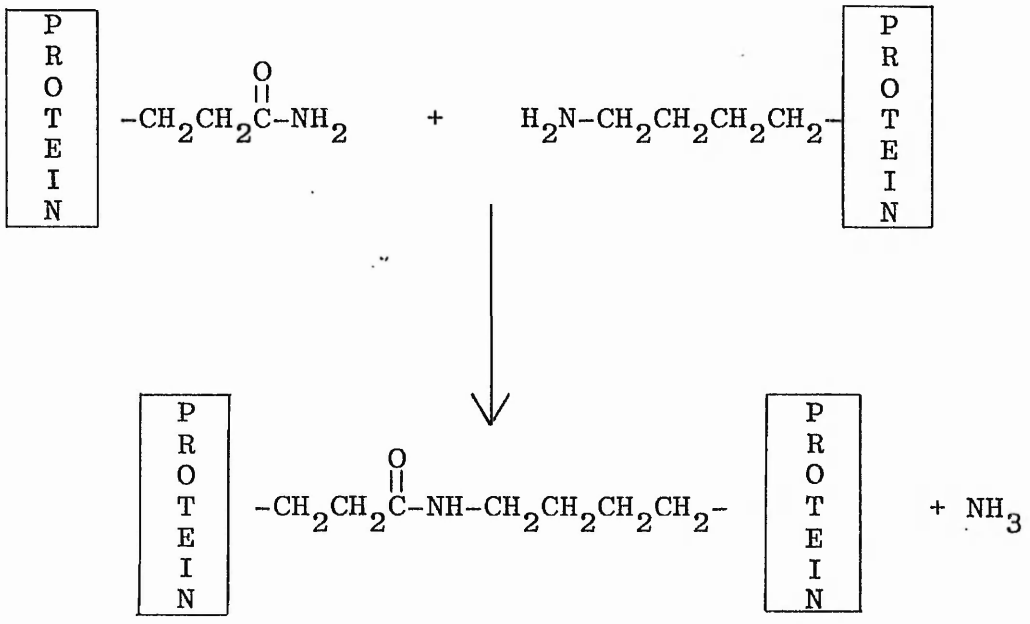


Figure 1.1 Reactions catalysed by transglutaminase

a. Incorporation of amines into proteins:



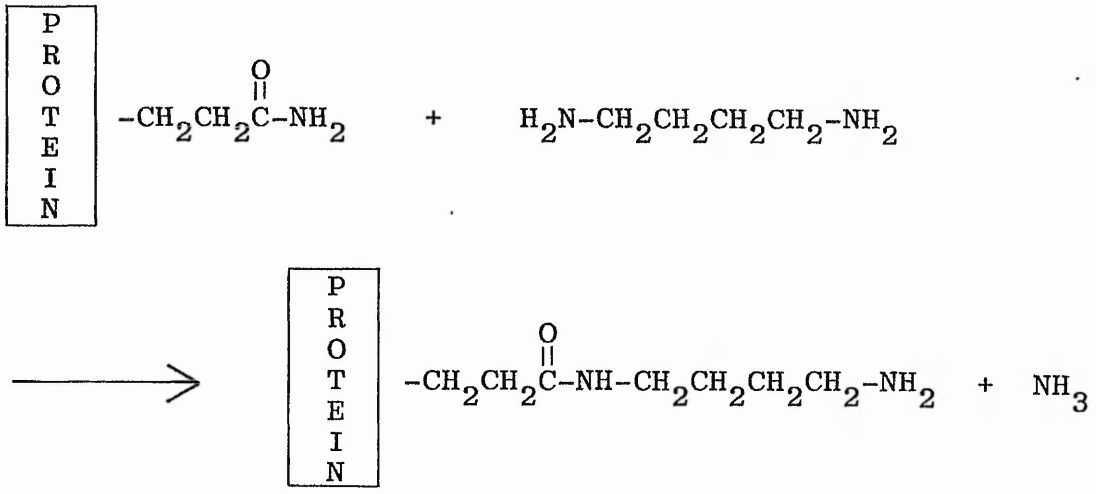
b. Formation of ε-(γ-glutamyl)lysine bonds: the cross-linking of proteins:



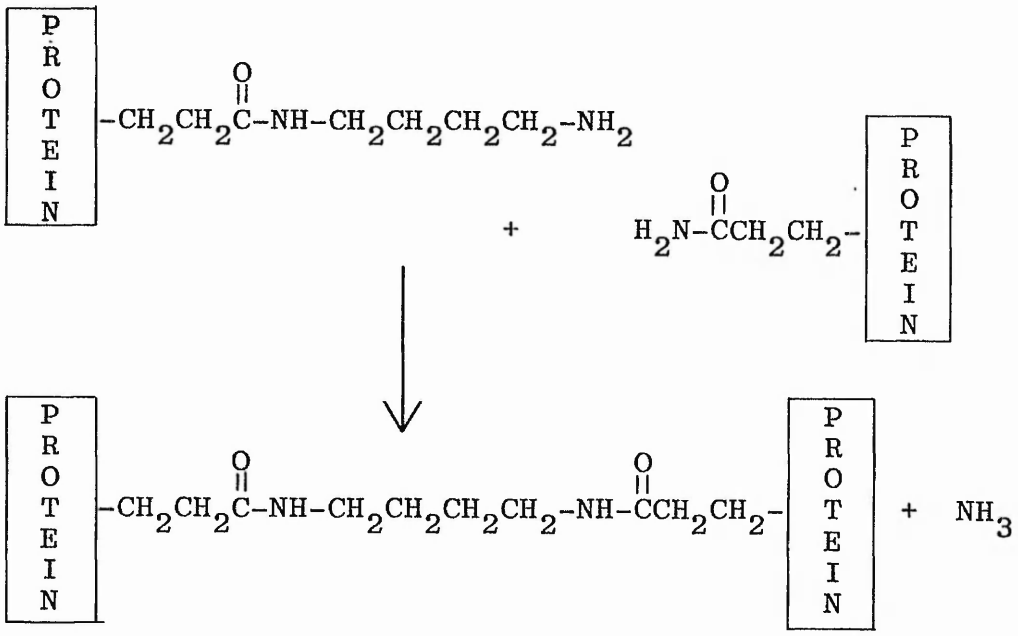
ε-(γ-glutamyl)lysine
cross-link

Figure 1.1 continued

c. Formation of diamine cross-links through polyamine (e.g. putrescine) incorporation:



N-(γ-glutamyl)putrescine



N,N-bis-(γ-glutamyl)putrescine

1.2.2. The mechanism of catalysis.

The reactions depicted in Table 1.1. proceed by a modified double-displacement reaction which for convenience can be divided into two stages:

i. the formation of a thioester linkage between amide, ester or peptide-bound glutamine and the cysteine -SH group at the enzyme active site;

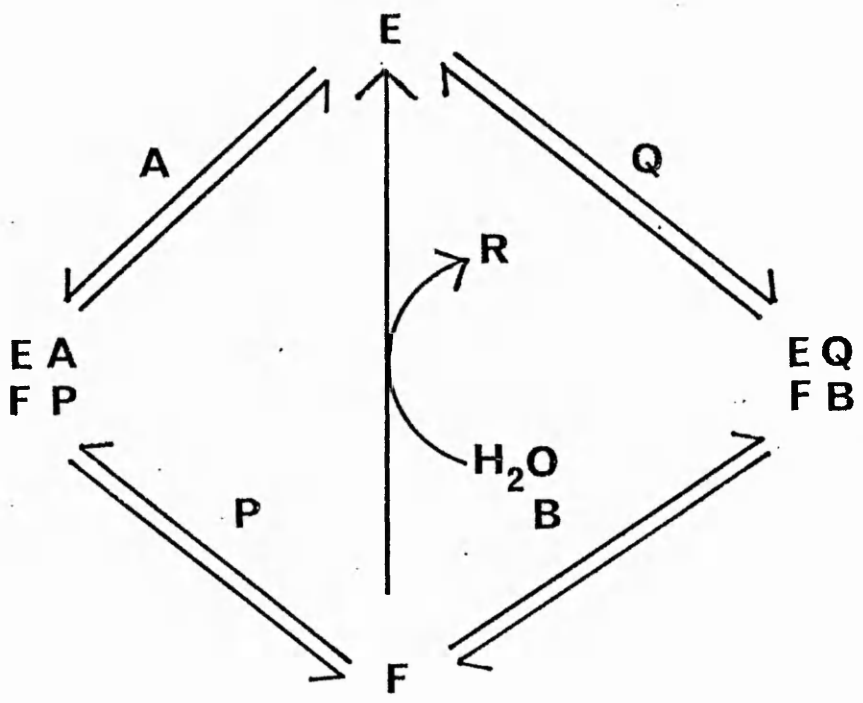
ii. lysis of the thioester bond by a nucleophile (water or primary amine).

The mechanism for reactions 1, 2 and 3 is illustrated in fig. 1.2. Evidence consistent with this mechanism was obtained for the guinea-pig liver enzyme and for Factor XIII (Chung and Folk, 1972; Gross and Folk, 1973; Chung et al., 1970; Gross and Folk, 1974; Folk, 1969; Curtis, et al., 1974 and Stenberg et al., 1975). Transglutaminases display a requirement for Ca^{2+} ions although other divalent cations can replace Ca^{2+} to varying extents (Folk et al., 1967a; 1967b). The role of Ca^{2+} is to bind the enzyme and induce a conformational change which exposes the active site.
The requirement for enzyme activity on a thiol group at the active site makes the enzyme sensitive to thiol-reactive compounds.
Thus, useful criteria for the definition of transglutaminase activity are:

i. thiol-dependence as judged by the inhibition of enzyme activity by such compounds as iodoacetamide, p-chloro-mercuribenzoic acid and 5,5'-dithiobis (2-nitrobenzoic acid) (Folk & Cole, 1966a; Connellan & Folk, 1969).

ii. Ca^{2} -dependence as judged by the inhibition of enzyme activity by Ca^{2+}-chelating agents such as EDTA and EGTA.

Figure 1.2. The Mechanism of Action of Transglutaminase



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-glutamic acid amide

1.2.3. The basis of substrate specificity.

Studies on substrate specificity have been carried out with guinea-pig liver transglutaminase (a tissue transglutaminase) and with Factor XIII, the plasma transglutaminase. It should thus be borne in mind that these two enzymes may not exhibit the same specificity as other transglutaminases. However, they will serve to illustrate the factors influencing substrate specificity which may be of significance to the biological role of transglutaminases. These factors have recently been reviewed by Folk (1983).

Substrate specificity in the case of transglutaminases may be viewed in respect of :

i. the requirements of the amide site where the acyl-enzyme intermediate forms;

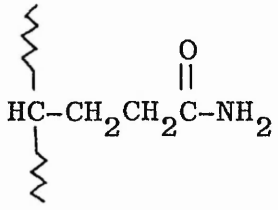
ii. the extended glutamine site where peptides containing glutamine bind to the enzyme;

iii. the amine site where nucleophiles interact with acyl-enzyme intermediates.

1.2.3.1. The amide site.

The requirements for amides to act as substrates for the incorporation of amines by guinea-pig liver transglutaminase were investigated in studies by Chung *et al.*, (1970), Chung, (1975) and Gross and Folk (1973). It was found that only L-glutamine residues in which the α-carbon NH_2 and COOH groups were peptide-bound could effectively participate in catalysis; peptide-bound asparagine (also with an amide side chain) did

not act as a substrate, reinforcing early findings by Waelsch and co-workers (Waelsch, 1962; Neidle and Acs, 1961). Thus, the structure below is an absolute requirement for amide substrates for amine incorporation:



A model was proposed which was compatible with the observed specificity of the enzyme for aliphatic amides in which the active site could accommodate all amides but only unbranched ones could alter the active site conformation suitably for catalysis to occur. In addition, a preference for uncharged groups on or near the α-carbon of peptide-bound glutamine was found (Folk & Cole, 1965; 1966b) suggesting that a hydrophobic domain existed near the active site. These observations were in accordance with "reporter" group-labeled studies (Gross & Folk, 1971).

1.2.3.2. The extended glutamine binding site.

The action of transglutaminase on a protein involves the interaction of amino acid side-chains around the enzyme active site with those in the vicinity of the substrate glutamine residue. This consideration suggests the possibility of a further basis for the substrate specificity of transgutaminases.

Studies on guinea-pig liver transglutaminase and Factor XIII demonstrated that whereas guinea-pig liver could utilise

benzoxycarbonyl-glutaminylglycine as a substrate, Factor XIII was not able to (Chung, 1972). Furthermore, patterns of cross-linking of fibrin, the natural substrate of Factor XIII (see below, Section 1.3.1.), were different for the two enzymes (Chung and Folk, 1972). This indicates that transglutaminases from different sources differ in their substrate specificity and probably do so in a way appropriate to their biological function.

The influence of variations in the sequence of amino acids on substrate effectiveness for guinea-pig liver enzyme were investigated by Gross et al., (1975) who found evidence suggesting that enzyme and substrate interacted over a sequence of at least five amino acid residues. A peptide fragment of β-casein with varying amino acid substitutions was used in similar studies with Factor XIII and liver transglutaminase (Gorman & Folk, 1980; 1981). It was found that certain amino acids greatly affected the affinity of the peptide for Factor XIII but were less important for amine incorporation catalysed by the liver enzyme. These studies also suggested that the active sites of these enzymes corresponded in size to at least nine or ten amino acid residues of the peptide substrates. These observations provide at least a partial basis for differences in specificity between transglutaminase enzymes.

1.2.3.3. The amine site.

The second stage of the catalytic mechanism the lysis of the thioester bond (Section 1.2.2.) involves a nucleophilic attack on the acyl-enzyme intermediate. For a catalytic goal to be achieved, the nucleophile must be strong enough to cause lysis of the

thioester linkage, and for aminolysis to occur, the amine must be in the uncharged form (Folk and Cole, 1966b). Therefore, in studies of the relative affinity of amine substrates for transglutaminase, the effective concentration of the amine, defined by its pKa, may be taken into account.

Similar considerations to those applied to glutamine substrates were applied to amine substrates in studies on their affinity for transglutaminases, that is, the nature of the moiety bearing the amine group and, in the case of peptide-bound lysine, the nature of the functional groups in its vicinity. It is also worth noting that the structure of an acyl intermediate may determine its specificity towards amines (Chung et al., 1970).

Lorand et al., (1979) synthesised a variety of primary amine substrates for guinea-pig liver transglutaminase and tested them for their ability to inhibit [^{14}C]-putrescine incorporation into N,N'-dimethylcasein to provide a measure of their substrate specificity. The best substrates possessed a large hydrophobic moiety attached to a 5-carbon alkylamine side chain (7.5Å long). Similarly, and using a different assay, Gross et al., (1977) determined the preference of guinea-pig liver for aliphatic amines to be for those which were unbranched unless the branch occurred at a distance 5 carbon atoms from the amine group. This structural similarity to the side chain of lysine led to studies on a series of α,w-diaminomonocarboxylic acid peptide derivatives which revealed the L-lysine analogue to be the most active. The suggestion from these three studies was that the transglutaminase active site displayed a preference for lysine as a second substrate.

The possibility of the existence of an extended lysine

site was investigated in studies with synthetic peptides (Gross et al., 1977). Only the amino acids adjacent to lysine seemed to affect the interaction with acyl-enzyme intermediates. However, a single L-leucine residue on the amino side of lysine considerably enhanced the specificity of leucine-containing peptides, suggesting the importance of a hydrophobic domain (Schrode & Folk, 1979) in accordance with the findings of Lorand et al., (1979).

1.2.3.4. Conclusion.

The studies with guinea-pig liver transglutaminase and Factor XIII which have been outlined demonstrated a common specificity of the enzymes for the amide and amine sites but a different specificity for the extended glutamine site. The preference for peptide-bound glutamine and lysine of these enzymes suggests that the formation of ε-(γ-glutamyl)lysine cross-links is likely to be the main reaction catalysed by these enzymes in vivo. Indeed, this is the case for Factor XIII, prostate transglutaminase, epidermal transglutaminase and hair follicle transglutaminase, and these enzymes are discussed below. Furthermore, it is significant that the lysine analogue monodansylcadaverine is always found to be a potent inhibitor of transglutaminase activity from a variety of sources of tissue.

There is clearly scope for variation in the substrate specificity of transglutaminases because these enzymes possess an extended glutamine site for the interaction of peptide substrate and enzyme. It is tempting to suggest that cells possess the means to express a variety of transglutaminse enzymes but only express those of appropriate substrate specificity to the tissue in question. However, it was pointed out by Folk (1983) that little has been learnt about the way in which transglutaminases operate upon macromolecular substrates in biological systems. It is to biological systems that attention is now focussed.

1.3. The Biological Role of the transglutaminases.

In this section, the transglutaminases, both intracellular and extracellular whose function or likely function is understood are discussed. The biological role of the tissue transglutaminases, whose function in cells is poorly understood, is discussed in the next section.

1.3.1. The plasma transglutaminase: Factor XIII.

The presence of a factor in blood responsible for the stabilisation of the fibrin clot during haemostasis has been known since 1944 (Robbins, 1944). This factor has since been identified as a transglutaminase enzyme and designated blood coagulation Factor XIII constituting the final link in the cascade of reactions in the blood coagulation system. Although a role for this transglutaminase in the stabilisation of the fibrin clot is now recognised its precise mode of action is yet to be fully elucidated. The structure and function of Factor XIII was recently reviewed by Lorand et al., (1980).

The Factor XIII zymogen exists as a multimer of 300,000 molecular weight comprised of 2 a subunits (each of 75,000 molecular weight) and 2 b subunits (each of 80,000 molecular weight) (Schwartz et al., 1973; Chung, 1972). Activation of Factor XIII is achieved by proteolytic cleavage by thrombin of a 4,000 molecular weight peptide from the a chain N-terminus of the a subunit (Takagi & Doolittle, 1974) and subsequent dissociation of the a'and b subunits (Lorand et al., 1974). The a' subunits

released under the influence of Ca^{2+} are catalytically active and are designated Factor XIIIa. Like other transglutaminases,Factor XIIIa requires the presence of Ca^{2+} ions for activity. This aspect of Ca^{2+}-activation should not be confused with the requirement of thrombin-catalysed proteolysis for Ca^{2+}.

Fibrin clots formed in the presence of Factor XIII and Ca^{2+} are more rigid and chemically resistant than clots formed in their absence. In particular, clots formed in the presence of Factor XIII and Ca^{2+} are resistant to dilute acids and bases and to strong solutions of urea (Laki & Lorand, 1948), suggesting a covalent interaction in the stabilisation of fibrin. Unequivocal evidence that stabilisation of fibrin involved the formation of ε(γ-glutamyl)lysine crosslinks catalysed by a transglutaminase was provided by the isolation of the dipeptide in proteolytic digests of cross-linked fibrin (Matacic & Loewy, 1968; Pisano et al., 1968; Lorand et al., 1968).

Examination of the symptoms of diseases caused by Factor XIII deficiency have also been useful in defining the physiological role of the enzyme. In this respect Duckert was able to propose three roles for Factor XIII catalysed protein cross-linking in haemostasis (Duckert et al., 1960; Duckert, 1972):

i. the production of a rigid fibrin clot;

ii. the production of a clot resistant to proteolysis;

iii. the support of tissue repair.

The polymerisation of fibrin catalysed by Factor XIII therefore plays an important role in haemostasis and wound healing.

The physiological substrate of Factor XIII, fibrin, is formed from a precursor, fibrinogen which consists of three

polypeptide chains α, β and γ of molecular weights 70,000, 60,000 and 50,000 respectively and each of which is found in duplicate in the fibrinogen molecule (Mossesson & Finlayson, 1976). Fibrin is produced by the thrombin-catalysed cleavage of two peptides from the amino-terminus of the fibrinogen polypeptides to yield a structure designated $(\alpha' \beta' \gamma')_2$. Fibrin monomers assemble to form a gel whose cross-linking may then be catalysed by Factor XIIIa. The cross-linking of the γ subunits rapidly forms a hemostatic plug whilst the slower α polymerisation leads to the production of a stable, long-term clot (Finlayson & Aronson, 1974). The β subunits do not appear to participate in the polymerisation.

Recent evidence suggests that the generation of Factor XIIIa is enhanced by fibrinogen. The Ca^{2+}-dependence of the dissociation of a'_2b_2(Factor XIII) is considerably lowered in the presence of fibrinogen (Credo et al., 1981). Furthermore, the thrombin-mediated activation of Factor XIII (i.e. $a_2b_2 \rightarrow a'_2b_2$) was enhanced by fibrinogen (Janus et al., 1983). This evidence, together with the evidence for the close physical association of fibrinogen and Factor XIII in plasma (Greenberg & Schuman, 1982) suggests a molecular basis for the co-ordination of the stabilisation of the fibrin clot by plasma transglutaminase.

1.3.2. Prostate transglutaminase.

The anterior prostate gland of rodents contains an enzyme activity responsible for catalysing the clotting of seminal plasma, a process which increases the efficiency of fertilisation (Mann & Lutwak-Mann, 1981). The extreme insolubility of a basic protein was responsible for the clotting in the guinea-pig (Nitoides & Williams-Ashman, 1967) and it was demonstrated that the clotted material contained a large number of ε(γ-glutamyl)lysine cross-links (Williams-Ashman et al., 1972), implicating a transglutaminase in the clotting process. A transglutaminase enzyme was later identified in the coagulating gland of guinea-pig prostate and was found to be Ca^{2+}-dependent and thiol-dependent and to have a molecular weight of 70,000 (Wing et al., 1974). Further investigation, however, suggested the existence of two forms of guinea pig prostate transglutaminase, both of which exhibited properties distinct from other transglutaminases (Wing, 1977; Tong, 1980). These results contrasted with those of Chung (1977) who identified two transglutaminases in guinea pig prostate which shared characteristics similar to those of Factor XIII and guinea pig liver (tissue) transglutaminase.

Rat seminal vesicle secretion proteins were also found to be cross-linked by ε(γ-glutamyl)lysine bonds (Williams-Ashman et al., 1977). In this system, two unique enzymes responsible for semen coagulation were identified in the coagulating gland of rats (Wilson et al., 1979; Williams-Ashman et al., 1980). These enzymes were found to be distinct from rat liver transglutaminase and plasma Factor XIII on the basis of their electrophoretic mobility at pH 7.4

and on the basis of their substrate specificity towards methylated seminal vesicle proteins (Williams-Ashman et al., 1980). They suggested that since seminal vesicle secretions contain high levels of the polyamines putrescine, spermidine and spermine, the polyamine cross-link may play a role in the stabilisation of the post-coital plug as well as the ε(γ-glutamyl)lysine bond. As an alternative, it was also suggested that the presence of polyamines may retard the cross-linking through the formation of ε(γ-glutamyl)lysine in urethra of the male where it would be inappropriate (Williams-Ashman et al., 1980).

Another role for the prostate transglutaminase was recently suggested by the observation in vitro that transglutaminase, in combination with uteroglobulin, can suppress the antigenicity of spermatozoa in the female reproductive tract (Mukherjee et al., 1983). Thus, prostate transglutaminase may mask cell surface antigens by cross-linking uteroglobulin to the spermatozoan antigenic determinants.

In conclusion, although the nature and identity of the prostate transglutaminases is uncertain, these enzymes are important in the stabilisation of the post-coital plug in rodents, and are therefore an aid to fertilisation. In addition, it is possible that prostate transglutaminase may aid fertilisation by masking antigenic determinants on the spermatozoa.

1.3.3. Epidermal transglutaminase.

A transglutaminase which is distinct from the tissue enzyme and which is also found in cells has been located in the epidermis and cultured epidermal cells. Estimates for the molecular weight of this enzyme were 55,000 for that derived from cow snout epidermis (Buxman and Wuepper, 1975) and 56-58,000 for the enzyme from the epithelium of new born rats (Peterson and Buxman, 1981). The enzyme was found to be localised in areas of the epidermis where keratinisation takes place (Buxman and Wuepper, 1975), suggesting that this enzyme may play a role in the manufacture of the cornified envelope possessed by fully differentiated keratinocytes.

Homogenates of human and rat epidermis were able to incorporate radiolabelled putrescine into high molecular weight proteins (Hanigan & Goldsmith, 1978), whilst in cultured cells of human epidermis such incorporation into a protein of 92,000 molecular weight was followed by crosslinking of this soluble protein into the cornified envelope (Rice and Green, 1979). Perhaps the most definitive proof of transglutaminase involvement in the process of formation of the cornified envelope was the increase in ε-(γ-glutamyl)lysine crosslinks which accompanied Ca^{2+}-induced formation of the envelope (Hennings et al., 1981). Two protein substrates of transglutaminase have been characterised in human keratinocytes (Kubilus &Baden, 1982). These similar, immunologically related proteins designated A (molecular weight 125,000) and B (molecular weight 12,000) were both convertible to higher molecular weight forms when acted upon by transglutaminase.

The evidence therefore favours the view that epidermal

transglutaminase has the specialised function of cross-linking cornified envelope precursor proteins with ε(γ-glutamyl)lysine bonds in order to produce the resistant structure required by the organism as an external barrier.

1.3.4. Hair follicle transglutaminase.

During investigations into the nature of the insolubility of hair proteins, it was postulated that the ε(γ-glutamyl)lysine cross-link found in citrulline-containing proteins of hair contributed to the crosslinking and consequent insolubility of these proteins (Harding & Rogers, 1971; 1972; 1976). Steps were therefore undertaken to investigate the role of transglutaminase in the formation of these crosslinks.

Hair follicles from guinea-pig, rat and sheep were found to contain a transglutaminase enzyme of a form peculiar to hair follicles (Chung & Folk, 1972; Harding & Rogers, 1972; Peterson & Buxman, 1981) in addition to the tissue transglutaminase. The hair follicle transglutaminase was shown to be able to catalyse the cross-linking of fibrin (Chung & Folk, 1972) with the formation of ε(γ-glutamyl)lysine cross-links (Harding & Rogers, 1972). The molecular weight of the enzyme (52-54,000) resembled that of epidermal transglutaminase, but in contrast existed as a dimer of subunits of 27,000 molecular weight (Chung & Folk, 1972; Peterson & Buxman, 1981). Furthermore, these two enzymes are immunochemically distinct (Buxman & Wuepper, 1976).

Although these studies are as yet incomplete, the suggestion is that hair follicle transglutaminase plays a role in the

cross-linking of hair proteins, conferring upon them the properties of insolubility and mechanical strength.

1.3.5. Conclusion:

The feature common to the transglutaminases Factor XIII, epidermal transglutaminase, hair follicle transglutaminase and prostate transglutaminase is that they catalyse the formation of ε(γ-glutamyl)lysine crosslinks between proteins which then acquire the property of mechanical and chemical resistance. This activity is the basis of the biological role of these enzymes which therefore provide a means of post-translational modification of proteins so as to influence the mechanical and chemical stability of tissues.

1.4. The biological role of tissue transglutaminase.

Tissue transglutaminase has been identified in a large number of tissues including liver, spleen, muscle, kidney, lung adrenal gland, brain, testis and pancreas (Chung, 1972). Such a wide distribution of transglutaminase indicates that the enzyme is ubiquitous in tissues. The tissue transglutaminase has been purified from guinea pig liver (Folk & Cole, 1966a), rabbit liver (Abe et al., 1977), rat liver (Barnes, 1980) and human erythrocytes (Brenner & Wold, 1978). In each case the enzyme was found to be a non-zymogenic monomer of molecular weight 70-90,000. Comparative studies which were based on chemical, physical, immunological and enzymatic properties indicated that the enzyme from different tissues in the same organism is identical (Chung, 1972; 1977).

1.4.1. The potential importance of tissue transglutaminase to cellular function.

The reactions catalysed by transglutaminases involving peptide-bound glutamine represent possible ways in which post-translational modifications of proteins may be achieved. Such enzyme-mediated modifications can regulate protein function as was evident in the case of the ε(γ-glutamyl)lysine cross-link. The ubiquity of transglutaminase activity in tissues and cells therefore opens up the possibility that the enzyme may represent a universal means of regulating protein function in cells. This is a particularly pertinent consideration, since the activity of the enzyme is dependent upon the presence of Ca^{2+}

which is itself a major regulator of intra-cellular events.

As pointed out by Folk (1983) and Lorand and Stenberg (1975), the catalysis of ε(γ-glutamyl)lysine cross-link formation may not necessarily be the basis of the biological role for the tissue transglutaminase, for there are other potentially important reactions which the enzyme is capable of catalysing. Indeed, even comparatively small modifications such as the incorporation of primary amines may conceivably lead to large changes in the behaviour of a protein. This can be readily appreciated by consideration of phenomena such as regulation of enzymes by protein phosphorylation and the malfunction of haemoglobin in sickle cell anaemia caused by the alteration of a single amino acid residue (Ingram, 1957).

With these considerations in mind, the possible cellular function of tissue transglutaminase in those systems where it has been studied will now be discussed.

1.4.2. Receptor-mediated endocytosis.

The involvement of transglutaminase in the process whereby cells internalise surface-bound ligands (reviewed by Besterman & Low, (1983); Pastan & Willingham, (1981a); Goldstein et al., 1979 and Willingham & Pastan, 1981b) has attracted much attention and has been the cause of some controversy.

The observation by Maxfield et al., (1979a; 1979b) that primary amines and ammonium ion blocked the clustering and internalisation of epidermal growth factor and α_2-macroglobulin by cultured fibroblasts led them to suggest that cellular transglutaminase may be involved in the process of cross-linking of receptors for these ligands into coated pits. This was a particularly attractive proposal, for receptor-mediated endocytosis is, in common with transglutaminase activity, a Ca^{2+}-dependent process. Furthermore, it was consistent with the observed specificity of transglutaminases for the cross-linking of proteins.

This proposal was reinforced by the same workers in studies of the endocytosis of rhodamine-labelled α_2-macroglobulin and epidermal growth factor by fibroblasts, Chinese hamster ovary cells and normal rat kidney cells. A wide range of compounds that were inhibitors of transglutaminase activity including monodansylcadaverine, alkylamines and bacitracin inhibited clustering and internalisation with a potency which correlated with the potency of these compounds as inhibitors of CHO transglutaminase activity (Davies et al., 1980; Levitzki et al., 1980). Also in accordance with the above was the observation

that internalisation of ^{125}I-labelled epidermal growth factor was inhibited by monodansylcadaverine (Haigler et al., 1980). Throughout these studies it was borne in mind that amines, by virtue of their basicity could raise the internal pH of the cell (Ohkuma & Poole, 1978) and inhibit endocytosis by virtue of this mechanism. However, the correlation between inhibition of transglutaminase activity and inhibition of clustering and internalisation argued that the effects of the compounds used were a result of inhibition of transglutaminase activity.

A major contradiction of the observations above emerged when it was reported by King et al., (1980) that the internalisation of ^{125}I-labelled epidermal growth factor was not inhibited by alkylamines. These studies suggested that the degradation of internalised epidermal growth factor was inhibited by alkylamines and that the increased association of ^{125}I-epidermal growth factor with treated cells was a result of this effect and not the result of inhibition of internalisation as suggested by Haigler et al., (1980). Furthermore, it was suggested that clusters of rhodamine-labelled epidermal growth factor were visible in the presence of alkylamines and that the procedures used by Maxfield et al., (1979a) were insufficiently sensitive. In accordance with the observations of King et al., Yarden et al., (1981) showed that primary amines did not inhibit the endocytosis of fluorescent and ^{125}I-labelled epidermal growth factor or fluorescent α_2-macroglobulin. Similarly, the internalisation of ^{125}I-chorionic gonadotropin by ovine luteal cells was not inhibited by a range of primary amines, but its degradation was inhibited (Ahmed and Niswender, 1981). The common picture that did emerge from these and other studies (Ascoli & Puett,

1978; McKanna et al., 1979) was that primary amines inhibited degradation of internalised ligand, probably by virtue of their ability to inhibit lysosomal function (Ohkuma & Poole, 1978; Poole & Ohkuma, 1981).

Primary amines were found to inhibit endocytosis in a number of other systems including β-adrenergic receptor endocytosis by frog erythrocytes (Chaung, 1981), receptor-mediated uptake of 3,3',5-triiodo-L-thyronine by fibroblasts (Cheng, et al., 1980), endocytosis of Pseudomonas toxin (Fitzgerald et al., 1980) and endocytosis of Shigella toxin (Keusch 1981). In the case of endocytosis by frog erythrocytes, a correlation between inhibition of endocytosis and inhibition of transglutaminase activity was observed.

The effects of amines on the endocytosis of a_2-macroglobulin were studied in further detail. Van Leuven et al., (1980) found that inhibition of endocytosis of α_2-macroglobulin by methylamine and dansylcadaverine could be dissociated from any lysosomotropic effects of these compounds and it was suggested that the decreased rate of uptake observed in the presence of these compounds was the result of inhibition of receptor recycling which may be dependent upon transglutaminase activity. This idea was consistent with the work of Kaplan & Keogh (1981) who determined that whilst one round of internalisation of α_2-macroglobulin could take place in the presence of primary amines (c.f. Yarden et al., 1981) subsequent rounds of internalisation were prevented. This was thought to be due to the depletion of receptors for α_2-macroclobulin which could not return to the cell surface in conditions under which

transglutaminase was inhibited.

By monitoring the binding and internalisation of ^{125}I-α_2-macroglobulin with an assay which distinguished between surface-bound and internalised ligand, Dickson et al., (1981) demonstrated the presence of two classes of receptor of high and low affinity. Bacitracin, monodansylcadaverine and a p-nitrophenyl ester were found to inhibit the binding of ligand to the high affinity class and to inhibit internalisation of α_2-macroglobulin. The authors suggested that transglutaminase activity was involved in the accumulation of low affinity receptors in coated pit regions where they acquired a high affinity.

It is clear that the involvement of transglutaminase in receptor-mediated endocytosis still requires some elucidation. From the evidence above, it seems that the process of clustering and internalisation per se may not require transglutaminase but that the enzyme may be involved in subsequent intracellular steps. The possibility exists that the enzyme may only be involved in the endocytosis of certain receptor-bound ligands. It has been established that some receptors including those for α_2-macroglobulin are recycled whereas others such as those for epidermal growth factor are not reutilised (Herzog, 1981). Thus the involvement of transglutaminase in endocytosis may be restricted to those systems where recycling of receptors occurs. This resolves the conflict which was apparent in the case of epidermal growth factor, the endocytosis of which may not involve transglutaminase. However, there may still be some disagreement over the inhibition of internalisation of α_2-macroglobulin and the point at which transglutaminase may be involved in this process.

1.4.3. Tumour growth.

The first suggestion of a role for transglutaminase in the growth of tumours came from Yancey and Laki (1972). These workers observed that the establishment of YPC-1 tumour in mice was inhibited by compounds which inhibited fibrin clot stabilisation with a consequent increase in host survival time. It was postulated that transglutaminase activity in tumours was required for their establishment. This idea was re-stated by the same workers when they correlated growth and metastasis of tumours with their transglutaminase content and with the transglutaminase content of recipient organs for metastases (Laki et al., 1977). Furthermore, an inverse correlation was found between mean survival time of mice and the transglutaminase content of transplanted tumours. The suggestion was made that transglutaminase interacted with substrate proteins on the cell surface such as fibrin and fibronectin in a way which favoured the growth of the tumour.

The conflicting view that transglutaminase activity is required to maintain a tissue in a non-proliferating state was suggested by studies of other workers. Birckbichler et al., (1976) demonstrated that Novikoff hepatoma and primary hepatoma induced by 3'-methyl-4-dimethylaminoazobenzene possessed a lower activity of transglutaminase than liver and that the subcellular distribution of the activity changed to become more particulate in the tumours. Similarly, the same workers demonstrated that a number of transformed cell lines possessed lower transglutaminase activity when compared to their normal counterparts (Birckbichler

et al., (1977a). The content of ε-(γ-glutamyl)lysine cross-links in virus transformed cells was also lower than in non-transformed cells, indicating a role for transglutaminase-catalysed protein crosslinking in the maintenance of the non-proliferating state (Birckbichler et al., 1978). From these observations it was suggested that cell membrane architecture was important to the growth of tumours and that transglutaminase may regulate this (Birckbichler & Patterson, 1978; 1980).

In later studies, it was further demonstrated that cultured L-cells grown in the presence of monodansylcadaverine or cystamine contained fewer ε(γ-glutamyl)lysine cross-links than untreated cells and that this correlated with an increased membrane fluidity (Haugland et al., 1982), emphasising the possible role of transglutaminase-catalysed formation of ε(γ-glutamyl)lysine in the stabilisation of cell membranes. The possible role of transglutaminase as a primary regulator of proliferation was also suggested when an increase in markers of proliferation was observed when transglutaminase was inhibited in cells (Birckbichler et al., 1981). More recently, evidence in support of that of Birkbichler and co-workers was obtained by Vanella et al., (1983) who observed a reduction in transglutaminase activity in Yoshida Ascites tumour cells and in Ascites tumour-bearing liver compared to normal liver.

1.4.4. Activation of lymphocytes and macrophages.

The involvement of transglutaminase in lymphocyte activation was suggested when it was found that transglutaminase activity was enhanced in human peripheral lymphocytes treated with concanavalin A or phytohaemagglutinin (Novogrodsky et al., 1978). The increase in enzyme activity occurred within 10-30 min of the exposure of the cells to the phytomitogens and was dependent on their binding to the cell surface. In later studies by Julian et al., (1983) and Günzler et al (1982) it was shown that the stimulation of B-lymphocytes by antigen (lectin or zymosan) to produce antibody-secreting cells was inhibited by primary amine inhibitors of transglutaminase activity. In the studies by Julian et al., it was found that the potency of inhibition of activation of lymphocytes by these compounds correlated with their potency as inhibitors of transglutaminase activity. These results therefore suggest that transglutaminase may be essential in the mechanism of B-cell activation.

A variety of macrophages were tested for their content of transglutaminase activity by fluorescence staining with monodansylcadaverine and by assay by [^{14}C]-putrescine incorporation into α-casein (Schroff et al., 1981). It was found that expression of transglutaminase activity depended on both the differentiation state and the type of cell studied and it was suggested that transglutaminase was a marker for macrophages in a particular state of activation or differentiation.

The activation of transglutaminase in mouse peritoneal

macrophages treated with soluble immune complexes or haemolysin-sensitised erythrocytes was detected by Fesüs et al., (1981). In the same study it was demonstrated that incorporation of [^{14}C]-methylamine into intact cells accompanied activation of macrophages and that methylamine could inhibit binding of immune complex and concomitant lateral reordering of membrane lipids. Similarly, it was found that inflammatory macrophages possessed 30-100 fold greater activity of transglutaminase than resident macrophages (Leu, et al., 1982). With reference to the studies on receptor-mediated endocytosis described previously (Section 1.4.2.), Leu et al. suggested a role for transglutaminase in Fc-mediated phagocytosis which was inhibited by inhibitors of transglutaminase activity. This suggestion was also consistent with the evidence obtained by Fesüs et al., (1981). In a further investigation of the activation of inflammatory macrophages, Murtaugh et al., (1983) demonstrated that transglutaminase activation was the result of accelerated synthesis of the enzyme which occurs within 90 min of stimulation of cells by a heat-labile serum component.

In conclusion, the evidence above is consistent with a role for transglutaminase in the activation of lymphocytes and macrophages. In macrophages the enzyme appears to be under transcriptional control and may be rapidly induced. Activation of the enzyme in lymphocytes may occur by means of an increase in the cytosolic concentration of Ca^{2+} ions which occurs when lymphocytes are stimulated (Whitney & Sutherland, 1972; Tsien et al., 1982). The point of involvement of transglutaminase in the activation process is unknown but may bear a relation to increased endocytotic activity or endomembrane flow in stimulated

cells. Alternatively, the enzyme may participate generally in the regulation of the growth or proliferation of cells, possibly by transduction of signals at the cell membrane.

1.4.5. Erythrocyte dysfunction.

The introduction of Ca^{2+} into erythrocytes by Ca^{2+}-transporting ionophores leads to a loss in deformability concomitant with changes in membrane polypeptides which include the cross-linking of proteins. The transglutaminase enzyme found in erythrocytes which is a typical tissue transglutaminase (Brenner & Wold, 1978) was implicated in the cross-linking of erythrocyte membrane proteins in Ca^{2+}-loaded erythrocytes when it was found that primary amines could inhibit the process (Lorand et al., 1976; Anderson et al., 1977). It was also shown that primary amines were specifically incorporated into membrane proteins of the erythrocyte (Lorand et al., 1975). Further evidence for the role of transglutaminase in the cross-linking of erythrocyte membrane proteins was obtained when the high molecular weight polymers found in Ca^{2+}-loaded erythrocytes were found to be rich in $\varepsilon(\gamma$-glutamyl)lysine dipeptide (Lorand et al., 1978; Seifring et al., 1978). Analysis of the cross-linked polymer produced indicated that it was comprised of a number of proteins including spectrin, band 2.1 and band 3 proteins (Bjerrum et al., 1981).

Although the Ca^{2+}-loaded erythrocyte represents an artificial model system, these results are of significance as a possible explanation for some of the membrane changes occurring in aged erythrocytes and in the irreversibly sickled erythrocyte in sickle-cell anaemia. The failure of the outwardly-directed Ca^{2+}-pump in aged erythrocytes may lead to stiffening of the aged red cell membrane as a result of transglutaminase-catalysed cross-linking of membrane and cytoskeletal proteins. The shape changes in

erythrocytes which are induced by the polymerisation of abnormal haemoglobin in the sickled red cell may have a component resulting from uncontrolled Ca^{2+} influx. Indeed, unpublished data suggest that Ca^{2+}-dependent proteolysis and transglutaminase may act in concert in sickled cells causing alterations in proteins in the erythrocyte membrane (Dr. M. Griffin, personal communication).

The specificity of erythrocyte transglutaminase for cross-linking of membrane proteins may also be significant in indicating the specificity of tissue transglutaminases in general, particularly as non-erythroid cells contain very similar membrane proteins (such as spectrin, actin and Band 4.1.) to those in erythrocytes (Cohen, et al., 1982).

1.4.6. Lens cataract formation.

The observation that human lens cataracts contained protein polymers which could not be dissociated into smaller subunits by treatment with urea and SDS led Lorand and co-workers to investigate the involvement of transglutaminase in the formation of these polymers (Lorand et al., 1981; Hsu et al., 1981). A transglutaminase enzyme similar to that found in liver was found in lens tissue of a variety of species, the highest activity being in rabbit lens cortex. Protein substrates of the enzyme in lens were identified as β_1 and β_2 crystallins by specific incorporation of [^{14}C]-putrescine and fluorescent dansylcadaverine. It was furthermore found that $\varepsilon(\gamma$ -glutamyl)lysine cross-links were present in the nuclei of human cataracts.

The β-crystallin substrates for lens transglutaminase were investigated in more detail by Berbers and co-workers (Berbers et al., 1983). These workers were able to demonstrate the selective incorporation by endogenous transglutaminase of calf lens cortex of [^{14}C]-putrescine and dansylcadaverine into three different β-crystallin chains. The precise site of attachment of amines incorporated into the predominant β-crystallin, βB_p, was identified as a single glutamine residue (position no. 7) in the N-terminal domain of βB_p.

The cross-linking by transglutaminase of specific lens proteins may therefore play a role in the stabilisation of this tissue. However, uncontrolled cross-linking (such as may occur in the aged or Ca^{2+}-loaded erythrocyte, Section 1.4.5.) may contribute to cataract formation in the senile lens.

1.4.7. The interaction of polyamines with transglutaminase.

The naturally occurring polyamines putrescine, spermidine and spermine are ubiquitous in animal cells and possibly also in plants and bacteria. A vast amount of literature has been published in which the polyamines were implicated in a number of cellular processes, and in particular those associated with cell proliferation (for reviews see Goyns, 1982; Heby, 1981; Pegg & McCann, 1982; Tabor & Tabor, 1976).

The structures of putrescine, spermidine and spermine are illustrated in Figure 1.3. which depicts the biosynthetic pathway of the polyamines in animal cells. It is important to appreciate the biosynthetic interrelation of these compounds, for their levels are regulated in cells in response to certain stimuli and inhibitors of polyamine biosynthesis are often used as probes for the study of the cellular function of these compounds. The inhibitor of polyamine biosynthesis whose use is most favoured is α-difluoro-methylornithine (DFMO) (Metcalf et al., 1978) an enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC) and the rate-limiting enzyme of the pathway for polyamine biosynthesis. The use of DFMO as an inhibitor of polyamine biosynthesis is now favoured as a result of its proven specificity for ODC as a cellular target (Pösö et al., 1983).

Since polyamines possess primary amine groups, they may act as substrates for transglutaminase. Indeed, the ability of putrescine, spermidine and spermine to become incorporated into proteins via transglutaminase-catalysed reactions has been demonstrated amply in extracellular and intracellular systems

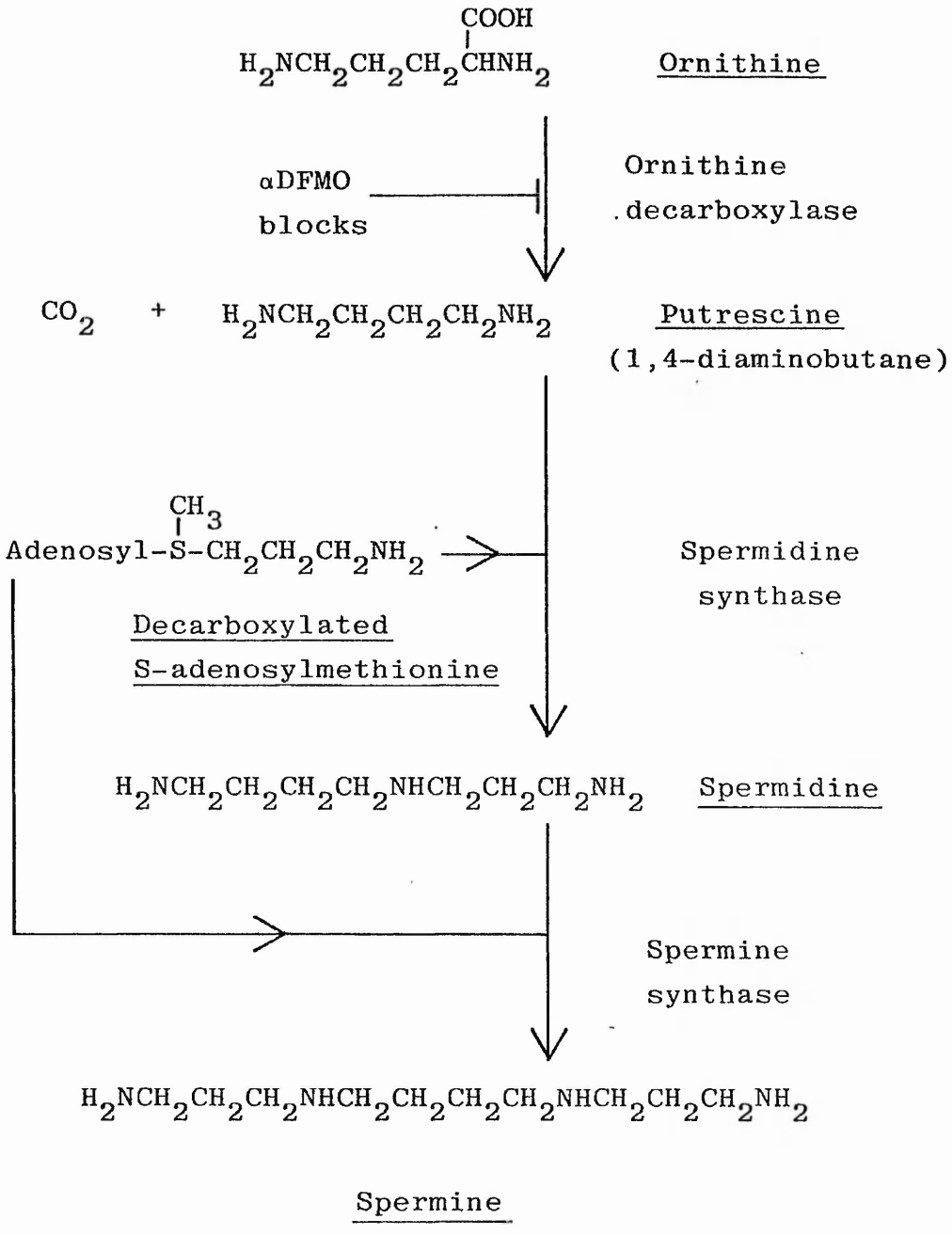
(Williams-Ashman & Cannelakis, 1980 for review). The incorporation of a polyamine into a protein through one of its primary amine groups may be followed by a similar reaction of the other amine group on the same molecule. Thus, both amine groups of the polyamine may become bound to γ-(glutamyl) residues of two different polypeptides forming a bis-(γ-glutamyl)polyamine cross-link, or diamine cross-link. These reactions which are illustrated in Fig. 1.1. have been demonstrated to occur in some systems (Folk et al., 1980; Williams-Ashman et al., 1980) and were briefly discussed in a previous section (1.3.2.). The formation of bis-(γ-glutamyl)polyamine cross-links may thus represent an alternative or additional means of transglutaminase-catalysed protein cross-linking.

The ubiquity of transglutaminase and polyamines in tissues suggests that they may interact with each other in cells and that their functions may be inter-dependent. Some workers have investigated the possible interaction of transglutaminase with polyamines in intact cells, and it was demonstrated that putrescine, spermidine and spermine can be incorporated into proteins of HTC cells (Cannelakis et al., 1981), WI-38 cells (Patterson et al., 1982) and activated lymphocytes (Folk et al., 1980).

It is interesting that a rise in the intracellular levels of polyamines (Fillingame & Morris, 1973) and in the activity of their biosynthetic enzymes (Bachrach et al., 1981) occurs during lymphocyte activation. As was discussed in an earlier section (1.4.3.), transglutaminase activity also rises during lymphocyte activation. Folk et al., (1980) demonstrated that incorporation of exogenously applied radiolabelled putrescine accompanied activation of

lymphocytes and that radiolabel was recovered not only in N-(γ-glutamyl)putrescine but also in N^1-(γ-glutamyl)spermidine and N^8-(γ-glutamyl)spermine following exhaustive proteolytic digestion of cellular proteins. These observations suggest a possible role for transglutaminase catalysed incorporation of polyamines into proteins during activation of lymphocytes.

Figure 1.3. Biosynthesis of the polyamines putrescine, spermidine and spermine



1.4.8. Conclusion

Most of the evidence described above some of which is controversial, is as yet inconclusive, in so far as a general cellular role for tissue transglutaminase has not been defined or proven. However, a common factor that emerges between most of the evidence is the suggestion of a role for transglutaminase in regulating functions associated with the cell membrane. A few rather isolated reports concerning the substrate specificity of transglutaminase also favour this idea. Thus, transglutaminase has been shown to cross-link the intracellular portion of HL-A and -B antigens (Pober & Strominger, 1981), membrane proteins of mouse erythrocytes, rabbit sarcoplasmic reticulum (Dutton & Singer, 1975), human β_2-microglobulin (Fesüs et al., 1981) and membrane proteins of rabbit liver (Linnoila et al., 1978).

It is also an intriguing possibility that transglutaminase regulates polyamine function or vice versa and that polyamine incorporation by transglutaminase may be important in the regulation of protein function by post-translational covalent modification.

1.5. The aims of the thesis: the study of model systems.

Most of the evidence for a role for the tissue transglutaminase in cells points to the involvement of the enzyme in cell membrane-mediated phenomena. It was logical to pursue this line of investigation in the present thesis in which two model systems were studied:

i. tumour growth;

ii. the exocytotic mechanism of insulin release from the pancreatic β-cell.

The potential importance of transglutaminase in tumour growth was discussed above and does not bear repetition here. The study of the involvement of transglutaminase in the mechanism of secretion is, however, a new proposal and the important relevant features of the mechanism of insulin release are discussed below.

1.5.1. The Mechanism of Glucose-Stimulated Insulin Release.

The peptide hormone insulin is important in the maintenance of glucose homeostasis in an organism and may be said to represent the hormone of fuel storage. The pancreatic β-cell releases insulin in response to challenges of glucose and other metabolic fuels and is acutely sensitive to changes in the medium glucose concentrations in the physiological range (5-10mM). Therefore, the function of the pancreatic β-cell is to regulate the synthesis, storage and release of insulin in order to maintain glucose homeostasis.

The cellular pathway along which secretion of insulin proceeds is well established (Howell et al., 1969; Orci et al., 1971) and is the same as the pathway determined for exocrine pancreas in the classic studies of Jamieson and Palade (Jamieson & Palade, 1967a; 1967b). Thus, synthesis of insulin on the rough-endoplasmic reticulum is followed by transfer to the Golgi complex where the hormone is processed and packaged into secretory vesicles (Steiner et al., 1974). Release of insulin finally proceeds by exocytosis involving the fusion of the secretory vesicle with the plasma membrane (Orci et al., 1973). This system of endomembrane flow may be regulated by microtubules and microfilaments involved in the guidance and translocation of vesicles to their appropriate destinations (Malaisse-Lagae et al., 1979; Boyd III et al., 1982; Boyd III, 1982; Howell & Tyhurst, 1982). However, it is not known how the precise interaction of membrane-bound vesicles is achieved by the cell. Furthermore the mechanism by which secretagogues such as glucose are able to mobilise the secretory apparatus is to a large

extent obscure.

The mechanism of glucose-stimulated insulin release has attracted much attention from researchers particularly since the introduction of methods for the isolation of intact, functional islets of Langerhans (for recent review see Hedeskov, 1980). Although islets are not a pure β-cell preparation, they are very amenable to experimental manipulation and have provided much information as to the mechanism of stimulus-secretion coupling. Other tissues particularly useful for biochemical studies where large quantities of material are required are islet β-cell tumours (Masiello et al., 1982; Sopwith et al., 1981).

It is now well established that the metabolism of glucose by the β-cell is required for glucose to elicit the secretory response (Ashcroft, 1980). The glucose-sensor is therefore a metabolic signal and this is the basis of the action of a number of other secretagogues which can act as metabolic fuels ("the Fuel Hypothesis" Malaisse et al., 1979).

It is also well established that, in common with many other systems of stimulus-response coupling, a rise in the cytosolic concentration of Ca^{2+} ions mediates the response of the pancreatic β-cell to glucose (Wollheim & Sharp 1981). The factors coupling glucose metabolism to Ca^{2+} mobilisation have been suggested to be levels of reduced pyridine nucleotides (Panten et al., 1973; Sener et al., 1984), phosphoenolpyruvate (Sugden & Ashcroft, 1977) and intracellular pH (Smith & Pace, 1983; Lindstrom & Sehlin, 1984). However, the identity of such coupling factors remains to be confirmed and their mode of action elucidated.

The prime function of Ca^{2+} in the β-cell, as in so many other

systems therefore appears to be as a second messenger. How the rise in cytosolic Ca^{2+} elicits the mobilisation of the secretory apparatus, however, is unknown. Attempts to identify possible targets for Ca^{2+} in the pancreatic β-cell have recently centered on the calcium-dependent regulator protein, calmodulin. Recent evidence suggests that calmodulin present in the islets of Langerhans (Sugden et al., 1979) and in β-cell tumours (Schubart et al., 1980; Hutton et al., 1981) may be essential for the mechanism of glucose-stimulated insulin release (Gagliardino et al., 1980; Henquin, 1981; Niki et al., 1982). Of particular interest is the action of calmodulin in stimulating a protein kinase which can phosphorylate specific islet cell proteins (Gagliardino et al., 1980; Harrison & Ashcroft, 1982; Colca et al., 1983). Some of these studies have suggested that the microtubule proteins a- and b-tubulin may be major substrates for Ca^{2+}/calmodulin-dependent phosphorylation in the β-cell (Colca et al., 1983). Apart from this, no specific proteins have been identified as possible targets for phosphorylation reactions involved in the stimulation of insulin release.

In addition to Ca^{2+}, cyclic 3', 5' adenosine monophosphate (cAMP) plays a role as a positive modulator of insulin release (Montague & Howell, 1976; Sharp, 1979; Hedeskov, 1980; Wollheim & Sharp, 1981). However, the involvement of cAMP is not thought to be essential for stimulus-secretion coupling in the pancreatic β-cell. A role for cAMP in the β-cell may lie in the regulation of protein phosphorylation catalysed by cAMP-dependent protein kinases found in rat islets (Christie & Ashcroft, 1984).

Evidence has accumulated that, as in many other systems of stimulus-response coupling, an enhanced turnover of phospholipids

(phosphatidylinositol, phosphatidylinositol-4-phosphate, phosphatidylinositol-4,5-bisphosphate) occurs in islets in response to secretagogues and other stimuli (Best & Malaisse, 1983; Axen et al., 1983; Clements et al., 1981). Although much of the evidence regarding the function of this effect appears contradictory, the metabolism of phosphatidylinositol (PI) may play a role in the regulation of Ca^{2+} mobilisation. In addition, one of the products of PI metabolism, diacylglycerol, is thought to regulate the activity of a Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) (Nishizuki & Takai, 1981). Phosphorylation reactions mediated by phospholipid metabolism may play a role in the regulation of insulin release (Brocklehurst & Hutton, 1984; Zawalich et al., 1983), particularly in view of the ability of the diacylglycerol analogue, 12-O-tetradecanoylphorbol-13-acetate (TPA) to stimulate insulin release (Malaisse et al., 1980; Zawalich et al., 1983).

The importance of post-translational modification of proteins by phosphorylation reactions in the mechanism of insulin release is further suggested by the observed stimulation of protein phosphorylation observed in islets stimulated by glucose (Suzuki et al., Colca et al., 1984) and by glucagon and cAMP (Suzuki et al., 1983). Since the site of action of these reactions may include secretory granules (Brocklehurst & Hutton, 1983; 1984) and microtubule proteins (Colca et al., 1983), protein phosphorylation mediated by Ca^{2+}/calmodulin, cAMP and phospholipid metabolism may regulate the interaction of components of the secretory apparatus.

1.5.2. The mechanism of insulin release from the pancreatic β-cell as a model system for the study of the cellular role of transglutaminase.

Exocytotic insulin release from the pancreatic β-cell is a Ca^{2+}-dependent and cell membrane-mediated process, and the mechanism whereby Ca^{2+} triggers endomembrane flow in this system is not known. It is therefore tempting to postulate a role for transglutaminase as a target for Ca^{2+} in this system because the enzyme is regulated by Ca^{2+} and because evidence favours a role for the enzyme in cell membrane-mediated processes. Furthermore, the observed specificity of transglutaminase for post-translational modification of proteins through cross-linking reactions can be envisaged as being a possible mechanism for regulating the interaction of cellular membranes during the secretory process. In conjunction with this, there are certain methodological advantages in studying this system as a model secretory system:

i. Problems of interferences by drugs with receptor binding do not exist in pharmacological studies of glucose-stimulated insulin release, since the glucoreceptor is a metabolic signal;

ii. Stimulus-secretion coupling is easily measured in studies using intact islets of Langerhans;

iii. Islets of Langerhans consist mainly of one cell type, the β-cell.

Therefore, the exocytotic mechanism of insulin release from the pancreatic β-cell represents a system which is amenable to the study of the cellular role of transglutaminase.

2. MATERIALS AND METHODS.

2.1. MATERIALS.

All of the work pertaining to the studies on islets of Langerhans and on the passaged tumour lines MC3 and CC5 was carried out in the laboratories of the Department of Life Sciences, Trent Polytechnic. Most of the work on hepatocellular carcinomata induced by diethylnitrosamine and 6-p-dimethylaminophenylazobenzothiazole and all analysis of polyamines by HPLC was carried out in the Central Toxicology Laboratories of I.C.I. Ltd., Alderley Park, Macclesfield, Cheshire.

Chemicals used for buffers and physiological media were of Analar grade; other chemicals were of reagent grade. The following list identifies the sources of chemicals;

Primary amines: methylamine hydrochloride; ethylamine; propylamine and putrescine dihydrochloride: Sigma Chemical Co. Ltd., Missouri, U.S.A.

Monodansylcadaverine: Fluka, Buchs, Switzerland.

Tyramine, cystamine: Aldrich Chemical Co., Gillingham, Dorset.

Trizma base: Sigma Chemical Co. Ltd., Missouri, U.S.A.

Hanks Balanced Salt Solution: Gibco Bio-cult Diagnostics, Paisley, Scotland.

Hepes buffer (1M): Flow Laboratories, Irvine, Scotland.

Bovine Serum Albumin, essentially fatty acid free: Sigma Chemical Co. Ltd., Missouri, U.S.A.

Rat insulin standard; anti-insulin antibody: Novo, Bagsvaerd, Denmark.

Collagenase from Clostridium histolyticum: Serva Feinbiochimica, West Germany.

Human Albumin: Hoechst Pharmaceuticals, Hounslow, Middlesex.

Chemicals for electrophoresis: acrylamide; N,N'-methylenebisacrylamide; sodium dodecylsulphate; N,N,N',N'-tetramethylenediamine (TEMED): British Drug Houses, Poole, Dorset.

Protein molecular weight standards:

Sigma Chemical Co. Ltd., Missouri, U.S.A.

Dibutylphthalate; dinonylphthalate:

Aldrich Chemical Co., Gillingham, Dorset.

Standard DNA from calf thymus: Boehringer, Lewes, Sussex.

Radiochemicals were obtained from Amersham International, Amersham, Bucks., except for ^{125}I-labelled insulin which was obtained from Novo, Bagsvaerd, Denmark. The stock supplies of radiochemicals were as follows:

i. [1,4(n)-^{14}C] putrescine dihydrochloride	109-113 Ci/mol
ii. [^{14}C] methylamine hyrochloride	56 Ci/mol
iii. D- [U-^{14}C] glucose	279 Ci/mol
iv. n-[1-^{14}C] hexandecane	30-60 µCi/mol
v. [1,4(n)-^{3}H] putrescine dihyrochloride	19 Ci/mmol
vi. L-[4,5-^{3}H] leucine	55-60 Ci/mmol
vii. [6,6'(n)-^{3}H] sucrose	9.8 Ci/mmol
viii. n-[1,2(n)-^{3}H] hexadecane	167µCi/mol
ix. ^{125}I-insulin	20-30 mCi/mg

Plastic vials and inserts for liquid scintillation counting were obtained from Sarstedt, West Germany; microcentrifuge tubes were obtained from Beckman, Irvine, California, U.S.A.

Measurement of pH was carried out using a Pye-Unicam PW9409 pH meter (Pye Unicam, Canbridge, U.K.).

Absorbance measurements were made in a Pye-Unicam SP-400 ultra-violet, variable wavelength spectrophotometer.

Fluorescence measurements were made with a Spectra-Physics Fluorimeter (Spectra-Physics, La Jolla, California, U.S.A.).

For sonication, a Soniprep 150 sonicater (M.S.E. Scientific Instruments, Crawley, Sussex) was used.

Centrifugation was carried out in the centrifuges listed below which were fitted with the following rotors and used at the relative centrifugal forces stated:

Centrifuge	Rotor	Relative centrifugal force (g av)
M.S.E. Superspeed 50	8x25 ml angle (r_{av}= 5.84 cm)	71,000
	8x25 ml angle fitted with 3 ml adaptors (r_{av}= 6.22 cm)	71,000
M.S.E. Major	24x30 ml swingout (r_{av}= 23 cm)	600
M.S.E. Centaur 1 (bench)	-	1,100
Beckman microfuge	-	8,700 (1.8 ml tubes)
(microcentrifuge)		9,390 (0.4 ml tubes)

2.2. ANIMALS.

For the supply of pancreatic tissue for isolation of Islets of Langerhans, Sprague-Dawley rats of both sexes weighing 200-300g were used.

For the passage of the tumour lines CC5 and MC3, AS rats of both sexes and weighing 200-300g were used.

For chemical carcinogenesis leading to the production of hepatocellularcarcinomata, male Alderley Park (AP) Wistar-derived rats were used throughout. The age of these rats are given in the dosing regimes detailed in the methodology for the tumour studies (section 2.7.1.2.).

2.3. CHEMICAL DETERMINATIONS.

2.3.1. Protein assay: method of Lowry.

The method used was a modification of the method of Lowry, et al (1951), and used SDS at a final concentration of 0.8% (w/v) as a means of making otherwise insoluble protein available for the colour reaction.

Stock solutions comprised of 4% (w/v) NaOH, 2.0% (w/v) Na_2CO_3 and 0.02% sodium tartrate (solution A) and 0.5% $CuSO_4 . 5H_2O$ (solution B) were prepared in distilled water. To the sample of protein in 100µl was added 100µl of 10% (w/v) SDS. The stock solutions were then mixed in the ratio 12.25 A : 0.25 B and 1.0 ml of this solution added to the protein samples in SDS. The samples were vortexed and after 20 min at room temperature, 0.1 ml of Folin-Ciocalteau reagent (freshly diluted 1:1 with distilled water) was added. The samples were vortexed and left a further 20 min before the measurement of absorbance at 750 nm. Standards of bovine serum albumin in the range 50 - 1000µg/ml were treated similarly.

2.3.2. Measurement of DNA: method of Burton.

Samples for DNA assay by the method of Burton (1956) were prepared by addition of 2.6ml ice-cold 10% (w/v) TCA to 0.4 ml of the test sample of homogenate. The resulting precipitate was pelleted by centrifugation at 2000g for 10 min in a bench centrifuge, the pellet resuspended in ice-cold 10% TCA and re-centrifuged. The pellet was then washed twice in 5 ml ice-cold 95% (w/v) ethanol by centrifugation and resuspension and thoroughly drained. Hydrolysis of the DNA in the pellet was then carried out in 3 ml 5% (w/v) TCA at 90°C for 10 min. After centrifugation of the lysate, a suitable sample of the supernatant was diluted to 1.0 ml with 5% (w/v) TCA. To the sample was added 2 ml of fresh diphenylamine reagent prepared by dissolving 1.5g of diphenylamine

in 100ml of glacial acetic acid (Analar) with the addition of 1.5ml concentrated sulphuric acid and 0.5ml of 16mg/ml acetaldehyde (diluted in distilled water). The samples were vortexed and left overnight at room temperature before the measurement of absorbance at 600nm. Standard DNA was prepared in 5% (w/v) TCA and used to construct a standard curve ranging from 10-100 µg DNA per ml.

2.3.3. Determination of radioactivity.

The radioisotopes ^{14}C and ^{3}H were determined by liquid scintillation counting mainly in a Packard Tri-Carb 460C liquid scintillation spectrometer. A small proportion of samples were also counted in a Packard Tri-Carb 300 liquid scintillation spectrometer. For the determination of ^{125}I, samples were counted directly in plastic stoppered tubes in a Packard Autogamma 500 gamma counter. For liquid scintillation counting, however, the following sample preparation procedures were required, depending on the type of sample.

2.3.3.1. Filter papers.

Samples deposited on Whatman 3MM filter paper were counted in scintillation cocktail O (B.D.H.) or in Fisofluor 2 (Fisons), cocktails suitable for dry heterogeneous samples. Squares of filter paper ($1cm^2$) prepared as described in the transglutaminase assay method were placed in 5ml polythene scintillation vial inserts and counted in 2ml of scintillation cocktail. Filter paper discs of 21 mm diameter prepared as described in the experiments on amine incorporation into islet protein were placed in scintillation vials and counted in 10ml of scintillation cocktail.

2.3.3.2. Solid tissue and protein samples.

Tissue slices and proteinaceous precipitates required solubilisation before an accurate determination of radioisotope could be performed for a homogeneous sample.

For tissue slices and precipitates prepared from tissue slices, 1.0 ml Soluene 350 (Packard) was used to dissolve the sample. Protein precipitates prepared from islets of Langerhans were dissolved in 0.1ml Soluene 350. Samples of precipitate were re-hydrated with a minimum of water before addition of the solubiliser. All samples were left overnight at room temperature in solubiliser before the addition of Dimilume 30 (Packard) to 10 times the volume of solubiliser. In the case of samples contained in 1.8ml microcentrifuge tubes, solubilisation and counting were performed in these same tubes.

2.3.3.3. Aqueous samples.

Samples consisting of solutions containing radioisotopes were counted in 10ml Instagel (Packard). In order to eliminate the contribution of chemiluminescence to the counts obtained, the samples were either acidified before counting or left at $4^{o}C$ overnight and counted.

2.3.3.4. Polyacrylamide gels.

Polyacrylamide gels prepared as described in the methodology for electrophoresis were carefully cut into 2mm sections along the tracks of electrophoresed protein with a razor blade. The sections were placed in scintillation vials and dried at $60^{o}C$ in a vacuum oven. The sections were then dissolved in 0.5ml of a solution of hydrogen peroxide (30 vols)/ammonia solution (specific gravity 0.88) 19:1(v/v) by incubation at $40^{o}C$ for 2-3 h. Each sample was then counted in 10ml Instagel after the addition of 0.1ml glacial acetic acid.

2.3.3.5. Determination of counting efficiency.

For dried filter paper samples,"total counts"samples (10µl) were taken from vials containing a known amount of radioactivity, air dried on filter papers and counted. For ^{14}C, the counting efficiency was regularly found to be 75-80%.

The Quench Indication Parameter of Packard Inst. was used for most other determinations of counting efficiency. A set of vials containing the same known amount of radioactivity but with varying amounts of a quenching agent were prepared and counted in the Packard 460C liquid scintillation counter according to the manufacturers' instructions. The quenching agent used was chloroform, except in the studies on uptake of radiolabelled compounds where a mixture of dibutylphthalate and dinonylphthalate (10:3) was used. The data obtained from the quench standards was then used by the counter to calculate the counting efficiency of each unknown sample.

Samples containing ^{14}C in Instagel were not very susceptible to quenching by chloroform, so the method of internal standardisation was used to calculate the counting efficiency of these samples and was found to be regularly 80%.

2.4. FRACTIONATION PROCEDURES.

2.4.1. Homogenisation.

2.4.1.1. Potter-Elvejhem.

Excised tissue was weighed and washed in ice-cold sucrose medium consisting of 0.25M sucrose, 1mM Tris, 1mM EDTA, pH 7.4. The tissue was then chopped and homogenised in 3 volumes of this medium with 6 passes of a glass/teflon Potter-Elvejhem homogeniser (clearance 0.61 mm) mechanically driven at 1700 r.p.m.. Tissues were kept on ice before, during and after homogenisation. If not required within 24 h, homogenates were stored frozen at -20°C.

2.4.1.2. Ultraturrax.

The mincing of tissue slices was achieved by use of a variable speed ultraturrax (Janke and Kundel, KG).

2.4.1.3. Ultrasonication.

This method of disrupting cells was found to be suitable for homogenising islets of Langerhans.

Islets of Langerhans isolated as described elsewhere (Section 2.6.2.) were placed in 1.5 ml plastic vials and excess isolation medium removed with a finely drawn pasteur pipette. The islets were then washed 5 times with ice-cold sucrose medium (0.25M sucrose, 1mM Tris-Cl, 1mM EDTA, pH 7.4) to remove all traces of HBSS. To the islets was then added between 100 and 300 µl of ice-cold sucrose medium, depending on the number of islets isolated, so that the concentration of islets was 1-2 per microlitre sucrose medium. Islets were then homogenised in this buffer by sonication with two bursts of 5 s each at 3µ peak-to-peak.

The probe of the sonicator was previously cooled to 4°C and the islets kept on ice during sonication. Thereafter, the homogenate was stored either at 4°C if required on the same day or at -20°C if required on subsequent days. When desired, homogenates were pooled when large amounts of material were required.

2.4.2. Fractionation of tissues by differential centrifugation.

Following homogenisation of tissues, a cell fractionation scheme illustrated in Fig 2.1. was used in some cases which was based on the methods of De Duve et al., (1955). This procedure yielded a 600g 'nuclear pellet' (N) a 71,000g 'mitochondrial, lysosomal and microsomal' pellet (MLP) and a particle-free supernatant fraction (S). Most work was, however, performed with only a 71,000g pellet (P) and particle-free supernatant (S) obtained from homogenates centrifuged at 71,000g av. for 45 min. at 4°C (see also Fig. 2.1.). In this case, the pellet contained all cellular debris, cell membranes and organelles. The preparation of particle-free supernatant from homogenates of islets of Langerhans necessitated the use of 3ml adaptors for an 8 x 25ml rotor due to the small volume (up to 1.0 ml) of homogenates.

The pellets prepared by differential centrifugation were resuspended in 3 volumes sucrose medium/g tissue by the use of a hand-held Dounce homogeniser, or in the case of 71,000g pellets from islet homogenates, by ultrasonication in 50-100µl sucrose medium.

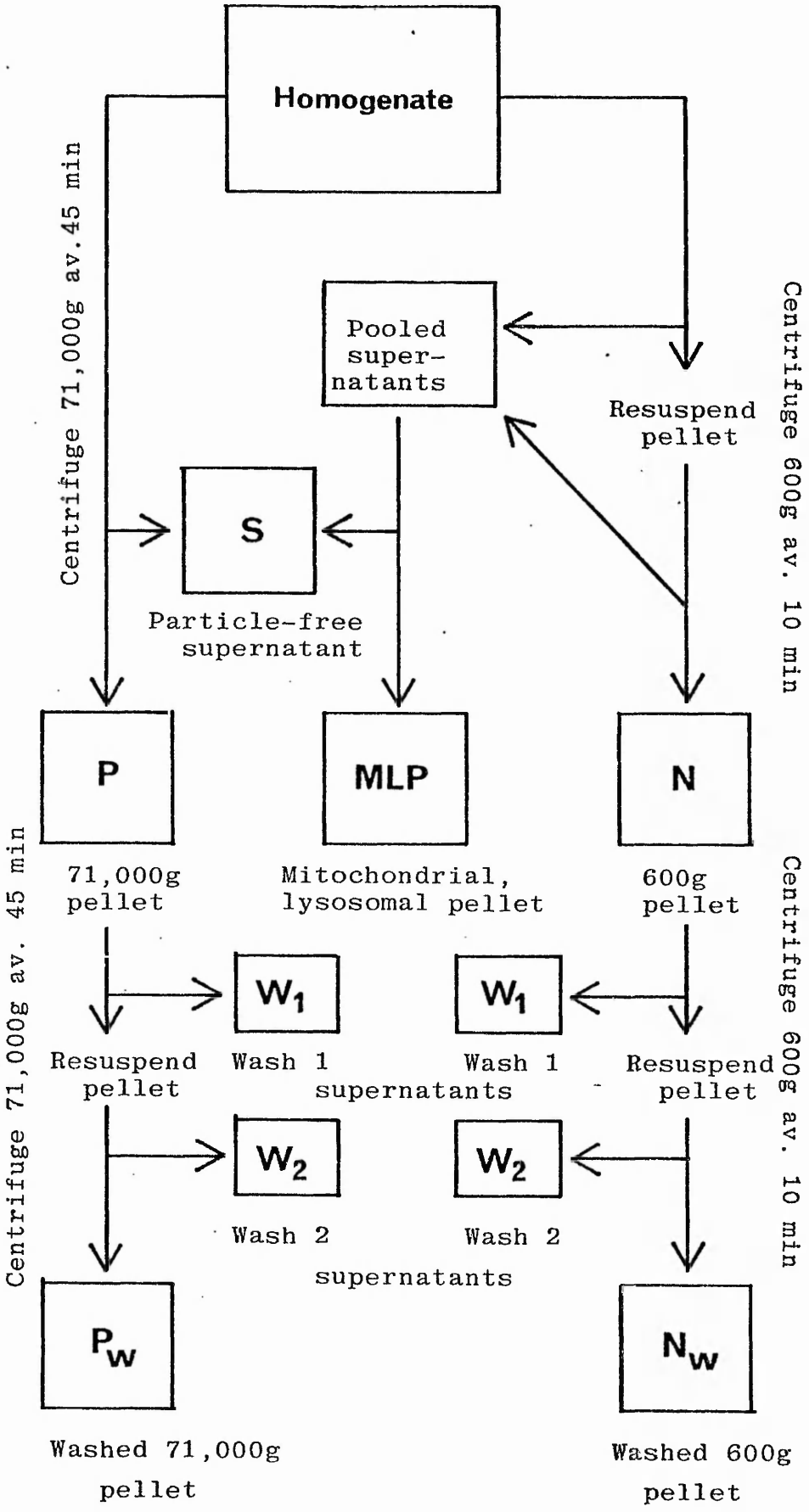
The procedure for studies on the leaching of transglutaminase activity from N and P pellet fractions is also illustrated in Fig. 2.1.. Following centrifugation, pellets were resuspended in the original volume of sucrose medium with a Dounce homogeniser.

All the procedures above were carried out at 4°C and all fractions stored on ice.

2.4.3. SDS-Polyacrylamide gel electrophoresis.

The method employed was a modification of that described by Laemmli (1979) for use with a vertical slab gel apparatus which is widely used for separation of proteins from tissue or cell extracts.

Figure 2.1. Fractionation Scheme



2.4.3.1. Solutions.

The solutions required were :

Tris-glycine electrode buffer (25mM Tris, 192mM glycine)	15.15 g Tris base 72.0 g glycine 5.0 g SDS in 5000ml pH 8.3
Tris-SDS stock solution,pH 6.8 (0.25M Tris)	3.03g Tris base 0.2 g SDS in 100ml pH 6.8
Tris-SDS stock solution,pH 8.8 (0.75M Tris)	9.08g Tris base 0.2 g SDS in 100ml pH 8.8
Sample buffer (62.5mM Tris)	25ml Tris-SDS stock, pH 6.8 2g SDS 10ml glycerol 5ml 2-mercaptoethanol 0.1 ml 1% bromophenol blue made up to 100ml
Acrylamide stock	30g acrylamide 0.8 g N'-N methylene-bisacrylamide in 100ml
Ammonium persulphate	0.1g in 10ml
N,N,N',N'-tetramethylethylenediamine (TEMED)	

2.4.3.2. Sample preparation.

Samples containing 50-100 µg of protein were dissolved in 50-100µl of SDS sample buffer (62.5mM Tris, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.01mg/ml bromophenol blue, pH 6.8). Prior to electrophoresis, samples were immersed in boiling water for 10 min to ensure that proteins were dissolved.

2.4.3.3. Procedure.

The gel mould apparatus for vertical slab gel electrophoresis (L.K.B., Bromma, Sweden) was assembled according to the manufacturer's instructions after the glass plates were thoroughly cleaned with detergent, followed by distilled water and filtered ethanol. The apparatus was set up to produce 1mm thick gels.

The resolving gel was prepared first by mixing 19.8 ml of acrylamide stock solution, 30 ml Tris-SDS stock solution pH 8.8 and 10.2ml distilled water in a clean Buchner flask. This solution was de-gassed for 30 min. Immediately before the gels were to be made, 1.5ml of freshly made 1% (w/v) ammonium persulphate and 15µl TEMED were added and this final gel solution mixed thoroughly without the introduction of air bubbles. Gels were then poured to a height of approx. 12 cm and overlayered with 2-butanol to exclude air and to create a flat top to the gel. Polymerisation was allowed to proceed for at least 2hr at room temperature. Before setting the stacking gel, the overlay of 2-butanol was removed and the surface of the gel washed with 50% (v/v) Tris-SDS stock, pH 8.8 and any excess buffer carefully absorbed with filter paper.

The stacking gel solution was prepared by the combination of 2 ml acrylamide stock, 10ml Tris-SDS stock solution, pH 6.8 and 8ml distilled water. Following de-gassing for 10-15 min and thorough mixing, 0.5ml of fresh ammonium persulphate and 7µl TEMED were added and the mixed solution poured on top of the resolving gel to within 2mm of the top of the gel mould which contained a comb for the formation of sample wells. Polymerisation was allowed to proceed for at least 1h at room temperature before sample addition.

The gels so prepared had the following compositions:

	resolving	stacking
acrylamide	10% (w/v)	3% (w/v)
N',N methylenebis-acrylamide	0.27% (w/v)	0.08% (w/v)
SDS	0.1% (w/v)	0.1% (w/v)
Tris	0.375M	0.125M

Electrode buffer was added to the sample wells and sample was then added to the bottom of the wells beneath the electrode buffer using a microlitre syringe. The rest of the electrophoresis apparatus was assembled according to the manufacturer's instructions and electrophoresis carried out at 16^{o}C and at a constant current of 20mA per gel for approximately 5h until the tracking dye was approximately 1cm from the edge of the gels. Standards of proteins of known molecular weights (Sigma) were also electrophoresed beside the samples on the same gel to allow calibration for determination of protein molecular weights. The protein standards used were β-galactosidase (92,000), bovine albumin (66,000), egg albumin (45,000), trypsinogen (24,000) and lysosyme (14300).

2.4.3.4. Fixing, staining and destaining of gels.

Following electrophoresis, the distance of the dye front from the top of the resolving gel was measured and the gel carefully removed from the glass mould. Gels were fixed for 30-60 min in a solution containing 10% (w/v) TCA and 3.4% (w/v) sulphosalicylic acid and stained for 2h or overnight in 1 litre of a stain containing 1.25g Page 83 (B.D.H.), 18% (v/v) methanol and 5% (v/v) glacial acetic acid. Stain was prepared freshly and not re-used.

Destaining of gels was achieved by their immersion in 500ml of a solution containing 18% (v/v) methanol and 5% (v/v) glacial acetic acid with several changes until background gel was free of colour. This process could be hastened by gentle heating and agitation of the solution.

If desired gels were preserved by drying in an L.K.B. gel drier or by immersion in a solution of 7% (v/v) glacial acetic acid containing 10% (v/v) glycerol.

2.5. TRANSGLUTAMINASE ASSAY.

Enzyme activity was measured by the method of [^{14}C]-putrescine incorporation into N,N'-dimethylcasein using the filter paper assay of Lorand et al., (1972).

The reaction mixture at 37°C and pH 7.4 contained the following:

Tris-Cl	28 mM
Dithiothreitol	3.85 mM
$CaCl_2$	2.5 mM
[^{14}C]-putrescine	1.2 mM (3.97 mCi/mmol)
N,N'-dimethylcasein	5 mg/ml

Control incubations were also performed in which EDTA (5 mM) replaced $CaCl_2$. For investigations into the Ca^{2+}-activation of transglutaminase, all solutions were prepared in glassware which had been washed in concentrated nitric acid followed by distilled and deionised water.

The assay components were in a final volume of either 100µl or 50µl after the addition of 45µl or 22.5µl respectively of enzyme sample. A final volume of 100µl was used when sufficient quantities of enzyme sample were available (i.e. with liver and tumour homogenates and samples of islet homogenates). When particle-free supernatant extracts of islets of Langerhans were used, however, the final volume was 50µl in order to conserve material.

The incubations were carried out in 1.0ml plastic microcentrifuge tubes in a water bath at 37°C. The reaction was started by the addition of enzyme samples; thereafter, samples (10µl) were taken at time intervals and deposited on pencil-labelled squares ($1cm^2$) of Whatman 3MM filter paper which were immediately placed in stirred ice-cold 10% (w/v) TCA for at least 10 min. The filter paper samples were then freed of unbound radioactivity by transfer through a washing procedure that was comprised of :

3 x 10 min ice-cold 5% (w/v) TCA;

1 x 5 min acetone/ethanol (1:1 (v/v));

1 x 5 min acetone.

The washing media were stirred and maintained at a volume in millilitres greater than five times the number of filter paper samples. The apparatus used to accommodate the samples during the washing stages consisted of a perforated beaker containing the samples placed inside a larger beaker containing a magnetic stirrer and the washing medium. This arrangement allowed for the handling of large numbers of samples and was used in other experiments where TCA-insoluble radioactivity was measured (Sections 2.6.4.5. and 2.6.5.). The filter paper samples were air-dried overnight and counted for radioactivity (Section 2.3.3.1.). Background was determined with filter paper squares which were taken through the washing procedure with the radioactive samples. Rates of Ca^{2+}-dependent incorporation of putrescine were then calculated after the subtraction of control values obtained from EDTA control vials. Units of enzyme activity were then expressed as nmol putrescine incorporated / h under the conditions of assay.

2.6. ISLET STUDIES.

2.6.1. Physiological media.

During studies on islets of Langerhans, two physiological media were used: Hanks Balanced Salt Solution (HBSS) used in the isolation of islets and Krebs Ringer Bicarbonate Medium (KRB) used during the subsequent experimental incubation of islets. These were both used in forms modified from those forms originally described.

HBSS (Hanks and Wallace, 1949) was prepared from concentrated stock solutions (10 x, without addition of phenol red or sodium bicarbonate) in the following manner:

A 100ml volumetric flask containing 100ml 10 x HBSS was washed out into a 1000ml beaker to which was added 200mg bovine serum albumin (essentially fatty acid free). The solution was made up to approximately 900ml with distilled water at 4°C and adjusted to pH 7.4 with approximately 3ml 7.5% (w/v) sodium bicarbonate. The HBSS was finally made up to 1000ml in a volumetric flask and stored at 4°C until used. This medium had the following composition:

	mg/l	mM
NaCl	8000	137
$CaCl_2$	140	1.27
KCl	400	5.37
KH_2PO_4	60	0.44
$MgSO_4.7H_2O$	100	0.41
$NaHCO_3$	350	4.17
$Na_2HPO_4.2H_2O$	60	0.34
glucose	1000	5.55
bovine albumin	200	0.003

HBSS was not used if more than 2 days old. The quantities used above were halved if less medium was required.

The KRB medium (Krebs & Henseleit, 1932) used in the present work was prepared as follows:

A stock solution of KRB was made by combining the following salt solutions in a screw-capped bottle (150ml):

0.9% (w/v) NaCl	100ml
1.3% (w/v) $NaHCO_3$	21ml
1.15% (w/v) KCl	4ml
1.22% (w/v) $CaCl_2.2H_2O$	2ml
3.82% (w/v) $MgSO_4.7H_2O$	1ml

This solution was gassed with O_2/CO_2 (19:1) for at least 30 min after which 640mg bovine serum albumin (essentially fatty acid free) was added together with 1.28ml 1.0M HEPES, pH 7.4. From this stock solution, the glucose-containing solutions used in the incubations were prepared by the addition of the appropriate volume of 50mg/ml (280mM) glucose in KRB stock solution and 100mM sodium phosphate buffer, pH 7.4 to volumetric flasks of the required final volume (10,25 or 50 ml) so that the final concentration of glucose was either 2.8, 30.5 or 34.2mM and the phosphate concentration was 1mM. These media were made up to volume with KRB stock solution, pH 7.4. The final composition of KRB medium was:

	mg/l	mM	
NaCl	6962	119.0	
$CaCl_2.2H_2O$	189	1.29	
KCl	356	4.77	
$MgSO_4.7H_2O$	295	1.24	
$NaHCO_3$	2111	25.14	
$NaH_2PO_4.2H_2O$	144	0.81	} 1mM
$Na_2HPO_4.2H_2O$	30	0.19	}
HEPES		10.0	
glucose	various	various	
bovine albumin	5000	0.076	

For the preparation of incubation media containing test compounds, a stock solution of the compound at 100 x the final concentration required was prepared in KRB stock solution and the pH adjusted to 7.4. Then 50µl or 100µl of this solution was added to 4.95 or 9.9ml respectively of KRB medium containing the desired glucose concentration.

KRB medium was prepared fresh daily and stored at 37°C in tightly capped containers.

2.6.2. Siliconisation of glassware.

The siliconisation of glassware was required in the islet studies to make glass surfaces hydrophobic, preventing the adherence of islets and other material.

The siliconising reagent used (Sigmacote, Sigma Chemical Co. Ltd., Missouri, U.S.A.) was placed in contact with the item of glassware for a few seconds, discarded and the glassware dried in a drying cabinet. In the case of pasteur pipettes, siliconising reagent was drawn into the pipette so that it contacted most of the inside and was then ejected.

When articles had thoroughly dried for a few hours, they were washed out with distilled water several times to remove any residue of siliconising reagent.

2.6.3. Isolation of islets of Langerhans.

The method used for the isolation of intact pancreatic islets of Langerhans by collagenase digestion was essentially that described by Lacy et al., (1972) which is itself a refinement of an earlier method (Lacy & Kostianovsky, 1967). Sprague-Dawley rats of both sexes and weighing 200-300g were used for the isolation of islets using the following procedure.

Following sacrifice of rats by cervical fracture, the duodenum was ligatured on either side of the point of entry of the common bile duct by using a surgical clamp. A cannula consisting

of a 0.6mm diameter plastic tube attached to a 31 x 0.6mm needle was then inserted into the bile duct via a small incision made in the bile duct. The pancreas was then inflated by injection of approximately 20ml of ice-cold Hanks' Balanced Salt Solution (HBSS) through the cannula until the pancreas was turgid. This part of the procedure served both to visualise and gently disrupt the pancreatic tissue. Next, the inflated pancreas was excised and placed in a clean glass scintillation vial and stored briefly on ice until required. Two pancreati obtained in this way were combined in a scintillation vial for each isolation of islets. Excess HBSS was poured off the combined pancreati which were then chopped continuously in the residual HBSS (approximately 10ml) with scissors for exactly 10 min. The chopped pancreatic tissue was washed five times with ice-cold HBSS and all excess HBSS removed with a pasteur pipette after the final wash. To effect release of pancreatic islets from this tissue, 8mg of collagenase was added to the chopped and washed pancreatic tissue in the scintillation vial which was then tightly capped. This mixture was shaken by hand with a semi-circular motion at a rate of 60-70 strokes/min. in a water bath at 37^{o}C. The time of this digestion was varied according to the activity of the batch of collagenase used: this was 10 min for 0.58 Units/mg collagenase and 8 min for 0.787 Units/mg collagenase. The digestion was stopped by the addition of approximately 20ml ice-cold HBSS which served both to cool the digestion and to dilute out the collagenase. The mixture was allowed to settle for 4-5 min and the supernatant removed. This washing procedure was repeated twice more to ensure that the digestion had ceased. Samples of the sedimented material (approximately 1ml each) were dispersed in approximately 30ml HBSS contained in a

plastic petri dish which was painted black on the outer surface. Intact islets of Langerhans released by digestion were then visualised with a dissecting microscope at a magnification of 10 or 20 x under incident illumination with a 60W bulb. Islets then appeared distinctly as dense white spherical or ovoid objects. Islets were removed from the dish using a finely drawn and siliconised pasteur pipette and placed in another blackened petri dish containing HBSS where they were ready for subsequent treatment. Using this procedure, usually 200-250 islets but sometimes as few as 100 or as many as 400 islets could be obtained from a pair of pancreati.

Subsequent to their isolation from the digest, islets were treated differently depending on the nature of the experiment to be performed with them. For the studies involving incubation of intact islets in physiological media, islets were incubated at 37^{o}C immediately after isolation in 1-2ml KRB medium containing 16.8mM glucose for 5 min followed by 60 min in KRB medium containing 2.8mM glucose in an atmosphere of O_2/CO_2 (19:1) before grouping for the experimental incubations. These pre-incubations were carried out in a 10ml beaker stoppered with a self-sealing rubber bung. For the experiments using islet homogenates, islets were not pre-incubated in KRB but extreme care was taken to elimate contamination of the islets by acinar tissue. This was achieved by repeated removal of islets to clean HBSS in blackened petri dishes using the dissecting microscope at high magnification (x 20) and a finely drawn siliconised pasteur pipette attached to an aspirator.

2.6.4. Treatment of islets.

2.6.4.1. Grouping of islets for experiments on intact islets.

Following their pre-incubation after isolation, islets were transferred to 15-20 ml KRB containing 2.8mM glucose contained in a blackened plastic petri dish. The islets which had

clumped together with residual acinar tissue could, whilst being viewed under a dissecting microscope, be cleanly separated by the use of finely drawn siliconised pasteur pipettes. The grouping procedure was intended to minimise the effects of variation in islet size which may lead to variation in the metabolic parameters being studied. Thus, each group of islets was created as far as possible to be comparable in distribution of islet size with other groups created from the same isolated batch. This was achieved by the removal of single islets to another dish containing KRB so that the largest islets of the population formed the first member of each group with subsequent members becoming progressively smaller. These manipulations were performed with siliconised finely drawn pasteur pipettes connected to an aspirator mouthpiece, making possible the fine control over manipulation which was required.

2.6.4.2. Apparatus for incubation of islets.

For studies on insulin release, glucose utilisation and protein synthesis, incubations of islets were performed inside 10ml glass beakers stoppered with self-sealing rubber bungs (Suba-seal, No. 49) and carried out at 37^{o}C in a constant temperature water bath. This system allowed for additions to be made to the incubations and for gassing to be carried out without the removal of the stoppers. Additions to the medium were made with disposable syringes and needles and with microlitre syringes which were used to inject media through the tops of the rubber bungs. Gassing with O_2/CO_2 (19:1) was achieved by the insertion of two needles (40 x 0.8mm) through the tops of the bungs, one needle carrying a slow stream of gas in and the other acting as an outlet to avoid build-up of pressure. Then after gassing for 5-6 min the needles were removed, leaving an atmosphere of 95% oxygen and 5% carbon dioxide inside the sealed beakers. During experiments when many

incubations were performed simultaneously, gassing was conveniently managed with a gas manifold consisting of rubber tubing attached to several 40 x 0.8mm needles.

2.6.4.3. Protocol for incubation of islets.

For the experiments on insulin release, glucose utilisation and protein synthesis, islets were incubated for a total period of 105-165 min which consisted of a pre-incubation period of 45 min with 2.8mM glucose followed by a period of usually 60 min but sometimes 90 or 120 min with 16.8mM glucose when glucose-stimulated parameters were measured. When incubations contained concentrations of test compounds, these compounds were present throughout the whole period of incubation, i.e. 105, 135 or 165 min. The pre-incubation period of 45 min was intended to allow test compounds to enter the islet cells by diffusion before stimulation of islets by increasing the glucose concentration of the medium to 16.8mM. The change from the low glucose medium (2.8mM glucose) to the high glucose medium (16.8mM glucose) was achieved by the addition to the pre-incubation medium of an equal volume of the same medium containing 30.5mM glucose to give a final concentration of glucose of 16.8mM.

2.6.5. Experiments on intact islets of Langerhans.

2.6.5.1. Studies on insulin release.

2.6.5.1.1. Incubation of islets.

Batches of 5 islets were placed in 10ml glass beakers and excess medium removed using a finely drawn siliconised pasteur pipette. Without delay, 0.5ml of KRB containing 2.8mM glucose together with the appropriate final concentration of test compound was added to the islets. The beakers were stoppered with self-sealing rubber bungs and gassed for 6 min with O_2/CO_2 (19:1) at the start of the incubation period. Following a pre-incubation period of 45 min at 37°C without shaking 0.5ml of KRB containing 30.5mM glucose and

the appropriate final concentration of test compound was added to give a final concentration of glucose of 16.8mM in 1.0ml medium. For the effects of test compounds on basal release, 0.5ml KRB containing 2.8mM glucose together with test compound was added to the existing medium. Following a further 60 min incubation at 37°C, also without shaking, aliquots of medium were sampled, and stored at -20°C until radioimmunoassay was performed to determine insulin release.

2.6.5.1.2. Radioimmunoassay for immunoreactive insulin.

Total immunoreactive insulin released into the incubation medium was measured by radioimmunoassay using an ethanol precipitation method (Heding, 1972). In each assay, standards of rat insulin were prepared ranging from 0 to 60 μU/ml insulin (20μU/ng). The rat insulin standards were prepared in NaFAM buffer which consisted of 40mM sodium phosphate, pH 7.3-7.5 containing 6% (w/v) human albumin, 0.6% sodium chloride and 0.24 mg/ml sodium merthiolate ('thimerosal', Sigma Chemicals Co. Ltd., Missouri, U.S.A.). Prior to radioimmunoassay, duplicate dilutions ranging from 1 in 5 to 1 in 50 were performed on the unknown samples in order to ensure that their values fell on the linear part of the standard curve. Standards, together with the unknown samples were diluted in NaFAM buffer to a final volume of 100μl in 12 x 75mm disposable glass test tubes and kept on ice.

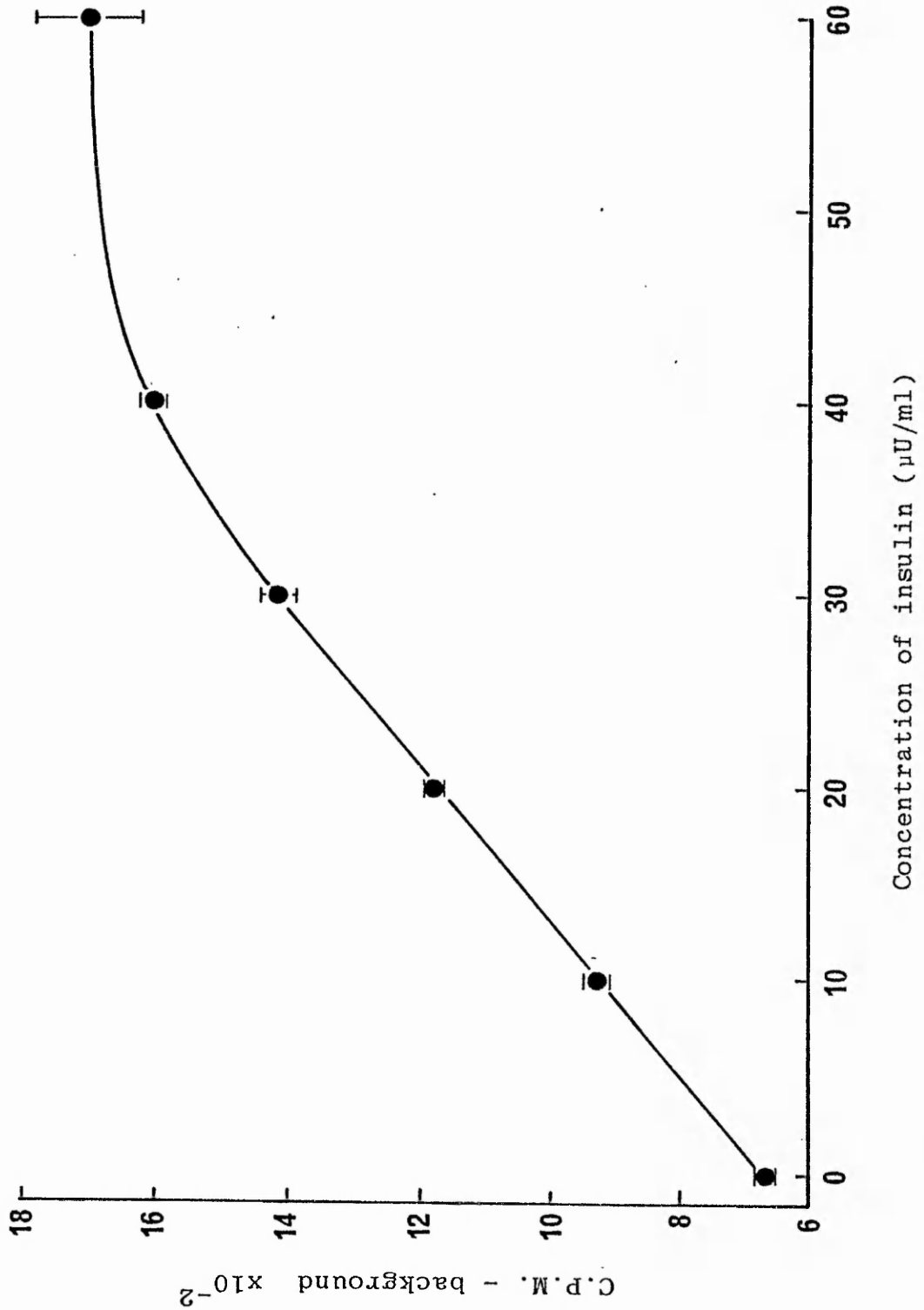
To each of the unknown sample tubes and standards thus prepared was added 100μl of anti-porcine guinea pig serum prepared in FAM buffer which consisted of 40mM sodium phosphate, pH 7.3-7.5, containing 1mg/ml human albumin and 0.24mg/ml sodium merthiolate. The original antibody-containing serum was diluted 1:1800 before its addition to the insulin sample tubes. The tubes were vortexed and incubated at 4°C for 20-24 h. Following incubation, 100μl of ^{125}I-labelled porcine insulin (20-30μU/ng and 20-30 mCi/mg) prepared in FAM buffer was added to each of the tubes and to three more tubes

containing 100µl each of FAM and NaFAM buffers. These three tubes served as total counts controls. The tubes were vortexed and incubated at 4°C for a further 4h. With the tubes still at 4°C, 1.6 ml of 95% (v/v) ethanol which was at room temperature was added to each of the tubes by means of an automatic dispenser ('Distrivar', Gilson, France) and the tubes vortexed and maintained on ice. The tubes were then centrifuged for 10 min in a Chilspin 2 centrifuge (M.S.E., Crawley, Sussex) at 1800g av. and 10°C and the supernatant decanted into plastic tubes for counting in a gamma counter. From the data obtained, a standard curve of counts per minute vs. insulin concentration was plotted and the values for the unknown sample tubes falling on the linear part of the curve were extrapolated. An example of a typical standard curve is shown in Fig. 2.2.. For each sample, an average value for insulin concentration in the undiluted sample was then calculated from the values obtained for each of the tubes prepared for that sample. For incubations of islets at low glucose (2.8mM) dilutions of 1 in 5 and 1 in 10 were made, both of which were normally in the linear region of the standard curve, whilst for the incubations at high glucose (16.8mM) dilutions of 1 in 10, 1 in 20 and 1 in 50 were used, at least two of which were normally on the linear part of the standard curve. Thus, values for each sample were a mean of at least four values with a standard error of less than 10%.

2.6.5.2. Measurement of islet glucose utilisation.

Glucose utilisation was measured by estimation of the amount of $^{14}CO_2$ released from [U-^{14}C] -glucose, a method described by Malaisse et al., (1974). Groups of 10-20 islets were placed in 1.5ml plastic vials and excess medium removed from them. Without delay, 50µl of KRB medium containing 2.8mM glucose and the appropriate final concentration of test compound was added to the

Figure 2.2. Insulin standard curve



islets in the vials which were placed in 10ml glass beakers. The beakers were stoppered with self-sealing rubber bungs and then gassed for 6 min. with O_2/CO_2 (19:1) at the start of the incubation period. Islets were preincubated at 37°C for 45 min before the addition of 45µl KRB medium containing 34.2mM glucose together with the appropriate final concentration of test compound and 5µl of [U-^{14}C]-glucose (1µCi) to give a final concentration of 16.8mM. For the determination of basal levels of glucose utilisation, 45µl of KRB containing 2.8mM glucose was added with 5µl [U-^{14}C] -glucose. Following a further incubation period of either 60 or 120 min., 0.2ml of hyamine hydroxide (methylbenzethonium hydroxide, Sigma Chemical Co. Ltd., Missouri, U.S.A.) was added to the glass beaker to absorb any released $^{14}CO_2$ and 50µl 0.2N HCl injected into the medium containing islets to release any trapped $^{14}CO_2$. This amount of acid proved to be adequate to lower the pH of the medium to below 1.0 even in the incubations containing test compounds at their highest concentrations. Following a further incubation period at 37°C of 30 min after the addition of the acid, the plastic wells were removed and the bottom of the outer surface of the wells washed with 400µl of distilled water into the glass beakers containing hyamine hydroxide. The diluted hyamine hydroxide was transferred to a scintillation vial and the beakers washed into the scintillation vial with a further 400µl distilled water. The hyamine hydroxide, now diluted to 1.0ml, was counted for radioactivity. For determination of background counts, control incubations containing radioactive medium but no islets were treated as above.

2.6.5.3. Measurement of islet total protein synthesis.

Protein synthesis in intact islets was measured by the incorporation of[^{3}H]-leucine into trichloroacetic acid precipitable protein. Batches of 10-20 islets grouped according to section 2.6.4.1.were placed in small plastic vials (1.5ml) and

all excess medium removed from them. Without delay, 50µl KRB containing 2.8mM glucose together with test compound at the correct final concentration was added to the islets in the vials which were placed in 10ml glass beakers. The beakers were stoppered with self-sealing rubber stoppers, gassed for 6 min as described previously and incubated at 37°C in a water bath without shaking for 45 min. After this preincubation period, 45µl KRB containing 34.2mM glucose together with the appropriate concentration of test compound and 5µl[^{3}H]-leucine(5µCi) were added to the islets through the self-sealing rubber bungs using microlitre syringes. This gave a final concentration of glucose of 16.8mM and leucine of 0.9µM. Following either a further 60 or 90 min incubation period at 37°C, the plastic vials containing islets were removed and 100µl of ice-cold 20% (w/v) trichloroacetic acid added. The mixture was sonicated at 4°C with two 5 s bursts at 3µ and the resulting suspension of precipitate transferred to 1.8ml microfuge tubes (Beckman). The tubes were centrifuged in a microfuge (Beckman) for 4 min at 8,740 g av. and the supernatant decanted. The pellet was washed three times in ice-cold 5% (w/v) trichloroacetic acid containing 2mM 'cold' leucine by resuspension and centrifugation and similarly, once in acetone/ethanol (1:1 v/v) and once in acetone. The precipitate was then dried and counted for radioactivity. Background was determined using blanks containing incubation medium with [^{3}H]-leucine present but no islets and were taken through the entire procedure above.

2.6.5.4. Measurement of uptake of radiolabelled compounds.

The uptake of [^{3}H]-putrescine, [^{14}C]-methylamine and [^{3}H]-leucine by islets and the determination of islet intercellular space using [^{3}H]-sucrose as an extracellular marker were measured using a method essentially that of Wollheim et al., (1978).

Incubations of islets were carried out in 400µl polyethylene

microcentrifuge tubes (Alpha Laboratories Ltd.) containing 20µl 6M urea which was overlayered with 200µl of a mixture of dibutylphthalate and dinonylphthalate (10:3). For the measurement of uptake of [^{3}H]-putrescine and [^{14}C]-methylamine and the determination of sucrose space, groups of 10-25 islets were placed in the tubes above the phthalate ester layer and excess medium removed with a finely drawn pasteur pipette. To the islets was then added 50µl KRB medium containing 2.8mM glucose together with the appropriate concentration of radiolabelled putrescine, methylamine or sucrose. Care was taken to ensure that an air space separated the medium from the phthalate ester layer. The concentrations and specific radioactivity of the compounds used were as follows:

	Final concentration of compound (mM)	Specific activity (Ci/mmol)
[^{3}H]-putrescine	2.0	0.1
	20.0	0.005
[^{14}C]-methylamine	1.78	0.056
[^{3}H]-sucrose	0.0102	9.8
	20.0	0.01042

The microcentrifuge tubes with their lids open and containing islets were quickly placed in 25ml Erlenmeyer flasks previously gassed with O_2/CO_2 (95:5) in a water bath at 37^{o}C. The flasks were closed with ground glass stoppers and incubations carried for times ranging from 0 to 45 min.. Following incubation the tubes were removed from the flasks with forceps and their lids closed. The tubes were then centrifuged at 9,390 g av. for 3 s in a microcentrifuge to terminate the incubation by sedimentation of the islets through the phthalate ester layer and into the urea at the bottom of the tubes. The bottoms of the tubes containing islets were then snipped off with scissors into scintillation vials, left for 30 min to allow for dissolution of the islets and then counted for radioactivity after

the addition of 10ml Instagel. Background was determined from incubations containing radioactive media but no islets and was subtracted from the counts obtained from incubations containing islets.

The uptake of [^{3}H]-leucine was determined in the same manner except that the incubations consisted of a preincubation period of 45 min in KRB medium containing 2.8mM glucose in the presence of the appropriate concentration of test compounds and in the absence of radiolabel followed by 60 min in KRB containing 16.8mM glucose together with test compound and 5μCi [^{3}H]-leucine (55 Cimmol^{-1}). In order to equalise the effects test compounds may have upon the "metabolic pull" from protein synthesis, 0.1mM cycloheximide was included throughout the period of all the incubations (45 + 60 min).

The sucrose space of islets was calculated from the counts obtained from incubations containing radiolabelled sucrose and the specific activity and concentration of the radiolabel. The amount of putrescine, methylamine or leucine represented by this space was then subtracted in the calculation of the uptake of these compounds.

2.6.5.5. Incorporation of [^{14}C]-methylamine into protein of intact islets.

Groups of 75 or 100 islets were placed in 1.5ml plastic vials and freed of excess medium. Then 50μl (for 75 islets) or 100μl (for 100 islets) of KRB medium containing 2.8mM glucose and 1.78mM [^{14}C]-methylamine (56 mCi/mmol) was added to the islets in the vial. The vial was placed in a 10ml beaker which was stoppered with a self-sealing rubber bung and gassed for 6 min with O_2/CO_2 (19:1) at the start of incubation at 37^{o}C. After 45 min preincubation with 2.8mM glucose, 50μl or 100μl of KRB containing 2.8mM or 30.5mM glucose and 1.78mM [^{14}C]-methylamine (56 mCi/mmol) was added to the incubation which was continued for a further 60 min. Following incubation, the vials were removed from the glass beakers and the incubation medium removed from the islets. The medium was retained

for the determination of background counts as described below. Two procedures were then employed for the removal of unbound radioactivity from the islet protein before preparation of sample for liquid scintillation counting.

i. Islets were first washed in KRB medium containing 2.8mM glucose and then homogenised by sonication in 100µl of this medium. The homogenate was then distributed on 21mm diameter Whatman 3MM filter paper discs which were placed in ice-cold 10% (w/v) trichloroacetic acid. Unbound radioactivity was then washed from the filters by the method described earlier (section 2.4.1.). Blank discs and discs on which were spotted 50µl aliquots of the radioactive incubation medium were also taken through the washing procedure and acted as background determinations. After drying, the discs were counted for radioactivity.

ii. Ice-cold 10% (w/v) trichloroacetic acid (200µl) was added to the islets, the mixture sonicated (two bursts of 5 s at 3µ peak-to-peak) and transferred to a 1.8 ml microcentrifuge tube. The precipitate was pelleted by centrifugation in a microcentrifuge for 4 min at 8,740g and washed three times with ice-cold 5% (w/v) trichloroacetic acid containing 2mM 'cold' methylamine. The lid of the microcentrifuge tube was cut away so that a piece of dialysis membrane caught between the lid and the tube formed the barrier between the medium in which the precipitate was suspended and a dialysis medium consisting of 2mM methylamine in ice-cold 5% (w/v) TCA. Dialysis of the precipitate was then performed overnight after which, the precipitate was pelleted, washed once in acetone/ethanol (1:1 v/v) and once in acetone. The dried precipitate was then counted for radioactivity. Backgrounds consisting of aliquots of medium which contained bovine albumin (5 mg/ml) were prepared in a similar manner.

2.6.6. Incorporation of radiolabelled amines into islet homogenate protein.

The incubation of radiolabelled amines with islet homogenates was used to measure rates of transglutaminase-catalysed incorporation of these amines into islet proteins and to prepare samples for subsequent resolution of labelled proteins by SDS-polyacrylamide gel electrophoresis.

The incubations for amine incorporation were carried out in 1.0 ml microcentrifuge tubes for the measurements upon rate of incorporation and in 1.8ml microcentrifuge tubes for the preparation of samples for electrophoresis. The incubation cocktail at 37°C and pH 7.4 contained 2.5mM $CaCl_2$, 3.85mM dithiothreitol and 18mM Tris-Cl and one of the following concentrations of amine:

	conc. (mM)	final specific activity ($mCimmol^{-1}$)	µCi per incubation
[^{14}C]-methylamine	1.78	56.0	10.0
[^{14}C]-putrescine	1.78	7.02	1.25
[^{3}H]-putrescine	2.0	50.0	10.0

These components were contained in a final volume of 100µl following the addition of 65µl of islet homogenate (50-150 µg protein) which started the reaction. Control incubations were also set up which contained 5mM EDTA instead of 2.5mM $CaCl_2$.

For the measurement of the rate of incorporation of amine into islet protein, samples of 20µl of the cocktail were taken at time intervals up to 60 min after addition of islet homogenate and were distributed on pencil-labelled Whatman 3MM filter paper discs (21mm diameter). These were immediately placed in ice-cold 10% (w/v) trichloroacetic acid and washed according to the procedure described in the transglutaminase assay method (section 2.4.1.).

For the preparation of incubated homogenate samples for SDS-polyacrylamide gel electrophoresis, incubations were carried out for up to 5h, following which 100µl of ice-cold 20% (w/v) trichloroacetic acid was added to the cocktail which was then shaken gently and kept on ice. The resulting precipitate was pelleted by centrifugation for 4 min in a microfuge and washed 3 times with ice-cold 5% (w/v) trichloroacetic acid containing 2mM cold putrescine or methylamine, as appropriate. The precipitate was then treated for subsequent electrophoresis as described in section 2.4.3.2..

2.6.7. Polyamine analysis.

2.6.7.1. Extraction of polyamines from islets.

Following incubation of islets in KRB medium contained in 1.5ml plastic vials, medium was removed and 200µl of 10% (w/v) TCA in 20% (v/v) methanol/distilled water was added. The islets were then sonicated with 2 bursts of 5 s at 3 µ peak-peak and the mixtures transferred to 1.8ml microcentrifuge tubes. TCA precipitable material was separated from the soluble material by centrifugation at 8,740g for 5 min in a microcentrifuge. The supernatants were transferred to small glass tubes and extracted 5 times by vortexing with 1.0ml of water-saturated diethyl ether after the addition of 25µl of 8 µM (200 pmol) 1,7-diaminoheptane as an internal standard. The TCA extract was then lyophilised in the glass tubes.

2.6.7.2. Analysis of polyamines by high-pressure liquid chromatography.

The method used was virtually identical to the ion-pair reverse-phase chromatographic procedure of Seiler (1983) using fluorescent detection following post-column derivatisation with σ-phthalaldehyde/ mercaptoethanol reagent.

The HPLC pumps (Altex, model 100A) used for the delivery of buffers were controlled by an automatic gradient programmer (Altex, model 420). Fluorescence reagent was also delivered with an Altex 100A HPLC pump.

Separation of the polyamines putrescine, spermidine and

spermine was achieved by using a C18 μ-Bondapak 3.9mm (internal diameter) x 300mm long column (10μm particles, Waters Associates Ltd,France) which was protected by a 3.9mm (internal diameter) x 60mm long guard column containing C18-bonded glass beads (30-38 μm dia. The μ-Bondapak column was maintained at constant temperature of 35°C.

Two buffers were used in the gradient elution procedure. Buffer A was 0.1M sodium acetate,10mM octane sulphonate, pH 4.5 and Buffer B was a mixture of 10 parts 0.2M sodium acetate, 10mM octane sulphonate, pH 4.5 and 3 parts acetonitrile. The pH of the buffers was adjusted to 4.5 with glacial acetic acid. Buffers were filtered through a 0.45μm membrane filter (Millipore) before the addition of similarly filtered acetonitrile. Buffers were then de-gassed by sonication for 5 min.

The σ-phthalaldehyde fluorescent reagent was prepared by dissolving 50g boric acid and 44g potassium hydroxide in 1000ml distilled water after the addition of 3ml of a solution of Brij 35 (30% (w/v), Sigma Chem. Co. Ltd., Missouri, U.S.A.). To this solution was added 400mg of σ-phthalaldehyde dissolved in 5ml methanol and 2ml 2-mercaptoethanol. This reagent was prepared freshly each day and maintained at 35°C in a water bath.

Elution was carried out at a constant flow rate of 1.5ml/min after equilibration of the column with Buffer A for 5 min. The elution conditions were as follows :

initial: 100% Buffer A

0-25 min: linear gradient of 0-100% Buffer B in Buffer A;

25-30 min: 100% Buffer B;

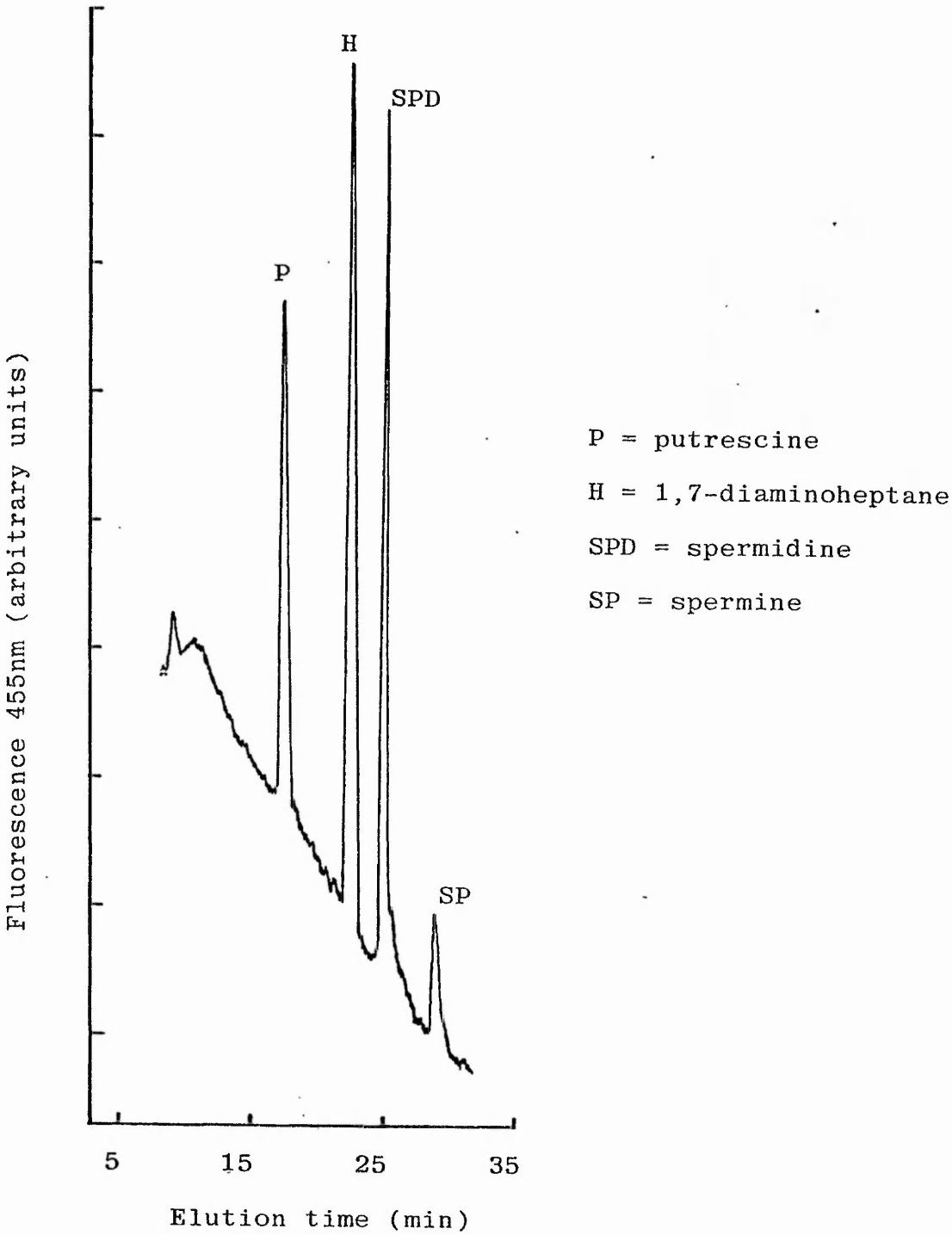
Polyamines were detected following their derivatisation with σ-phthalaldehyde reagent which was at a constant temperature of

35°C and mixed with the column eluent in a 1:1 ratio. Fluorescence produced by excitation at 340nm was monitored continuously at 455nm using a fluorimeter connected to a chart recorder.

Samples containing polyamines were dissolved in 70µl Buffer A and 60µl injected into an injector (Rheodyne, Cotati, California, U.S.A.) fitted with a 50µl injection loop, from which samples were applied to the column.

Polyamine levels were quantitated by reference to peak heights of standards (156 pmol each of putrescine, spermidine, spermine and 1,7-diaminoheptane). The shapes of the peaks were Gaussian and the response of the system to different amounts of polyamines measured by peak height was linear. An example of the traces obtained from standard runs of putrescine, spermidine, spermine and 1,7-diaminoheptane is shown in Fig. 2.3..

Figure 2.3. Elution profile for polyamines



2.7. TUMOUR STUDIES.

2.7.1. Sources of tumour tissue.

Tumour tissue was obtained by two means: passage of existing transplantable tumour lines and by induction of primary hapatocellular carcinoma with chemical carcinogens.

2.7.1.1. Passage of tumour lines MC3 and CC5.

The sources of these tumours were described by Moore,(1972).MC3 is a sarcoma of soft tissue origin and was induced chemically by methylcholanthrene. CC5 is an osteosarcoma induced with cupric-chelated-N-hydroxy-2-acetylaminofluorene.

Tumours were propagated in AS rats of both sexes by subcutaneous implantation of approximately $5mm^3$ of freshly excised tumour tissue. Rats were anaesthetised with diethyl ether, their flanks shaved and sterilised with Hibitane spray (I.C.I. Ltd.) and a small (3-4mm) incision made in the skin of the flank. A small subcutaneous pocket was then gently made to receive the tumour implant. Following implantation, the incision was sealed with a metal surgical clip, sterilised once more with Hibitane spray and rats allowed to recover. Tumours were then allowed to grow for periods of up to four weeks before harvesting.

2.7.1.2. Induction of hepatocellular carcinomata with diethylnitrosamine (DEN) and 6-p-dimethylamino-phenylazobenzothiazole (6-BT).

2.7.1.2.1. Carcinogenesis with DEN.

In one set of animals, male AP (Wistar-derived) rats which were 4-5 weeks old at the start of dosing were continuously administered 550 µg DEN per rat per day for 5 days/week. DEN was dissolved in water and administered by gavage; controls were similarly administered water only. Rats were sacrificed 32 weeks after the start of dosing.

In a second set of animals, dosing was carried out as above except that dosing ceased after 27 weeks and animals were sacrificed after 35 weeks from the start of dosing.

The use of DEN as a carcinogen has previously been described by Argus & Hoch-Ligeti, (1961).

2.7.1.2.2. Carcinogenesis with 6-BT.

Male AP rats 6 weeks old at the start of dosing were administered 5mg/kg 6-BT/day, 7 days/week for 2 months. 6-BT was administered in corn oil by gavage and controls were similarly administered corn oil alone. Rats were sacrificed 12 months after the start of dosing.

A second set of animals was similarly dosed, except that 7.5 mg/kg was administered instead of 5mg/kg.

The use of 6-BT as a carcinogen has previously been described by Elliot et al., (1983).

2.7.2. Cell fractionation and leaching studies.

The fractionation of tumour and liver tissue by centrifugation and studies on the leaching of transglutaminase activity from pellet fractions was described in an earlier section (2.4.2. and Fig. 2.1.).

3. RESULTS AND DISCUSSION : ISLET STUDIES.

The main aim of the studies on islets of Langerhans was to test the hypothesis that tissue transglutaminase may be involved in the mechanism of insulin release from the pancreatic β-cell. The work followed three major lines of investigation:

1. Identification of islet transglutaminase;
2. Testing the hypothesis for the involvement of islet transglutaminase in insulin release using a pharmacological approach;
3. Examination of the substrates for transglutaminase in islets.

These three lines of investigation will be considered separately in sections 3.1, 3.2. and 3.3. respectively.

3.1. CHARACTERISATION OF ISLET TRANSGLUTAMINASE.

The purpose of this investigation was firstly to demonstrate the existence of a transglutaminase enzyme in pancreatic islets and secondly to define the catalytic properties of this enzyme. The properties defined could subsequently be used in the construction of a hypothesis for defining a common role of the enzyme in cells. Throughout this work, it should be borne in mind that islets may only be composed of 60-70% β-cells: the properties observed below may partly reflect those of non-β-cell transglutaminase.

3.1.1. TRANSGLUTAMINASE ACTIVITY IN ISLET HOMOGENATES.

Homogenates of islets of Langerhans were assayed for enzyme activity by [^{14}C]-putrescine incorporation into N,N'-dimethylcasein in the presence of either 2.5mM $CaCl_2$ or 5mM EDTA.

Figure 3.1 demonstrates the presence in islets of a Ca^{2+}-dependent transglutaminase enzyme. Putrescine incorporation in the presence of 2.5mM $CaCl_2$ proceeds in a reasonably linear fashion for up to 10 min from the start of the reaction, but thereafter proceeds at a non-linear rate. Background incorporation in the presence of 5mM EDTA is detectable but proceeds at a very slow rate. This level of incorporation may be the result of a failure of the chelator to remove all free Ca^{2+} from the enzyme. Alternatively, a major proportion of this apparent activity may be the result of the failure of the washing procedure of the assay to eliminate all unbound radioactivity from the samples. The latter explanation is more likely in view of the almost constant level of 'incorporation' above background over 5 - 30 min in the EDTA containing vials.

Rates of incorporation of putrescine were calculated for islet homogenates and for homogenates of other rat tissues assayed in a similar manner. The specific activity of transglutaminase measured in this way and expressed per mg protein was found to be of a similar order of magnitude in these tissues to that found in islets of Langerhans (Table 3.1.).

Figure 3.1.

Transglutaminase activity in homogenates of islets of Langerhans

Homogenates of islets were assayed for transglutaminase activity as described in the Methods section (2.5.). Background values obtained from blank filter paper squares which were taken through the washing procedure were subtracted (30-40 c.p.m.) before the calculation of n mol putrescine incorporated per mg protein.
Closed circles denote assay system containing 2.5mM $CaCl_2$; open circles denote assay system containing 5.0mM EDTA.
For all points, n=6 except * where n=3. Bars represent S.E.M.

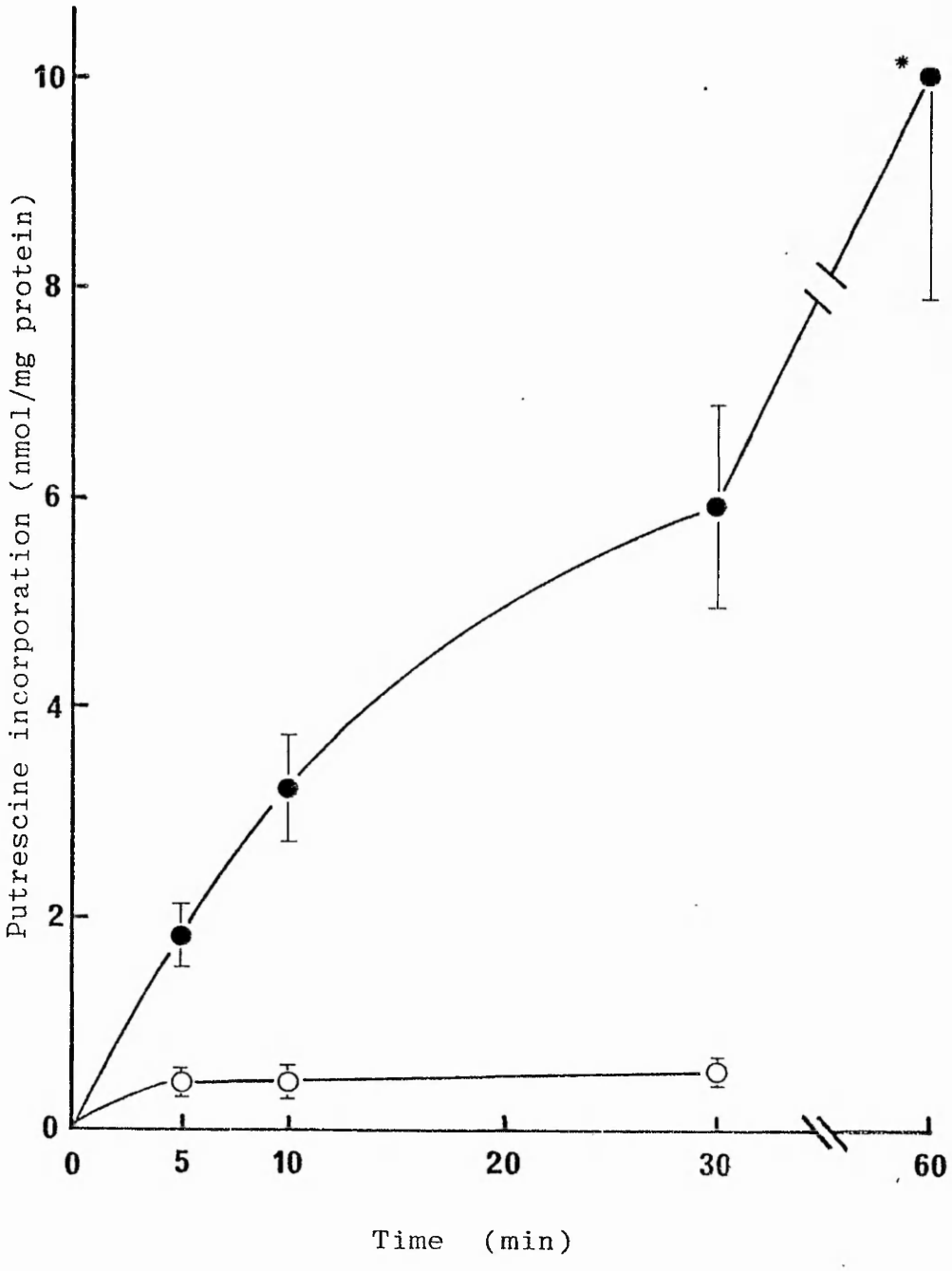

Putrescine incorporation (nmol/mg protein)
10
8
6
4
2
0
*
0
5
10
20
30
60
Time (min)

Table 3.1.

Transglutaminase activity in homogenates of various rat tissues.

	U/mg protein	
Liver	33.2 ± 3.5	(3)
Lung	31.5 ± 4.2	(4)
Spleen	30.0 ± 0.6	(3)
Pancreatic islets	20.3 ± 3.0	(9)

Homogenates of liver, lung and spleen were prepared in ice-cold 0.25M sucrose, 1mM Tris, 1mM EDTA, pH 7.4 with a Potter-Elvejhem homogeniser and islet homogenates were prepared by sonication in the same medium. These homogenates were assayed for transglutaminases activity by [^{14}C]-putrescine incorporation into N,N'dimethylcasein. Rates of putrescine incorporation were calculated and expressed per milligramme homogenate protein. One unit of activity corresponds to one nmol putrescine incorporated per hour under the conditions of assay. Results are mean ± S.E.M. of the number of observations shown in parentheses.

3.1.2. SUBCELLULAR DISTRIBUTION OF ISLET TRANSGLUTAMINASE.

Islet homogenates were centrifuged for 45 min at 71,000g av and the pellet and supernatant fractions produced were assayed for transglutaminase activity. This fractionation procedure (De Duve et al., 1955) sediments all cellular membranes leaving a particle-free supernatant containing only soluble cell protein. Using this technique it was found that 94 $\pm$ 3% (n=3) of the total enzyme activity was present in the supernatant fraction. Comparison of the specific activity of this fraction with that of islet homogenates (Table 3.1.) indicated an increase in specific activity of approximately two-fold (Table 3.2.).

It would be desirable to carry out any characterisation of an enzyme on purified or semi-purified preparations. In the present work this was not possible due to the small amounts of islet material available. In view of the restriction of islet transglutaminase activity to the soluble fraction of islet homogenates and the slight increase in specific activity obtained by this procedure, this supernatant extract was used for the following experiments aimed at characterisation of the enzyme.

Table 3.2.

Subcellular distribution of islet transglutaminase

	% Total activity (3)		% Total protein (3)		Specific activity (U/mg protein) (2)	% Homogenate total activity (2)
Supernatant	94	± 3	70	± 9	56.8 ± 10.6	77 ± 0
Pellet	6		30		13.9 ± 1.7	2 ± 0.2

Homogenates of islets in 0.25M sucrose, 1mM Tris, 1mM EDTA, pH 7.4 were fractionated by centrifugation at 71,000g for 45 min and the resuspended pellet and supernatant fractions assayed for transglutaminase activity (section 2.4.1.).

Total activity and total protein refer to the sum of the activity or protein in the fractions.

3.1.3. ACTIVATION OF ISLET TRANSGLUTAMINASE BY Ca^{2+} IONS.

The dependence of transglutaminase activity on the concentration of free Ca^{2+} ions was investigated by titration of enzyme activity with varying concentrations of free Ca^{2+} in the range 25 - 2000 µM. The incorporation of putrescine into N,N'-dimethylcasein at these free concentrations of Ca^{2+} at three different time intervals is shown in Fig. 3.2.. Small additions of Ca^{2+} readily activate the enzyme with 100 µM Ca^{2+} giving rise to 70% of maximal velocity. Additions of Ca^{2+} above 100µM resulted in only a relatively small increase in activity with saturation of enzyme activity occurring at around 500 µM Ca^{2+}. The data were calculated to reveal initial rates of putrescine incorporation and were re-plotted as a double reciprocal plot after the method of Lineweaver and Burk (1934) (Fig. 3.3.). From this plot, the Km for Ca^{2+} for islet transglutaminase as measured in this system was calculated as 39µM.

The effect of Mg^{2+} ions on enzyme activity at saturating levels of Ca^{2+} was investigated as well as the effect of Mg^{2+} in the absence of Ca^{2+}. The results illustrated in figure 3.4. indicate that at 2mM, $MgCl_2$ did not activate the enzyme in the absence of Ca^{2+}, nor did it affect the velocity of the reaction in the presence of 2.5mM $CaCl_2$.

Figure 3.2.

Ca^{2+}-activation of islet transglutaminase

Islets were homogenised as described in section 2.5.1.3. except that 1mM EGTA was substituted for 1mM EDTA in the homogenisation medium. Supernatant extracts were then prepared as described in section 2.5.2. The extract was assayed for transglutaminase activity (section 2.4.1.) in the presence of free Ca^{2+} concentrations ranging from 25μM to 2.0mM. These concentrations were maintained with the use of Ca/EGTA buffers created by the addition of varying amounts of $CaCl_2$ to the assay in the presence of 0.45mM EGTA which was present in the assay following addition of islet extract. For the calculation of free Ca^{2+} concentrations, an association constant at pH 7.4 of $10^{7.12}$ for $[Ca.EGTA] / [Ca][EGTA]$ was used (Schatzman, 1973). Putrescine incorporation was calculated after the subtraction of background counts obtained from vials containing 5mM EGTA.

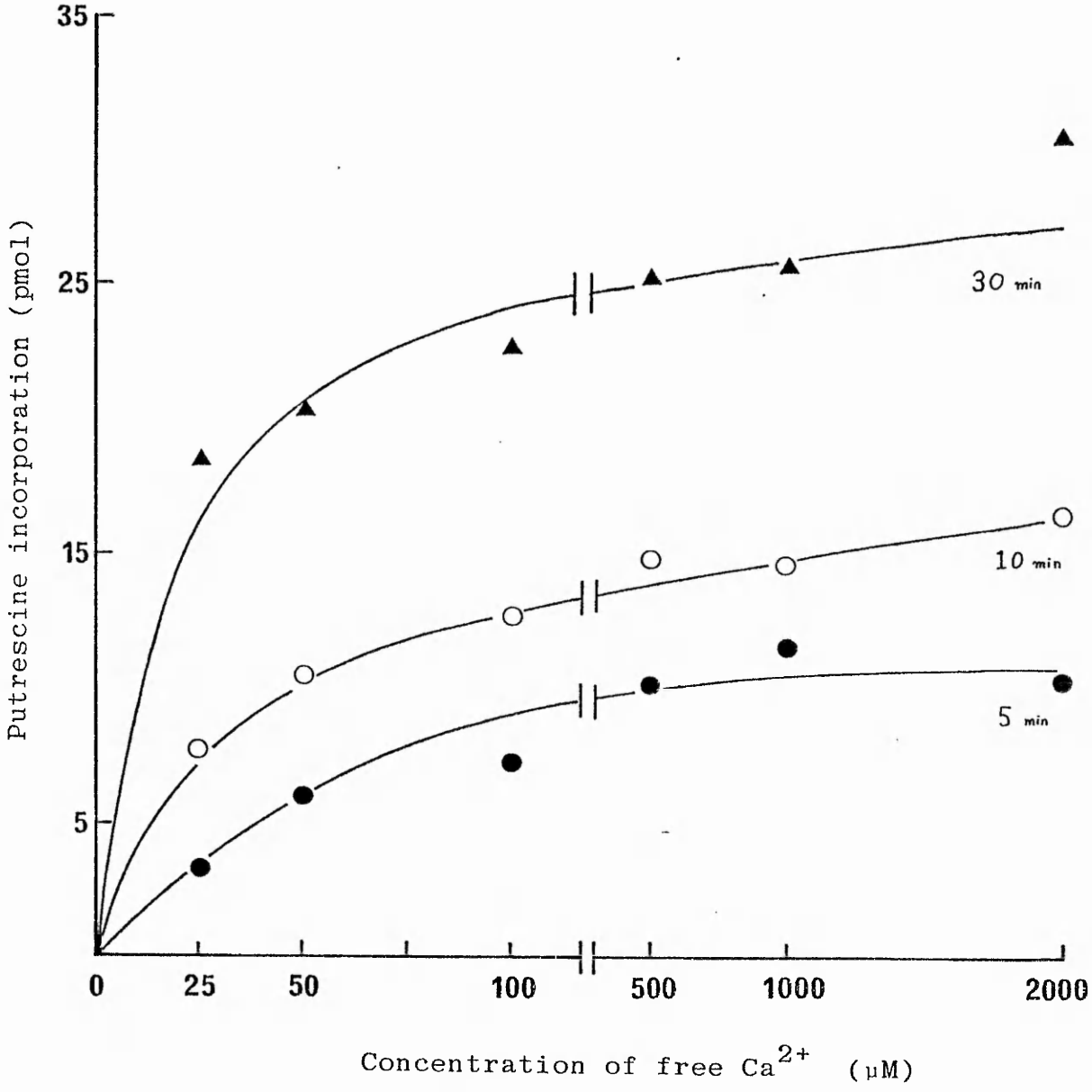

35
25
15
5
Putrescine incorporation (pmol)
30 min
10 min
5 min
0
25
50
100
500
1000
2000
Concentration of free Ca2+ (μM)

Figure 3.3.

Calculation of the apparent Km for Ca^{2+} of islet transglutaminase.

Initial rates of putrescine incorporation into N,N'-dimethylcasein were calculated from the data in Fig. 3.2.. A reciprocal plot of $\frac{1}{v}$ (v measured in nmole putrescine incorporated / h) against $\frac{1}{[Ca^{2+}]}$ was made whose intercept on the $\frac{1}{[Ca^{2+}]}$ axis represented $-\frac{1}{Km}$, the reciprocal of the Km for Ca^{2+} for islet transglutaminase as measured in this system.

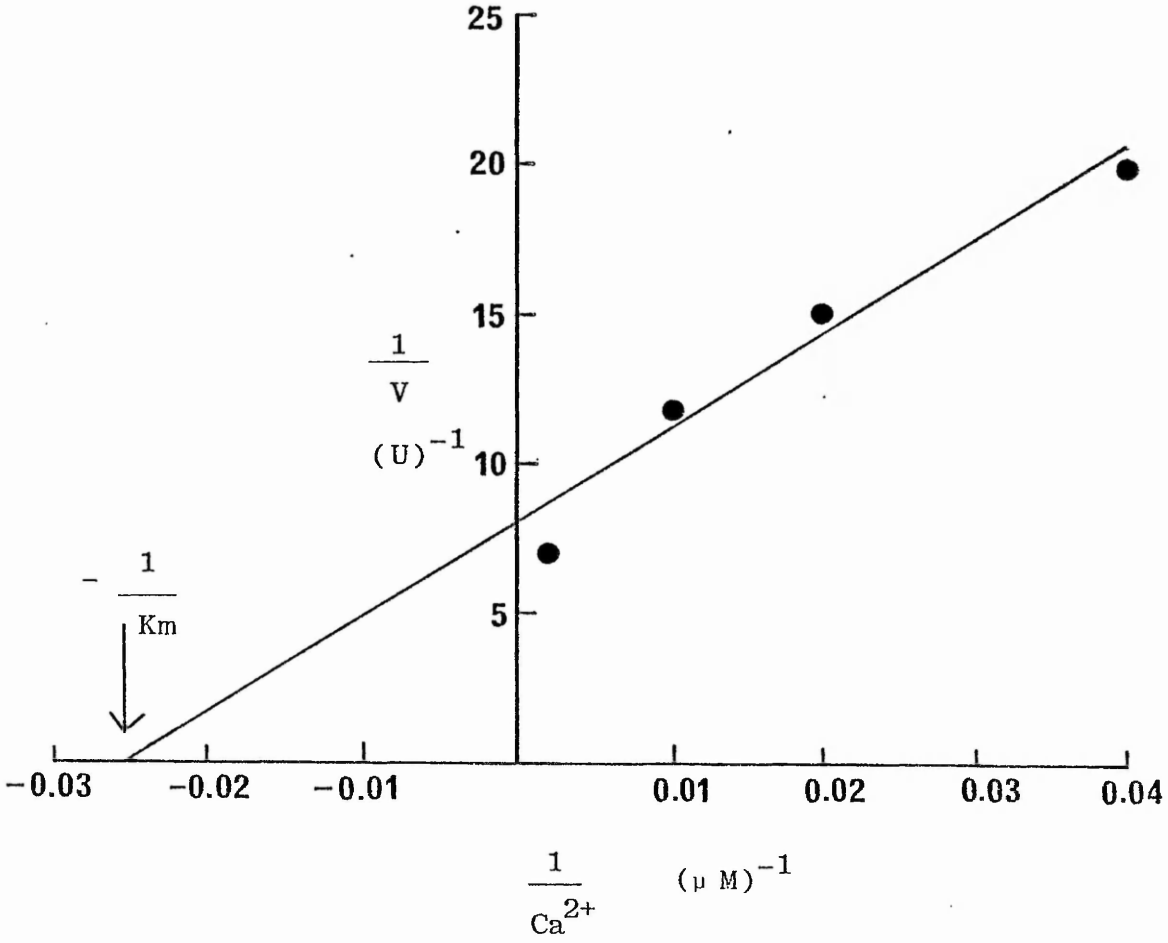

25
20
15
10
5
1/V
(U)^-1
-1/Km
-0.03
-0.02
-0.01
0.01
0.02
0.03
0.04
1/Ca2+
(μ M)^-1

Figure 3.4.

Effect of Mg^{2+} ions on islet transglutaminase activity.

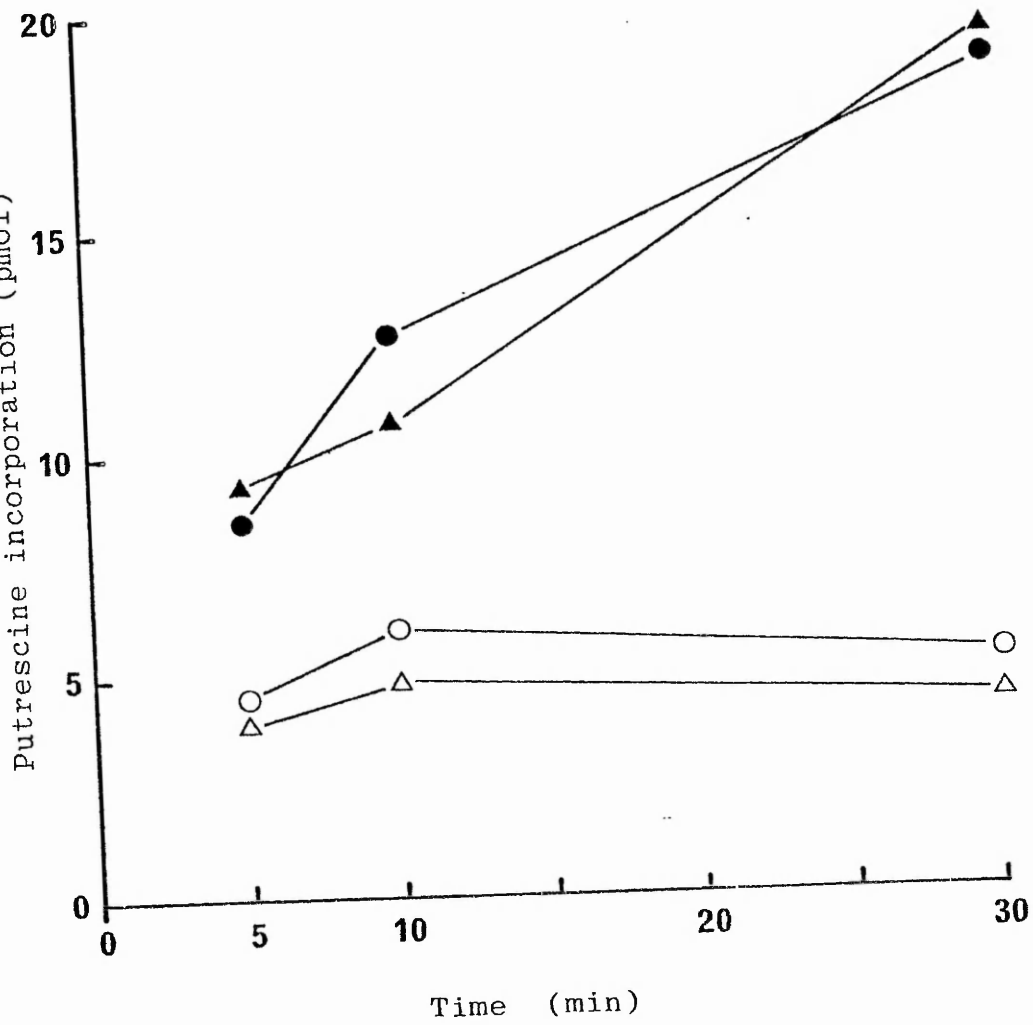
Extracts of islets (71,000g av supernatant) were assayed for transglutaminase activity in the presence of either $CaCl_2$ (2.5mM), $MgCl_2$ (2.0mM), $CaCl_2$ (2.5mM) + $MgCl_2$ (2.0mM) or EDTA (5mM). The incorporation of putrescine (pmol) was calculated after the subtraction of background counts and was an average of two values except in the case of EDTA which was a single value.

Open circles refer to EDTA (5mM);

closed circles refer to $CaCl_2$(2.5mM);

open triangles refer to $MgCl_2$ (2.0mM);

closed triangles refer to $CaCl_2$ (2.5mM) + $MgCl_2$ (2.0mM).


20
15
10
5
0
0
5
10
20
30
Putrescine incorporation (pmol)
Time (min)

3.1.4. SULPHYDRYL GROUP (-SH) DEPENDENCE OF ISLET TRANSGLUTAMINASE.

The -SH dependence of islet transglutaminase was investigated by titration of enzyme activity with varying concentrations of the thiol-reactive compounds, iodoacetamide, p-chloro-mercuribenzoic acid (p-CMB) and the thiol-active site directed inhibitor of transglutaminase,cystamine (Siefring et al., 1978). In each case measurement of enzyme activity was carried out in the absence of dithiothreitol. This omission alone led to a 40% loss in enzyme activity in control incubations when compared to those containing 3.85mM dithiothreitol. The effects of iodoacetamide, p-CMB and cystamine are illustrated in Fig. 3.5a and b. Iodoacetamide and p-CMB were found to be potent inhibitors of islet transglutaminase activity with a concentration of 2μM iodoacetamide and 15 μM p-CMB being sufficient to reduce enzyme activity by 80%. Cystamine was much less potent, a concentration of 600 μM being required for 50% inhibition of enzyme activity. With each of the different inhibitors, the response of enzyme activity to these compounds was non-linear. In the case of iodoacetamide and p-CMB there was an initial rapid reduction in activity with increasing concentration of inhibitor, whilst the 20% of activity remaining was less sensitive to subsequent increases in inhibitor concentration. For cystamine, 30% of enzyme activity was inhibited by relatively small additions of cystamine, whereas the remainder of the activity required much greater concentrations of cystamine for further inhibition. This difference in the response of enzyme activity to these compounds may result from either their differing

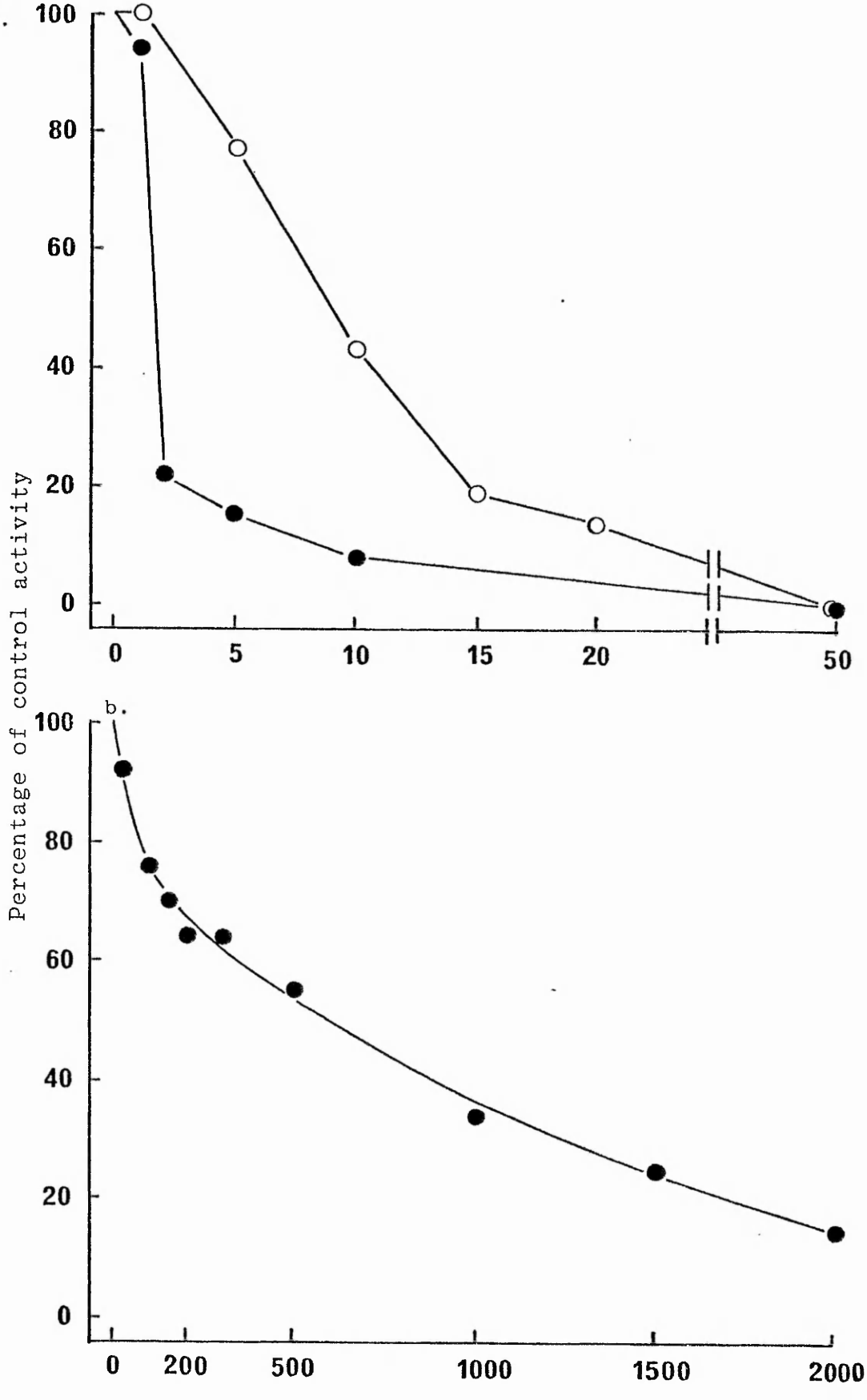
potency as inhibitors and/or from a difference in their mode of action. The mode of action of cystamine is probably complex and is discussed elsewhere.

Figure 3.5.a. and 3.5.b.

Sulphydryl-dependence of islet transglutaminase.

Supernatant extracts of islets were assayed for transglutaminase activity as described in Section 2.4.1. except that dithiothreitol was omitted from all incubations in order that the thiol-reactive compounds should retain their full reactivity. Iodoacetamide and cystamine were prepared in 50mM Tris and adjusted to pH 7.4 at 37^{o}C before serial dilutions were carried out in the same buffer. p-Chloromercuribenzoic acid was prepared in 100mM glycylglycine pH 8.0 and diluted in this buffer. The use of this buffer instead of 50mM Tris did not affect transglutaminase activity. Rates of putrescine incorporation were calculated after the subtraction of counts obtained from vials containing 5mM EDTA and are expressed as a percentage of the control system without inhibitor. The effects of iodoacetamide and p-chloromercuribenzoic acid on transglutaminase activity are shown in Fig. 3.5.a. where open circles refer to the assay system containing p-chloromercuribenzoic acid and closed cirles refer to the system containing iodoacetamide. The effect of cystamine is shown in Fig. 3.5.b.

3.5.a.


100
80
60
40
20
0
0
5
10
15
20
50
b.
100
80
60
40
20
0
0
200
500
1000
1500
2000
Percentage of control activity
Concentration of inhibitor (μM)

3.1.5. KINETIC EVALUATION OF PRIMARY AMINE INHIBITORS OF ISLET TRANSGLUTAMINASE ACTIVITY.

In order to characterise a number of primary amine substrates of transglutaminase (inhibitors of transglutaminase-catalysed protein cross-linking) for use in later studies, various primary amines were kinetically evaluated for their effects on [^{14}C]-putrescine incorporation into N,N'-dimethylcasein catalysed by islet transglutaminase. Extracts of islets (71,000 g supernatant) were assayed for transglutaminase activity in the presence of varying concentrations of both putrescine and primary amine inhibitor whilst the concentration of N,N'-dimethyl-casein was maintained constant at 5 mg/ml. The method of Lineweaver and Burk (1934) was then applied to the data to yield reciprocal plots from which the appropriate kinetic parameters could later be calculated. The plots obtained for the control systems without amine present yielded Km values for putrescine incorporation into N,N'-dimethylcasein between 0.2 and 1.1 mM with a mean of 0.59 ± 0.1 mM (n=8). Without further investigation this variability is difficult to explain, although it may bear some relation to the time and conditions of storage of the islet homogenate before its use. Vmax for putrescine incorporation into N,N'-dimethylcasein was also variable as would be expected when different preparations of enzyme are used; the average value of Vmax was 116.6 ± 11.3 pmol putrescine incorporated/h per 22.5µl enzyme sample.

The primary amines which were tested for their effects on transglutaminase activity were monodansylcadaverine, methylamine, ethylamine, propylamine, cystamine and tyramine. The results

for monodansylcadaverine, methylamine, ethylamine, propylamine and cystamine are shown in Figs. 3.6., 3.7., 3.8., 3.9. and 3.10. respectively. The plots obtained for monodansylcadaverine, methylamine, ethylamine and propylamine intersected at $\frac{1}{V_{max}}$ on the $\frac{1}{V_o}$ axis, indicating that these compounds are competetive inhibitors of transglutaminase activity in this system.

Cystamine exhibited behaviour characteristic of a mixed-type inhibitor of enzyme activity. This compound has previously been reported to be an active-site directed inhibitor of transglutaminase (Siefring et al., 1978), inhibition involving the formation of a disulphide bond between the enzyme active site cysteine residue and cysteamine with the dissociation of the two half-cystamine molecules. Cystamine or cysteamine may also act as primary amine substrates of transglutaminase, so that a competetive component as well as an irreversible component contribute to inhibition of enzyme activity. The result obtained for cystamine in the present work is consistent with this idea.

Tyramine was tested for its effects on islet transglutaminase activity and was found to be ineffective as an inhibitor up to a concentration of at least 5mM. Tyramine has been shown to be a substrate of guinea-pig liver transglutaminase (Pincus & Waelsch, 1968). It was not however a good substrate, possessing a Km for incorporation into the synthetic tripeptide carbobenzoxy-L-alanyl-L-glutaminyl-L-valine ethyl ester of 23.8mM. This poor affinity may explain the lack of effect of the compound when used at 1-5mM.

For determination of the potency of the primary amine inhibitors of islet transglutaminase activity the Ki value

Figures 3.6., 3.7., 3.8., 3.9. and 3.10.

Effects of monodansylcadaverine, methylamine ethylamine, propylamine and cystamine on islet transglutaminase activity.

Islet extracts (71,000g supernatant) were assayed for transglutaminase activity in duplicate in the presence of varying concentrations of putrescine and primary amine. The concentrations of putrescine used in the cases of monodansylcadaverine, methylamine, ethylamine and cystamine were 1.2, 0.5, 0.3 and 0.2mM and in the case of propylamine were 1.2, 0.6, 0.3 and 0.1mM. In the case of cystamine, dithiothreitol was omitted from all incubations. The amines were prepared in 50mM Tris, pH 7.4 at 37^{o}C and serial dilutions were prepared in this buffer. Initial rates of putrescine incorporation were calculated after the subtraction of background counts which were obtained from samples incubated in the presence of 5mM EDTA. Reciprocal plots of velocity (nmol putrescine incorporated / h) against substrate concentration (mM putrescine) were drawn for each concentration of primary amine.

Key to symbols used in the diagrams:

Fig. 3.6. : Concentration of monodansylcadaverine △ 5 μM

○ 10 μM

□ 20 μM

Fig. 3.7. : Concentration of methylamine △ 2 mM

○ 5 mM

□ 10 mM

Fig. 3.8. : Concentration of ethylamine △ 1 mM

○ 2 mM

□ 5 mM

Fig. 3.9. : Concentration of propylamine △ 1 mM

○ 5 mM

□ 10 mM

Fig. 3.10. : Concentration of cystamine △ 25 µM

○ 100 µM

□ 200 µM

● represents the control system without addition of inhibitory amine.

Fig. 3.6.

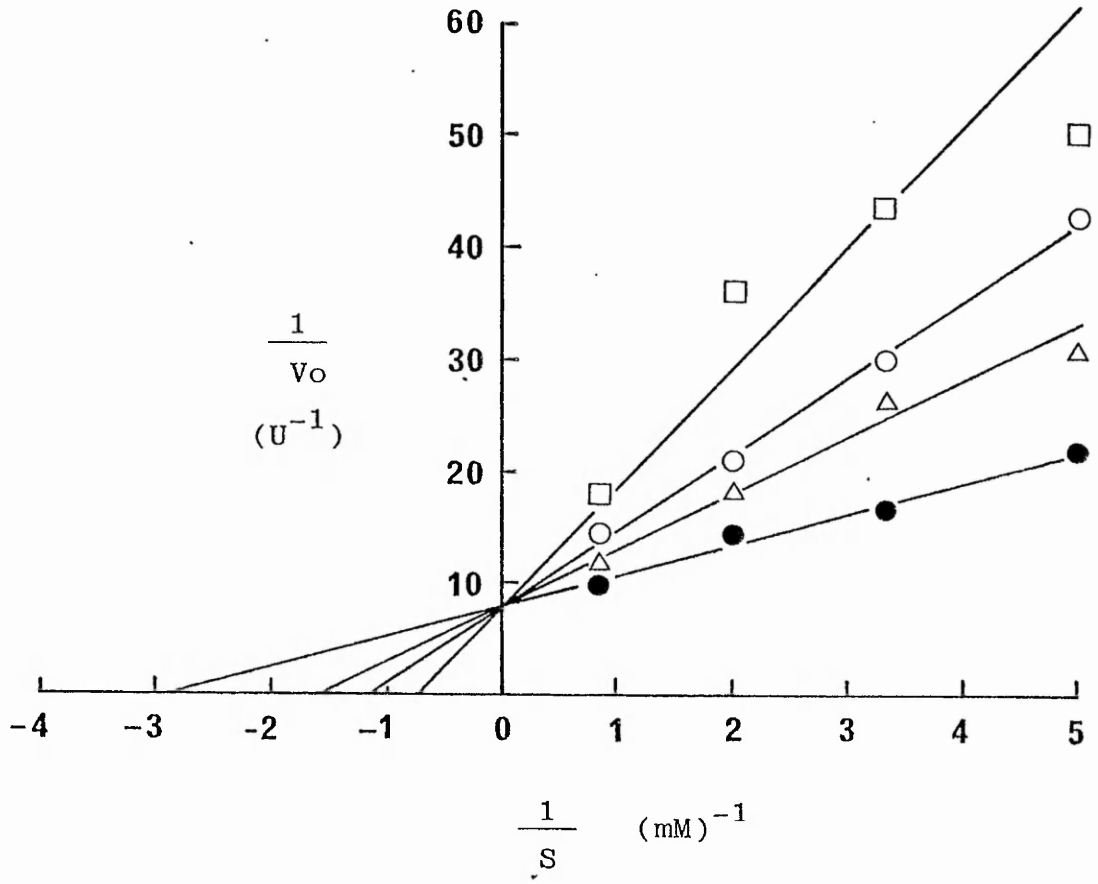


Fig. 3.7.

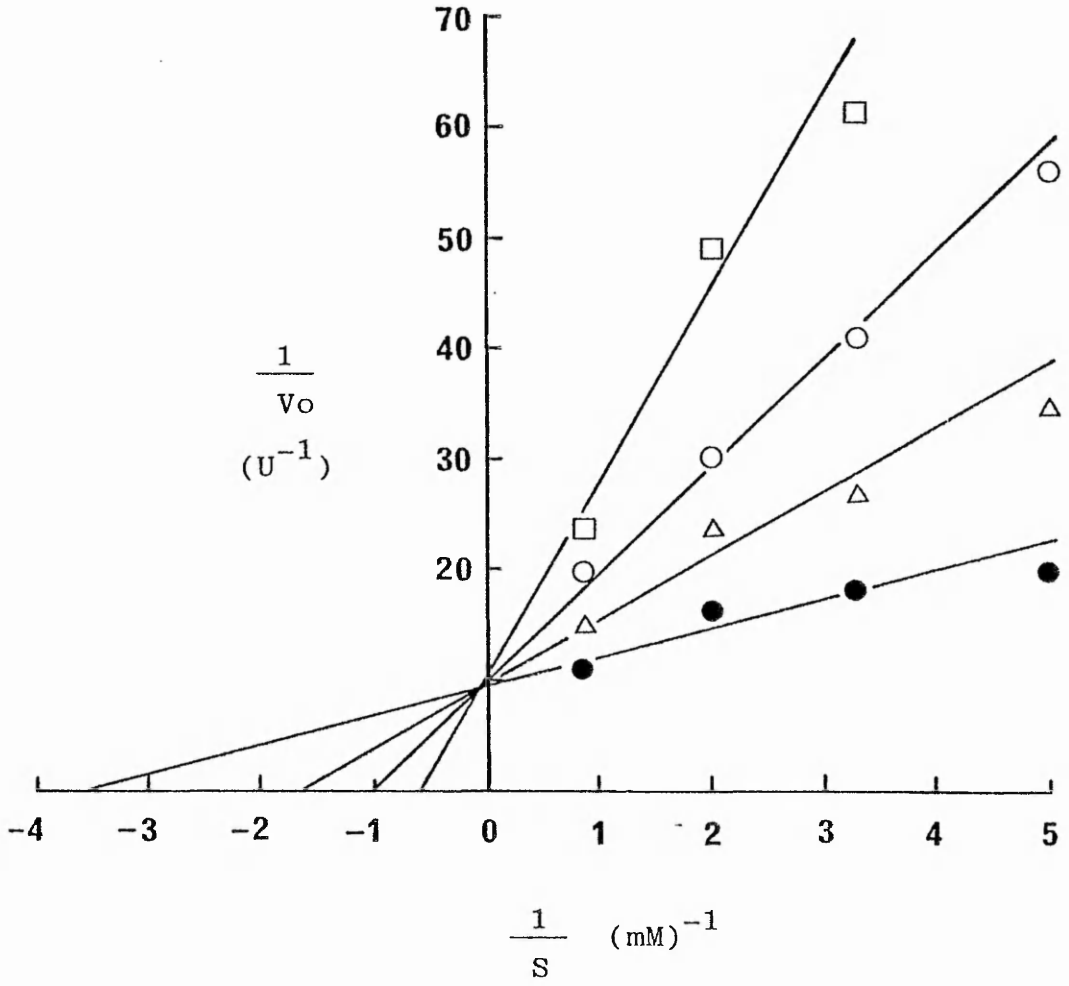


Fig. 3.8.

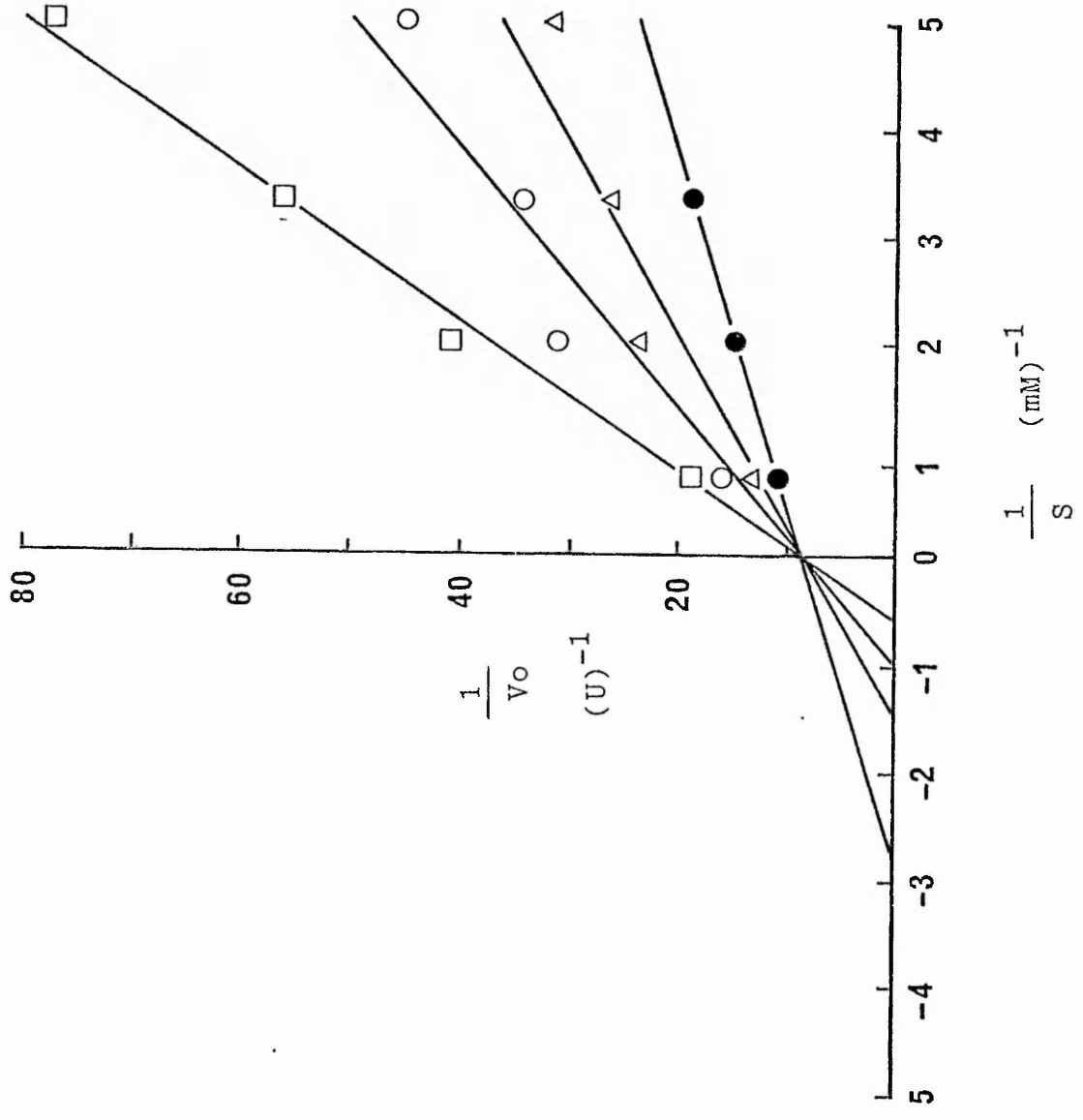


Fig. 3.9.

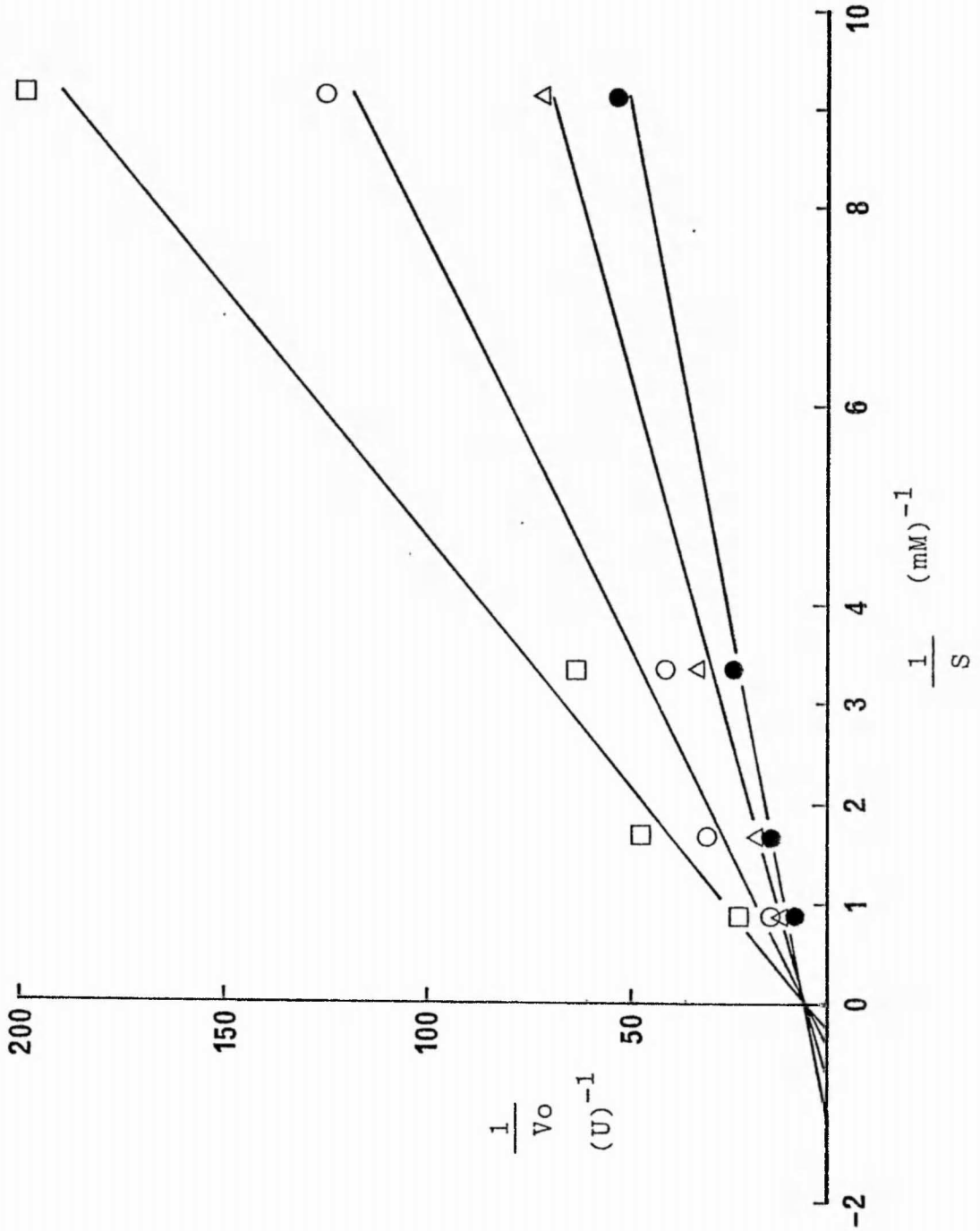
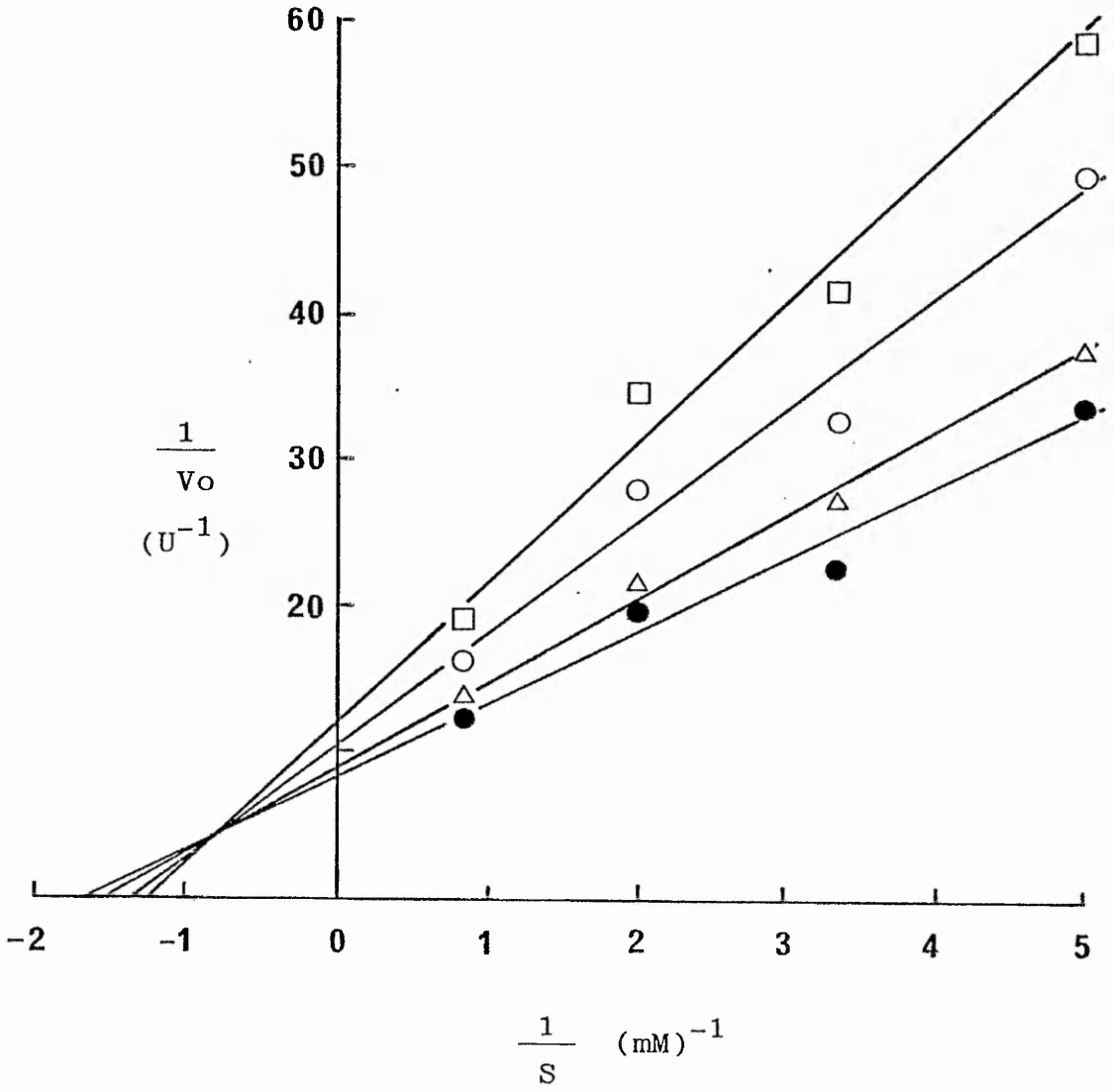


Fig. 3.10.



(inhibition constant) was determined which was calculated from the Lineweaver-Burk plots by the method of Dixon (1964). These re-plots are shown in Figs. 3.11, and 3.12. In the case of cystamine, two kinetic constants for inhibition may be calculated. However, due to the likely irreversible component contributing to inhibition by this compound, the value for Ki calculated would be meaningless.

The most potent inhibitor of islet transglutaminase activity was monodansylcadaverine, with a Ki of 7.3μM. The alkylamines methylamine, ethylamine and propylamine were far less potent with Ki values of 2.0mM, 1.75mM and 3.8mM respectively. Cystamine, for reasons stated above is not directly comparable in this regard.

Figures 3.11.a. and b and 3.12.a. and b.

Calculation of Ki for monodansylcadaverine, methylamine, ethylamine and propylamine by the method of Dixon.

The data in Figs. 3.6. and 3.7., 3.8. and 3.9. were re-plotted to yield graphs of $\frac{1}{Vo}$ against [I], the concentration of primary amine inhibitor at a fixed concentration of the substrate, putrescine. The concentration of inhibitor at the intercept on the [I] axis raised to the level on the $\frac{1}{Vo}$ axis of $\frac{1}{Vmax}$ is the Ki for that compound. The plots for monodansylcadaverine, methylamine, ethylamine and propylamine are shown in Figures 3.12.a., 3.12.b., 3.13.a. and 3.13.b. respectively.

Fig. 3.11.a.

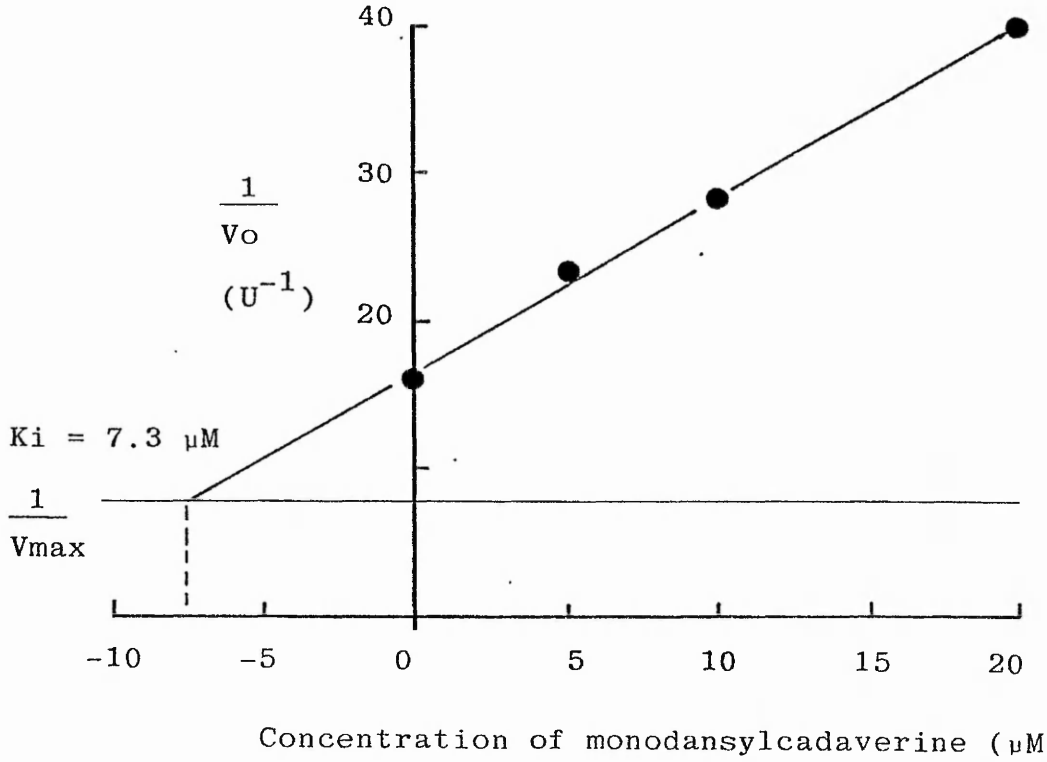


Fig. 3.11.b.

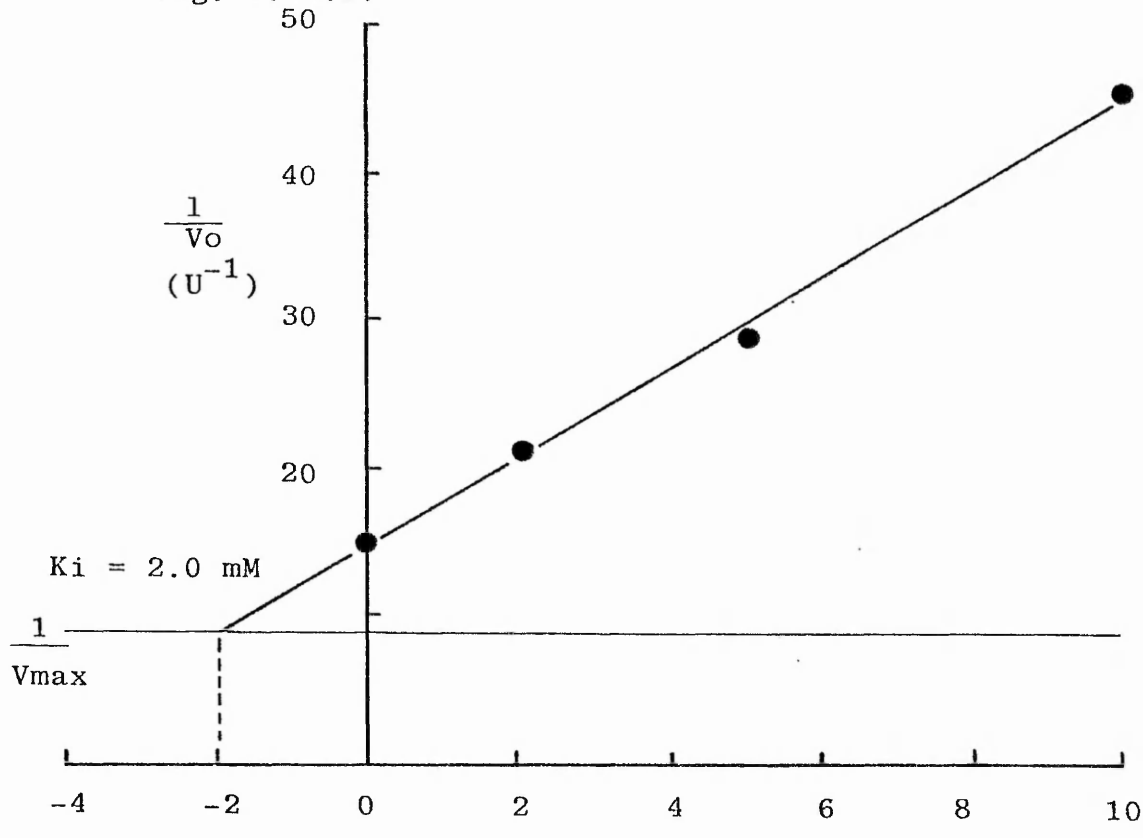


Fig. 3.12.a.

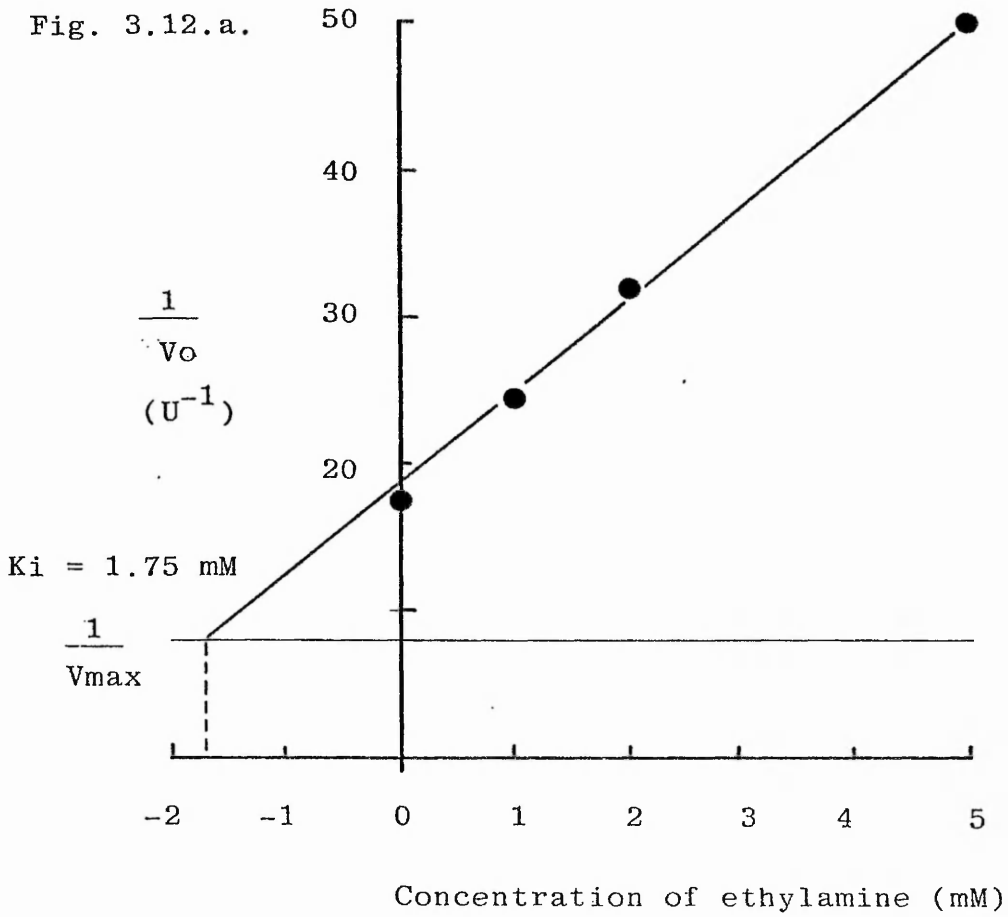
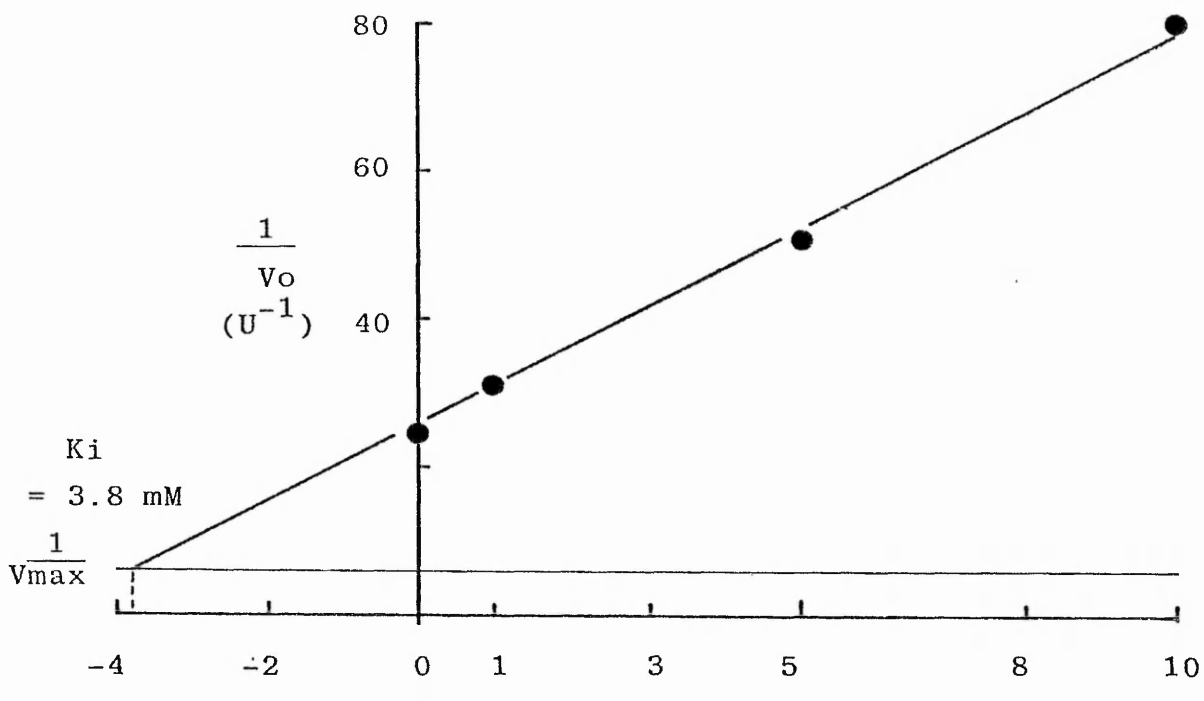


Fig. 3.12.b.



3.1.6. DISCUSSION.

Homogenates of islets of Langerhans catalysed the incorporation of [^{14}C]-putrescine into N,N'-dimethylcasein; this activity was inhibited by the Ca^{2+}-chelating agents EDTA and EGTA and by the thiol-reactive compounds iodoacetamide,p-chloromercuri-benzoic acid and cystamine. Therefore, the following criteria for the identification of transglutaminase activity were satisfied:

i. catalysis of amine incorporation into protein;

ii. requirement of enzyme activity for Ca^{2+};

iii. thiol-dependence of activity.

Apart from the importance of this conclusion to the present thesis, these results help to confirm the ubiquity of transglutaminase in mammalian cells.

The activity of islet transglutaminase was almost entirely restricted to the 71,000g supernatant obtained from homogenates of islets. Although the sonication procedure used to prepare homogenates may affect the distribution of activity, this result may reflect a cytosolic distribution of transglutaminase in the intact β-cell.

The sensitivity of the islet transglutaminase to Ca^{2+} concentration was investigated and revealed a half-saturation value for Ca^{2+} of 39 μM. Tissue transglutaminase from other sources has been reported to be less sensitive to Ca^{2+} than the islet enzyme. For example, erythrocyte transglutaminase requires 0.3mM Ca^{2+} for half-maximal velocity (Siefring *et al.*, 1978) and in early studies of guinea-pig liver transglutaminase it was found that approximately 1mM Ca^{2+} was required for maximal activity (Clarke *et al.*,

1959). However, the islet enzyme displayed a similar activation by Ca^{2+} to the enzyme isolated from rat liver which was found to give 40% of its maximal activity at 40 μM Ca^{2+} (Barnes, 1980).

Shortly after the completion of these studies, a report was published describing a similar investigation to that undertaken for this thesis (Gomis et al., 1983). These workers obtained similar results for the Ca^{2+}-activation of islet transglutaminase with a Km of 70 μM when the radiolabelled amine substrate was [^{14}C]-methylamine (0.5mM) and a Km of 90 μM when the amine was (2,5)-[^{3}H]-histamine (0.5mM). Furthermore, it was found that Mg^{2+} (1.0mM) failed to affect enzyme activity in the presence of 0.5mM Ca^{2+}.

Divalent cations of similar charge density to that of Ca^{2+}, including Mg^{2+}, Ba^{2+}, Mn^{2+} and Sr^{2+} have been reported to activate guinea-pig liver transglutaminase (Clarke et al., 1959; Folk & Cole, 1966). It appears from the results of these workers and from results reported in this thesis however, that islet transglutaminase is not activated by Mg^{2+} and furthermore that millimolar concentrations of Mg^{2+} which have been reported to exist in cells (Lehninger, 1980) are unlikely to interfere with a maximally active islet transglutaminase (see Section 3.1.3.).

The consideration of the Ca^{2+}-activation for islet transglutaminase is important for its implication in the Ca^{2+}-mediated mechanism of stimulus-secretion coupling in the pancreatic β-cell. The responsiveness of transglutaminase to Ca^{2+} may make the enzyme one of many possible targets for Ca^{2+} through which this cation mobilises the cell for periods of stimulated insulin release. In the resting β-cell, the cytosolic

free Ca^{2+}-concentration has been estimated at 0.1 μM (Wollheim & Sharp, 1981) suggesting that islet transglutaminase is inactive under these conditions. Although the half-saturation value for Ca^{2+} of islet transglutaminase may be rather high even compared to cytosolic Ca^{2+} concentrations in the stimulated ß-cell, release of Ca^{2+} from intracellular stores (e.g. endoplasmic reticulum, secretory vescicles) resulting in localised concentrations of Ca^{2+} in the vicinity of the enzyme may contribute to its activation. The sensitivity of islet transglutaminase to Ca^{2+} may therefore be compatible with a role for the enzyme in the mechanism of insulin release.

The inhibition of islet transglutaminase by the thiol-reactive compounds iodoacetamide, p-CMB and cystamine demonstrated the -SH dependence of the islet enzyme. The response of the enzyme to these compounds was non-linear. Similarly, Folk & Cole (1966) found that during inhibition of guinea-pig liver transglutaminase with pCMB and 5,5'-dithiobis(2-nitrobenzoic acid), the first 5 -SH groups of the enzyme reacted rapidly with these compounds, accounting for 85-95% of the total transglutaminase activity; thereafter, reaction with further -SH groups was slower. In the same study, a value for -SH content of 16-17 moles per mole of enzyme was determined. Total inhibition of enzyme activity was found to coincide with the reaction of one of these groups, the active site cysteine residue. Inhibition of transglutaminase by thiol reagents is therefore likely to result not only from their reaction with the active site thiol of the enzyme but with thiol groups elsewhere in the enzyme molecule.

During kinetic analysis of islet transglutaminase, the enzyme displayed a variable Km for putrescine incorporation into

N,N'-dimethylcasein with a mean value calculated as approximately 0.6mM. This value indicates that putrescine is a good substrate for islet transglutaminase.

The effects of a number of primary amine substrates of transglutaminase upon islet transglutaminase activity measured by [^{14}C] -putrescine incorporation into N,N'-dimethylcasein were kinetically evaluated. The effects of monodansylcadaverine, methylamine, ethylamine and propylamine were consistent with the well established view that the nature of inhibition of transglutaminase activity by these amines is competitive (Lorand et al., 1979). Furthermore, their relative potency as inhibitors was similar to that previously reported. The effect of cystamine on transglutaminase activity was characteristic of mixed type inhibition. The mode of action of this compound is probably complex and has been discussed (Section 3.1.5.).

In summary, a typical transglutaminase enzyme exists in islets of Langerhans whose sensitivity to Ca^{2+} may be compatible with a role in the secretory mechanism in the pancreatic β-cell. In addition, several primary amine substrates of the enzyme have been characterised for their ability to inhibit transglutaminase activity. A knowledge of the relative potency of these inhibitors was useful in the interpretation of the results of the studies on insulin release described in later studies for it allowed a comparison to be made between the potency of the effects of the amines on insulin release and transglutaminase activity.

3.2. PHYSIOLOGICAL STUDIES ON INTACT PANCREATIC ISLETS.

These studies were undertaken to test the hypothesis for the involvement of islet transglutaminase in the mechanism of insulin release from the pancreatic β-cell. This hypothesis demands that interference with transglutaminase activity in islets would lead to inhibition of insulin release by intact islets. Therefore, the effects on insulin release of inhibitors of transglutaminase activity were determined and the specificity of these effects with regard to transglutaminase investigated.

3.2.1. EXPERIMENTS ON INSULIN RELEASE.

3.2.1.1. Stimulation of insulin release from intact islets.

When islets were incubated in batches as described in section 2.6.5.1.1., changing the concentration of glucose in the medium from 2.8mM to 16.8mM led to a 6 to 20 fold increase in the rate of insulin release over a 1 h period. The variability in the secretory response of islets to glucose was the result of the use of different isolated populations of islets for each experiment. Therefore in the experiments in the following section, rates of basal and stimulated insulin release were determined for untreated islets which acted as controls in each experiment. Over the course of these experiments, average rates of insulin release for islets incubated at 2.8mM glucose were 8.9 $\pm$ 0.8 μU/h/islet (n=56) and for islets incubated at 16.8mM glucose were 95.4 $\pm$ 6.2 μU/h/islet (n=49).

3.2.1.2. Effects of primary amines on insulin release.

Table 3.3.

Effects of primary amines upon basal insulin release.

Compound tested	Concentration (mM)	Insulin release (μU/h/islet)	
Monodansyl-cadaverine	-	15.5 ± 2.4	(4)
	0.02	16.9 ± 2.5	(4) N.S.
	0.1	12.8 ± 1.7	(3) N.S.
Methylamine	-	5.5 ± 0.4	(6)
	15.0	8.8 ± 0.4	(4) $p \leq 0.001$
Propylamine	-	4.4 ± 1.5	(4)
	10.0	5.9 ± 1.0	(4) N.S.
Cystamine	-	5.5 ± 0.4	(6)
	1.0	6.0 ± 0.6	(5) N.S.

Batches of five islets were incubated at 37°C for 45 min in 0.5ml KRB medium containing 2.8mM glucose followed by 60 min in 1.0ml of the same medium. Samples of medium were taken and their insulin content determined by radioimmunoassay. Rates of insulin release over the total period of incubation are expressed as μU insulin/h/islet ± S.E.M., and figures in parentheses refer to number of batches of islets. The significance of difference from control incubations performed in each experiment was assessed using the Students' t-test. N.S. denotes no significant difference from controls.

The effects of monodansylcadaverine, methylamine, propylamine and cystamine on insulin release from islets incubated at 2.8mM glucose are shown in Table 3.2. Monodansylcadaverine at (0.02 and 0.1mM), propylamine (10mM) and cystamine (1.0mM) did not significantly affect basal insulin release. Methylamine, however, displayed a small but highly significant stimulation of insulin release, amounting to a 60% increase over the control rate.

The effects of primary amines on glucose-stimulated insulin release at 16.8mM glucose are shown in Table 3.3. The amines tested inhibited glucose-stimulated insulin release with a potency which varied with the amine tested. Inhibition was dose-dependent in the cases of monodansylcadaverine, methylamine, ethylamine and cystamine. Incubation of methylamine (1.5mM), ethylamine (1mM) and cystamine (0.1mM) with rat insulin standards during the radioimmunoassay indicated that these compounds did not interfere with the measurement of insulin. Therefore, the inhibition of insulin release observed was not due to the interference of the amines with the insulin assay. Monodansylcadaverine was the most potent inhibitor of insulin release and was effective in the concentration range 10 to 100 μM. Of the other amines, cystamine was effective over the range 0.1 to 1.0 mM, whilst methylamine, ethylamine and propylamine were of a similar potency to each other and effective in the range 1 - 5 mM.

The naturally occuring diamine putresine, which was used as the radiolabelled amine substrate in the assay for transglutaminase was also tested for its effect on glucose-stimulated insulin release. At the relatively high concentration of 20 mM, this compound proved to be fairly ineffective in the inhibition of insulin release (Table 3.3). Some reports suggest that

Table 3.4.

Effects of primary amines on glucose-stimulated insulin release from islets of Langerhans.

Batches of 5 islets were incubated at 37°C for 45 min in 0.5 ml KRB containing 2.8mM glucose followed by 60 min in 1.0 ml KRB containing 16.8 mM glucose. Samples of medium were taken and insulin released into the medium was determined by radioimmunoassay. The concentrations of inhibitors stated were present throughout the total period of incubation (105 min). Control incubations using islets from the same isolation were carried out in the absence of primary amines in each experiment. Results are expressed as a percentage inhibition $\pm$ S.E.M. of stimulated insulin release obtained in control incubations after the subtraction of basal rates of insulin release. Figures in parentheses refer to numbers of batches of islets. The significance of difference from controls was determined by the Students' t-test; N.S. denotes no significant difference. * denotes that 16.8mM glucose was present over 45 min instead of 60 min and a pre-incubation period of 75 min at 2.8mM glucose was carried out in the presence and absence of primary amine.

Test Compound	Concentration (mM)	Percentage inhibition of glucose-stimulated insulin release		
Monodansyl-cadaverine	0.01	31 ± 8	p≤0.05	(4)
	0.02	41 ± 1	p≤0.01	(4)
	0.05	90 ± 12	p≤0.01	(6)
	0.10	91 ± 8	p≤0.001	(4)
	0.10	85 ± 8	p≤0.001	(5)
Cystamine	0.10	47 ± 17	p≤0.25	(6)
	0.50	79 ± 10	p≤0.05	(6)
	1.0	98 ± 4	p≤0.002	(6)
Methylamine	1.0	51 ± 5	p≤0.001	(6)
	5.0	78 ± 8	p≤0.01	(4)
	15.0	99 ± 6	p≤0.001	(4)
Propylamine	0.5	61 ± 6	p≤0.01	(5)
	1.0	47 ± 11	p≤0.05	(4)
	5.0	95 ± 2	p≤0.001	(5)
Ethylamine	1.0	50 ± 9	p≤0.005	(7)
	5.0	81 ± 8	p≤0.001	(6)
Putrescine	20.0	30 ± 5	p≤0.45	(4)
	20.0 *	20 ± 7	N.S.	(5)

putrescine does not freely enter cells (Pohjanpelto, 1976; Davies et al., 1980). Therefore, in another experiment, the preincubation period at 2.8mM glucose was extended by 30 min to 75 min in an effort to allow more putrescine to enter the islet cells before stimulation of islets by glucose. However, no significant inhibition of insulin release by putrescine at 20mM was observed over a stimulation period of 45 min at 16.8mM glucose following this preincubation (Table 3.4).

3.2.1.3. Correlation of inhibition of insulin release with inhibition of transglutaminase.

The range of concentration over which primary amines inhibited glucose-stimulated insulin release was compared with their Ki values for inhibition of transglutaminase activity. This comparison, shown in Table 3.5., shows that the relative potency of the amines for inhibition of transglutaminase activity correlates well with their relative potency with regard to their inhibition of glucose-stimulated insulin release. This correlation represents good evidence for the involvement of islet transglutaminase in the mechanism of glucose-stimulated insulin release.

Putrescine was not a potent inhibitor of glucose-stimulated insulin release in spite of the fact that it was found to be a good substrate for islet transglutaminase (section 3.1.5.). In this respect, this compound does not follow the correlation described above. Whilst one may argue that this observation detracts from the evidence for the involvement of transglutaminase in insulin release, the

possibility already mentioned that putrescine does not enter the islet β-cell may account for this apparent anomaly. A more intriguing explanation is that putrescine may itself be involved in the secretory response of islets to glucose. These possibilities were tested and the results are presented and discussed in sections 3.2.4., 3.2.5. and 3.2.6.

Table 3.5.

Correlation of inhibition of insulin release with inhibition of islet transglutaminase.

Compound	Ki for inhibition of putrescine incorporation into N,N'-dimethylcasein	Inhibition of insulin release	
		Range of % inhibition	Range of inhibitory concentration
Monodansyl-cadaverine	7.3 μM	31 - 91	10 - 100 μM
Methylamine	2.0 mM	51 - 99	1 - 15 mM
Ethylamine	1.75 mM	50 - 81	1 - 5 mM
Propylamine	3.8 mM	61 - 95	0.5 - 5 mM

3.2.2. THE NON-SPECIFIC TOXIC EFFECTS OF PRIMARY AMINES ON ISLETS.

It is possible that the amines tested and used in the previous studies are toxic to cells and inhibit insulin release by other non-specific mechanisms. In the case of islet β-cells, this consideration is particularly important in view of the intimate involvement of islet β-cell metabolism in their secretory activity (Hedeskov, 1980). Glucose metabolism is widely accepted as constituting the 'glucoreceptor' in the mechanism of glucose-stimulated insulin release and for sustained insulin release, a supply of insulin destined for export must be maintained by the β-cell. Therefore, two parameters, glucose oxidation and protein synthesis, both of which are stimulated in islets by glucose, were chosen for investigation in these toxicity studies.

3.2.2.1. Effects of primary amines upon glucose-stimulated glucose utilisation

During these studies in which release of $^{14}CO_2$ from [U-^{14}C] glucose was used as a measure of glucose oxidation, increasing the concentration of glucose in the incubation medium led to approximately a 10 - fold increase in the rate of glucose oxidation. However, different isolated batches of islets differed in their ability to utilise glucose. Therefore, in common with the insulin release studies, batches of islets were incubated in the absence of test compounds in each experiment to yield control values of glucose utilisation.

The effects of monodansylcadaverine, methylamine, ethylamine, propylamine and cystamine on islet glucose-stimulated glucose oxidation are shown in Table 3.6. When monodansylcadaverine was tested at 100μM, the highest concentration of this compound used in the insulin release studies, it did not significantly affect islet glucose oxidation. However, all of the other amines tested inhibited this metabolic process to varying degrees. Cystamine was a potent inhibitor, effective over the same concentration range with which it was effective in the inhibition of glucose-stimulated insulin release. The importance of glucose metabolism in the mechanism of glucose-stimulated insulin release suggests that cystamine could exert its effects on insulin release solely by virtue of this non-specific effect. Therefore, it is diffcult to separate the toxicity of cystamine from any effect it may have upon transglutaminase activity in the β-cell with regard to inhibition of insulin release. High concentrations of the alkylamines methylamine (15mM) and propylamine (5mM) inhibited glucose utilisation by approximately 50%, whilst ethylamine at 5mM had no significant effect. In the cases of methylamine and propylamine, however, it was possible to define concentrations of these compounds which did not inhibit glucose oxidation but which still gave rise to inhibition of glucose-stimulated insulin release. These concentrations were 5mM in the case of methylamine and 1mM in the case of propylamine.

3.2.2.2. Effects of primary amines on synthesis of total islet protein.

In the course of these studies, protein synthesis

Table 3.6.

Effects of primary amines on glucose-stimulated glucose utilisation.

Batches of 10-20 islets were incubated with and without the concentrations of compounds shown in the table. These were present throughout an incubation period consisting of 45 min in 50µl KRB containing 2.8 mM glucose and a further 60 min in 100µl KRB containing 16.8 mM [U-^{14}C]- glucose (0.595mCi mmol^{-1}). $^{14}CO_2$ released by islets was collected with hyamine hydroxide after acidification of the incubation medium and the radioactivity in the hyamine determined by scintillation counting (see section 2.6.4.2.). Rates of glucose utilisation were expressed as pmol glucose/ h/10 islets and results are expressed as a percentage inhibition $\pm$ S.E.M. of control rates determined for untreated islets in the same experiment. Figures in parentheses refer to numbers of batches of islets; the significance of difference from controls was determined using the Students' t-test. N.S. denotes no significant difference from controls.

* denotes that $^{14}CO_2$ release was measured over 120 min.

Typical rates of islet glucose utilisation were, for islets at 2.8mM glucose 28.9 $\pm$ 4.6 pmol /h/10 islets (n=4) and for islets at 16.8mM glucose 306 $\pm$ 21 pmol/h/10 islets (n=31).

Compound tested	Concentration (mM)	Percentage inhibition of glucose-stimulated glucose utilisation		
Monodansyl-cadaverine	0.1	0	(4)	N.S.
	0.1	*19.8 ± 17.0	(3)	N.S.
Methylamine	5.0	5.1 ± 7.1	(4)	N.S.
	15.0	55.4 ± 4.0	(5)	$p \leq 0.001$
Ethylamine	5.0	8.7 ± 6.9	(4)	N.S.
Propylamine	1.0	19.0 ± 19.0	(4)	N.S.
	5.0	48.0 ± 8.0	(3)	$p \leq 0.01$
Cystamine	0.1	23.8 ± 11.0	(5)	$p \leq 0.25$
	1.0	90.3 ± 9.0	(4)	$p \leq 0.002$

measured by [^{3}H]-leucine incorporation into total islet TCA precipitable material was increased 3 to 4 fold when the medium glucose concentration was raised from 2.8 to 16.8mM. Control batches of islets were included in each experiment in order to minimise the effects of variation between islet populations in their ability to synthesise protein.

The effects of primary amines upon glucose-stimulated [^{3}H]-leucine incorporation into total islet protein are shown in Table 3.6. At the concentration of 100μM, monodansylcadaverine did not significantly affect this metabolic process. However, methylamine and ethylamine inhibited [^{3}H]-leucine incorporation to varying degrees. These effects were more potent than those on glucose oxidation: concentrations of 2mM methylamine and 1mM ethylamine were sufficient to significantly inhibit [^{3}H]-leucine incorporation. Propylamine was tested at 1mM and at this concentration did not give rise to significant inhibition of [^{3}H]-leucine incorporation. In the case of methylamine, it was possible to define a concentration (1mM) which did not inhibit [^{3}H]-leucine incorporation but which still gave rise to inhibition of insulin release over the same experimental time period. This was not possible, however, in the case of ethylamine. Experiments were therefore undertaken whose aims were, firstly to determine whether inhibition of [^{3}H]-leucine incorporation represented a direct measurement of inhibition of protein synthesis and secondly to determine whether inhibition of protein synthesis leads to inhibition of insulin release under the conditions of these experiments. These experiments are described below (Sections 2.2.2.3. and

Table 3.7.

The effects of primary amines on synthesis of total islet protein in glucose-stimulated islets.

Batches of 10-20 islets were incubated at 37°C in 50µl KRB containing 2.8mM glucose and the appropriate concentration of test compound for 45 min before an incubation of 60-90 min also with test compound in 100µl KRB containing 16.8 mM glucose and 5µCi [^{3}H] -leucine (55-60 Ci/mmol). Following incubation, the TCA precipitable material in the incubations was washed free of unbound radioactivity, solubilised and the bound radioactivity determined by scintillation counting (see Section 2.6.4.3.). Rates of glucose-stimulated incorporation of [^{3}H]-leucine were expressed as d.p.m./h/10 islets and the results expressed in the table as a percentage inhibition $\pm$ S.E.M. of control rates determined for untreated islets in the same experiment. Figures in parentheses refer to numbers of batches of islets; the significance of difference from controls was determined using the Students' t-test.

N.S. denotes no significant difference from controls.

* denotes measurement of [^{3}H]-leucine incorporation over 90 min.

Typical control rates of [^{3}H]-leucine incorporation were at 2.8mM glucose 72,671 $\pm$ 5634 d.p.m./h/10 islets (n=20) and at 16.8mM glucose 306,471 $\pm$ 21,392 d.p.m./h/10 islets (n=35).

Test compound	Concentration	Percentage inhibition of glucose-stimulated [^{3}H]-leucine incorporation into islet protein	
Monodansyl-cadaverine	0.1	* 0	(3) N.S.
Methylamine	1.0	10.2 ± 3.2	(4) N.S.
	2.0	25.5 ± 5.5	(5) $p \leq 0.1$
	5.0	79.3 ± 4.0	(5) $p \leq 0.02$
Ethylamine	1.0	38.2 ± 7.1	(5) $p \leq 0.01$
	5.0	49.0 ± 12.6	(4) $p \leq 0.05$
Propylamine	1.0	19.5 ± 18.0	(3) N.S.

3.2.2.4.).

3.2.2.3. Effects of amines on [^{3}H]-leucine uptake by islets.

An apparent inhibition of protein synthesis measured by [^{3}H]-leucine incorporation could result from an inhibition of [^{3}H]-leucine uptake leading to an alteration in the specific activity of [^{3}H]-leucine in the islet cells. In order to test this possibility, the effects of methylamine and ethylamine on [^{3}H]-leucine uptake were measured. In these experiments, 0.1mM of the inhibitor of protein synthesis, cycloheximide was included in all incubations in order to equalise the effects of the drain of [^{3}H]-leucine into synthesised protein.

3.2.2.3.1. Calculation of sucrose space of islets.

In studies of uptake of radiolabelled compounds, allowance must be made for the contribution of intercellular spaces to the apparent uptake. The use of [^{3}H]-sucrose as an extracellular marker has been successful (Wollheim et al., 1977; 1978) and was used in these studies to enable a correction to be made for the islet intercellular space.

Islets were incubated for either 30 or 45 min in KRB containing 2.8mM glucose in the presence of either 10.2μM or 20mM [^{3}H]-sucrose according to the method described in Section 2.6.4.4.. The assumption was made that, after subtraction of background, any apparent uptake of sucrose detected by this method was the result of the permeation of the compound between and not into the islet cells. The sucrose space was found to be independent of the incubation times used

and the final concentration of radiolabel. An average value of 2.84 ± 0.27 nl/islet (n=11) was calculated for the islet sucrose space. This compares with values found by other workers of 6.4 nl/islet (Morgan and Montague, 1982) and 1.22 ± 0.10 nl/islet (Wollheim et al., 1978). The value obtained was used to calculate a value for the subtraction of extracellular [^{3}H]-leucine in the studies described below.

3.2.2.3.2. Effects of methylamine and ethylamine on uptake of [^{3}H]-leucine by islets.

In a time-course identical to that over which protein synthesis was measured, 5mM of methylamine or ethylamine gave rise to 35± 17% and 48 ± 9% inhibition of [^{3}H]-leucine uptake respectively (Table 3.7.). When these concentrations were reduced to 1mM for ethylamine and 2mM for methylamine, no significant inhibition of [^{3}H]-leucine uptake could be detected. Therefore at the higher concentrations of these amines, inhibition of [^{3}H]-leucine incorporation may include a component which is due to inhibition of [^{3}H]-leucine uptake. However, at lower amine concentrations, inhibition of uptake of [^{3}H]-leucine probably does not contribute to inhibition of [^{3}H]-leucine incorporation which may then reflect true inhibition of islet protein synthesis.

3.2.2.4. Assessment of the effect on insulin release of inhibition of protein synthesis.

The results described previously for the effects of methylamine, ethylamine and propylamine on [^{3}H]-leucine incorporation into islet protein suggest that inhibition of

Table 3.8.

Effects of methylamine and ethylamine on [^{3}H]-leucine uptake in glucose-stimulated islets.

In two separate experiments, a and b, batches of 10 or 12 islets were incubated at 37^{o}C in 400µl microcentrifuge tubes in 50µl KRB containing 2.8mM glucose and the appropriate concentration of test compound followed by 60 min in the same medium containing 16.8mM glucose, 5µCi [^{3}H]-leucine (55 Ci/mmol) and test compound. Cycloheximide was included in all incubations at a final concentration of 0.1mM for the entire period of incubation. Incubations were terminated by separation of the islets from the medium by centrifugation through phthalate esters in a microcentrifuge (see Section 2.6.4.4.). The radioactivity remaining in the islets was determined by scintillation counting and the uptake expressed as pmol /h/10 islets after subtraction of the sucrose space equivalent. The significance of difference from controls was determined using the Students' t-test; N.S. denotes no significant difference from controls. Figures in parentheses refer to numbers of batches of islets.

Concentration of amine (mM)	Uptake of leucine (pmol/h/10 islets)		
a.			
-	0.317 ± 0.059	(5)	
Methylamine 5mM	0.205 ± 0.055	(5)	$p \leq 0.25$
Ethylamine 5mM	0.164 ± 0.029	(5)	$p \leq 0.05$
b.			
-	0.570 ± 0.123	(3)	
Methylamine 2mM	0.662 ± 0.222	(3)	N.S.
Ethylamine 1mM	0.619 ± 0.147	(4)	N.S.

protein synthesis still occurs even at low concentrations of these alkylamines. This non-specific effect could conceivably contribute to inhibition of insulin release by these amines. So that the contribution, if any, of protein synthesis inhibition to inhibition of insulin release could be assessed, the effects of the protein synthesis inhibitor, cycloheximide on [^{3}H]-leucine incorporation and insulin release were measured. Experimental protocols identical to those used for the studies on primary amines were maintained.

The results of this investigation are shown in Table 3.9 Cycloheximide was a potent inhibitor of protein synthesis when tested at concentrations ranging from 10μM to 1.0mM. At a concentration of 10μM, this compound reduced glucose-stimulated [^{3}H]-leucine incorporation to levels comparable to those found for un-stimulated islets. At the same concentration, cycloheximide was found not to significantly affect glucose-stimulated insulin release from islets (Table 3.9.). Therefore, even drastic inhibition of protein synthesis is unlikely to contribute significantly to inhibition of glucose-stimulated insulin release over the time course of the experiments performed in these studies. In a similar investigation by Morris and Korner (1970), 200 μg/ml cycloheximide inhibited 85% of total islet protein synthesis whilst it failed to inhibit insulin release from islets in the first hour after raising the medium glucose concentration from 2.0 mM to 20mM. These results are in accord with those presented here.

Table 3.9.

Effect of cycloheximide on glucose-stimulated synthesis of total islet protein and glucose-stimulated insulin release.

Concentration of cycloheximide (μM)	Incorporation of [^{3}H]-leucine (d.p.m./h/10 islets		Insulin release (μU/h/islet)	
-	280,279 ± 20,067	(4)	86.6 ± 21.1	(4)
10	74,779 ± 9,766	(4) *	71.4 ± 5.8	(5) N.S.
-	302,976 ± 7,620	(4)		
100	33,975 ± 1,451	(4) *	N.T.	
-	118,069 ± 14,022	(3)		
1000	6,531 ± 285	(3) *	N.T.	

The experimental protocols for measurement of[^{3}H]-leucine incorporation into total islet protein and insulin release were identical to those used in the studies on primary amines. The results shown are from four separate experiments in each of which control incubations containing no cycloheximide were carried out. The significance of difference from controls was determined using the Students' t-test: * denotes $p \leq 0.001$; N.S. denotes no significant difference from controls. Figures in parentheses refer to numbers of batches of islets. N.T. denotes not tested.

3.2.3. DISCUSSION

The results which have been described demonstrate that primary amines inhibited glucose-stimulated insulin release from islets of Langerhans with the same relative potency with which these compounds inhibited islet transglutaminase activity. It is therefore tempting to speculate that inhibition of insulin release by primary amines is the result of their interference with a transglutaminase-dependent step in the mechanism of glucose-stimulated insulin release from the pancreatic β-cell.

Studies on the effects of these amines on islet glucose oxidation and protein synthesis, however, suggested that methylamine, ethylamine, propylamine and cystamine displayed varying degrees of toxicity to islets. Indeed, cystamine was such a potent inhibitor of glucose utilisation that this effect alone could account for the inhibition of insulin release by this compound. Whilst this does not rule out the possibility of a specific interaction of cystamine with transglutaminase, no involvement of the enzyme can be inferred from the effects of cystamine in this system. It was possible to define concentrations of methylamine, ethylamine and propylamine which still gave rise to inhibition of glucose-stimulated insulin release in the absence of any significant effect on glucose oxidation. Experiments with cycloheximide suggested that even substantial inhibition of glucose-stimulated protein synthesis in islets does not significantly affect rates of insulin release under the experimental conditions employed. Therefore, although methylamine, ethylamine and propylamine have non-specific effects

at high concentrations, these effects cannot account for inhibition of insulin release at concentrations below which glucose oxidation is not affected. It is suggested that this component of inhibition is due to inhibition of transglutaminase activity in the islet β-cell.

These results serve to illustrate the need for care in the interpretation of similar pharmacological studies with regard to the specificity of the effects of compounds on cellular events. This is particularly relevent in the case of studies where effects of amines on receptor-mediated endocytosis were measured (e.g. Davies et al., 1980; King et al., 1981).

In the case of studies aimed at the implication of transglutaminase in cellular events, specific substrates or other inhibitors of transglutaminase should be used wherever possible (e.g. monodansylcadaverine, tosyl-cadaverine). Such compounds may be used at far lower concentrations than less potent inhibitors like alkylamines and thus may exert inhibitory effects in the absence of non-specific effects. This is well illustrated in the present work by monodansylcadaverine. Such specific compounds have been characterised by Lorand et al., (1979) but unfortunately most of these compounds are not commercially available.

There may be other cellular processes which are inhibited by amines besides glucose oxidation and protein synthesis. Indeed, some inhibition of leucine uptake was observed at millimolar concentrations of methylamine and ethylamine. This may be a direct effect on leucine uptake or an indirect effect reflecting other non-specific effects of these compounds. These effects could be explained by the existence of charged forms of

of the amines. At physiological pH, a proportion of the total concentration of amine is protonated, the proportion of cation present depending on the pKa of the amine. These molecules could displace membrane-bound cations (e.g. Ca^{2+}, Mg^{2+}) from anionic sites (Ganguly & Bradford, 1982; Koenig et al., 1983) or directly interfere with ion transport by competition. In this context, the slight stimulation of insulin release at 2.8mM glucose by methylamine may be relevent. Protonated methylamine may be able to displace sufficient Ca^{2+} from anionic binding sites or intracellular stores to cause a small rise in the cytosolic free concentration of Ca^{2+}. This rise may be sufficient to cause an increase in the rate of insulin release which is regulated by this cation. Whether the stimulation of insulin release by methylamine resembles the first phase of glucose-stimulated insulin release associated with release of Ca^{2+} from intracellular stores requires investigation. It is also possible that interference with the fluxes of other ions may lead to a slight stimulation of insulin release by methylamine.

By virtue of their nature as weak bases, amines may alter the internal pH of cells, particularly in organelles with acidic interiors. Amines enter cells in their uncharged forms and are protonated in regions of low pH. The protonation of amines inside vesicles of low pH (e.g. lysosomes, endosomes) leads to their accumulation inside these vesicles resulting from the impermeability of membranes to the charged amine species. Such accumulation leads to an increase in the intra-vesicular pH (Ohkuma &Poole, 1978) and consequent interference with lysosomal function. The interference of amines with membrane flow as a

result of this mechanism was the subject of a recent review (Dean et al., 1984). In the case of insulin secretion from the pancreatic β-cell, however, there is no evidence to suggest that lysosomes are involved in the mechanism of endomembrane flow, recycling of membrane taking place through the Golgi complex (Orci et al., 1978).

Also relevent in this context is the observation that the weak bases ammonium ion (20mM) and imidazole (10mM) inhibit insulin release from islets by up to 70%. These compounds raise the internal pH of the islet β-cell and may affect the coupling between glucose metabolism and insulin release (Smith and Pace, 1983). However, the concentrations of amines used in the present work which do not affect islet glucose utilisation or protein synthesis were much lower than those used to lower intracellular pH and may not alter internal pH. Furthermore, the dimethylated analogue of monodansylcadaverine, dimethyldansylcadaverine, which is more basic than monodansylcadaverine only inhibits glucose-stimulated insulin release from islets by about 30% at 100μM (Dr. M. Griffin personal communication).

The conclusion of these arguments is that inhibition of insulin release by primary amines is a function of their specificity for transglutaminase and not of their basicity.

An apparently anomalous observation of the context of the argument above was that putrescine, a good substrate of islet transglutaminase failed to act as an effective inhibitor of glucose-stimulated insulin release. Two main possibilities arise as explanations:

i. Putrescine does not enter the islet β-cell and therefore

cannot act upon an intracellular transglutaminase-dependent step;

ii. Putrescine is involved in the process of insulin secretion either in the mechanism of stimulation by glucose or in subsequent steps in the secretory pathway or both.

The first possibility is suggested by reports mentioned previously that some cells are not freely permeable to putrescine. Also noteworthy is the point that if putrescine entered islet cells to the high concentrations used in the insulin release experiments it is possible that it may inhibit insulin release by non-specific mechanisms, considering the toxicity of the alkylamines methylamine, ethylamine and propylamine.

The second possibility was suggested by the fact that putrescine is a natural substrate for transglutaminases. Polyamines (putrescine, spermidine and spermine) have been shown to be substrates of transglutaminases (Williams-Ashman et al., 1980; Williams-Ashman & Cannelakis, 1980; Chan et al., 1981) and can be incorporated into proteins of intact cells (Folk et al., 1980; Cannelakis et al., 1981; Patterson et al., 1982). In studies by Folk et al., (1980), Schrode & Folk (1978) and Williams-Ashman et al., (1980), the existence of diamine cross-links (bis-(γ -glutamyl) putrescine, bis-(γ -glutamyl) spermidine and bis-(γ -glutamyl)spermine)as a result of transglutaminase activity was demonstrated in rat seminal plasma in which both primary amine groups of the polyamines participated in acyltransfer reactions (see Fig. 1.1. and section 1.4.7.). It is therefore possible that transglutaminase-catalysed cross-linking reactions involving polyamines may occur in the presence of high concentrations of these compounds. Putrescine may occur naturally in the pancreatic β-cell and may mediate cross-linking

reactions essential for the secretory mechanism. As an alternative, incorporation of putrescine into proteins by transglutaminase by one amine group, without cross-linking but which would lead to an alteration of the charge of a protein may be part of the secretory mechanism of the pancreatic β-cell.

In conjunction with this, it is interesting to consider the evidence presented by Koenig et al., (1983) that polyamines, including putrescine, are involved in Ca^{2+}-mediated stimulation of kidney cells by testosterone. These workers demonstrated that stimulation was dependent upon the rapid synthesis of putrescine, the intracellular level of which was increased approximately two-fold within 5 min of treatment of kidney slices with hormone. This system is analogous to the Ca^{2+}-mediated mechanism of glucose-stimulated insulin release and a similar investigation in islets was worthwhile in an attempt to define any role of putrescine and other polyamines(for which putrescine is the precursor)in the mechanism of insulin release. An involvement of putrescine in this mechanism would presumably preclude the inhibitory action of the compound.

The next part of this thesis describes experiments which were aimed at testing the permeability of islets to putrescine and at testing the hypothesis for the involvement of this diamine in the mechanism of glucose-stimulated insulin release.

3.2.4. UPTAKE OF PUTRESCINE AND METHYLAMINE BY ISLETS.

These studies were undertaken to determine whether the failure of putrescine to act as an effective inhibitor of insulin release was likely to be due to its failure to gain entry to the islet ß-cell. With regard to the involvement of transglutaminase in insulin release, the impermeability of islet cells to putrescine would preclude any interference of this compound with a transglutaminase-dependent step. The uptake of putrescine was measured and compared to the uptake of methylamine, a permeant amine which inhibited glucose-stimulated insulin release.

3.2.4.1. Uptake of methylamine by islets.

In these uptake studies, account was taken of the amount of radiolabelled compound associated with the islet intercellular space. The calculation of this space was described in the investigation of the effects of primary amines on [^{3}H]-leucine uptake and the value for sucrose space which was determined was used for the studies on uptake of methylamine and putrescine.

The uptake of [^{14}C]-methylamine at a concentration of 1.78mM by islets over a time-course of 30 min is shown in Fig. 3.13.. This compound readily entered islets in excess of the sucrose space with a rate that gradually decreased between 2 and 15 min. After 15 min of incubation, uptake appeared to continue in a linear fashion.

Assuming an average intracellular islet volume of 3 nl (Sener & Malaisse, 1978), the uptake of methylamine after 30 min would represent an accumulation in the islet cells to a concentration

of around 19mM, a figure well in excess of the extracellular concentration in the incubation medium. This accumulation may be the result of the sequestration of methylamine in areas of low pH in the cell. The mechanism and possible consequences of this phenomenon have been discussed (see section 3.2.3.) and have been reviewed (Dean et al., 1984). Although the cytosolic concentration of methylamine cannot be determined with any degree of accuracy in this experiment, the suggestion is that the 45 min pre-incubation period used in the experiments to test the effects of amines on insulin release was sufficient to allow for equilibration of permeant compounds across islet cell membranes.

3.2.4.2. Uptake of putrescine.

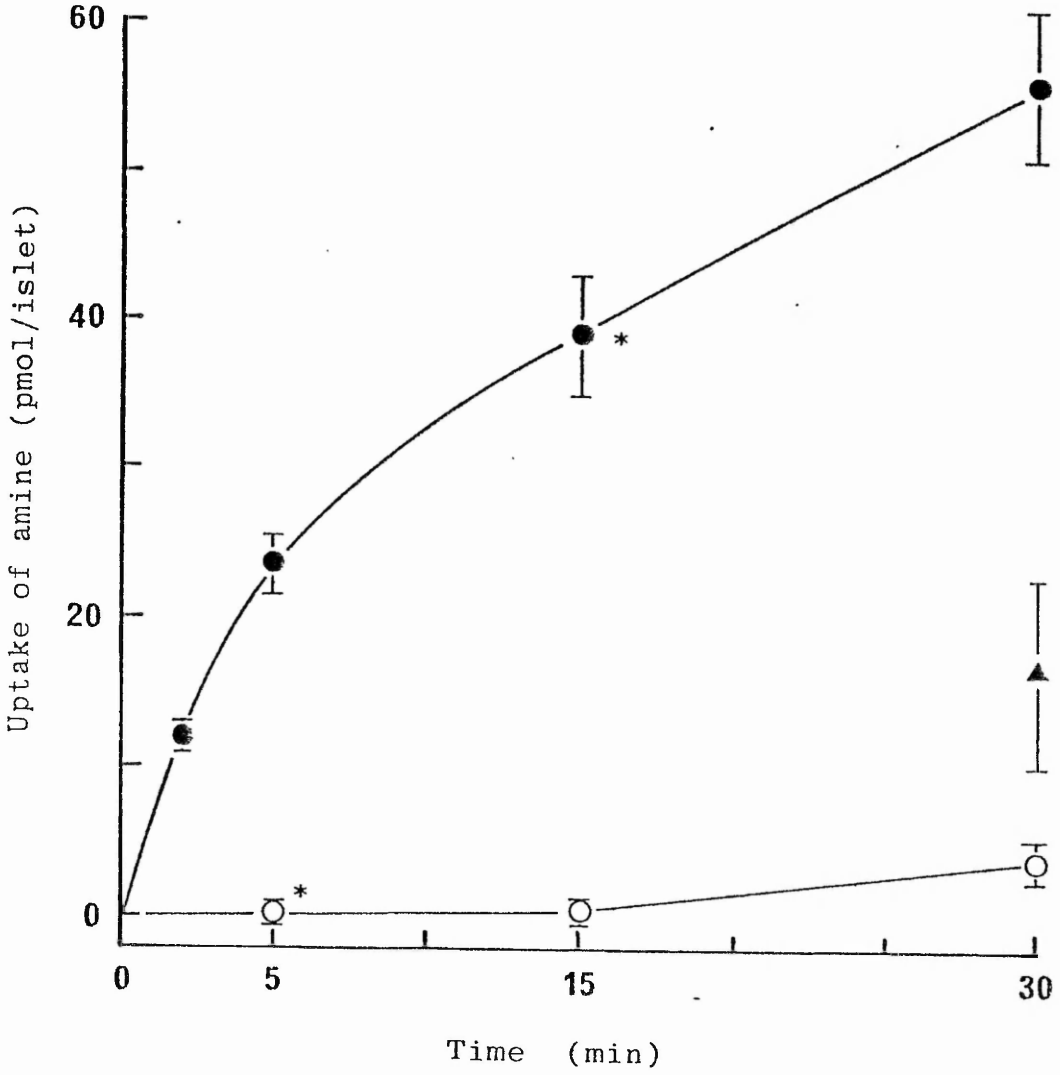
The entry of [^{3}H]-putrescine into islets was studied in similar experiments to those involving methylamine. At 2.0 mM, putrescine uptake above the sucrose space was barely detectable for up to 15 min incubation. At 30 min, uptake was only marginal (4.29 $\pm$ 0.71 (n=6) pmol/islet) compared to that of methylamine at a similar concentration. Uptake of putrescine at 20 mM, the concentration used in the insulin release studies, was also measured. After 30 min incubation, uptake was 16.73 $\pm$ 6.6 p mol putrescine per islet (n=8). These levels were well below those obtained for methylamine at one tenth the concentration. Assuming an average islet intracellular volume of 3 nl, the accumulation of putrescine after 30 min when used at a final concentration of 20mM could represent a cytosolic concentration approaching 6mM. However, the contribution to uptake of protonated putrescine trapped inside vescicles of low pH cannot be estimated in this experiment. Furthermore, a proportion of the

Figure 3.13.

Uptake of methylamine and putrescine by islets of Langerhans.

Batches of 10 to 20 islets were incubated with[^{14}C]-methylamine at a concentration of 1.78mM or [^{3}H]-putrescine at 2.0mM in 400µl microcentrifuge tubes as described in the Methods section (2.6.4.4.). Incubations were terminated at various time intervals by sedimentation through a layer of phthalate esters and the amount of radiolabel remaining in the islets determined by scintillation counting. Uptake was expressed as pmol compound per islet and was calculated after the subtraction of the amount of compound present in the islet extracellular space (sucrose space). The value of the islet sucrose space was assumed to be 2.84 nl and its calculation was described in the studies on [^{3}H]-leucine uptake (Section 3.2.2.3.). Points represent the mean of determinations from 6 batches of islets except for * where the mean is from 5 observations. Bars represent S.E.M. ▲, represents the uptake of [^{3}H]-putrescine at a concentration of 20mM.

Closed circles represent uptake of methylamine; open circles represent uptake of putrescine.


60
40
20
0
Uptake of amine (pmol/islet)
*
*
0
5
15
30
Time (min)

putrescine which entered the islets may be metabolised or bound to cellular membranes. Therefore the free cytosolic concentration of putrescine under these conditions cannot be estimated, but may be much lower than this calculation suggests.

The main conclusion that can be drawn from these experiments is that islet cells are not as permeable to putrescine as they are to methylamine. This observation may account for the difference between these compounds in their ability to inhibit insulin release from islets. It is possible that the relatively small amount of putrescine which appears to enter islets incubated with 20mM putrescine may account for the small amount of inhibition of glucose-stimulated insulin release from islets incubated at the same concentration of the compound.

3.2.5. PERMEABILISATION STUDIES.

In their studies on receptor-mediated endocytosis, Davies et al., (1980) found that permeabilisation of fibroblasts was required before putrescine was able to inhibit internalisation of α_2-macroglobulin. These workers judged cell permeability by trypan blue exclusion which was lost upon incubation of cells with 10% (w/v) dimethylsulphoxide (DMSO) which they used as a permeabilising agent. In the following studies, the same compound was used in an effort to permeabilise islets to putrescine. The effects of DMSO on the ability of putrescine to inhibit insulin release and to enter islets was then investigated.

3.2.5.1. Effect of putrescine on insulin release from islets treated with DMSO.

Before testing the effect of putrescine on insulin release in the presence of DMSO, the effect of DMSO alone on insulin release by islets was determined (Table 3.10.a.). At a concentration of 10% (w/v), DMSO did not significantly affect the average rate of insulin release from islets incubated at either 2.8 or 16.8 mM glucose. However, the response to 16.8mM glucose appeared to be more erratic in the presence of DMSO than in untreated islets, as indicated by the high standard error of the value obtained for DMSO treated islets. Since the response of islets to glucose may depend upon the permeability barrier presented by the β-cell membrane, a non-specific alteration in permeability may affect the response of the islet β-cell to glucose in an unpredictable manner. Given that islets

are clumps of cells, there may be much variation in the accessibility of DMSO to β-cells when islets of different sizes and shapes are exposed to the compound. Thus, the effect of DMSO may vary as a result of its non-specificity and according to islet size and shape.

With this in mind, the effect of putrescine on glucose-stimulated insulin release of DMSO-treated islets was investigated (Table 3.10.b.). In these conditions, 20mM putrescine gave rise to 48 $\pm$ 20% inhibition of glucose-stimulated insulin release. It is tempting to suggest, therefore, that permeabilisation of islets with DMSO allows sufficient putrescine to enter the β-cell to interfere with a transglutaminase - dependent step in the mechanism of insulin secretion. However, the amount of inhibition observed in this experiment, although substantial, was not statistically very significant ($p \leq 0.25$). Although the indication is that putrescine causes inhibition of glucose-stimulated insulin release under these conditions, the variability manifest in this experiment and those previously discussed creates difficulty for its interpretation.

3.2.5.2. Effect of dimethylsulphoxide on putrescine uptake.

In order to determine whether DMSO does indeed permeabilise islets to putrescine, the uptake of putrescine was measured after 30 min incubation in medium containing 10% (w/v) DMSO and 2.0mM [^{3}H]-putrescine. In the presence of DMSO, uptake was 8.54 $\pm$ 1.72 pmol/islet (n=4) compared with the value obtained previously for untreated islets of 4.29 $\pm$ 0.71 (n=6). This difference was significant to a level of $p \leq 0.05$. Therefore, under the conditions

of measurement of uptake, DMSO appears to significantly enhance the uptake of putrescine by islets by a factor of two. However, the enhanced uptake was still much lower than the uptake of methylamine over the same time period (Figure 3.12). Therefore, treatment of islets with 10% DMSO over 30 min is not sufficient to allow putrescine to enter islet cells to the same extent as methylamine. However, this treatment was designed to be comparable to the uptake experiments with [^{14}C]-methylamine and is not comparable to the conditions used in the insulin release experiments. It is possible that the longer treatment used in the insulin release experiments in the presence of 20mM putrescine may allow sufficient putrescine to permeate the islet cells to interfere with islet transglutaminase activity.

Table 3.10.

Effects of dimethylsulphoxide and putrescine on insulin release.

The effects of dimethylsulphoxide (DMSO) at a concentration of 10% (w/v) and of putrescine in the presence of 10% (w/v) DMSO were determined in two separate experiments (a. and b.). These experiments were identical in format to those performed in the studies on the effects of primary amines on insulin release. The incubation period of 105 min consisted of a period of 45 min in 2.8mM glucose containing medium followed by 60 min in 16.8mM glucose containing medium; when included, test compounds were present throughout the entire incubation period. In experiment b., 10% DMSO was included in all incubations, including controls. Results are expressed as μU insulin/h/islet $\pm$ S.E.M. with numbers in parentheses referring to numbers of batches of islets. The significance of difference from controls was assessed using the Students' t-test; N.S. denotes no significant difference from controls. The values which were compared statistically are represented in brackets.

Treatment	Glucose conc. (mM)	Insulin release (μU/h/islet)		
a.				
-	2.8	7.4 ± 3.7		(4)
10% DMSO	2.8	7.9 ± 0.91	N.S.	(4)
-	16.8	85.5 ± 9.3		(4)
10% DMSO	16.8	75.6 ± 29.3	N.S.	(4)
b.				
10% DMSO	2.8	9.0 ± 1.8		(4)
10% DMSO	16.8	46.4 ± 8.6		(5)
10% DMSO + 20mM putrescine	16.8	28.5 ± 7.6	$p \leq 0.25$	(6)

3.2.6. THE INVOLVEMENT OF POLYAMINES IN GLUCOSE-STIMULATED INSULIN RELEASE.

The failure of putrescine to act as an effective inhibitor of insulin release previously demonstrated may be a result of the limited permeability of the islet β-cell to this diamine. The other possibility to be considered is that putrescine may participate in the secretory mechanism, an eventuality that would presumably preclude inhibition of secretion by this compound. To test this possibility, the following investigations were undertaken:

i. the effect on glucose-stimulated insulin release of the inhibitor of polyamine biosynthesis, α-difluoromethylornithine (DFMO), was assessed;

ii. the levels of putrescine, spermidine and spermine in islets were examined after treatment of islets with DFMO and before and after stimulation with glucose.

These experiments would indicate whether rapid synthesis of the polyamines in the islet cells is involved in the secretory mechanism.

3.2.6.1. The effect of DFMO on glucose-stimulated insulin release.

The effect of DFMO on insulin release from islets was determined in an experimental protocol similar to that used in the studies conducted with primary amines (Table 3.11.). Glucose-stimulated insulin release was unaffected by this compound at a final concentration of 4mM when it was incubated with islets for 45 min in medium containing 2.8mM glucose followed by 60 min

Table 3.11.

The effect of DFMO on glucose-stimulated insulin release.

Treatment	Glucose concentration	Insulin release (μU/h/islet)	
-	2.8	3.4 ± 0.6	(5)
-	16.8	126.3 ± 6.8	(5)
4mM DFMO	16.8	142.9 ± 13.3	(7) N.S.

Batches of 5 islets were incubated at 37°C for 45 min in 100μl KRB containing 2.8mM glucose followed by 60 min in 200μl KRB containing 16.8mM glucose. Samples of medium were taken and insulin released into the medium was determined by radioimmunoassay. The concentration of DFMO stated was present throughout the total period of incubation (105 min). Results are expressed as rates of insulin release ± S.E.M. over one hour after subtraction of basal levels of insulin release for the 45 min pre-incubation period. Figures in parentheses refer to numbers of batches of islets. The significance of difference from controls incubated in the absence of DFMO was determined using the Students' t-test; N.S. denotes no significant difference from controls.

in medium containing 16.8mM glucose. If it is assumed that this treatment was sufficient to substantially inhibit ornithine decarboxylase in the islet β-cell, the result obtained suggests that rapid synthesis of polyamines is not involved in the mechanism of glucose-stimulated insulin release.

3.2.6.2. Levels of polyamines in islets.

The islet content of polyamines putrescine, spermidine and spermine was examined by HPLC analysis of TCA extracts of islets which had been incubated at either 2.8mM glucose or at 16.8mM glucose in the presence and absence of 4mM DFMO.

Interestingly, islets were found to contain readily detectable levels of spermidine and spermine as well as lower levels of putrescine. This is the first observation of these compounds in islets of Langerhans.

The levels of putrescine, spermidine and spermine found in islets are displayed in Table 3.12. The incubation of islets for 5 min at 16.8mM glucose did not significantly affect the islet content of any of these polyamines when statistical comparison (Students' t-test) was made to levels of these compounds in islets maintained at 2.8mM glucose. Assuming that the β-cells are the main contributors to levels of polyamines in islets, this observation lends further credence to the idea that rapid synthesis of polyamines is not involved in the mechanism of stimulation of insulin release in the pancreatic β-cell.

The effect of DFMO on levels of polyamines in glucose-stimulated islets was examined by pre-incubation of islets for 45 min in KRB containing 2.8mM glucose and 4mM DFMO before a

Table 3.12.

Levels of polyamines putrescine, spermidine and spermine in islets of Langerhans.

	Polyamine content (pmol/islet)		
Compound	2.8mM glucose (6)	16.8mM glucose (8)	16.8mM glucose + 4mM DFMO (5)
putrescine	0.40 ± 0.18	0.59 ± 0.18	0.35 ± 0.11
spermidine	3.41 ± 0.98	3.48 ± 0.42	2.71 ± 0.50
spermine	2.63 ± 0.73	2.67 ± 0.51	2.17 ± 0.35

Groups of 50-100 islets were incubated with the concentrations of glucose shown for 5 min in 200µl KRB. The medium was removed and the islets sonicated in 200µl 10% (w/v) TCA in 20% (v/v) methanol before centrifugation at 9,320 g av.. The supernatant containing polyamines was extracted with ether and lyophilised. In the case of incubations containing DFMO, a pre-incubation period of 45 min at 2.8mM glucose was included before stimulation of islets with 16.8mM glucose. For HPLC lyophilised extracts were re-dissolved in 70µl HPLC buffer A (see methods section) and applied to a C_{18} µ-Bondapak column for HPLC resolution of polyamines as described in the Methods section (2.6.7.). The inclusion of internal standard (1,7-diaminoheptane) in the TCA extracts allowed the proportion of the total extract injected to be calculated. Results are expressed as pmole polyamine/islet ± S.E.M. with the number of batches of islets shown in parentheses.

stimulation period at 16.8mM glucose of 5 min also in the presence of 4mM DFMO (Fig. 3.12.). Although the levels of putrescine, spermidine and spermine were slightly lower than the corresponding levels of these compounds in islets incubated at 16.8mM glucose or 2.8mM glucose in the absence of DFMO, these differences were not statistically significant as judged by the Students' t-test.

The failure of DFMO to exert any significant effect on the levels of any of the polyamines putrescine, spermidine and spermine in islets may be interpreted in two ways:

1. DFMO under the conditions used did not inhibit synthesis of polyamines. This may be due to a failure of the compound to enter the islet cells or to a failure of the compound to effectively inhibit ODC, whose regulation in cells is probably complex (Bachrach, 1984).
2. DFMO did inhibit ODC, but this was not manifested due to a slow turnover rate of the polyamines.

The validity of these alternatives requires further investigation.

The report (Koenig et al., 1983) which prompted the experiments above detailed good evidence that synthesis of polyamines was involved in the rapid response of kidney cells to testosterone:

a. Levels of putrescine, spermidine and spermine in kidney tissue rose 1.5 to 2-fold within 5 min of dosing of mice with testosterone.
b. Activation of ornithine decarboxylase was detectable within 2 min of activation of kidney slices.
c. DFMO (5mM) inhibited the activation by testosterone of hexose transport, amino acid transport and endocytosis as well as the

rise in polyamine levels accompanying activation.

d. Putrescine added exogenously could reverse the effects of DFMO.

In the same investigation, it was found that DFMO suppressed the mobilisation of Ca^{2+} in stimulated kidney cells and this led to the postulation that a mechanism of cation exchange by the polyamines was involved in the regulation of Ca^{2+} mobilisation in this system.

The similar, but less thorough investigation undertaken in this thesis into the involvement of polyamines in the mechanism of glucose-stimulated insulin release from the pancreatic β-cell suggested that ornithine decarboxylase and polyamines are probably not involved in an analogous rapid system of stimulus-response coupling to that observed for kidney cells. Therefore, the polyamines may not play a major role in the mechanism by which glucose mobilises Ca^{2+} in the islet β-cell. It is possible, however, that these compounds may play a more restricted role which does not involve their rapid synthesis, but possibly their re-distribution in the β-cell. This last consideration may be relevant to the role of transglutaminase in the β-cell, in view of the fact that polyamines are transglutaminase substrates.

3.2.7. Discussion.

Measurement of the uptake of putrescine and methylamine suggested that methylamine rapidly accumulated in islets, whereas putrescine did not do so. The difference in the ability of putrescine and methylamine to enter islet cells is probably attributable to the possession by putrescine of two amine groups which may be protonated at physiological pH. Thus, the proportion of putrescine in a cationic form with either one or two charged amine groups will be greater than the proportion of cationic methylamine, since the pKa of the amino groups of methylamine and putrescine are similar (pKa methylamine = 10.16-10.79; pKa putrescine = 8.83-9.35 and 10.26-10.80). Since cell membranes are impermeable to charged molecules, the proportion of a given concentration of putrescine that may permeate the cell membrane will be less than the corresponding proportion of methylamine.

Treatment of islets with DMSO (10% w/v) increased their permeability to putrescine approximately two-fold over a period of 30 min. Although such treatment appeared to increase the ability of putrescine to inhibit glucose-stimulated insulin release, DMSO appeared to make islets respond erratically to glucose, making this experiment difficult to interpret. It is possible that the relatively low permeability of islets to putrescine may account for the relatively poor ability of the diamine to cause inhibition of insulin release by interference with an intracellular transglutaminase-dependent step.

However, uptake studies also suggested that islets incubated

with 20mM putrescine for 30 min may contain concentrations of the compound sufficient to participate effectively in trans-glutaminase-catalysed reactions in the β-cell. Since 20mM putrescine was found to be fairly ineffective in the inhibition of glucose-stimulated insulin release, it is possible that putrescine is a natural β-cell substrate for transglutaminase, and may participate in the mechanism of insulin release.

It should also be borne in mind that the metabolism of putrescine which enters the β-cell may act to decrease the intracellular concentration of the compound. Putrescine may be metabolised by diamine oxidases (Andersson et al., 1980)or by enzymes of the pathway of polyamine synthesis (see Introduction, Section 1.4.6.). The products of such metabolism may not interfere with transglutaminase activity in the β-cell.

The hypothesis for the involvement of putrescine and other polyamines in the mechanism of stimulus-secretion coupling in the pancreatic β-cell was tested. The observations that DFMO did not inhibit glucose-stimulated insulin release and that levels of the polyamines putrescine, spermidine and spermine did not significantly change when islets were stimulated with glucose argued against a mechanism involving the rapid synthesis of polyamines being involved in the response of the β-cell to glucose (c.f. Koenig et al., 1983). Therefore, the failure of putrescine to effectively inhibit insulin release may not be a result of the involvement of the diamine in such a mechanism.

However, these observations do not preclude the involvement of polyamines in the mechanism of insulin release. Indeed, the mere presence of these compounds in islets may implicate them

in a mechanism in conjunction with transglutaminase. Kinetic studies of islet transglutaminase activity (Section 3.1.) indicated that the Km of the enzyme for putrescine incorporation into N,N'-dimethylcasein was in the range 0.2-1.0mM. Assuming a mean intracellular islet volume of 3nl (Sener & Malaisse, 1978) the concentrations of putrescine, spermidine and spermine may be estimated to be 0.2mM, 1.2mM and 1.0mM respectively. Although such estimates are complicated by the possible binding and sequestration of polyamines in cells, the free cytosolic concentrations of these compounds may be high enough for them to participate effectively in transglutaminase-catalysed reactions in the β-cell. This consideration gives added credence to the idea that the failure of putrescine to be very effective in the inhibition of glucose-stimulated insulin release may reflect its involvement in transglutaminase-catalysed reactions essential for the secretory mechanism of the pancreatic β-cell. The possible significance of such reactions involving the formation of protein-polyamine conjugates in the β-cell is discussed in a later section (Section 5.).

In conclusion, the results obtained suggested the possibility that polyamines may play a role in the insulin secretory mechanism in conjunction with islet transglutaminase. Although this may reflect the poor ability of putrescine to cause inhibition of glucose-stimulated insulin release, the low permeability of islet cells to putrescine may be a contributing factor. The function of polyamines in islets is a subject for further investigation but is probably not connected with a rapid, Ca^{2+}-mediated response of the β-cell to glucose which is dependent upon rapid polyamine synthesis.

3.3. SUBSTRATE STUDIES

The proposal has been made that inhibition of insulin release by primary amines is mediated by islet transglutaminase (Section 3.2.2.5.). This mechanism of inhibition would involve the covalent incorporation of amines into glutamine residues of proteins normally involved in transglutaminase-catalysed reactions in the islet β-cell which are essential for the continuous operation of the mechanism of glucose-stimulated insulin release. The studies below aimed to demonstrate the presence of substrate proteins for transglutaminase in islets and to identify these as far as possible.

3.3.1. INCORPORATION OF PUTRESCINE AND METHYLAMINE INTO ISLET HOMOGENATES.

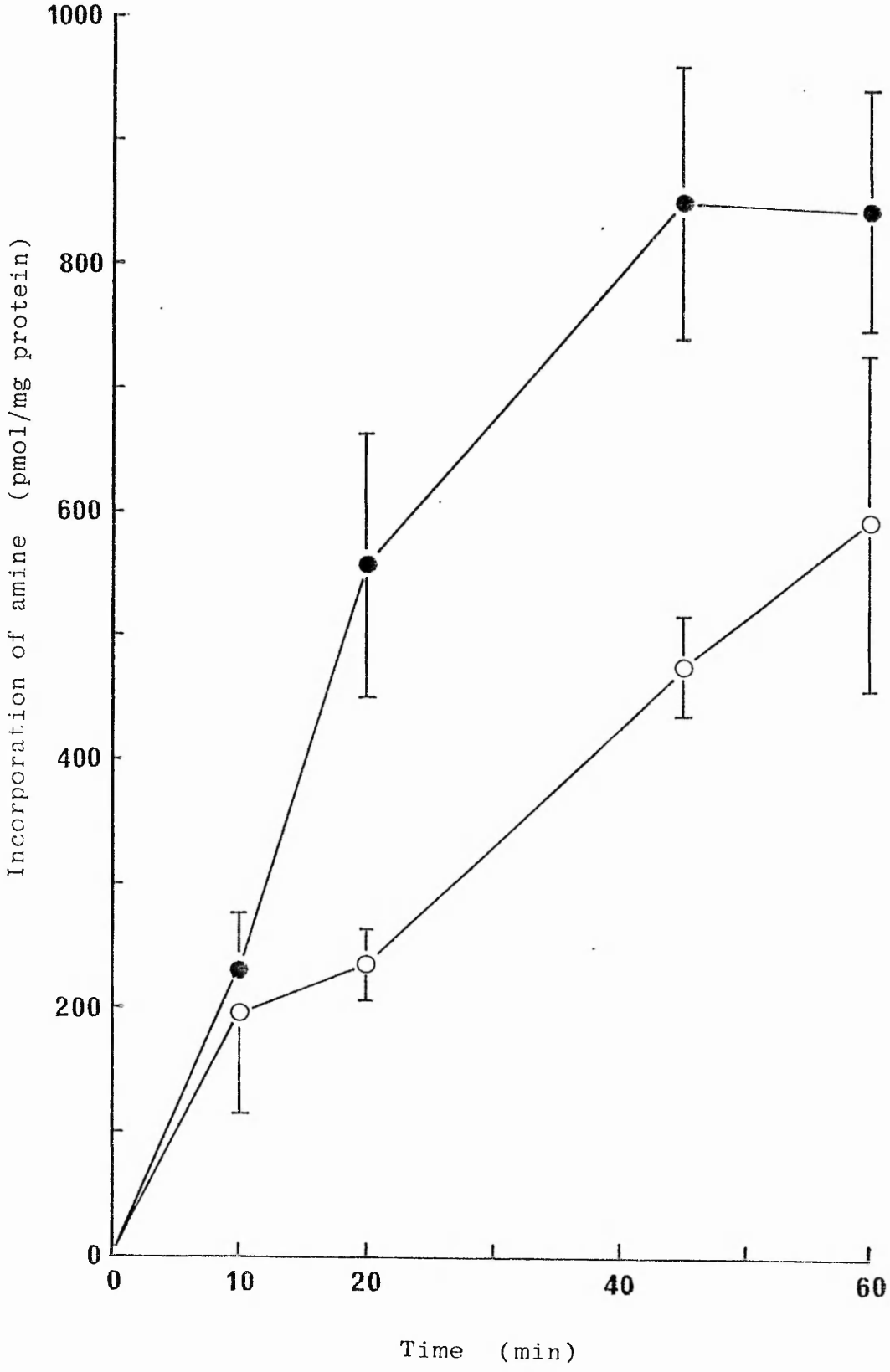
The presence of substrate proteins for transglutaminase in islet homogenates was demonstrated by incubation of islet homogenates with [^{14}C]-putrescine and [^{14}C]-methylamine each at a concentration of 1.78mM. The time-dependent and Ca^{2+}-dependent incorporation of these radiolabelled amines catalysed by endogenous islet transglutaminase activity is shown in Fig. 3.14. The initial rates of incorporation of these amines (from 0-10 min) appear similar, but over the next 50 min, putrescine incorporation was significantly greater at each of the time intervals sampled. After 45 min, the incorporation of putrescine appeared to have reached a saturation point. It is noteworthy that even up to 60 min, the incorporation of methylamine was still proceeding, indicating that loss of enzyme activity is unlikely to account

Figure 3.14.

Incorporation of methylamine and putrescine into islet homogenates catalysed by endogenous transglutaminase.

Islet homogenates (approx. 50 µg protein) were incubated at 37°C with 1.78mM of either [^{14}C]-putrescine (7.02 mCi/mmol) or [^{14}C]-methylamine (56 mCi/mmol) in a buffer containing 18mM Tris, 3.85mM dithiothreitol and 2.5mM $CaCl_2$ or 5mM EDTA, pH 7.4

Samples (20µl) were taken at the time intervals indicated, washed free of unbound radioactivity and counted. Protein concentration was determined by the method of Lowry. Results are expressed as pmole of amine incorporated per mg homogenate protein after the subtraction of counts obtained from vials containing EDTA (5mM). All points are a mean of three observations and bars represent S.E.M.
Closed circles represent the incorporation of putrescine; open circles represent the incorporation of methylamine.


1000
800
600
400
200
0
Incorporation of amine (pmol/mg protein)
0
10
20
40
60
Time (min)

for the cessation of incorporation observed for putrescine.

These results indicate that islets contain proteins which possess γ-glutamyl residues which can act as substrates for endogenous transglutaminase. The apparent saturation of these substrates within 45 min by putrescine under the conditions of assay indicates the the number of available γ-glutamyl residues is limited. The overall higher rate of incorporation of putrescine over that of methylamine suggests that putrescine is a better substrate of islet transglutaminase than methylamine.

3.3.2. SUBCELLULAR DISTRIBUTION OF PROTEIN SUBSTRATES.

In one experiment, islet homogenate was incubated with [^{3}H]-putrescine at a final concentration of 2.0mM, centrifuged at 71,000 gav for 45 min and the amount of radiolabel incorporated into each of the resulting fractions determined (Table 3.13). Ca^{2+}-dependent incorporation of radiolabel was observed in the pellet and supernatant fractions but the majority (70%) was found in the pellet fraction. This result suggests that most of the substrates for transglutaminase in islets are associated with particulate material which would be composed mainly of cellular membranes. The identity of the membrane components containing these proteins requires further investigation.

Table 3.13.

Subcellular distribution of protein substrates for transglutaminase in islets.

	d.p.m. incorporated	
	$CaCl_2$	EDTA
Pellet	11482	1303
Supernatant	6013	3220

Homogenates of islets prepared by sonication in ice-cold 0.25M sucrose, 1mM Tris, 1mM EDTA, pH 7.4 were incubated with 2.0mM [^{3}H]-putrescine (50 mCi/mmol) for 3h at 37^{o}C with either 2.5mM $CaCl_2$ or 5mM EDTA. Following incubation, homogenates were centrifuged at 71,000 g av for 45 min and the pellet resuspended in 100 µl 0.25M sucrose, 1mM Tris, 1mM EDTA, pH 7.4. The pellet and supernatant fractions were precipitated with an equal volume of 20% (w/v) TCA and washed 5 times with 5% TCA containing 2mM 'cold' putrescine to remove unbound radiolabel. Radiolabel bound to the TCA-precipitable material was counted after solubilisation of samples and counting efficiency was determined using the Quench Indicating Parameter of Packard Inst..

3.3.3. INCORPORATION OF[^{14}C]-METHYLAMINE INTO PROTEINS OF INTACT ISLETS.

When islets were incubated with[^{14}C]-methylamine (1.78mM, 56mCi/mmol) for 60 min at either 2.8 or 16.8mM glucose following a 45 min preincubation period at 2.8mM glucose in the presence of the radiolabel, no incorporation of radiolabel above background levels was detectable. Therefore, although peptide-bound γ-glutamyl substrates for transglutaminase exist in islets as demonstrated for islet homogenates,they are not available for acyl transfer reactions with exogenously applied amine to the same extent intact islets. This assumes that in this experiment methylamine was present in the islet cells at a similar available concentration to that used in the studies with homogenates of islets.

3.3.4. IDENTIFICATION OF PROTEIN SUBSTRATES FOR TRANSGLUTAMINASE IN ISLETS.

3.3.4.1. Studies with [^{3}H]-putrescine

Homogenates of islet of Langerhans were incubated with [^{3}H]-putrescine (2mM) and the radiolabelled proteins were separated by SDS-polyacrylamine gel electrophoresis and detected by scintillation counting of gel slices. Using this procedure, incorporation of radiolabel was restricted to only a few densely labelled gel sections in each experiment undertaken. However, it was not possible to obtain a reproducible pattern of labelling of islet homogenate proteins with [^{3}H]-putrescine. Furthermore, incorporation of radiolabel appeared to have occurred in proteins incubated in the presence of EDTA (5mM) before electrophoresis, indicating that incorporation may not have been transglutaminase-mediated. When replicate samples of the same homogenate which was incubated with [^{3}H]-putrescine in the presence of $CaCl_2$ (2.5mM) were compared after SDS polyacrylamide gel electrophoresis, different patterns of labelling of gel slices were observed. These observations suggest that tritium, which is known to be exchangeable under certain conditions (Waterfield et al., 1968) may have been transferred between molecules in a non-specific manner under the conditions of these experiments, leading to spurious incorporation of radiolabel. These results therefore provided no information as to the nature of the protein substrates containing available γ-glutamyl residues in islets.

3.3.4.2. Studies with [^{14}C]-putrescine.

Islet homogenates were incubated with [^{14}C]-putrescine (2.0mM) and treated in the same manner as described for the experiments using [^{3}H]-putrescine and the result shown in fig. 3.15. Calibration of the gel with molecular weight standard proteins (Fig. 3.17a.) indicated that the most densely labelled band of the gel had a molecular weight of 68-76,000 with smaller amounts of label in bands of molecular weight 34-36,000, 27-28,000 and 20-21,000 as indicated in Fig. 3.15. It is noteworthy that although the specific activity of ^{14}C used in this experiment was only half that used in the experiments with [^{3}H]-putrescine, the amount of incorporated label was a factor of 100 less. This supports the suggestion that tritium may be transferred between molecules, under the conditions of these experiments leading to spurious incorporation.

3.3.4.3. Studies with [^{14}C]-methylamine.

In similar experiments to those described above, islet homogenates were incubated with [^{14}C]-methylamine (1.78mM) and labelled proteins resolved by SDS-polyacrylamide gel electrophoresis. Ca^{2+}-dependent incorporation was restricted mainly to a single band (approx. 50% of total incorporated radiolabel) (Fig. 3.16.). Calibration of the gel (Fig. 3.17.b.) indicated that the molecular weight of this band was 47-49,000 and that significant labelling occurred in proteins of molecular weight 20-21,000 and above 92,000 (out of calibration range) as well as at the dyefront (Fig. 3.16.).

Figure 3.15. Labelling of islet homogenate proteins with [^{14}C] -putrescine.

Homogenates of islets were incubated at 37^{o}C for 3h in the presence of [^{14}C] -putrescine (2.0mM; 25mCi/mmol) as described in the Methods Section (Section 2.6.6.). Islet homogenate proteins (approximately 75 µg protein) were then precipitated with TCA and dissolved in 50 µl SDS sample buffer. Proteins were separated by SDS-polyacrylamine gel electrophoresis using a 10% resolving gel and a 3% stacking gel. Following fixing and staining of gels, protein tracks were sectioned at 2mm intervals, the sections dissolved in hydrogen peroxide/ammonia solution and counted for radioactivity. Results are plotted as histograms of counts per minute in each section after subtraction of background versus migration distance. The molecular weights of labelled bands of protein were calculated by reference to relative migration distances of standard molecular weight proteins run on the same gel (see Fig. 3.17a.).

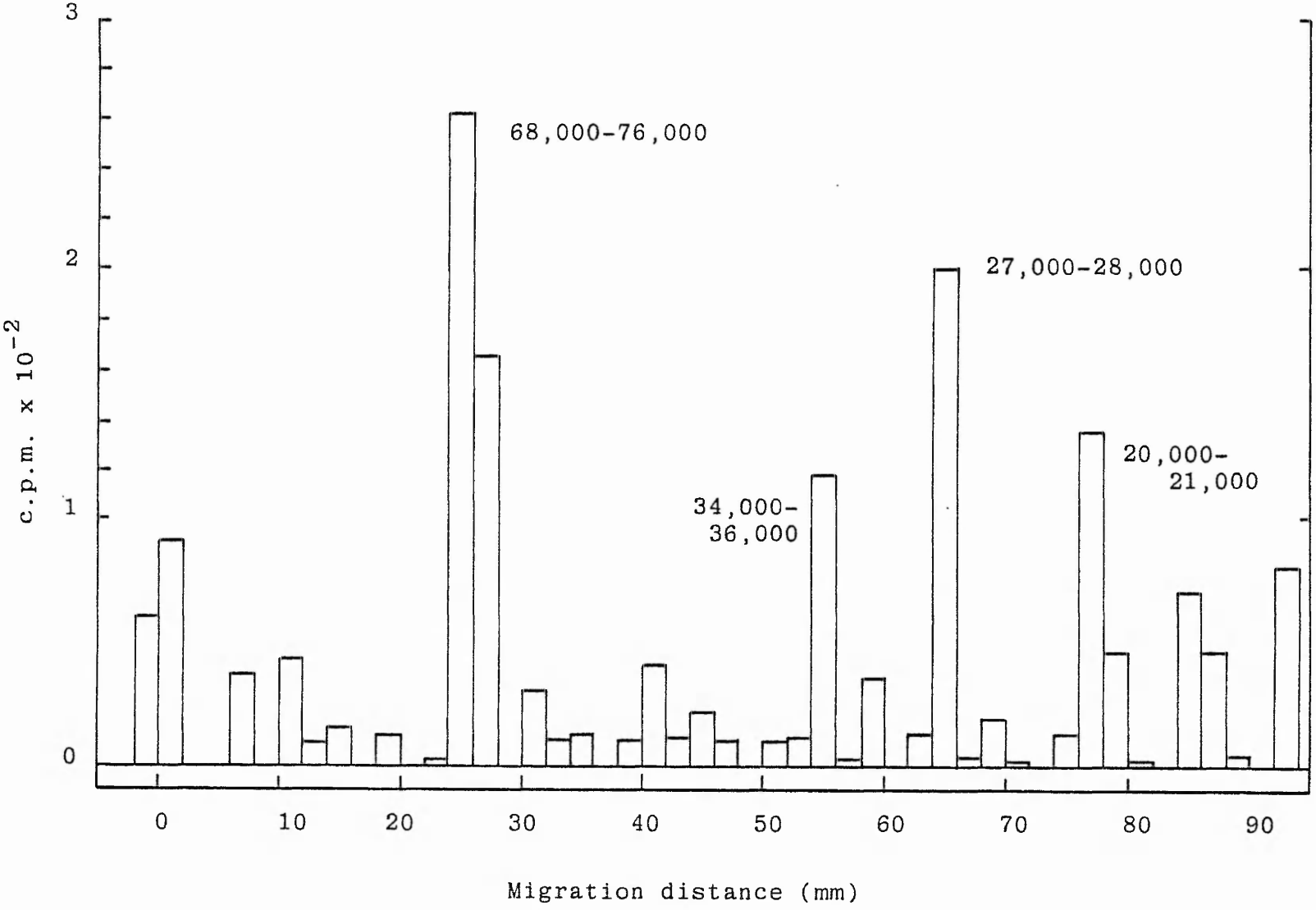
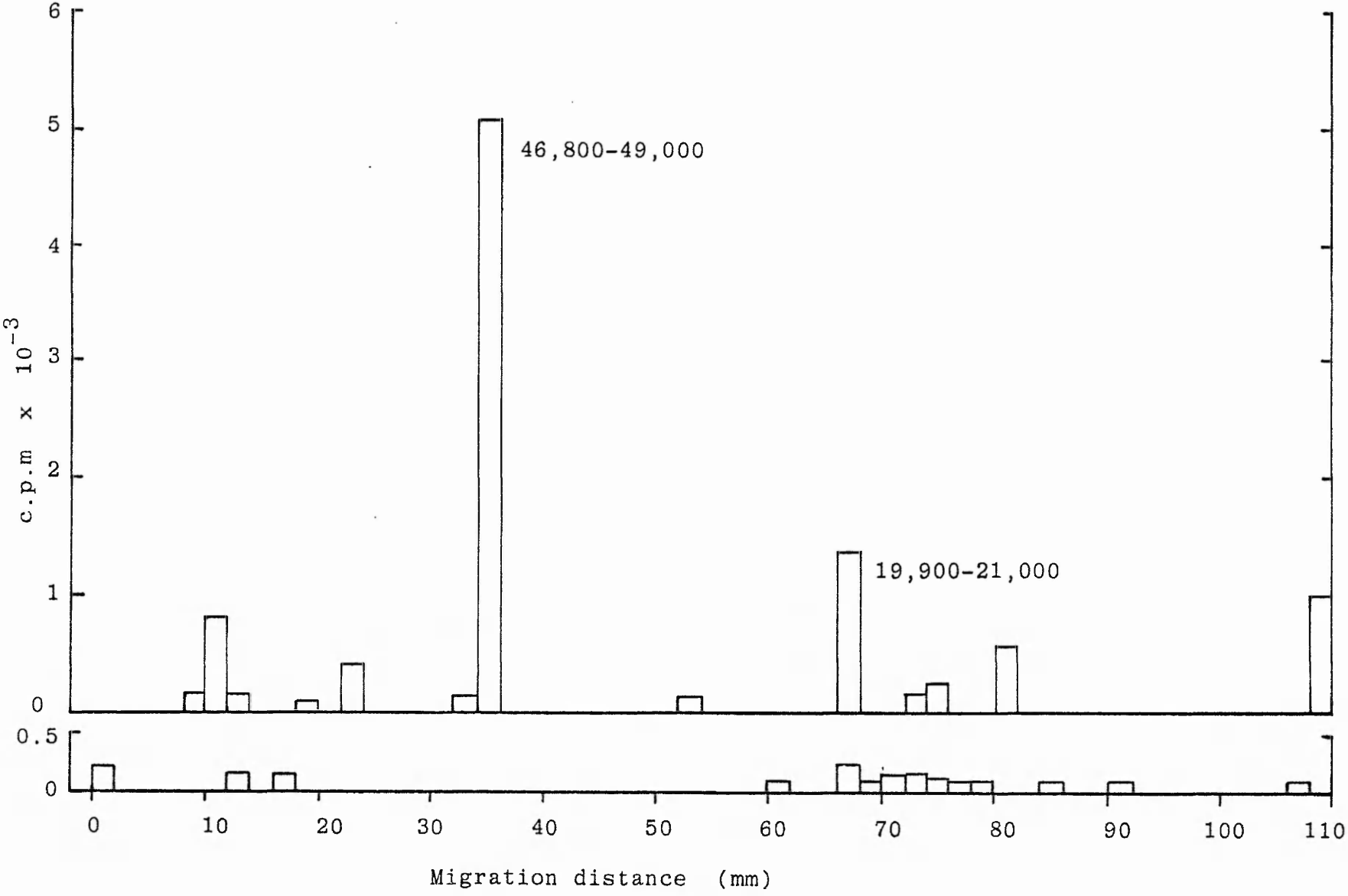

3
2
1
0
c.p.m. x 10^{-2}
68,000-76,000
27,000-28,000
20,000-
21,000
34,000-
36,000
0
10
20
30
40
50
60
70
80
90
Migration distance (mm)

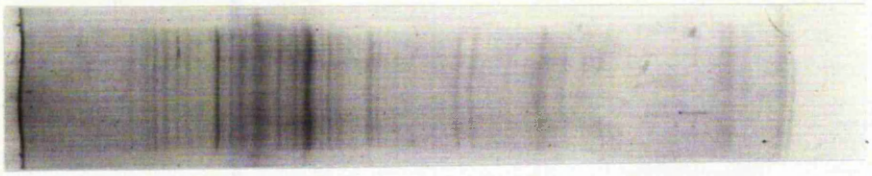
Figure 3.16 Labelling of islet homogenate proteins with [^{14}C] -methylamine.

Homogenates of islets were incubated at 37^{o}C for 2h in the presence of [^{14}C]-methylamine (1.78mM; 56mCi/mmol) as described in the Methods Section. Control incubations were included in which 5mM EDTA replaced 2.5mM $CaCl_2$ in the incubation cocktail. Following incubation, islet homogenate proteins (approximately 75 µg protein) were precipitated with TCA, washed free of unbound radioactivity and dissolved in 50 µl of SDS sample buffer. Proteins were separated by SDS polyacrylamide gel electrophoresis using a 10% resolving gel and a 3% stacking gel. After fixing and staining of gels, protein tracks were sectioned at 2mm intervals, the sections dissolved in hydrogen peroxide/ammonia and counted for radioactivity. Results are plotted as histograms of counts per minute after subtraction of background versus migration distance. The result for control incubations (5mM EDTA) is shown in the lower part of the figure. The molecular weights of labelled protein bands were calculated by reference to the relative migration distances of standard molecular weight proteins run on the same gel (see Fig. 3.17b.).

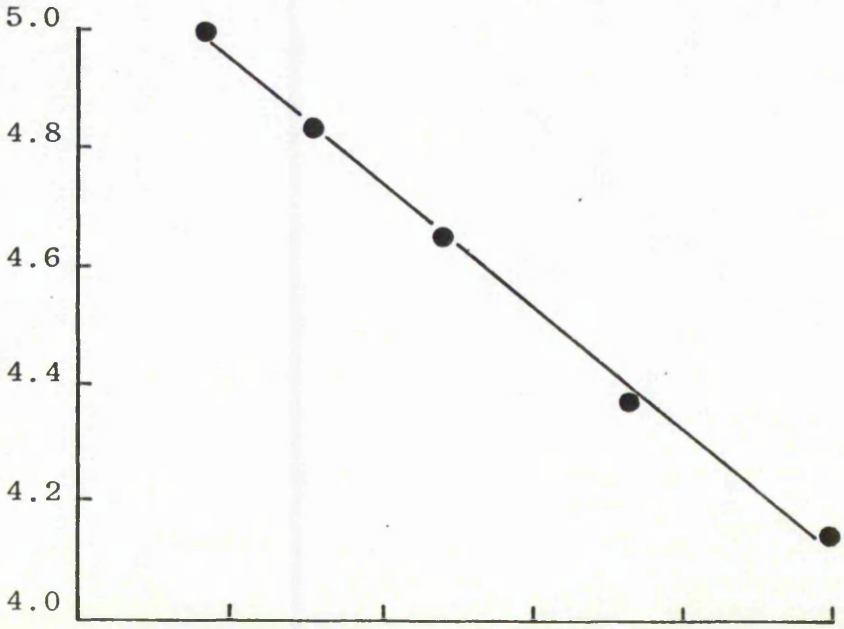

6
5
4
3
2
1
0
0.5
0
c.p.m x 10^{-3}
46,800-49,000
19,900-21,000
0
10
20
30
40
50
60
70
80
90
100
110
Migration distance (mm)

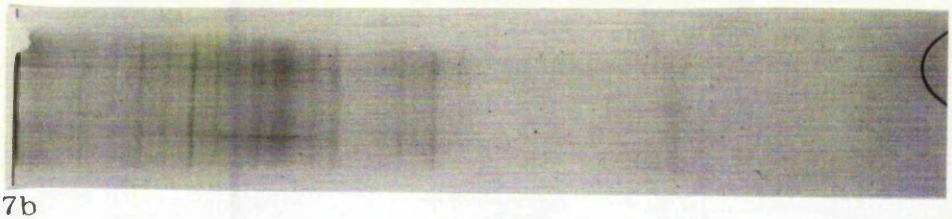
Figures 3.17a. and 3.17b. Calibration of polyacrylamide gels for estimation of protein molecular weight.

Protein molecular weight standards (approximately 10μg each of β-galactosidase (92,000), bovine serum albumin (66,000), ovalbumin (45,000), trypsinogen (24,000) and lysosyme (14,300) were electrophoresed on the same gels used for the separation of islet proteins (Figs. 3.15 and 3.16). Migration distance relative to the migration distance of lysosyme was plotted against $\log_{10}$ molecular weight. Photographs of the islet protein tracks used for determination of [^{14}C]- putrescine incorporation (3.17a.) and [^{14}C]-methylamine incorporation (3.17b.) are shown above their respective calibration graphs.

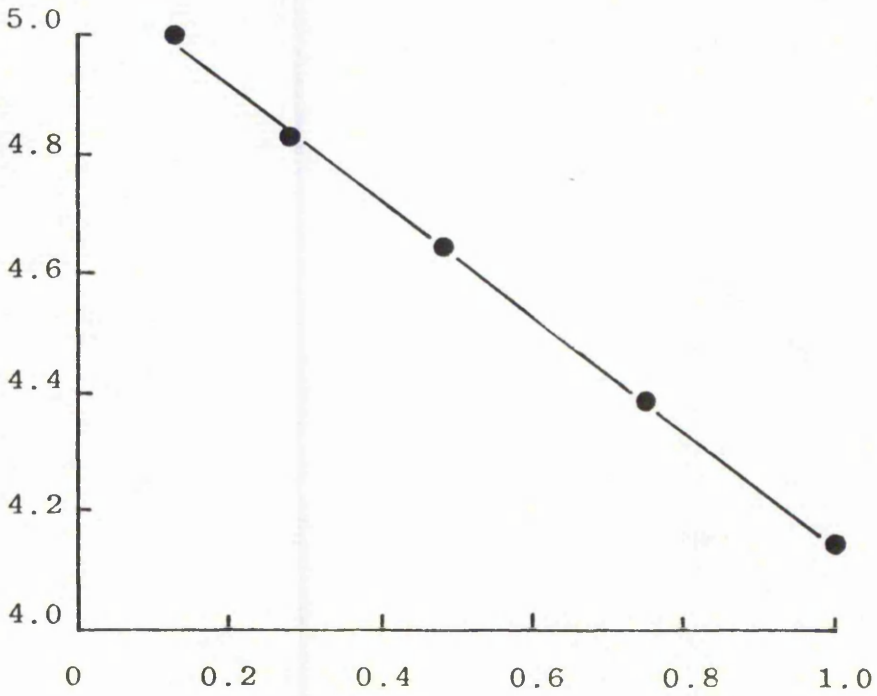


3.17a


5.0
4.8
4.6
4.4
4.2
4.0



3.17b


Log 10 Molecular Weight
5.0
4.8
4.6
4.4
4.2
4.0
0
0.2
0.4
0.6
0.8
1.0
Relative mobility

3.3.5. DISCUSSION OF SUBSTRATE STUDIES.

The Ca^{2+}-dependent incorporation of radiolabelled amines into the TCA-precipitable material of islet homogenates indicated the presence in islets of proteins which contain γ-glutamyl residues available for transamidating reactions. Fractionation of homogenates after incorporation of radiolabel suggested that most of these substrates existed in the particulate fraction of islets. These observations are consistent with a role for islet transglutaminase in the membrane interactions occurring during periods of glucose-stimulated insulin release.

However, further investigation of these substrate proteins suggested that they may not represent putative transglutaminase substrates which participate in the mechanism of secretion in the intact β-cell. Firstly, incorporation of [^{14}C]-methylamine into proteins of intact islets was not detectable in a similar amount of islet material to that used in the studies with homogenates. Methylamine readily permeates islet cells (see uptake studies, Section 3.2.4.) and would be expected to be available for transglutaminase-catalysed reactions under these conditions. Therefore, the number of substrate proteins available to transglutaminase in the intact β-cell may be very small, and those which were detected in homogenates may not be available to the enzyme *in vivo*. Secondly, the patterns of labelling of protein by [^{14}C]-methylamine, [^{14}C]-putrescine and [^{3}H]-putrescine were not reproducible between experiments. In the case of [^{3}H]-putrescine, this effect may be explained by the exchange of the radiolabel between protein molecules. This explanation

is, however, unlikely in the cases of [^{14}C]-putrescine and [^{14}C]-methylamine. Three other possibilities arise as explanations for the difference in patterns of labelling of proteins by these radiolabelled compounds:

i. Limited proteolysis of radiolabelled proteins by endogenous islet proteases may occur during the incubations with radiolabelled amines. The degree and specificity of such proteolysis may vary between experiments.

ii. The specificity of islet transglutaminase may be variable under the conditions of these experiments, possibly as a result of limited proteolysis of its substrates.

iii. [^{14}C]-putrescine may participate in the formation of diamine (bis-(γ-glutamyl)putrescine) cross-links between protein molecules and increase the apparent molecular weight of proteins in this way. Incorporation of [^{14}C]-methylamine can only lead to the formation of N-(γ-glutamyl)methylamine conjugates and will not alter the apparent molecular weight of substrate proteins for transglutaminase.

The last possibility is intriguing particularly since the sum of the molecular weights of the bands most densely labelled by methylamine is in the range (approximately 70,000) of the band most densely labelled by putrescine. The labelling of the 68-76,000 molecular weight band by putrescine may therefore be the result of the formation of diamine cross-links between 47-49,000 and 20-21,000 molecular weight proteins. However, these experiments will bear repetition in order to determine the reproducibility of these patterns of protein labelling and their possible significance to the function of islet transglutaminase.

3.4. Conclusion to the islet studies

These studies were undertaken in order to test the hypothesis that tissue transglutaminase may be involved in the mechanism of insulin release from the pancreatic β-cell. The following evidence was obtained which was consistent with this hypothesis:

i. Islets of Langerhans contained a typical Ca^{2+} - and thiol-dependent transglutaminase enzyme whose activity was inhibited by primary amines.

ii. The sensitivity of islet transglutaminase to Ca^{2+} was such that small changes in Ca^{2+} in the micromolar range could activate the enzyme.

iii. Primary amine inhibitors of transglutaminase activity inhibited glucose-stimulated insulin release from pancreatic islets with a potency which matched their relative potency as inhibitors of islet transglutaminase activity.

iv. Putrescine, which was a good substrate of islet transglutaminase, was relatively ineffective in the inhibition of insulin release. However, this anomaly may be explained by the failure of the diamine to rapidly gain entry to the β-cell or by the involvement of putrescine in the mechanism of insulin release.

v. Although methylamine, ethylamine and propylamine displayed toxic effects (inhibition of islet glucose utilisation and protein synthesis) which may themselves lead to inhibition of insulin release, a component of inhibition by these amines was identified which was independent of these effects.

Although proteins containing γ-glutamyl residues available for

transglutaminase-catalysed reactions were detected in islet homogenates, the nature of these substrates for transglutaminase in islets remained unresolved.

Experiments with the irreversible inhibitor of ornithine decarboxylase, α-difluoromethylornithine indicated that rapid synthesis of putrescine was unlikely to be involved in the mechanism of glucose-stimulated insulin release.

Interestingly, islets were found to contain readily detectable levels of spermidine and spermine and smaller amount of putrescine. The function of these compounds in islets is open to speculation, but it is possible that they may interact with islet transglutaminase and participate in reactions catalysed by the enzyme.

4.0 RESULTS AND DISCUSSION: TUMOUR STUDIES.

The evidence implicating transglutaminase in tumour growth and metastasis and in cell proliferation was summarised in the introduction (Section 1.4.3.). Most of the evidence suggested that the activity of transglutaminase decreases in tumours and proliferating tissues with respect to normal and non-proliferating tissue. A role for the enzyme in a mechanism involving the stabilisation of the cell membrane was suggested (Birckbichler & Patterson, 1978) on the basis of comparisons made between the activity of the enzyme and levels of its products in transformed and normal tissues and cells. It was therefore of interest to examine the levels of activity and subcellular distribution of transglutaminase in tumours obtained directly by carcinogenesis and in established transplantable tumour lines in order to determine the relationship of transglutaminase activity to the proliferative state of tissues.

4.1. LEVELS OF TRANSGLUTAMINASE ACTIVITY IN TUMOURS.

4.1.1. Transglutaminase activity in passaged tumours MC3 and CC5.

Tumours propogated in AS rats were harvested at 27 or 30 days after implantation and transglutaminase activity was determined in tissue homogenates using the standard assay. The results are shown in Table 4.1. and are compared to the activity similarly measured in normal tissues of AS rats (lung, liver and spleen). Transglutaminase activity in MC3 and CC5 at either 27 or 30 days of age was much lower than in any of the normal tissues of AS rats. This was the case irrespective of whether the enzyme

Table 4.1. Transglutaminase activity in tumour lines MC3 and CC5 and in normal tissues of AS rats.

Tissue	Tumour age (days)	U/mg protein	U/mg DNA	U/g tissue
MC3 (3)	27	3.19 ± 0.76	86.7 ± 22.6	379 ± 91
MC3 (5)	30	1.97 ± 0.23	52.6 ± 5.7	228 ± 32
CC5 (3)	27	4.72 ± 1.0	138 ± 31	773 ± 296
CC5 (4)	30	0.92 ± 0.11	26 ± 3	124 ± 9
Liver (6)	-	33.4 ± 1.9	4004 ± 122	6188 ± 241
Lung (5)	-	26.3 ± 1.75	915 ± 117	3544 ± 246
Spleen (5)	-	28.8 ± 2.9	610 ± 150	4472 ± 357

Tissues were homogenised in 3 volumes 0.25M sucrose, 1mM Tris, 1mM EDTA, pH 7.4 at 4°C and assayed for transglutaminase activity. Protein was determined by the method of Lowry (1951) and DNA by the method of Burton (1956). One unit (U) of enzyme activity equals one nmol putrescine incorporated per hour under the conditions of assay. Results are the mean ± S.E.M. with the number of observations shown in parentheses.

The tumours aged 27 and 30 days were obtained from separate sets of animals transplanted at different times.

activity was expressed on the basis of per milligramme protein, per milligramme DNA or per gramme wet weight of tissue. In both the tumour lines, transglutaminase activity appeared to be reduced at 30 days after implantation compared to that measured 27 days after implantation. This was the case irrespective of the way in which activity was expressed. Whilst it may be possible that this change in enzyme activity was the result of the different age of the tumours, the difference may only reflect the fact that the two sets of tumours were passaged from different parent tumours.

4.1.2. Transglutaminase activity in hepatocellular carcinomata induced with D.E.N. and 6-B.T.

Primary tumours were induced in the liver of AP rats in four separate experiments, two of which utilised D.E.N. as the carcinogen and two of which utilised 6-B.T. as the carcinogen. Since the dosing regimes were slightly different in these four experiments, each one is treated separately with its own set of control values. In each study, homogenates of hepatocellular carcinoma, normal (untreated) liver and liver tissue surrounding the tumours (tumour-bearing or pre-neoplastic liver) were prepared for transglutaminase assay.

4.1.2.1. Tumours induced with D.E.N.

The activity of transglutaminase measured in homogenates of tumours induced with D.E.N. is shown in Table 4.2. a. and b.

In the first experiment (4.2.a.), a significant reduction in enzyme activity was observed in hepatocellular carcinomata compared to that measured in normal liver. The reduction was significant ($p \leq 0.05$) when transglutaminase activity was expressed on the basis of per milligramme protein, per milligramme DNA or per gramme of tissue. The magnitude of the reduction was approximately 60%, irrespective of the manner of expression of enzyme activity. Although there appeared to be a slight reduction in transglutaminase activity in the tumour-bearing liver, this was found not to be statistically significant.

In the second study (Table 4.2.b), no significant reduction in enzyme activity with respect to normal tissues was observed in either the hepatocellular-carcinomata or the tumour-bearing liver. A noticeable feature of this experiment was the variability in the enzyme activity measured in the hepatocellular carcinomata particularly when expressed per milligramme protein. This may reflect the variation in the morphology and histology of these tumours, some of which were of a soft type and others of which were hard and fibrous.

4.1.2.2. Tumours induced with 6-B.T.

The transglutaminase activity measured in homogenates of hepatocellular carcinomata induced with 6-B.T. is shown in Table 4.3.a and b. When enzyme activity was expressed per milligramme DNA or per gramme tissue, a reduction in activity was observed in the hepatocellular carcinomata compared to normal liver in both these experiments. The reduction in enzyme activity was significant to a level of $p < 0.1$ in both sets of animals when activity was

expressed per unit DNA, and to a level of $p \leq 0.25$ and $p \leq 0.05$ when activity was expressed per g tissue in the first and second sets respectively. Activity expressed per milligramme protein was almost identical to the control value in both experiments. In both experiments, the enzyme activity in the tumour-bearing liver appeared to be intermediate between hepatocellular carcinoma and normal liver when activity was expressed as U/mg DNA or U/g tissue. However, this situation only represented a partially significant reduction in enzyme activity ($P \leq 0.25$) in one experiment, when activity was expressed per gramme tissue and per milligramme DNA in the second set of animals (Table 4.3.b.).

Table 4.2.

Levels of transglutaminase activity in hepatocellular carcinomata induced with D.E.N.

Rats were dosed with DEN as described in the Methods section (Section 2.7.1.2.1.). Table 4.2.a. shows the results from animals sacrificed after continuous dosing for 32 weeks and Table 4.2.b. shows the results from animals dosed for 27 weeks and sacrificed after 35 weeks. Control animals were treated separately for each set of animals. Samples of tumour tissue, surrounding liver and control liver were excised and homogenised in 0.25M sucrose, 1mM Tris, 1mM EDTA, pH 7.4 at $4^{\circ}C$ and assayed for transglutaminase activity. Protein was determined by the method of Lowry et al., (1951) and DNA by the method of Burton (1956). One unit of enzyme activity equals one nmol putrescine incorporated/h under the conditions of assay. Results are the mean $\pm$ S.E.M. with the number of observations shown in parentheses. The significance of difference from controls (normal liver) was assessed using the Students' t-test; N.S. denotes no significant difference from controls. Allowance was made in the t-test for samples with unequal variances.

4.2.a.

Tissue	U/mg protein	U/mg DNA	U/g tissue
Hepatocellular-carcinoma (3)	14.0 ± 1.3	2533 ± 401	2725 ± 510
	$P \leq 0.05$	$P \leq 0.05$	$P \leq 0.05$
Tumour-bearing liver (3)	28.8 ± 1.5	5699 ± 378	6034 ± 510
	N.S.	N.S.	N.S.
Normal liver (6)	33.4 ± 5.8	6060 ± 1031	7648 ± 1520

4.2.b.

Hepatocellular-carcinoma (4)	62.2 ± 16.3	4635 ± 1306	6434 ± 1961
	N.S.	N.S.	N.S.
Tumour-bearing liver (4)	36.3 ± 3.3	9166 ± 264	8553 ± 1031
Normal liver (4)	41.4 ± 2.7	6544 ± 1247	7388 ± 660

Table 4.3.

Levels of transglutaminase activity in hepatocellular carcinomata induced with 6-BT.

Rats were dosed with 6-BT as described in the Methods section (Section 2.7.1.2.2.). Table 4.3.a. shows the results from animals administered 7.5 mg 6-BT/kg and Table 4.3.b. shows the results from animals dosed with 5.0 mg 6-BT/kg. Control animals were treated separately for each set of animals. Samples of tumour tissue, surrounding liver tissue and control liver were homogenised in 3 volumes 0.25M sucrose, 1mM Tris, 1mM EDTA, pH 7.4 at $4^{o}C$ and assayed for transglutaminase activity. Protein was determined by the method of Lowry et al., (1951) and DNA by the method of Burton (1956). One unit (U) of enzyme activity equals one nmole putrescine incorporated per hour under the conditions of assay. Results are the mean $\pm$ S.E.M. with the number of observations shown in parentheses. Significance of difference from controls was determined using the Students' t-test; N.S. denotes no significant difference. Allowance was made in the t-test for samples with unequal variances.

4.3.a.	Tissue	U/mg protein	U/mg DNA	U/g tissue
	Hepatocellular carcinoma (4)	30.0 ± 7.2	3269 ± 972	4369 ± 1586
		N.S.	$P \leq 0.1$	$P \leq 0.25$
	Tumour-bearing liver (3)	33.7 ± 3.6	4376 ± 808	6388 ± 480
		N.S.	N.S.	N.S.
	Normal liver (4)	30.9 ± 1.3	5741 ± 818	6679 ± 536
4.3.b.	Hepatocellular carcinoma (5)	27.1 ± 11.6	2496 ± 1009	3192 ± 955
		N.S.	$P \leq 0.1$	$P \leq 0.05$
	Tumour-bearing liver (2)	27.2 ± 8.2	3139 ± 754	4225 ± 667
		N.S.	$p \leq 0.25$	$P \leq 0.25$
	Normal liver (5)	28.2 ± 3.0	4707 ± 559	5881 ± 659

4.1.3. Discussion.

The passaged tumour lines MC3 and CC5 possessed substantially lower levels of transglutaminase activity than any of the normal tissues (liver, lung and spleen) of the same strain of rats. In the absence of a control tissue for a strictly valid comparison to be made with these tumours, it is not possible to say that the enzyme activity in these tissues was reduced. However, the magnitude of the difference in transglutaminase activity between these tumours and normal tissues (sometimes as great as 200-fold) would suggest a link between the neoplastic state and reduced levels of transglutaminase activity.

In the hepatocellular carcinomata induced with DEN and 6-BT, a reduction in transglutaminase activity was usually observed in these tissues when compared to normal liver. (Tables 4.2a, 4.3a and b). The reduction in enzyme activity was seen to be most significant when it was expressed per unit DNA or per unit wet weight of tissue. Expressing enzyme activity per unit DNA may be the most informative way, for if it is assumed that the amount of DNA per cell is equal in liver and hepatocellular carcinoma, enzyme activity per milligramme DNA represents enzyme activity per cell of the tissue. Changes in protein content and fluid content of the tissue during the growth of a tumour may mask changes in transglutaminase activity which may occur in cells.

In two of the sets of animals, one set treated with DEN and one set treated with 6-BT, the tumour-bearing liver

appeared to possess a transglutaminase activity when expressed as U/g tissue or U/mg DNA which was intermediate between those of hepatocellular carcinoma and normal liver (Table 4.2a and 4.3b). Although this situation represented a reduction of activity in pre-neoplastic liver compared to normal liver, the difference was only approaching significance ($p \leq 0.25$) in one case (Table 4.3b.). This observation suggests that in some cases transglutaminase has a tendency to fall as liver tissue approaches a neoplastic state.

In general, the results reported here agree with those obtained by other workers who observed a decreased transglutaminase activity in tumour tissue (Birckbichler & Patterson, 1978; Vanella et al., 1983). However, a reduction in enzyme activity in hepatocellular carcinomata when compared to normal liver was not a universal phenomenon (see Table 4.2b). A large variation in enzyme activity between tumours obtained in the same experiment was sometimes observed. Heterogeneity in the type or growth state of the tumours may account for this, particularly in view of the heterogeneity in morphology and histology that was observed.

It is of interest to note that the transglutaminase activity measured in the established, transplantable tumours MC3 and CC5 was so much lower than in any other tissues assayed in a similar manner. These tumours were so well adapted to uncontrolled growth after subcutaneous implantation that the success rate for growth after transplantation was close to 100% (data not shown). It is likely that the propagation of these tumours has led to a selection of cell type within them

which is best adapted to the conditions of implantation. Thus, these tumours may represent homogeneous tissues adapted for uncontrolled growth and the very low levels of transglutaminase activity in these tissues may be a reflection of this characteristic.

The mechanism by which a reduction in transglutaminase activity occurs in tumours is a matter for speculation and further investigation. It is possible that expression of transglutaminase activity is altered by mutations in the gene for the enzyme, either in structural element(s), leading to the synthesis of less active enzyme molecules, or in regulatory elements, leading to the synthesis of fewer enzyme molecules. Alternatively, altered expression of enzyme activity may result from co-ordinated regulatory events associated with neoplasia, which involve the suppression of transglutaminase activity. In connection with the latter mechanism, it is worth noting that the enzyme in macrophages appears to be under transcriptional control (Murtaugh et al., 1980). Enzyme activity may also be affected by post-translational means (e.g. protein phosphorylation) or by the inclusion of natural inhibitors derived from tumour tissue (e.g. polyamines) in the assay system. However, this last possibility may be discounted by the observation that the activity of rat liver homogenate was unaffected by the addition of particle-free extract of tumours (Dr. R.N. Barnes, personal communication).

Without further investigation, it is not possible to say whether lowered transglutaminase activity in tumours represents a defect with respect to the enzyme or whether a reduction in transglutaminase activity may be viewed as one of a series of

biochemical changes required for neoplastic growth or cell proliferation. Observations by other workers suggest that the relationship between levels of transglutaminase activity and cell proliferation is not simple. For example, in CHO cells transglutaminase activity doubles within 3h after their release from quiescence, falls and re-peaks at 6h and 9h thereafter (Scott & Haddock-Russell, 1982). Rises in transglutaminase activity have also been associated with the proliferation of liver tissue following partial hepatectomy (Remington & Russell, 1982). Therefore, a rise or fall in transglutaminase activity may be associated with proliferative growth, depending on the type of tissue. This is also illustrated by the data in Table 4.2b. in which the enzyme activity in tumours induced with DEN did not appear to fall significantly compared to normal liver.

4.2. SUBCELLULAR DISTRIBUTION OF TRANSGLUTAMINASE ACTIVITY IN TUMOURS.

The subcellular distribution of transglutaminase activity may be important to the function of the enzyme in the cell. Studies by Birckbichler et al,(1977) suggested that transglutaminase activity was associated with the membrane-containing fraction in hepatoma to a greater extent than in normal liver. Furthermore, it has been suggested that restriction of transglutaminase activity to the particulate fraction in cells represented a means of limiting the activity of the enzyme (Barnes, 1980). The subcellular distribution of transglutaminase activity in the tumours whose homogenate transglutaminase activity was discussed in the previous section was examined to study further the changes in transglutaminase activity during tumour growth.

4.2.1. Subcellular distribution of transglutaminase activity in the tumour lines MC3 and CC5.

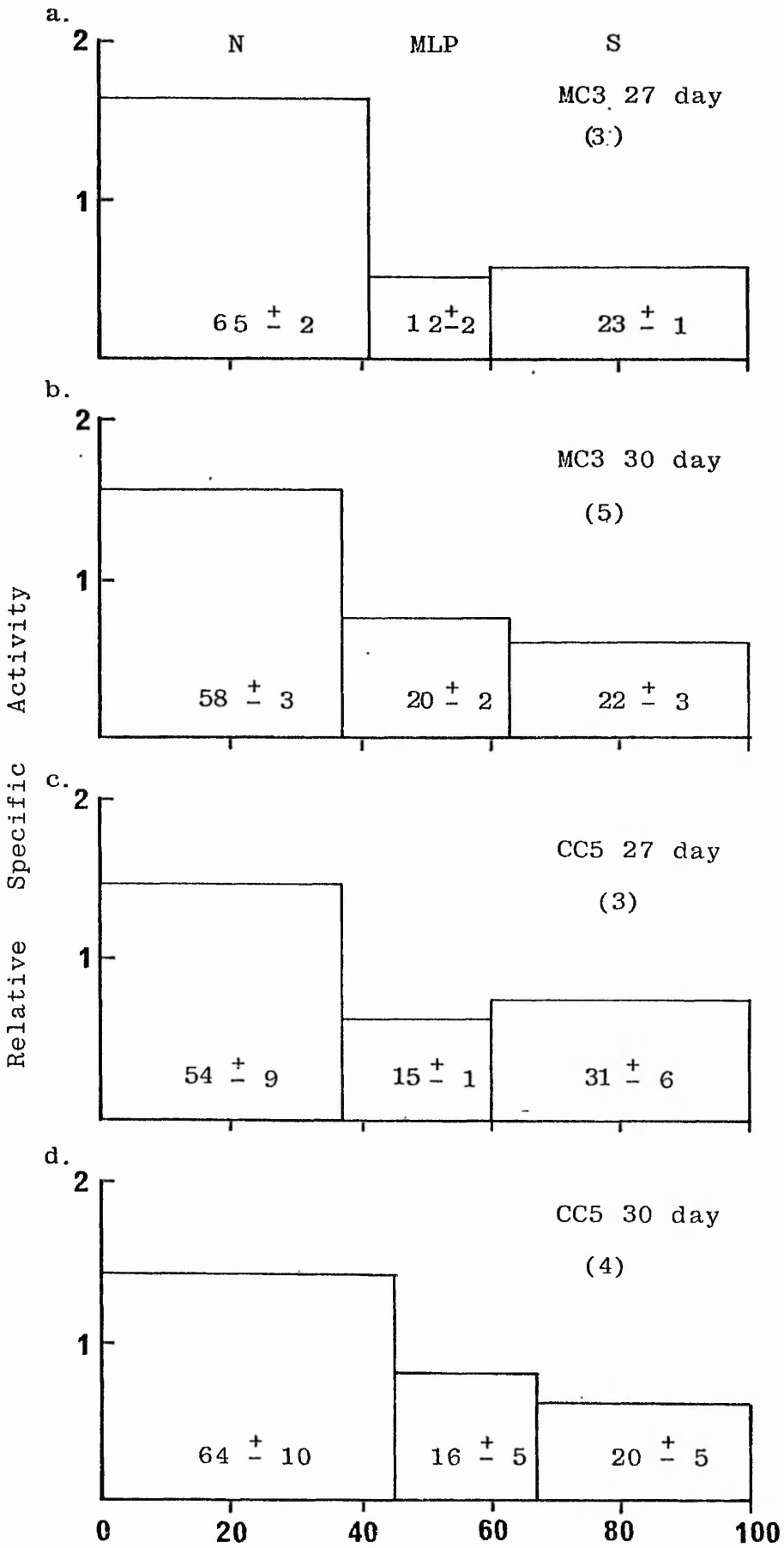
Homogenates of the tumours MC3 and CC5 were fractionated by centrifugation to produce a 600g 'nuclear' pellet (N), a 71,000g 'microsomal' pellet (MLP) and a particle-free supernatant (S) which were assayed for transglutaminase activity. The results are shown in Fig. 4.1.a. and b. The distribution of enzyme activity was very similar in the two tumours with most of the activity present in the membrane-containing fractions N and MLP which together accounted for some 70-80%

Figure 4.1. Distribution of transglutaminase activity in the passaged tumours MC3 and CC5.

Tumours were harvested at either 27 or 30 days after implantation into AS rats, homogenised in 3 volumes ice-cold 0.25M sucrose, 1mM Tris, 1mM EDTA, pH 7.4 and fractionated into N, MLP and S fractions as described in the Methods section. Transglutaminase activity in each of the fractions was assayed and calculated for each fraction and expressed per gram of original tissue. Protein was determined by the method of Lowry and also expressed per gram of original tissue. Results are presented as histograms of relative specific activity (percentage distribution of enzyme activity/percentage distribution of protein) vs. percentage distribution of protein in each fraction. Figures in parentheses refer to numbers of observations and numbers in bars refer to the % total recovered activity for that fraction.

The tumours aged 27 and 30 days were transplanted at different times in separate sets of animals.

4.1.


a.
N
MLP
S
MC3 27 day
(3)
2
1
65 ± 2
12±2
23 ± 1
b.
MC3 30 day
(5)
58 ± 3
20 ± 2
22 ± 3
c.
CC5 27 day
(3)
54 ± 9
15 ± 1
31 ± 6
d.
CC5 30 day
(4)
64 ± 10
16 ± 5
20 ± 5
Relative Specific Activity
0
20
40
60
80
100
Percentage protein

of the total activity recovered. The largest proportion of activity (approx. 60% of total recovered) was associated with the N pellet which was assumed to contain plasma membrane fragments and nuclei (De Duve et al., 1955).

There were small but noticeable differences in the distribution of transglutaminase activity in tumours aged 30 days compared to those aged 27 days. In MC3 and CC5 tumours aged 30 days, the relative specific activity of the MLP fraction was higher than that of the S fraction, whereas in tumours aged 27 days the relative specific activity of the S fraction was the greater (Fig. 4.1.). Also, in CC5 tumours, a slight re-distribution of enzyme activity (10%) from the S fraction to the N fraction appeared to have occurred at 30 days compared to 27 days. Although these changes may be related to the different ages of the tumours, they may only reflect differences in the tumours used for propagation, which were different for the tumours of different ages.

4.2.2. Distribution of transglutaminase activity in hepatocellular carcinomata.

The subcellular distribution of enzyme activity in primary tumours was determined using a short fractionation procedure which provided a membrane-containing 'particulate' fraction (P) and a particle-free supernatant (S). The data from four separate sets of animals are shown separately with controls for each set.

4.2.2.1. Distribution in tumours induced with DEN

The distribution of transglutaminase activity in tumours induced with DEN is shown in fig. 4.2.a. and b. In both sets of animals enzyme activity was distributed approximately equally in each fraction of normal liver. In the hepatocellular carcinomata, however, the greater proportion of enzyme activity was present in the P fraction containing cell membranes. This re-distribution with respect to controls was extreme in the first set of animals (Fig. 4.2.a.) and very substantial in the second set (Fig. 4.2.b.) and in neither case did it correlate with a gross re-distribution of protein between the fractions. In the first set of animals, there was also a substantial re-distribution of activity towards the particulate fraction of the tumour-bearing livers which appeared to represent an intermediate stage between the normal and transformed liver. However, this situation was not borne out in the second set of results in which the

distribution of transglutaminase activity was similar in control and tumour-bearing liver.

4.2.2.2. Subcellular distribution of transglutaminase activity in tumours induced by 6-BT.

Examination of Fig. 4.3.a. and b. reveals that, in common with the tumours induced by DEN, those induced with 6-BT displayed a re-distribution of transglutaminase activity to the particulate fraction compared to untreated controls. In neither case could a re-distribution of total protein of the fraction account for the re-distribution of enzyme activity. The effect was most marked in the second set of animals (Fig. 4.3.b.) although it was not as substantial in either set as in either of the sets of tumours induced with DEN. The distribution of enzyme activity in the tumour-bearing livers of both sets of animals treated with 6-BT did not appear to differ from that observed in untreated controls.

Figure 4.2. and 4.3. Subcellular distribution of transglutaminase activity in hepatocellular carcinomata induced with D.E.N. and 6-B.T.

Tumours, tumour-bearing liver and normal liver from untreated animals were excised and homogenised in ice-cold 0.25M sucrose, 1mM Tris, 1mM EDTA, pH 7.4 and fractionated by centrifugation at 71,000 gav. for 45 min. The resulting pellet and supernatant (P and S fractions) were assayed for transglutaminase activity. Protein was determined by the method of Lowry et al., (1951) and results are expressed as histograms of relative specific activity vs % protein for each fraction. Figures in parentheses refer to numbers of observations and numbers in bars refer to % distribution of total recovered enzyme activity for the two fractions.

The results for the four experiments which used different dosing regimes are presented as follows :

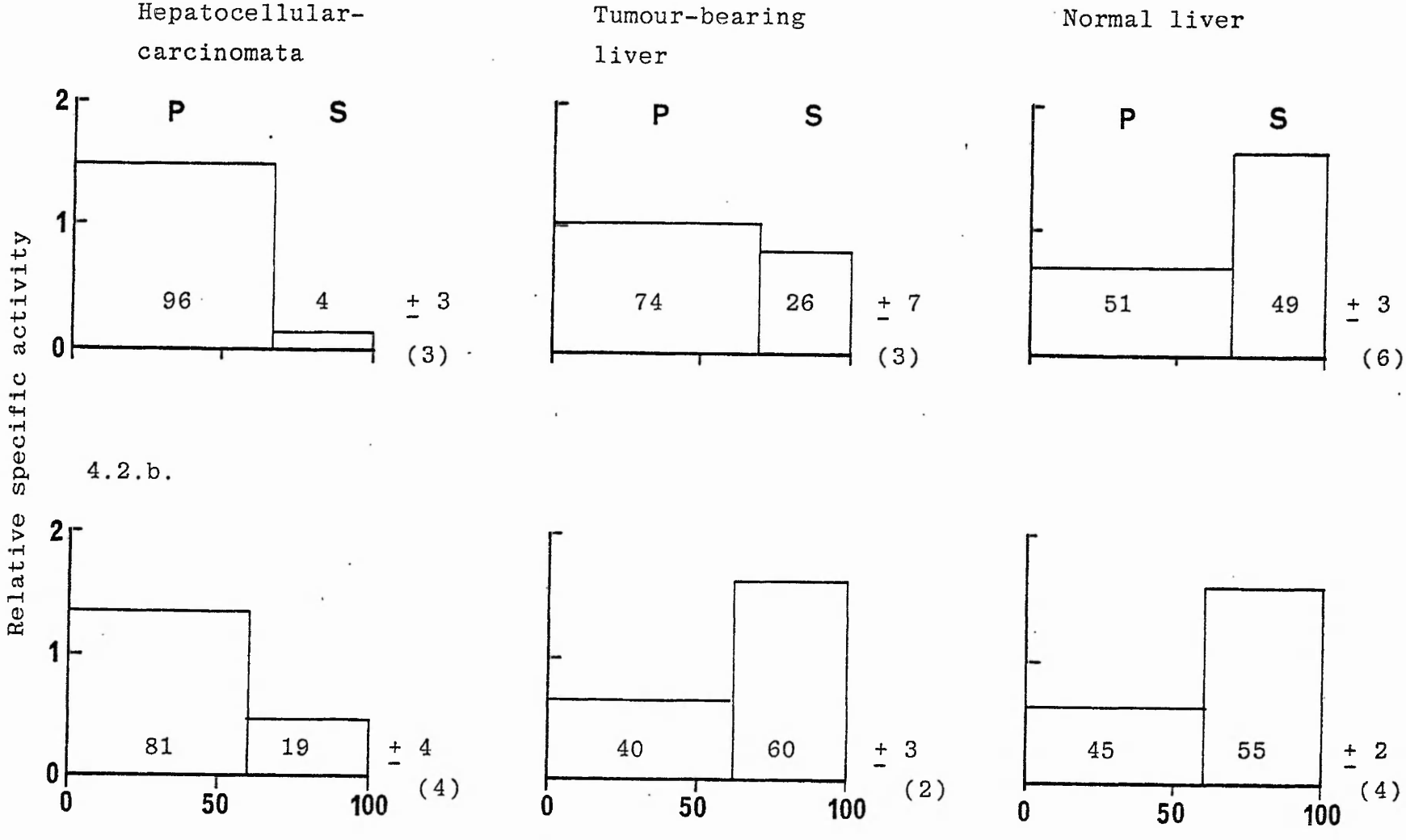
4.2.a. DEN continuous dosing for 32 weeks;

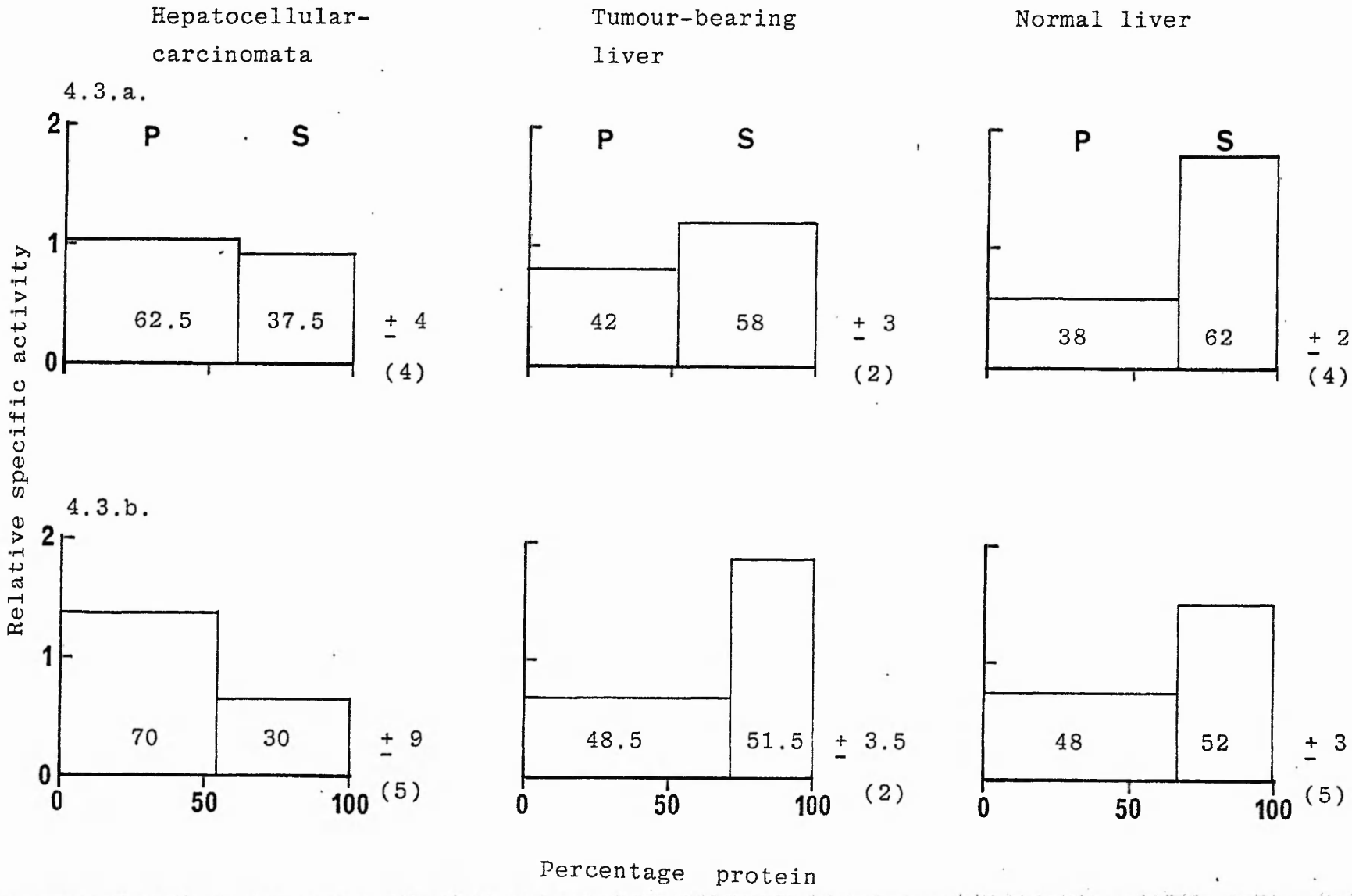
4.2.b. DEN 27 weeks' dosing and sacrificed after 35 weeks;

4.3.a. 6-BT 7.5mg/Kg

4.3.b. 6-BT 5.0mg/Kg

4.2.a.


Hepatocellular-carcinomata
Tumour-bearing liver
Normal liver
P
S
2
1
0
96
4
± 3
(3)
74
26
± 7
(3)
51
49
± 3
(6)
4.2.b.
81
19
± 4
(4)
40
60
± 3
(2)
45
55
± 2
(4)
0
50
100
Relative specific activity
Percentage protein


Hepatocellular-carcinomata
Tumour-bearing liver
Normal liver
4.3.a.
4.3.b.
P
S
62.5
37.5
± 4
(4)
42
58
± 3
(2)
38
62
± 2
(4)
70
30
± 9
(5)
48.5
51.5
± 3.5
(2)
48
52
± 3
(5)
Relative specific activity
Percentage protein
0
1
2
50
100

4.2.3. Discussion.

In all the tumour types studied, which included primary hepatoma and passaged tumour lines, most of the transglutaminase activity recovered after fractionation of homogenates of these tissues was associated with the particulate or membrane-containing fractions. In the case of the hepatocellular carcinomata, the association of enzyme activity with the 71,000 g pellet was a feature which distinguished this neoplasm from normal liver. Although the degree of re-distribution of transglutaminase activity to the particulate fraction varied, it was substantial and statistically significant in all four sets of experiments undertaken ($p \leq 0.05$). These findings are in accordance with those of Birckbichler et al., (1976) who showed that whereas in normal liver 90% of transglutaminase activity was found in the 105,000 g supernatant, only 30% of activity was recovered in this fraction in Novikoff hepatoma and primary hepatoma induced with 3'-methyl-4-dimethylaminoazobenzene. Therefore, subcellular re-distribution of transglutaminase activity may be a marker of neoplasms.

Although it is possible that the distribution of transglutaminase activity in the tumour lines MC3 and CC5 reflects the neoplastic state of these tissues, a similar conclusion cannot be reached from the results obtained for these tissues. A true control tissue was not available for a comparison to be made with MC3 and CC5 and the distribution observed in these tissues may only reflect that of their parent tissues.

The significance of the subcellular distribution of

transglutaminase activity is not known, but studies have been undertaken to investigate it (Soret et al., 1982; Remington & Haddock-Russell, 1982; Bruce et al., 1983; Barnes, 1980). The occurrence of a re-distribution of transglutaminase activity in the pathological state of neoplasia may be a result of the transformation of the tissue and this may reflect or contribute to the properties and behaviour of the tumour. The increased association of transglutaminase with the particulate fraction of tumour tissue may bear some relationship to the fall in enzyme activity sometimes observed in tumours when compared to normal liver. This is in accordance with ideas already stated that the enzyme may be stored in a less active form by association with cell membranes (Barnes, 1980).

What transpires from studies on the subcellular distribution of transglutaminase activity is that in normal tissues, a component of the total transglutaminase activity is usually associated with the particulate fraction of the cell. For example, in normal lung, 90% of the total transglutaminase activity was associated with particulate material (Griffin et al., 1978). Therefore, a predominantly particulate distribution for the enzyme is not a feature peculiar to tumours and may not necessarily determine the proliferative state of a tissue.

The suggestion that it is possible to make is that a tissue may require a certain subcellular localisation of transglutaminase for the enzyme to express its activity in a manner appropriate to the requirements of that tissue. The change in enzyme distribution on transformation of liver tissue may therefore reflect the different requirements of tumour tissue as distinct

from those of normal liver.

By way of caution, it is worth considering that the observed distribution of transglutaminase activity may have been an artefact of the homogenisation procedure. Non-specific interactions of the enzyme with cell membranes, cell surfaces and cell debris may influence its apparent subcellular distribution and any differences observed between liver and tumour may only reflect, for instance, changes in cell surface properties which may bear no relevance to the function of transglutaminase in the cell.

This caution aside, it is interesting to consider how the subcellular distribution of transglutaminase may be regulated in response to conditions inside the cell. Studies on AS rat liver (Barnes, 1980) suggested that the enzyme associated with the 600g pellet was readily leached from the pellet by resuspension in sucrose media. However, when washing medium contained a combination of putrescine and Ca^{2+} the enzyme became firmly bound to the pellet and could not be leached out. Inclusion of either Ca^{2+} or putrescine alone did not have this effect. It was demonstrated that guinea pig liver transglutaminase could crosslink its own protein (Birckbichler et al., 1977) so the interesting possibility arose that rat liver enzyme was immobilised in the 600g pellet by a covalent interaction involving a diamine crosslink (see Introduction Fig. 1.1.). A similar mechanism may be involved in the re-distribution of transglutaminase in tumour tissue, particularly in view of the reports suggesting that levels of putrescine in liver increase during carcinogenesis (Milano et al., 1981) and the general association of polyamines with the growth of tissues (Bachrach, 1978; Bachrach et al., 1981; Dipasquale et al., 1978; Prakash et al., 1980; Goyns, 1982; Heby, 1981; Haddox

& Haddock-Russell, 1981).

In the work that follows, the association of transglutaminase with particulate fractions of tumours was investigated in an effort to further understand the mechanism of re-distribution of transglutaminase activity in tumour tissue.

4.3. The association of transglutaminase activity with the membrane fraction of tumours: leaching experiments.

The results obtained in the previous section (4.2.) suggested that a re-distribution of transglutaminase activity to the particulate fraction of cells may be a marker for neoplastic growth and suggests a possible involvement of the enzyme in tumour growth. The aim of the investigation below was to determine the strength of the association of transglutaminase activity with the pellet fractions of tumours.

4.3.1. Leaching of transglutaminase activity from the 600g pellet of MC3 and CC5.

The N pellet fraction obtained by centrifugation of MC3 and CC5 tumour homogenates at 600 g av. was used in experiments to study the association of enzyme activity with the membrane fraction of these tumours. The pellets were washed twice in sucrose medium by centrifugation and then resuspended. Transglutaminase activity in the pellet, washed pellet and washes was then determined. The N pellet obtained from rat liver was treated similarly and used for comparative purposes in Table 4.4.. In the tumour MC3 at either 27 or 30 days after implantation, approximately 85% of the total activity obtained after the washing procedure was still associated with the N pellet. The enzyme activity in the pellets of CC5 at 27 and 30 days after implantation appeared to be slightly less tightly bound, with some 65-75% of the total activity recovered after the washing procedure remaining in the pellet. The transglutaminase

activity in the N pellet of liver appeared less tightly bound than in either MC3 or CC5, with approximately 50% of the total activity recovered remaining in the N pellet after washing. Therefore, in both tumour lines, but particularly MC3, the transglutaminase activity in the N pellet appears to be less easily leached from this fraction than in normal rat liver.

4.3.2. Leaching of transglutaminase activity from the 71,000g pellet of hepatocellularcarcinomata.

In similar experiments to those described above, the amount of transglutaminase activity that could be leached from the pellet (P) obtained by centrifugation of homogenates of tumours induced with DEN at 71,000g av. was investigated. These tumours were the same ones whose transglutaminase activity was studied earlier (Table 4.2.b.; Fig. 4.2.b.).

Examination of Table 4.5. indicates that in these hepatocellular carcinomata, in the tumour-bearing liver obtained from the same animals and in normal liver, approximately 60% of the total enzyme activity recovered after washing remained in the P fraction. Therefore, the pellet-associated transglutaminase activity appeared to be bound with a similar degree of tenacity in these tissues.

Table 4.4. Leaching of transglutaminase activity from the N pellet of MC3 and CC5 tumours.

The 600g pellet (N pellet) prepared from homogenates of MC3 and CC5 tumours harvested at 27 and 30 days after transplantation were washed twice with ice-cold 0.25M sucrose, 1mM Tris, 1mM EDTA, pH 7.4 by resuspension and centrifugation in the original volume of buffer (see Methods Section, Fig. 2.1.). Transglutaminase activity was then determined in the homogenate (H), N pellet (N), washes (W_1 and W_2) and washed N pellet (Nw) and expressed as specific activity (U/mg protein) and total activity (U/g tissue) for each fraction. The experiment was performed in the same manner using the N pellet obtained from homogenates of rat liver for the purposes of comparison. One unit of enzyme activity equals one nmol putrescine incorporated per hour under the conditions of assay. Results are the mean ± S.E.M. with the number of observations shown in parentheses.

Tissue	Fraction	U/mg protein	U/g tissue	% activity recovered after washing	% activity of original N pellet
MC3 (2)	H	2.45 ± 0.30	290 ± 25		
(27 days)	N	4.07 ± 0.32	186 ± 0.5	98	
	Nw	3.73 ± 0.55	159 ± 24	84	85
	W1	2.35 ± 0.07	19 ± 0.5	10	10
	W2	3.30 ± 2.22	11 ± 1.5	6	5
MC3 (5)	H	1.97 ± 0.23	288 ± 32		
(30 days)	N	4.11 ± 1.02	125 ± 37	73	
	Nw	5.65 ± 0.99	148 ± 10	87	118
	W1	1.85 ± 0.49	13 ± 3	8	10
	W2	4.05 ± 1.01	10 ± 2	5	8
CC5 (3)	H	4.72 ± 0.99	773 ± 296		
(27 days)	N	9.39 ± 1.84	350 ± 39	51	
	Nw	14.0 ± 2.76	440 ± 64	64	126
	W1	16.18 ± 4.79	134 ± 57	19	38
	W2	48.96 ± 17.37	115 ± 38	17	33
CC5 (4)	H	0.92 ± 0.11	124 ± 9		
(30 days)	N	2.84 ± 0.87	157 ± 47	131	
	Nw	2.20 ± 0.50	89 ± 16	74	57
	W1	2.68 ± 0.93	17 ± 1	14	11
	W2	5.91 ± 2.67	14 ± 6	12	9
Liver (2)	H	24.31 ± 4.17	4814 ± 745		
	N	17.37 ± 2.48	2324 ± 263	94	
	Nw	22.89 ± 2.66	1297 ± 57	53	56
	W1	22.31 ± 2.17	769 ± 102	31	33
	W2	19.81 ± 2.65	402 ± 96	16	17

Table 4.5 Leaching of transglutaminase activity from the 71,000g pellet (P fraction) of hepatocellular carcinomata induced with DEN.

The 71,000g pellets (P) prepared from homogenates of hepatocellular carcinomata, tumour-bearing liver and normal liver were washed twice with ice-cold 0.25M sucrose, 1mM Tris, 1mM EDTA pH 7.4 by resuspension and centrifugation in the original volume of buffer (see Methods Section, Fig. 2.1.). Transglutaminase activity was then determined in the homogenate (H), pellet (P), washes (W_1 and W_2) and washed pellet (P_w) and expressed as specific activity (U/mg protein) and total activity (U/mg tissue) for each fraction. One unit of enzyme activity equals one nmol putrescine incorporated per hour under the conditions of assay. Results are the mean ± S.E.M. with the number of observations shown in parentheses.

Sample		U/mg protein	U/g tissue	% 'washed' activity	% activity of original P pellet
Hepatocellular-carcinoma (4)	H	62.2 ± 16.3	6434 ± 1961		
	P	83.9 ± 20.0	5195 ± 1509	112	
	Pw	54.5 ± 8.1	2705 ± 718	58.5	52
	W1	171.7 ± 39.8	1145 ± 223	24.5	22
	W2	259.5 ± 38.3	777 ± 232	17	15
Tumour-bearing liver (2)	H	33.9 ± 7.5	7717 ± 1713		
	P	29.8 ± 6.9	6622 ± 1829	121	
	Pw	38.3 ± 2.7	3690 ± 341	67	56
	W1	129.4 ± 6.8	1372 ± 342	25	21
	W2	325.4 ± 162.5	421 ± 58	8	6
Normal liver (2)	H	38.4 ± 0.5	7455 ± 909		
	P	60.9 ± 3.9	4277 ± 189	145	
	Pw	44.8 ± 0.2	1755 ± 36	60	41
	W1	124.4 ± 5.1	601 ± 21	20	14
	W2	323.2 ± 65.1	599 ± 88	20	14

4.3.3. Discussion.

The results described above indicated that the transglutaminase activity in the 600g pellet prepared from homogenates of the tumour lines MC3 and CC5 was less easily leached from the pellet by resuspension in sucrose medium than was the case for normal liver. This was particularly noticeable in the case of MC3. In the hepatocellular carcinomata induced by DEN and the tumour-bearing liver of the same animals, the transglutaminase activity associated with the 71,000g pellet could be washed from the pellets as easily as in normal liver. Therefore, the association of transglutaminase activity with the particulate material appeared to be stronger in the case of MC3 and CC5 than for the 71,000g pellet of tumours induced by DEN. Although the pellet fractions investigated in these tumours were different, this indicates that the mechanism of binding of transglutaminase activity to the particulate fraction in these tumours may be different. It is possible that the stronger association of transglutaminase activity with the N pellet of MC3 and CC5 compared to normal liver may be the result of a covalent interaction involving a diamine cross-link as described previously (see Section 1.4.) which would not allow the easy release of transglutaminase during resuspension of the pellet. Alternatively, the association may involve interactions of a weaker nature (e.g. ionic interactions or hydrophobic interactions) and difference in the tenacity of pellet-associated transglutaminase activity may reflect an altered number of binding sites for the enzyme. This may result from alterations in the particulate material (e.g. cell membranes) or from alterations in the enzyme itself.

The hepatocellular carcinomata used in these studies on leaching of transglutaminase activity displayed a re-distribution of transglutaminase activity to the 71,000g pellet without a fall in total or specific activity compared to normal liver (Fig. 4.2.b. and Table 4.2.a.). The transglutaminase activity measured in MC3 and CC5 was very low compared to normal tissues (Table 4.1.) and most of the total enzyme activity was found to be present in the particulate fractions of these tumours. Therefore it is possible that the strength of association of transglutaminase activity with the particulate material bears a relation to the magnitude of transglutaminase activity in the tissue and the proliferative state of the tissue.

4.4. CONCLUSION TO THE TUMOUR STUDIES.

In the passaged tumour lines MC3 and CC5, transglutaminase activity was substantially lower than that found in the normal rat tissues liver, lung and spleen. In hepatocellular carcinomata induced with DEN and 6-BT, transglutaminase activity was in most cases lower than that measured in normal liver. However, in these primary tumours, a fall in transglutaminase activity was not found to be a universal feature associated with neoplasia.

In all the tumours studied, the greater proportion of transglutaminase activity recovered after fractionation of tumour homogenates was associated with particulate material. In the primary tumours induced with DEN and 6-BT this situation represented a significant re-distribution of transglutaminase activity with respect to normal liver. Such a re-distribution of enzyme activity may therefore be a marker of neoplasia.

The pellet-associated activity in hepatocellular carcinomata induced with DEN did not appear to adhere to the pellet prepared by centrifugation of tissue homogenates at 71,000g any more strongly than in the case of normal liver. In the case of MC3 and CC5, however, transglutaminase activity associated with the pellet prepared by centrifugation of tissue homogenates at 600g appeared to bind more tenaciously than in the case of normal liver.

These results suggest that changes in the magnitude and subcellular distribution of transglutaminase activity occur during tumour growth and indicate that those changes may be important in the behaviour of neoplastic tissues.

5. GENERAL DISCUSSION

The aim of this thesis was to help to elucidate the cellular function of the tissue transglutaminase. In the course of these studies, several original findings were made which are important in respect of the stated aim:

i. Transglutaminase activity was detected and partially characterised in pancreatic islets of Langerhans;
ii. Evidence was obtained which suggested that islet transglutaminase may be involved in the mechanism of glucose-stimulated insulin release from the pancreatic β-cell;
iii. The polyamines putrescine, spermidine and spermine were detected in islets and their presence implied a possible joint cellular role with transglutaminase;
iv. A predominantly particulate distribution of transglutaminase appeared to be a marker of tumour tissue.

Some of these findings have been presented in a published form in addition to this thesis (Bungay et al., 1982; Bungay et al., 1984a; Bungay et al., 1984b; Barnes et al., 1984).

The evidence obtained in this thesis for the involvement of islet transglutaminase in the mechanism of insulin release from the pancreatic β-cell is not conclusive but provides the basis for further experimentation. In particular, the effects on insulin release of highly specific substrates for transglutaminase should be investigated in order to define more closely a correlation between inhibition of transglutaminase activity and insulin release.

Definitive evidence for the involvement of transglutaminase in insulin release requires the demonstration of γ-glutamyl conjugates (ε-(γ-glutamyl) lysine, (γ-glutamyl) polyamines and bis-(γ-glutamyl) polyamines) whose formation would be essential in the secretory mechanism.

The proposal that tissue transglutaminase may be involved in the secretory mechanism is a new idea but it is not unrelated to suggestions made by other workers on the role of transglutaminase in cells which pointed towards a role for the enzyme in cell membrane-mediated events (see Introduction, Sections 1.4 and 1.5). Thus, the role of transglutaminase in glucose-stimulated insulin release may be to regulate the interactions between cellular membranes during secretion. The β-cell must possess the means for maintaining specificity in the interactions between membrane components during their passage between organelles in the cell (Jamieson, 1972) but it is not known how this specificity is achieved or maintained (Palade,1982). Transglutaminase-catalysed post-translational modification of membrane proteins may play a role in this process by identifying and altering areas of membrane before their association with other membranes and making their specific interaction possible. A role for protein cross-linking can also be envisaged as being a possible mechanism for stabilisation of membranes, preventing their intermixing during the process of endomembrane flow and making possible their subsequent non-random removal. Therefore it is possible to identify potential roles for transglutaminase in membrane recognition and in the segregation of membranes after their fusion, maintaining chemical specificity.

Of particular relevance is the proposal that the role of

transglutaminase in receptor-mediated endocytosis may be in the recycling of receptors (Van Leuven et al., 1980). It is known that during periods of secretory activity, membrane which is inserted into the plasma membrane by exocytosis is retrieved by endocytosis and reutilised (Farquhar, 1982). This process occurs in the pancreatic β-cell during periods of glucose-stimulated insulin release (Orci et al., 1978). Therefore, the process of recycling of membrane components is a common feature of exocytosis and endocytosis. It is therefore tempting to speculate that the role of tissue transglutaminase in the pancreatic β-cell is in the recycling of membranes during periods of high exocytotic activity and that furthermore,this may be a common role for the enzyme in cells.

The observations made in this thesis that basal rates of insulin release (in the presence of 2.8mM glucose) were not inhibited by treatment of islets with primary amine inhibitors of transglutaminase activity (see Section 3.2.1.) supports the suggestion of a role for islet transglutaminase in membrane recycling. Basal insulin release probably does not proceed at rates high enough to require the recycling of secretory vesicle membrane inserted into the plasma membrane and therefore may not require transglutaminase activity. It may also be concluded that since basal insulin release proceeds (presumably by exocytosis) in conditions under which transglutaminase activity is inhibited, the process of exocytosis per se may not require transglutaminase.

The consideration of the involvement of transglutaminase in glucose-stimulated insulin release should take into account possible interactions with other potential or known regulators of the secretory mechanism, since the action of such regulators must be

finely co-ordinated.

The mobilisation of the secretory apparatus probably involves many carefully regulated interactions between membranes, membrane infrastructures (e.g. clathrin) and cytoskeletal proteins (e.g. tubulin, actin). Evidence has recently accumulated that such interactions may be regulated by post-translational modification of proteins involving phosphorylation of proteins mediated by protein kinases which themselves are regulated by Ca^{2+}/calmodulin, cAMP and phospholipid metabolism (see Introduction, Section 1.5.). The observation that some phosphorylated proteins are better substrates for transglutaminase than their non-phosphorylated counterparts (Iwanij, 1977) indicates the possibility that transglutaminase-catalysed reactions may act in concert with protein phosphorylation during activation of the secretory mechanism.

In addition, the involvement of phospholipid metabolism in glucose-stimulated insulin release (Axen et al., 1983; Best & Malaisse, 1982; Tooke et al., 1984) may have some bearing on the ability of membrane proteins to act as substrates for transglutaminase. Alteration of membrane phospholipids by phospholipases may alter the microenvironment and hence conformation of membrane proteins in ways which alter their substrate specificity for transglutaminase. The products of phospholipid metabolism (diacylglycerol, cyclic-4,5-inositol phosphate, polyphosphoinositides) are thought to be important in the regulation of cellular responses associated with Ca^{2+} (Berridge, 1982; 1984). The possibility arises that transglutaminase activity in the β-cell may be influenced by such molecules, particularly in the vicinity of the plasma membrane which is the postulated site of action of the enzyme.

The post-translational modification of proteins situated on or in membranes may lead to alteration in the interactions between membrane compartments such that membrane traffic is directed towards the exocytosis of insulin. It is interesting that, under basal conditions, a large proportion of the total insulin synthesised in the β-cell is degraded, whereas under conditions of stimulation of insulin release almost none is degraded (Halban & Wollheim, 1980). The switch from the degradative to the exocytotic pathway would presumably involve a change in the direction of membrane traffic towards the plasma membrane and away from the lysosomes where intracellular degradative processes take place. This re-direction of membrane traffic could involve transglutaminase activity in the way suggested above (i.e. in membrane recognition and segregation). It is interesting to note in this context that the degradative pathway of endocytosis (e.g. for epidermal growth factor) may not involve transglutaminase activity (see Introduction, Section 1.4.2.).

The stimulation of insulin release by glucose involves the release of Ca^{2+} from intracellular stores and the influx of Ca^{2+} across the cell membrane, both processes contributing to a rise in cytosolic Ca^{2+} concentration. It is thought that the release from intracellular stores is the first process to contribute to the stimulation of exocytosis and this is followed by the influx of Ca^{2+} (Wollheim & Sharp, 1981). It is therefore possible that an intracellular target for Ca^{2+} such as transglutaminase could at least in part be responsible for eliciting a further rise in cytosolic Ca^{2+} concentration by stimulating Ca^{2+} influx or by blocking Ca^{2+} efflux. Indeed, calmodulin, which may be a factor involved in the coupling of a rise in cytosolic Ca^{2+} to mobilisation

of the secretory apparatus, may also be involved in the regulation of Ca^{2+} fluxes in islets (Wollheim et al., 1981; Janjic et al., 1981; Pershadsingh et al., 1980) as well as in other systems (Klee et al., 1980). Therefore, at this stage it is not possible to rule out the involvement of islet transglutaminase in steps involved in the mediation of cation fluxes in the β-cell.

Although no direct evidence for the nature of the catalytic role of islet transglutaminase was obtained in this thesis, the possibility of an interaction with the polyamines putrescine, spermidine and spermine was suggested by the high islet content of these organic cations. The specificity of other transglutaminases whose function is known has been found to be for the formation of ε-(γ-glutamyl) lysine cross-links (e.g. Factor X111; epidermal transglutaminase). The potency of monodansylcadaverine, which is an analogue of lysine, as an inhibitor of islet transglutaminase activity(Ki = 7.3 μM) may suggest a similar catalytic role for the enzyme in the β-cell. However, the microenvironment of the cell may favour the formation of (γ-glutamyl) polyamine conjugates by transglutaminase. Furthermore, it is possible that both ε(γ-glutamyl) lysine and polyamine conjugates may occur, depending on the orientation or proximity of lysine-containing substrate proteins and polyamines in the vicinity of transglutaminase in the intact cell.

Indeed, it is possible that certain advantages may accrue from cross-linking via the incorporation of polyamines as opposed to ε(γ-glutamyl) lysine cross-links. The spacing between polypeptide chains in the case of the diamine cross-link is greater than that in the case of the ε(γ-glutamyl) lysine cross-link. This may allow for greater ease of cross-link formation between membrane proteins

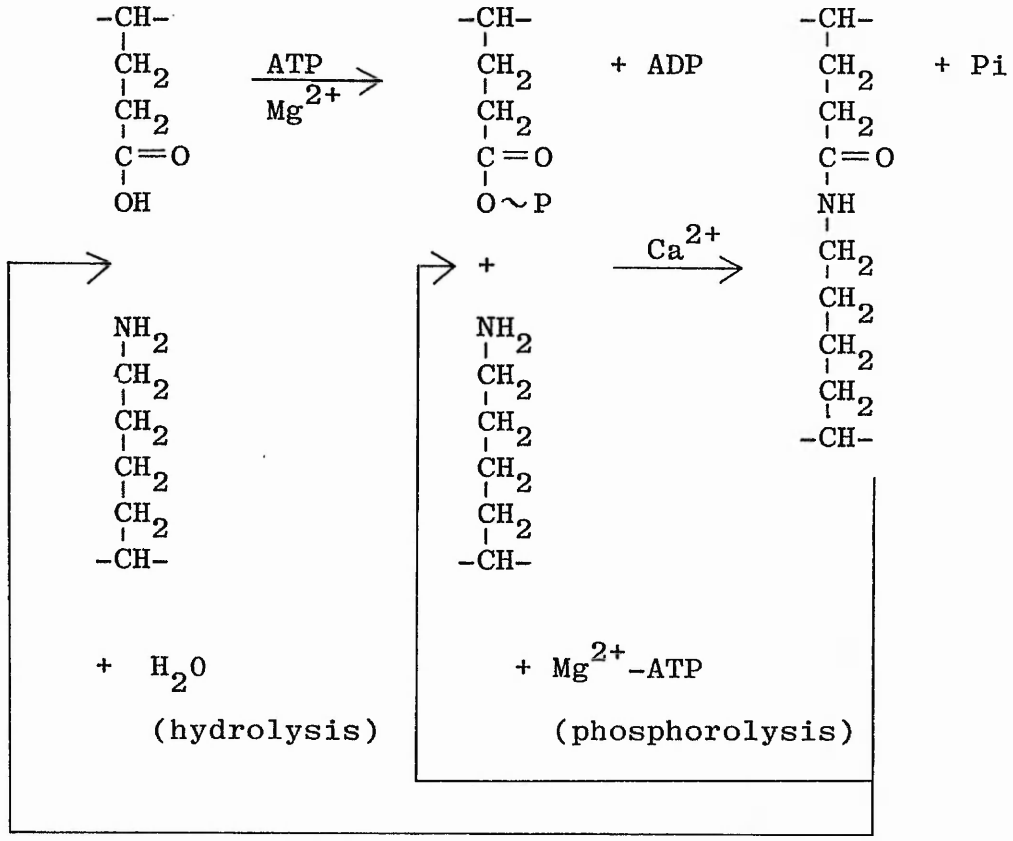
whose conformation does not favour their close approach in the lipid bilayer. This is a particular pertinent consideration in the cases of spermidine which possess a span of 6 C-atoms with a N-atom (approximately 11Å) and spermine which possesses a span of 10 C-atoms with 2 N-atoms (approximately 16Å) and which are the most abundant polyamines in islets. However, although formation of polyamine conjugates in cells has been demonstrated (Folk et al., 1980), the formation of diamine cross-links by transglutaminase has only been demonstrated in extracellular systems (Folk et al., 1980; Schrode & Folk, 1978; Williams-Ashman et al., 1980) or tentatively in Aplysia neuronal cells (Ambron & Kremzner, 1982).

The alternative possibility exists that transglutaminase-catalysed incorporation of polyamines may occur through only one amine group of these molecules. This action may alter the charge and possibly the conformation of a protein, and in the case of membrane proteins, could lead to changes in the charge and interactions of cellular membranes. Of particular interest here are reports suggesting that spermidine and spermine are able to influence the aggregation and fusion of phospholipid vesicles (Schuber et al., 1983; Hong et al., 1983). The mechanism of this action of spermidine and spermine is thought to involve interactions between positively charged amine groups on the polyamines and negatively charged phosphate groups on the phospholipids (Schuber et al., 1983). Such interactions may serve to stabilise domains of phospholipids and thus may alter interactions between vesicles. Therefore, polyamines could regulate the interactions between cellular membranes in the process of endomembrane flow. Attachment of polyamines to a number of membrane proteins by transglutaminase could serve to create

discrete and stable areas of membrane with ordered arrays of phospholipids held in place by ionic interactions with polyamines which themselves are anchored to membrane proteins. Such a mechanism represents a means of transglutaminase-mediated membrane stabilisation which does not involve protein cross-linking.

A potential difficulty for the suggestion that transglutaminase may participate in the regulation of membrane interactions in the β-cell is that the reactions catalysed by the enzyme may not be readily reversible. A γ-glutamylamine cyclotrasferase capable of degrading ε(γ-glutamyl) lysine and (γ-glutamyl) polyamines to form free amines and 5-oxo-L-proline has been identified in kidney and other tissue (Fink et al., 1980). However, no such enzyme has been found which acts upon products of transglutaminase catalysis in intact proteins. This situation is in contrast to that of post-translational modification of proteins by phosphorylation reactions which may be readily reversible by phosphatases without the need for protein degradation. The difficulty in the case of transglutaminase may be resolved by postulating that during periods of stimulated insulin release, proteins modified by transglutaminase activity may remain modified until rates of secretion decrease when these proteins may be degraded. Furthermore, it is possible that cross-links formed by the action of transglutaminase may be rapidly modulated in the β-cell. In studies on a variety of cell types including plasmodia of Physarum polycephalum and chick skeletal muscle, it was shown that the ε(γ-glutamyl) lysine content of membrane and cytoskeletal proteins could be rapidly modulated in vivo by temperature changes and in vitro by treatment with Mg^{2+}-ATP and Mg^{2+}-ATP + Ca^{2+} (Loewy & Matacic, 1981; Loewy et al., 1981). These workers suggested that cycling of ε(γ-glutamyl) lysine cross-links may occur under the influence of a transglutaminase-like enzyme activity

involving the formation of an activated acyl-intermediate with the hydrolysis of ATP :



Although the enzyme responsible for these reactions may not be a typical transglutaminase, these studies indicate the possibility that cross-links formed by the action of transglutaminase could be rapidly modulated in the pancreatic β-cell.

The work of Loewy and Matacic serves to illustrate the dearth of knowledge concerning the nature of the enzymes responsible for the formation of ε(γ-glutamyl) lysine cross-links in cells. Since a number of catalytic roles are possible for transglutaminase which would reflect the physiological role of these enzymes in tissues, a diversity of transglutaminase-like enzymes may exist to perform these roles.

Indeed, one report suggests that the enzyme purified from activated rat chondrosarcoma cells may be activated by limited proteolysis and thus may exist as two cellular forms (Chang & Chung, 1982). It is possible that several forms of tissue transglutaminase exist in cells and that more than one function is carried out by these enzymes.

Bearing this in mind, it is interesting to compare the properties of transglutaminase from rat islets of Langerhans with those of the enzyme from rat liver. Purified rat liver transglutaminase was found to be half-maximally active at approximately 40 μM Ca^{2+} and possessed a Km for putrescine incorporation into N,N'-dimethylcasein of 0.5mM (Barnes, 1980). These values are comparable to those obtained similarly for the islet transglutaminase (Km for Ca^{2+} = 39 μM; Km for putrescine = 0.59mM). It is therefore tempting to speculate that the enzyme from rat liver and pancreatic islets is the same and may perform a similar or identical physiological role in these tissues. Such a consideration is consistent with the postulation that the enzyme may play a common role in tissues, which was stated in the Introduction (Section 1.4.). Since a role for tissue transglutaminase in the membrane interactions occuring during the secretion of insulin was suggested, and since similar interactions occur in most, if not all, cell types, a common role for the enzyme may lie in the regulation of membrane traffic and, more specifically, in membrane recycling (Van Leuven et al., 1980; Bungay et al., 1984).

Work in this thesis suggested that the expression of transglutaminase activity in tumours was altered with respect to normal tissues. In the passaged tumour lines MC3 and CC5, transglutaminase activity measured in homogenates of these tissues

was substantially lower than in the normal rat tissues liver, lung and spleen. In the primary hepatocellular carcinomata induced with DEN and 6-BT, levels of transglutaminase activity were variable, usually being lower but sometimes being higher than in normal liver. A reduction in transglutaminase activity compared to normal tissues therefore did not appear to be a universal feature of neoplasia. With reference to the work of Laki et al., (1977) and Birckbichler et al., (1976; 1977), it is possible that certain tumours require a reduced transglutaminase activity for their progression while others require higher levels. In this respect, it is interesting that the passaged tumour lines possessed such low levels of enzyme activity, for this may reflect their established proliferative behaviour.

Comparison of the subcellular distribution of transglutaminase activity in tumours with that of normal tissues indicated that in all the tumours studied, the greater proportion, of transglutaminase activity recovered was associated with the particulate fractions. The re-distribution of transglutaminase activity to the 71,000g pellet of tumours induced with DEN and 6-BT was a feature which distinguished these tissues from normal liver, from which they were derived. As previously suggested (Section 4), transglutaminase activity may be limited in the cell by its sequestration on cellular membranes.

In the light of these results, it is interesting to consider how changes in the magnitude and distribution of transglutaminase activity may contribute to the altered behaviour of tumour tissues, particularly in the light of a possible common role for the enzyme in the mediation of endomembrane flow.

Alterations in the expression of transglutaminase activity in tumours may be expected to lead to a deficiency in the regulation of

membrane traffic in these tissues. Assuming that changes in transglutaminase activity are universal in tumours, reports documenting the ability of islet β-cell tumours to secrete insulin in response to stimuli (Hutton et al., 1981; Masiello et al., 1982) and the ability of cultured HepG2 hepatoma cells to recycle surface receptors (Ciechanover et al., 1983) suggest that changes in transglutaminase activity may not necessarily lead to complete breakdown of membrane recycling or secretory mechanisms. However, it is possible that such mechanisms may require only a small proportion of the total cellular transglutaminase activity for their operation. Furthermore, a change in the subcellular distribution of transglutaminase may not alter the ability of the enzyme to regulate membrane flow to such an extent that the process is inhibited.

A change in the subcellular distribution of transglutaminase activity may be one of a series of co-ordinated events required for neoplasia or cell proliferation. This may reflect a reduced requirement of proliferating cells for membrane flow phenomena which could interfere with the main activities of these cells (e.g. cell division, DNA synthesis). This idea would be consistent with the view that transglutaminase may be stored in a latent form on cellular membranes, in which case, a re-distribution of transglutaminase activity to the particulate fraction would accompany a fall in enzyme activity.

The significance of changes in transglutaminase activity to tumour cell function may be more qualitative in nature. If the enzyme plays a role in membrane recognition and segregation as previously suggested in this section, the flow of membranes in the tumour cell may be misdirected as a result of changes in

transglutaminase activity. This could lead to a defect in the processing of cell surface proteins or glycoproteins destined for export. Cell surface glycoprotein components may play a role in maintaining functional contact between cells. Changes in these components may therefore contribute to the failure of tumours to regulate their proliferation (Emmelot, 1973; Patterson, 1974) and the ability of certain tumours to undergo metastatic growth.

The stabilisation of cell membrane architecture with ε(γ-glutamyl) lysine cross-links has been proposed to be important in the maintenance of the non-prolifetating state (Birckbichler & Patterson, 1978). This idea is also consistent with a role for transglutaminase in regulating membrane flow. The manufacture and insertion into the plasma membrane of areas of membrane stabilised by transglutaminase-catalysed reactions (cross-linking of proteins or incorporation of polyamines) would represent a similar process to that of the exocytosis of recycled membrane and would involve recognition and segregation functions for the enzyme.

By way of conclusion, the word 'stabilisation' may point to the key role of tissue transglutaminase in cells. Furthermore, stabilisation may represent the key role of all types of transglutaminases, since this is the function of Factor X111 in blood coagulation, of epidermal transglutaminase in terminal diffentiation and of prostate transglutaminase in semen coagulation. However, our knowledge of the catalytic and physiological role of the tissue transglutaminase is still poor. More attention to the study of this ubiquitous enzyme may, at the same time as clarifying the role of the enzyme, help to elucidate cellular functions whose own mechanisms are obscure.

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The inhibition of glucose-stimulated insulin secretion by primary amines

A role for transglutaminase in the secretory mechanism

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1. Rat pancreatic islets contain a Ca^{2+}-activated and thiol-dependent transglutaminase (EC 2.3.2.13) comparable in activity with that found in rat liver, lung and spleen. The Ca^{2+}-dependence of this enzyme is such that half-maximal velocity was obtained in the region of 40 μM. 2. Preincubation of rat islets with primary-amine substrates of transglutaminase (monodansylcadaverine, methylamine, ethylamine, propylamine and cystamine) led to an inhibition of glucose-stimulated insulin release by these amines. 3. Kinetic analysis of the competitive substrates methylamine, monodansylcadaverine, propylamine and ethylamine for their ability to inhibit islet transglutaminase activity indicated a potency that matched their ability to inhibit glucose-stimulated insulin release. 4. When these amines were tested for their effects on glucose-stimulated protein synthesis and glucose utilization, the most potent inhibitor of insulin release, monodansylcadaverine, had no effect on either process at 100 μM. The amines cystamine, ethylamine, methylamine and propylamine had variable effects on these metabolic processes. For ethylamine, methylamine and propylamine, concentrations were found which inhibited glucose-stimulated insulin release in a manner which was found to be independent of their effects on either glucose oxidation or protein synthesis. 5. Primary amines may therefore inhibit insulin release through their incorporation by islet transglutaminase into normal cross-linking sites. A role for protein cross-linking in the secretory mechanism is suggested.

Reactions catalysed by the Ca^{2+}-dependent enzyme transglutaminase (protein-glutamine: amine γ-glutamyltransferase, EC 2.3.2.13) lead to the post-translational modification of a protein either through the specific incorporation of amines into the γ-carboxamide groups of glutamine, or by the cross-linking of polypeptide chains by ε-(γ-glutamyl)lysine bridges (Mycek *et al.*, 1959; Lorand & Stenberg, 1976). Although transglutaminase enzymes occur widely in Nature (Folk & Finlayson, 1977), the function of the most widespread group of these enzymes, the 'tissue transglutaminase' (Chung, 1972), is still poorly understood. Studies with this transglutaminase are now suggesting that it may play an important role in the mediation of events at the cell membrane. Observations supporting this idea include the demonstration of ε-(γ-glutamyl)lysine cross-links in cell-membrane proteins (Birckbichler *et al.*, 1973), the specificity of erythrocyte transglutaminase in the cross-linking of membrane proteins and associated structures (Siefring *et al.*, 1978; Bjerrum *et al.*, 1981), the cross-linking of fibronectin (Birchbichler & Patterson, 1978) and the cross-linking of β_2-microglobulin (Fésüs *et al.*, 1981).

The physiological significance of these observations is as yet still unclear, but a functional role has been indicated for the enzyme in the membrane interactions involved in receptor-mediated endocytosis (Davies *et al.*, 1980). Evidence for this role was first suggested by the ability of competitive amine substrates of the enzyme to inhibit the receptor-mediated endocytosis of α_2-macroglobulin and epidermal growth factor. This finding was later extended to the receptor-mediated endocytosis of low-density lipoprotein (Pastan &

Abbreviations used: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; KRB medium, Krebs–Ringer bicarbonate medium (Krebs & Henseleit, 1932).

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Willingham, 1981), phage T3 (Cheng *et al.*, 1980) and *Shigella* toxin (Keusch, 1981).

Although some controversy exists over the site and mode of action of primary amines in this process (King *et al.*, 1980; Kaplan & Keogh, 1981), a functional role for transglutaminase in the receptor-recycling mechanism has now been indicated (Van Leuven *et al.*, 1980).

To test the involvement of transglutaminase in another cellular event involving specific membrane interactions, we have investigated a role for transglutaminase in the Ca^{2+}-mediated mechanism of stimulus–secretion coupling in the pancreatic β-cell (Wollheim & Sharp, 1982). Like receptor-mediated endocytosis, a rise in the cytosolic concentration of free Ca^{2+} plays a key role in this cellular event, but the mechanism whereby Ca^{2+} elicits the exocytotic release of insulin and its overall role in the secretory cycle is still unknown.

Evidence to suggest that transglutaminase may be involved in the membrane interactions occurring during glucose-stimulated insulin secretion has been indicated by our initial investigations, which have demonstrated the inhibition of glucose-stimulated insulin release from pancreatic islets by primary-amine substrates of transglutaminase (Griffin *et al.*, 1982; Bungay *et al.*, 1983). In the present paper we have extended this work and provided further evidence to suggest that the pancreatic-islet transglutaminase may play an important role in the secretory mechanism for insulin release.

Experimental

Materials

Collagenase used in the isolation of pancreatic islets was obtained from Serva Feinbiochemica, Heidelberg, W. Germany. Radiochemicals (D-[U-^{14}C]glucose, L-[4,5-^{3}H]leucine, [1,4-^{14}C]putrescine and [6,6′(*n*)-^{3}H]sucrose) were purchased from Amersham International, Amersham, Bucks., U.K., except for ^{125}I-labelled insulin, which was obtained from Novo, Bagsvaerd, Denmark. Methylamine hydrochloride, ethylamine, propylamine, cycloheximide and bovine serum albumin were obtained from Sigma Chemical Co., Poole, Dorset, U.K., Cystamine was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K., and monodansylcadaverine was from Fluka A.G., Buchs, Switzerland. The dimethylated analogue of monodansylcadaverine was kindly given by Professor Laszlo Lorand, Northwestern University, Evanston, IL, U.S.A. Standard rat insulin and insulin antibody were obtained from Novo, and human albumin was from Hoechst (U.K.), Hounslow, Middx., U.K.

Methods

Isolation of islets of Langerhans. Islets of Lange hans were isolated from the pancreas of 200–300 Sprague–Dawley rats by a collagenase digestic technique similar to that of Lacy & Kostianovs (1967). For the studies of insulin release, gluco oxidation and protein synthesis, islets were inc bated at 37°C immediately after isolation in KR medium containing 16.8mM-glucose for 5mi followed by 60min in the same medium containi 2.8mM-glucose in an atmosphere of O_2/CO_2 (19: before grouping for the experimental incubation

Studies on insulin release. Batches of five isle were incubated at 37°C in KRB medium co taining bovine serum albumin (5mg/ml) in a atmosphere of O_2/CO_2 (19:1). The incubatio were for 45min in 0.5ml of KRB containi 2.8mM-glucose, followed by 60min in 1.0ml KRB containing 16.8mM-glucose. The change the higher glucose concentration was achieved the addition of 0.5ml of KRB containing 30.5m glucose. For the effects of test compounds on bas rates of insulin release, 0.5ml of KRB containi 2.8mM-glucose was added instead. Experiments determine the effects of test compounds on insul release were performed with the concentrations the test compounds shown in the Tables a Figures throughout the text; test compounds we present throughout the incubation period 105min. The preincubation period of 45min w intended to allow test compounds to enter the isl cells before stimulation. After incubation, sampl of medium were taken and stored at −20°C un radioimmunoassay was performed to determi insulin release (Heding, 1972). Before radi immunoassay, serial dilutions ranging from 1:5 1:50 were carried out on the test samples. Th allowed insulin measurements to be made on t linear part of the standard curve, and also test for linearity between the respective dilutions ma and the amount of insulin present. This acted as preliminary screen for any interference by te compounds on the insulin assay.

Measurement of glucose oxidation by islets. Gl cose oxidation was measured by determining t amount of $^{14}CO_2$ released from [U-^{14}C]glucose, method essentially that of Malaisse *et al.* (1974 10–20 islets were incubated in small plastic we suspended in 10ml glass beakers, which we sealed with self-sealing rubber stoppers. Islets we preincubated in the presence or absence of te compounds for 45min in 50μl of KRB incubati medium containing 2.8mM-glucose in an atmo phere of O_2/CO_2 (19:1) before the final addition 50μl of 30.5mM-glucose in KRB containing [U ^{14}C]glucose (final sp. radioactivity 0.595Ci/mo After incubation for 1h, 0.2ml of Hyami hydroxide was added to the glass beaker to abso

any released $^{14}CO_2$, and 50μl of 0.2M-HCl was injected into the islet incubation medium to release any further trapped $^{14}CO_2$. This amount of HCl proved to be adequate for lowering the pH of the medium to below 1.0 even in the incubations containing test compounds at their highest concentrations. After a further incubation period of 30min after addition of the acid, the plastic wells were removed and the Hyamine hydroxide was diluted to 1.0ml with distilled water and then counted for radioactivity after addition of 10ml of Instagel (Packard). Counting efficiency was determined by internal standardization.

Measurement of total islet protein synthesis. Protein synthesis in intact islets was measured by the incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable protein. Batches of 10–20 islets were incubated at 37°C for 45min in 50μl of KRB containing 2.8mM-glucose in the presence of test compound, followed by 60–90min in 16.8mM-glucose/KRB medium containing 5μCi of [^{3}H]-leucine (55 or 60Ci/mmol) in a final volume of 100μl. After incubation, 100μl of ice-cold 20% (w/v) trichloroacetic acid was added, the mixture was sonicated (two bursts of 5s, 3μm peak-to-peak) and the resulting precipitate washed three times in 5% (w/v) ice-cold trichloroacetic acid containing 2mM-leucine, once in acetone/ethanol (1:1, v/v) and once in acetone. The dried precipitate was solubilized in 0.1ml of Soluene 350 (Packard) and counted for radioactivity in 1.0ml of Dimilume (Packard). Counting efficiency was determined by using the Quench Indicating Parameter of Packard.

Measurement of [^{3}H]leucine uptake into islets. Uptake of leucine was measured by a method essentially that of Wollheim *et al.* (1978). Incubations were performed in 400μl polyethylene microcentrifuge tubes (Alpha Laboratories). Tubes contained 20μl of 6M-urea, which was overlayered with 200μl of a mixture containing dibutyl phthalate and dinonyl phthalate (10:3, v/v). KRB medium (50μl) containing batches of 10–20 islets was placed above the phthalate ester layer but separated from it by a small air space. Incubations were for 45min in 50μl of KRB medium containing 2.8mM-glucose, followed by 60min in 100μl of KRB medium containing 16.8mM-glucose and 5μCi of [^{3}H]leucine (55Ci/mol). The appropriate concentration of amine together with cycloheximide (100μM) was present throughout each period of incubation of 45+60min. Incubations were terminated by sedimenting the islets for 3s at 9390$g_{av.}$ in a Beckman microcentrifuge. The bottoms of the tubes containing the islets were cut off into suitable counting vials, left for 30min to allow dissolution of the islets and then counted for radioactivity after addition of 10ml of Instagel. To enable the true uptake of [^{3}H]leucine into the islet cells to be calculated, the apparent uptake corresponding to islet extracellular space was also determined and subtracted accordingly. Islet extracellular space was measured with [^{3}H]sucrose as an extracellular marker. Batches of 15 islets were incubated with 5μCi of [^{3}H]sucrose (9.8Ci/mmol) for 30 and 45min in KRB medium (50μl) containing 2.8mM-glucose. The methodology used was essentially that described for measurement of [^{3}H]-leucine uptake. The average sucrose space was calculated as 2.84 ± 0.27nl/islet ($n = 11$). Counting efficiency was determined by using the Quench Indicating Parameter of Packard. Statistical significance was determined by Student's t test.

Homogenization of tissues. Homogenates of islets of Langerhans were prepared by sonication (two bursts of 5s, 3μm peak-to-peak) in ice-cold 0.25M-sucrose/1mM-Tris/HCl/1mM-EDTA, pH7.4 (one to two islets/μl). For cellular-distribution studies, homogenates were centrifuged at 71000$g_{av.}$ for 45min to obtain particulate and particle-free-supernatant fractions. Homogenates of rat liver, lung and spleen were prepared in the same sucrose buffer with a Potter–Elvehjem homogenizer.

Transglutaminase assay. Transglutaminase activity was measured by the incorporation of [^{14}C]-putrescine into *NN'*-dimethylcasein (Lorand *et al.*, 1972). The incubation mixture, at 37°C, consisted of 28mM-Tris/HCl, pH7.4, 3.85mM-dithiothreitol, 2.5mM-$CaCl_2$ or 5mM-EDTA, *NN'*-dimethylcasein (5mg/ml) and 1.2mM-[^{14}C]putrescine (3.96Ci/mol) and 22.5μl of islet preparation in a final volume of 50μl. Samples (10μl) were taken for the counting of trichloroacetic acid-insoluble radioactivity at appropriate time intervals, and rates of putrescine incorporation were determined after subtraction of EDTA controls. For enzyme assays investigating the effect of thiol-reactive inhibitors on the enzyme, dithiothreitol was omitted from the assay. Protein was determined by the method of Lowry *et al.* (1951).

Measurement of the effect of Ca^{2+} on transglutaminase activity. For this, islet supernatants were prepared as described above, except that 1mM-EGTA was substituted for EDTA in the homogenization buffer. Addition of 22.5μl of islet preparation to a final assay volume of 50μl then gave a final concentration of EGTA in the assay mixture of 0.45mM. Different amounts of $CaCl_2$ were then added to the assay medium in excess of the EGTA present, so that the free Ca^{2+} concentrations present ranged from 25μM to 2.0mM. For calculation of the free Ca^{2+} concentrations, an association constant at pH7.4 of $10^{7.12}$ for [CaEGTA]/[Ca][EGTA] was used (Schatzman, 1973).

Results

Characterization of islet transglutaminase

When islet homogenates were assayed for transglutaminase activity by [^{14}C]putrescine incorporation into *NN'*-dimethylcasein, a Ca^{2+}-dependent enzyme of activity comparable with that found in homogenates of rat liver, lung and spleen could be demonstrated (Table 1). The islet transglutaminase displayed no observable activity when Mg^{2+} (2.5mM) was substituted for Ca^{2+} in the enzyme assay, indicating the Ca^{2+}-specificity of this enzyme.

Fractionation of the islet homogenate ($n = 4$) into a particulate and a cytosolic fraction by centrifugation at 71000$g_{av.}$ for 45min indicated that most of the enzyme activity ($94 \pm 3\%$) was present in the cytosolic fraction. Subsequent studies on the enzyme were therefore performed with this supernatant fraction.

Further characterization of the islet enzyme as a typical transglutaminase was performed by investigation of the thiol-dependence and Ca^{2+}-activation of the enzyme. Measurement of enzyme activity in the presence of different concentrations of the thiol-reactive compounds *p*-chloromercuribenzoic acid or iodoacetamide (1–50 μM) indicated that the enzyme was very susceptible to these two compounds, with concentrations of 2 μM-iodoacetamide and 15 μM-*p*-chloromercuribenzoic acid giving approx. 80% loss of enzyme activity. Further, when enzyme activity was measured in the presence of different concentrations (0.025–2.0mM) of the amine cystamine, an inhibitor of the enzyme which is thought to undergo a disulphide exchange with the active thiol group of transglutaminase enzymes (Siefring *et al.*, 1978), a progressive decrease in enzyme activity was observed with increasing cystamine concentrations, with a 50% loss of enzyme activity occurring at 0.6mM-cystamine.

Table 1. *Activity of transglutaminase in homogenates of various rat tissues*

Homogenates of rat liver, lung, spleen and islets of Langerhans were prepared in 0.25M-sucrose/1mM-Tris/HCl/1mM-EDTA, pH7.4, and assayed for transglutaminase activity as described in the Experimental section. The numbers of determinations are shown in parentheses. Unit of enzyme activity is 1nmol of putrescine incorporated/h.

Tissue	Transglutaminase activity (units/mg of protein)
Liver	33.2±3.5 (3)
Lung	31.5±4.2 (4)
Spleen	30.0±0.6 (3)
Pancreatic islets	26.2±5.7 (6)

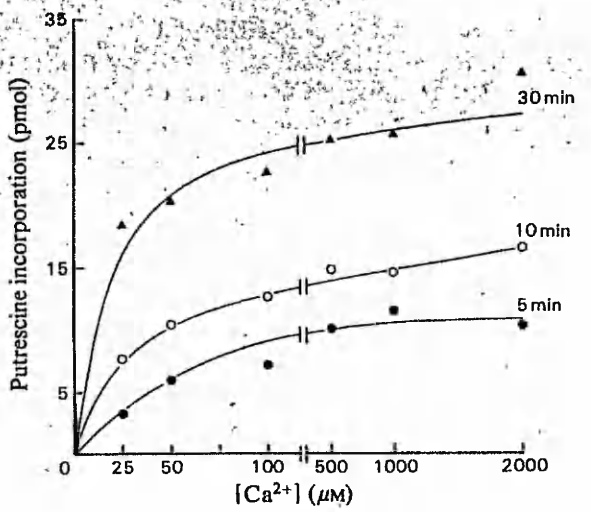

Fig. 1. *Ca^{2+} activation of pancreatic-islet transglutaminase* Transglutaminase activity was measured by incorporation of [^{14}C]putrescine into *NN'*-dimethylcasein at 5 (●), 10 (○) and 30min (▲) with variable concentrations of free Ca^{2+} from 25 to 2000 μM as described in the Experimental section.

The demonstration of Ca^{2+} as an activator of islet transglutaminase was performed by titrating enzyme activity with various concentrations of free Ca^{2+} in the range 25 μM–2mM. As demonstrated in Fig. 1, the islet enzyme was readily activated by small additions of Ca^{2+} in the concentration range 25–100 μM. If it is assumed that the *NN'*-dimethylcasein present in the assay has negligible effect on the amount of free Ca^{2+} present, then treatment of the data by the Lineweaver–Burk method gave the enzyme an apparent half-saturation value for Ca^{2+} of 39 μM.

Inhibition of islet transglutaminase activity by primary amines

To determine the potency of potential inhibitors of islet transglutaminase activity, which could later be compared with the inhibitors' effects on glucose-stimulated insulin release, various primary-amine substrates of the enzyme were tested for their effects on [^{14}C]putrescine incorporation into *NN'*-dimethylcasein catalysed by islet transglutaminase. The apparent inhibition constants (K_i) for each of the compounds monodansylcadaverine, methylamine, ethylamine and propylamine were calculated by the method of Dixon (Dixon & Webb, 1964) from data prepared from Lineweaver–Burk plots. The apparent K_m for putrescine for the islet transglutaminase was calculated as 0.59 ± 0.10mM ($n = 8$) from these data. Example plots for the results obtained for monodansylcadaverine and the alkylamine methylamine are illustrated in Fig. 2. The plots obtained for mono-

dansylcadaverine, methylamine, ethylamine and propylamine with three different concentrations of each inhibitor were all found to intersect at $1/V_{max.}$ on the $1/v$ axis of the Lineweaver–Burk plot, a result in keeping with the findings of others (Lorand *et al.*, 1979) for the competitive nature of these amines when measured under these conditions. Dixon plots of $1/v$ against [I] for calculation of K_i values were linear in each case.

Of the amines tested, monodansylcadaverine was the most potent inhibitor of islet transglutaminase (K_i 0.0073mM), followed by ethylamine (K_i 1.75mM), methylamine (K_i 2.00mM) and propylamine (K_i 3.8mM).

Effects of primary-amine inhibitors of transglutaminase on glucose-stimulated insulin release from intact islets

To test the hypothesis that the pancreatic-islet transglutaminase may be involved in the mechanism of insulin release from the islet β-cell, primary-amine inhibitors of islet transglutaminase activity were tested for their effects on glucose-stimulated insulin release. Insulin release measured in batch-type incubations of islets was normally increased 6–10-fold when the glucose concentration in the incubation medium was raised from 2.8 to 16.8mM, the variation in the capacity of islet populations to respond to glucose being a function of different islet populations used from different isolations. To minimize this inconsistency when examining the effects of different inhibitors on insulin release, control rates of basal and stimulated insulin release were always determined for each batch of islets that was tested.

The effects of primary amines on insulin release are shown in Table 2. With reference to the work of Davies *et al.* (1980), the amines were chosen not only for their ability to inhibit transglutaminase but also on the basis of their likelihood to gain entry to the islet β-cell.

Of the amines tested, all appeared to inhibit glucose-stimulated insulin release from islets incubated at 16.8mM-glucose in a dose-dependent manner. Incubation of cystamine, ethylamine or methylamine with rat insulin standards during the radioimmunoassay indicated that these amines had no effect on the insulin assay when tested at the maximum concentrations used in the inhibition studies. This rules out the possibility that the observed inhibition of insulin release by these amines might be due to interference with insulin measurement. When the amines were tested for their effects on the basal release of insulin in the presence of 2.8mM-glucose for a total time period of 105min (45+60min), monodansylcadaverine (0.1mM), cystamine (1mM) and propylamine (10mM) had no significant effect. Methylamine, however showed a small but significant stimulation of insulin release when tested under these conditions (60% increase over control basal rates; $n=4$; $P \leqslant 0.001$), a result that we are unable to explain at present. The most potent inhibitor of transglutaminase activity, monodansylcadaverine, also proved to be the most potent inhibitor of glucose-stimulated insulin release, and was effec-

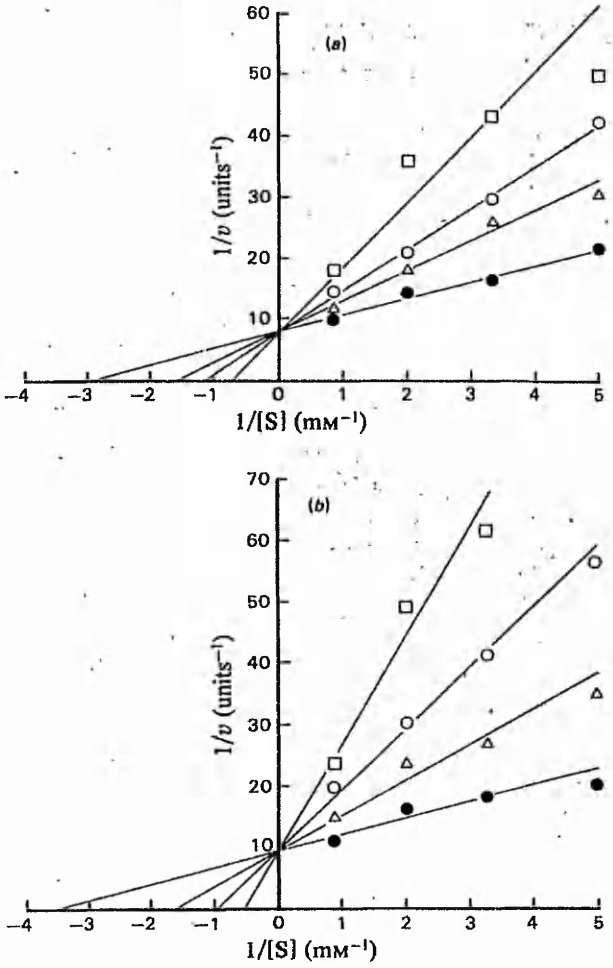

Fig. 2. *Lineweaver–Burk plots for the inhibition of pancreatic-islet transglutaminase by monodansylcadaverine and methylamine*

Islet supernatant prepared by the protocol described in the Experimental section was assayed for transglutaminase activity by [^{14}C]putrescine incorporation into *NN'*-dimethylcasein by using different concentrations of putrescine (1.2, 0.5, 0.3 and 0.2mM). (*a*) Transglutaminase activity in the presence of various concentrations of monodansylcadaverine: △, 5μM; ○, 10μM; ▲, 20μM. (*b*) Transglutaminase activity in the presence of various concentrations of methylamine; △, 2mM; ○, 5mM; ▲, 10mM. The symbol ● represents the control system without inhibitor. Unit of enzyme activity is 1nmol of putrescine incorporated/h.

Table 2. *Effects of test compounds on glucose-stimulated insulin release*

Batches of five islets were incubated in KRB medium, in the presence or absence of the concentrations of primary amines stated, according to the protocol described in the Experimental section. Insulin released into the medium was determined by radioimmunoassay. Stimulation of insulin release in control and test incubations over a 60min period was determined by subtracting basal rates of insulin release in control incubations performed simultaneously over the same total time period of 45+60min. The inhibition of glucose-stimulated release for each amine tested is then expressed as the mean percentage (±S.E.M.) of that in control incubations, for the numbers of determinations shown in parentheses. The significance of difference from controls was determined by Student's *t* test; N.S. denotes no significant difference from controls. Typical rates of insulin release (μunits/h per islet) from isolated islets were 8.9±0.8 ($n=56$) at 2.8mM-glucose and 95.4±6.2 ($n=49$) at 16.8mM-glucose.

Test compound	Concn. (mM)	Inhibition of glucose-stimulated insulin release (%)
Monodansylcadaverine	0.01	31±8 (4) $P\leqslant0.05$
	0.02	41±1 (4) $P\leqslant0.01$
	0.05	90±12 (6) $P\leqslant0.01$
	0.10	91±8 (4) $P\leqslant0.001$
Cystamine	0.10	47±17 (6) $P\leqslant0.25$
	0.50	79±10 (6) $P\leqslant0.05$
	1.0	98±4 (6) $P\leqslant0.002$
Methylamine	1.0	51±5 (6) $P\leqslant0.001$
	5.0	78±8 (4) $P\leqslant0.01$
	15.0	99±6 (4) $P\leqslant0.001$
Propylamine	0.5	61±6 (5) $P\leqslant0.01$
	1.0	47±11 (4) $P\leqslant0.05$
	5.0	95±2 (5) $P\leqslant0.001$
Ethylamine	1.0	50±9 (7) $P\leqslant0.005$
	5.0	81±8 (6) $P\leqslant0.001$
Cycloheximide	0.01	19±7 (5) N.S.
N'-Dimethyldansylcadaverine	0.1	24±9 (3) $P\leqslant0.1$

tive over the concentration range 10–100μM. The dimethyl analogue of monodansylcadaverine, *N'*-dimethyldansylcadaverine, showed only 24±9% ($n=3$; $P\leqslant0.1$) inhibition of glucose-stimulated insulin release at 100μM. Of the other amines tested, cystamine proved to be an effective inhibitor of insulin release over the concentration range 0.1–1.0mM, whereas methylamine, propylamine and ethylamine were of a similar potency to each other, being effective in the concentration range 1.0–5.0mM. Reference to the K_i values calculated for monodansylcadaverine, methylamine, ethylamine and propylamine for their effects on islet transglutaminase activity indicates that these concentration ranges are in the same order of magnitude with regard to their effects on insulin release.

Effects of primary amines on islet glucose oxidati and synthesis of total islet protein

To exclude the possibility that primary amin may inhibit insulin release by non-specific mec anisms, the effects of amines on two other glucos stimulated parameters, namely glucose oxidatic and protein synthesis, were investigated. In o experiments, increasing the glucose concentratic from 2.8mM to 16.8mM stimulated glucose oxid tion 7–10-fold and protein synthesis 3–4-fold. T minimize errors between experiments, contr rates of glucose oxidation and protein synthes were always determined for untreated islets in eac investigation.

Table 3 shows that the amines displayed variation in their effects on $^{14}CO_2$ release from [U ^{14}C]glucose and on [^{3}H]leucine incorporation in total islet protein when tested at the concentratio used in the experiments on insulin release. A 100μM, monodansylcadaverine did not signi cantly inhibit either glucose oxidation or prote synthesis in stimulated islets. Cystamine, howeve was a potent inhibitor of glucose oxidatio causing almost total inhibition of the stimulat rate at 1.0mM and 24% inhibition at 0.1m Methylamine, ethylamine and propylamine al inhibited glucose oxidation in glucose-stimulat islets, but with a far lower potency than cystamin Only at concentrations below 5mM for methy amine and ethylamine and 1mM for propylami did these amines show no significant effect c glucose utilization in stimulated islets.

When these primary amines were tested for the effects on glucose-stimulated incorporation [^{3}H]leucine into total islet protein, only at 1mM d methylamine and propylamine show no significa inhibitory effect on this process. At the same co centration, however, ethylamine still gave 38 inhibition of [^{3}H]leucine incorporation. Whe glucose-stimulated uptake of [^{3}H]leucine into isle was measured in the presence and absence methylamine and ethylamine, but in the presen of 0.1mM-cycloheximide to equalize any 'met bolic pull' from protein synthesis, inhibition [^{3}H]leucine uptake was observed when the amin were present at 5mM. Comparison with the rate uptake of [^{3}H]leucine measured for contro (0.317±0.059pmol/h per 10 islets, $n=5$) ga percentage inhibition values of 48±9 ($n=$ $P\leqslant0.05$) for 5mM-ethylamine and 35±17 ($n=$ $P\leqslant0.25$) for 5mM-methylamine. When the co centrations of these amines in the incubatic medium were decreased to 1mM-ethylamine ar 2mM-methylamine, no significant effect [^{3}H]leucine uptake into islets was detectable. may be concluded therefore that the observ inhibition of glucose-stimulated [^{3}H]leucine inco poration by these amines when tested at the

Table 3. *Effects of test compounds on islet glucose oxidation and protein synthesis*

Glucose oxidation by islets was determined by $^{14}CO_2$ release from [U-^{14}C]glucose as described in the Experimental section. Incubations were carried out for 1 h after the addition of stimulatory concentrations of glucose, except for those incubations containing monodansylcadaverine, which were carried out for both 1 h and 2 h(*). For each concentration of amine tested, control values were determined for untreated islets from the same isolation to accommodate any variability seen between different islet preparations in their ability to utilize glucose. Total islet protein synthesis was assessed by [^{3}H]leucine incorporation into trichloroacetic acid-precipitable material. Control batches of islets were used in each experiment from the same preparation, for the same reasons given for the experiments on glucose oxidation. The inhibition of glucose oxidation and protein synthesis by test compounds is expressed as a mean percentage (±S.E.M.) relative to control incubations; the numbers of determinations are shown in parentheses. The significance of the difference from controls was determined by Student's *t* test; N.S. denotes no significant difference from controls, N.T. denotes not tested. Typical values for glucose-stimulated total protein synthesis were 306471 ± 21392 d.p.m./h per 10 islets ($n = 35$) and for glucose utilization 306 ± 21 pmol/h per 10 islets ($n = 31$).

Test compound	Concn. (mM)	Inhibition (%) of glucose-stimulated: Glucose utilization	Total protein synthesis
Monodansylcadaverine	0.1	0 (4) N.S.	0 (3) N.S.
	0.1	*19.8 ± 17 (3) N.S.	–
Cystamine	0.1	23.8 ± 11 (5) $P \leqslant 0.25$	N.T.
	1.0	90.3 ± 9 (4) $P \leqslant 0.002$	N.T.
Methylamine	1.0	N.T.	10.2 ± 3.2 (4) N.S.
	2.0	N.T.	25.5 ± 5.5 (5) $P \leqslant 0.1$
	5.0	5.1 ± 7.1 (4) N.S.	79.3 ± 4 (5) $P \leqslant 0.02$
	15.0	55.4 ± 4 (5) $P \leqslant 0.001$	N.T.
Propylamine	1.0	19 ± 19 (4) N.S.	19.5 ± 18 (3) N.S.
	5.0	48 ± 8 (3) $P \leqslant 0.01$	N.T.
Ethylamine	1.0	N.T.	38.2 ± 7.1 (5) $P \leqslant 0.01$
	5.0	8.7 ± 6.9 (4) N.S.	49 ± 12.6 (4) $P \leqslant 0.05$
Cycloheximide	0.01	N.T.	73.3 ± 3.5 (4) $P \leqslant 0.001$
	0.1	N.T.	88.8 ± 0.5 (4) $P \leqslant 0.001$

higher concentrations may in part be due to inhibitory effects on [^{3}H]leucine uptake. At lower concentrations of these amines, however, our results suggest that inhibition of [^{3}H]leucine uptake does not contribute to inhibition of [^{3}H]leucine incorporation, which may therefore represent true inhibition of protein synthesis. To determine what effect the inhibition of glucose-stimulated protein synthesis would have on insulin release over the time course of our experiments, cycloheximide was incubated with islets by using an experimental protocol identical with that used with primary amines. At a final concentration of 10 μM, cycloheximide inhibited glucose-stimulated protein synthesis by 73% of the stimulated rate, a value comparable with non-stimulated rates of protein synthesis. At this same concentration cycloheximide did not significantly inhibit glucose-stimulated insulin release (Table 2). It therefore appears that, under the conditions of assay, the nature of inhibition of glucose-stimulated insulin release by methylamine, ethylamine and propylamine at concentrations that do not significantly affect glucose-stimulated glucose utilization is independent of the effects these primary amines may have on protein synthesis.

Discussion

These studies were undertaken to demonstrate the presence of a typical transglutaminase enzyme in islets of Langerhans and to test the hypothesis that this enzyme may be involved in the Ca^{2+}-dependent mechanism of insulin release from the islet β-cell.

Our results indicate that pancreatic islets do contain a typical thiol-dependent transglutaminase enzyme which requires Ca^{2+} for its activation. Initial studies on the Ca^{2+}-dependence of the enzyme have demonstrated that half-maximal velocity of the enzyme occurs in the region of 40 μM-Ca^{2+}.

If it is assumed that the Ca^{2+} concentration in the cytosol of the resting β-cell is in the region of 0.1 μM (Wollheim & Sharp, 1982) and that other localized stores are not available to the enzyme, then the suggestion is that the islet transglutaminase may be latent under these conditions and that its activation may occur during periods of β-cell stimulation, when an increase in the cytosolic Ca^{2+} concentration takes place (Wollheim & Sharp, 1982).

In order to test the hypothesis for trans-

glutaminase involvement in the Ca^{2+}-mediated stimulatory mechanism of insulin release, known primary-amine substrates of the enzyme (inhibitors of transglutaminase-catalysed protein cross-linking) were tested for their effects on insulin release from glucose-stimulated pancreatic islets.

Of the primary amines tested, monodansylcadaverine was by far the most potent inhibitor of glucose-stimulated insulin release and was approx. 50 times more potent than methylamine, ethylamine or propylamine, which were of similar potency to each other. These relative potencies were found to correlate well with the relative potency of the amines when tested for their effects on islet transglutaminase activity.

When amines were tested for their effect on two other metabolic processes intimately associated with the secretory response of the β-cell, namely glucose oxidation and protein synthesis, the most potent inhibitor of insulin release, monodansylcadaverine, had no significant effect on either of these processes when tested at a concentration of 100 μM. Methylamine, ethylamine and propylamine did, however, show variable effects on these two metabolic processes, a finding that should be borne in mind by other investigators when using these alkylamines in studies related to receptor-mediated endocytosis (King *et al.*, 1980). Similarly, cystamine, the thiol-directed active-site inhibitor of transglutaminase (Siefring *et al.*, 1978), which, of the compounds tested, proved to be the second most potent inhibitor of glucose-stimulated insulin release, was also a potent inhibitor of stimulated islet glucose oxidation when tested at concentrations of 0.1 and 1.0 mM. Considering the importance of increased glucose utilization in glucose-stimulated insulin release, it is difficult to separate this non-specific effect of cystamine from any effect this compound may have on islet transglutaminase activity in the intact islet.

For methylamine, ethylamine and propylamine, however, concentrations of these alkylamines were found (5 mM for methylamine and ethylamine and 1 mM for propylamine) that gave significant inhibition of glucose-stimulated insulin release but did not affect glucose oxidation. At 1 mM, methylamine and propylamine were also found not to affect glucose-stimulated incorporation of [^{3}H]leucine into islet protein, although ethylamine when tested at the same concentration still gave a significant inhibition of this process, which could not be attributed to any effect of the amine on [^{3}H]leucine uptake. However, comparable experiments performed with the protein-synthesis inhibitor cycloheximide indicated that, in the time course of the experiments carried out, inhibition of glucose-stimulated protein synthesis is unlikely to affect insulin release. This result compares favourably with those obtained by Morris & Korner (1970).

Our results therefore suggest that monodansylcadaverine, methylamine, ethylamine and propylamine affect events catalysed by islet transglutaminase which are involved in the glucose-stimulated mechanism of insulin release. This is further substantiated by the observation that the dimethyl analogue of monodansylcadaverine, which is not a substrate of transglutaminase, possessed only a small percentage (24%) of the inhibitory power of the primary-amine analogue, indicating that the effect of monodansylcadaverine is mainly a consequence of the primary-amine group.

Primary amines when incubated with cells can, however, affect other processes, which may feasibly lead to inhibition of the secretory process, and it is important that these should be considered. For example, it has been shown that the weak bases imidazole (10 mM) and NH_4^+ (20 mM) can inhibit glucose-stimulated insulin release by up to 70%. This is thought to be due to an increase in the intracellular pH of the β-cell, which in turn may affect the coupling between glucose utilization and insulin secretion (Smith & Pace, 1983). Primary amines, being weak bases, may therefore inhibit glucose-stimulated insulin release by increasing the internal pH of the β-cell. However, three points argue against this. Firstly, the amine concentrations used in our experiments, which were found not to inhibit either glucose oxidation or protein synthesis, were relatively low (20 μM–1 mM) compared with those used in the studies on pH alteration, and thus may not affect intracellular pH. Secondly, although monodansylcadaverine and methylamine are of similar basicity, monodansylcadaverine is some 50 times more potent as an inhibitor of insulin release, suggesting that specificity for the transglutaminase enzyme is the differentiating factor. Thirdly, the basic dimethylated analogue of monodansylcadaverine has only a small effect on insulin release.

Our findings therefore suggest that the membrane interactions involved in the exocytotic mechanism of insulin release are dependent on reactions that may be inhibited by primary amines. The evidence suggests that the nature of this inhibition is consistent with a role for transglutaminase in the secretory mechanism. Such a role for the enzyme may lie in the translocation and/or exocytosis of the secretory granule. In other systems studied, a role for transglutaminase has been suggested in the mechanism of receptor recycling (Van Leuven *et al.*, 1980). Membrane recycling is now thought to be a necessary feature of continued exocytotic activity (Farquhar, 1981), and such a process will occur in the β-cell during glucose-stimulated insulin release (Orci *et al.*,

1978; Herzog, 1981).

During membrane recycling, it is at present not known how chemical specificity is achieved or maintained by interacting membranes of different compartments (Palade, 1982), although stabilization by membrane infrastructures has received much attention (Pearse & Bretscher, 1981). The cross-linking of membrane proteins or associated structures of the endocytosed membrane by transglutaminase would provide an alternative or additional mechanism of membrane stabilization, thus limiting the intermixing of membrane components on fusion and making possible their subsequent non-random removal from membrane components such as the Golgi body in the recycling process. A role for membrane-protein stabilization by the ε-(γ-glutamyl)lysine cross-link in this mechanism is therefore an attractive proposal, and its importance in glucose-stimulated insulin release requires further investigation.

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Abstracts

47. Evidence for the Involvement of Transglutaminase in Insulin Secretion in the Rat

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Transglutaminase is found in many tissues and may cause protein cross-linkage via $\varepsilon(\gamma$-glutamyl)-lysine bonds, a process which can be competitively inhibited by primary amines. Islets of Langerhans isolated from rat pancreas contained a Ca^{2+}-dependent transglutaminase (endo-γ-glutamine: -lysine transferase) which can be measured in homogenates of islets by ^{14}C-putrescine incorporation in N,N'-dimethylcasein. Expressed in terms of protein, the specific activity is comparable to that found in the liver. Glucose-stimulated insulin release from isolated rat islets was inhibited by incubation with the following inhibitors of transglutaminase: monodansylcadeverine (80%–90% inhibition at 0.1 mmol/l and 50% inhibition at 0.02 mmol/l), methylamine (80% inhibition at 15 mmol/l) and propylamine (60% inhibition at 10 mmol/l). Further investigation of monodansylcadaverine action demonstrated no effect on basal insulin release, on U-^{14}C-glucose oxidation by islets or on protein synthesis measured by ^{3}H-leucine incorporation into total islet protein. These results demonstrate the presence of a calcium-dependent transglutaminase within the islets of Langerhans in rats and suggest that this transglutaminase could be involved in the exocytotic secretory mechanisms associated with glucose-stimulated insulin release.

The involvement of transglutaminase and polyamines in insulin release from islets of Langerhans

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Tissue transglutaminase is a Ca^{2+}-dependent enzyme which, like other transglutamines, catalyses the cross-linking of proteins via ε-(γ-glutamyl)lysine bonds. This enzyme also catalyses the incorporation of polyamines into protein-bound glutamine residues which may lead to the cross-linking of proteins by bis-(γ-glutamyl)polyamine bridges. Various roles for this enzyme have been suggested in a number of membrane-mediated events (Siefring *et al.*, 1978; Birckbichler & Patterson, 1978; Davies *et al.*, 1980; Van Leuven *et al.*, 1980) and more recent evidence has suggested the importance of transglutaminase in the Ca^{2+}-mediated mechanism of stimulus–secretion coupling in the pancreatic β-cell during glucose-stimulated insulin release (Griffin *et al.*, 1982; Gomis *et al.*, 1983).

Polyamines, which are potential physiological substrates of transglutaminase (Folk *et al.*, 1980), have recently been implicated in the Ca^{2+}-mediated stimulus–response coupling in kidney cells after stimulation by testosterone (Koenig *et al.*, 1983). The suggestion is that increased levels of polyamines in stimulated cells (via synthesis *de novo*) may lead to a cation-exchange reaction which involves mobilization of intracellular Ca^{2+} and increased Ca^{2+} influx. The connection between these observations led us to investigate the possible role of polyamines in the mechanism of glucose-stimulated insulin release from the pancreatic β-cell in an attempt to both clarify the role of transglutaminase and to identify any role for polyamines in this mechanism.

When putrecise was tested for its effect on glucose-stimulated insulin release in batch-type incubations of isolated islets (Bungay *et al.*, 1984), only 3% inhibition of this process could be effected at a putrescine concentration as high as 20 mM. This is in contrast to the inhibition of glucose-stimulated insulin release observed for monoamine substrates of transglutaminase (Bungay *et al.*, 1984). Methylamine, for example, gave rise to a dose-related inhibition of glucose-stimulated insulin release (51–99% inhibition) when tested over the concentration range 1–15 mM.

In order to determine the suitability of putrescine as an endogenous substrate of the islet transglutaminase, islet homogenates (prepared in ice-cold 0.25 M-sucrose/1 mM-Tris/HCl/1 mM-EDTA, pH 7.4) were incubated in the presence of Ca^{2+} (2.5 mM) or EDTA (5 mM) and in the presence of [Ca^{2+}]putrescine. Measurement of the trichloroacetic acid-insoluble incorporation of radiolabel into islet protein indicated a rapid, Ca^{2+}-dependent incorporation of putrescine which was approximately twice the rate measured for [^{14}C]methylamine when incubated under identical conditions (Fig. 1*b*).

Since the poor ability of putrescine to inhibit glucose-stimulated insulin release may be attributable to its failure to gain entry to the islet β-cell, the permeability of islets to putrescine and methylamine was investigated. Batches of islets were incubated with either [^{14}C]methylamine (1.78 mM) or [^{3}H]putrescine (2.0 mM) for various times and their u assessed from the counts obtained from islets after separation from incubation media by centrifu through phthalate esters (Wollhein *et al.*, 1978). The a

Abbreviation used: DFMO, α-difluoromethylornithine.

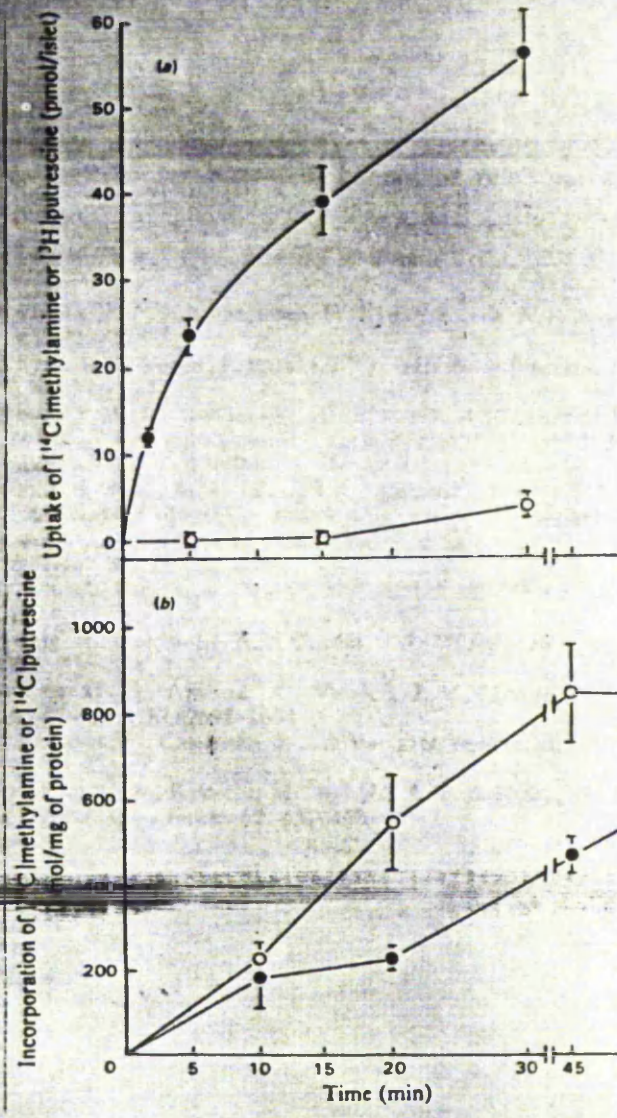

Fig. 1. *Uptake by intact islets and incorporation into isl teins of methylamine and putrescine*

(*a*) Uptake of [^{14}C]methylamine (1.78 mM) (●) [^{3}H]putrescine (2.0 mM) (○) was measured by incubat batches of 10–20 islets in 50 μl of Krebs bicarb medium and uptake assessed using the method of Wo *et al.* (1978). (*b*) The Ca^{2+}-dependent incorporati [^{14}C]methylamine (●) and [^{14}C]putrescine (○) int homogenate proteins was assessed at 37°C and pH 7. both compounds at a final concentration of 1.78 mM

ent uptake corresponding to islet extracellular space was also determined using [^{3}H]sucrose and subtracted accordingly. The data illustrated in Fig. 1*a* suggest that, in contrast to methylamine which rapidly accumulated in islets, putrescine does not freely permeate islet cells.

Since islets appear to be relatively impermeable to exogenous supplies of putrescine, the possibility that intracellylar polyamine levels may be regulated by their rapid synthesis *de novo* during glucose-stimulated insulin release was also investigated. Batches of islets were incubated with 4mM-DFMO, an enzyme-activated, irreversible inhibitor of ornithine decarboxlyase, the enzyme responsible for intracellular synthesis of putrescine. DFMO failed to affect glucose-stimulated insulin release from islets over a period of 60min at 16.8mM-glucose in the presence of DFMO after a preincubation period at 2.8mM-glucose of 45min also in the presence of DFMO.

These results are therefore consistent with the notion that the failure of putrescine to act as an effective inhibitor of glucose-stimulated insulin release is likely to be due to the failure of the compound to enter islet β-cells. Whilst our results suggest that rapid synthesis of polyamines is not involved in glucose-stimulated insulin release, their involvement in the secretory mechanism through mobilization from cellular compartments cannot be ruled out at this stage.

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4 Changes in transglutaminase activity during tumour growth and metastasis

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The Ca^{2+}-dependent enzyme transglutaminase (EC 2.3.2.13) catalyses the acyl-transfer reaction between peptide-bound glutamine residues and primary amine groups, resulting in the incorporation of amines into proteins, or if the amine is peptide-bound lysine, the cross-linking of proteins via ε-(γ-glutamyl)-lysine bridges (Folk & Finlayson, 1977). The cellular function of tissue transglutaminase is poorly understood, but evidence for its importance in the mediation of events at the cell membrane has been reported (Lorand *et al.*, 1978; Davies *et al.*, 1980). Birckbichler & Patterson (1978) have suggested that lowered transglutaminase activity is a requirement for cellular proliferation and have reported lower amounts of ε-(γ-glutamyl)-lysine cross-links in transformed cells compared with their normal counterparts. Others, however, have suggested that transglutaminase activity is necessary for stabilization of the fibrin network associated with solid tumours (Laki *et al.*, 1977), and that transglutaminase activity is necessary for the establishment of metastases.

In this study we have examined the levels and distribution of transglutaminase activity in the established solid tumour lines P7, P8, MC3 and CC5 (Moore, 1972) during their growth and metastasis, and in the development of primary liver tumours induced by diethylnitrosamine.

Tumour lines passaged subcutaneously in AS rats were harvested at various times after transplantation, and the peripheral non-necrotic tissue was homogenised in 0.25M-sucrose/1mM-Tris/HCl/1mM-EDTA, pH7.4, and assayed for transglutaminase activity by [^{14}C]putrescine incorporation into *NN'*-dimethylcasein (Lorand *et al.*, 1972). Separation of tissue homogenates into particulate and particle-free fractions was performed by centrifugation at 71000$g_{av.}$ for 45min.

The transglutaminase activities (total and specific) present in the homogenates of tumour lines were always substantially lower than those found in the normal rat tissue homogenates of liver, lung and spleen prepared in the same manner (Table 1*a*).

Variations in enzyme activity between tumours existed, but no initial pattern was evident that would distinguish between metastasising and non-metastasising types of growth. There was, however, a significant decrease in transglutaminase activity in the primary growths of the metastasising lines P7 and P8 when secondary growths from these tumours were detectable in the lungs of host animals. Under these conditions a 4-fold decrease occurred in P7 cells and a 7-fold decrease in P8 cells when activity was expressed per unit weight of DNA, an approximate measure of activity per cell.

So that we could also determine the magnitude and distribution of transglutaminase activity in the development of primary tumours, hepatocellular carcinomas (confirmed histologically) were induced in Wistar-derived rats by using diethylnitrosamine (Argos & Hoch-Ligeti, 1961). After the development of the primary growth (224 days) the tumour was excised, together with a portion of the surrounding liver. The tumour, a portion of the surrounding (pre-neoplastic) liver and a portion of control rat liver were homogenised separately and fractionated as described above. Measurement of transglutaminase activity in these homogenates indicated what appeared to be a progressive decrease in both total and specific activity occurring from normal liver to the pre-neoplastic liver and finally to the hepatocellular carcinoma (Table 1*b*). This decrease in transglutaminase activity was accompanied by a progressive redistribution of enzyme activity from the soluble to the particulate fraction of the cell. The significance of this change in enzyme distribution is unclear at present, but the concomitant decrease in activity in the development of the tumour suggests that enzyme immobilization may be a

Table 1. *Transglutaminase activity in normal rat tissues, rat sarcomas and in an hepatocellular carcinoma induced by diethylnitrosamine*

For measurement of percentage distribution of activity, homogenates were centrifuged for 45min at 71000$g_{av.}$ to give particulate (P) and particle-free (S) fractions. * Denotes that metastases to the lung were detected. The unit of enzyme activity represents nmol of putrescine incorporated/h. Results are mean values ± S.E.M. for *n* determinations. Abbreviation: N.T., not tested.

Tissue	Tumour age (days)	Transglutaminase activity (units/mg of protein)	(units/mg of DNA)	(units/g of tissue)	Percentage distribution of activity (P/S)
(*a*) P7 ($n=4$)	21	2.56±0.15	297±9	499±16	85.2/14.8±5
P7*	55	1.31±0.06	81±2	219±6	79.1/20.9±5
P8 ($n=4$)	22	1.40±0.08	350±13	148±4	75.2/24.8±7
P8*	39	0.176±0.01	50±2	32±1	44.0/56.0±8
MC3 ($n=3$)	27	1.88±0.12	136±5	137±5	77.2/22.8±0.9
MC3	43	1.14±0.06	115±5	108±4	N.T.
CC5	27	4.72±1.0	138±31	773±296	71.5/28.5±7.1
Normal liver ($n=6$)	—	33.4±1.9	4004±122	6188±241	30.1/69.9±6
Lung ($n=5$)	—	26.3±1.75	915±117	3544±246	95.0/5.0±5
Spleen ($n=5$)	—	28.8±2.9	610±150	4472±357	86.8/13.2±6.5
(*b*) Hepatocellular carcinoma ($n=3$)	—	14.0±1.3	2533±401	2725±502	95.7/4.3±3.4
Tumour-bearing liver ($n=3$)	—	28.8±1.5	5699±378	6034±510	74.3/25.7±6.7
Control liver ($n=6$)	—	33.4±5.8	6060±1031	7648±1520	50.7/49.3±3.1

means of limiting enzyme activity in the neoplastic cell.

Our results are therefore consistent with the theory that lowered transglutaminase activity is required in proliferating tissues, and furthermore suggest that the decreases in transglutaminase activity in a tissue may be a potential marker for both tumourigenesis and tumour progression.

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A study of the pulmonary toxicity induced by the anti-tumour compound bleomycin

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Bleomycin is a mixture of glycopeptides used in cancer chemotherapy (Umezawa, 1974). Unfortunately a major side effect of bleomycin therapy is the development of pulmonary fibrosis (Delena *et al.*, 1972). The pulmonary toxicity of bleomycin, although limiting the clinical use of the drug, has led to its use as an injurious agent in the development of animal models of pulmonary fibrosis (Snider *et al.*, 1978). The mechanism of toxicity, however, is not fully understood.

Several studies have suggested that bleomycin exerts its anti-tumour activity by binding to DNA and causing strand scission (Suzuki *et al.*, 1969). The mechanism, as suggested by studies *in vitro* (Oberley & Buettner, 1979), involves the formation of a Fe(II)–bleomycin complex which in the presence of oxygen and a suitable reducing agent is thought to lead to the cyclic generation of reactive oxygen species such as $O_2^{-\cdot}$ and $OH^{\cdot}$.

The present study was designed to give information on the question of whether activated oxygen species play a significant role in bleomycin-induced pulmonary toxicity. The work includes a study of the effect of bleomycin on the lungs' oxidant-defence systems after the instillation of bleomycin into rat lungs, and also an examination of the effects of hyperoxia on bleomycin-induced pulmonary fibrosis.

The effects of bleomycin on the depletion of cellular NADPH either directly in the cyclic generation of reactive oxygen species or indirectly through depletion of glutathione in the lungs' oxidant-defence systems was tested by measuring stimulation of the pentose phosphate pathway. This was done by measuring any change in the rates of the oxidation of [1-^{14}C]glucose and [6-^{14}C]glucose in lung slices (Keeling *et al.*, 1982) from the left lungs of rats previously dosed intrabronchially with a fibrogenic dose of bleomycin (1.0 unit of bleomycin in 0.1 ml of iso-osmotic saline). The results (Fig. 1*a*) indicated that no great changes in the oxidation rates of [1-^{14}C]-glucose and [6-^{14}C]glucose could be detected that would be indicative of stimulation of the pentose phosphate pathway when measured in lung slices between 3 and 48 h after treatment. A similar study with the herbicide paraquat did, however, result in a 6-fold change in the C-1/C-6 ratio when lungs were examined 3 h after treatment.

When the oxidant-defence enzymes superoxide dismutase and catalase were measured (Beauchamp & Fridovich, 1971; Lück, 1965) in extracts of similarly treated lungs a equivalent times after bleomycin instillation, no increase i enzyme activities could be detected. A significant decreas in superoxide dismutase occurred at both 24 and 48 h afte dosing (Fig. 1*b*). Measurement of glucose-6-phosphate de hydrogenase activities (Lohr & Waller, 1974), however

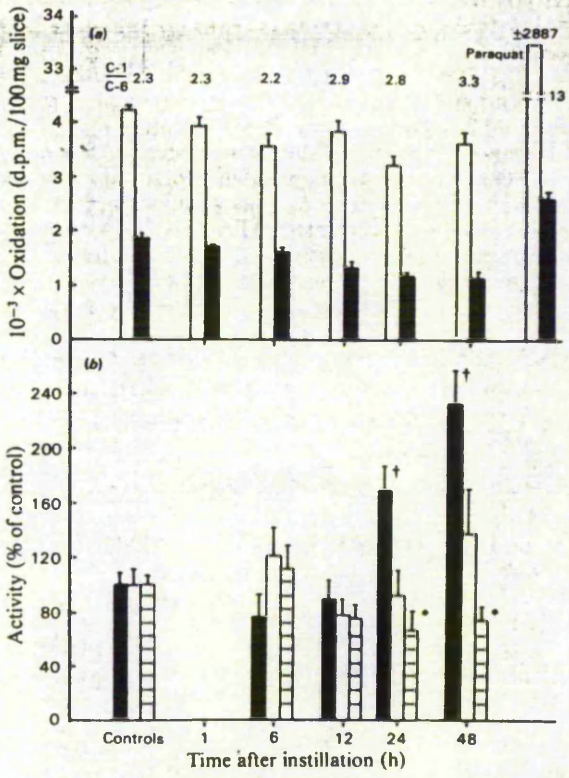

Fig. 1. *Effect of intrabronchial instillation of bleomycin on (a oxidation of [1-^{14}C]glucose* (□) *and [6-^{14}C]glucose* (■) *by ra lung slices and (b) lung activities of superoxide dismutase* (▤) catalase (□) *and glucose-6-phosphate dehydrogenase* (■)

Rats (180–200 g) received 1.0 unit of bleomycin in 0.1 ml o isotonic saline into the left lung, except for controls, whic received saline alone. Rats instilled with paraquat instead o bleomycin received 3 mg of paraquat in 0.1 ml of saline Results show the mean values ± S.E.M. ($n = 4$). *$P \leq 0.0$ and †$P \leq 0.02$ for significance of differences from controls