A RELATIONSHIP BETWEEN TRYPSIN AND PLASMIN INHIBITORS AND SIALYLTRANSFERASE ACTIVITY

SHEILA NADKARNI

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by

Sheila Nadkarni, B.Sc.(Hons), DMLT, M.Sc.

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Department of Biochemistry
Memorial University of Newfoundland
St. John's, Newfoundland, Canada

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ABSTRACT

The incubation of rat jejunal slices in Kreb's-Ringer bicarbonate buffer (KRB) resulted in a time dependent release of soluble sialyltransferase (STase) into the incubation medium for up to 6 h. However, the STase released was susceptible to proteolysis and in order to measure the STase activity released there was a requirement for either heat-inactivated serum, α_1 proteinase inhibitor (A1PI) or α_2 antiplasmin in the incubation medium. Trypsin and plasmin activities were higher in medium obtained from KRB incubations, compared to incubations where KRB was supplemented with either heat-inactivated horse serum (HHS) or heat inactivated rat serum (HRS).

Addition of heparin to jejunal incubations supplemented with HRS or HHS resulted in decreased STase activity and increased trypsin and plasmin activities in the medium. It was determined that the heparin-binding fraction (HBF) from HHS or HRS was the serum component required in order to measure STase activity in the medium.

HBF exhibited inhibitory activity towards trypsin and plasmin, but did not inhibit either elastase, thrombin, chymotrypsin, kallikrein or papain. A trypsin-binding protein (TBP) was isolated from HBF by trypsin agarose affinity

chromatography. TBP was able to inhibit trypsin and plasmin and on SDS-Page showed a single major band and an apparent molecular weight of 67 kDa. When TBP was used to supplement jejunal incubations, it was as effective as HBF in protecting the STase activity released during jejunal incubations.

Galactosyltransferase (GTase), which was also released in the soluble form during jejunal incubations, was not dependent on the proteolytic activity of the medium. GTase activity in the incubation medium remained similar whether incubations were carried out in KRB alone or in KRB supplemented with either HHS, HRS, HBF or TBP. Addition of heparin to incubations in either KRB or KRB supplemented with HRS did not cause a decrease in GTase activity, further suggesting that, GTase in contrast to STase was not dependent on the proteolytic activity of the medium.

Heat-inactivated serum from turpentine treated rats had higher trypsin and plasmin inhibitory activities compared to heat-inactivated control rat serum. When heat-inactivated serum from turpentine treated rats was used to supplement KRB during jejunal incubations there was an increase in the STase activity released into the medium compared to incubations where heat-inactivated serum from control rats was used. In contrast, GTase activity remained similar whether incubations were carried out in KRB alone or in KRB

supplemented with heat-inactivated serum from either control or turpentine treated rats.

Trypsin and plasmin when added individually to a mixture of pure STase(s) and GTase preferentially inhibited STase activity. This effect was observed for both the STases used (α2-6[N] and α2-3 [O]). TBP was able to protect STase against the action of trypsin and plasmin.

Serum from turpentine treated and control rats when incubated for 4 h at 37 °C showed a progressive decline in STase activity over the time of incubation. Trypsin and plasmin inhibitory activities also decreased over the 4 h of incubation. Serum STase, as well as trypsin and plasmin inhibitory activities remained higher in the turpentine treated rats compared to control rats. However, serum GTase activity was similar in both groups.

In incubations with hepatocytes, STase activity released into the medium was also dependent on the balance between trypsin and plasmin inhibitory/ trypsin and plasmin activities of the incubation medium. STase activity was higher in the medium when heat-inactivated serum from either turpentine treated or control rats, HBF or TBP were used as supplements compared to incubations carried out in buffer alone. GTase activity remained similar when incubations were carried out in buffer alone or in buffer supplemented with antiproteases.

The results indicate that increased STase activity was associated with increased trypsin and plasmin inhibitory activities or decreased trypsin and plasmin activities. This effect was not observed for GTase activity. Soluble STase is susceptible to proteolysis and trypsin and plasmin inhibitors therefore play a key role in determining measurable STase activity.

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LIST OF PUBLICATIONS

Some of the work presented in this thesis has been published and the publications are listed below:

Nadkarni, S. and Mookerjea, S. (1993) Role of a trypsin-binding protein in determining the levels of sialyltransferase activity. Submitted to Enzyme.

Nadkarni, S. and Mookerjea, S. (1993) Relationship between trypsin and plasmin inhibitory activity and sialyltransferase activity. Submitted to Comp. Biochem. Physiol.

Nadkarni, S. and Mookerjea, S. (1993) Role of antiproteolytic heparin-binding serum protein(s) in modulating the levels of sialyl- and galactosyltransferase activity released during the incubation of rat jejunal slices. Int. J. Biochem. 25, 731-738.

Nadkarni, S., Hunt, H., Ratnam, S., Nagpurkar, A. and Mookerjea, S. (1990) Heparin-binding protein(s) is required for the protection of sialyltransferase released during the incubation of rat jejunal slices. Biochem. J. 280, 687-693.

Mookerjea, S., Hunt, D., Nadkarni, S., Ratnam, S., Collins-Francis, J. and Nagpurkar, A. (1990) Regulation of sialyltransferase activity in intestinal segments of rats. Indian. J. Biochem. Biophys. 27, 446-451.

Mookerjea, S., Nadkarni, S., Scaplen, D. and Nagpurkar, A. (1988) Secretion of sialyltransferase by rat intestinal slices, in Sialic Acids (Proceedings of the Japanese-German Symposium on Sialic Acids), Berlin, Germany, 112-113.

TABLE OF CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

ACTH.....adrenocorticotrophic hormone
ADH.....alcohol dehydrogenase
ADP.....adenosine diphosphate
AGP.....α_1 acid glycoprotein
A1PI.....α_1 proteinase inhibitors
ANOVA.....analysis of variance
ASTI.....acid stable trypsin inhibitor
Arg.....arginine
Asn.....asparagine
AT.....antithrombin
ATP.....adenosine triphosphate
BAEE.....Nα-benzoyl-l-arginine-p-nitroanilide
BAPNA.....Nα-benzoyl-DL-arginine-p-nitroanilide
bpti.....bovine pancreatic trypsin inhibitor
BSA.....bovine serum albumin
CHO.....chinese hamster ovary
CMP.....cytidine monophosphate
ConA.....concanavalin A
Da.....Dalton
DS.....desialylated
DSG.....desialylated-degalactosylated
Dol.....dolichol
DNA.....deoxyribonucleic acid
ER.....endoplasmic reticulum
Fuc.....fucose
Gal.....galactose
GalNAc.....N-acetylgalactosamine
GlcNAc.....N-acetylglucosamine
Glc.....glucose
GTase.....galactosyltransferase
HBF.....heparin-binding fraction
HHS.....heat-inactivated horse serum
HPLC.....high performance liquid chromatography
HRS.....heat-inactivated rat serum
KRB.....Kreb's-Ringer bicarbonate buffer
Lys.....lysine
Man.....mannose
MES.....2-(N-morpholino) ethane sulfonic acid
Met.....Methionine
NAD.....nicotinamide adenine dinucleotide
NeuAc.....N-acetylneuraminic acid or sialic acid
NMR.....nuclear magnetic resonance
PA.....plasminogen activator
PAGE.....polyacrylamide gel electrophoresis
PAI.....plasminogen activator inhibitor
PTA.....phosphotungstic acid
PSTI.....pancreatic secretory trypsin inhibitor

RER.....rough endoplasmic reticulum
RNA.....ribonucleic acid
SD.....standard deviation
SDS.....sodium dodecyl sulphate
Ser.....serine
SER.....smooth endoplasmic reticulum
STase.....sialyltransferase
SUPHEPA.....N-succinyl-L-phenylalanine-p- nitroanilide
TAT.....tumour associated trypsin
TATI.....tumour associated trypsin inhibitor
TCA.....trichloroacetic acid
Thr.....threonine
TBP.....trypsin-binding protein
UDP.....uridine diphosphate

CHAPTER I. INTRODUCTION

SECTION 1. GLYCOPROTEINS: FUNCTIONS, SYNTHESIS AND STRUCTURE

I.1.1. Glycoproteins: occurrence and functions

Glycoproteins are the products ensuing from the covalent association via a glycosidic linkage, between a carbohydrate (or glycan) and a protein [Marks et al., 1962; Neuberger et al., 1966]. Glycoproteins are ubiquitous compounds found in a large variety of living organisms (Table 1.1). They occur in membrane-bound as well as soluble forms, in intracellular and extracellular fluids. Examples include enzymes, hormones, immunoglobulin, lectins, circulatory and membrane proteins [Spiro, 1963, 1966; Sharon and Lis, 1981; Schachter, 1984; Cumming, 1992].

The carbohydrate moieties of glycoproteins have been implicated in a number of biochemical functions [Sharon and Lis, 1981; Berger et al., 1982; Schachter et al, 1982; Schachter, 1984; Cumming, 1992; Roth, 1993]. These functions include (1) influence on the physical properties of proteins, (2) determination of blood group antigenicity, (3) protection against proteolysis of the polypeptide moiety, (4) control of the lifetime of circulatory proteins, (5) role in biological functions such as membrane recognition and uptake of glycoproteins by cells and (6) involvement in cell-cell interactions and cellular differentiation.

TABLE 1.1. Examples of glycoproteins. Examples of commonly occurring glycoproteins are shown in the table. [Adapted from Sharon and Lis, 1981].

Glycoproteins	Source	Molecular weight	Carbohydrate content (%)
enzymes alkaline phosphatase	mouse liver	130 000	18
hormones erythropoietin	human urine	34 000	29
membrane proteins glycophorin	human erythrocytes	31 000	60
serum proteins IgG immunoglobulin	human serum	150 000	10
structural proteins collagen	rat skin	300 000	0.4

I.1.2. Glycopeptide-linkages and glycan structures

I.1.2.A. Types of glycopeptide-linkages

Glycoproteins are classified according to the linkage formed between the oligosaccharide (or glycopeptide) and the amino acid. Two types of linkages predominate, namely the N-type and O-type glycosidic linkages respectively. In N-linked glycoproteins, an N-acetylglucosamine (GlcNAc) residue in the glycopeptide is linked N-glycosidically to an asparagine (Asn) residue in the polypeptide (Figure 1.1). Most N-linked glycoproteins share a common pentasaccharide core comprised of three mannose (Man) residues and two GlcNAc residues, linked to an Asn (Figure 1.1). Examples of N-linked glycoproteins include serum proteins (such as α_1 proteinase inhibitor [A1PI], transferrin, α_1 acid glycoprotein {AGP}), enzymes (such as alkaline phosphatase, amylases), hormones (such as thyroglobulin, chorionic gonadotrophin), membrane proteins (such as glycosyltransferases) and receptor proteins (such as rhodopsin) [Kornfeld and Kornfeld, 1980; Sharon and Lis, 1981; Berger et al., 1982; Schachter et al., 1982; Cumming, 1992].

The most prevalent O-type linkage is that which occurs between N-acetylgalactosamine (GalNAc) O-glycosidically linked to the hydroxyl groups of either a serine (Ser) or threonine (Thr) residue on the polypeptide (Figure 1.2). Examples of molecules with this kind of oligosaccharide linkage include

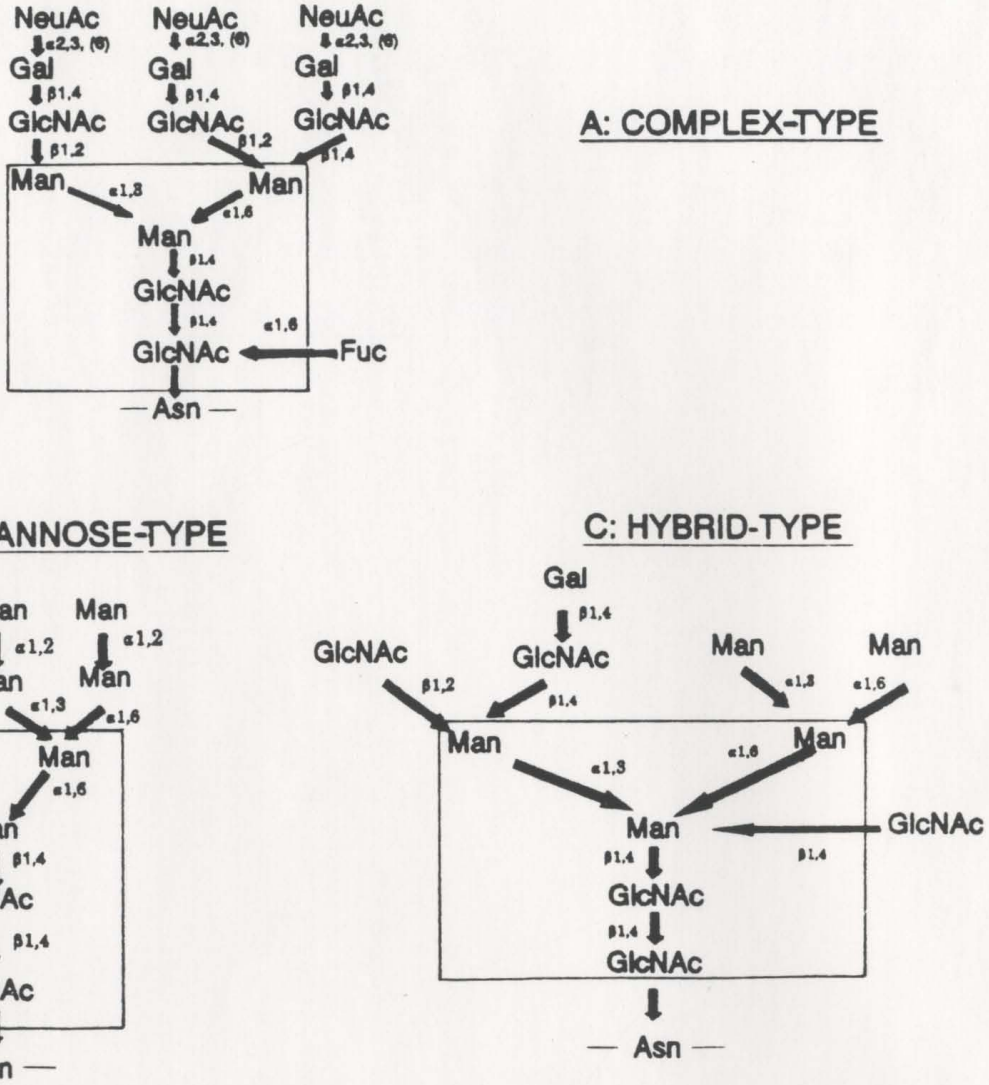


Figure 1.1. Typical structures of N-linked carbohydrate chains

Figure 1.1. Typical structures of N-linked carbohydrate chains. Examples of a complex-type glycan (A), high mannose-type glycan (B), hybrid-type glycan (C) are shown in the figure and are the type of the glycan structures found in N-linked glycoproteins. N-linked glycoproteins share a common pentasaccharide core comprising of $Man_3GlcNAc_2$, which is indicated in the boxed area. [Adapted from Sharon and Lis, 1981; Roth, 1987].

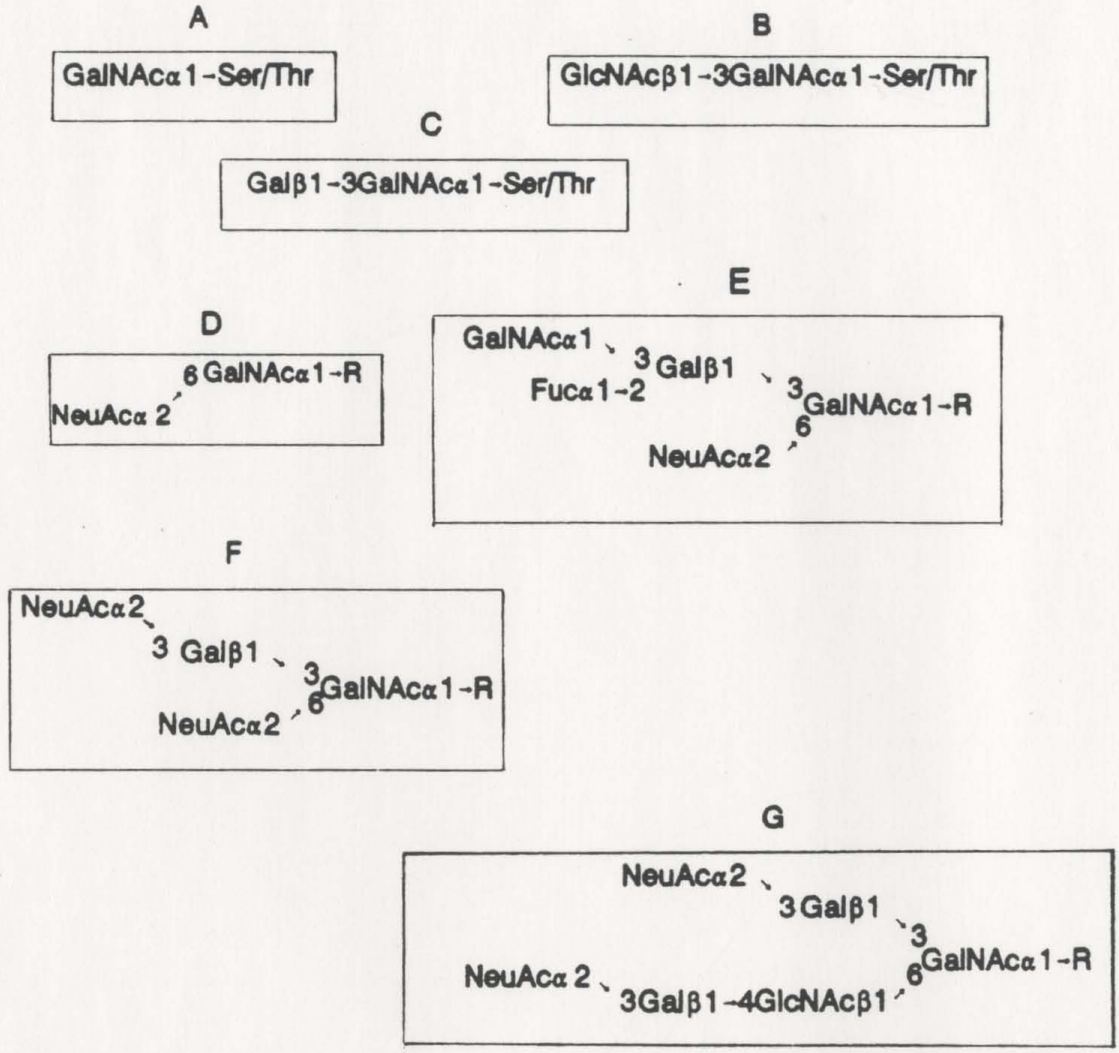


Figure 1.2. Typical structures of O-linked carbohydrate chains

Figure 1.2. Typical structures of O-linked carbohydrate chains. A, B and C denote the typical core structures found in O-linked oligosaccharides, whereas D, E, F and G show the structures of various O-linked glycan chains. [Adapted from Roth, 1987].

the mucins found lining the mucous epithelia of the respiratory, genito-urinary and gastro-intestinal systems [Berger et al., 1982; Schachter et al., 1982]. This type of O-linkage can also occur together with a N-glycosidic linkage in a glycoprotein (for example fetuin and immunoglobulin). Other O-glycosidic type linkages include those occurring in collagen and basement membranes, consisting of short carbohydrate chains, such as the glucosyl-galactosyl-hydroxylysine and galactosyl-hydroxylysine linkages respectively. Another O-glycosidic linkage is that seen in proteoglycans where oligosaccharides are attached to polypeptides by a xylosyl-Ser linkage. This introduction will focus mainly on some of the general features of N- and O-linked glycoproteins and/ or oligosaccharides.

I.1.2.B. Glycan structure

Studies elucidating the oligosaccharide structure of a particular glycoprotein are complicated by a number of factors [Sharon and Lis, 1981; Kornfeld and Kornfeld, 1980, 1985; Schachter, 1984; Cumming, 1992; Roth, 1993]. One of these factors is the complex nature of the glycopeptide chains resulting from the linkages the glycan residues form. For instance, many of the sugar chains are branched and do not necessarily show repeating patterns. Another factor is "microheterogeneity" which is a common phenomenon, whereby a particular glycoprotein can occur in forms that vary from each

other by the structure of one or more of their glycopeptide chains.

In the past few decades, advances have been made in the methods available for the study of glycopeptide structure [Kornfeld and Kornfeld, 1980; Sharon and Lis, 1981; Berger et al., 1982; Schachter, 1984]. In order to accomplish the detailed characterisation of a particular oligosaccharide unit a combination of techniques is generally used. A first step usually involves purification of the glycoprotein, followed by proteolytic digestion and isolation of the oligosaccharides. The glycan moieties of glycoproteins can be isolated using chromatographic methods such as gel filtration, ion exchange, gas-liquid, high performance and affinity chromatography [Kornfeld and Kornfeld, 1980; Sharon and Lis, 1981; Blake and Goldstein, 1982; Finne and Krasius, 1982; Wells et al., 1982; Yamashita et al, 1982; Cumming, 1992]. Affinity chromatography using lectins as adsorbents is widely used for the isolation of glycoproteins. Among the most frequently used lectins are concanavalin A (Con A) which is specific for mannose (Man) and glucose (Glc) residues; soybean agglutinin which is specific for GlcNAc and galactose (Gal) residues; wheat germ agglutinin which is specific for GlcNAc and sialic acid (also known as N-acetylneuramininc acid {NeuAc}) and peanut agglutinin which is specific for Gal.

One of the easiest features to elucidate is probably the

carbohydrate-peptide linkage. This is because the N- and O-glycan linkages differ in their stability towards hydrolysis by acid or alkali [Takasaki et al., 1982]. O-linked glycans can be easily released by mild treatment with alkali/ borohydride. N-linked glycans generally require stronger conditions such as alkaline hydrolysis, trifluoroacetolysis and hydrazinolysis.

Methylation analysis, initially introduced by Haworth and coworkers in the 1930s and later modified by Hakamori and coworkers, is commonly used in the elucidation of glycopeptide structure [Hakamori and Jeanloz, 1961: reviewed by Sharon and Lis, 1981 and references cited therein; Berger et al 1982; Kornfeld, 1982]. This technique involves methylation of all free hydroxyl groups, followed by acid hydrolysis during which the glycosidic linkages are cleaved, whereas the methyl linkages remain intact. The product formed is a mixture of partially methylated sugars with free hydroxyl groups. These hydroxyls mark the position where the sugars were linked in the starting material. Further analysis of the sugars and sugar linkages can then be carried out using other techniques, including chromatography, electroscopic methods or mass spectroscopy [Sharon and Lis., 1981; Wells et al., 1982; Schachter, 1984].

The sequence of sugars in glycopeptides as well as their configuration can also be determined using glycosidases

[Kornfeld and Kornfeld, 1980; Schachter et al., 1983; Takasaki et al., 1982]. Exoglycosidases such as sialidase, β-galactosidase and α-mannosidase sequentially remove the corresponding monosaccharides from the non reducing end of glycan chains. Endoglycosidases can cleave a N-linked oligosaccharide from a glycoprotein leaving a GlcNAc residue attached to the polypeptide.

Other techniques such as NMR spectrometry are also used to study the anomeric linkages, nature and relative proportions of the sugars [Dabrowski et al., 1980; Carver and Grey, 1981; Schachter, 1984]. One of the major advantages of these techniques is their non-destructive character.

I.1.3. Glycosyltransferases and glycoprotein synthesis

I.1.3.A. Glycosyltransferases

The glycosyltransferases are a group of enzymes which play a key role in the synthesis of glycopeptide chains. Inside the cell these enzymes are present bound to membranes in the Golgi apparatus and endoplasmic reticulum (ER) [Beyer et al., 1979, 1981; Berger et al., 1982]. The function of these enzymes is to catalyse the transfer of a sugar residue from a donor to an acceptor. The donor is generally a nucleotide diphospho sugar except for the reaction catalysed by sialyltransferase (STase) where the donor is CMP-NeuAc (CMP-sialic acid). The acceptor can be an oligosaccharide either in the free form or linked covalently to protein

[Berger et al., 1982; Schachter, 1984; Hirschberg and Snider, 1987; Roth, 1987; Finne and Colley, 1989]. A typical reaction catalysed by a glycosyltransferase with STase as an example is shown in Figure 1.3. These enzymes operate in a concerted fashion, the product of each glycosyltransferase reaction serving as the substrate for the next enzyme. The nature of these enzymes and the reactions they catalyse ensures that out of a large number of structures theoretically possible only a limited set of glycopeptide chains are actually produced. On the basis of the type of sugar transferred, glycosyltransferases can be grouped into families (eg. STases, galactosyltransferases [GTases], fucosyltransferases etc.). The individual enzymes in each family can be distinguished by their specificity for the acceptor substrates and the type of anomeric linkage formed in the product. The reaction shown in Figure 1.3 is typical for STase which catalyses the addition of the terminal NeuAc onto N-linked oligosaccharides. The enzyme preferentially utilises the sequence Galβ1→4GlcNAc in the acceptor and is not active for other sequences such as Galβ1→3GlcNAc or Galβ1→3GalNAc [Beyer et al., 1981; Joziasse et al., 1987].

I.1.3.B. Subcellular localization of oligosaccharide synthesis

Some differences exist between the synthesis of N- and O-linked glycans which will be addressed along with glycopeptide synthesis in the following sections. The enzymes involved in

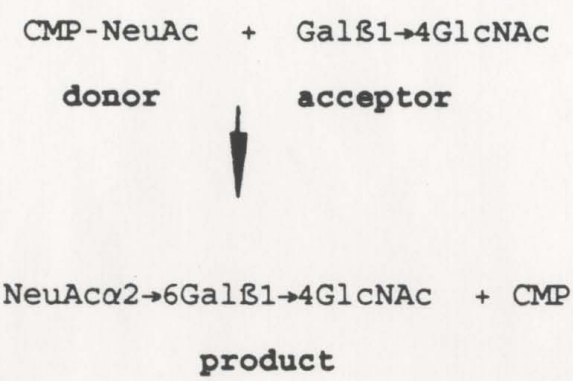


Figure 1.3. Example of a reaction catalysed by STase

Figure 1.3. Example of a reaction catalysed by STase. The reaction shown is typical for the α2→6 STase which catalyses the addition of terminal NeuAc onto N-linked glycan chains, resulting in the formation of NeuAc α2→6 Gal linkage. [Adapted from Beyer et al., 1981].

the early stages of glycan synthesis are localized in therough endoplasmic reticulum (RER) whereas those associated with the later stages of glycan synthesis are localized in the Golgi apparatus [Schachter, 1978; Roth et al., 1985b; Roth, 1987]. N-Glycosylation is believed to be cotranslational or to occur shortly after completion of the polypeptide chain [Li et al., 1978 a,b; Schachter, 1978, 1984; Kornfeld and Kornfeld, 1985]. Unlike N-glycosylation, the events involved in O-glycosylation are not clearly defined. It is believed that O-glycosylation is a cotranslational event, which takes place in the smooth endoplasmic reticulum (SER) and Golgi [Sharon and Lis 1981; Berger et al., 1982]. The later events associated with the maturation of both N- and O- linked glycans take place in the ER and Golgi apparatus. Translational and initial glycosylation occurs in the RER after which the glycoproteins translocate via the SER to the Golgi apparatus. The glycoprotein moiety undergoes processing and may undergo modification by phosphorylation and further glycosylation [Reitman and Kornfeld, 1981; Goldberg and Kornfeld, 1983]. Lastly near or at the trans region of the Golgi, sorting takes place and the proteins are packaged for secretion to the blood stream, or transport to either the lysosomes or other membrane locations (Figure 1.4) [Berger et al., 1982; Hirschberg and Snider, 1987].

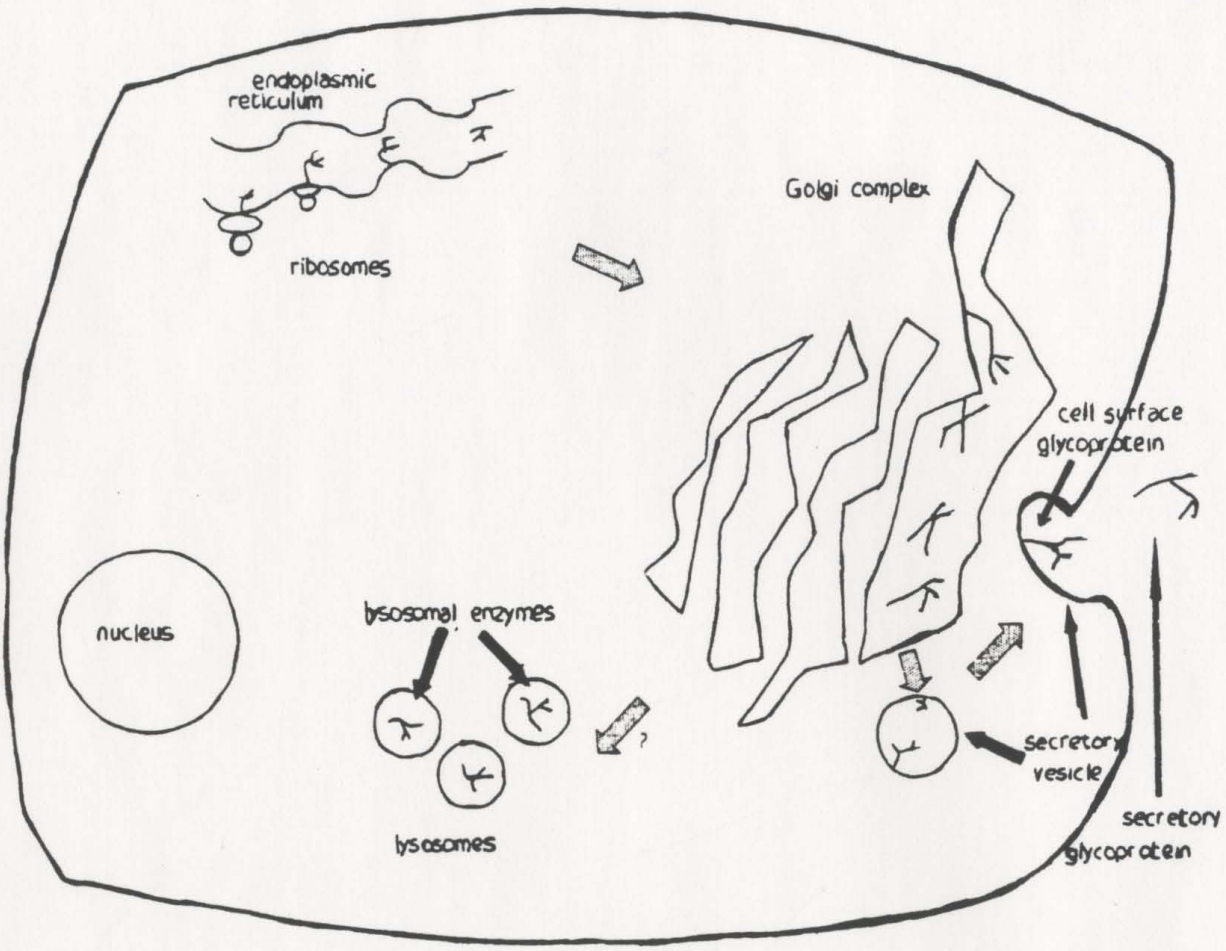


Figure 1.4. Subcellular localization of glycopeptide synthesis

Figure 1.4. Subcellular localization of glycopeptide synthesis. The various cellular compartments wherein glycopeptide synthesis occurs are shown in the figure. [Adapted from Berger et al., 1982].

KEY:- (⊢<) glycoprotein.

I.1.3.C. Synthesis of N-linked glycans

(i) Synthesis of the lipid-linked precursor chain: The assembly of the precursor oligosaccharide takes place on a 'lipid-carrier' known as dolichol (Dol), an α-saturated polyisoprenoid [Chapman et al., 1979; Parodi and Leloir, 1979; Berger et al., 1982; Schachter, 1984]. The oligosaccharide is linked to Dol via a pyrophosphate group. Dol is also involved in the synthesis of the sugar donors, Dol-P-Man and Dol-P-Glc which are lipid linked sugars required in the later stages of the biosynthesis of the precursor sugar chain [Spiro et al., 1976 a,b]. The first steps in the synthesis of the lipid linked oligosaccharide are the formation of GlcNAc-PP-Dol and its conversion to GlcNAcβ1→4GlcNAc-PP-Dol. The next step involves the addition of a β Man residue donated by GDP-Man giving rise to the product Manβ1→4GlcNAcβ1→4GlcNAc-PP-Dol. Elongation of this trisaccharide takes place by the highly ordered addition of α Man and α Glc residues. The end product is a lipid linked species Glc_3 Man_9 $GlcNAc_2$-PP-Dol [Figure 1.5]. The completed oligosaccharide is transferred en bloc to the protein.

(ii). Glycosylation of proteins: The transfer of the oligosaccharide to an Asn residue on a nascent polypeptide chain occurs in the RER [Czichi and Lennarz, 1977; Schachter, 1978; Kornfeld and Kornfeld, 1985]. It is thought that the

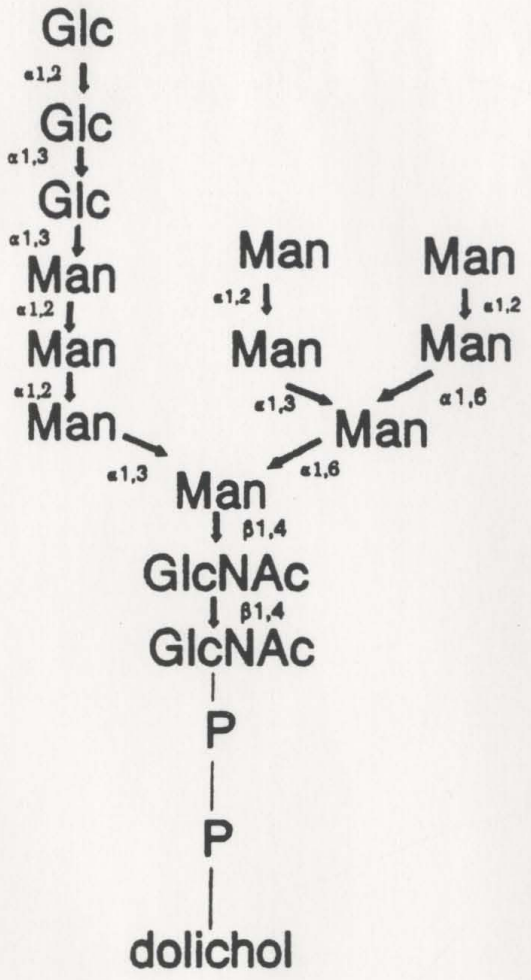


Figure 1.5. Proposed structure of the lipid-linked oligosaccharide precursor of Asn-linked glycans

Figure 1.5. Proposed structure of the lipid-linked oligosaccharide precursor of Asn-linked glycans.

The structure of the dolichol-linked oligosaccharide precursor involved in the synthesis of N-linked glycoprotein chains is shown in the figure. [Adapted from Roth, 1987].

terminal Glc residues on the saccharide function as a signal for transfer to the protein [Kaplan et al., 1987; Robbins et al., 1977; Truco and Robbins, 1979; Allen et al., 1984]. This reaction is catalysed by oligosaccharyltransferase, an enzymewhich preferentially acts on glycosylated lipid-linked liposaccharides compared to unglycosylated lipid derivatives.

In order for the carbohydrate transfer or attachment to occur, there is a requirement for the sequence Asn-X-Ser(Thr) on the polypeptide chain where X can be any amino acid. This however is not the sole requirement as other factors including sufficient exposure of the tripeptide sequence of the acceptor protein are also considered to influence carbohydrate attachment. Fragmented or denatured peptides are considered to be better acceptors for glycosylation compared to folded polypeptide chains [Schachter, 1978; Berger et al., 1982; Green, 1982; Pollack and Atkinson, 1983; Trimble et al., 1983; Schachter, 1984; Kornfeld and Kornfeld, 1985].

(iii). Processing of the protein-linked oligosaccharide: Once the oligosaccharide is transferred onto the polypeptide chain, the carbohydrate portion of the "newly formed glycoprotein" undergoes extensive processing (Figure 1.6). The processing begins with the removal of the Glu residues by glucosidases [Grina and Robbins, 1979; Ugalde et al., 1980]. The distal glucose is removed by microsomal α-glucosidase I, α-glucosidase II then removes the two remaining Glu residues

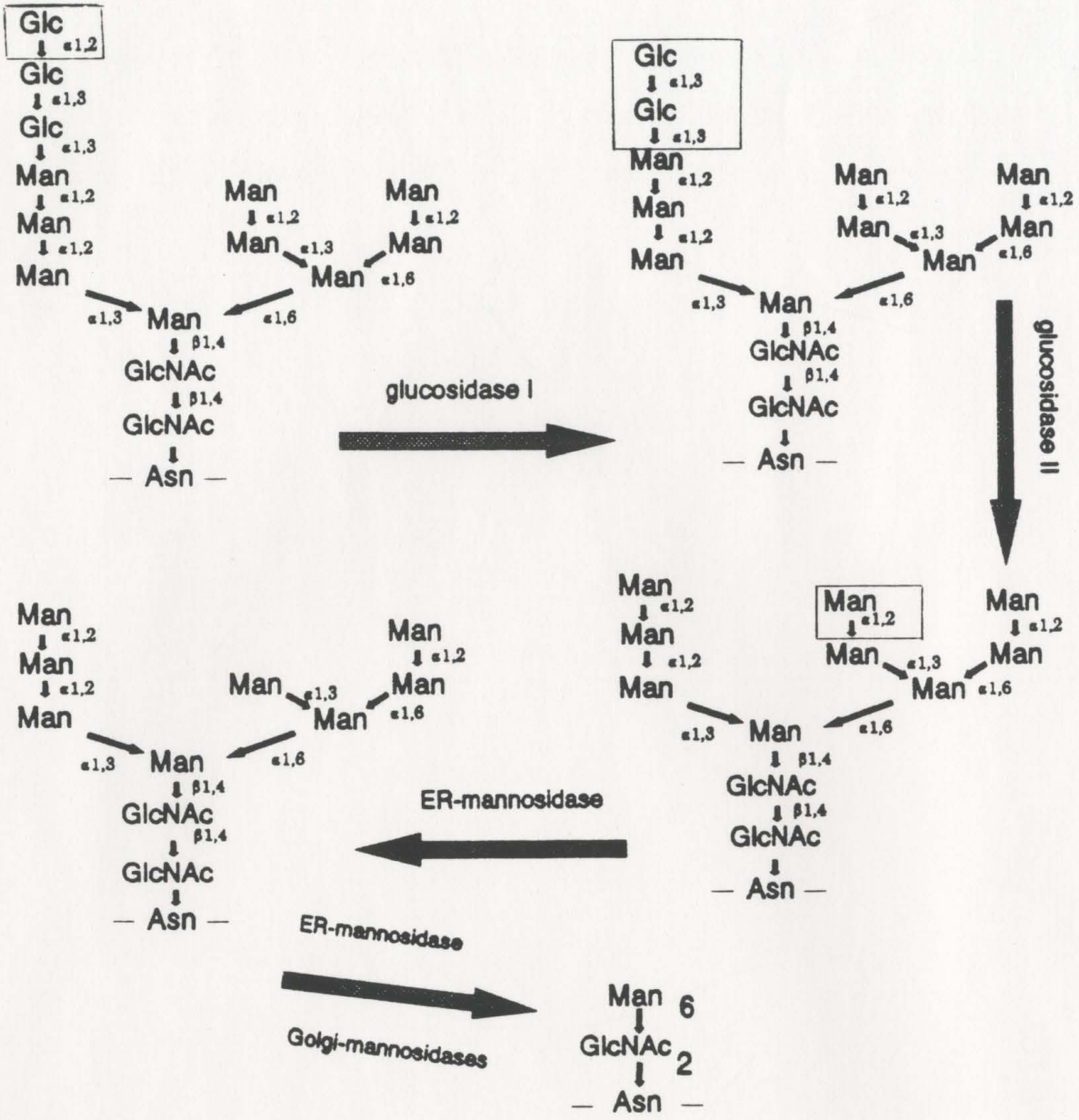


Figure 1.6. Processing of the Asn-linked oligosaccharides

Figure 1.6. Processing of the Asn-linked oligosaccharides. Schematic diagram showing the processing reactions occurring in the ER during the synthesis of Asn-linked oligosaccharides. [Adapted from Roth, 1987].

giving a glycopeptide with the structure $Man_9GlcNAc_2$. A large number of glycoproteins at this stage go through " mannose-trimming". The $Man_9GlcNAc_2$ intermediate undergoes processing by the removal of the $\alpha 1 \rightarrow 2$ linked mannose residues, catalysed by α-mannosidases which are located in the Golgi. In the order for the $Man_5GlcNac_2$ pentasaccharide or core structure to be formed two different mannosidases are needed [Tabas et al., 1981; Tabas and Kornfeld, 1979; Tulsiani et al., 1982; Allen et al., 1984]. The $Man_5GlcNAc_2$ glycopeptide linked to protein can be converted into either a complex-type or a hybrid-type glycan (Figures 1.7 and 1.8). Some glycoproteins contain carbohydrate moieties which after the removal of Glc do not get processed to the same extent as complex- and hybrid-type glycans. This results in the formation of high-Man structures containing between 5 to 9 Man residues [Trimble et al., 1983]. A number of factors govern the extent and processing of the individual glycosylation stages [Wilson et al., 1981; Kornfeld, 1982; Schachter, 1984]. These include (1) the conformational exposure of the glycosylation site, (2) the activity of the enzymes catalysing the various reactions and (3) the transit time through the ER and Golgi.

(iv). Conversion to either complex-type or hybrid-type structures: GlcNAc-transferase I plays a key role in the formation of complex- and hybrid-type glycans from the

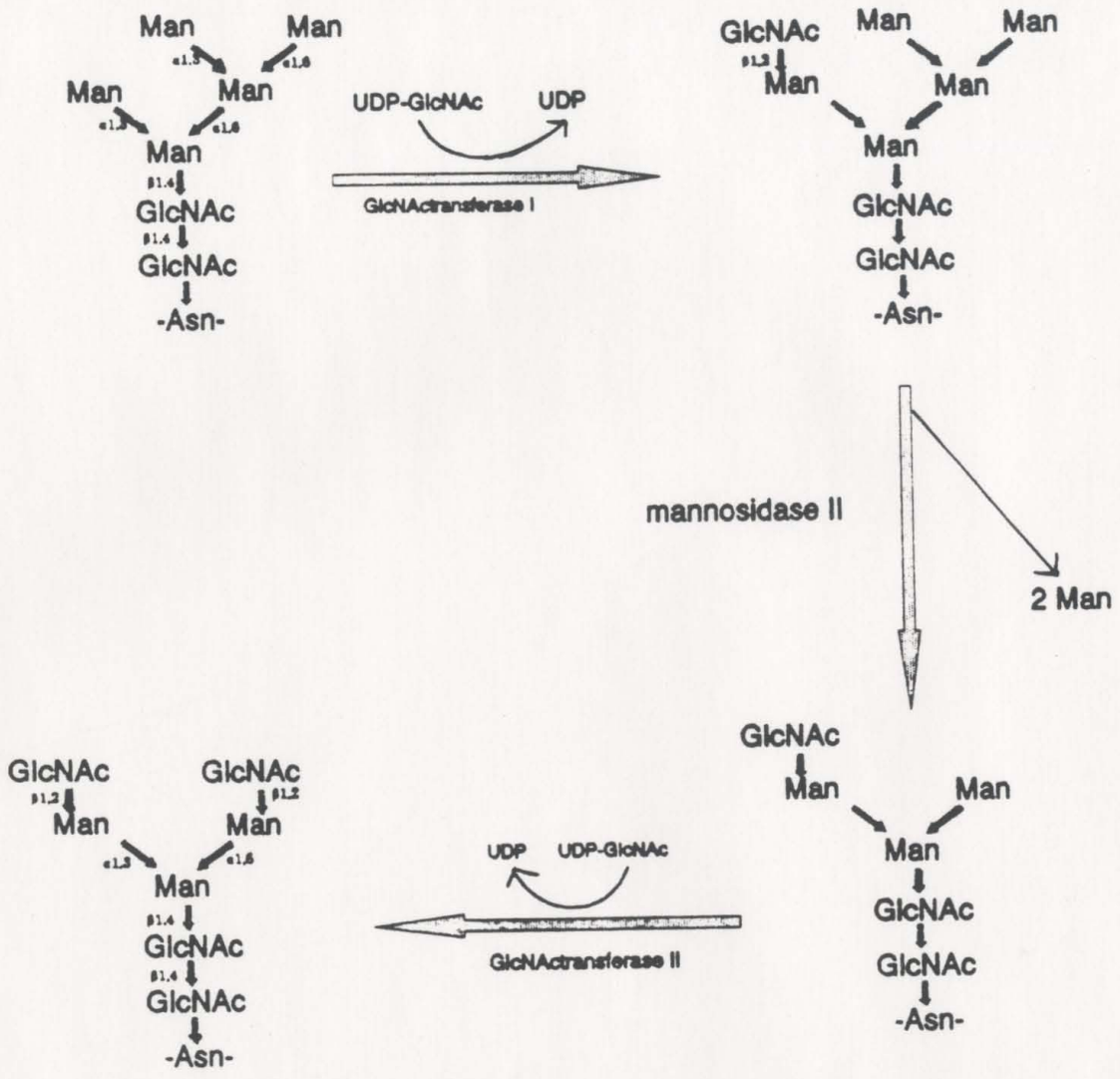


Figure 1.7. Terminal steps involved in the synthesis of complex-type glycans

Figure 1.7. Terminal steps involved in the synthesis of complex-type glycans. Some of the terminal steps involved in the formation of complex-type glycans are shown in the figure. [Adapted from Roth, 1987].

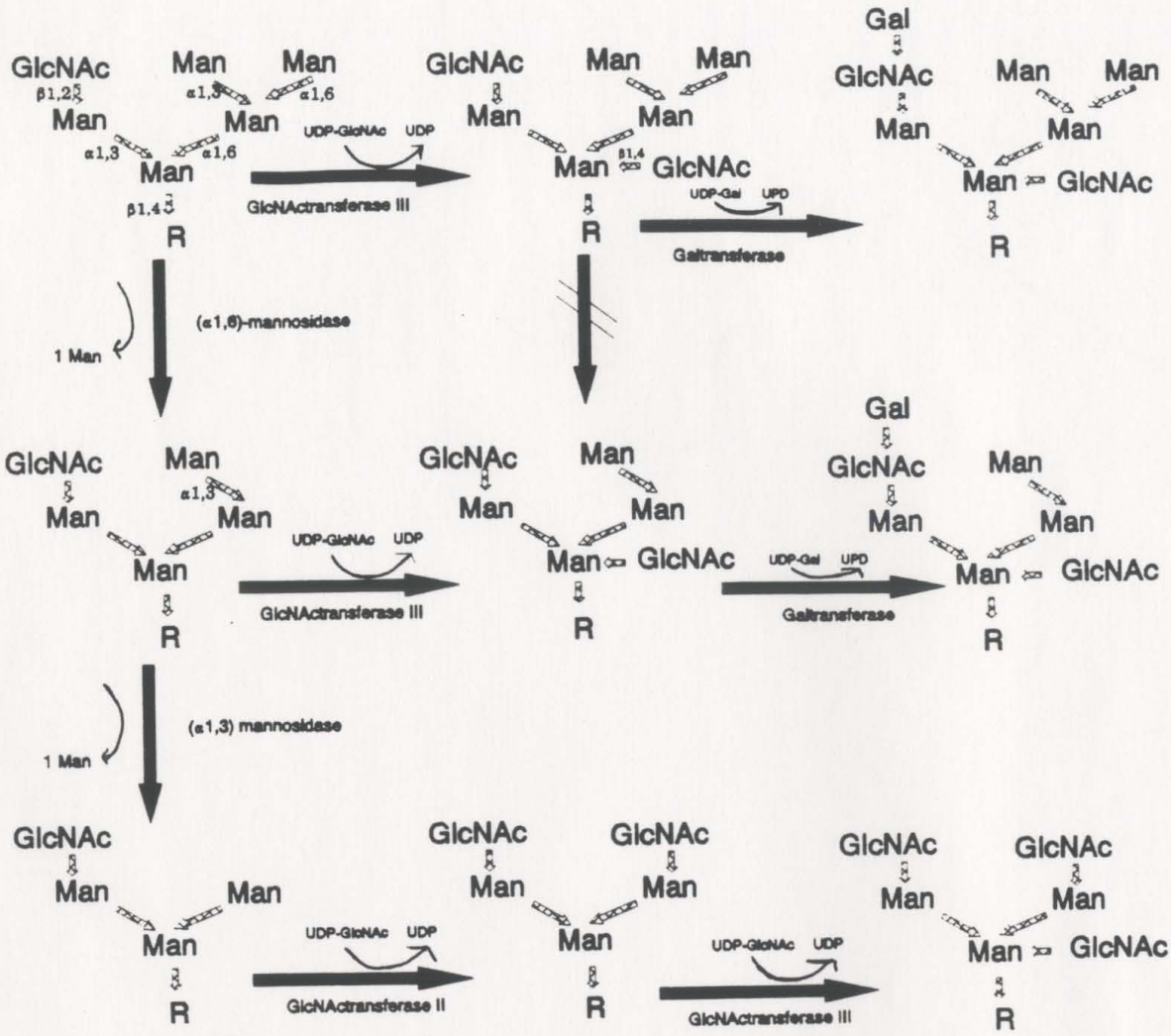


Figure 1.8. Proposed mechanism involved in the formation hybrid-type glycans

Figure 1.8. Proposed mechanism involved in the formation of hybrid-type glycans. The steps leading to the formation of hybrid-type glycans are shown in the figure. [Adapted from Kornfeld, 1982].

$Man_5GlcNAc_2$ intermediate. This enzyme attaches GlcNAc in a $\beta 1 \rightarrow 2$ linkage to Man (Figure 1.7) to give the structure GlcNAc-Man_5-$GlcNAc_2$-R. At least five of the enzymes that take part in the synthesis of the glycan chain require prior action by GlcNAc transferase I [Harpaz and Schachter, 1980 a,b; Hasilik et al., 1981]. The formation of complex type glycans involves α-mannosidase II which catalyses the removal of the two remaining α-linked Man residues. The resulting glycopeptide $GlcNAcMan_3GlcNAc$-R is converted by GlcNAc-transferase II into a biantennary structure [Bendiak and Schachter, 1987 a,b]. Further branching takes place through the action of GlcNAc-transferases IV and V [Schachter, 1978, 1984]. Gal is then added in a $\beta 1 \rightarrow 4$ linkage to GlcNAc at the Man $\alpha 1 \rightarrow 3$ branch of the biantennary glycans [Hanover and Lennarz, 1981; Hubbard and Ivatt, 1981; Berger et al., 1982; Hirschberg and Snider, 1987]. The complex-type structures can undergo fucosylation at the core GlcNAc. Finally NeuAc (in either a $\alpha 2 \rightarrow 3$ or $\alpha 2 \rightarrow 6$ linkage) is added to the terminal Gal residues, or L-Fuc is added to the subterminal GlcNAc residue in a $\alpha 1 \rightarrow 3$ linkage. Addition of αNeuAc, αFuc or αGal blocks the further extension of the sugar chains [Paulson et al., 1978].

Hybrid-type carbohydrate structures are produced in those tissues where GlcNAc-transferase III is active (Figure 1.8). The enzyme transfers GlcNAc in $\beta 1 \rightarrow 4$ linkage to the

branching β Man. This bisecting GlcNAc hinders access to the $GlcNAc_5$ recognition site [Harpaz and Schachter, 1980 a,b; Narasimhan, 1982]. This in turn prevents action of α mannosidase II resulting in the synthesis of hybrid-type oligosaccharides.

(v). Is there a requirement for elaborate processing?

The complex processing pathway(s) are thought to be a mechanism whereby the cell is able to generate a variety of glycan structures, without the need for developing new pathways for the biosynthesis of lipid-linked oligosaccharides [Berger et al., 1982; Kornfeld, 1982; Roth, 1987]. The processing pathway is therefore a means by which at least five different classes of N-linked glycans can be produced from a single precursor [Figure 1.9].

I.1.3.D. Synthesis of O-linked glycans

The biosynthesis of O-linked glycans differs from N-linked glycans [Schachter, 1978; Berger et al., 1982; Roth, 1987]. O-linked carbohydrate chains are synthesized one residue at a time directly onto the protein backbone. So far there seems to be no evidence to indicate involvement of a lipid-linked intermediate. The factors which ultimately control and determine the structures of O-glycopeptides are not clearly defined. Some of the reactions involved in the synthesis of O glycans have been studied and these studies

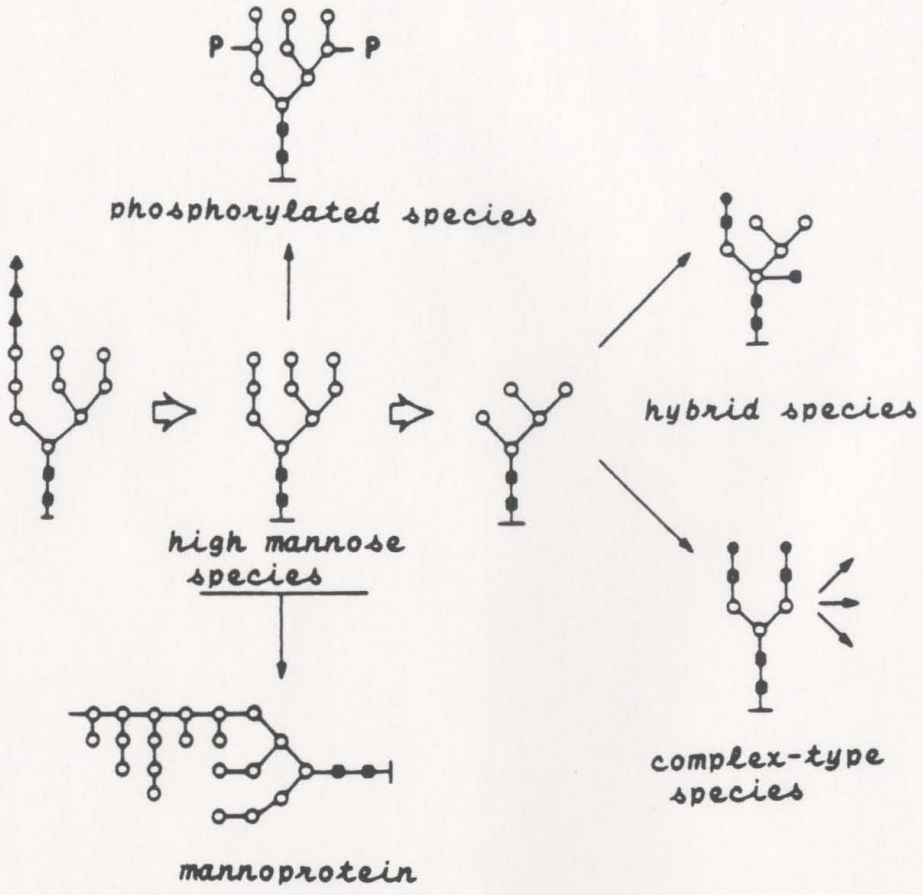


Figure 1.9. Different kinds of glycans formed from a common lipid-linked oligosaccharide

Figure 1.9. Different kinds of glycans formed from a common lipid-linked oligosaccharide. The figure shows a summary of the different types of glycans which are known to be formed from a common lipid-linked oligosaccharide. [Adapted from Kornfeld, 1982].

KEY: (▲) glucose, (○) mannose, (■) N-acetylglucosamine, (●) galactose, (⊥) asparagine.

have provided valuable information regarding the synthetic process(es) of O-linked oligosaccharides.

The initial step is the transfer of GalNAc from UDP-GalNAc to either a Ser or Thr residue on the polypeptide chain of a protein and this reaction is catalysed by the enzyme UDP-GalNAC: polypeptide transferase. [Hill et al., 1977; Schachter, 1978; Berger et al., 1982, Roth, 1987].

UDP-GalNAc + HO-(Thr/Ser) ⟶ GalNAcα-O-(Thr/Ser) + UDP

The GalNAc-transferase involved in O-glycosylation is mainly located in the SER and Golgi apparatus and recognises Ser and Thr residues [Hill et al., 1977]. The product of one glycosyltransferase serves as the acceptor substrate for the next reaction.

In a particular tissue, the structure of O-linked glycans is determined by the relative activities of the glycosyltransferases and the substrate specificities of the particular tissue. The ordered addition of sugar residues results in the formation of a well defined glycan structure. In mucins the synthesis of sialylated oligosaccharides proceeds in an ordered fashion by the additions of Fuc, GalNAc and NeuAc to Galβ1→3GalNAc-R chains [Hill et al., 1977; Berger et al., 1982]. The addition of NeuAc to the oligosaccharide GalNAcα1-R appears to block elongation of this chain. The

length of the glycan chain thus depends on the stage where NeuAc is introduced into the glycan [Berger et al., 1982]. A proposed scheme for the biosynthesis of the porcine mucin pentasaccharide is shown in Figure 1.10. After the formation of the glycan chain GalNAcαThr/Ser, either Gal or NeuAc can be transferred next. It has been shown that Gal requires to be added next, and that the addition of NeuAc before Gal terminates the oligosaccharide as a disaccharide [Schachter, 1978; Schachter and Roseman, 1980]. The steps involved in glycosylation after the formation of the sequence Galβ1→3 GalNacαThr/Ser have been studied [Beyer et al., 1981]. Three homogeneous glycosyltransferases purified from porcine submaxillary glands were used and the possible tri- and tetrasaccharide intermediates en route to the completed pentasaccharide were tested as substrates. The glycosyltransferases used were those which catalysed the addition of the non-reducing terminal NeuAc, Fuc and GalNAc and the results are summarized in Figure 1.10 (B). These studies indicated that the preferred order of addition is NeuAc, Fuc and GalNac respectively. The NeuAc was added first as fucosylated substrates are poor substrates for STase and the GalNAc added last since GalNAc-transferase requires the Fucα1→2Gal sequence as a substrate.

Some studies indicate that there is genetic control at the level of the expression of the number and type of

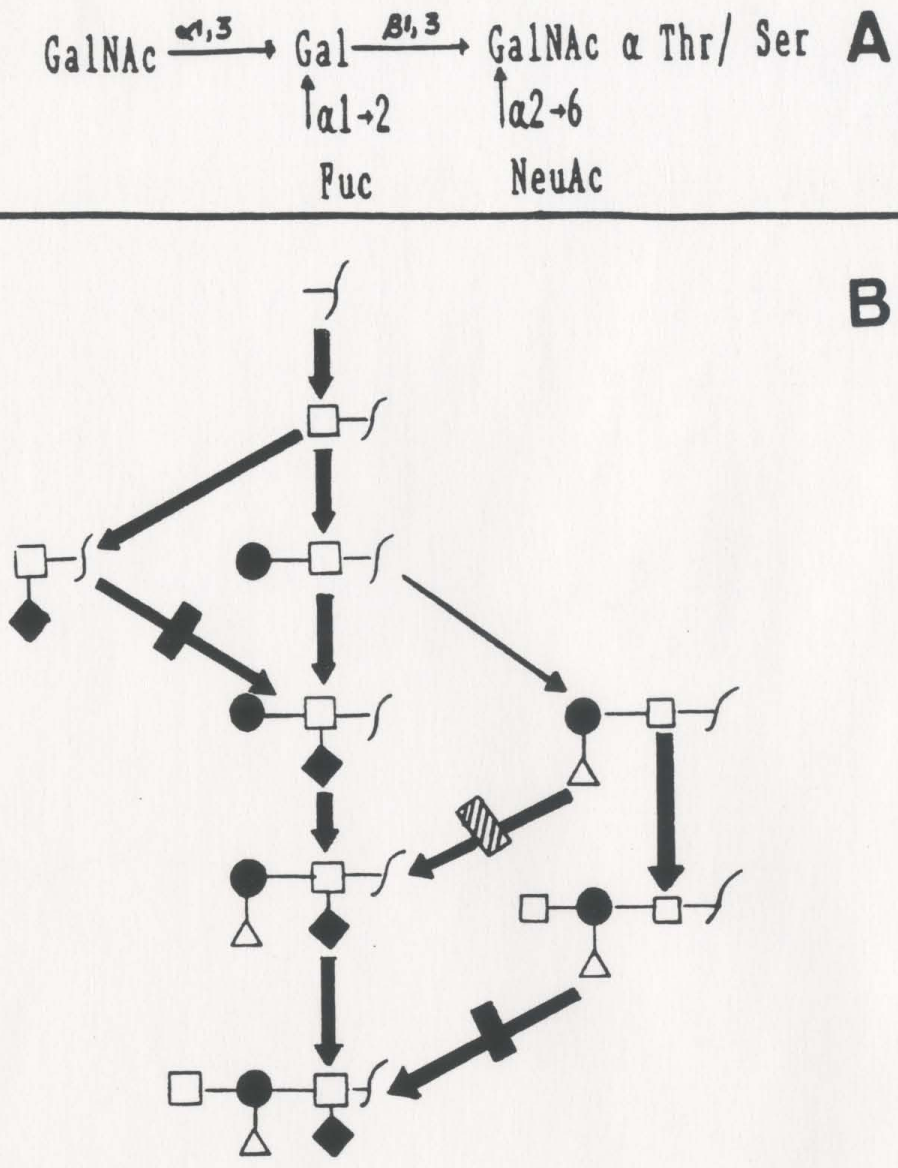


Figure 1.10. Proposed stages in the synthesis of O-linked oligosaccharide chains

Figure 1.10. Proposed stages in the synthesis of O-linked oligosaccharide chains. Panel A shows the structure of the completed mucin pentasaccharide. Panel B shows the biosynthesis. The preferred addition of sugars is shown in the centre. Alternate pathways branching to the sides result in dead end products because of the inability of glycosyltransferases to carry out the reactions shown by hatched or solid blocks. [Adapted from Berger et al., 1982].

glycosyltransferases which are produced in a cell. The genetic basis for the A, B and O blood groups is determined by the carbohydrate structures present on the red cell glycoconjugates [Schachter and Roseman, 1980; Beyer et al., 1981; Keshavara et al., 1992]. All three blood group structures share a common terminal precursor sequence Fucα1→2Gal. Blood group A is determined by an additional GalNAc residue GalNAcα1→3 (Fucα1→2)Gal and blood group B by an additional Gal residue Galα1→3(Fucα1→2)Gal.

SECTION 2. SIALYLTRANSFERASES (STases)

I.2.1. Nature and cellular localization of STases

I.2.1.A. Specificity of STases

STases are a family of glycosyltransferases which catalyse the transfer of NeuAc from CMP-NeuAc, to usually terminal positions on the carbohydrate residues of suitable acceptors [Beyer et al., 1981]. The acceptor can be an oligosaccharide either in the free form or attached to a protein.

The hallmark of glycosyltransferases is thought to be their specificity which forms the basis of the one-enzyme one-linkage hypothesis. Each STase exhibits high specificity for the protein acceptor, the nucleotide sugar, the linkage which is formed (α2→3, α2→6, α2→8) and the structure of the acceptor molecule. The reaction shown in Figure 1.11 is typical for the

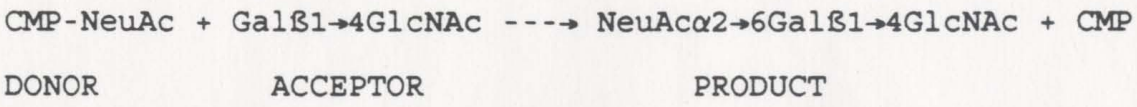


Figure 1.11. Example of a reaction catalysed by STase

Figure 1.11. Example of a reaction catalysed by STase. An example of a reaction catalysed by STase is shown. CMP-NeuAc is the nucleotide sugar donor, Galβ1→4GlcNAc is the required sugar sequence in the acceptor. The product formed will have NeuAc linked α2-6 to a Gal residue. [Adapted from Beyer et al., 1981].

STase which catalyses the addition of NeuAc onto N-linked oligosaccharides. The enzyme preferentially utilises the sequence Galβ1→4GlcNAc in the acceptor and is not active for other sequences such as Galβ1→3GlcNAc and Galβ1→3GalNAc [Beyer et al., 1981; Bergh et al., 1983; Joziasse et al., 1987; Van den Eijnden et al., 1977, 1980; Nemansky et al., 1992]. The specificity of STase is believed to extend to structural features beyond the terminal N-acetyllactosamine units on the oligosaccharide chains of acceptor proteins. Studies by Van den Eijnden and coworkers [1980] showed that β-galactoside α2→6STase from bovine colostrum preferentially transferred NeuAc to the Galβ1→4GlcNAcβ1→2Manα1→3Man branch of α_1AGP. The branch with the sequence Galβ1→4GlcNAcβ1→4Manα1→3Man was of intermediate preference, whereas the least preferred branch was that with the sequence Galβ1→4GlcNAcβ1→2Manα1→6Man. Evidence not conforming to the one-enzyme one-linkage hypothesis also exists in the literature. For example studies have shown that the Galβ1→4GlcNAcα2→6 STase from rat liver was able to sialylate the disaccharide Manβ1→4 GlcNAc and the trisaccharide Manβ1→4GlcNAcβ1→4GlcNAc [Van Pelt et al.,1989].

In eucaryotic cells five NeuAc linkages predominate namely (1)NeuAcα2→6Gal (2)NeuAcα2→3Gal (3)NeuAcα2→6GalNAc (4)NeuAcα2→6GlcNAc and (5)NeuAcα2→8NeuAc [Sadler et al., 1979; Beyer et al., 1981; Weinstein et al., 1982 a,b; Finne et al.,

1983; McCoy et al., 1985; Broquet et al., 1991].

I.2.1.B. Distribution of STase within the cell

STases in cells are found located predominantly in the Golgi apparatus, but enzyme activities have also been detected in ER and plasma membranes [Berger and Hesford, 1985; Roth et al., 1986; Taatjes et al., 1987; Bosshart and Berger, 1992; Tang et al., 1992]. There is evidence that in mouse and rat hepatocytes Galα2-6 STase is found within the trans Golgi cisternae and the trans tubular network [Carey and Hirschberg, 1981; Roth, 1987]. In intestinal goblet cells, STase was found to be distributed in the trans cisternae of the Golgi apparatus stack, brush border and plasma membranes [Roth et al., 1986: Stitcher et al., 1991]. The presence of two O-glycan STases have been reported in rat brain, with the α2-3 STase located towards the lumen and the α2-6 STase located towards the cytoplasmic side of the Golgi membranes [Baubichon-Cortay et al., 1986b].

Drugs and related agents can cause redistribution of STase in cellular membranes. For instance, in experiments with cultured hepatocytes it was observed that malignant transformation caused a reorganization of (trans) Golgi apparatus elements containing STase, leading to a more extensive distribution compared to intact hepatocytes [Taatjes et al., 1987]. Experiments by Tang and coworkers (1992) with CHO cells transfected with cDNA of the rat STase have revealed

that the STase expressed was found localized in the trans-Golgi, but upon treatment with brefeldin A was redistributed into the ER. STases have been found in the outer membranes of mouse liver mitochondria [Gateau et al., 1980], rat liver nuclei [Richard et al., 1975] and synaptosomes [Breen and Regan, 1986].

STases have also been found localised to internal membranes other than the Golgi and ER. The presence of ecto-STases has been demonstrated in a variety of cells including platelets [Bauvois et al., 1981], lymphocytes [Hoflack et al., 1979], lymphoblasts [DucDeudon et al., 1984], neuronal cells [Matsui et al., 1983, 1986] and mucosal cells of the small intestine [Taatjes et al., 1988].

I.2.2. STases in tissues and extracellular fluids

I.2.2.A. Distribution of STase in tissues

STase activities have been detected in a variety of mammalian tissues including rat mammary glands [Jourdian et al., 1963; Roseman et al., 1966; Carlson et al., 1973a], sheep submaxillary glands [Roseman et al., 1966; Carlson et al., 1973b], embryonic chicken brain [Roseman et al., 1966], rat and calf liver [Van den Eijnden and Schiphorst, 1981; Paulson et al., 1982; 1984], rat and chicken brain [Dall'Olio, 1990; Van den Eijnden and Schiphorst, 1981] and mouse kidney [Bardos et al., 1980]. STases are also present in blood cells such as platelets [Bauvois et al., 1982], leucocytes and

lymphocytes [Hoflack et al., 1979; Baker et al., 1987]. It is thought that in order to synthesize all the sialo-oligosaccharide sequences known, more than 15 STases are required [Schauer, 1982]. A number of STases have been purified including βGalα2→6 STase from rat liver [Weinstein et al., 1982a,b], βGalNAcα2→6 STase from porcine submaxillary glands [Sadler et al., 1979], βGalα2→3 STase from human placenta [Joziasse et al., 1985b], βGalα2→6 and βGalα2→3 STases from human platelets [Bauvois et al., 1982].

The primary sequence of βGal α2→6 STase (E.C.2.4.99.1) from rat liver has been determined from the nucleotide sequence of cDNA and was compared with five other cloned glycosyltransferases [Weinstein et al., 1987; Paulson and Colley, 1989]. The six enzymes studied by these researchers showed almost no sequence homology. However, they shared a homologous amino terminal NH_2 tail on the cytoplasmic side of the membrane, a 16-20 amino acid anchor domain in the membrane and a large carboxyl terminal catalytic domain on the lumenal side of the Golgi membrane (Figure 1.12).

The stem region is thought to act as a flexible hold leaving the catalytic region exposed, thereby enabling it to glycosylate carbohydrate moieties of membrane-bound proteins as well as other proteins of the secretory pathway enroute through the Golgi apparatus [Weinstein et al., 1987; Paulson

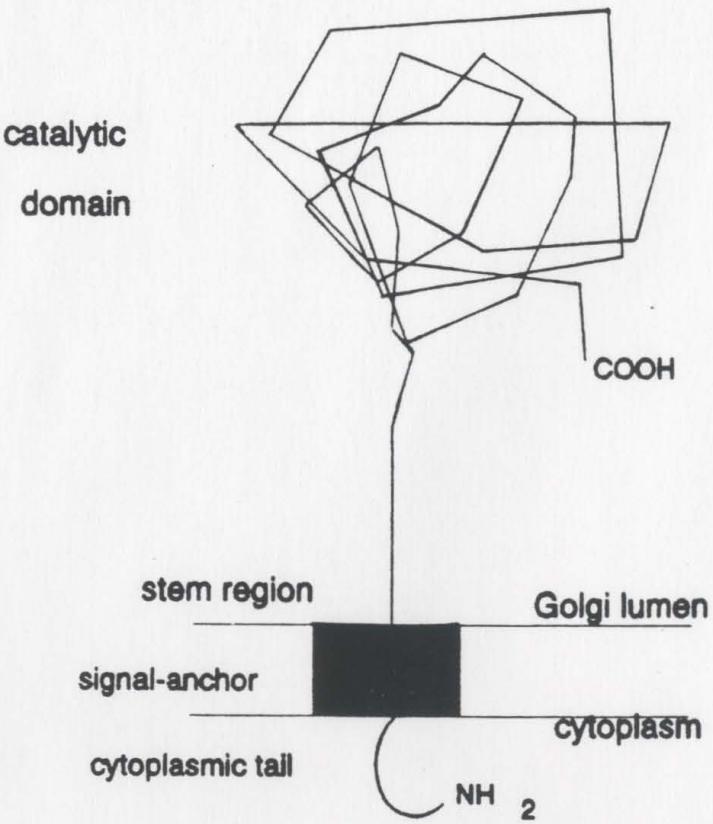


Figure 1.12. Proposed topography of STase

Figure 1.12. Proposed topography of STase. Terminal glycosyltransferases are believed to share a common topography in the Golgi apparatus. The enzyme molecule is thought to consist of a short NH_2-terminal cytoplasmic tail, a signal anchor domain which spans the membrane, an extended stem region, and a large COOH-terminal catalytic domain oriented within the lumen of the Golgi cisternae. Proteolytic cleavage of the membrane anchor is believed to release the molecule in the soluble form. [Adapted from Paulson and Colley, 1989].

and Colley, 1989]. Recent studies suggest that the signal anchor domain as well as a second region of Galα2→6 STase, probably the stem region, contain sequences which play a role in the Golgi apparatus localization [Colley et al., 1989, 1992].

I.2.2.B. Soluble STases

Soluble forms of STases exist in body fluids including milk, serum, colostrum and intestinal lymph [Hudgin and Schachter, 1971a,b; Kim et al., 1972a,b; Mookerjea et al., 1972; Paulson et al., 1977; Joziasse et al., 1987; Ratnam et al., 1981]. These enzymes are believed to originate due to proteolytic release from their membrane-bound forms [Paulson and Colley, 1989; Broquet et al., 1991]. Results obtained from NH_2 terminal analysis of the soluble forms of STase have revealed a lumenal stem region which separates the catalytic domain from the transmembrane domain and is exposed to proteases [Weinstein et al., 1987; Colley et al., 1989; Gillespie et al., 1992]. Other studies have shown that a cathepsin-D-like protease is involved in the release of STase from hepatocyte membranes [Lammers and Jamieson, 1988, 1990; McCaffrey and Jamieson, 1993].

Some pathological conditions are associated with increases in activities of STase(s) in both tissue(s) and body fluids. Turpentine induced inflammation in the rat is

accompanied by increased STase activity in liver and serum [Kaplan et al., 1983; Fraser et al., 1984]. Hepatocytes and liver slices in culture have been shown to release soluble STase into the incubation medium [Kaplan et al., 1983; Van Dijk et al., 1986]. In the rat colchicine injection resulted in increased STase activity in intestine, intestinal lymph and serum [Fraser et al., 1980; Ratnam et al., 1981, 1987]. Chu and coworkers [1988] have demonstrated that thermal injury in the rat caused increased STase activity in small intestine and serum. Liver is thought to be the major source of soluble STase activity in tissue fluids especially blood. The presence of STase in blood cells such as platelets [Bauvois et al., 1981, 1982], granulocytes and lymphocytes [Hoflack et al., 1979; Baker et al., 1987] suggests that these may also contribute to the STase activity present in blood. The increased activity of STase observed in small intestine as well as serum, following thermal injury and colchicine injection suggest that the intestine could be a possible source of soluble STase activity.

Although it is clear that STase activity can be detected in extracellular fluids, as well as in cell/tissue culture medium, the factors which control the levels of soluble STase are not clearly defined. Also, the conditions which govern the release of the membrane-bound STase and the subsequent levels of soluble STase activity are poorly understood.

The release of STase from the membranes could be due to a number of factors. For example, the STase activity detected in tissue fluids or incubation media could be the result of release of surface materials from membranes. It has been proposed that STase is a secretory protein which may explain its association with plasma membranes [Bosshart and Beyer, 1992]. Release of STase into the incubation media and/ or tissue fluids could also occur as the result of cell damage, due to either pathological processes or normal wear and tear of cells. STases however are membrane-bound proteins, and detection in the soluble form would still require these membrane-bound molecules to be dislodged from the membrane. There is evidence that proteolytic cleavage of the STase occurs near the membrane domain which causes the molecule to be released from the membranes. This protease activity has been described as a cathepsin-D like activity [Lammers and Jamieson, 1986,1988, 1990].

I.2.3. Regulation of STase activity

A number of pathophysiological conditions are associated with changes in sialylation and/or STase activities [Paulson and Colley, 1989; Broquet et al., 1991; Jamieson et al., 1993]. Changes in sialylation occur during development and differentiation as is evident in the rat intestine [Taatjes et al., 1987; Chu et al., 1988; Taatjes and Roth, 1988] and in oncogenic transformation, resulting in modified circulating or

membrane glycoproteins [Miyagi et al., 1988; Passaniti and Hart, 1988]. There are many ways whereby regulation of STase can occur, some of which are described below.

(a). Effect of cytokines and glucocorticoids: During turpentine induced inflammation in the rat, there is an increase in serum βGal α2→6 STase activity [Kaplan et al., 1983; Fraser et al., 1984]. Jamieson and his coworkers have reported that a cathepsin D-like protease activity in Golgi membranes is increased during the acute phase response, which in turn caused an increased release of STase from hepatocyte membranes [Lammers and Jamieson, 1986, 1988, 1990; McCaffrey and Jamieson, 1993].

Dexamethasone caused increased release of soluble STase activity during experiments with cultured hepatocytes [Van Dijk et al., 1986] and intestinal tissue [Kolinska et al., 1990]. Wang and coworkers have demonstrated that in hepatoma cells dexamethasone caused an increase in STase activity [Wang et al., 1989, 1990a,b]. The effect of glucocorticoids and cytokines on STase is thought to be due to the increased synthesis of mRNA [O'Hanlon et al., 1989; Wang et al., 1989, 1990a]. In experiments with FAZA cells, dexamethasone caused an increase in STase activity in both the cells and incubation medium [Harder et al., 1990].

(b). Drugs and other agents: Drugs and other reagents which cause "specific effects" on metabolism are also known to

affect STase activity. It has been shown that STase activities in liver and serum were increased when FAZA hepatoma cells were treated with phorbol ester [Harder et al., 1990]. Colchicine injection in the rat resulted in increased STase activity in intestine, intestinal lymph and serum [Fraser et al., 1980; Ratnam et al., 1981, 1987]. Retinoic acid is thought to stimulate STase production in melanoma cell lines [Deutsch and Lotan, 1983; Lotan et al., 1988].

(c). Regulation of STase through gene expression: The cloning of the Galβ1→4GlcNAcα2→6 STase [Weinstein et al., 1987], Galβ1→ 3(4)GlcNAcα2→3 STase [Wen et al., 1992] and Galβ1→3GalNAc α2→3 STase [Gillespie et al., 1992] have helped in better understanding the regulation of STase. Lee and coworkers [1989] have demonstrated that the terminal sequence of Chinese Hamster ovary (CHO) cell glycoproteins can be altered by expression of a Gal$\alpha\beta$2→6 STase. The wild type cells normally produce oligosaccharide chains terminating in an α2→3 NeuAc linkage and do not possess the βGal α2→6 STase. A modified cell line of CHO transfected with cDNA coding for βGalα2→6STase was able to synthesise the α2→6 linkage by competing with the α2→3 STase.

Recent studies by Wang et al [1990b] have shown that the hepatic STase transcript and the related kidney mRNAs are transcribed from different initiation sites. Also physically distinct promoter elements regulate the transcriptional

initiation of the STase isoforms.

(d) Effect of developmental factors: Changes associated with tissue development are often accompanied by changes in either STase or NeuAc. For example in the newborn rat α2→3 STase activity in liver was high compared to α2→6 STase activity, when lactose was used as an acceptor [Hudgin and Schachter, 1972]. In the adult rat however α2→6 STase predominated indicating that a shift from α2→3 STase to α2→6 STase occurred during aging. Assays with lactosamine, revealed a higher proportion of α2→6 STase, compared to α2→3 STase in large intestine of the newborn rat [Dall'Olio et al., 1990]. During maturation the α2→6 STase decreased resulting in a reversal of the ratio.

Experiments have shown that in the suckling rat, NeuAc in the small intestine was distributed in the apical and basolateral plasma membranes of the epithelial cells, as well as the mucous of the goblet cells [Taatjes and Roth, 1990]. During the weaning period NeuAc was detectable throughout the villus. In the adult rat, NeuAc could not be detected in the epithelial cells but was present in the goblet cell mucous of the lamina propia as well as in the smooth muscle cells [Taatjes and Roth, 1990]. In contrast, the distribution of Fuc was opposite to that of NeuAc. In the suckling rat, Fuc in the small intestine was restricted to the goblet cells, whereas in the adult, Fuc was present in the apical and basolateral

plasma membranes of both epithelial and goblet cells. The results showed that a shift from sialylation to fucosylation occurred during the weaning phase of development.

(e) Effectors acting directly on STase: Studies by Scudder et al., (1982) have shown that the α 2-6 STase of bovine colostrum was stimulated by a bovine heart lectin. Five plant lectins tested showed very little or no activation of the α2-6 STase activity.

Detergents such as Triton X-100 have been shown to activate the Gal α2→3 STase. A similar effect on the Gal α2→3 STase was observed with lysophosphatidylcholine [Westcott and Hill, 1985]. Other studies have indicated that the same lipid could have opposite effects on STases from the same tissue. For example it has been shown that lysophosphatidylcholine (1) inhibits the Gal α2→3 STase sialylating the O-glycans from rat brain and (2) strongly activates the GalNAc-Ser α2→6 STase [Baubichon-Cortay et al., 1986a]. The GalNAc-Ser α2→6 STase on the other hand was inhibited by thiol reagents such as N-ethylmaleimide unlike the Gal α2→3 STase [Baubichon-Cortay et al., 1989].

(f) Regulation through enzyme specificity: Regulation can also occur as a result of enzyme specificity. Purified α2→3 and α2→6 STases from rat liver were able to sialylate N-glycans from rat liver but could not sialylate O-glycan branches [Paulson et al, 1982]. Though each of the enzymes was able to

sialylate the Gal β1→4GlcNAc sequence only the α2→3 STase was able to sialylate the Galβ1→3GlcNAc structure. Studies by Joziasse et al [1985a, b] have shown that the α2→6 STase from bovine colostrum preferentially sialylated the Manα1→3Man branch of a biantennary glycopeptide of the lactosamine type. For bi, tri and tetrasaccharides the Galβ1→4GlcNAcβ1→2Manα1→3 branch was preferentially sialylated [Joziasse et al., 1987].

In O-linked glycoprotein structures such as Galα1→3 GalNAc-Ser, the sialylation of the GalNAc residue through the α2→6 NeuAc generally occurs following sialylation of the Gal residue by an α2→3 linkage. This has been demonstrated in fetal calf liver [Bergh et al., 1983; De Heig et al., 1986] and rat brain [Baubichon-Cortay et al., 1986a,1989].

(g) Effect of serum on STase activity released during cell/tissue culture experiments: In experiments with hepatocytes it was observed that the STase activity released into the incubation medium was increased when heat-inactivated serum was used to supplement incubations [VanDijk et al., 1986]. Ratnam et al., [1987] reported that the addition of heat-inactivated serum to the incubation medium caused a release of STase from rat intestinal slices. Other researchers have shown that in experiments with intestinal cultures, an increased release of STase into the culture medium was observed when the incubations were supplemented with heat-inactivated serum [Kolinska et al., 1990].

I.2.4. STase in small intestine

I.2.4.A. The small intestine

The small intestine stretches from the pylorus to the ileocecal valve and is divided into the duodenum, jejunum and ileum [Cheng, 1981a,b; Cheng and Leblond, 1981a,b,c; Moog, 1981; Neutra and Padykula, 1984]. The anatomy of the small intestine is similar in all mammalian species. The mucosa forms the innermost layer and is in contact with the lumen (Figures 1.13 and 1.14). The submucosa underlays the mucosa and is rich in blood vessels and lymphatics. The next layer is a muscular layer made up of smooth or involuntary muscles. The outer surface is covered over most of its area by the visceral peritoneum or serous layer (serosa), in which run the blood vessels and lymphatics. The arterial supply comes from the branches of the superior mesenteric artery. The venous drainage leads into the portal vein, which takes the blood from the intestine to the liver (Figures 1-13 and 1-14).

The mucosa is made up of folds and from it project numerous tiny finger like projections known as the villi (Figures 1-13 and 1-14). The crypts of Liberkuhn lie between the bases of the villi. It is from the crypts that the columnar epithelial cells which cover the surface of the villi arise. These cells which are constantly produced in the crypts, move up the villus and are ultimately shed from its tips. Many of the epithelial cells have an absorptive

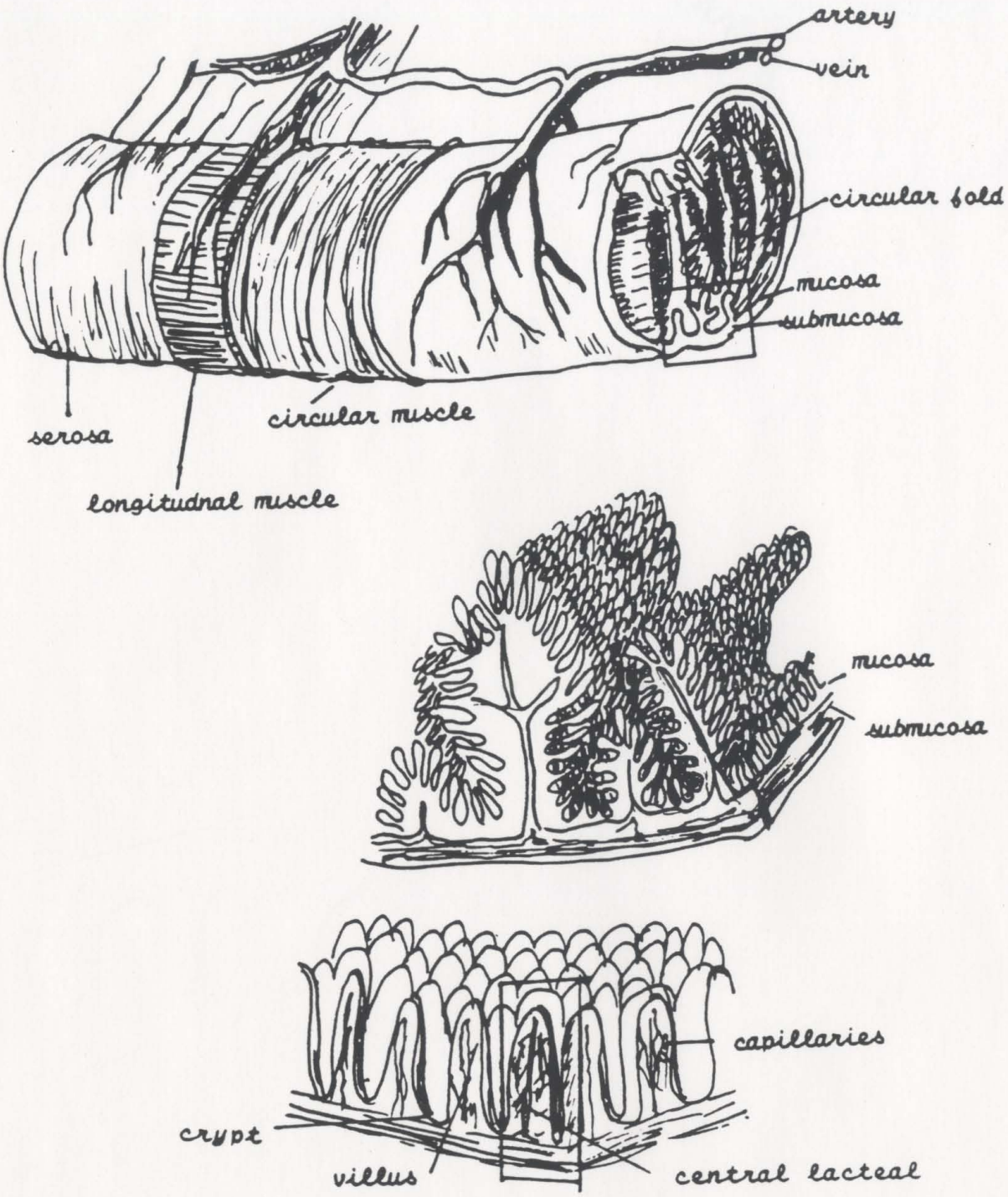


Figure 1.13. Anatomical structure of the small intestine

Figure 1.13. Anatomical structure of the small intestine. The wall of the intestine is made up of four concentric layers, comprised of three outer layers:- the serosa, the muscularis and the submucosa, which surround the innermost layer the mucosa. Several projections the villi extend from the intestinal surface increasing its absorptive area. [Adapted from Moog, 1981].

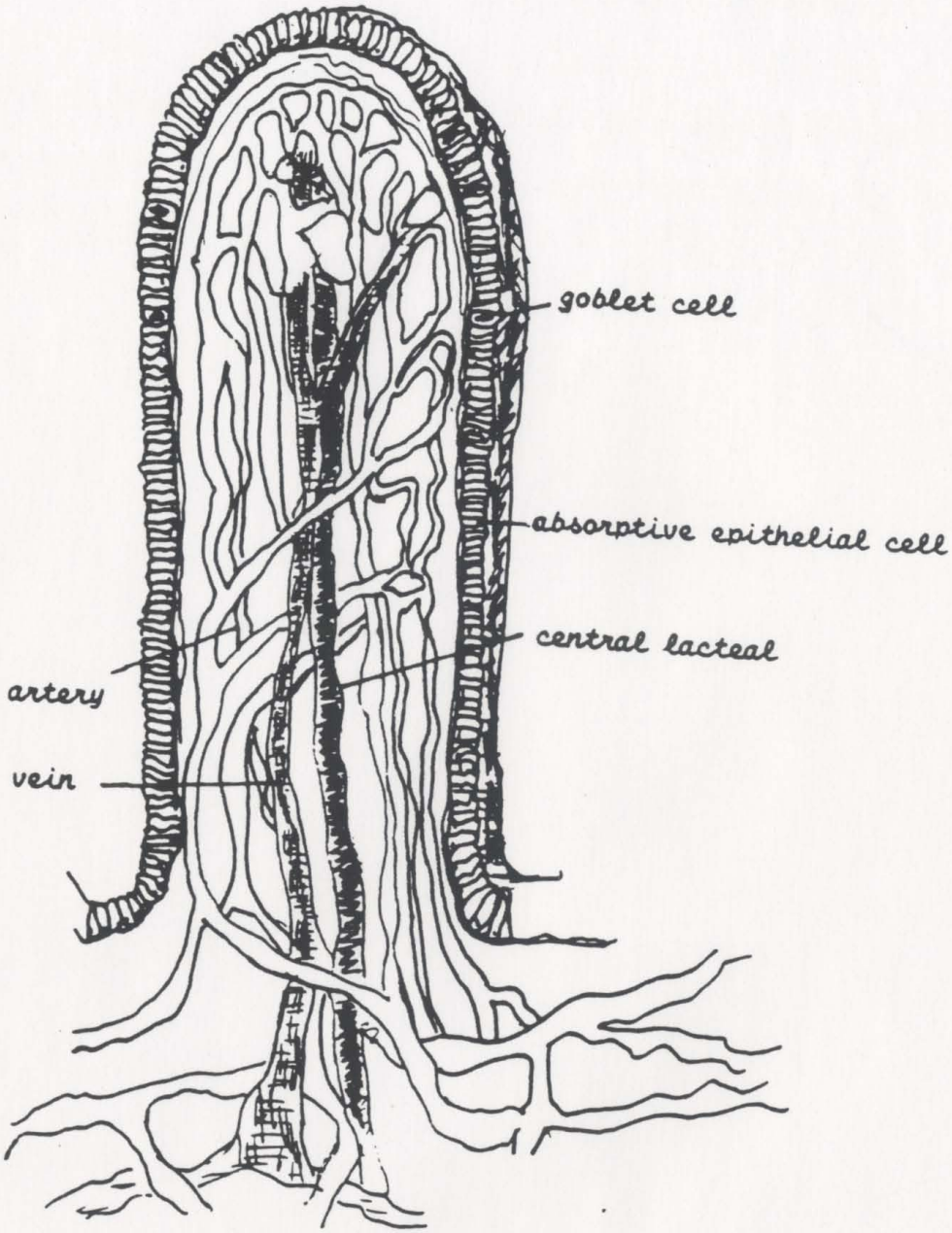


Figure 1.14. Structure of a villus

Figure 1.14. Structure of a villus. The structure of a villus is shown in the figure. The villi extend into the interior space from the surrounding surface, inside each villus is a dense network of blood and lymph vessels. [Adapted from Moog, 1981].

function.

The main functions of the intestine are (1) to allow enzymatic breakdown of the larger dietary components such as proteins, fats and polysaccharides, a process occurring largely in the lumen, (2) to complete digestion by breakdown of the smaller molecules resulting from the preliminary digestive processes, a process occurring mainly in the brush border and (3) to absorb the final products of digestion. A major part of luminal digestion is brought about by the enzymes of pancreatic secretions which act upon the products of digestion. The mucosal cells in the duodenum and upper jejunum release enterokinase which converts trypsinogen to the active enzyme trypsin by the removal of a terminal hexapeptide. Trypsin is also an important activator of other proteolytic enzyme precursors. The brush border enzymes include maltase, isomaltase, sucrase, lactase and τ amylase, which are involved in carbohydrate digestion; aminopeptidases and several dipeptidases, which are involved in oligopeptide digestion.

I.2.4.B. The small intestine as a model for the study of STase/ sialoglycoconjugates

The intestine is an interesting tissue for the study of STase and/ or NeuAc for a number of reasons. Glycoconjugates are both abundant and widespread in the intestine. The mucosal and epithelial cells of the gastrointestinal tract are rich in

both secretory and structural glycoproteins [Rubio et al.,1964; Galand and Forstner, 1974; Neutra and Padykula, 1984]. The digestive enzymes including maltase, sucrase and alkaline phosphatase are glycoproteins [Rubio et al., 1964; Yeh and Moog, 1975]. The lining of the intestinal cells has a high mucin content. Gastrointestinal mucins are glycoproteins with structural diversity whose main function is to provide protection. Glycoconjugates in the intestine are also constituents of both plasma and endoplasmic membranes, as well as being cellular components of the mucous.

STases and NeuAc are thought to play important roles in cell differentiation and recognition, which is yet another reason that the intestine is a good model for their study. The rate of cell turnover in the intestinal mucosa is rapid as surface epithelial cells are shed by being sloughed off into the lumen and are replaced by new cells proliferating and migrating from the crypts [Cheng, 1981a,b; Cheng and Leblond, 1981a,b,c].

I.2.4.C. Expression of STase/ sialoglycoconjugates in small intestine

A number of investigators have sought to measure and compare STase activity between differentiated villus and undifferentiated crypt cells. The method used by Weiser (1973b) involved the use of citrate and EDTA to separate as well as dissociate cells resulting in epithelial cell

fractions which defined a gradient from villus tips to crypts. The results indicated that higher STase activity was present in the villus cells compared to crypt cells. Kim and coworkers in 1975 used a method based on planar tissue sections to separate crypt and villus cells and found that STase was enriched in crypt cells. The discrepancy between the two groups is thought to be the result of the different methods employed for the separation of crypt and villus cells. Other studies have confirmed the results of Kim et al., [1975], indicating that the crypt cells contained higher STase compared to villus cells [Martin and Louisot, 1976, Biol et al., 1987].

The studies cited above did not make any distinction between the type of STase activity measured (that is α2→6 or α2→3 STase). A number of more recent studies have focused on studying the distribution of specific STases in the intestine. In the small intestine STase was found to be predominantly that which catalysed the α2→6 NeuAc linkage on lactosamine which was used as the oligosaccharide acceptor [Dall 'Olio et al., 1990]. By contrast in the large intestine the STase activity was of the α2→3 type. Immunochemical studies using an antibody directed towards the polypeptide portion of the α2→6Galβ1→4 GlcNAc STase molecule have shown that the enzyme was present in a number of post-Golgi apparatus structures including mucous droplets and plasma

membranes [Taatjes et al., 1988]. The presence of STase in the goblet cell mucous and the absorptive cells are thought to be markers for the differentiated state of the small intestinal epithelium [Taatjes et al., 1988]. These results suggest that both the "ecto-STase" in plasma membranes of intestinal cells and that released from Golgi membranes could be secreted with the goblet cell mucous.

Bennett and coworkers [1987] have shown that NeuAc was distributed throughout the small intestine at the microvillar surface of the crypt and villus columnar cells. Other studies have shown that in the lower half of the crypt region NeuAc was present in the apical and basolateral membranes.

I.2.4.D. Changes in STase/ sialoglycoconjugates during development

Numerous studies have shown that in the pre-weaning rat, STase activity in the small intestine was increased compared to the adult and then declined after weaning [Chu and Walker, 1986; Biol et al., 1987, 1992; Taatjes and Roth, 1990]. These studies also revealed that during the weaning phase there was a shift from sialylation to fucosylation in the intestinal microvillar glycoconjugates. Immunological studies using a lectin specific for the NeuAcα2→6Gal have shown that in the suckling rat, NeuAc was present in the apical as well as basolateral plasma membranes, in both epithelial and goblet cells throughout the villi [Taatjes and Roth, 1990]. In the

adult rat NeuAc was not detectable in the plasma membranes of epithelial cells but abundant staining was detected in the goblet cell mucous, cells of the lamina propia and smooth muscle cells.

I.2.4.E. Alterations in small intestinal STase

The role of NeuAc/sialoglycoconjugates in colon during pathological conditions has been more extensively studied compared to small intestine [Kim and Isaacs, 1975; Whitehead et al., 1979; Irimura et al., 1988; Dall'Olio et al., 1992]. This is due to the fact that STase and NeuAc metabolism are altered during colon cancer, a disorder which is associated with a high mortality rate.

There are indications that STase in small intestine also plays an important role in pathophysiological conditions. Increased STase activity in small intestinal mucosa and serum have been observed following thermal injury in the rat [Chu et al., 1988]. Colchicine injection in the rat, resulted in increased STase activity in small intestine, intestinal lymph and serum [Fraser et al., 1980; Ratnam et al., 1987]. Increases in STase activity and sialylation, in small intestine have been reported following hydrocortisone injection in the rat [Kolinska et al., 1988, 1990]. Dexamethasone also increased the amount of soluble STase activity which was released during jejunal culture experiments [Kolinska et al., 1989, 1990].

SECTION 3. GALACTOSYLTRANSFERASES (GTases)

I.3.1. Membrane-bound GTases: Golgi and cell surface enzymes

GTases catalyse the transfer of Gal from UDP-Gal onto suitable carbohydrate residues on glycoproteins [Schachter et al., 1970; Schachter and Roseman, 1980; Beyer et al., 1981: Berger et al., 1987a; Yadav and Brew, 1991]. Among mammalian glycosyltransferases, GTases are the most widely studied enzymes with β1→4GTase (β1→4GlcNAc GTase) being the most extensively studied glycosyltransferase.

GTases are widely distributed in both mammalian tissues as well as extracellular fluids, existing in both membrane-bound and soluble forms [Kim et al., 1972a,b; Pierce et el., 1980; Pestalozzi et al., 1982; Ram and Mungal, 1985]. In the cell, GTases are found on Golgi and cell membranes. For example, in human ovarian adenocarcinoma cells two distinct subcellular populations of GTase were observed, one within the trans-Golgi compartment and one on the plasma membrane [Sichel et al., 1991]. Some studies have shown that a longer GTase protein containing a unique 13 amino acid peptide was preferentially targeted to the plasma membrane, whereas the shorter GTase protein without the 13 amino acid peptide was retained in the Golgi apparatus [Lopez et al., 1991]. The cytoplasmic (or N-terminal) half of the transmembrane domain of GTase is believed to play a key role in the Golgi retention signal [Aoki et al., 1992]. In this region the Cys29 and His32

were found to be critical for the GTase to be retained in the Golgi.

Studies have shown that in liver cells, GTase was present in Golgi membranes [Schachter et al., 1970; Fraser and Mookerjea, 1977; Taatjes et al., 1987]. In HeLa cells GTase was found in the trans Golgi network [Taatjes et al., 1987]. Other studies have shown that the distribution of GTase in Golgi membranes was similar in HeLa and CaCO2 cells and that GTase was found throughout the trans Golgi membranes [Watzele et al., 1991]. The function of the Golgi GTase is primarily to catalyse the reactions involved in the synthesis of glycoproteins. Traditionally GTase is considered to be a marker enzyme for the Golgi membranes.

The localization of GTase on numerous cell-membranes has been shown using immunological, cytochemical and biochemical techniques [Roseman, 1970; Shur and Roth, 1975: Pierce et al., 1980; Runyan et al., 1988]. GTase has been detected on the surface of cell-membranes in absorptive intestinal cells [Roth et al., 1985a], human ovarian adenocarcinoma cells [Lopez et al., 1991; Evans et al., 1993], Swiss 3T3 fibroblasts [Evans et al., 1993], HeLa cells [Roth and Berger, 1982] and rat phaeochromatocytoma cells [Begovac and Shur, 1990]. Roseman in 1970 put forth the hypothesis that cell surface GTase played a key role in cell adhesion and recognition. In view of Roseman's hypothesis other authors have since then reviewed

the role of cell surface GTase [Shur, 1984; Bayna et al., 1986; Shur and Roth,1975; Shur, 1991]. Cell surface GTase is thought to serve as a receptor in cell-cell interactions during murine fertilisation [Miller et al., 1992], neurite growth [Begovac et al., 1991], mesenchymal cell migration [Hathaway and Shur, 1992], cell adhesion [Shur, 1983] and morula compaction [Bayna et al., 1988].

A number of GTases have been purified including β1→4 GTase from malignant ascitic fluid [Boyle et al., 1988], β1→4 GTase from Ehrich ascites tumour cells [Elices and Goldstein, 1988], β1→4 GlcNAc GTase from human embryonal carcinoma cells [Suganuma et al., 1987], β1→4 GTase isoenzymes from serum of cancer patients [Podolsky and Weiser, 1979], β1→4 GTase from human malignant pleural effusion [Ram and Mungal, 1984: Boyle et al., 1988], β1→4 GTase from rat small intestine [Weiser et al., 1987], β1→4 GTase from rat liver microsomes [Fraser and Mookerjea, 1977; Kawano et al., 1992] and β1→4 GalNAc GTase from rat small intestine [Wilson et al., 1987].

I.3.2. GTase in the small intestine

The distribution of GTase in the small intestine has been the focus of many investigations [Weiser 1973 a,b; Lau and Carlson, 1981; Wilson et al., 1984; Kim et al., 1975]. Weiser [1973 a,b] reported that GTase activity was higher in the crypt cells compared to the villi in the small intestine of rat. However, in a later study it was reported that the villus

cells were rich in GTase and that GTase was largely concentrated in the basolateral plasma membranes of villus cells [Weiser et al., 1978; Wilson et al, 1984]. According to studies by Kim et al [1975], GTase was equally distributed in the crypt and villus cells. These discrepancies are thought to be due to the different techniques employed to separate the crypt and the villus cells. Using immunocytochemical techniques, the localisation of GTase has been studied in human small intestinal enterocytes [Pestalozzi et al., 1982; Roth et al., 1985a]. GTase was found on the trans Golgi and plasma membranes of absorptive cells, being more concentrated along the brush border membrane. The lateral plasma membrane was also labelled and the intensity of the label decreased progressively towards the basal portion of the enterocytes. The glycocalyx extending from the microvillus tips was intensely labelled whereas the microvillus core showed very little label.

The presence of ecto-GTase or extracellular GTase in small intestinal epithelial cells suggests that GTase in intestinal cells besides its usual role in glycoprotein synthesis, may have an additional role in cell-cell interaction [Weiser, 1973 a,b; La Mont et al., 1974; Vegt et al., 1981].

I.3.3. GTase in other tissues (cells)

Immunological techniques have been used to study the

distribution of GTase in rat hepatocytes [Taatjes et al., 1987]. In intact liver cells GTase was found to be spread out in the trans-Golgi and appeared as a spot like fluorescence. In cultured hepatocytes GTase was extensively distributed throughout the Golgi tubular network. These studies indicated that there was a difference in the Golgi localisation of GTase between intact and cultured hepatocytes. GTase has been purified from rat liver microsomes [Fraser and Mookerjea, 1977; Kawano et al., 1992]. The presence of GTase activity has also been demonstrated in bovine liver [Hudgin and Schachter, 1971b]. In hepatic disease elevated levels of serum GTase have been observed [Kim et al., 1972 a,b]. Other researchers have reported that in the rat chronic ethanol consumption lead to decreased GTase activity in liver and Golgi fractions [Gausch et al., 1992]. In the rat, streptozotocin induced diabetes resulted in decreased GTase activity in liver [Tepperman et al., 1983].

GTase has also been studied in other tissues and cells. In porcine thyroid cells, thyrotropin caused an increase in cellular GTase activity [Franc et al., 1984]. Leukaemic L1210 cells showed the ability to release soluble GTase activity into the medium [Klohs et al., 1984]. In leukaemia cells obtained from patients, GTase in cell membranes was increased compared to normal cells [Rossowski and Srivastava, 1983]. In studies with human ovarian adenocarcinoma cells a release of

soluble GTase was observed into the culture medium [Sichel et al., 1991]. The GTase activity released as well as the cell surface GTase was dependent on cellular adhesion and proliferation. It was further observed that though intracellular GTase was unchanged, both the cell surface and the soluble GTase were increased in adhering cells compared to non adhering cells. The presence of cell surface GTase activity has also been demonstrated in hamster fibroblasts, where it was observed that dividing but not resting fibroblasts released GTase into the medium [LaMont et al., 1977].

I.3.4. Soluble GTases

Soluble GTases occur in a wide variety of mammalian tissue fluids and have been detected in human serum [Kim et al., 1972a,b; Podolsky and Weiser, 1979; Davey et al., 1984], rat serum [Fraser and Mookerjea, 1977], porcine serum [Hudgin and Schachter, 1971b] and hamster serum [Podolsky et al., 1977]. Other extracellular fluids where GTase activity has been detected include human milk [Gerber et al., 1979; Amano et al., 1991], bovine milk [Babad and Hassilid, 1964; Magee et al., 1973], pleural effusions [Kim et al., 1982] and human ascitic fluid [Gerber et al., 1979].

The human [Masri et al., 1988; Uejima et al., 1992], bovine [Narimatsu et al., 1986; Shaper et al., 1986; Masiby and Qasba, 1989] and murine [Nakazawa et al.,1988; Shaper et

al., 1988] cDNAs encoding GTase have been cloned and sequenced. Recently the properties of the enzyme produced by cDNA expression have been studied [Masiby and Qasba, 1989; Nakazawa et al., 1988, 1991]. It is believed that the soluble forms of GTase in body fluids occur due to proteolytic release from the membrane anchor [Paulson and Colley, 1989].

During pathological conditions, especially cancer, increases in GTase activity have been observed in serum [Podolsky et al., 1977, 1978 and 1981; Capel et al., 1982], ascitic fluid [Gerber et al., 1979; Boyle et al., 1988] and pleural effusions [Ram and Mungal, 1984]. The possibility of serum GTase being a marker enzyme for cancer has been the subject of a number of investigations [Capel et al., 1982; Chatterjee et al., 1980, 1985; Davey et al., 1983; 1984; Ram and Mungal, 1985]. Serum GTase activity has been elevated in cancer, particularly ovarian cancer [Davey et al., 1984; Chatterjee et al., 1980 and 1985]. However other benign diseases can also lead to elevated serum GTase levels [Kim et al., 1972a, b; Kessel et al., 1977]. Also there is a broad overlap in serum GTase levels between cancer patients and normal controls [Kessel et al., 1977; Chatterjee et al., 1980, 1985; Davey et al., 1984]. Another problem stems from the fact that increases in serum GTase activity usually occur in the later stages of the disease. Therefore with all these factors taken into consideration the measurement of serum

GTase activity is thought to be inadequate as a diagnostic test for cancer.

A cancer associated isoform of GTase (GTase II) in human serum was initially reported by Weiser and coworkers [1976] and later characterised [Podolsky et al., 1977, 1978]. GTase II was found to be slower moving in non-denatured PAGE compared to GTase I (the normal isoform of GTase). The presence of GTase II was shown to correlate positively with the presence of cancer [Podolsky et al., 1978, 1981]. Two isoforms of GTase have also been separated from malignant ascitic fluid [Boyle et al., 1988]. Like serum there was a close similarity between the forms in terms of their kinetic properties. GTase II had a higher level of sialic acid residues.

Though liver is considered to be the major source of soluble GTase activity in blood and other extracellular fluids, the presence of GTase in other tissues indicates that liver may not be the sole source of soluble GTase activity.

SECTION 4. PROTEASES AND PROTEASE INHIBITORS IN DISEASE STATES

I.4.1. Proteases

I.4.1.A. Serine proteinases

Serine proteinases are the largest and best studied family of proteolytic enzymes. Proteases play vital roles in numerous physiological processes including digestion,

complement activation, blood coagulation, fibrinolysis, release of physiologically active peptides and pregnancy [Neurath, 1984; Powers and Harper, 1986; Nilsson, 1991; Mignati and Rifkin, 1993]. Though the physiological importance of proteinases cannot be disputed, the fact remains that proteinases can also prove extremely hazardous.

Uncontrolled proteolysis can destroy the protein component of cells and ultimately lead to cell death, which probably accounts for the fact that more than 10% of the circulatory blood proteins are protease inhibitors [Powers and Harper, 1986]. Proteinases have been implicated in pathological processes including tumour invasion, inflammation, pancreatitis, renal disease and septicemia [Powers and Harper, 1986; Pepper et al., 1990; Steven et al., 1992a,b; Techner et al., 1992; Wada et al., 1993 a,b; Zamir et al., 1993]. Examples of some serine proteinases and their functions in pathophysiology are shown in Table 1.2.

In a serine proteinase the active site is typically made up of two regions which are the catalytic site and the substrate binding site(s). Most inhibitors of serine proteinases interact with both of these regions [Steitz and Shulman, 1982; Powers and Harper, 1986].

Three major classes of serine proteinases can be distinguished based upon their primary substrate specificity. These include (1) trypsin-like proteinases, (2) chymotrypsin-

Table 1.2. Examples of serine proteinases. Examples of serine proteinases and the pathological processes they are involved in are shown in the table. [Adapted from Powers and Harper, 1986].

Normal function	Representative enzymes	Processes
Blood coagulation	factor IXa factor Xa factor XIa factor XIIa factor VIIa thrombin activated protein C plasma kallikrein	vascular clotting, cerebral infarction, coronary infarction
Digestion	trypsin chymotrypsin elastase (pancreatic) enterokinase	pancreatitis
Fibrinolysis	plasmin plasminogen activator	tumour invasion
Phagocytosis	elastase (granulocyte) cathepsin G chymases (mast cell) tryptases(mast cell)	inflammation, emphysema, adult respiratory distress syndrome, rheumatoid arthritis

like proteinases and (3) elastase-like proteinases. Trypsin proteinases will cleave substrates which have positively charged amino acid residues such as Arg (arginine) and Lys (lysine). Chymotrypsin and elastase will preferentially act on substrates with aromatic or large aliphatic and smallaliphatic side chains respectively [Powers and Harper, 1986].

I.4.1.B. Plasmin and plasminogen activators (PAs)

Plasmin is a broad spectrum proteinase found in mammalian blood capable of degrading components of the extracellular matrix including laminin, fibronectin and fibrin [Norman, 1958; Edy and Collen, 1977; Matsuda et al., 1980]. It has been long recognised that plasmin has a high affinity for fibrin and fibrinogen, and that blood contains a large excess of plasmin inhibitors [Norman, 1958; Christensen and Clemmensen, 1977, 1978]. Plasmin is a trypsin-like endopeptidase which attacks the lysyl-Lys bond in a variety of proteins. The many possible substrates for this enzyme include casein, protamine, cell-membrane proteins, immunoglobulin, ACTH, glucagon, complement components and factors V, VII and XI of the clotting system [Edy and Collen, 1977; Moroi and Aoki, 1977].

Plasminogen activators (PAs) are serine proteinases which convert plasminogen to plasmin [Clemmensen et al., 1981: Saksela and Rifkin, 1988; Sitrin et al., 1990]. In humans the process of plasminogen activation is thought to be a critical

component of diverse biological systems including the complement and clotting systems. In vivo this reaction is catalysed by PAs and results in the conversion of plasminogen to plasmin. Plasmin plays an important role in the maintenance of vascular potency by converting insoluble fibrin to soluble fibrin degradation products [Thorsen et al., 1981; Sakata and Aoki, 1982; Blasi et al., 1987; Saksela and Rifkin, 1988; Sitrin et al., 1990; Vassalli et al., 1991]. Vertebrate PAs can be divided into four main groups, circulating PAs found in blood, tissue PAs, urinary PAs (urokinase) and tissue culture PAs [Levin and Loskutoff, 1982; Sprenger and Kluft, 1987: Janicke et al., 1991]. In plasma the predominant PAs include tissue type and urokinase PAs. The molecular weights of the circulatory PAs (98000 - 165000 Da) are different from the urinary and tissue PAs not in circulation (which have molecular weights between 50 000 - 80 000 Da). This difference has been attributed to the formation of aggregates in plasma.

In the maintenance of normal physiological conditions, control of the integrity of a cell's environment is an important factor. This in turn depends on the control of both anabolic and catabolic mechanisms. Removal of the extracellular matrix within a connective tissue is controlled by proteinases like collagenase and plasminogen once it has been converted into its active form plasmin. It is therefore not surprising that plasmin and PA have been implicated in

numerous physiological and pathophysiological processes including embryogenesis, angiogenesis, ovulation, inflammation and tumour metastasis [Busso et al., 1987; Sugihara et al., 1989; Vassalli et al., 1991]. Dexamethasone caused reduction in PA activity in the rat brain tumour cells [Wolf et al., 1993]. Highly metastatic cells are known to produce classes of degenerative enzymes including PA and release them at higher concentrations than normal cells [Liotta et al., 1986]. A tissue type PA is thought to be an initiator of tumour cell invasion during metastasis [Dano et al., 1985; Jankun et al., 1991]. Plasmin and PAs also play vital roles in the maintenance of vascular potency by converting fibrin to soluble fibrin degradation products [Thorsen et al., 1981; Sakata and Ayoki, 1982; Vassalli et al., 1991; Sane et al., 1993].

I.4.1.C. Trypsin

Trypsin is thought to play a key role in the onset and development of pancreatitis [Morgan et al., 1968; Creutzfeldt and Schmidt, 1970; Steer, 1986; Arais et al., 1993]. It is believed that in the acute stage of pancreatitis, trypsin reactivates itself and other pancreatic enzymes, resulting in increased levels of trypsin and decreased levels of trypsin-inhibitors in plasma.

Other conditions are also known to affect trypsin levels in plasma and/or urine. Alcohol has been shown to affect

pancreatic secretions in animals [Singh, 1983; Tsukamoto et al., 1986] and man [Varcaigne et al., 1980]. This probably accounts for the observed increase in both pancreatic trypsinogen and plasma trypsin in rats exposed to alcohol [Tsukamoto et al., 1986]. Increased trypsin activity in plasma has been reported in patients with uraemia [Shannon et al., 1985]. Recent studies by See and Smith [1992] suggest that urinary trypsin levels/ activity play a significant role on the physiologic processes involved in haemostasis and susceptibility towards urinary tract infections. The active urinary trypsin was shown to promote fibrinolysis in vitro and bacterial adherence to urothelial surface in vivo. Trypsin also plays a role in the pathophysiology of lung tissue. In the rat, trypsin injection caused emphysema and decreased trypsin inhibitory activity in plasma [Reichart et al., 1991, 1992].

Very little is otherwise known regarding the role of trypsin in inflammatory processes and cancer. Two tumour-associated trypsins (TATs) have been isolated from mucinous ovarian cyst fluid [Koivunen et al., 1989]. TAT is thought to account for the proteolytic activity of a number of human tumour cell lines [Koivunen et al., 1990, 1991a,b].

I.4.2. Protease inhibitors

I.4.2.A. Serpins

The serpins are a superfamily of *ser*ine *p*roteinase

*in*hibitors present in plasma [Carrell and Travis, 1985, Carrell and Boswell,1986]. This family includes a large group of homologous proteins, including α_1 antitrypsin (also known as α_1 proteinase inhibitor {A1PI}), α_2 antiplasmin, antithrombin III, heparin cofactor II, rat angiotensin, C1-inhibitor and ovalbumin [Peterson et al., 1979; Carrell et al., 1979, 1982]. A number of factors complicate the discussion and understanding of this family of inhibitors. Firstly, α_1 antitrypsin is not primarily an inhibitor of trypsin. Its main target is leucocyte elastase, although it can also inhibit other proteinases such as trypsin, plasmin, chymotrypsin and kallikrein. These factors have resulted in α_1 antitrypsin being renamed A1PI. Also murine α_1 chymotrypsin is an inhibitor of trypsin-like proteinases rather than chymotrypsin. Yet another anomaly is the fact that some members of this family such as egg white ovalbumin show no inhibitory activity [Hunt and Dayhoff, 1980].

The shared characteristics of this group of proteins include their common derivation from an ancestral serine proteinase inhibitor, as well as similarities in their tertiary structure and reactive centres. Serpins are generally small glycoproteins (with molecular weights between 40 000 and 80 000 Da) containing a single polypeptide chain and a variable number of oligosaccharide side chains: four in

antithrombin, three in A1PI and one in ovalbumin [Carrell et al., 1982; Carrell and Travis, 1985]. The serpins show sequence similarities indicative of a common tertiary structure.

I.4.2.B. α_2 Antiplasmin

Two main types of plasmin inhibitors are known to exist in mammalian blood, those which inhibit plasmin (also known as antiplasmins) and those which inhibit the activation of plasminogen (also known as antiactivators) [Norman 1958]. Blood contains at least five proteins other than α_2 antiplasmin and PA-inhibitors which have the ability to inhibit plasmin. These include A1PI, inter α trypsin inhibitor, α_2 macroglobulin, antithrombin III heparin complexes and C1 inhibitor.

The major plasmin inhibitor in human plasma is α_2 antiplasmin which has a molecular weight of approximately 70 000 Da and was independently identified by four groups [Bagge et al., 1976; Collen, 1976; Moroi and Aoki, 1976; Mullertz and Clemmenson, 1976]. Wiman and coworkers (1979) demonstrated that different plasminogen fragments containing Lys binding sites were able to compete with plasmin for interaction with α_2 antiplasmin [Wiman and Collen, 1978 a,b; Wiman et al., 1978; 1979; Wiman, 1981]. It was also found that a high-affinity binding site in the first three triple-loop

structures of plasminogen is mainly responsible for this interaction [Wiman 1981; Lijnen et al., 1983]. Several researchers have shown that α_2 antiplasmin inhibits the binding of plasminogen to fibrin [Moroi and Aoki, 1977; Aoki and Sakata, 1980; Suenson and Thorsen, 1981]. It has also been demonstrated that the binding ability towards fibrin of an isolated plasminogen fragment containing the high affinity lysine-binding site is decreased by $\alpha_2$antiplasmin [Aoki and Sakatai, 1980: Suenson and Thorsen, 1981]. Later studies revealed that human serum contains one form of antiplasmin which binds to plasminogen and another form that does not bind (40% of total antiplasmin) [Christensen and Clemmensen, 1978; Wiman, 1981]. The non-plasminogen-binding form lacks a 26 residue peptide from the C terminus [Sasaki et al., 1983].

In purified systems α_2 antiplasmin has been shown to react with trypsin [Wiman, 1981]. It reacts slowly with urokinase [Holmberg et al., 1980; Samama et al., 1980], kallikrein, Factor Xa, thrombin [Saito et al., 1979] and tissue-type plasminogen [Koringer and Collen, 1981]. In normal plasma α_2 antiplasmin is able to bind plasmin and to a lesser extent trypsin. However, α_2 antiplasmin does not bind to thrombin, chymotrypsin, tissue type PA [Edy and Collen, 1977] or papain [Moroi and Aoki, 1976, 1977]. Therefore it appears that α_2 antiplasmin behaves differently in purified systems

compared to blood. α_2 Antiplasmin can exert an inhibitory effect on fibrinolysis at three levels, (1) rapid inactivation of plasmin, (2) interference with the adsorption of plasminogen to fibrin and (3) by cross-linking to fibrin [Weitz et al., 1993].

When blood is clotted in the presence of calcium ions, α_2 antiplasmin is cross-linked to the fibrin [Collen, 1976; Wiman and Collen, 1978 a, b; Sakata and Aoki, 1982]. When fibrin is formed, tissue type PA and plasminogen adsorb to the clot in a sequential and well ordered manner. The affinity of tissue-type PA for plasminogen is high [Hoylaerts et al., 1982]. The plasmin formed on the fibrin surface has both its Lys binding sites and its active site occupied and is only slowly inactivated by α_2 antiplasmin. In normal plasma, significant activation of plasminogen by tissue-type PA is negligible and α_2 antiplasmin will rapidly bind any free plasmin, thereby preventing proteolysis of other plasma proteins such as fibrinogen [Ohlsson and Collen, 1977].

Proteases and their inhibitors are involved in a number of disease processes. α_2 Antiplasmin is an acute phase reactant whose concentration in blood is increased during the acute phase response [Matsuda et al., 1980]. Increased α_2 antiplasmin levels have been observed in patients with thrombosis [Teger-Nilsson., et al 1978, Samana et al., 1980].

Major surgery has been associated with significant decreases in plasma levels of plasminogen and α_2 antiplasmin [Frisch,1980; Buller et al., 1981]. In cardiac disorders plasma concentrations of $\alpha_2$antiplasmin were decreased [Aoki et al., 1977; Frisch, 1980]. Some studies have found that α_2 antiplasmin levels in blood were decreased in the microembolism syndrome in man and rat [Bagge et al., 1976]. In patients with septic shock there was an increase in serum plasminogen and α2 antiplasmin which was related to mortality [Gallimore et al., 1980]. In hepatic disease, particularly cirrhosis, increased fibrinolysis is a well known fact and is often accompanied by decreased levels of circulating α_2 antiplasmin [Aoki and Yamanaka, 1978; Teger-Nilsson et al., 1978].

I.4.2.C. Plasminogen-activator inhibitors (PAIs)

The actions of plasmin and PAs are regulated to a great extent by their protease inhibitors. Four kinds of plasmin activator inhibitors (PAIs) are present in mammals, namely endothelial PAI-1, placental/macrophage PAI-2, urinary PAI-3 or protein-C-inhibitor and protease nexin [Ericksson et al., 1985; Blasi et al., 1987; Loskutoff et al., 1989; Seebacher et al., 1992] .

The primary inhibitor of PA is PAI-1 which is also the most researched PAI and is a serpin [Loskutoff et al., 1989].

PAI-1 is present in blood, platelets, tissues and can rapidly form inactive 1:1 inhibitor-protease complexes [Ericksson et al., 1985; Sawdley and Loskutoff, 1991]. Also PAI-1 has been implicated in a number of pathological conditions. High plasma levels of PAI-1 have been associated with higher rates of mortality in patients suffering from septicemia and septic shock [Kruithof et al., 1993]. Phorbol myristate ester caused increased production and release of PAI-1 in rabbit fibroblasts [Murphy et al., 1993]. Tumour necrosis factor caused an increased release of PA and PAI in granulocytes [Logan et al., 1992].

I.4.2.D. α_1 Proteinase inhibitor (A1PI)

A1PI is the most researched proteinase inhibitor in human blood [Travis and Salvesen, 1983; Heidtmann and Travis, 1986]. The principle reason for this is the prevalence of the genetically caused deficiency of A1PI [Laurell and Ericksson, 1963; Martin et al., 1973]. The clinical manifestation of A1PI deficiency appears in the form of liver disease and/ or emphysema in deficient patients. The emphysema is caused by alterations in the balance of proteinase-proteinase inhibitor activity which results in increased elastase activity. The major target of A1PI is leucocyte elastase and therefore decreases in A1PI generally result in tissue damage [Ericksson 1984].

Human A1PI makes up the major portion of the α band in serum or plasma following gel electrophoresis. The protein is produced mainly in the liver parenchymal cells, secreted into blood and distributed to other parts of the body. The mature protein (molecular weight ~ 52 000 Da) consists of a single polypeptide chain of 394 amino acid residues, containing one free cysteine residue, no disulphide bridges and three carbohydrate side chains [Carrell et al., 1982].

A1PI contains a single reactive site, concentrated around a Met-Ser sequence of 36 amino acid residues from the C terminus [Johnson and Travis, 1978; Boswell et al., 1983]. Proteinases are inhibited by a 1:1 complex formation, which is thought to involve cleavage of the reactive site peptide bond of the inhibitor. These complexes are reported to be very stable and cannot be dissociated by treatment with denaturing agents. Evidence for this comes from the crystallographic studies done with cleaved A1PI, which indicated that A1PI was a globular protein with an ordered structure, 30% of the molecule being in the helical form and 40% in β sheets [Lobermann et al., 1984].

The occurrence of multiple variants of A1PI has been well documented [Fagerhol and Laurell, 1970; Martin et al., 1973; Dietz et al., 1974; Brewerton., 1984; Hinney et al., 1992] . The genetic variants of the inhibitor generally exhibit slight differences in their isoelectric points [Pierce et al., 1976;

Lorier and Hawes, 1984]. At least 20 different forms of A1PI have been separated on the basis of their isoelectric points. The genes for the isoforms are assigned a letter according to the Pi (Proteinase inhibitor) system eg PiM [Fagerhol and Laurell, 1970; Heidtman and Travis, 1986]. The phenotype present in plasma reflects the equal expression of paternal and maternal alleles, such as PiMZ. The most common allele is PiM and the protein has intermediate electrophoretic mobility, whereas PiZ has the highest isoelectric point of all the known variants.

Microheterogeneity between isoforms of A1PI is common and is due largely to the differences in the glycan side chains [Vaughan et al., 1982]. Human A1PI contains three glycan side chains, all at Asn residues attached to the polypeptide chain [Hodges et al., 1979; Mega et al., 1980]. Major isoforms of PiM have been isolated and found to contain different combinations of the two possible chains with variable amounts of the terminal NeuAc residues.

The most researched Pi type is the ZZ variant which is always associated with a deficiency of plasma A1PI. Heterozygous PiMZ individuals have serum A1PI levels about 50% of normal and homogenous individuals (PiZZ) have A1PI levels of less than 15% of normal. An extensive clinical study carried out by Bruce et al. (1984), revealed that the PiMZ genotype often leads to an early onset of emphysema. The basic

defect in A1PI deficiency appears to be in the secretion of the molecule since in liver tissue inclusion bodies containing A1PI were present [Liebermann et al., 1972; Bathurst et al., 1984]. Active A1PI can be extracted from these globules suggesting that in PiZZ individuals, A1PI is synthesised but not properly secreted into blood. Analysis of serum A1PI from PiM and PiZ patients revealed that the carbohydrate content of these variants was similar [Hercz, 1984]. The A1PI present in hepatic globules however had a higher Man content compared to serum A1PI [Bathurst et al., 1984]. It was determined that the impaired secretion of A1PI, in PiZ individuals is caused by intrinsic errors in the post-translational oligosaccharide modification due to incomplete glucose trimming in secretory proteins [Gross et al., 1983].

The first function attributed to A1PI was its ability to inhibit trypsin which lead to the protein being named α_1 antitrypsin [Schultze et al., 1962]. However since then a large number of serine proteinases have been shown to have the ability to form complexes with A1PI. These proteinases include neutrophil and pancreatic elastases, chymotrypsin, plasmin, thrombin, acrosin [Panell et al., 1974; Heidtman and Travis, 1986] and tissue kallikreins [Hirano et al., 1984]. The main target of the human A1PI is neutrophil elastase [Ericksson, 1984; Savolainen and Berode, 1988].

A variety of pathophysiological conditions are associated with alterations in either the levels or functional ability of circulating A1PI. Inherited deficiency of A1PI is associated with predisposition towards the early onset of a variety of diseases in children and adults [Laurell and Ericksson, 1963; Martin et al., 1973: Ericksson., 1984; Propst et al., 1992]. These include pulmonary emphysema, chronic pancreatitis, liver disease and viral infections.

Exposure to cigarette smoke has been shown to lead to decreased proteinase inhibitory activity in serum [Janoff et al., 1980; Dooley and Pryor, 1982; Evans and Pryor, 1992: Silverman et al., 1992]. The decreased inhibitory activity can be attributed to lowered elastase inhibitory activity of A1PI in blood. This imbalance between proteases and protease inhibitors is thought to increase the risk of pulmonary emphysema in smokers compared to non smokers. Dogs treated with chloramine showed impaired function in serum A1PI [Abrahms et al., 1981].

Increased serum levels of A1PI have been observed in hepatic carcinoma [Lee et al., 1992] and chronic liver disease [Trischitta et al., 1991]. Serum A1PI is an acute phase reactant and increased serum levels of A1PI have been observed in inflammation [Travis and Salvesen, 1983; Heidtman and Travis, 1986; Trischitta., et al 1991]. The role of A1PI is thought to be inhibition of the proteolytic enzymes which are

particularly active during the acute phase response.

1.(IV).2.E. Antithrombin III (AT)

Antithrombin III (AT) is a serum glycoprotein, with a molecular weight of approximately 58 000 Da which plays a major role in controlling serine proteases in the intrinsic coagulation system [Abilgaard, 1969,1981]. Though thrombin is the main target of AT, it also inhibits other serine proteinases including plasmin, trypsin and kallikrein. The mechanism of inhibition involves the formation of an equimolar, stable complex between the inhibitor and enzyme such that the active site of the protease is not accessible to substrates. The inhibitory effect of AT towards thrombin is enhanced by heparin [Abilgaard., 1969; Rosenberg and Damus, 1973]. The major role of AT is in the regulation of blood clotting. Individuals with a congenital deficiency of AT have reduced plasma levels of the inhibitor and run an increased risk of developing deep vein thrombosis [Abildgaard, 1981].

I.4.2.F. Acid stable trypsin inhibitors (ASTIs)

Muller in 1908, followed by Bauer and Reich in 1909 originally reported the presence of a trypsin inhibitor in human urine. Since then acid stable trypsin inhibitors (ASTIs) have been detected and purified from human plasma and urine [Shulman, 1955; Sumi et al., 1987]. A number of ASTIs have been detected in other body fluids including bile [Yamamoto et al., 1986], bronchial mucous [Hochstrasser et al., 1973],

ascites of malignancy and tumour effusions [Akazawa et al., 1983]. The ASTIs purified have molecular weights ranging from between 23 000 to 86 000 Da [Akazawa et al., 1983, Maruyama et al, 1984; Okumichi et al., 1984: Yamamoto et al., 1986].

These proteins also share similar antigenicity and chemical properties. ASTIs exhibit a broad spectrum of inhibition towards a number of proteases, including trypsin, plasmin and leucocyte elastase [Sumi et al., 1987]. Proksch et al [1973] purified an ASTI from urine and reported that it was antigenically similar to inter α trypsin inhibitor, and that the latter may serve as a precursor for ASTI. However Clavey and coworkers [1979] purified an ASTI from urine which did not share any antigenicity with inter α trypsin inhibitor. Therefore some controversy exists as to whether inter α trypsin inhibitor serves as a precursor for ASTI.

Increased levels of ASTI(s) in urine and plasma have been reported in kidney disorders [Toki and Sumi, 1982; Maruyama et al., 1984, 1991]. ASTI is present in kidney tissue and it is believed that ASTI is produced by the kidneys and excreted into urine by the proximal tubules [Maruyama et al., 1984, 1991]. Increased production of ASTI by the kidney was observed during inflammation in the rat, which lead to subsequent increases in serum and urinary levels of ASTI [Sugiki et al., 1991]. Other researchers have also reported increased serum levels of ASTI during inflammation and ASTI is thought to be

an acute phase marker [Jonsson et al., 1982]. The kidney is thought to be one of the main sources of ASTI in urine and plasma. An ASTI has also been detected in liver [Odum et al., 1987] and human hepatoma cells have been shown to release an endothelial cell growth factor similar to ASTI [McKeehan et al., 1986]. The presence of ASTI(s) has been demonstrated in tumours of the kidney, stomach, colon, cerebrum [Yoshida et al., 1989] and lung [Okumichi et al., 1984]. Increased levels of ASTI in urine have been detected in patients with malignant tumours [Onitsuka et al., 1985].

I.4.2.G. Tumour associated trypsin inhibitor (TATI) or pancreatic secretory trypsin inhibitor (PSTI)

The acinar cells in the pancreas produce and secrete a trypsin inhibitor referred to as pancreatic secretory trypsin inhibitor (PSTI) or Kazal inhibitor [Kazal et al., 1948]. It is a small peptide with a molecular weight of 6 000 Da. Tumour associated trypsin inhibitor (TATI) was detected initially in the urine of patients suffering from ovarian cancer [Stenman et al., 1982]. Since then the sequencing of this peptide has revealed it to be identical to PSTI. Tomita et al [1987] have shown that PSTI and ASTI are encoded by the same gene, confirming the fact that they are indeed identical. TATI/PSTI is a protease inhibitor and its expression in malignancy is thought to be indicative of protease activity in tumour tissue.

TATI/PSTI is produced by the pancreas and very high concentrations are present in pancreatic fluid [Kazal et al., 1948; Sheving, 1983]. Very low levels of TATI/PSTI are normally detected in plasma and urine [Kitahara et al., 1980]. Increased levels of TATI/PSTI have been observed in tumour fluid [Halila et al., 1985; Tomita et al., 1987] and production of TATI/PSTI has been demonstrated in many tumour cell lines [Ogata, 1988; Koivunen et al., 1991a,b]. Increased serum levels of TATI/PSTI have been observed in tumours of the pancreas, ovary, oesophagus and bladder [Ueda et al., 1989; Taccone et al., 1991], malignant gastric disease [Higashiyama et al., 1990 a,b; 1992; Gion et al., 1991; Toricaguena et al., 1991] and colorectal cancer [Tomita et al., 1990; Catarino and Conde, 1991]. Pancreatitis and pancreatic cancer are associated with increased concentrations of TATI/PSTI in serum [Aroasio and Piantino, 1991].

TATI/PSTI has been detected in the mucosa of the gastrointestinal tract and is secreted into the gastric juice [Bohe et al., 1986a; Freeman et al., 1990a, b; Shibata et al., 1986]. Severe infections and trauma can lead to elevated serum levels of TATI/PSTI [Kitahara et al., 1980; Matsuda et al., 1985; Ogawa et al., 1985] and it has been hypothesised that TATI/PSTI behaves as an acute phase marker [Ogawa, 1988]. In pancreatic diseases such as acute pancreatitis, the preactivation of zymogen proteases particularly trypsin is

believed to play a vital role in the development of disease [Creutzfeldt and Schmidt, 1970; Steer, 1986]. The physiological role of TATI/PSTI is thought to be the prevention of activation of trypsinogen in the pancreatic duct [Funukoshi et al., 1992]. TATI is able to inhibit trypsin [Huhtala et al., 1982; 1983] and acrosin [Huhtala, 1984] which are the main targets of the inhibitor in physiology. TATI/PSTI shows weak inhibition towards other serine proteinases such as plasmin and PAs [Turpeinen et al., 1988].

I.4.2.H. Role of trypsin and plasmin inhibitors in pathophysiology of small intestine

Trypsin and plasmin inhibitors have been implicated in a number of pathophysiological processes associated with the intestine. Though liver and blood cells are considered to be the main sites of production of A1PI the protein has also been detected in other tissues, including the epithelial lining of the small and large intestine [Geboes et al., 1982; Bohe et al., 1986 a,b, 1987, 1988; Rijsinghani et al., 1993]. The presence of A1PI has also been demonstrated in intestinal carcinomas [Kitas et al., 1982; Damajnov et al., 1983; Katoaka et al., 1989]. It is believed that A1PI is synthesised in neoplastic tissues such as human intestinal epithelium during intestinal carcinoma as a result of expression of the A1PI gene [Kitas et al., 1982]. TATI/PSTI have been found in small intestinal mucosa in Paneth and goblet cells [Bohe et al.,

1986a,b, 1987, 1988; Fukayama et al., 1986; Freeman et al., 1990a,b]. There is some evidence suggesting that the presence and/ or expression of trypsin inhibitors such as A1PI and TATI is related to the progression of gastrointestinal cancer [Catarino and Conde, 1991; Gion et al., 1991; Piatino and Aroasio, 1991].

Protease inhibitors particularly A1PI are believed to play important roles in intestinal diseases including inflammatory disease, Crohn's disease and protein-losing enteropathy. During inflammatory bowel disease regions of the intestine particularly the intestinal wall are affected by the inflammatory processes [Beck, 1987]. Though the serum levels of most acute phase proteins are increased, the serum levels of proteinase inhibitors are either unchanged or decreased [Bohe et al., 1986a,b; Strygler et al., 1990]. It is thought that the severe inflammatory processes in the intestine are accompanied by pronounced tissue destruction where proteases are involved which in turn leads to decreased circulatory levels of inhibitors. Protein losses are a common event in inflammatory intestinal disease and Crohn's disease. The faecal clearance of plasma A1PI is used as a measure of protein leakage into the intestinal tract [Mizon et al., 1988; Strygler et el., 1990]. Other studies have shown that the A1PI excreted in the faeces of patients with inflammatory disease had different molecular forms compared to normal individuals

[Mizon et al., 1991; El Yamini et al., 1992]. The A1PI in faeces of patients with intestinal disease had molecular weights of 51 000 and 45 000 Da respectively compared to a molecular weight of 38 000 Da found in normal subjects.

SECTION.5. PURPOSE OF PRESENT RESEARCH

Previously it was reported that heat-inactivated serum when added to incubations of intestinal slices caused a release of soluble STase into the incubation medium [Ratnam et al., 1987]. According to these authors in order for the release of STase to occur there was an absolute requirement for heat-inactivated serum in the incubation medium. Other studies have shown that colchicine treatment in the rat resulted in increased STase activity in intestine, intestinal lymph and serum [Fraser et al., 1980; Ratnam et al., 1980 and 1981]. Thermal injury in the rat also resulted in increased STase activity in small intestine [Chu et al., 1988]. These researchers have hypothesised that the intestine could serve as a source of STase activity in body fluids. However, very little is known regarding the release of STase from intestine and the factors which affect this process.

The primary purpose of the present research was to study the release of soluble STase from jejunal slices and to determine the role played by heat-inactivated serum in the release process. Initial studies during the course of this

thesis revealed that the STase was being released into the incubation medium even in the absence of serum supplements, but the enzyme released was subject to proteolysis. It was determined that the heparin-binding fraction (HBF) was the serum component which was antiproteolytic and was required to measure STase activity. The additional objectives of this research were

(1) to characterize the protease inhibitory activity of HBF,

(2) to investigate the relationship between protease-inhibitory/ protease activity and STase activity and

(3) to determine whether GTase activity was also influenced by the levels of protease-inhibitory/ protease activity.

CHAPTER II. MATERIALS AND METHODS

II.1. Materials

II.1.1. Rats

Male Sprague-Dawley rats (body weight 150-350 g) were obtained from Charles River Canada Inc., La Prairie, Quebec. Unless stated otherwise rats were fasted for 18 hours and had free access to drinking water prior to the experiments.

II.1.2. Chemicals and reagents

Unless specified otherwise, the chemicals/ reagents used were of commercial origin and of the highest grade available.

Adenosine-5′-diphosphate (ADP), alcohol dehydrogenase ({ADH}, equine liver, 1-2 units/ mg), antipain, bovine AGP, bovine fetuin, bovine pancreatic trypsin inhibitor (bpti), bovine serum albumin (BSA), Nα-benzoyl-L-arginine-ethyl ester (BAEE), α-benzoyl-DL- arginine-p-nitroanilide (BAPNA), α-casein, chymotrypsin (type II, bovine pancreas, 40-60 units/mg protein), glutaminase (grade V was from E. coli; 50-200 units/ mg protein), glutamate dehydrogenase (type IV bovine liver; 40 units per mg protein); GTase (Gal β1→4 GlcNAc, bovine milk, 5-15 units /mg protein), elastase (human plasma, 50 units/ mg protein), heparin (porcine intestinal mucosa low molecular mass, Na salt 4-6 kDa), heparin agarose (contained 410 μg heparin/ ml packed gel), A1PI (human plasma), α_2 antiplasmin (human plasma), kallikrein (human plasma, 5-15

units/ mg protein), leupeptin, nicotamide-adenine-dinucleotide (NAD), orcein-elastase, papain (papaya latex, 50 units/ mg protein), plasmin (human plasma, 3-6 units/ mg protein), N-succinyl-l-phenylalanine-p-nitroanilide (SUPHEPA), trypsin (type XI, bovine pancreas, 6000-9000 BAEE units/mg protein) and trypsin-agarose (was in the form of insoluble enzyme attached to cross linked beaded agarose, 50-100 units/ ml packed gel) were purchased from Sigma (St. Louis, MO, USA).

Horse serum and Waymouth's MB 752/1 medium (1X) were from Gibco Diagnostics (Burlington, Ontario, Canada).

Sialyllactosamine isomers [(α2→3) and (α2→6)] were from Oxford Glycosystems (Rosedale, N.Y., USA).

Pure STase α2-6[N] (E.C.2.4.99.1., from rat liver) and STase α2-3[O] (E.C.2.4.99.4., from porcine liver) were purchased from Boehringer Manheim (Laval, Quebec, Canada).

II.1.3. Radioisotopes

CMP[^{14}C]NeuAc (1.8mCi/mmol), UDP[^{14}C]Gal (300mCi/mmol), [^{14}C]glucosamine hydrochloride (54.2 mCi/mmol) and [^{14}C]leucine (52mCi/mmol) were purchased from New England Nuclear (Lachine, Quebec, Canada).

II.1.4. Buffers and solutions

Acetate buffer: (0.5 M; pH 5.0), $CH_3COONa.3H_2O$ (6.8g) was dissolved in 100 ml distilled water (Solution 1). Glacial acetic acid (2.9 ml) was diluted with deionised water (Solution 2). 67.8 ml of solution (1) was mixed with 32.2 ml of solution (2).

Glycine NaOH buffer: (pH 9) NaCl (100 mM) and glycine (100 mM) were mixed 1:1 and 885 ml of the resulting solution was diluted to 1000 ml with 100 mmol/l of NaOH.

Hanks' buffer: (pH 7.4) NaCl (0.137 M), $NaHCO_3$ (26 mM), Na_2HPO_4 (0.34 mM), KH_2PO_4 (0.44mM), EGTA (0.5 mM), 0.8 mM $MgSO_4.7H_2O$, KCl (5.4 mM).

Kreb's-bicarbonate buffer: (pH 7.4) NaCl (0.154 M), KCl (0.154 M), $CaCl_2$ (0.11 M), KH_2PO_4 (0.154 M), $MgSO_47H_2O$ (0.15 M), $NaHCO_3$ (0.15 M).

Kreb's-bicarbonate/BSA: (pH 7.4) NaCl (118 mM), KCl (4.8 mM), $CaCl_2$ (2.9 mM), KH_2PO_4 (0.95 mM), $MgSO_4.7H_2O$ (1.2 mM), $NaHCO_3$ (23.8 mM), 0.25% BSA.

Lysine-phosphate buffer: (pH 7.4) l-lysine (0.15 M), KH_2PO_4(0.1M).

Waymouths Medium/BSA: (pH 7.4) Waymouth's MB 752/1 medium plus 0.2% (w/v) BSA.

2-(n-morpholino) ethane sulfonic acid (MES): (pH 6.8) MES 1.25M.

Phosphate buffer: (pH 7.6) K_2HPO_4 (0.1 M).

Triethanolamine buffer (TRA): (pH 7.8) TRA (0.2M), $CaCl_2$ (20 mM).

Tris buffer: (pH 7.4) 5 mM Tris-HCl.

II.2. Analytical Procedures

II.2.1. Electrophoresis

The PhastSystem electrophoresis unit (Pharmacia) was used to analyze samples by SDS-PAGE. Typically samples of HHS, HRS, HBF and TBP at concentrations ranging from between 0.5- 5 mg/ml were analyzed. Gels were run as per the instructions of the manufacturer. The electrophoresed samples were stained with Coomassie Blue R 250 dye (Pharmacia Blue R) with the aid of the development unit of the Phastsystem. The molecular weights of the SDS-denatured proteins were determined by comparison with low-molecular-mass-standards (Pharmacia).

II.2.2. High performance liquid chromatography (HPLC)

The purity of TBP was determined by HPLC on a Perkin-Elmer series 4 HPLC system using a TSK-250 gel filtration column (7.5x300mm; Bio-Rad). Protein samples were filtered through 0.45 μm filters to remove particulate matter. Typically 20-30 μg of protein was injected into the column, which had been equilibrated with 10 bed volumes of 0.05 M Na_2SO_4, 0.02 M NaH_2 PO_4 buffer (pH 6.8) at a flow rate of 1.0 ml/min. The eluent from the column was continuously monitored at 280 nm using a LC-95 UV/visible spectrophotometer detector (Perkin-Elmer). The area under the absorbance peak was determined using a LCI-100 Laboratory Computing Integrator (Perkin-Elmer) and the area % used as an indicator of protein

purity.

HPLC was also used to analyze the product (sialyl-lactosamine isomers) of assays for STase when lactosamine was used as an acceptor. The procedure followed was similar to a previously published method [Ratnam et al., 1987]. The residue obtained from assays (section II.7.1.B.) was dissolved in 50μl water containing approximately 50% α2-3′ and 50% α2-6′ sialyllactosamine (50μg) and the solution filtered through 0.45 μM filters to remove particulate matter. Aliqouts (10-20 μl) of this mixture were chromatographed on a Perkin Elmer series 4 HPLC system using a Lichrosorb NH_2 column (4 x 250 mm; particle size, 5 μm). The column was equilibrated isocratically with a 18:7 (v/v) mixture of acetonitrile and 15 mM KH_2 PO_4 (pH 5.2) at a flow rate of 2ml/min. Absorbance of the eluent was measured at 195 nm. The volume of the eluent corresponding to the areas under the absorbance peaks was collected and fractions counted for radioactivity.

II.2.3. Affinity chromatography

II.2.3.A. Isolation of the heparin-binding fraction (HBF) from heat inactivated serum

Heparin-agarose was packed into a column (20 x 2 cm) and the column equilibrated with at least 10 bed volumes of Tris buffer (5mM; pH 7.4) at a flow rate of 30ml per hour. HRS or HHS was passed through the column (1ml serum per 2ml packed

gel) and fractions of the eluent collected. The unbound fractions resulting after each application of serum were pooled and concentrated by ultrafiltration to the original volume of serum applied. Absorbance was monitored at 280 nm using an LKB Unicord S Detector. The column was washed thoroughly with 5mM Tris buffer until the absorbance of the eluent was less than 0.05 at 280 nm. The heparin-bound fraction (HBF) was eluted with 1M NaCl and the eluent fraction containing the protein peak collected. HBF was dialysed exhaustively against deionised water (thrice). HBF was lyophilised and stored at -20° C dissolved in either KRB or Tris-buffer, until use. The absorbance was monitored at 280nm.

HHS was also applied to a Sepharose column using the above procedure. The unbound fractions were pooled and concentrated by ultrafiltration.

Protein measurements were carried out according to the procedure of Lowry et al., (1951).

II.2.3.B. Isolation of trypsin-binding protein (TBP)

Trypsin-agarose (4 x 50 units) was mixed with 20 ml agarose, packed into a column (20 x 2 cm) and equilibrated with approximately 10 bed volumes of 5 mM Tris buffer (pH 7.4), at a flow rate of 30ml per hour. HBF (40mg dissolved in 2 ml 5mM Tris-HCl) was applied to the column. The unbound-trypsin fraction was collected, concentrated to half its

volume by ultrafiltration, dialysed against deionised water (thrice) and lyophilised. The column was washed intensively with 5mM Tris buffer until the absorbance was less than 0.05 at 280 nm. The bound fraction was eluted with 20 mM HCl, the volume under the protein peak was collected, dialysed against deionised water (thrice), lyophilised, dissolved in Tris-HCl or KRB and stored at -20°C.

II.2.3.C. Concanavalin A (Con-A) affinity chromatography

Con-A agarose was packed into a column (10 x 2 cm) and the column equilibrated with at least 10 bed volumes of 0.5M Tris/ 1 mM $MgCl_2$/ 1mM $CaCl_2$ (pH 8). Chromatography was carried out according to the procedures described by Koj et al, (1982) and Pos et al, (1989). TBP (500 μg) dissolved in 5mM Tris-buffer was passed through the column which was washed thoroughly with the equilibrating buffer until the absorbance of the effluent was less than 0.05 at 280 nm. The bound protein was eluted with 100 mM methyl mannoside and the fraction under the protein peak collected, dialysed against deionised water (thrice), lyophilised and stored at -20°C dissolved in Tris-buffer.

II.2.4. Assay Conditions

Assay conditions for all enzyme assays, in particular the concentrations of substrates, protein concentrations, incubation times and optimal pH for all enzyme assays were

established prior to experiments. Protein concentrations and incubation times were determined so that the rate of formation of product was directly proportional. Substrate concentrations used were not a limiting factor in product formation. All assays were done in duplicate.

II.3. Preparation of serum samples

II.3.1. Control rat serum

Rats were exsanguinated under light ether anaesthesia by withdrawing blood from the abdominal aorta. Serum was recovered from clotted blood by centrifugation at 1240 x g for 5 minutes.

II.3.2. Serum from turpentine treated rats

For some experiments rats were injected with commercial grade turpentine (0.5 ml/ 100 g body weight), subcutaneously in the thigh region [Fraser et al., 1984]. Control rats received a similar volume of 0.9 % NaCl. Thirty h following injection, rats were exsanguinated under light anaesthesia and serum obtained from clotted blood as described above.

II.3.3. Preparation of heat-inactivated serum

Serum (either rat or horse) was heated by incubating in a water bath at 56°C for 30 min. This resulted in the inactivation of STase and GTase activities.

II.4. Preparation of desialylated and degalactosylated protein acceptors

AGP and fetuin were used as the protein acceptors for glycosyltransferase assays. Both proteins (1 g) were desialylated and degalactosylated according to the method of Spiro (1964). Briefly, desialylation was carried out by acid hydrolysis where the protein was incubated at 80 °C with 200 mls of 0.05M H_2SO_4 for 60 minutes, after which the protein solution was neutralised with approximately 1.5 - 2ml of NaOH. The solution was dialysed against 0.1M NaCl (once), and against deionised water (twice) before it was lyophilised.

In order to obtain the degalactosylated protein the dialysate after dialysis with water, was treated with 10 ml of 0.2M sodium metaperiodate plus 10 ml 1M sodium acetate for 8 hours at 22 °C. Following this 10 ml of ethylene glycol was added and the solution stirred for 1 hour at 4°C. The solution was then treated with 25 ml of 0.1 M BaH_4 (pH 8) and left for 8 hours at 4 °C, after which the pH was adjusted to 5 with acetic acid. The resulting solution was dialysed against 0.1M NaCl (twice), followed by deionised water (thrice). The solution was treated with 0.7 ml concentrated H_2SO_4, pH adjusted to 7 with 2 ml 0.1N NaOH, dialysed initially against 0.1 M NaCl (twice), followed by deionised water (thrice) before it was lyophilised.

II.5. Jejunal slices and medium

II.5.1. Preparation of jejunal medium

Rats were exsanguinated under light ether anaesthesia and blood drawn for serum samples as described (section II.3.1.). The small intestine was removed, the first 10 cm from the pyloric end of the stomach (duodenum) was discarded, the next 30 cm taken as the jejunum and used in experiments. The jejunal tissue was rinsed gently with ice cold 0.9% NaCl and then cut into 0.5 cm slices. Jejunal slices (10-15 slices; ~0.5 g of tissue) were incubated in oxygenated (mixture of 95% oxygen and 5% carbon dioxide) 25 ml stoppered Erlenmeyer flasks containing 2 ml KRB buffer with 25 mM glutamine. Unless stated otherwise, the flasks were incubated at 37°C in a water bath. The buffer used was KRB or KRB supplemented with either 20% (v/v) heat inactivated serum or 30 mg albumin or 20% (v/v) glycerol in a final volume of 2ml. At specified time intervals between 0 to 6 h, the clear medium obtained by decanting the incubation mixture was centrifuged at 12 000 x g for 10 min. and the supernatant assayed for STase and GTase activities.

In some experiments heparin was added to KRB and KRB-enriched incubations at concentrations between 0 and 200 µg/ 2ml.

In studies carried out to examine the effect of serum (HHS or HRS) and its fractions on the release of STase and

GTase, jejunal slices were incubated as above in KRB or KRB containing either HBF, TBP, unbound-heparin fraction or unbound-trypsin fraction. In some experiments jejunal slices were incubated as above in KRB or KRB containing 200-400 μg of antiprotease (leupeptin, antipain, bovine pancreatic trypsin inhibitor, α_2 antiplasmin or A1PI).

II.5.2. Studies on the viability of jejunal slices

II.5.2.A. Measurement of incorporation of glucosamine and leucine

The rates of protein and glycoprotein synthesis by jejunal slices during incubation were determined by measuring the incorporation of either [^{14}C]-leucine or [^{14}C]-glucosamine into TCA/PTA precipitable cellular proteins. Jejunal slices were incubated in oxygenated KRB or KRB containing 20% (v/v) HHS as described earlier. [^{14}C]Glucosamine-hydrochloride (0.5 μmol, 20μl, 0.5 μ Ci/mmol) or [^{14}C]leucine (0.5 μmol, 20μl, 0.5 μCi/mmol) was added to each set of five flasks at 0,2,3 and 4 hours. In each flask, the incorporation of radioactivity into jejunal proteins was monitored at 0, 10, 20, 60 and 120 min according to the method of Forstner (1970). After incubation, the jejunal slices were removed and washed with KRB containing a 10 fold excess of unlabelled leucine or glucosamine. The washed slices were homogenised in 2 ml KRB using a Polytron homogeniser (setting 7 for 2x10s). Protein was precipitated by

leaving the homogenates overnight at 4°C in a mixture of 10% TCA and 1% PTA (1:1; v/v). The mixture was centrifuged and the supernatant removed. The pellet was resuspended in TCA/ PTA and centrifuged for 10 minutes (12 000 g ; x3). The supernatant was removed and the pellet suspended in 2 ml chloroform/ methanol (3:1; v/v) and centrifuged for 10 minutes (12 000 g; x3). The pellet was dried under nitrogen, suspended in 1 M KOH and aliquots used for the measurement of radioactivity and protein.

II.5.2.B. Measurement of glutamine + glutamate

Jejunal slices were incubated in KRB or KRB supplemented with 20% (v/v) HHS for time intervals ranging from 0 to 4 hours (KRB contained 25 mM glutamine). Medium samples were prepared as described (section II.5.1). Perchloric acid extracts were prepared as described (section II.11.1) and the supernatant was then used in the assays. Glutamine + glutamate in the medium was measured using glutaminase and glutamate dehydrogenase, according to the procedures described by Lund [1974]. The principle of the method is outlined in reactions (1) and (2). Reaction (1) was allowed to proceed and a portion of the reaction mixture was taken for the glutamate assay.

glutaminase

(1) L-glutamine + H_2O ----------→ L-glutamate + NH_3

glutaminase

(2) L-glutamate + H_2O + NAD^+ ---------→ 2-oxoglutarate + NADH + NH_4

dehydrogenase

Briefly, the assay mixtures contained 0.4 ml of sample, 0.5 ml acetate buffer (0.5 M), 0.1 ml of glutaminase (0.005 units/ ml acetate buffer) and 0.4 ml deionised water. Standard incubations were carried out similarly except that 0.5 ml deionised water, 0.1 ml glutaminase and 0.5 ml l-glutamine (10 mM) were used. The glutamine free blank contained 0.5 ml buffer, 0.1 ml glutaminase and 0.4 ml deionised water. The reaction mixture was incubated at 37 °C for 1 h and the reaction stopped by cooling the tubes on ice. In the next stage, incubations contained 1 ml of the reaction mixture, 2 ml glycine-NaOH buffer (pH 9), 0.1 ml ADP (33.5 mM) and 0.2 ml NAD (27 mM). The contents of the tubes were mixed and the absorbance of the solution was measured at 340 nm. Glutamate dehydrogenase 0.05 ml (5mg/ml) was added, tubes were allowed to stand at room temperature for 45 minutes after which the absorbance was measured at 340 nm. The differences in the respective absorbance values were taken as a measure of glutamine + glutamate concentration.

II.6. Preparation of hepatocytes

Rat hepatocytes were prepared according to a

modification of the collagenase perfusion procedure previously described by Seglen [1973]. Rats were anaesthetized with ether and the abdominal cavity exposed. The liver was perfused through the portal vein with calcium-free Hank's buffer at 37 °C excised free of other tissue and placed in a reservoir. The perfusion solution was changed to Hank's buffer (120 ml containing 25 mg of collagenase, 35 mg of $CaCl_2.2H_2O$) and this solution was re-circulated through the liver at a flow rate of 30 ml/min at 37 °C for 15 min. The perfusion solution was oxygenated continuously with 95% oxygen and 5% carbon dioxide. After perfusion with the collagenase solution, the liver was placed in a 100 mm Petri dish on ice, rinsed with Waymouths medium/BSA and then squeezed in order to release the cells. The cell suspension was kept on ice for 10 min to lower the temperature to between 0-4°C and filtered through 8 layers of cheesecloth to remove aggregates. The filtrate was washed three times by centrifuging at 50 x g for 5 min. The final cell pellet was re-suspended in ice-cold Waymouths medium /BSA and used immediately. Cell viability which was assessed by 0.02% trypan blue exclusion, was found to be between 82-85 %. The concentrations of hepatocytes was determined by microscopy using a counting chamber (1/400 mm^2 x 1/10 mm deep) from Hausser Scientific. Hepatocytes (1 x 10^6 cells) were suspended in 2 ml Waymouths medium containing 25 mM glucose in

Erlenmeyer flasks. Some incubations were supplemented with either 20% (v/v) heat-inactivated serum, 250 μg TBP or 500 μg HBF. Flasks were oxygenated (95% oxygen and 5% carbon dioxide) and incubated at 37 °C for up to 4 h. At time intervals between 0 and 4 h flasks were removed from incubation, the contents decanted out and centrifuged at 10 000 x g for 10 minutes. The clear medium was removed and used in assays.

II.7. Glycosyltransferase assays

II.7.1. Assays for STase activity

II.7.1.A. Assays with desialylated protein acceptors

STase was assayed using DS-α1AGP or DS-fetuin according to the method described by Ratnam et al (1987). Briefly, the assay mixture contained 20 μl jejunal or hepatocyte medium (80-120 μg protein), 5 μl CMP [^{14}]C NeuAc (5.6 nmol; 27000 d.p.m.), 5 μl DS-AGP (250 μg), 5 μl MES buffer (pH 6.8), 15 μl deionised water, in a total volume of 50 μl. Assays were incubated at 37°C for 3 h (in the case of jejunal medium) or 1 h (in the case of hepatocyte medium), after which the reaction was stopped by the addition of 1 ml of an ice-cold mixture of 5% trichloroacetic acid (TCA) and 1% phosphotungstic acid [(PTA); 1:1, v/v]. The precipitate formed was filtered under suction through 2.4 cm diameter glass fibre filters (Whatman 934 AH), which were dried and counted for radioactivity using Ready Safe scintillation fluid in a Wallac

1209 Rackbeta Liquid Scintillation Counter. Control incubations were carried out without any exogenous acceptor.

Assays for serum STase were similar to that described for jejunal medium except that 10 μl sample was used and the reaction mixtures incubated for 30 min. In assays with the pure STases, the assay mixture contained 10μl sample (enzyme mixtures were prepared as described in section II.10), 10 μl CMP [^{14}]C NeuAc (11.2 n mol; 55000 d.p.m.); 10μl DS-AGP (for α2→6 STase; 500 μg) or 10 μl DS-fetuin (for α2→3 STase; 500 μg), 5 μl Triton X-110 (0.5%) and 15 μl deionised water in a total volume of 50 μl. Assays were incubated at 37°C for 30 min.

II.7.1.B. Assays with lactosamine as acceptor

In order to determine the specific linkage (NeuAc α2→3 Gal or NeuAc α2→6 Gal) STase was assayed using lactosamine as an acceptor. The α2→3 and α2→6 sialyllactosamine isomers formed were separated by HPLC, the volume under the sialyllactosamine peaks collected and aliquots counted for radioactivity as described (section II.2.2). Assays were carried out according to a previously published method [Dall'Olio et al., 1990]. Briefly, the assay mixture contained 30 μl of medium sample, 10 μl of sodium cacodylate buffer (80 mM, pH 6.5), 10 $MnCl_2$ (10 mM), 20 μl lactosamine (250 μg), 20 μl CMP [^{14}C]-NeuAc (80 pmol; 385714 d.p.m.), 10 μl deionised

water in a total volume of 100 μl. Assays were incubated at 37°C for 3 hours and the reaction terminated by the addition of chloroform/methanol (1:1), the protein precipitate was removed by centrifugation at 12 000 x g for 5 min in an Eppendorf microcentrifuge. The protein pellet was washed twice with chloroform/methanol (3ml totally). The combined supernatants were dried under nitrogen, the residue dissolved in sialyllactosamine and samples processed by HPLC as described (section II.2.2).

II.7.2. Assays for GTase activity

Assays for GTase were carried out according to the method previously described by Fraser and Mookerjea, (1977). Briefly the assays contained 20 μl of jejunal or hepatocyte medium, 5 μl UDP[^{14}C]Gal (3nmol; 17800 d.p.m.), 5 μl DSG-α1AGP or DGS-fetuin (250μg), 5 μl ATP (100 nmol), 5 μl $MnCl_2$ (625 nmol), 5 μl MES buffer, 5 μl deionised water in a total volume of 50 μl. Incubations were carried out at 37°C for 3 h (in the case of jejunal medium) or 1 h (in the case of hepatocyte medium), after which the reaction was stopped by the addition of 1 ml ice-cold TCA/PTA as described for STase. The precipitate formed was filtered under suction and counted for radioactivity as described for STase (section II.7.1.). Control incubations were carried out in the absence of exogenous acceptor.

Assays for serum GTase were similar except that 10 μl sample was used and the reaction mixture incubated for 30 min. In assays with pure GTase, the assay mixture contained 10 μl sample (enzyme mixture was prepared as described in section II.10), 10 μl DSG-AGP or DSG-fetuin (500 μg), 10 μl UDP[^{14}C]Gal (6 nmol, 36 000 d.p.m.), 5 μl MES buffer, 5 μl Triton X-100 (0.5%) and 15 μl deionised water in a total volume of 50 μl. Assays were incubated at 37 ° C for 30 min.

II.8. Sialidase and CMP-sialic acid hydrolase activities

II.8.1. Assays for sialidase activity

Sialidase activity was measured in 2 and 4 h jejunal medium samples obtained from incubations in KRB and KRB supplemented with 20% (v/v) HHS. The method used was similar to that described by Fraser et al., [1980]. The purpose of these experiments was to determine whether there was significant sialidase activity in the medium samples, which could cause in liberation of sialic acid from the sialylated protein acceptor (AGP) and thereby interfere with STase assays.

Rats were injected intraperitoneally with colchicine (0.5 mg/ 100 g of body weight). Colchicine was made up just before use by dissolving 5 mg in 1 ml of 0.9% NaCl. Rats were exsanguinated 18 h after injection under ether anaesthesia, jejunal tissue removed and washed thoroughly with 0.9% NaCl.

Mucosal cells were obtained by scraping the surface with a glass slide. The cells were washed twice with KRB by centrifugation at 12 000 g for 10 min after which the cells were resuspended in KRB, filtered through one layer of cheesecloth and homogenized using a Polytron homogenizer.

The homogenised cell fraction (2ml, 1.8 mg protein) was incubated at 37°C with 1 ml CMP [^{14}C] NeuAc (1.2 μmol, 2μCi), 2 ml rat serum, 2 ml MES buffer in a total volume of 10 ml. After 3 h of incubation the reaction mixture was centrifuged for 10 min at 12 000 x g and washed with Tris-HCl (50mM containing 0.25 M sucrose) to remove soluble proteins and labelled nucleotide sugar.

The insoluble labelled pellet (20μl, 18-20 μg protein) was used as substrate in an assay consisting of 20 μl jejunal medium, 10 μl of MES buffer (pH 6.8) in a total volume of 50 μl. Reactions were stopped at zero time and after a 3 h incubation at 37°C with 1ml TCA/PTA (5%/1%). Precipitates were filtered, washed and radioactivity measured as described (section II.7.1.). Release of labelled sialic acid from the pellet was used as a measure of sialidase activity.

II.8.2. Assays for CMP-NeuAc hydrolase activity

CMP-NeuAc acid hydrolase activity was measured in samples of jejunal medium obtained from incubations in either KRB or KRB plus HHS according to the method of Fraser and coworkers

(1980). The purpose of these experiments was to determine whether there was CMP-NeuAc hydrolase activity in medium samples which was causing hydrolysis of CMP-NeuAc thereby limiting its availability during STase assays.

Medium samples were obtained from 2 and 4 h jejunal incubations in KRB and KRB containing HHS respectively (section II.5.1.). Samples in a volume of 1 ml were incubated with CMP ^{14}C NeuAc (120 nmol, 100 μl, 0.2 μCi) for 4 h at 37 ° C. At 1-h time intervals aliquots of 200 μl were removed, the reaction stopped with an equal volume of cold 95% ethanol, the tubes were allowed to stand on ice for 30 min and the supernatants were removed. The respective supernatants were pooled, dried under nitrogen and dissolved in distilled water containing 50 μg of a mixture of CMP-NeuAc and NeuAc (1:1). An aliquot of the supernatant was injected into an Lichrosorb amine column connected to a Perkin Elmer HPLC system. HPLC was performed as described for the separation of sialyllactosamine isomers (section II.2.2.). Peaks corresponding to CMP-NeuAc and NeuAc were collected and fractions counted for radioactivity. Zero time samples and standard radioactive CMP-NeuAc samples were also chromatographed and in these runs there was no evidence of hydrolysis of CMP-NeuAc.

II.9. Stability of STase and GTase in sera from control and turpentine treated rats

Serum samples (200 - 500 μl) obtained from either control or turpentine treated rats were incubated for up to 4 h at 37°C, without TBP or supplemented with TBP (250 μg/ 2ml). At time intervals of 0, 1, 2 and 4 h samples were removed and stored at -20°C until assayed.

II.10. Experiments with pure STase and GTase

Pure STase (α2→6 or α2→3; 0.4 mU/10 μl) and pure GTase (0.4 mU/10 μl) were mixed 1:1 (v/v). Plasmin or trypsin (0 to 0.012 mg) were added to the glycosyltransferase mixture and samples incubated at 37 °C for 10 minutes, after which samples were removed from incubation and assayed for STase and GTase activities. In some experiments TBP (0.0 to 0.03 mg) was added in increasing amounts to the glycosyltransferase mixture along with 0.01 mg protease. Samples were incubated at 37 °C for 10 min and assayed for glycosyltransferase assays as described (sections II.7.1. and II.7.2).

II.11. Measurement of protease inhibitory and protease activities

II.11.1. Sample preparation

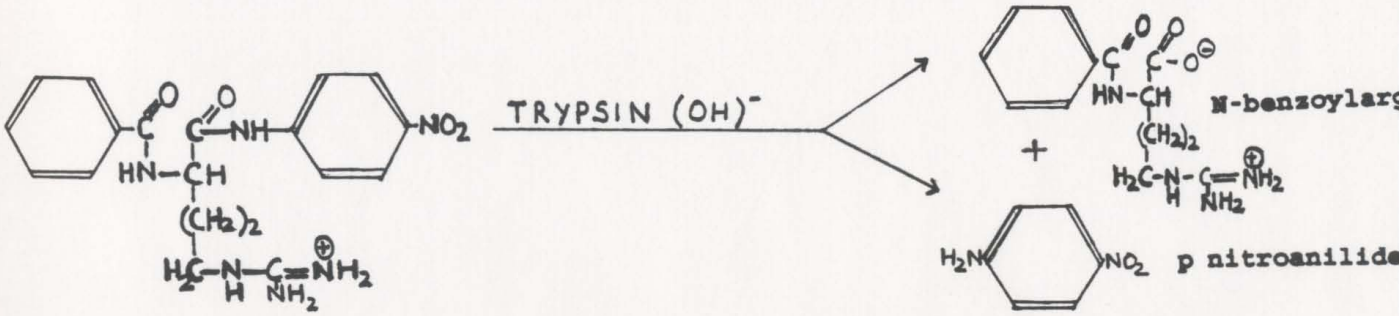
Perchloric acid extracts were prepared from serum and HBF according to the procedure described by Fritz et al (1974). HRS was mixed with an equal volume of 6% (w/v) perchloric acid

and the precipitated protein removed by centrifugation (1200 x g for 15 min.). The supernatant was treated by the drop wise addition of 5M K_2CO_3 until the pH was between 6 and 7. The precipitate was removed by centrifugation and the supernatant used in assays. Unless otherwise stated A1PI, BSA, HBF and TBP were used at concentrations of 30 μg/ ml.

II.11.2. Assays for trypsin inhibitory activity

II.11.2.A. Assays with BAPNA

Trypsin inhibitory activity was assayed using the peptide BAPNA, as described by Fritz et al., 1974. As shown in the reaction, BAPNA is converted to N-benzoylarginine and p nitroanilide. The p-nitroanilide formed is coloured and can be monitored by measuring the absorbance at 405 nm.



N-benzoylarginine-p-nitroanilide

(BAPNA)

Briefly, the assay mixture contained 0.2 ml trypsin (50 μg/ ml in 0.001N HCl), 1.7 ml TRA buffer, 0.1 ml inhibitor sample. Control assays were done with 0.2 ml trypsin (50 μg/ ml in 0.001N HCl) and 1.8 ml of TRA. The reactants were incubated at 25 °C for 5 minutes after which 1 ml BAPNA (2.2 mM) was added. The solutions were incubated for 30 minutes at 25 °C, the reaction was stopped by cooling the tubes on ice and the absorbance of the solutions was measured at 405 nm. The differences in the absorbance values between trypsin assays and trypsin plus inhibitor assays were taken as a measure of trypsin inhibitory activity. Concentrations of unbound-heparin fraction and unbound-trypsin fraction for up to 5 mg/ml did not show trypsin inhibitory activity.

When trypsin activity was measured, assays were similar and contained 1.0 ml of medium, 1 ml of TRA buffer and 1ml of BAPNA. Solutions were incubated for 30 minutes at 25 °C, after which the reaction was stopped and absorbance measured as described above.

II.11.2.B. Assays with α casein

Trypsin inhibitory activity using α casein as substrate was assayed according to the method of Fritz et al., (1974). The enzymatic breakdown of azocasein yields TCA-soluble hydrolysis products, the formation of which can be monitored by an increase in absorbance at 366 nm. A typical reaction

mixture contained 0.2ml of trypsin (50 μg/ ml), 0.7 ml of phosphate buffer, 0.1 ml of the inhibitor sample and 2 ml of α casein (2%). Control assays with trypsin were similar except that the incubations contained 0.2 ml trypsin, 0.8 ml phosphate buffer and 2 ml of casein. Reactants were mixed and incubated at room temperature for 10 minutes and 3 ml of TCA (5%) added. The contents of the tubes were mixed and the tubes allowed to stand for 30 minutes at room temperature. The tubes were centrifuged at 12 000 x g for 5 minutes, supernatants separated and absorbance measured at 405 nm. The differences in the absorbance values between trypsin assays and trypsin plus inhibitor assays were indicative of inhibitory activity towards trypsin.

Concentrations of unbound-heparin fraction and unbound-trypsin fraction for up to 5 mg/ml did not show inhibitory activity towards trypsin.

II.11.3. Assays for plasmin inhibitory activity

II.11.3.A. Assays with BAEE

Plasmin inhibitory activity using the peptide, BAEE, was measured according to the method described by Fritz et al., (1974). Hydrolysis of BAEE results in the liberation of ethanol which is measured enzymatically (reactions 1 and 2).

(1) Benzoyl-L-arginine ethyl ester ------→ benzoyl-L-arginine + ethanol

(2) Ethanol + NAD^+ --------→ acetaldehyde + NADH + H^+

The assay mixture contained 2.3 ml of glycine-NaOH

buffer, 0.2 ml of plasmin (50 μg/ml), 0.1 ml of inhibitor, 0.1 ml of NAD^+ (60 mg/ml), 0.02 ml of ADH (30 mg/ml) and 0.5 ml of BAEE (6 mM). Control incubations with plasmin contained 0.2 ml plasmin, 2.4 ml buffer, 0.1 ml of NAD^+, 0.12 ml ADH and 0.5 ml BAEE. The contents of the tubes were mixed and tubes were incubated for 20 min at 25°C. The reaction was stopped by cooling the tubes on ice after which the absorbance was measured at 364 nm. The differences in the absorbance values between plasmin assays and plasmin plus inhibitor assays were indicative of inhibitory activity towards plasmin. Concentrations of unbound-heparin fraction and unbound-trypsin fraction for up to 5 mg/ml did not show plasmin inhibitory activity.

When plasmin activity was measured assays were similar and contained 0.5 ml BAEE, 0.1 ml NAD^+, 0.02 ml ADH and 2.6 ml buffer.

II.11.3.B. Assays with α casein

Plasmin inhibitory activity using α casein as substrate was assayed as described for the measurement of trypsin-inhibitory activity (section II.11.2.[B]) except that plasmin (50 μg/ml) was used instead of trypsin and that the buffer used was lysine phosphate. Concentrations of unbound-heparin fraction and unbound-trypsin fraction for up to 5 mg/ml did not show plasmin inhibitory activity.

II.11.4. Assays for thrombin inhibitory activity

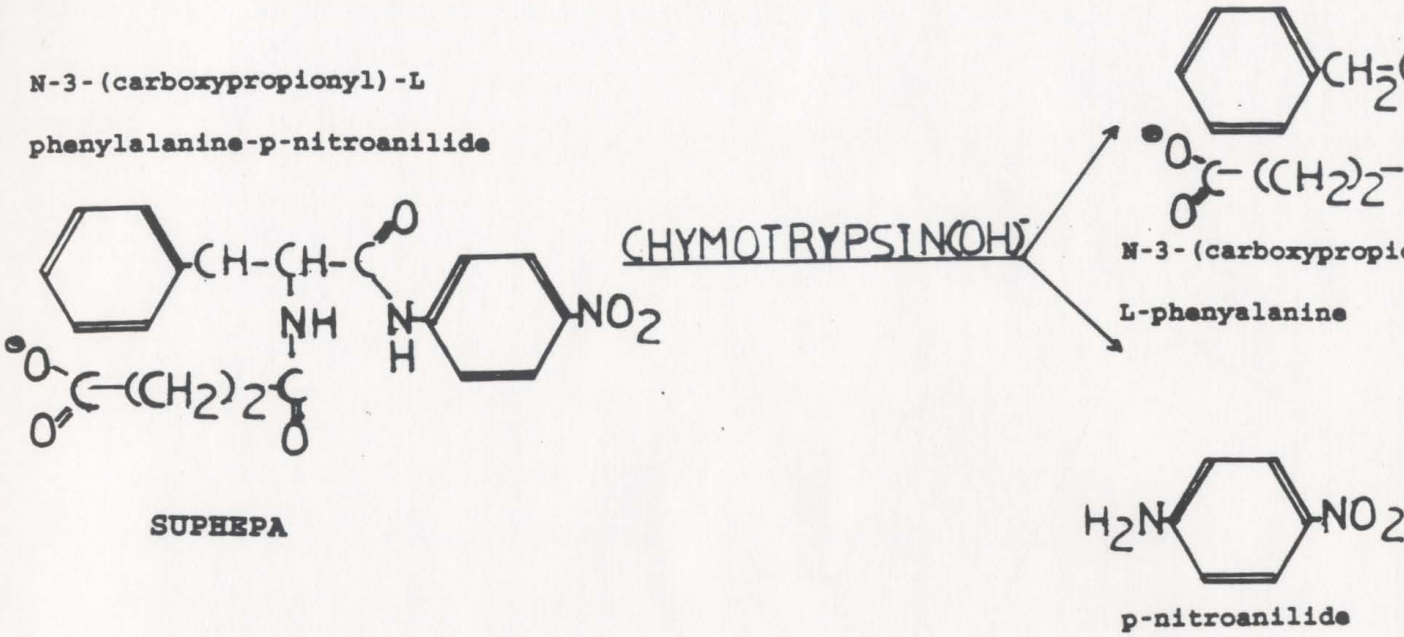
The procedure was similar to that used for the determination of plasmin inhibitory activity with BAEE as the substrate (section II.11.3 [A]) except that thrombin (50 μg/ml) was used instead of plasmin. Concentrations of HBF and TBP for up to 5 mg/ml did not show thrombin inhibitory activity.

II.11.5. Assays for kallikrein inhibitory activity

The method used was similar to that described for measurement of plasmin-inhibitory activity with BAEE (section II.11.3. [A]) except that kallikrein (50 μg/ml) was used. Concentrations of HBF and TBP for upto 5 mg/ml did not show kallikrein inhibitory activity.

II.11.6. Assays for chymotrypsin inhibitory activity

Chymotrypsin inhibitory activity was assayed using the peptide SUPHEPA as described by Fritz et al., (1974). Hydrolysis of SUPHEPA by chymotrypsin results in the formation of N-3-(Carboxypropionyl)-L-phenylalanine and p-nitroanilide. The liberation of p-nitroanilide is measured. The substrate N-succinyl-L-phenylalanine-p-nitoanilide (SUPHEPA) is also known as N-3-(Carboxypropionyl)-L-phenylalanine-p-nitroanilide.



Briefly the assay mixture contained 0.2 ml of chymotrypsin (0.1 mg/ml), 0.2 ml of inhibitor, 1.6 ml of TRA buffer (0.2 M). The reactants were mixed and incubated for 5 minutes at 25 °C after which 1 ml of SUPHECA (12.8 mM) was added and reactants were incubated for a further 20 minutes. Control incubations with chymotrypsin were similar and contained 0.2 ml of protease, 1.8 ml TRA and 1ml of SUPHECA. The reaction was stopped by cooling the tubes on ice and absorbance of the solution was measured at 405 nm. The differences in the absorbance observed between protease and protease plus inhibitor assays were indicative of inhibitory activity. Concentrations of HBF and TBP for upto 5 mg/ml did not show chymotrypsin inhibitory activity.

II.11.7. Assays for elastase inhibitory activity

Elastase inhibitory activity was assayed using orcein-elastin as the substrate according to the method of Appel (1974). The mucoprotein fraction of orcein-elastin is degraded by elastase giving soluble hydrolysis products. The amount of dye liberated is a measure of enzymatic activity.

orcein-elastin ------→ orcein + hydrolysis products.

Briefly the assay mixture contained 0.2 ml of elastase (50 μg/ml), 0.1 ml of inhibitor, 2.2 ml of phosphate buffer, 0.05 ml of orcein-elastin (5 mM). Control incubations with elastase were similar and contained 0.2 ml elastase, 2.3 ml of buffer and 0.5 ml of substrate. The reactants were mixed and incubated for 30 minutes at 37 °C after which the reaction was stopped by cooling the tubes on ice. The tubes were centrifuged at 12 000 x g for 15 minutes, supernatants separated and absorbance measured at 578 nm. The difference in absorbance between elastase assays and elastase plus inhibitor assays were indicative of inhibitory activity. Concentrations of HBF and TBP for upto 5 mg/ ml did not show chymotrypsin inhibitory activity.

II.11.8. Assays for papain inhibitory activity

Assays were similar to those described in section II.11.1. (A) except that papain (50 μg/ml) was used instead of trypsin. Concentrations of HBF and TBP for upto 5 mg/ml did

not show papain inhibitory activity.

II.12. Data presentation and statistical analysis

The computer program SigmaPlot 5 (Jandel Scientific) was used to plot figures. Data are reported as mean ± S.D. (standard deviation). Differences between means were tested for statistical significance by analysis of variance (ANOVA) and the Bonferroni method, with the aid of the computer program GraphPAD InStat. P values of 0.05 or less were taken to indicate a significant difference between means.

CHAPTER III. ROLE OF HEAT-INACTIVATED HORSE SERUM (HHS) ON THE SIALYLTRANSFERASE (STASE) RELEASED FROM JEJUNAL SLICES

III.1. Introduction

In the cell STases occur bound to the Golgi, ER and cell membranes [Roth et al., 1986; Taatjes et al., 1988]. STases also occur in the soluble form in body fluids, such as human and rat serum [Hudgin and Schachter., 1972; Mookerjea et al., 1972] and rat intestinal lymph [Ratnam et al., 1981]. Liver is generally considered to be the main source of soluble STase activity in serum and other body fluids. It has been proposed that other tissues such as intestine could also contribute to soluble STase activity and evidence to support this theory is present in the literature. For example colchicine treatment in the rat resulted in increased STase activity in intestinal lymph and serum [Fraser et al., 1980; Ratnam et al., 1981]. Also, thermal injury in the rat caused elevations in STase activity in small intestine and serum [Chu et al., 1988]. Considering these factors it is important to understand the release of STase from intestine.

Previous studies have revealed that the incubation of rat jejunal slices resulted in the release of soluble STase into the incubation medium [Ratnam et al., 1987]. In these studies

there was an absolute requirement for HHS in the incubation medium, in order to observe the release of STase from jejunal slices. Though it is clear that STases can occur in tissue fluids and incubation media due to the release of the membrane-bound enzymes from cell membranes, the factors which control the levels of soluble STase activity have not been clearly defined.

The purposes of the present study were therefore (1) to study the release of STase from rat jejunal slices and (2) to define the role of HHS in the release of STase.

III.2. Results

III.2.1. Effect of HHS on STase release from jejunal slices

Figure 3.1 shows the release of STase during the incubation of rat jejunal slices. When the incubation buffer KRB, was supplemented with 20% (v/v) HHS the STase activity in the medium increased with time for up to 6 h. However, when the jejunal slices were incubated in KRB alone or in KRB supplemented with either 20% glycerol or 30 mg BSA only trace amounts of STase were detected in the medium [Figure 3.1]. The concentration of BSA used (30 mg/ 2ml) corresponded to the amount of protein present in 20% (v/v) HHS. The levels of STase activity detected in medium samples obtained from 1, 2, 4 and 6 h incubation of jejunal slices in KRB + 20% (v/v) HHS, were increased compared to samples from jejunal incubations in KRB ($P < 0.001$ for the 1, 2 and 4 h time points). The

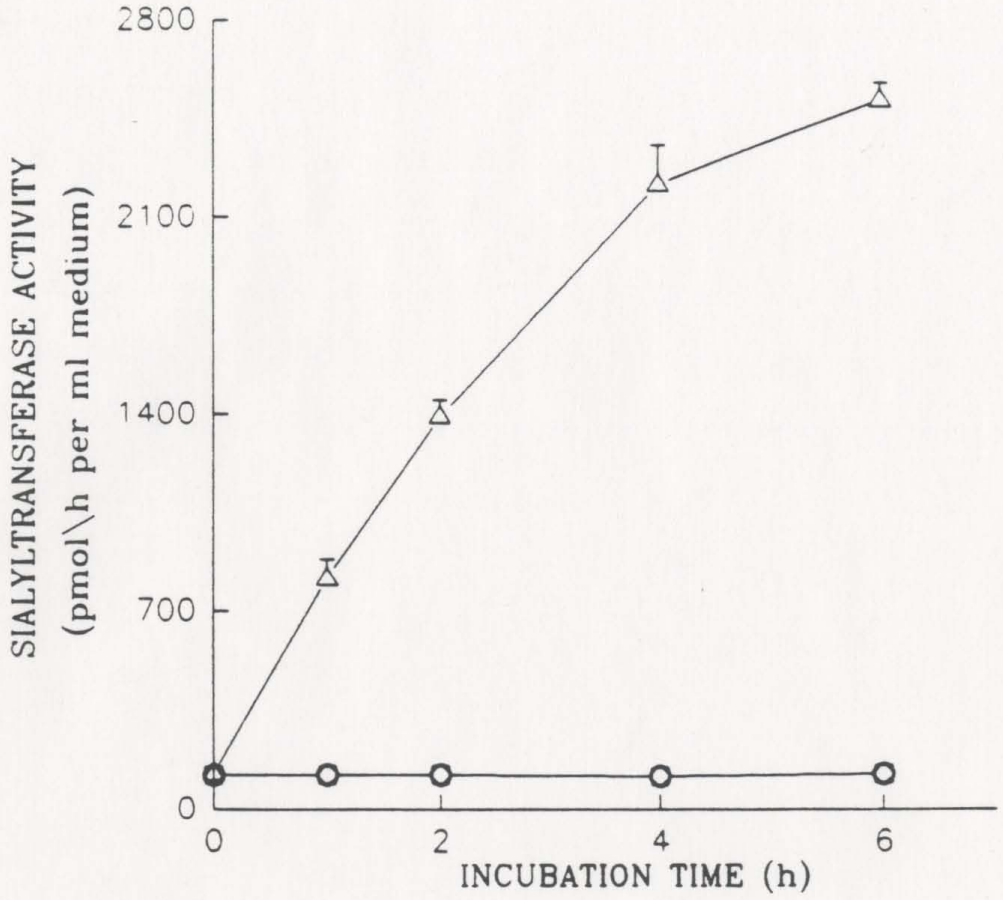


Figure 3.1. Effect of the presence of HHS in the incubation medium on STase release from jejunal slices

Figure 3.1. Effect of the presence of HHS in the incubation medium on STase release from jejunal slices. Jejunal slices were incubated in KRB (□), or in KRB supplemented with either 20% (v/v) HHS (△), 30 mg BSA (◇) or 20% (v/v) glycerol (○). At the indicated times, incubations were stopped, and the medium was decanted, centrifuged and assayed for STase activity as described in Materials and Methods (sections II.5.1 and II.7.1). STase activity is expressed as pmol of NeuAc transferred to DS-AGP/h per ml of incubation medium. Results represent means ± S.D. from four experiments.

progressive increase in the STase activity released into the medium, observed during incubations supplemented with HHS, was dependent on the concentration of HHS used, for up to 20% (v/v) of HHS [Figure 3.2].

The addition of 0.3% (v/v) Triton X-100 to the assays for STase in the medium obtained from KRB + 20% (v/v) HHS incubations, did not result in significant increases in the STase activity measured [Figure 3.3; $P > 0.05$ indicating that the means for the 1, 2, 4 and 6 h time points, were not significantly different]. This indicated that the STase released into the medium was soluble in nature.

When the jejunal incubations were carried out in KRB + 20% (v/v) HHS at 37° C, the levels of STase activity detected in the medium ranged from between 800 to 2300 units for the 2, 4 and 6 h time points [Figure 3.4: STase activity is expressed as p mol/h per ml medium]. However, when the incubations were carried out in KRB + 20% (v/v) HHS at 4°C, the levels of STase activity detected in the medium were less than 100 units for the 2, 4 and 6 h time points [Figure 3.4]. Likewise trace amounts of STase (< 100 units) were detected in the medium when jejunal slices were incubated in KRB at 4 °C.

Lactosamine was also used as an acceptor to assay for STase activity in 4 h medium samples obtained from jejunal incubations in KRB + HHS. The results showed that the STase

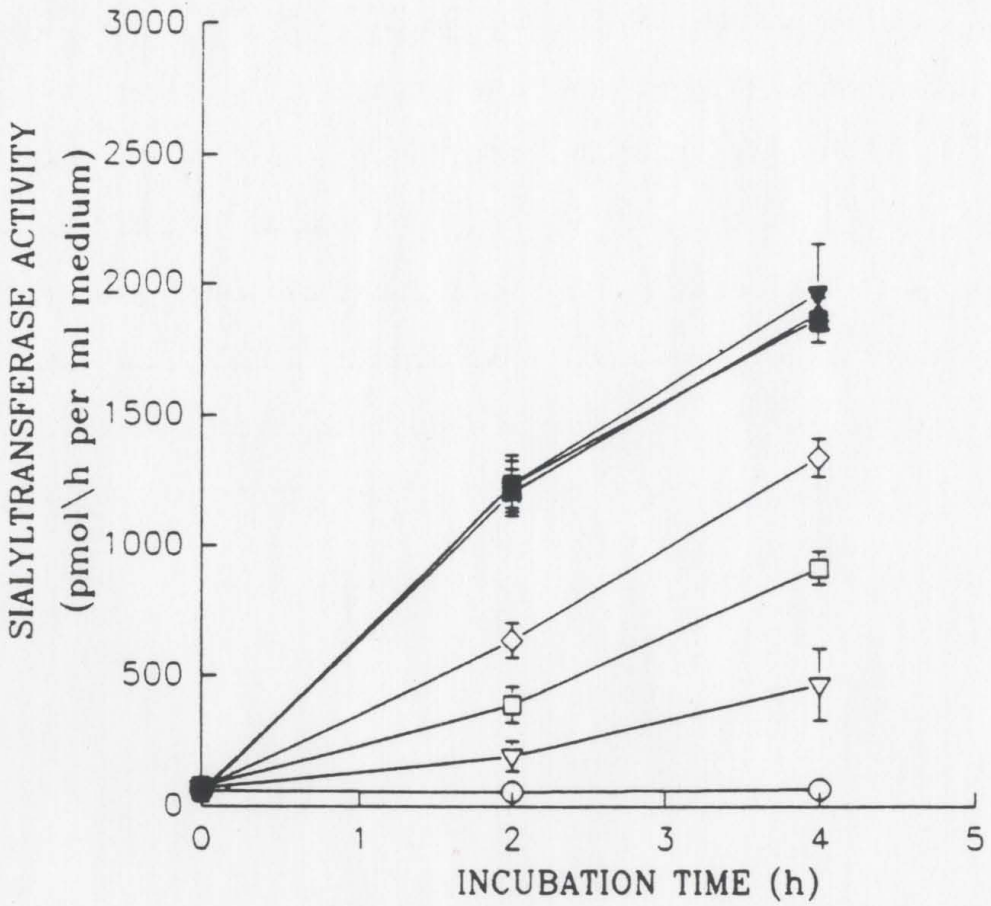


Figure 3.2. Effect of the concentration of HHS in the incubation medium on STase release from jejunal slices

Figure 3.2. Effect of the concentration of HHS in the incubation medium on STase release from jejunal slices. Jejunal slices were incubated in KRB (○) or in KRB supplemented with HHS at concentrations of either 5% [v/v] (▽), 10% [v/v] (□), 15% [v/v](◇), 20% [v/v] (▲), 30% [v/v] (■) or 40% [v/v] (▼). At the indicated times, incubations were stopped, medium samples were prepared and assayed for STase activity as described in Figure 3.1. STase activity is expressed as pmol of NeuAc transferred to DS-AGP/h per ml of incubation medium. Results represent means ± S.D. from three experiments.

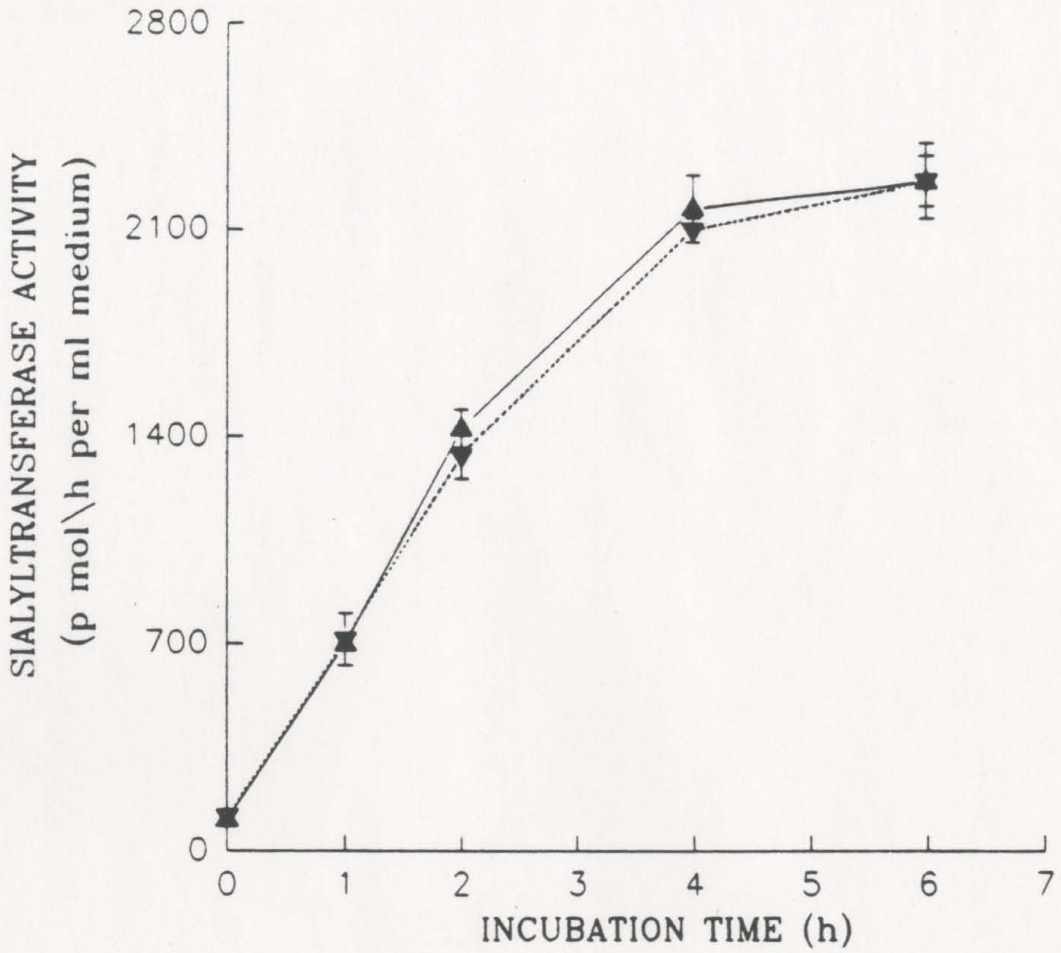


Figure 3.3. Effect of Triton X-100 on the medium STase activity

Figure 3.3. Effect of Triton X-100 on the medium STase activity. Jejunal slices were incubated in KRB + 20% (v/v) HHS and at the indicated times medium samples were prepared as described for Figure 3.1. Samples were assayed for STase activity in the presence (—▲—) or absence (·····▼·····) of 0.3% (v/v) Triton X-100 as described in Materials and Methods (section II.7.1). STase activity is expressed as pmol of NeuAc transferred to DS-AGP/h per ml of incubation medium. Results represent means ± S.D. from five experiments.

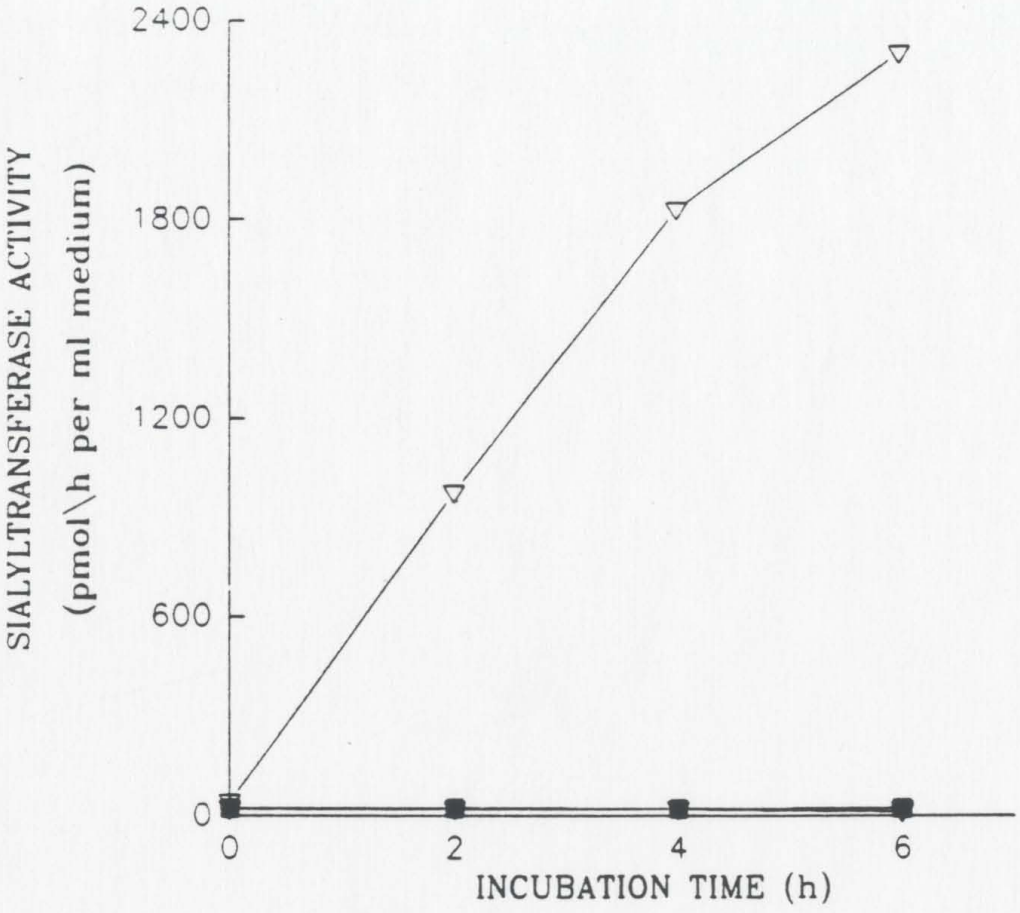


Figure 3.4. Effect of temperature on the release of STase

Figure 3.4. Effect of temperature on the release of STase.

Jejunal slices were incubated in either KRB [(■) at 4°C; (□) at 37°C], or in KRB + 20% (v/v) HHS [(▼) at 4°C; (▽) at 37°C], as described in Figure 3.1. Medium samples were prepared and assayed for STase activity as described for Figure 3.1. STase activity is expressed as pmol of NeuAc transferred to DS-AGP/ h per ml medium. Results represent means from two experiments.

present in the medium was predominantly that which catalysed the α2-6 linkage of NeuAc to the Gal residue of lactosamine, indicating that the enzyme was the α2→6 Galβ1→4 GlcNAc STase [Figure 3.5].

III.2.2. Viability of jejunal slices during incubation

The glutamate + glutamine in the incubation medium decreased over the 6 h of incubation [Figure 3.6]. There was no significant difference between the rates of decrease whether the incubations were carried out in KRB or in KRB + 20% (v/v) HHS [$P > 0.05$ indicating that the means were not significantly different]. As a control, flasks containing KRB or KRB + 20% (v/v) HHS were incubated without any jejunal tissue for up to 6 h and the glutamate + glutamine measured. In these flasks the decrease in glutamine + glutamate over the 6 h of incubation was less than 5%.

In other experiments jejunal slices were incubated in either KRB alone or in KRB + 20% (v/v) HHS for 0, 1, 3 or 4 h, at which times labelled glucosamine or leucine were added and the incubations further continued. In these incubations the rates of incorporation of [^{14}C]-glucosamine [Figures 3.7(a) and 3.7(b)] and [^{14}C]-leucine [Figures 3.7(c) and 3.7 (d)] into acid-insoluble proteins were measured for up to 120 min. The maximum incorporation of leucine into acid-insoluble proteins occurred within 30 minutes. In the case of glucosamine the

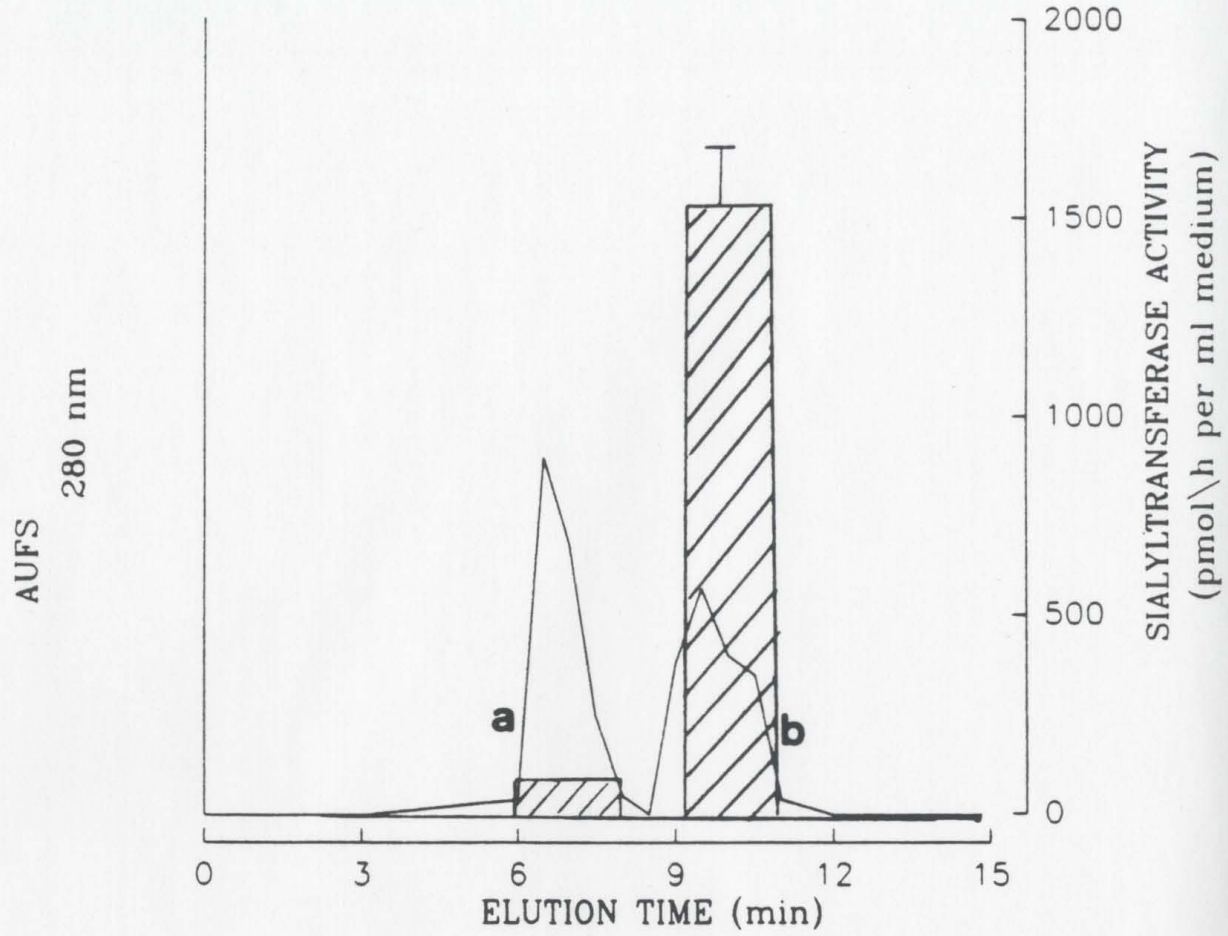


Figure 3.5. HPLC elution profile of sialyl-lactosamine isomers

Figure 3.5. HPLC elution profiles of sialyl-lactosamine isomers. Medium samples were prepared by incubating jejunal slices in KRB + 20% (v/v) HHS for 4 h as described in Figure 3.1. Samples were assayed using lactosamine as the acceptor as described in Methods and Materials (section II.7.1.B). Samples were processed and HPLC performed as described (section II.2.2). Fractions under the peaks for α2→3 (a) and α2→6 (b) sialyl-lactosamine were collected and counted for radioactivity. STase activity (▨) is expressed as p mol/h per ml medium. Results represent means ± S.D. from three experiments.

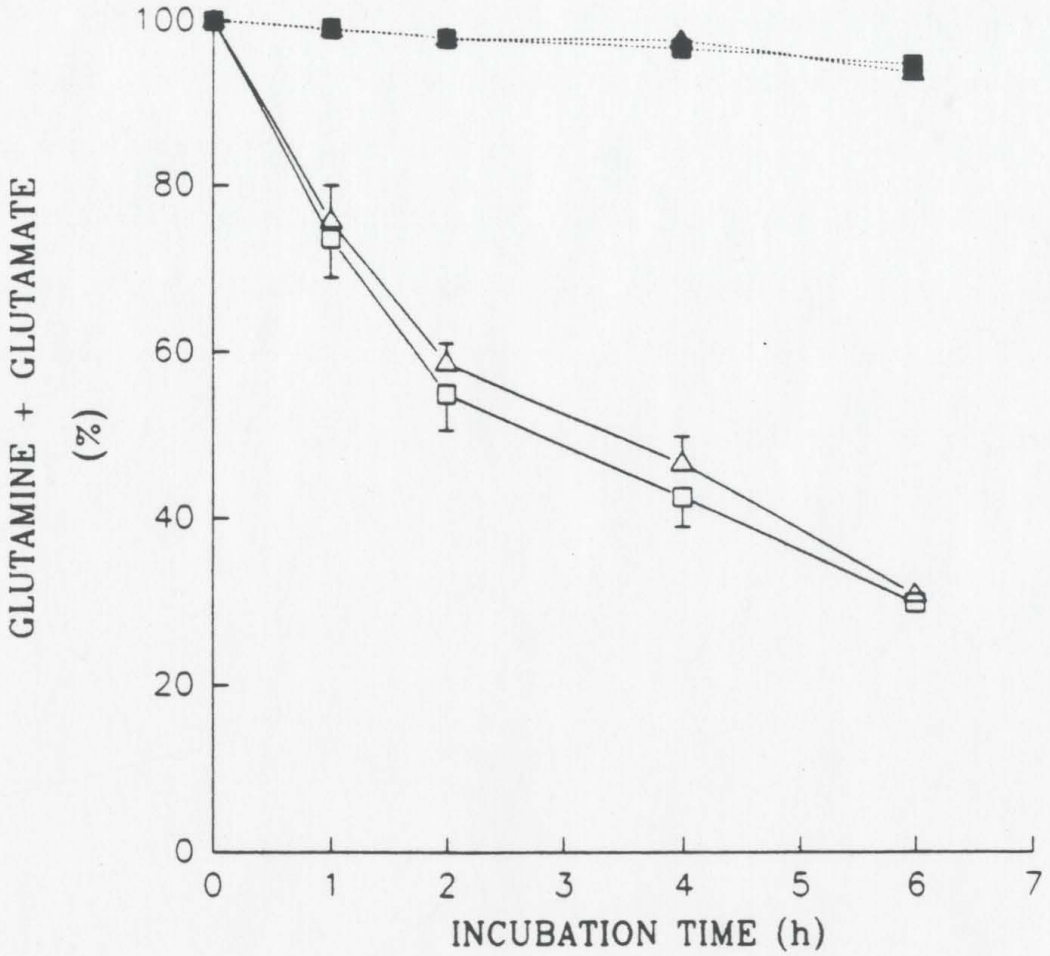


Figure 3.6. Glutamine + glutamate in the incubation medium

Figure 3.6. Glutamine + glutamate in the incubation medium.

Jejunal slices were incubated in either KRB (□) or KRB + 20% (v/v) HHS (△) as described in Figure 3.1. Control incubations were carried out in either KRB (···■···) or KRB + 20% (v/v) HHS (···▲···) without jejunal slices. At indicated times incubations were stopped, medium samples prepared and assayed for glutamine as described in Materials and Methods (section II.5.2.B.). Results are expressed as % of glutamate and glutamine left in medium. Results represent means ± S.D. from four experiments.

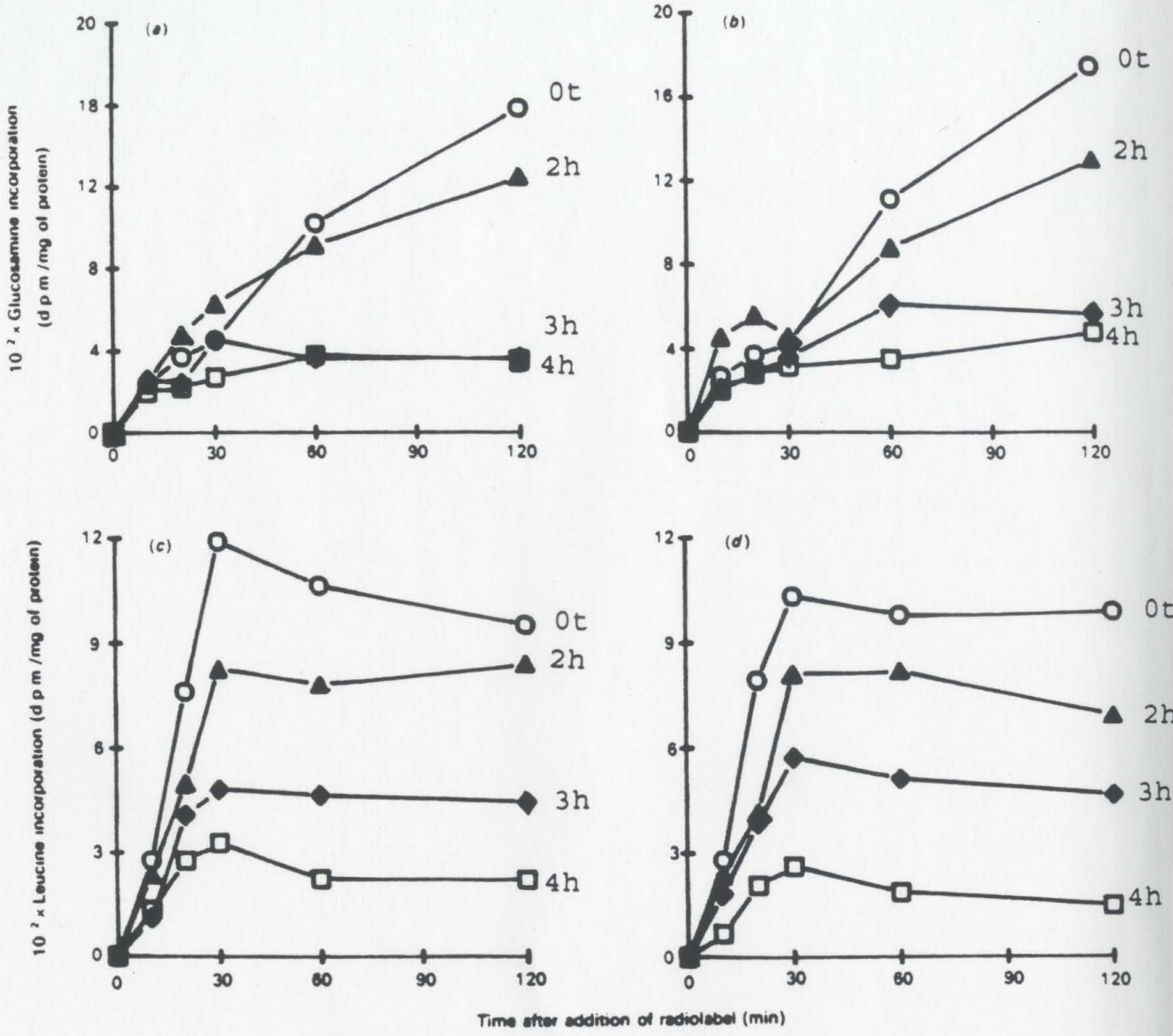


Figure 3.7. Incorporation of [^{14}C] glucosamine and [^{14}C] leucine into proteins by jejunal slices

Figure.3.7. Incorporation of [^{14}C]glucosamine and [^{14}C]leucine into proteins by jejunal slices. Jejunal slices were incubated in KRB (a and c) or in KRB + 20% (v/v) HHS (b and d) as described in Figure 3-1. [^{14}C] Glucosamine (a and b) or [^{14}C]leucine (c and d) were added after 0 h (○), 2 h (▲), 3 h (◆) or 4 h (□) to incubations which were further continued for a maximum of 120 min. At the indicated times these incubations were stopped, homogenates of the slices prepared and the incorporation of radiolabelled glucosamine or leucine into acid-insoluble proteins was measured as described in Materials and Methods (section II.5.2.A.). Results are expressed as d.p.m./mg of protein and represent mean values from two experiments.

rate of incorporation continued to increase for up to 120 min. Also, the overall rates of incorporation for both labelled glucosamine and leucine decreased progressively from 0 to 4 h of incubation. This suggested that the cells were less efficient in synthesising glycoproteins and proteins at 4 than at 0 h. The decreasing rates of glycoprotein and protein synthesis were similar whether the incubations were carried out in KRB alone or in KRB supplemented with HHS.

III.2.3. Sialidase and CMP-NeuAc hydrolase activities in medium

Sialidase activity was measured in 2 and 4 h medium samples obtained from jejunal incubations in either KRB or KRB + HHS respectively. Jejunal mucosal samples from colchicine treated rats were incubated with CMP [^{14}C]-NeuAc in order to prepare the labelled substrate for sialidase activity as described (section II.8.1). Studies by Fraser et al., [1980] have indicated that intestinal tissue homogenate samples prepared from colchicine treated rats are good endogenous acceptors for STase. The medium samples were incubated with the labelled substrate and the release of radiolabel from the substrate taken as a measure of sialidase activity. Release of radioactive label was lower in medium samples obtained from incubations in KRB compared to samples obtained from KRB + HHS incubations [Table 3.1].

Table 3.1. Sialidase activity in medium samples obtained from jejunal incubations. Jejunal homogenate samples from colchicine treated rats were incubated for 3h with CMP [^{14}C]NeuAc to prepare the labelled substrate for sialidase activity as described in the Materials and Methods (section II.8.1). The labelled substrate was then incubated for 3h with medium samples obtained from jejunal slice incubations in either KRB or KRB + 20% (v/v) HHS. The release of [^{14}C]NeuAc was monitored and sialidase activity is expressed as pmol of NeuAc released. Results represent means from two experiments.

sialidase activity

Incubation mixture	p mol NeuAc in pellet		p mol NeuAc released
	0h	3h	
KRB (2h)	731	730	1
KRB (4h)	763	753	10
KRB + 20% (v/v) HHS (2h)	641	621	20
KRB + 20% (v/v) HHS (4h)	700	681	19

CMP-NeuAc hydrolase activity was measured in 2 and 4 h medium samples obtained from incubations in either KRB or KRB supplemented with 20% (v/v) HHS [Table 3.2]. During these experiments the samples were incubated with CMP [^{14}C]-NeuAc for 3 h after which the radioactivity in the unhydrolysed CMP [^{14}C]-NeuAc and free NeuAc was measured. The free NeuAc released was lower in medium samples obtained from incubations in KRB compared to KRB + HHS samples [Table 3.2].

III.2.4. Function of HHS in the release of STase

In order to investigate the role of HHS in the release of STase during jejunal incubations, (1) 20% (v/v) HHS was added to KRB containing the jejunal slices throughout the incubation or (2) either 20% (v/v) HHS or 0.9% NaCl was added immediately to the decanted KRB medium obtained after 0, 1, 2 and 4 h of incubation respectively and STase activity was measured. These results indicated that the immediate addition of HHS to KRB partially restored STase activity [Figure 3.8]. In samples where HHS was added immediately to medium obtained from jejunal incubations in KRB, STase activity was significantly higher for the 1, 2 and 4 h time points, compared to samples where 0.9% NaCl was added [$P < 0.001$ for the 1, 2 and 4 h time points]. In further experiments where HHS was added at different times to KRB medium kept on ice there was a gradual loss of STase activity with time compared

Table 3.2. CMP NeuAc hydrolase activity in medium samples obtained from jejunal incubations. 2h and 4h medium samples from jejunal incubations in either KRB or KRB + 20% (v/v) HHS were incubated with CMP[^{14}C] NeuAc for 3h as described in Materials and Methods (section II.8.2.). Reactions were stopped by addition of 95% ethanol, samples centrifuged, supernatants separated and dried under nitrogen. The residue was dissolved in 50μl water containing 50 μg of a mixture of unlabelled CMP-NeuAc and NeuAc. CMP-NeuAc and NeuAc were separated by HPLC as described (section II.2.2) and the fractions under the respective peaks were counted for radioactivity. Results represent means from two experiments.

CMP NeuAc hydrolase activity

Incubation mixture	p mol NeuAc acid left unhydrolyzed	p mol NeuAc released
KRB (2h)	2700	213
KRB (4h)	2644	216
KRB + 20% (v/v) HHS (2h)	2713	280
KRB + 20% (v/v) HHS (4h)	2731	250

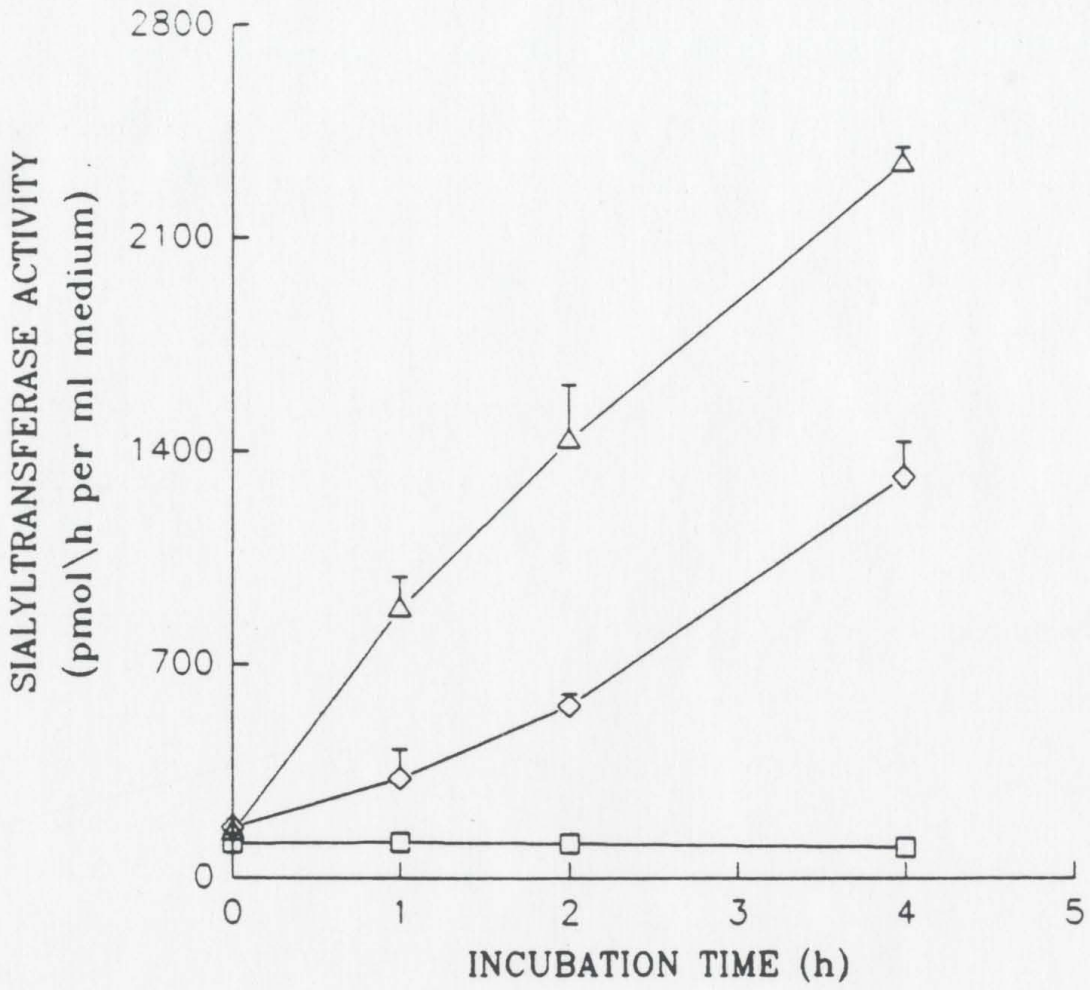


Figure 3.8. Effect of immediate addition of HHS to KRB medium on STase activity released from jejunal slices

Figure 3.8. Effect of immediate addition of HHS to KRB medium on STase activity released from jejunal slices. Jejunal slices were incubated in KRB (□) or in KRB + 20% (v/v) HHS (△) as described in Figure 3.1. At the indicated time intervals the incubations were stopped and the medium was decanted. 20% (v/v) of 0.9% NaCl was added immediately to the decanted medium obtained from jejunal incubations in KRB (□) or in KRB + HHS (△). 20% (v/v) HHS was also added to decanted medium samples from jejunal incubations in KRB (◇). The samples were then assayed for STase activity as described in Figure 3.1. STase activity is expressed as pmol of NeuAc transferred/ h to DS-AGP per ml medium. Results represent means ± S.D. from three experiments.

to samples where HHS was added immediately [Figure 3.9].

III.2.5. Effect of antiproteases/ proteases on STase released

Antipain, BPTI, leupeptin or pepstatin added to KRB during 2 and 4 h incubations resulted in 3 to 5 fold increases in STase activity at a 200 ug dose and 3 to 6 fold increases at a 400 ug dose, compared to incubations in KRB alone [Table 3.3]. Similar addition of human A1PI to KRB resulted in 11 to 20 fold increases for the 2 and 4 h incubations at a 200 ug dose and 22 and 33 fold increases for the 400 ug dose [Table 3.3]. α_2 Antiplasmin caused 13 and 19 fold increases for 2 and 4 h incubations at the 200 μg dose, and 20 and 30 fold increases at the 400 μg dose, in medium STase activity compared to incubations in KRB alone.

Trypsin activity in the medium was higher when jejunal slices were incubated in either KRB alone or KRB containing 30 mg BSA compared to KRB + HHS incubations [Figure 3.10].

The addition of increasing concentrations of KRB medium obtained after 4 h incubation of jejunal slices inhibited the STase activity of the 4 h KRB + HHS medium [Figure 3.11].

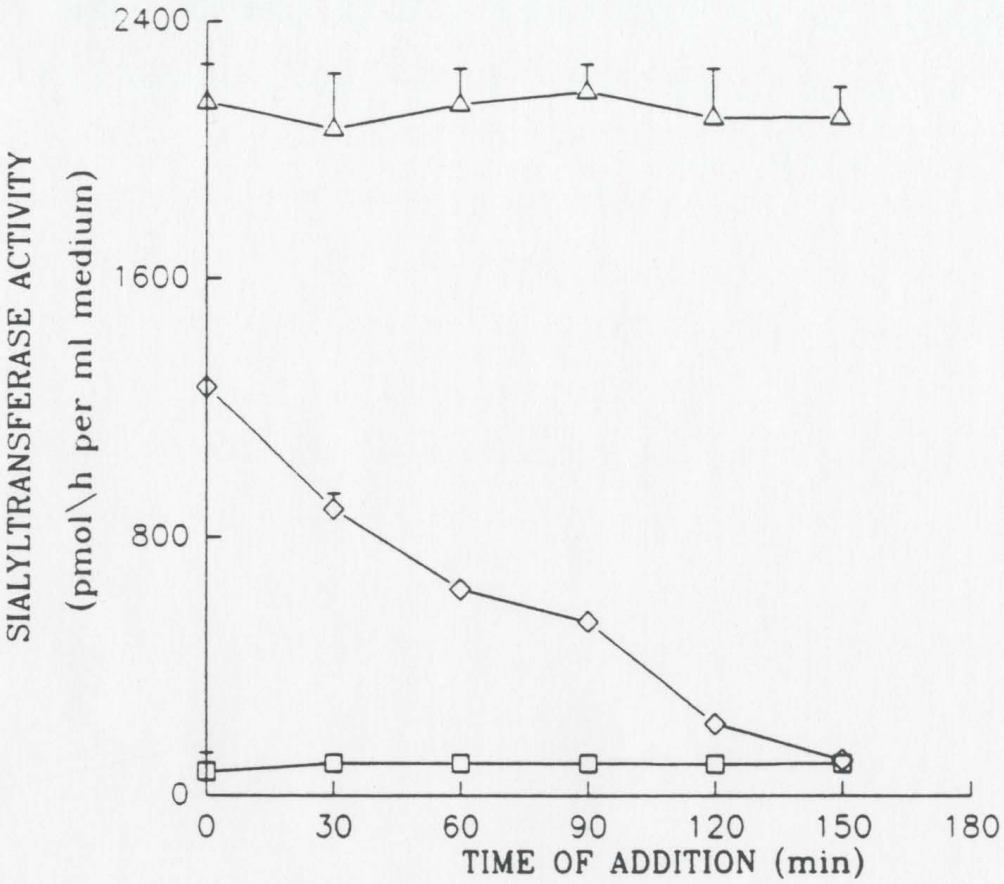


Figure 3.9. Effect of immediate addition of HHS to KRB medium on STase activity released from jejunal slices

Figure 3.9. Effect of immediate addition of HHS to KRB medium on STase activity released from jejunal slices. Jejunal slices were incubated in either KRB (□) or KRB + 20% (v/v) HHS (△) as described for Figure 3.1. After 2 h of incubation the medium samples were decanted into tubes which were kept on ice. At the indicated times 20% (v/v) of 0.9% NaCl was added to the decanted medium samples obtained from KRB (□) and KRB + HHS incubations (△). To another set of decanted samples from KRB incubations, 20% (v/v) HHS was added (◇). STase activity was measured as described in Figure 3.1. STase activity is expressed as pmol of NeuAc transferred/h to DS-AGP per ml incubation medium. Results represent means ± S.D. from four experiments.

Table 3.3. Effect of antiproteases on the STase activity released during jejunal incubations. Jejunal slices were incubated in KRB or in KRB containing either 20% (v/v) HHS or antiprotease [200 (Table 3a and Table 3b) or 400 μg of antipain, BPTI, leupeptin, pepstatin, α-2 antiplasmin or A1PI). Medium samples were prepared from 2 h and 4 h incubations and STase activity measured as described in Materials and Methods (sections II.5.1. and II.7.1.). STase activity is expressed as pmol of NeuAc transferred/h to DSG-AGP per ml of medium. Results represent means ± S.D. from experiments.

Incubation mixture	STase activity (p mol/h per ml medium)	
Antiprotease (μg/2ml)	2h	4h
no antiprotease	62.3 ± 26	70.4 ± 23.1
antipain (200)	256.8 ± 30.1	222.9 ± 187.3
antipain (400)	220.8 ± 156.7	239.0 ± 116
BPTI (200)	223.9 ± 87.4	239.0 ± 117.1
BPTI (400)	316.2 ± 59.9	355.3 ± 59.9
leupeptin (200)	347.0 ± 136.5	222.9 ± 187.3
leupeptin (400)	328.4 ± 102.2	464.2 ± 68.9
pepstatin (200)	287.5 ± 103.4	385.4 ± 123.2
pepstatin (400)	309.6 ± 87.5	385.4 ± 78.4
A1PI (200)	742.0 ± 124.0	1438.3 ± 95.6
A1PI (400)	1378.9 ± 526.0	2332.6 ± 193.7
α_2 antiplasmin (200)	812.9 ± 22.5	1272.3 ± 131.2
α_2 antiplasmin (400)	1272.6 ± 321.8	2147.8 ± 118.3
HHS 20% (v/v)	1444.9 ± 42.5	2478.5 ± 123.2

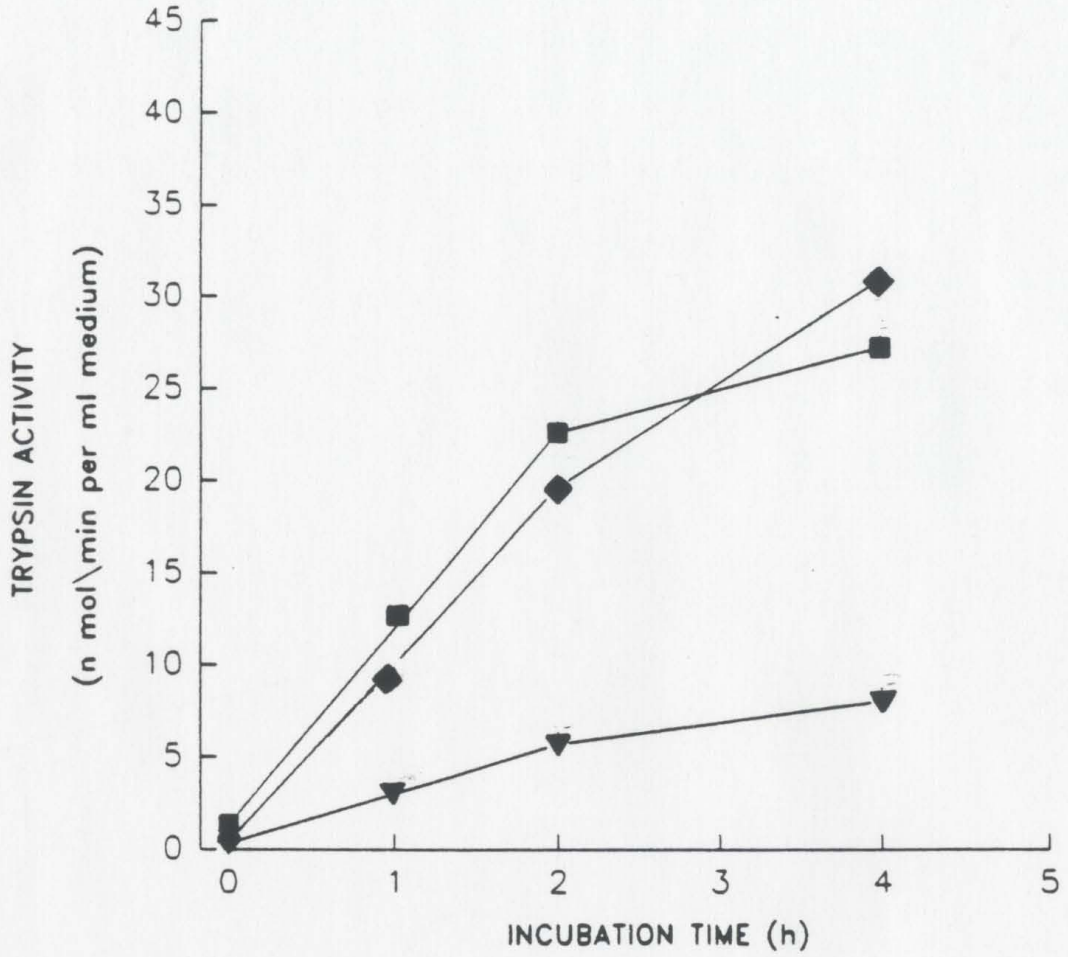


Figure 3.10. Trypsin activity in the medium

Figure 3.10. Trypsin activity in the medium. Jejunal slices were incubated in KRB (■) or in KRB supplemented with either 20% (v/v) HHS (▼) or 30 mg BSA (◆) as described for Figure 3.1. At the indicated times, incubations were stopped, the medium was decanted, centrifuged and assayed for trypsin activity as described in Materials and Methods (section II.11.2.[A]). Trypsin activity is expressed as nmol of substrate hydrolysed /min per ml medium. Results represent means from two experiments.

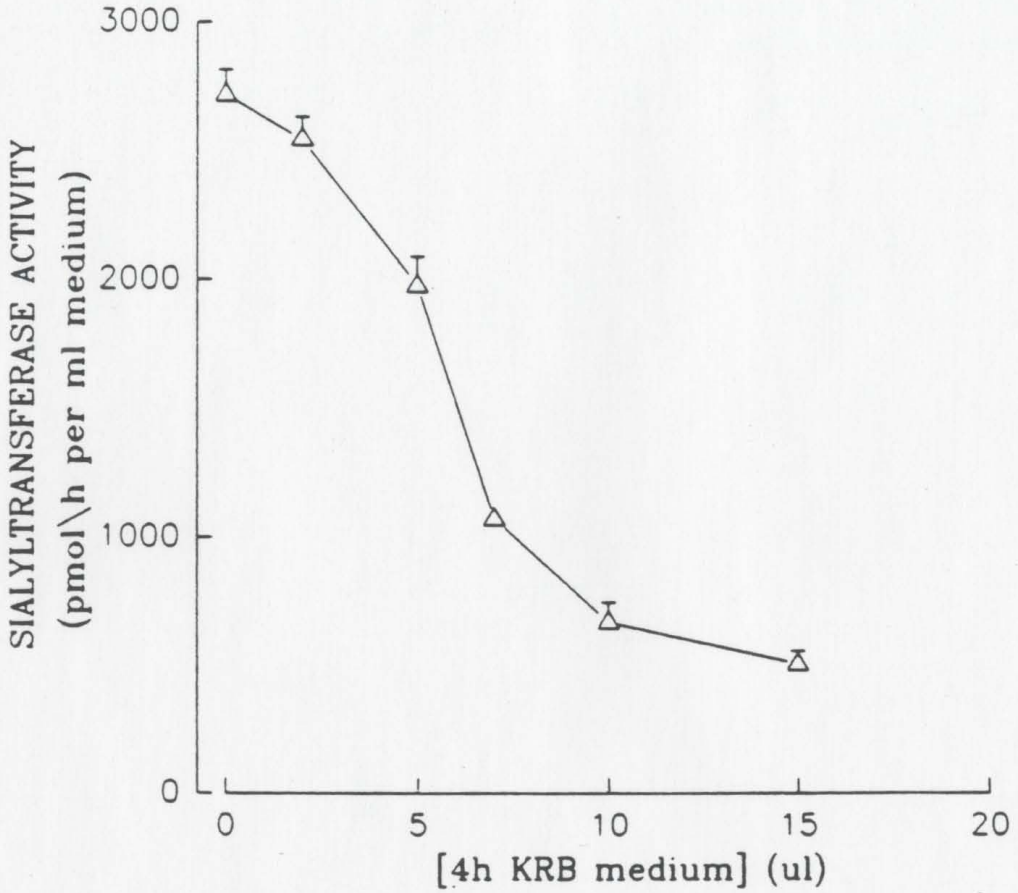


Figure 3.11. Effect of addition of 4h KRB medium on STase activity

Figure 3.11. Effect of addition of 4h KRB medium on STase activity. Jejunal slices were incubated for 4 h in KRB or in KRB supplemented with 20% (v/v) HHS as described for Figure 3.1 and medium samples were prepared. Increasing volumes of 4 h KRB medium was added to 4 h KRB + HHS medium and STase activity measured as described in Figure 3.1. STase activity is expressed as pmol of NeuAc transferred to DS-AGP/h per ml medium. Results represent means ± S.D. from four experiments.

III.2.6. Effect of the heparin and the heparin-binding fraction (HBF) of HHS on the STase activity released during jejunal incubations

Heparin when added to jejunal slice incubations at a concentration of 100 ug/ 2ml, decreased the activity of STase released in the presence of HHS [Figure 3.12; $P < 0.001$ for the 1, 2, 4 and 6 h time points]. Also, heparin in a dose dependent manner decreased the activity of STase released during jejunal incubations supplemented with HHS [Figure 3.13]. The addition of heparin [100 ug/2ml] to incubations resulted in 70 and 55% decreases in STase activity for the 4 and 2 h jejunal medium samples respectively. An increase in the heparin concentration to 200 μg/ ml further decreased STase activity by 79 and 76% respectively in the 4 and 2 h jejunal medium samples. Heparin when added directly to the assays at concentrations of 100 and 200 ug/ 2ml caused decreases in STase activity of 10, 20 and 25, 39 % for the 4 and 2 h jejunal medium samples respectively [Figure 3.14]. This suggested that when heparin was present during the incubation of jejunal slices it caused greater inhibition of STase activity or that it inhibited the release of STase compared to when heparin was added directly to the assays. This lead to further studies to determine whether heparin was binding to specific antiproteolytic protein(s) in HHS and if these protein(s) had an effect on STase activity.

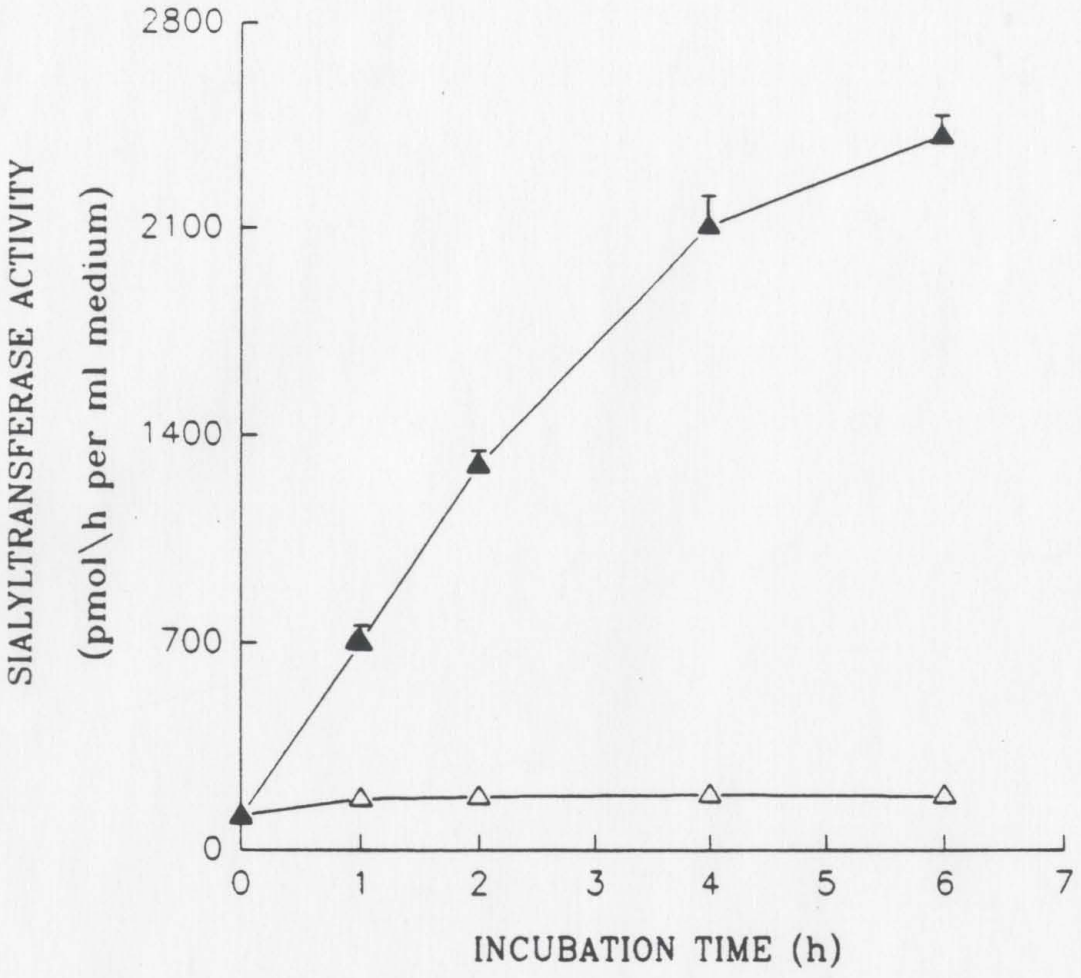


Figure 3.12. Effect of heparin on the STase activity released from jejunal slices

Figure 3.12. Effect of heparin on the STase activity released from jejunal slices. Jejunal slices were incubated in KRB + 20% (v/v) HHS as described for Figure 3.1. Heparin (100 μg/ml) was added at 0 h to one set of incubations (△). Control incubations were carried out in the absence of heparin (▲). At the specified times, medium samples were prepared and STase activity in the medium was measured as described in Figure 3.1. STase activity is expressed as pmol of NeuAc transferred/ h to DS-AGP per ml medium. Results represent means ± S.D. from five experiments.

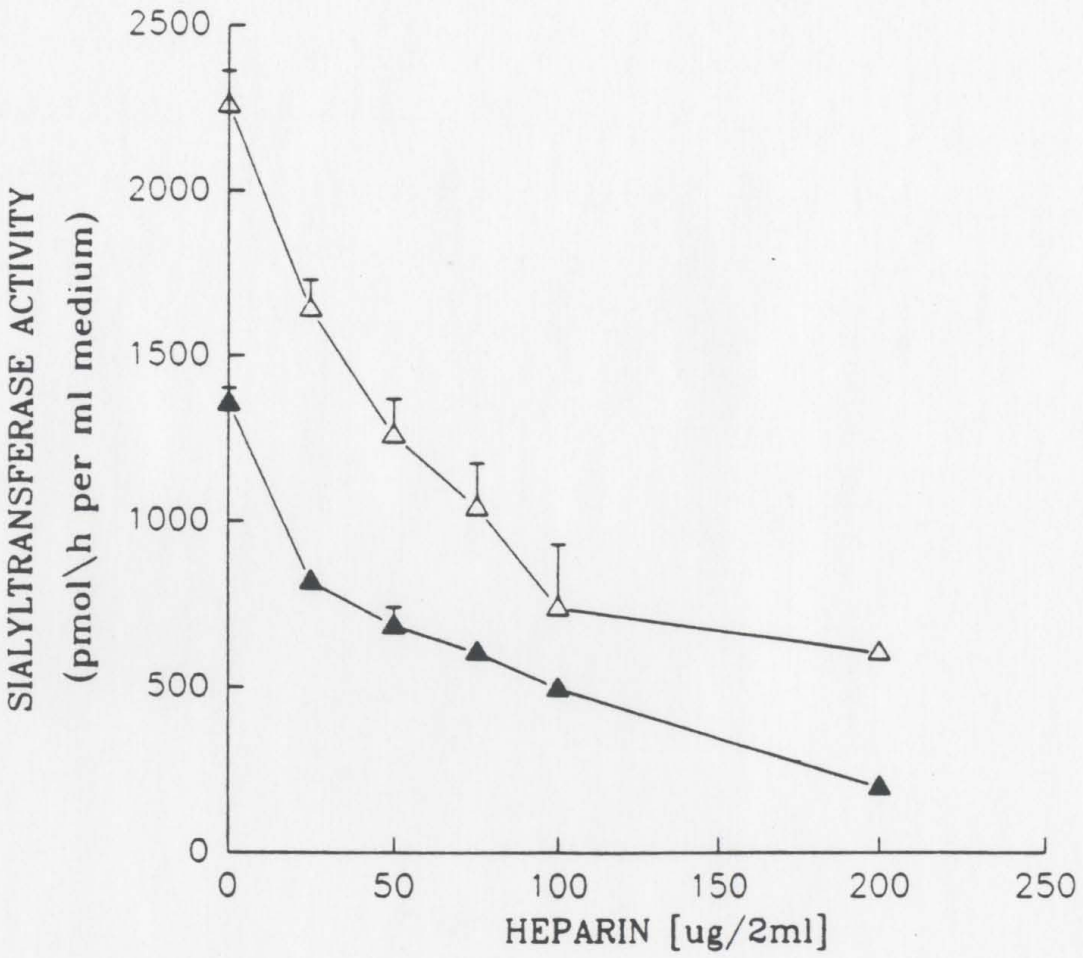


Figure 3.13. Effect of heparin concentrations on the STase activity released from jejunal slices

Figure 3.13. Effect of heparin concentrations on the STase activity released from jejunal slices. Increasing concentrations of heparin were added at zero time to jejunal incubations supplemented with 20% (v/v) HHS. The incubations were stopped at either 2 h (▲) or 4 h (△) and medium samples were analyzed for STase activity as described in Figure 3.1. STase activity is expressed as pmol/ h of NeuAc transferred to DS-AGP per ml of incubation medium. Results represent means ± S.D. from three experiments.

The unbound-heparin fraction and HBF were separated from HHS by affinity chromatography using heparin-agarose [Figure 3.15]. When the unbound-heparin fraction was used in incubations, the activity of STase in the medium was significantly decreased compared to incubations wherein complete HHS was used [Figure 3.16; $P < 0.001$ for the 1, 2 and 4 h time points].

In control experiments HHS was applied to an agarose column and the unbound-agarose fraction collected. When 20% (v/v) of the unbound-agarose fraction was added to incubations the activity of STase in the medium was similar to that observed when HHS was used [Figure 3.16: $P > 0.05$ indicating that the means were not significantly different for the 1, 2 and 4 h time points]. When HBF [500 ug/ 2ml] was added to incubations, STase activity detected in the medium was similar to that observed with HHS [Figure 3.16; $P > 0.05$ indicating that the means were not significantly different for the 1, 2 and 4 h time points]. Addition of increasing concentrations of HBF up to 1200 ug/ 2ml to the jejunal slice incubations resulted in a progressive increase of STase activity in the medium compared to incubations in KRB [Figure 3.17; $P < 0.001$ for the 200, 500, 800 and 1200 μg doses compared to KRB]. These results suggested that HBF was the fraction of HHS which was required in the incubation medium in order to measure the

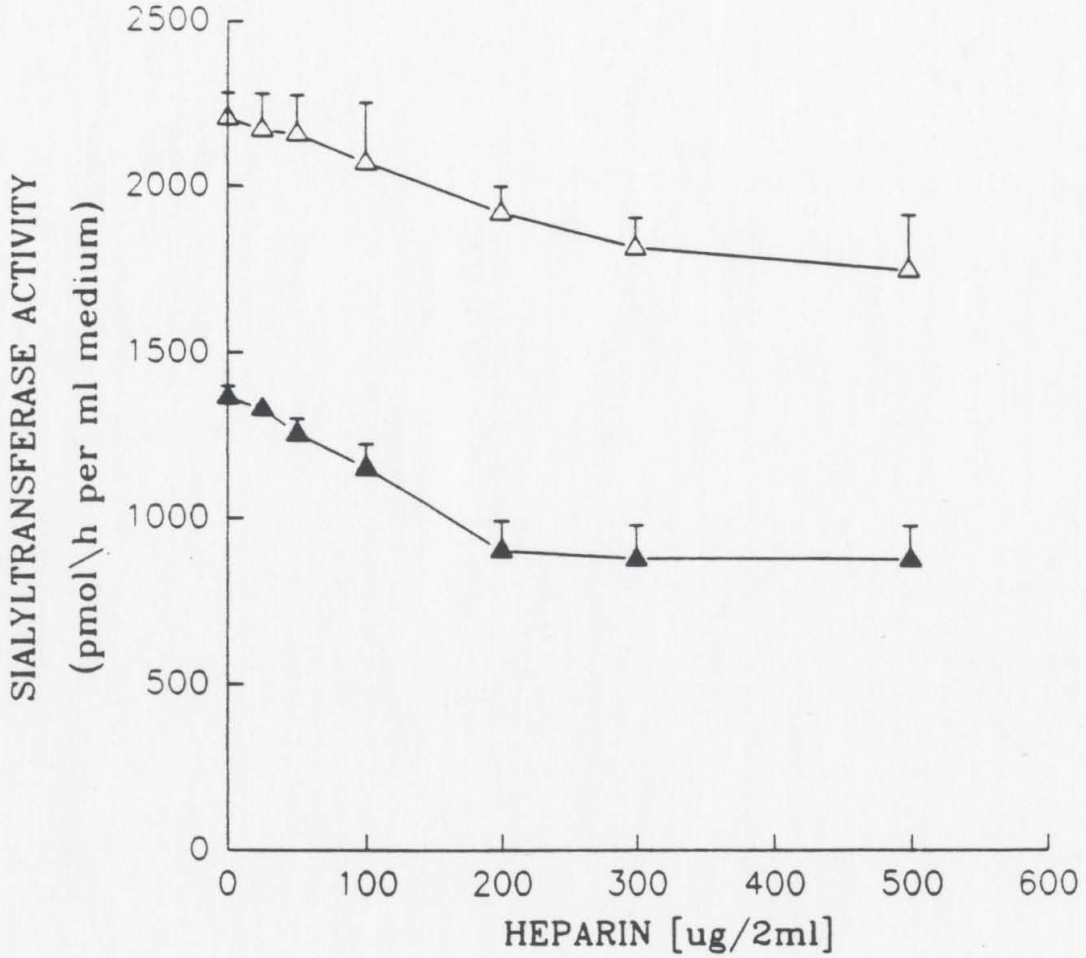


Figure 3.14. Effect of heparin on the assay for STase

Figure 3.14. Effect of heparin on the assay for STase. Jejunal slices were incubated in KRB + 20% (v/v) HHS. At 2 h (▲) and 4 h (△) incubations were stopped and medium samples processed as described in Figure 3.1. Samples were then assayed for STase activity in the presence of increasing concentrations of heparin. STase activity is expressed as pmol of NeuAc transferred/h to DS-AGP per ml incubation medium. Results represent means ± S.D. from four experiments.

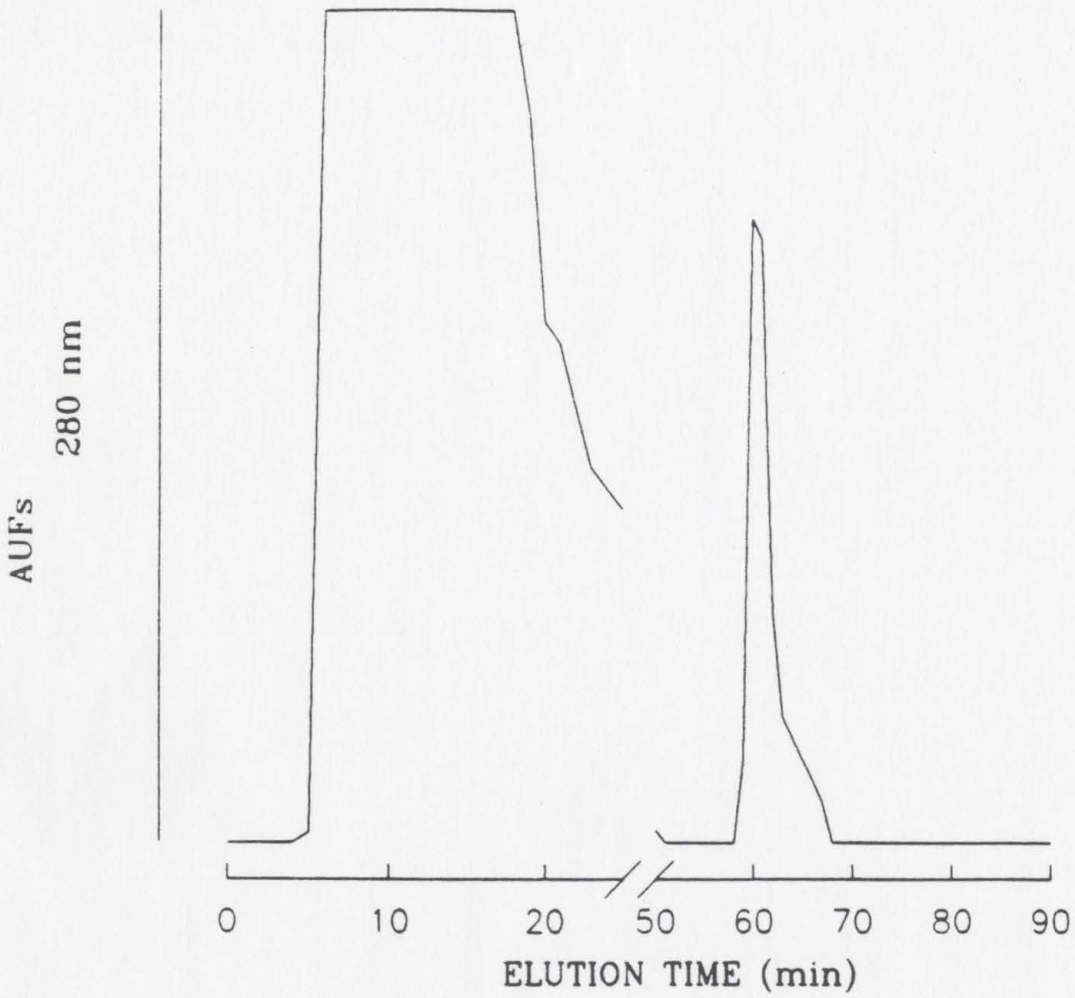


Figure 3.15. Affinity chromatography profile of the binding of HHS to heparin agarose: separation of the heparin-binding fraction (HBF)

Figure 3.15. Affinity chromatography profile of the binding of HHS to heparin-agarose: separation of the heparin-binding fraction (HBF). HHS was applied to a heparin-agarose column which had been equilibrated with 5 mM Tris buffer as described in Materials and Methods (section II.2.3.A). The column was then washed extensively with Tris-buffer until the absorbance of the eluent at 280 nm was less than 0.05. The bound fraction was eluted with 1 M NaCl in Tris-buffer and processed. This graph was regenerated by digitizing the elution profile with Sigmaplot 5.0.

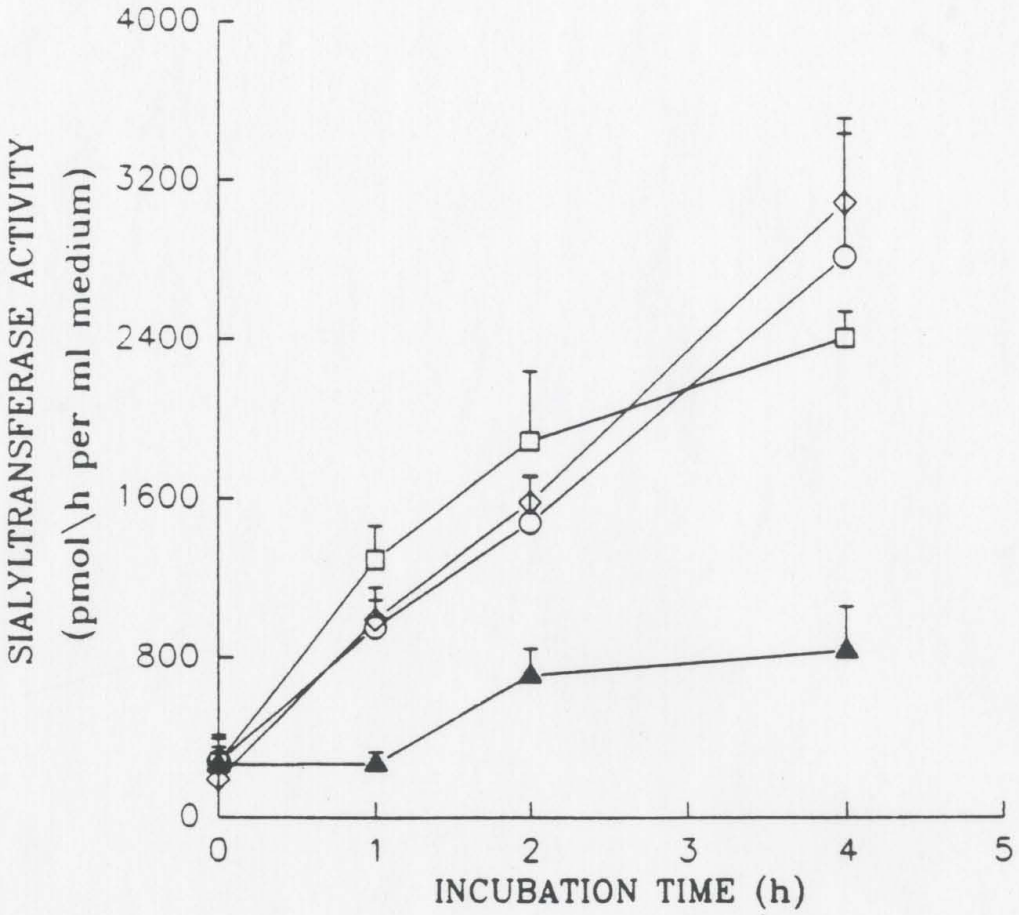


Figure 3.16. Effect of HBF and heparin-unbound fractions fractions of HHS on medium STase activity

Figure 3.16. Effect of HBF and heparin-unbound fractions of HHS on medium STase activity. Jejunal sections were incubated in KRB supplemented with either 20% (v/v) HHS (○), 20% (v/v) unbound-heparin fraction (▲), 20% (v/v) unbound sepharose fraction (□) or 500 μg HBF (◇) as described in Materials and Methods (sections II.2.3.A. and II.5.1). At the indicated times the medium samples were prepared and assayed for STase activity as described in Figure 3.1. STase activity is expressed as pmol of NeuAc transferred to DS-AGP/h per ml medium. Results represent means ± S.D. from four experiments.

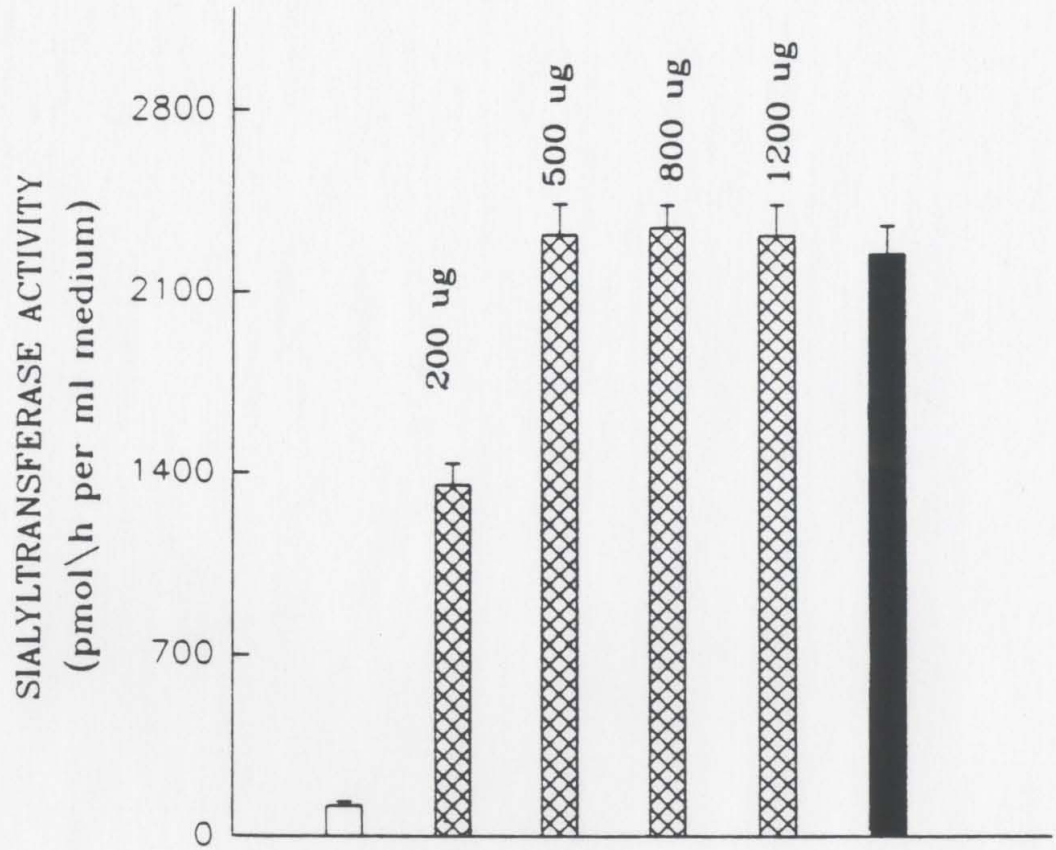


Figure 3.17. Effect of HBF on medium STase activity

Figure 3.17. Effect of HBF on medium STase activity. Jejunal sections were incubated in KRB (▭), or in KRB supplemented with either 20% (v/v) HHS (■) or 200 -1200 μg HBF (▩). After 4 h of incubation medium samples were prepared and STase activity measured as described in Figure 3.1 . STase activity is expressed as pmol of NeuAc transferred/h to DS-AGP per ml medium. Results represent means ± S.D. from four experiments.

STase activity released during jejunal incubations. HBF when added directly to the assays for STase did not increase STase activity indicating that HBF was required in the incubations in order to exert its protective effect on STase [Figure 3.18]. HBF was able to inhibit the hydrolysis of BAPNA by trypsin, indicating that HBF possessed antiproteolytic activity.

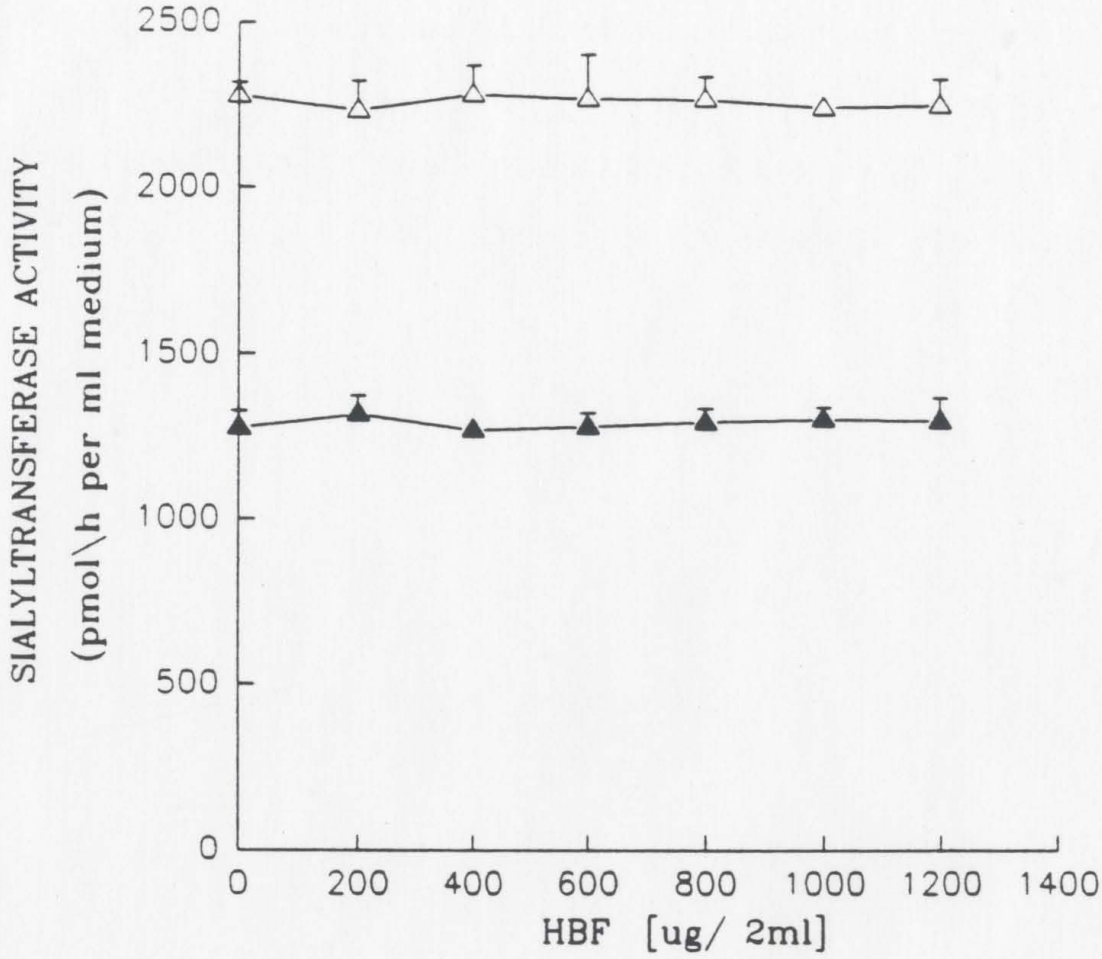


Figure 3.18. Effect of HBF on the assay for STase

Figure 3.18. Effect of HBF on the assay for STase. Jejunal slices were incubated in KRB supplemented with 20% (v/v) HHS. At 2 h (▲) and 4 h (△) medium samples were prepared as described in Figure 3.1. Increasing concentrations of HBF were added to samples which were assayed for STase activity. STase activity is expressed as pmol of NeuAc transferred/h to DS-AGP per ml medium. Results represent means ± S.D. from four experiments.

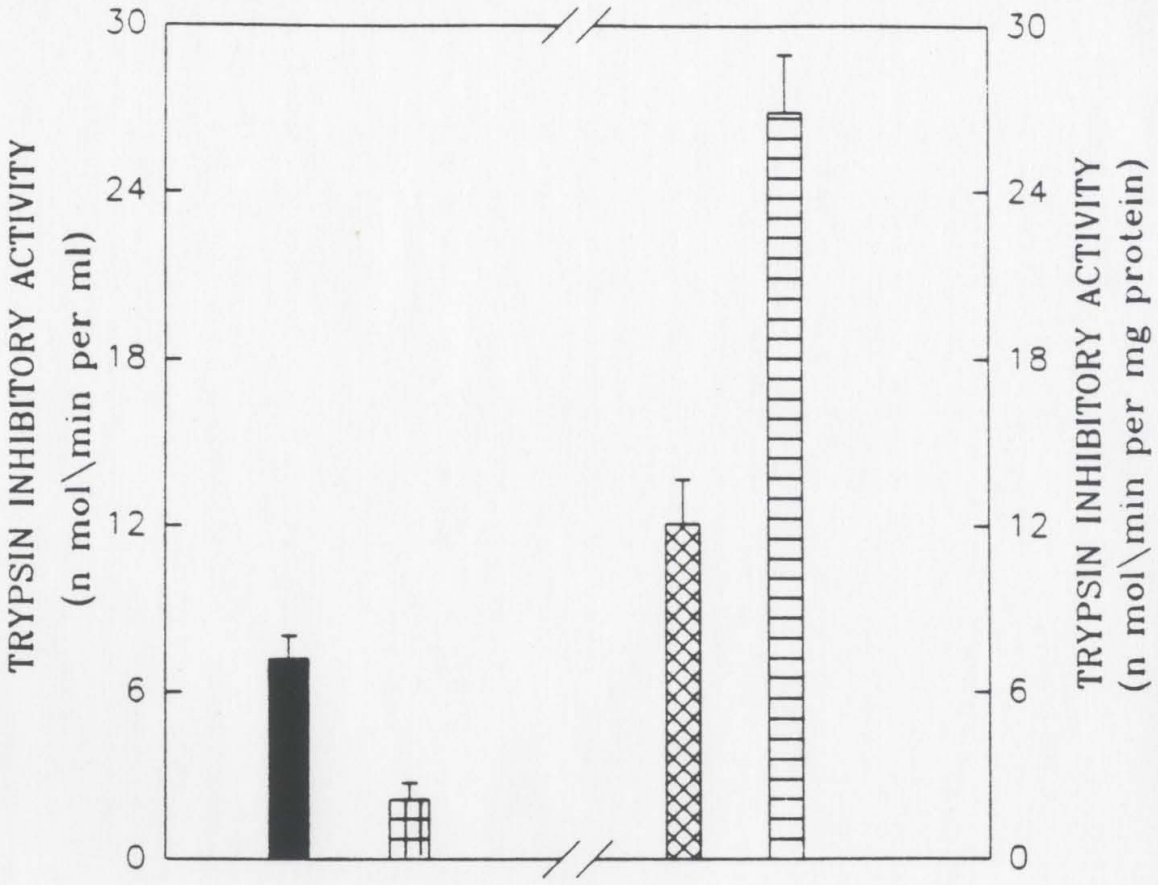


Figure 3.19. Trypsin inhibitory activity of HHS and its fractions

Figure 3.19. Trypsin inhibitory activity of HHS and its fractions. Inhibition of trypsin activity is compared between samples of HHS (■), unbound-heparin fraction (▦), HBF (▩) and A1PI (▤). Fraction preparation and assays for trypsin inhibitory activity were carried out as described in Materials and Methods using BAPNA as substrate (sections II.2.3.A and II.9.2.A). Trypsin inhibitory activity was obtained from the difference between activity in the presence and absence of the inhibitor and is expressed as nmol/min per ml serum for HHS and the unbound-heparin fraction, and as nmol/min per mg protein for HBF and A1PI. Results represent means ± S.D. from five experiments.

III.3. Discussion

The incubation of rat jejunal slices in KRB supplemented with 20% (v/v) HHS, lead to a time dependent appearance of STase activity in the medium for up to 6 h [Figure 3.1]. When the incubations were carried out in KRB alone or in KRB supplemented with either 30 mg BSA or 20(v/v) glycerol, only trace amounts of STase activity were detected in the medium. This indicated that among the different supplements used during incubations, HHS was the supplement which was required in order to measure STase activity in the incubation medium. These results suggested that HHS was exerting an effect either on the release process itself or on the STase activity released. The STase released was soluble in nature as the addition of Triton X-100 to the assays did not result in significant increases in activity [Figure 3.3]. These observations support those made by Ratnam et al., [1987] who reported that there was a need for HHS in the incubation medium in order to observe the release of STase during the incubation of jejunal slices.

Glutamine is the main fuel for intestinal cells [Neutra and Padykula, 1984], and was the nutrient source used during incubations. Therefore, the measurement of glutamine + glutamate during jejunal incubations was one of the criteria used to assess cell viability during incubations. Glutamine + glutamate in the incubation medium decreased with time of

incubation from 0 to 6 h [Figure 3.6]. This indicated that glutamine was either being utilized or broken down during incubations. In control experiments without jejunal tissue, wherein incubations were carried out in either KRB or in KRB + HHS, glutamine + glutamate decreased by approximately 5% over the 6 h of incubation. This suggested that the decrease in medium glutamine + glutamate during the 6 h of incubations in the presence of jejunal slices was probably the result of uptake of glutamine by the jejunal slices.

Other criteria used to determine the viability of the jejunal tissue slices during incubations, included measurement of the rates of incorporation of labelled glucosamine and leucine into acid-insoluble proteins. The results indicated that the cells were more efficient in synthesising proteins/ glycoproteins at 0 than at 4 h of incubation, though the cells were still synthesising proteins/ glycoproteins at 4 h [Figure 3.7]. It was also observed that there was no difference between the rates of glycoprotein or protein synthesis whether the jejunal slices were incubated in KRB alone or in KRB + HHS. Both the criteria used to assess viability namely the patterns of glutamine uptake and rates of glycoprotein/ protein synthesis showed similar results for incubations in KRB and KRB + HHS. Therefore the addition of HHS to KRB had no beneficial or detrimental effect on the viability of the jejunal slices. Also, the differential appearance of STase

activity in the presence of HHS could not be attributed to differences in viability between HHS-free and HHS-supplemented incubations.

Sialidase and CMP-NeuAc hydrolase activities were not higher in KRB incubations compared to incubations supplemented with HHS [Tables 3.1 and 3.2]. Therefore the low levels of STase activity detected in incubations carried out in KRB alone was not due to the increased activities of either CMP-sialic acid hydrolase and or sialidase.

According to the results presented in this section HHS or antiproteases were required in order for significant amounts of STase to be detected in the incubation medium. Among the antiproteases used A1PI and α_2 antiplasmin were the most effective in terms of reproducing the effect of HHS on STase activity [Table 3.3]. The medium obtained after jejunal incubations in KRB alone showed higher trypsin activity compared to medium obtained from HHS supplemented incubations [Figure 3.10]. This suggested that the higher proteolytic activity in KRB medium might be linked to the low levels of STase activity observed.

HHS when added immediately to medium obtained from jejunal incubations in KRB alone, caused a restoration of STase activity [Figures 3.8 and 3.9]. This indicated that STase was being released during incubations even in the

absence of HHS, but that HHS was required in order to measure STase activity. Also this raises the possibility that the proteases were less active in the presence of jejunal slices so it was only when the slices were removed that most of the proteolysis of STase occurred. Medium obtained from 4 h incubation of jejunal slices in KRB alone, when added to medium from 4 h HHS-supplemented incubations inhibited STase activity [Figure 3.11]. This suggested that there might be protease(s) in the incubation system which were destroying the STase activity released. This is supported by the observation that HHS, A1PI and α_2 antiplasmin were able to protect the STase.

The addition of heparin to jejunal incubations containing HHS decreased the STase activity released into the medium [Figures 3.12 and 3.13]. Though heparin also had an inhibitory effect on STase activity when added to assays for STase, the inhibition was greater when heparin was present during incubations. This led to further investigations for factor(s) in HHS which might bind to heparin and which had an effect on STase activity. Heparin is known to bind and interact with a number of molecules including growth factors [Quinkler et al., 1989; Zarnegar et al., 1990] and proteins [Raulais et al., 1991]. Using heparin-agarose HBF was separated from HHS [Figure 3.15]. HBF, when added to incubations, was able to

reproduce the effect of HHS on STase activity [Figures 3.16 and 3.17]. STase activity detected in the medium was markedly lower when the unbound-heparin fraction of HHS was used to supplement incubations, compared to incubations containing either HHS or HBF. Thus the removal of HBF by heparin resulted in the loss of protection of STase activity by HHS [Figure 3.16]. Furthermore HBF displayed trypsin inhibitory activity [Figure 3.19]. This implied that antiproteolytic serum components or proteins such as HBF, A1PI and α-2 antiplasmin played a vital role in determining the measurable STase activity released into the incubation medium.

A number of investigations have been directed towards studying STase in the small intestine [Kim et al., 1975; Biol et al., 1987: 1992]. Immunocytochemical studies have shown that STase was present in ER and Golgi membranes as well as in post-Golgi apparatus structures including mucous droplets and plasma membranes [Taatjes et al., 1988], indicating that STase is widely distributed in the membranes of intestinal cells. In these studies the focus has largely been towards defining the role of STase in cell renewal, differentiation and cell-cell interactions.

Other investigators have measured soluble STase and intestinal STase in pathological conditions. For instance, thermal injury in the rat resulted in increased STase activity in intestine and serum [Chu et al., 1988]. Colchicine

treatment in the rat resulted in increased STase activity in intestine, intestinal lymph and serum [Fraser et al., 1980; Ratnam et al., 1981, 1987]. These investigators have hypothesised that the intestine could contribute to the levels of soluble STase in extracellular fluids.

The factors which are involved in the release process are poorly understood. Since STases are membrane bound enzymes conversion to the soluble form would have to involve release from the membranes. The release of the STase molecule from the membrane is believed to occur due to proteolytic cleavage [Lammers and Jamieson, 1988, 1989, 1990; Colley et al., 1989; Jamieson et al., 1993: McCaffrey and Jamieson, 1993].

Ratnam and coworkers [1987] reported that there was an absolute requirement for HHS in the incubation medium in order to observe the release of STase from rat intestinal slices. The results presented in this chapter support this observation. However, this effect of HHS on STase activity was due most likely to the antiproteolytic properties of HHS. The results also indicate that HBF was the specific antiproteolytic component of HHS which governed the levels of STase released during jejunal incubations. Though HBF did not affect the release process directly, it is evident that the STase activity released was labile, HBF was able to protect STase and thereby permit the determination of STase levels in the incubation medium. The protease responsible for releasing

the soluble STase from the membrane is not the same as the protease which degrades STase because HHS did not prevent the release but only prevented degradation of the enzyme released. Also, HHS did not contain this protease because purified protease inhibitors (α_2 antiplasmin or A1PI) gave the same increase in STase as HHS did.

Among the antiproteases used A1PI and α-2 antiplasmin were most effective in reproducing the effect of HHS on STase activity [Table 3.3]. Trypsin activity was higher in medium obtained from KRB incubations compared to medium obtained from KRB + HHS incubations [Figure 3.10]. The addition of medium obtained after 4 h incubations of jejunal slices in KRB, to 4 h medium samples from KRB + HHS incubations caused an inhibition in STase activity. These results collectively suggest that protease(s) were present in the jejunal incubation system which were capable of destroying STase and that antiproteases when present were able to prevent proteolytic degradation of STase.

In conclusion the soluble STase activity released into the incubation medium during jejunal incubations was easily destroyed by proteases and antiproteases such as HBF, A1PI or α-2 antiplasmin were required in the incubation medium in order to measure the STase activity released. The results also imply that during jejunal incubations levels of protease

inhibitors and proteases may play key roles in determining the levels of STase activity released.

CHAPTER IV. RELATIONSHIP BETWEEN ANTIPROTEOLYTIC PROTEINS IN RAT SERUM AND THE GLYCOSYLTRANSFERASE ACTIVITIES RELEASED DURING JEJUNAL INCUBATIONS

IV.1. Introduction

GTases are enzymes which catalyse the transfer of Gal onto suitable glycoprotein or oligosaccharide acceptors [Schachter and Roseman., 1980; Beyer et al., 1981]. Gal residues on the glycopeptide chains usually serve as acceptors for the reaction catalyzed by STase, as NeuAc is usually added onto Gal residues. Increases in soluble GTase activity occur during cancer in extracellular fluids including serum [Podolsky et al., 1977,1978; Capel et al., 1982], ascitic fluid [Gerber et al., 1979] and pleural effusions [Ram and Mungal, 1984].

There are some pathophysiological conditions where, though increases in STase activity were observed, serum GTase activity was unchanged. For instance, during turpentine induced inflammation in the rat, increases were observed in liver activities of both STase and GTase [Kaplan et al., 1983; Fraser et al., 1984]. However in serum, though STase activity was increased, GTase remained unchanged. In the rat, following colchicine injection, increases in STase activity but not GTase activity, were observed in intestine, intestinal lymph

and serum [Fraser et al., 1980; Ratnam et al., 1981; Ratnam et al., 1987]. In both these conditions though soluble serum STase activity was increased GTase activity remained unchanged suggesting that the factors which control the levels of these two enzymes are different.

Results presented in the previous chapter indicated that STase released during the incubation of rat jejunal slices was labile. Also, antiproteolytic agents such as HHS, HBF isolated from HHS, A1PI or α-2 antiplasmin were required in the incubation medium in order to measure the STase activity released. The purposes of the experiments outlined in this chapter were

(1) to determine whether HBF from heat inactivated rat serum (HRS), like HBF from HHS had a role in protecting the STase activity released during jejunal incubations,

(2) to determine whether GTase was released during jejunal incubations and if GTase activity was dependant on HBF,

(3) to further study the relationship between protease inhibitory and STase activities and

(4) to characterise the protease inhibitory activity of HBF.

IV.2. Results

IV.2.1. Effect of HRS on the STase and GTase activities released during jejunal incubations

Figure 4.1 compares the release of STase and GTase from jejunal slices into the incubation medium, when the

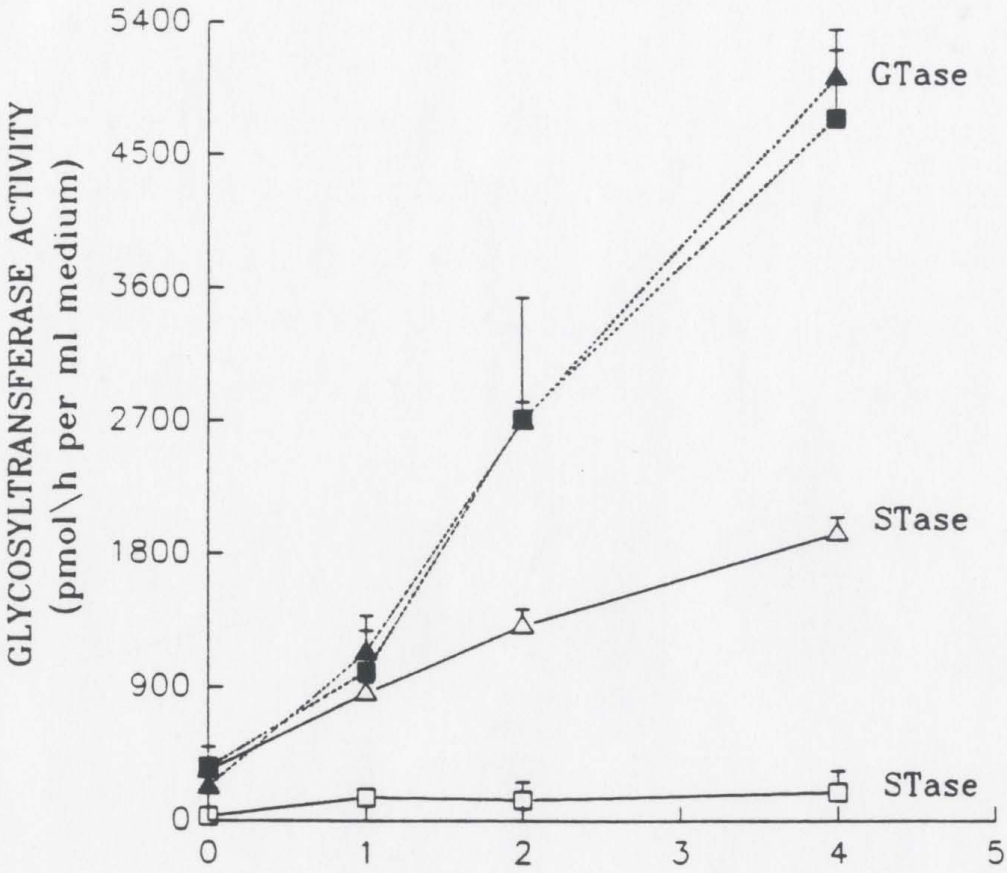


Figure 4.1. Effect of HRS in the incubation medium on STase and GTase release from jejunal slices

Figure 4.1. Effect of HRS in the incubation medium on STase and GTase release from jejunal slices. Jejunal slices were incubated in either KRB (□ , ■) or KRB + 20% (v/v) HRS (△ , ▲). At the indicated times, incubations were stopped, the medium decanted, centrifuged and assayed for STase and GTase activities as described in Materials and Methods (sections II.5.1., II.7.1. and II.7.2). STase (———) and GTase (·····) activities are expressed as pmol of NeuAc or Gal transferred to DS-AGP or DSG-AGP/h per ml medium. Results represent means ± S.D. from three experiments.

incubations were carried out in either KRB or KRB + 20% (v/v) HRS. The activities of STase and GTase in medium, increased with time for up to 4 h. Unlike STase, the increment of GTase activity was not dependent on the presence of HRS. STase activity, detected in the medium was significantly higher in samples obtained from KRB + HRS incubations, compared to incubations in KRB alone [Figure 4.1; $P < 0.001$ for the 1, 2 and 4 h time points]. In the case of GTase, activity remained similar in samples obtained from incubations in either KRB or KRB + HHS [Figure 4.1; $P > 0.05$ indicating that the means were not significantly different].

IV.2.2. Effect of HBF on STase and GTase activities released during jejunal incubations

Heparin when added to the incubations, inhibited the activity of the STase released in the presence of HRS [Figure 4.2; $P < 0.01$ for the 1, 2 and 4 h time points]. However, heparin did not have an effect on the GTase activity released into the medium during incubations in KRB or in KRB + 20% (v/v) HRS [Figure 4.3; $P > 0.05$ indicating that there was no significant difference between the means].

Using heparin-agarose the heparin-unbound fraction and HBF were separated from HRS [Figure 4.4]. When the unbound-heparin fraction (20%, v/v) was used to supplement incubations, STase activity in the medium was decreased compared to incubations supplemented with HRS (20%, v/v)

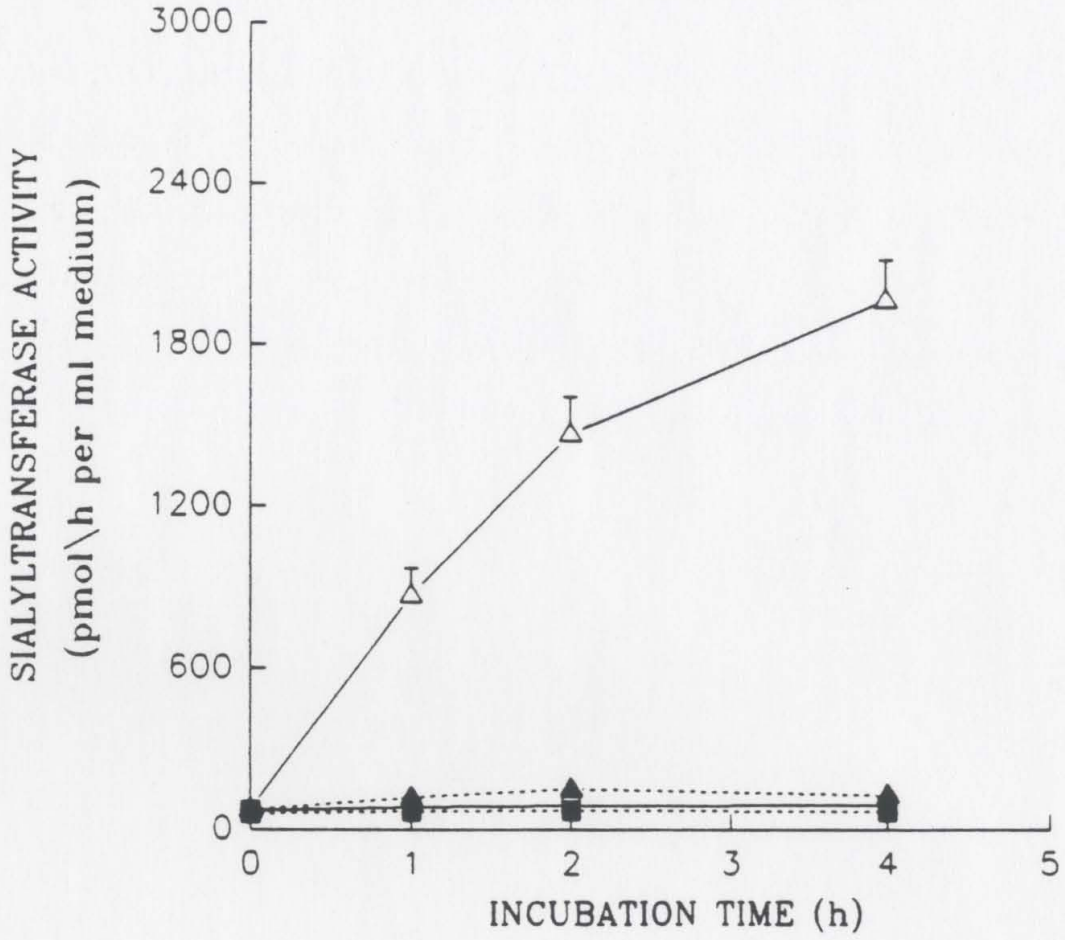


Figure 4.2. Effect of heparin on STase released from jejunal slices

Figure 4.2. Effect of heparin on STase released from jejunal slices. Heparin (100 μg/2ml) was added at 0 h to jejunal incubations carried out in KRB (····■····) or in KRB + 20% (v/v) HRS (····▲····). Control incubations were carried out in the absence of heparin in KRB (—□—) or KRB + 20% (v/v) HRS (—△—). At specified times, the medium samples were processed and STase activity measured as described in Figure 4.1. Results represent means ± S.D. from six experiments. STase activity is expressed as pmol of NeuAc transferred to DS-AGP/h per ml incubation medium.

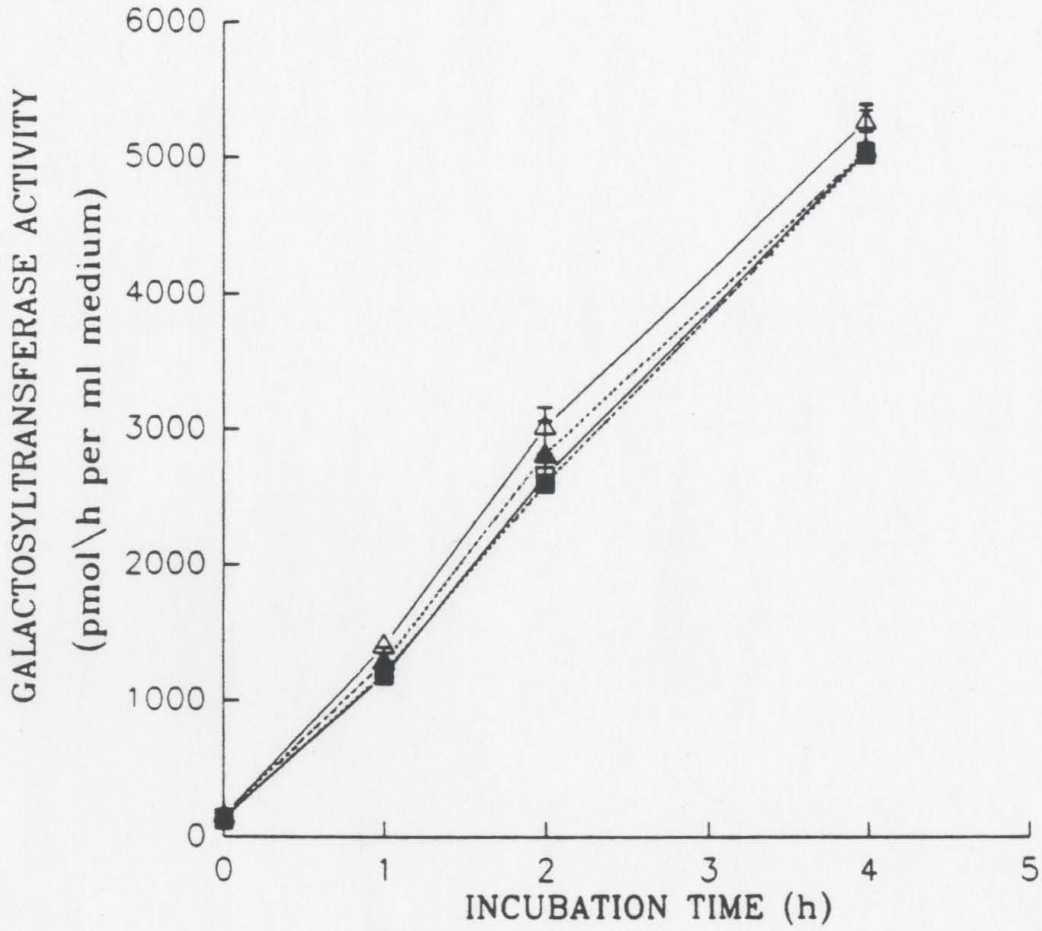


Figure 4.3. Effect of heparin on GTase released from jejunal slices

Figure 4.3. Effect of heparin on GTase released from jejunal slices. Heparin (100 μg/2ml) was added at 0 h to incubations carried out in KRB (···■···) or in KRB + 20% (v/v) HRS (···▲···). Control incubations were carried out without heparin in KRB (-□-) or in KRB + 20% (v/v) HRS (-△-). At the indicated times, the medium samples were processed and GTase activity measured as described in Figure 4.1. GTase activity is expressed as pmol of Gal transferred to DSG-AGP/h per ml medium. Results represent means ± S.D. from six experiments.

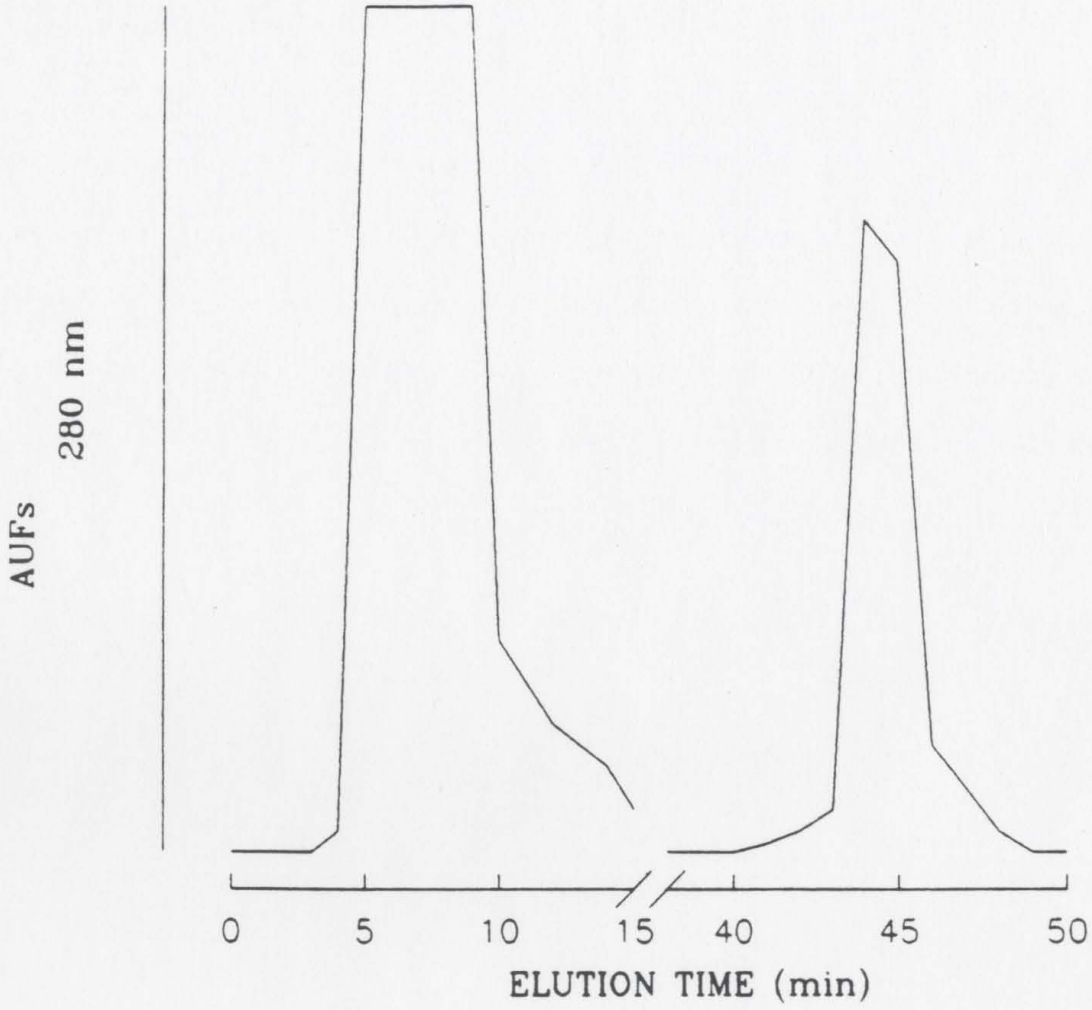


Figure 4.4. Affinity chromatography profile of the binding of HRS to heparin-agarose: separation of the heparin-binding fraction (HBF)

Figure 4.4. Affinity chromatography profile of the binding of HRS to heparin-agarose: separation of the heparin-binding fraction (HBF). HRS was applied to a heparin-agarose column which had been previously equilibrated with 5 mM Tris-buffer as described in Materials and Methods (section II.2.3.A). The column was then washed extensively with Tris-buffer until the absorbance of the eluent at 280 nm was less than 0.05. The bound fraction was eluted with 1 M NaCl in Tris-buffer and processed. This graph was regenerated by digitizing the elution profile with Sigmaplot 5.0.

[Figure 4.5; $P < 0.01$ for the 1, 2 and 4 h time points].When HBF (500 μg) was used to supplement KRB during incubations STase activity detected in the medium was similar to that observed when HRS was used as supplement [Figure 4.5; $P > 0.05$ indicating that the means for the 1, 2 and 4 h time points were not significantly different]. STase activity in 4 h medium was increased when HBF (from 500 - 1200μg) was used to supplement incubations, compared to incubations in KRB [Figure 4.6; $P < 0.001$ for incubations supplemented with either 200, 500, 800 or 1200 μg HBF compared to incubations without supplement].

In contrast to STase, GTase activity in the medium remained similar whether incubations were carried out in KRB or KRB supplemented with either HRS, HBF or unbound-heparin fraction [Figure 4.7; $P > 0.05$ indicating that for the 1, 2 and 4 h time points, there was no difference between samples from KRB incubations compared to samples from incubations supplemented with either HRS, HBF or the unbound-heparin fraction]. GTase activity was not affected by increasing the concentrations of HBF, as activity was similar between 4 h medium samples obtained from KRB incubations and incubations supplemented with 200 - 1200 μg HBF [Figure 4.8; $P > 0.05$ indicating that there was no significant difference between samples obtained from incubations in KRB, compared to samples obtained from incubations where KRB was supplemented with

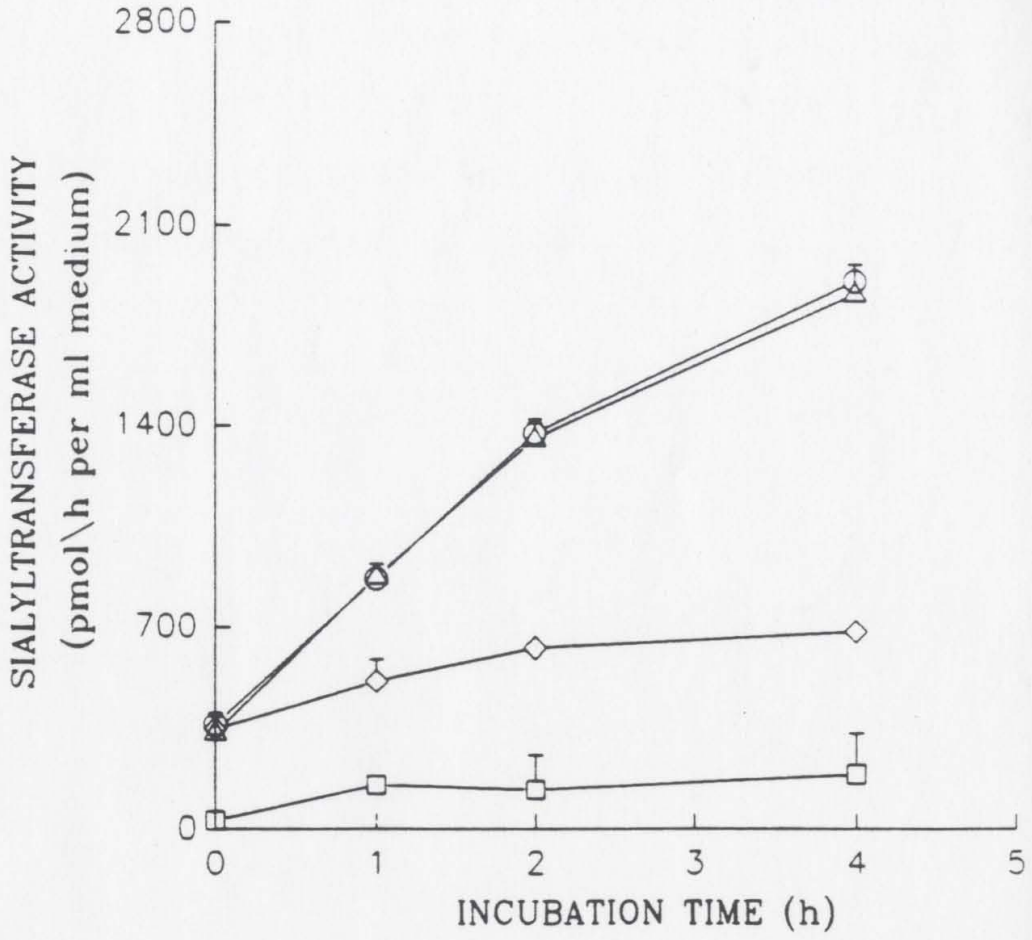


Figure 4.5. Effect of the HBF and heparin-unbound fraction of HRS on the medium STase activity

Figure 4.5. Effect of the HBF and heparin-unbound fraction of HRS on the medium STase activity. Jejunal sections were incubated in KRB (□) or in KRB supplemented with either 20% (v/v) HRS (△), 20% (v/v) unbound-heparin fraction (◇) or 500 μg HBF (○). At the indicated times medium samples were processed and STase activity measured as described in Figure 4.1. STase activity is expressed as pmol of NeuAc transferred to DSG-AGP/h per ml medium. Results represent means ± S.D. from four experiments.

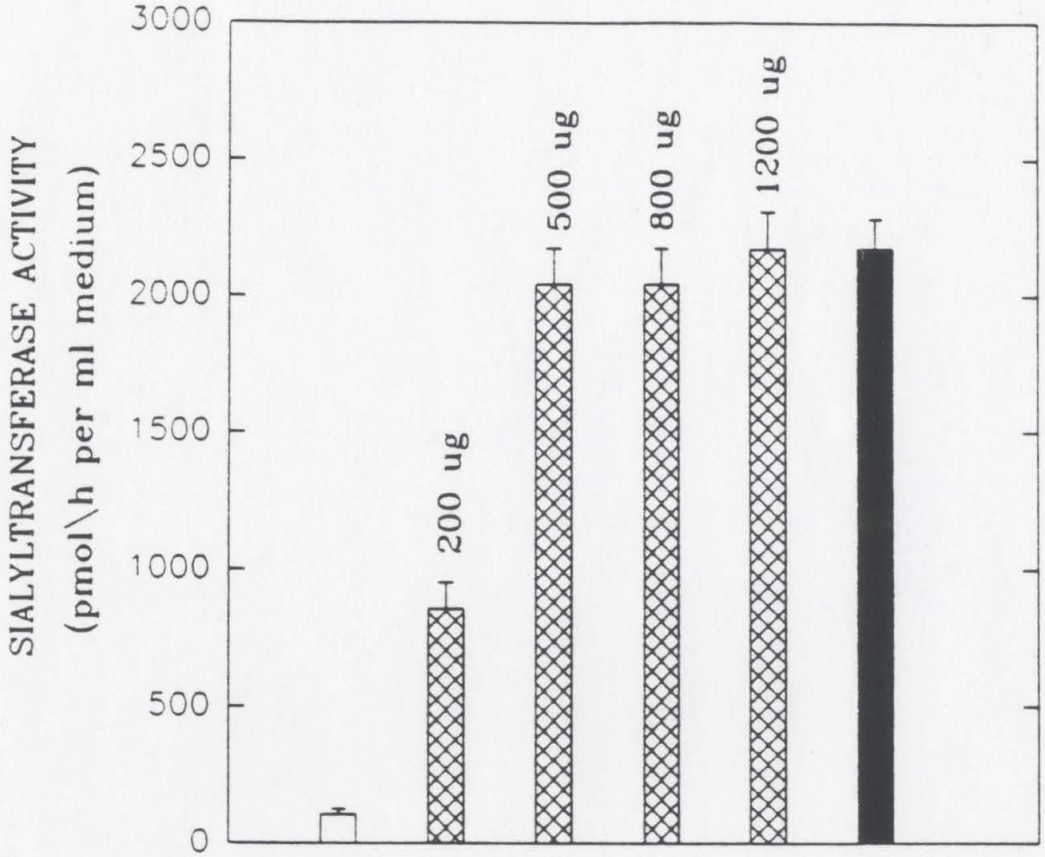


Figure 4.6. Effect of HBF on the medium STase activity

Figure 4.6. Effect of HBF on the medium STase activity. Jejunal slices were incubated in KRB (☐) or in KRB supplemented with either 20% (v/v) HRS (■) or increasing concentrations [200 to of 1200] μg HBF (⊠). Medium samples were prepared after 4 h of incubation and STase activity was measured as described in Figure 4.1. STase activity is expressed as pmol of NeuAc transferred to DS-AGP/h per ml incubation medium. Results represent means ± S.D. from four experiments.

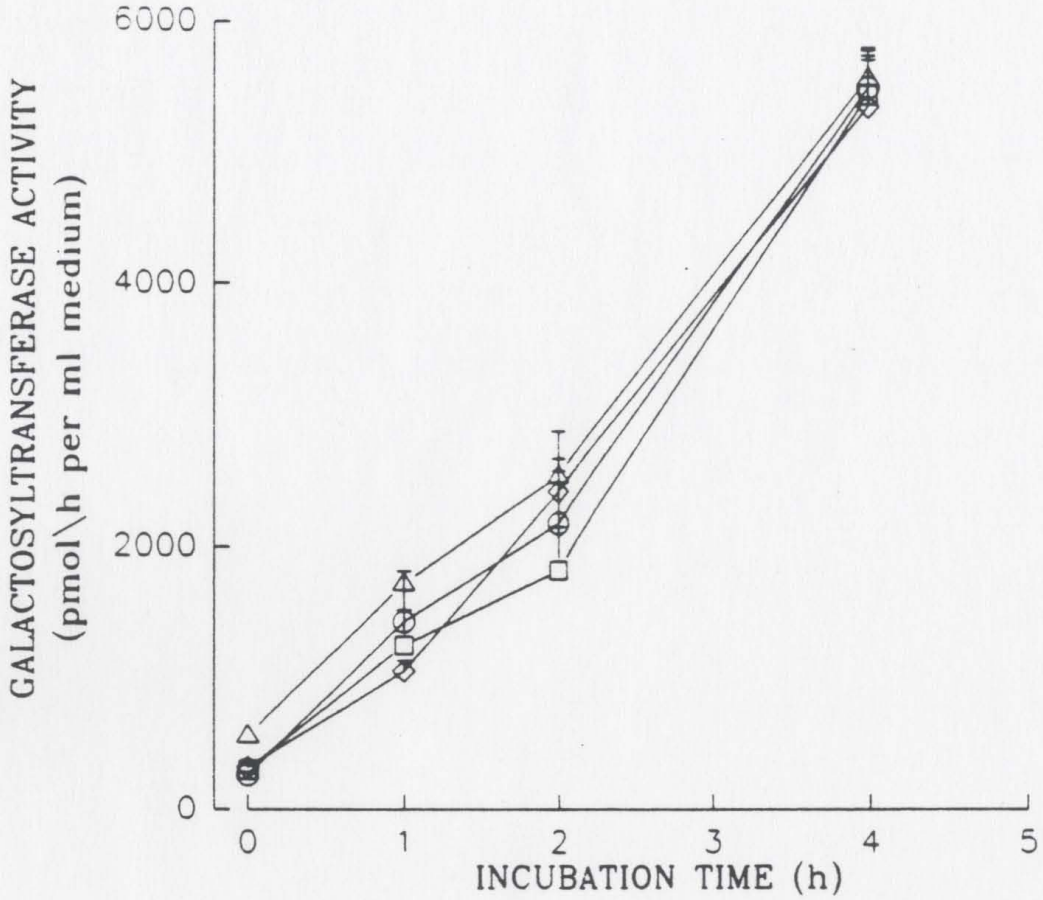


Figure 4.7. Effect of HBF and heparin-unbound fraction of HRS on the medium GTase activity

Figure 4.7. Effect of the HBF and heparin-unbound fractions of HRS on the medium GTase activity. Jejunal slices were incubated in KRB (□) or in KRB supplemented with either 20% (v/v) HRS (△), 500 μg HBF (◇) or 20% (v/v) unbound-heparin fraction (○). At the indicated times the incubations were stopped, medium samples processed and GTase activity measured as described in Figure 4.1. GTase activity is expressed as pmol of Gal transferred to DSG-AGP/h per ml medium. Results represent means ± S.D. from five experiments.

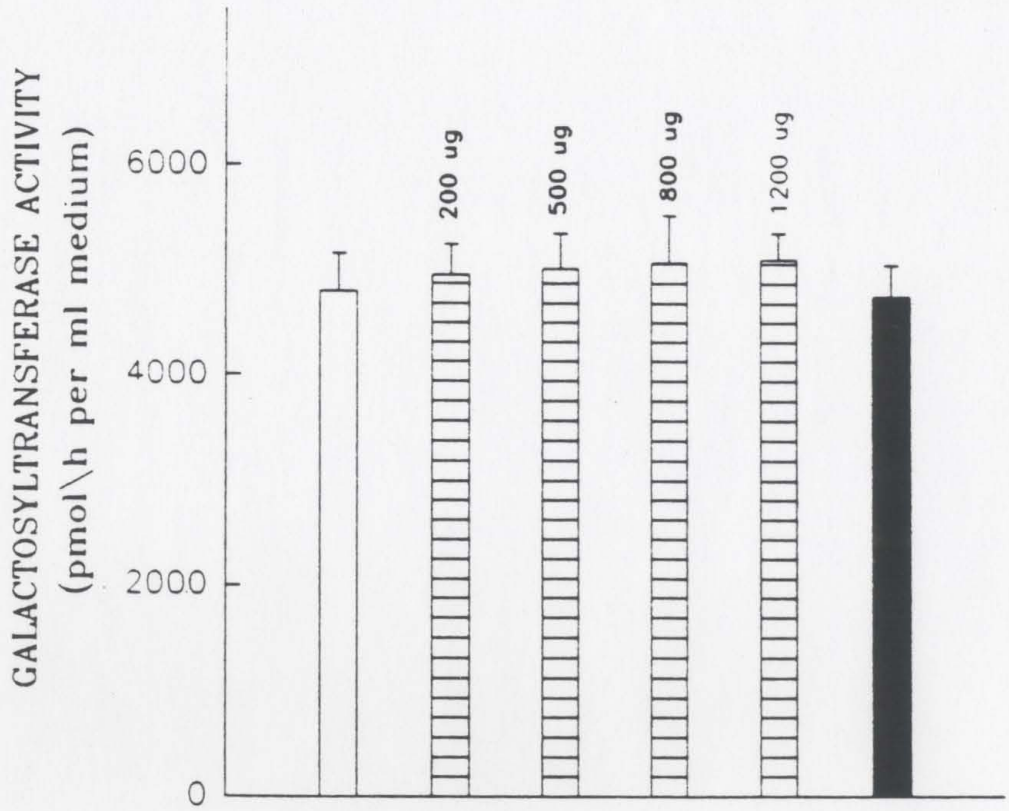


Figure 4.8. Effect of HBF on the medium GTase activity

Figure 4.8. Effect of HBF on the medium GTase activity. Jejunal slices were incubated in KRB (▭) or in KRB supplemented with either 20% (v/v) HRS (▬) or increasing concentrations [200 to of 1200] μg HBF (▤). Medium samples were prepared after 4 h of incubation and GTase activity was measured as described in Figure 4.1. GTase activity is expressed as pmol of Gal transferred to DSG-AGP/h per ml incubation medium. Results represent means ± S.D. from four experiments.

either 200, 500, 800 or 1200 μg HBF].

IV.2.3. Protease and protease inhibitory activities in the medium and their effect on STase and GTase activities

Trypsin and plasmin activities in the medium increased with time when the jejunal slices were incubated in either KRB alone or in KRB supplemented with either 30 mg BSA or 20% (v/v) HRS [Figures 4.9 and 4.10]. In incubations where KRB was supplemented with 20% (v/v) HRS, both trypsin and plasmin activities in the medium remained markedly lower compared to medium from incubations in either KRB or KRB + BSA [Figures 4.9 and 4.10]. For trypsin activity, $P < 0.001$ for the 1, 2 and 4 h time points for samples incubated in KRB and KRB + BSA respectively, compared to KRB + HRS incubations. Likewise, for plasmin activity $P < 0.001$ for the 1, 2 and 4 h time points for samples incubated in KRB and KRB + BSA respectively, compared to KRB + HRS incubations.

The addition of 500 μg of either A1PI or α_2 antiplasmin to jejunal incubations in either KRB, KRB + BSA or KRB + HRS resulted in decreased trypsin and plasmin activities in the medium compared to incubations without A1PI or α_2 antiplasmin. For trypsin activity in samples with and without A1PI, $P < 0.01$ for the 1, 2 and 4 h time points, when incubations were carried out in KRB + HRS, KRB + BSA and KRB respectively. Similarly, for plasmin activity in samples with and without α_2

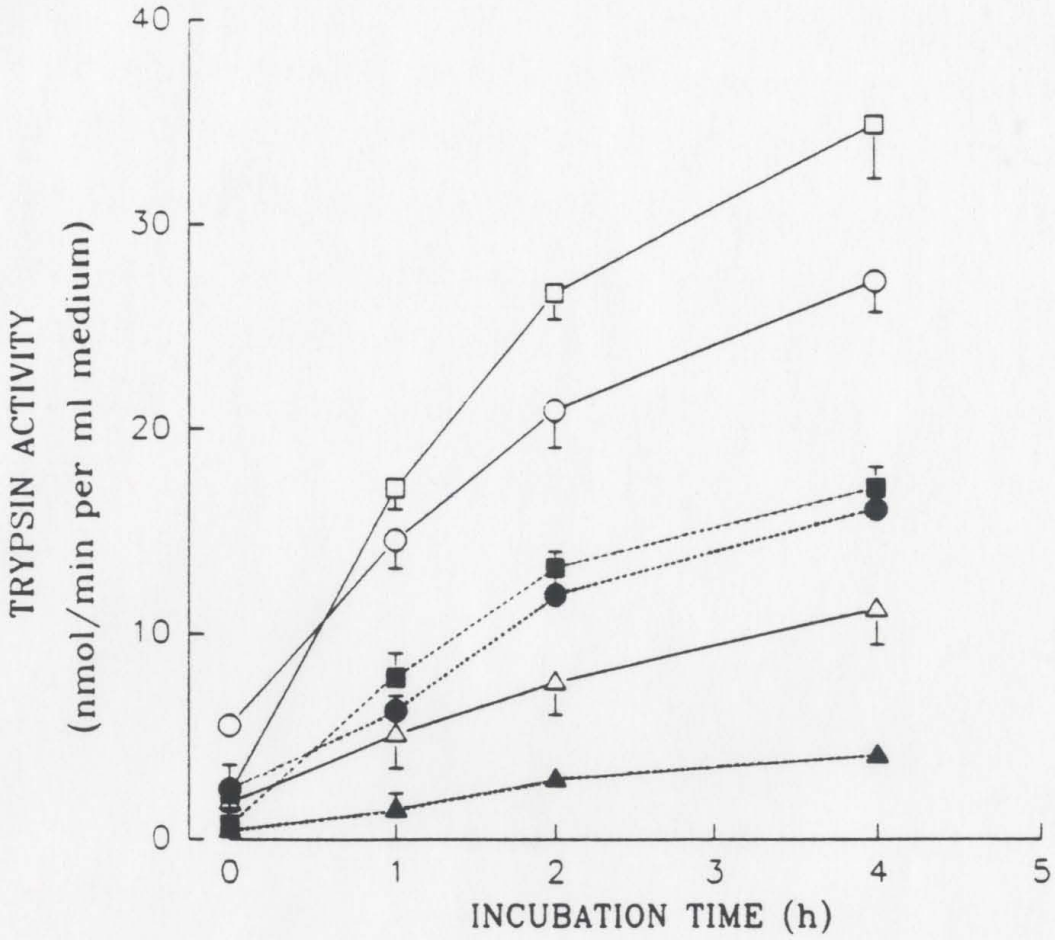


Figure 4.9. Trypsin activity in medium

Figure 4.9. Trypsin activity in medium. Jejunal slices were incubated in KRB (—□—), or in KRB supplemented with either 30 mg BSA (—○—) or 20% (v/v) HRS (—△—) as described for Figure 4.1. Parallel experiments were carried out wherein 500 μg A1PI was added to jejunal slices incubated in either KRB (···■···), KRB + 30 mg BSA (···●···) or KRB + 20% (v/v) HRS (···▲···). At the indicated times incubations were stopped, the medium was decanted, centrifuged and assayed for trypsin activity as described in Materials and Methods (section II.11.2.A). Trypsin activity is expressed as n mol of substrate hydrolysed/ min per ml medium. Results represent means ± S.D. from four experiments.

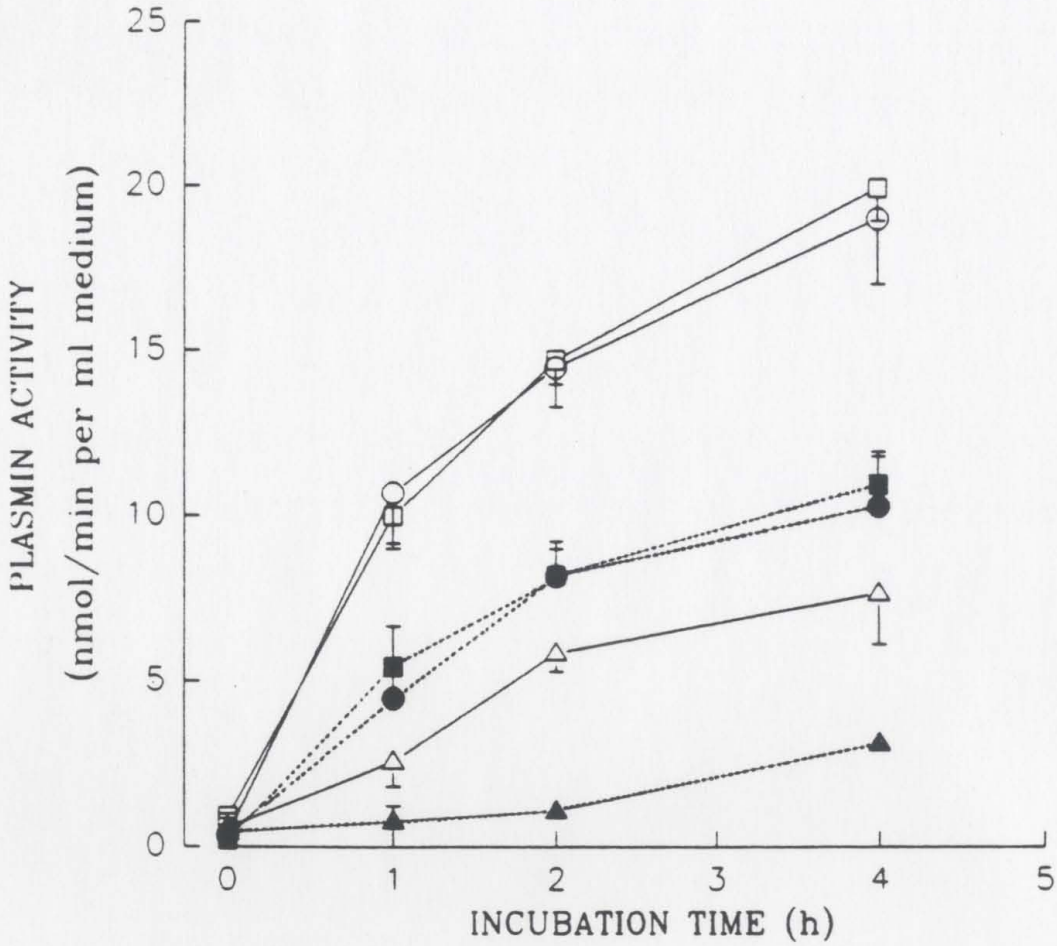


Figure 4.10. Plasmin activity in medium

Figure 4.10. Plasmin activity in medium. Jejunal slices were incubated in KRB (-□-) or in KRB supplemented with either 30 mg BSA (-○-) or 20% (v/v) HRS (-△-) as described for Figure 4.1. Parallel experiments were carried out wherein 500 μg α_2 antiplasmin was added to jejunal slices incubated in either KRB (··■··), KRB + 30 mg BSA (··●··) or KRB + 20% (v/v) HRS (··▲··). At the indicated times incubations were stopped, the medium was decanted, centrifuged and assayed for plasmin activity as described in Materials and Methods (section 2.11.3.[A]). Plasmin activity is expressed as n mol of substrate hydrolysed/ min per ml medium. Results represent means ± S.D. from four experiments.

antiplasmin, $P < 0.01$ for the 1, 2 and 4 h time points, when incubations were carried out in KRB + HRS, KRB + BSA and KRBrespectively.

STase activity was higher in medium obtained from incubations where KRB was supplemented with either A1P1 or α_2 antiplasmin, compared to incubations in KRB [Figure 4.11]. $P < 0.01$ for the 1, 2 and 4 h time points, when the incubations were supplemented with α_2 antiplasmin, A1PI and HRS respectively, compared to incubations in KRB. In the case of GTase, activity remained similar whether the incubations were carried out in KRB or in KRB supplemented with either HRS, A1PI or α_2 antiplasmin [Figure 4.12; $P > 0.05$ indicating that the means were not significantly different for the 1, 2 and 4 h time points].

Addition of 100 μg heparin to jejunal incubations containing HRS resulted in increases in trypsin and plasmin activities [Figures 4.13 and 4.14]. In the case of trypsin activity $P < 0.001$ for the 1, 2 and 4 h time points between samples obtained from KRB + HRS incubations compared to KRB + HRS + heparin incubations. Likewise, for plasmin activity $P < 0.001$ for the 1, 2 and 4 h time points between samples from KRB + HRS incubations compared to KRB + HRS + heparin incubations. The addition of increasing amounts of KRB medium which was obtained after 4 h of incubation, to medium obtained

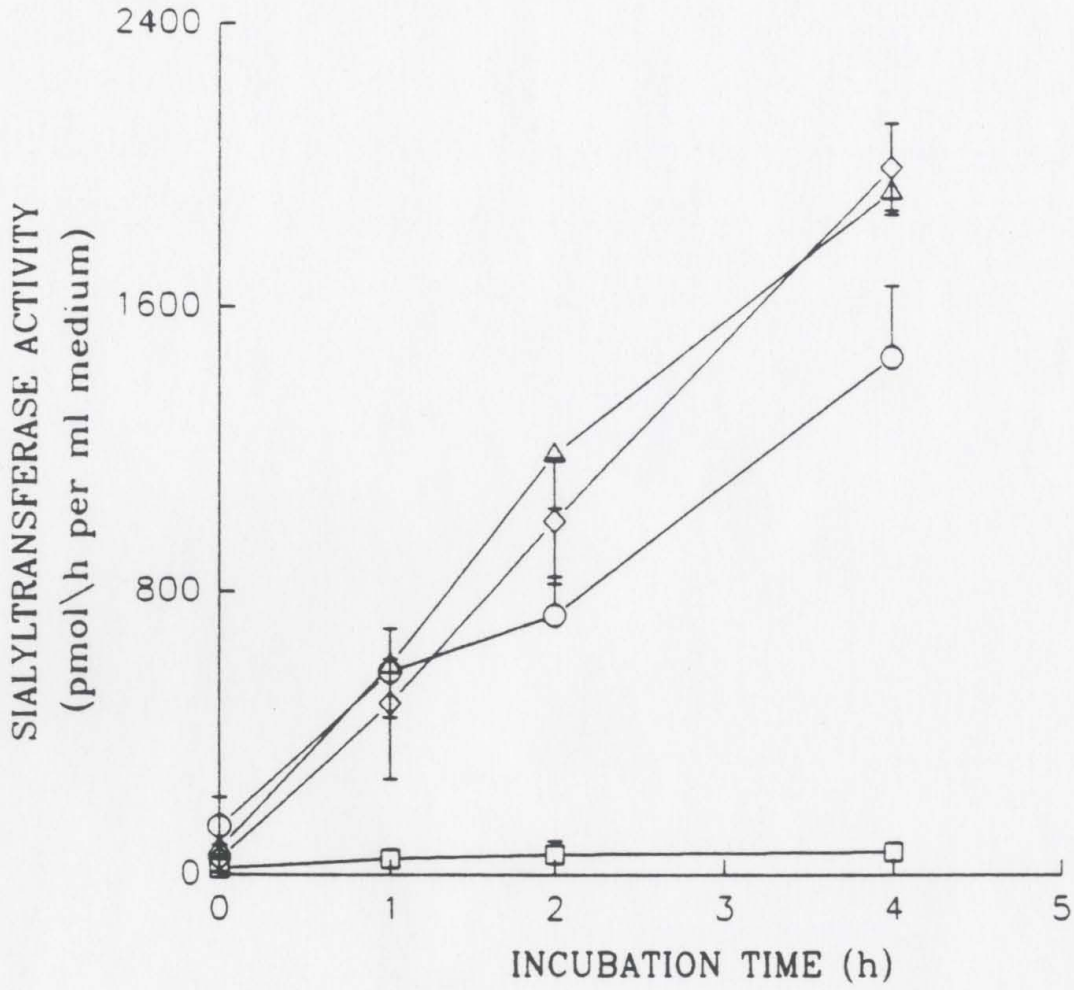


Figure 4.11. Effect of addition of A1PI and α2 antiplasmin to jejunal incubations on STase activity

Figure 4.11. Effect of addition of A1PI and α_2 antiplasmin to jejunal incubations on STase activity. Jejunal slices were incubated in either KRB (□) or in KRB supplemented with either 20% (v/v) HRS (△), 500 μg A1PI (◇) or 500 μg α_2 antiplasmin (○). Medium samples were prepared and STase activity measured as described in Figure 4.1 . STase activity is expressed as pmol of NeuAc transferred to DS-AGP/h per ml medium. Results represent means ± S.D. from four experiments.

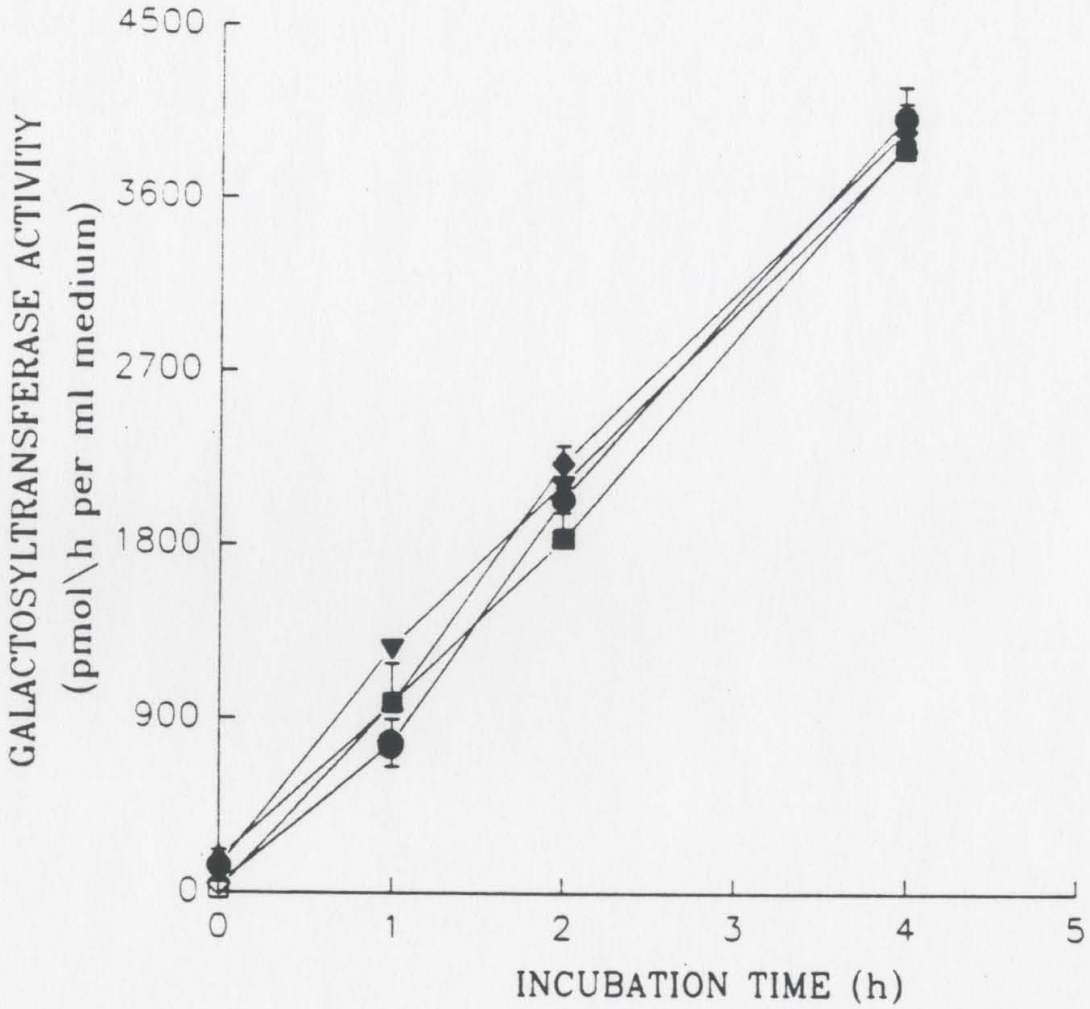


Figure 4.12. Effect of addition of A1PI and α2 antiplasmin to jejunal incubations on GTase activity

Figure 4.12. Effect of addition of A1PI and α-2 antiplasmin to jejunal incubations on GTase activity. Jejunal slices were incubated in either KRB (■) or in KRB supplemented with either 20% (v/v) HRS (▲), 500 μg A1PI (●) or 500 μg α_2 antiplasmin (◆). Medium samples were prepared and GTase activity measured as described in Figure 4.1. GTase is expressed as pmol of Gal transferred to DS-AGP/h per ml medium. Results represent means ± S.D. from four experiments.

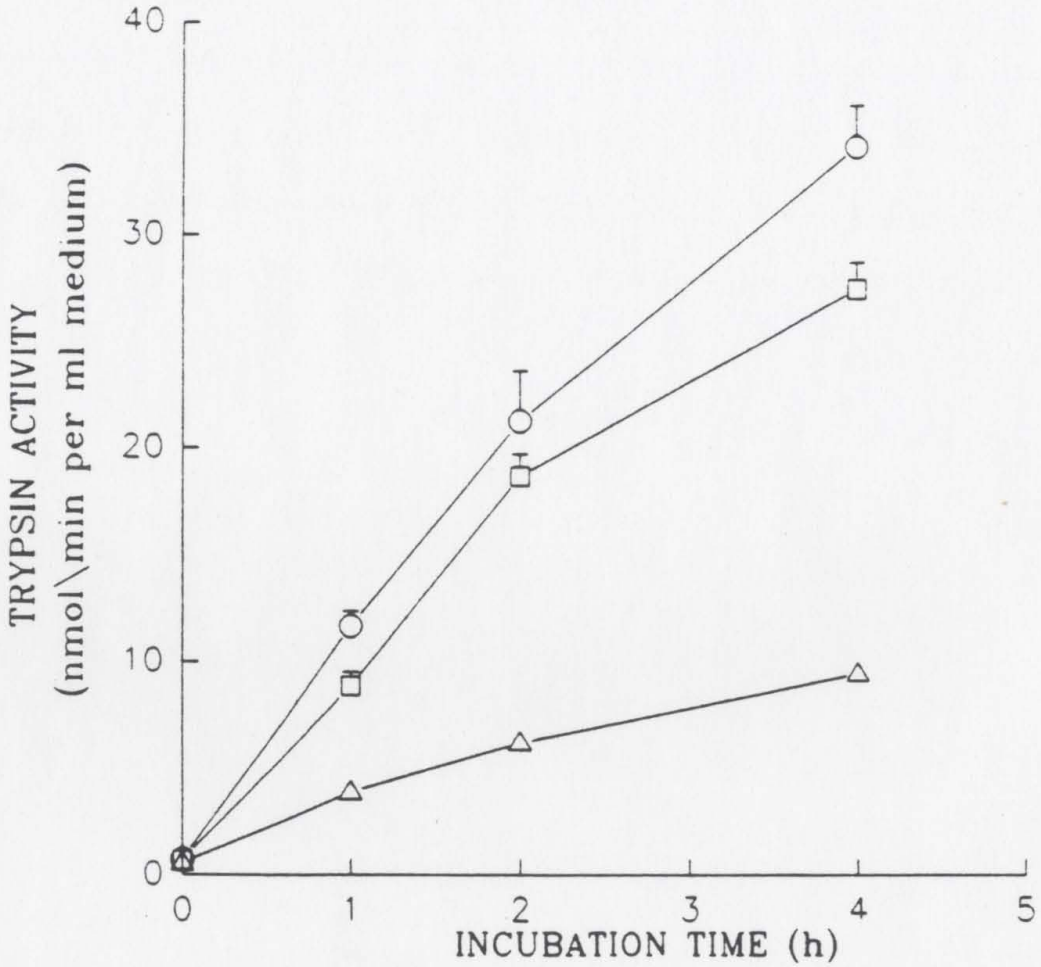


Figure 4.13. Effect of heparin on trypsin activity

Figure 4.13. Effect of heparin on trypsin activity. Jejunal slices were incubated in KRB (□) or in KRB supplemented with either 20% (v/v) HRS (△) or 20% (v/v) HRS + 100 μg heparin (○). At indicated times medium samples were prepared and assayed for trypsin activity as described in Figure 4.9. Trypsin activity is expressed as nmol of substrate hydrolysed/ min per ml incubation medium. Results represent means ± S.D. from four experiments.

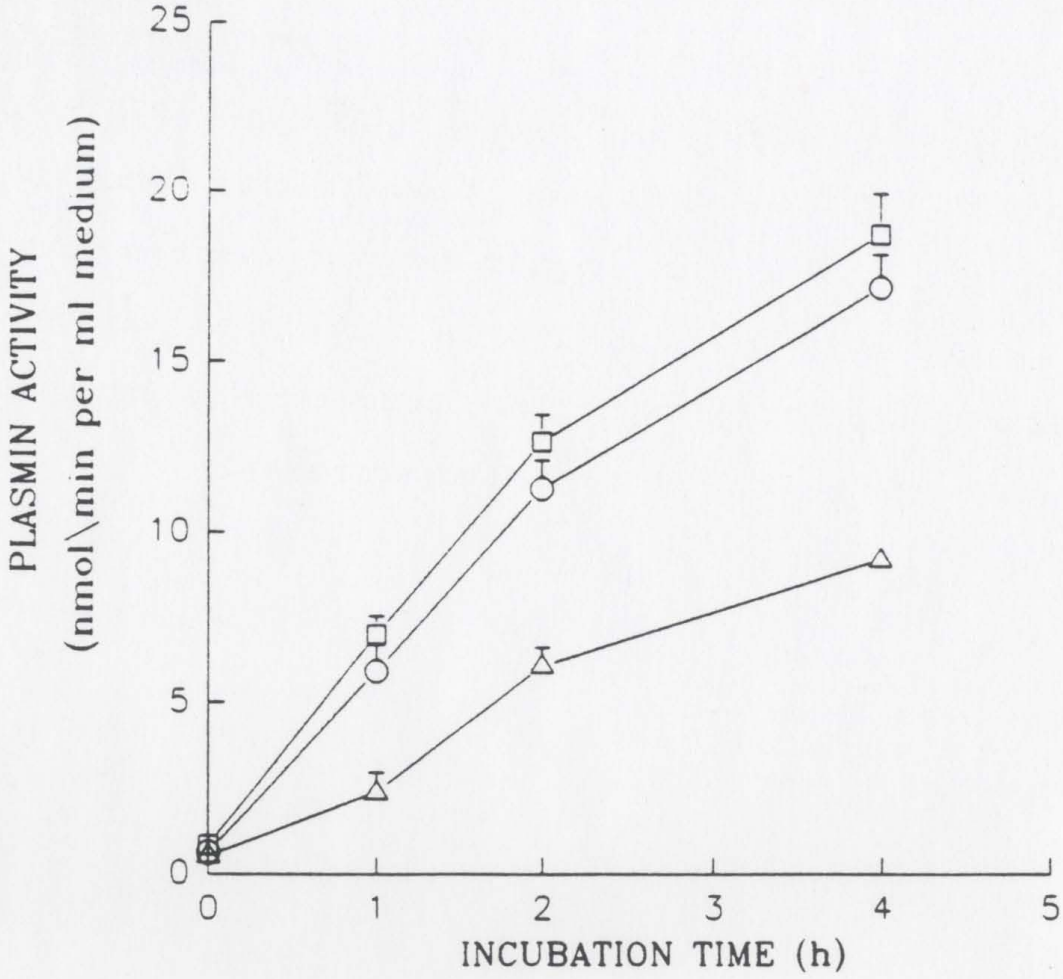


Figure 4.14. Effect of heparin on plasmin activity

Figure 4.14. Effect of heparin on plasmin activity. Jejunal slices were incubated in KRB (□) or in KRB supplemented with either 20% (v/v) HRS (△) or 20% (v/v) HRS + 100 μg heparin (○). At indicated times medium samples were prepared and assayed for plasmin activity as described for Figure 4.10. Plasmin activity is expressed as nmol of substrate hydrolysed/ min per ml incubation medium. Results represent means ± S.D. from four experiments.

from 4 h incubations in KRB + 20% (v/v) HRS, resulted in inhibition of STase activity [Figure 4.15]. Similar inhibition was not observed for GTase where the addition of 4 h KRB medium caused an increase in GTase activity in 4 h medium samples from KRB + HRS incubations [Figure 4.15]. This increase in GTase activity was due to the fact that GTase activity was present in the 4h KRB medium being added.

Heat-inactivated serum from turpentine treated rats had higher trypsin and plasmin inhibitory activities compared to heat inactivated serum from control rats [Figure 4.16; $P < 0.001$ for trypsin and plasmin inhibitory activities respectively, between heat inactivated sera from turpentine and control rats]. When heat-inactivated serum from turpentine treated rats was used to supplement incubations, trypsin activity in the 2 and 4 h medium was decreased compared to incubations where heat- inactivated serum from control rats was used [Figure 4.17; $P < 0.001$ for the 2 and 4 h time points]. Likewise the addition of heat-inactivated serum from turpentine treated rats resulted in decreased plasmin activity in medium compared to incubations containing heat-inactivated control serum [Figure 4.18; $P < 0.01$ for the 2 and 4 h time points].

When heat-inactivated control serum was used in incubations trypsin and plasmin activities were decreased in the 2 and 4 h medium samples compared to incubations where KRB

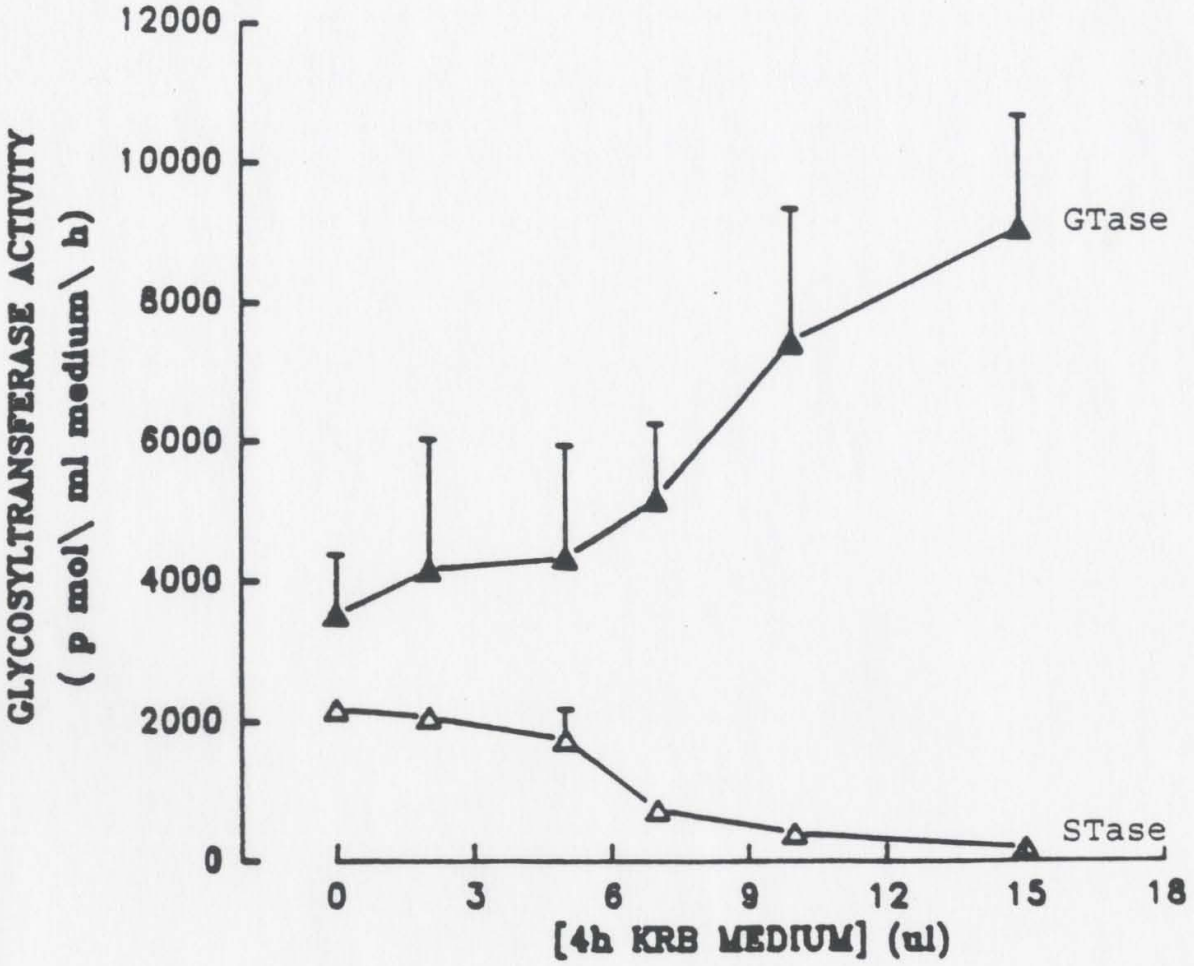


Figure 4.15. Effect of addition of 4 h KRB medium on STase and GTase activities

Figure 4.15. Effect of addition of 4 h KRB medium on STase and GTase activities. Jejunal slices were incubated for 4h in KRB alone or in KRB supplemented with 20% (v/v) HRS as described in Figure 4.1 and medium samples prepared. STase (△) and GTase (▲) activities were measured in HRS supplemented medium in the presence of increasing volumes of 4 h KRB medium as described. Glycosyltransferase activities are expressed as pmol of NeuAc or Gal transferred/h to DS-AGP and DSG-AGP respectively per ml of incubation medium. Results represent means ± S.D. from five experiments.

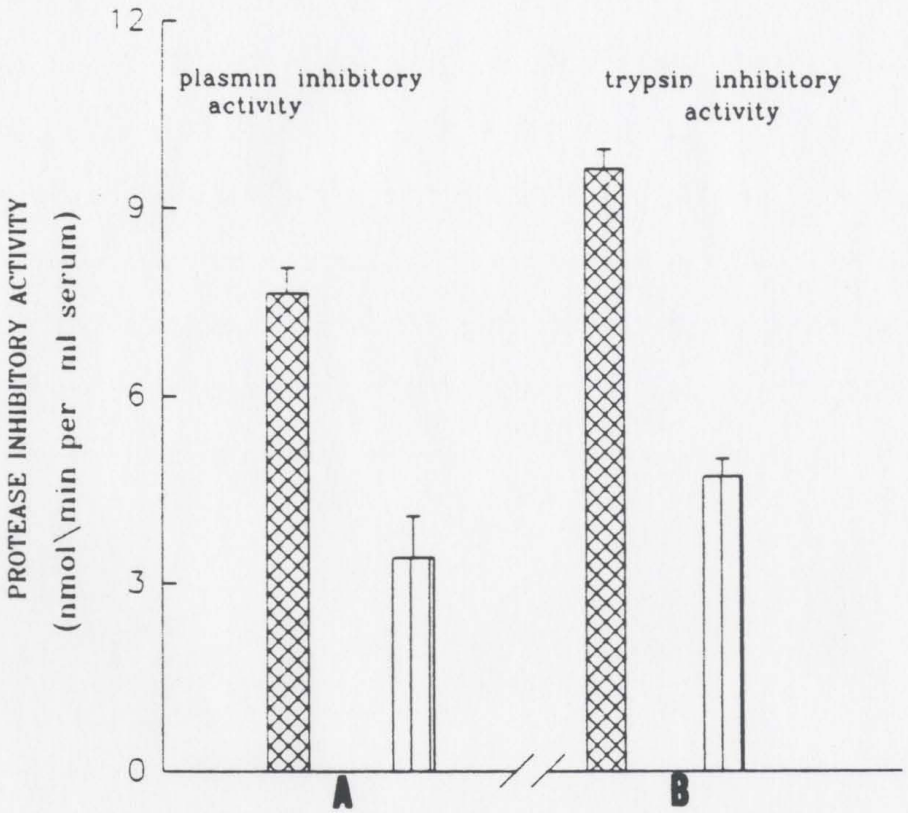


Figure 4.16. Trypsin and plasmin inhibitory activities in heat inactivated sera from turpentine treated and control rats

Figure 4.16. Trypsin and plasmin inhibitory activities in heat inactivated sera from turpentine treated and control rats. Serum was obtained from turpentine treated rats (▩) and control rats (□), trypsin and plasmin inhibitory activities were measured as described in Materials and Methods (sections II.3.1, II.3.2, II.11.2.A. and II.11.3.A. Trypsin (panel B) and plasmin (panel A) inhibitory activities were obtained from the differences between activities in the presence and absence of inhibitor as nmol/ min per ml serum. BAPNA and BAEE were the substrates used for trypsin and plasmin respectively. Results represent means ± S.D. from six experiments.

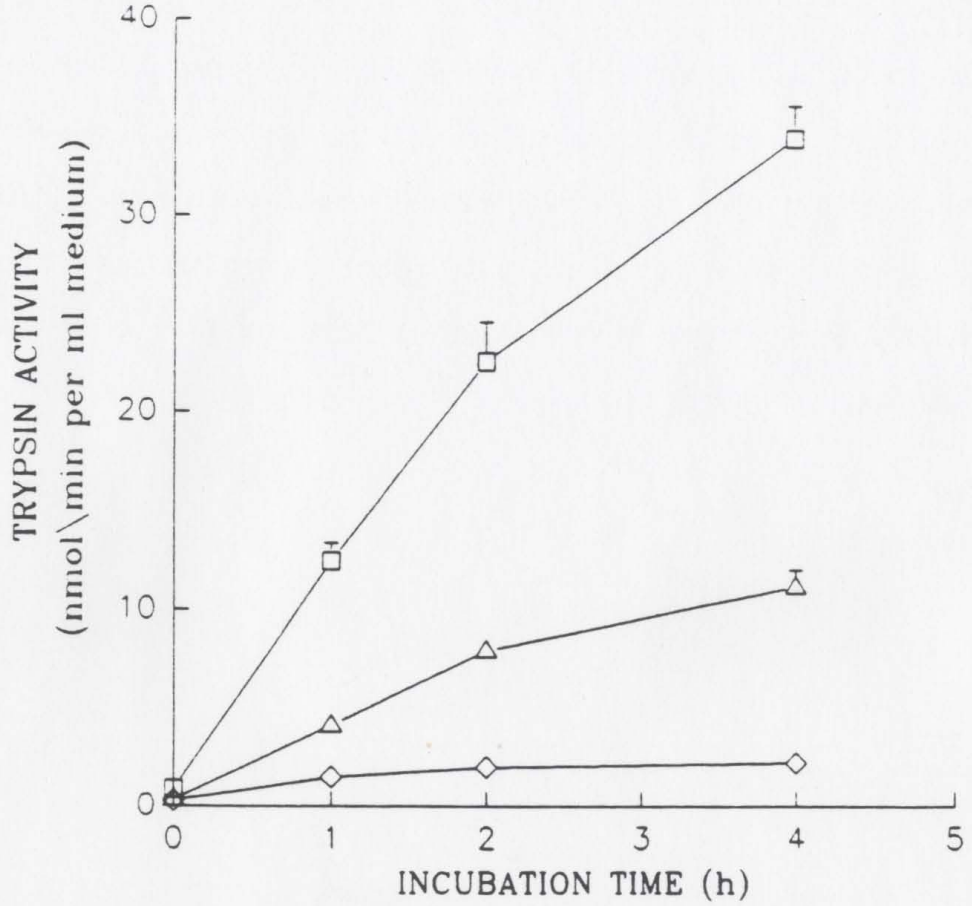


Figure 4.17. Trypsin activity in medium supplemented with heat-inactivated serum from turpentine treated rats

Figure 4.17. Trypsin activity in medium supplemented with heat-inactivated serum from turpentine treated rats. Jejunal slices were incubated in KRB (□) or in KRB supplemented with 20% (v/v) heat-inactivated serum from either turpentine treated rats (◇) or control rats (△) and medium samples prepared as described in Figure 4.1. Trypsin activity was assayed using BAPNA as substrate as described for Figure 4.9 and is expressed as n mol of substrate hydrolysed/ min per ml of incubation medium. Results represent means ± S.D. from six experiments.

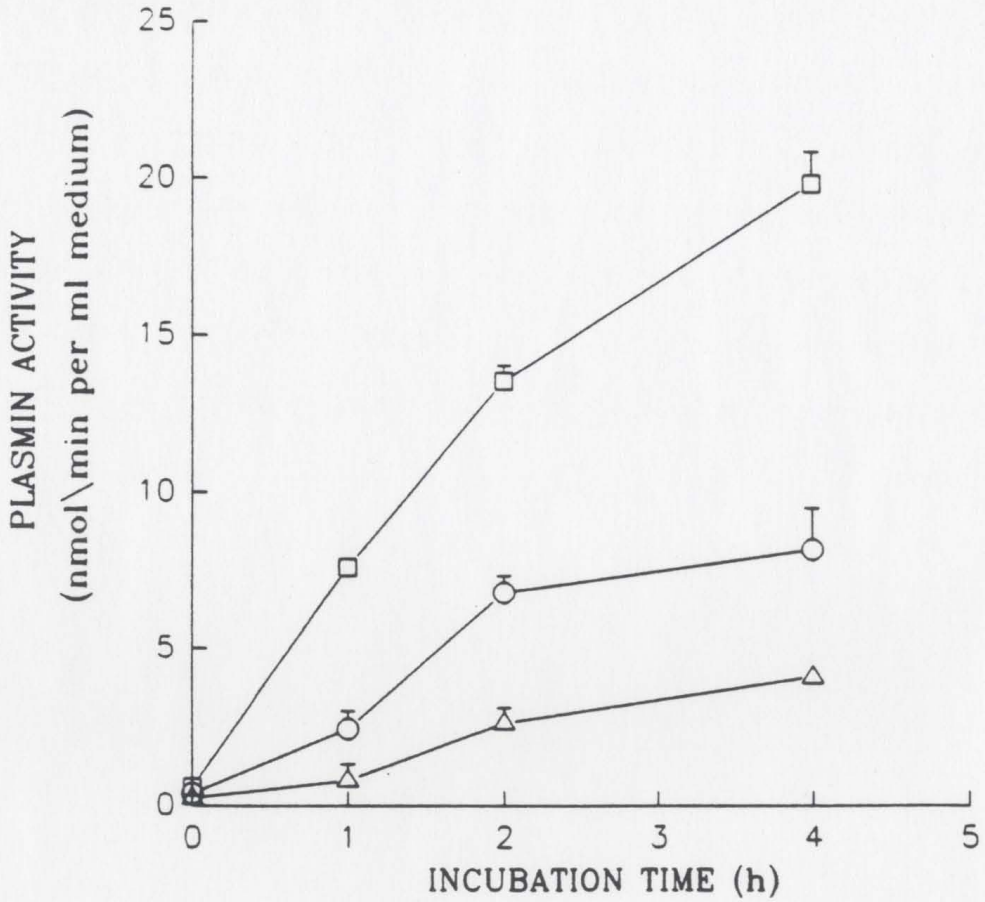


Figure 4.18. Plasmin activity in medium supplemented with heat-inactivated serum from turpentine treated rats

Figure 4.18. Plasmin activity in medium supplemented with heat-inactivated serum from turpentine treated rats. Jejunal slices were incubated in KRB (□) or in KRB supplemented with 20% (v/v) heat-inactivated serum from either turpentine treated rats (△) or control rats (○) and medium samples prepared as described in Figure 4.1. Plasmin activity was assayed using BAEE as substrate as described in Figure 4.10 and is expressed as n mol of substrate hydrolysed/ min per ml of incubation medium. Results represent means ± S.D. from six experiments.

was used [Figures 4.17 and 4.18; $P < 0.001$ for trypsin and plasmin respectively for the 2 and 4 h time points].

Heat-inactivated serum from turpentine treated rats when used to supplement incubations, resulted in increased STase activity for the 2 and 4 h medium samples compared to incubations carried out in heat-inactivated control rat serum [Figure 4.19; $P < 0.001$ for the 2 and 4 h time points]. GTase activity however remained similar whether the incubations were carried out in KRB alone or in KRB supplemented with heat-inactivated serum from turpentine treated or control rats [Figure 4.19; $P > 0.05$ for the 2 and 4 h time points, indicating that the means were not significantly different].

IV.2.4. Protease inhibitory activity of HBF

The protease-inhibitory activity of HBF towards various proteases was examined. HBF was able to inhibit: (1) hydrolysis of BAPNA and α-casein by trypsin [Table 4-1] and (2) hydrolysis of BAEE and α-casein by plasmin [Table 4.2]. In these experiments human AIPI and BSA were used as controls A1PI is a broad spectrum protease inhibitor known to inhibit trypsin, elastase, thrombin, plasmin, chymotrypsin and kallikrein [Travis and Salvesen, 1983]. HBF did not show any inhibitory activity towards elastase, thrombin, kallikrein, chymotrypsin and papain [Table 4.3].

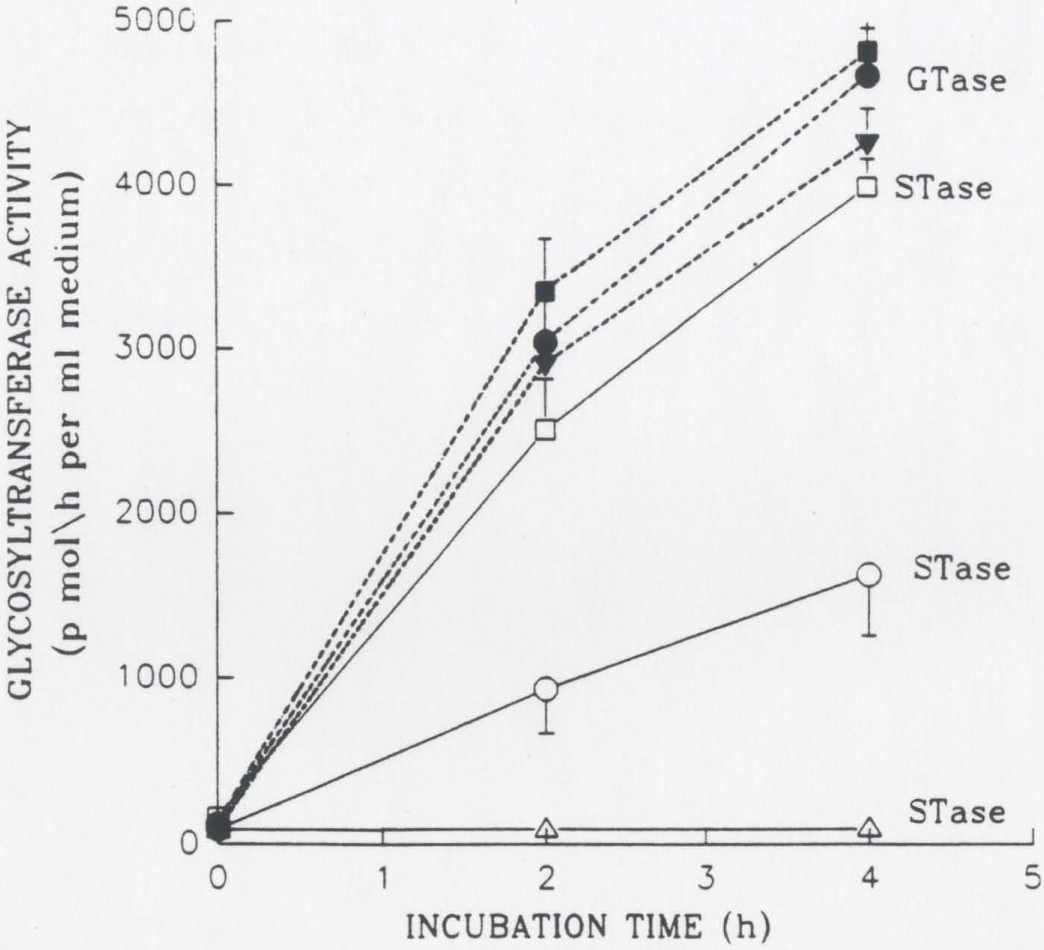


Figure 4.19. Effect of heat-inactivated serum from turpentine treated rats on the STase and GTase activities released during jejunal incubations

Figure 4.19. Effect of heat-inactivated serum from turpentine treated rats on the STase and GTase activities released during jejunal incubations. Jejunal slices were incubated in KRB (△ , ▼) or in KRB supplemented with 20% (v/v) of heat-inactivated serum from either turpentine treated rats (□ , ■) or control rats (○ , ●). Medium samples were prepared at indicated times and STase and GTase activities measured as described for Figure 4.1. STase (△, ○ , □) and GTase (▼ , ●, ■) activities are expressed as pmol of NeuAc or Gal transferred onto DS- or DSG-AGP/h per ml medium. Results represent means ± S.D. from six experiments.

Table 4.1. Trypsin inhibitory activity of HBF and TBP. Trypsin inhibitory activity of HBF, A1PI, BSA, TBP and the unbound-trypsin fraction was measured using either BAPNA or casein as substrates. Assays were carried out as described in Materials and Methods (sections II.2.3.A., II.11.2.A. and II.11.2.B. Trypsin inhibitory activity is expressed as the difference between activity in the presence and absence of the inhibitor as n mol/min per mg protein or % inhibition. Results represent means ± S.D. from five experiments.

	trypsin inhibitory activity	
	substrate:BAPNA	substrare casein
	nmol/min per mg protein	% inhibition
HBF	10.14 ± 0.9	44.40 ± 1.6
A1PI	9.12 ± 0.6	41.42 ± 5.4
BSA	no inhibition	no inhibition
TBP	35.45 ± 0.7	60.40 ± 6.7
unbound-trypsin fraction	no inhibition	no inhibition

Table 4.2. Plasmin inhibitory activity of HBF and TBP. Plasmin inhibitory activity of HBF, A1PI, BSA, TBP and the unbound-trypsin fraction was measured using either BAEE or casein as substrates. Assays were carried out as described in Materials and Methods (sections II.2.3.A. and II.11.3.A. and II.11.3.B. Plasmin inhibitory activity is expressed as the difference between activity in the presence and absence of the inhibitor as n mol/min per mg protein or % inhibition. Results represent means ± S.D. from five experiments.

plasmin inhibitory activity

	substrate:BAEE	substrate:casein
	nmol/min per mg protein	% inhibition
HBF	6.03 ± 0.9	20.30 ± 0.8
A1PI	16.70 ± 0.1	15.60 ± 2.7
BSA	no inhibition	no inhibition
TBP	15.50 ± 1.4	26.32 ± 1.8
unbound-trypsin fraction	no inhibition	no inhibition

Table 4.3. Inhibitory activity of HBF and TBP towards chymotrypsin, elastase, kallikrein, papain and thrombin. Inhibitory activity of HBF and TBP towards chymotrypsin, elastase, kallikrein, papain and thrombin was measured as described in Materials and Methods (sections II.11.4. to II.11.8). Inhibitory activity is expressed as the difference between activity in the presence and absence of the inhibitor and is expressed as n mol/min per mg protein and results represent means ± S.D. from four experiments.

Table 4.3

Protease inhibitory activity
(n mol/ min per mg protein)

Protease	Inhibitor			
	HBF	A1PI	BSA	TBP
chymotrypsin	0	7.2 ± 0.9	0	0
elastase	0	40.2 ± 0.7	0	0
kallikrein	0	4.3 ± 0.7	0	0
papain	0	1.6 ± 0.4	0	0
thrombin	0	4.1 ± 0.8	0	0

IV.2.5. Isolation of a trypsin-binding protein (TBP) from HBF

Using trypsin-agarose affinity chromatography a trypsin-binding protein [TBP] was separated from HBF [Figure 4.20]. TBP on SDS-PAGE showed a major band with an apparent molecular mass of 67 000 Da [Figure 4.21]. The gel filtration profile of TBP revealed a single peak and the purity of the sample was 98% as judged by the area under the HPLC profile [Figure 4.22]. TBP was able to bind to Con-A indicating it was a glycoprotein [Figure 4.23]. Human albumin which is not a glycoprotein and was used as a control did not exhibit binding to Con-A [Figure 4.23]. TBP showed inhibitory activity towards plasmin and trypsin [Tables 4.1 and 4.2]. However TBP did not show inhibitory activity towards either chymotrypsin, elastase, kallikrein, papain or thrombin [Table 4.3]. The unbound-trypsin fraction of HBF did not inhibit trypsin or plasmin [Tables 4.1 and 4.2].

IV.2.6. Effect of TBP on the STase and GTase activities released during jejunal incubations

TBP when added to KRB during jejunal incubations, had a similar effect as HBF on the STase activity released [Figure 4.24: $P < 0.001$ for the 1, 2 and 4 h time points, when samples from incubations containing HBF and TBP respectively were compared to samples from KRB incubations]. Thus, TBP like HBF was able to protect the STase activity released into the medium. STase activity in the medium was similar when the

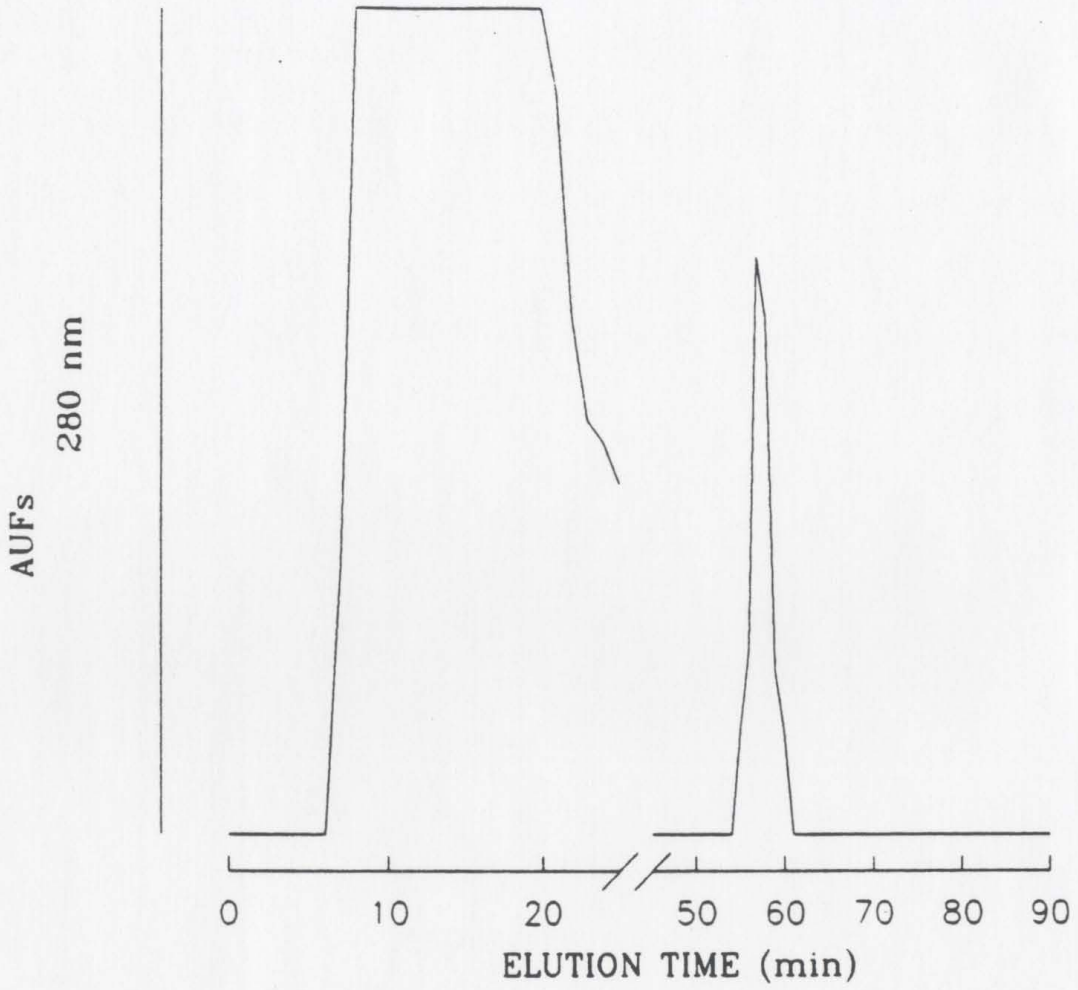


Figure 4.20. Affinity chromatography profile of the binding of HBF to trypsin-agarose: separation of TBP

Figure 4.20. Affinity chromatography profile of the binding of HBF to trypsin-agarose: separation of TBP. HBF was applied to a trypsin-agarose column which had been previously equilibrated with 5mM Tris-buffer as described in Materials and Methods (section II.2.3). The column was washed extensively with Tris-buffer until the absorbance of the eluent at 280 nm was less that 0.05. The bound fraction was eluted with 20 mM HCl and processed as described. This graph was regenerated by digitizing the elution profile with Sigmaplot 5.0.

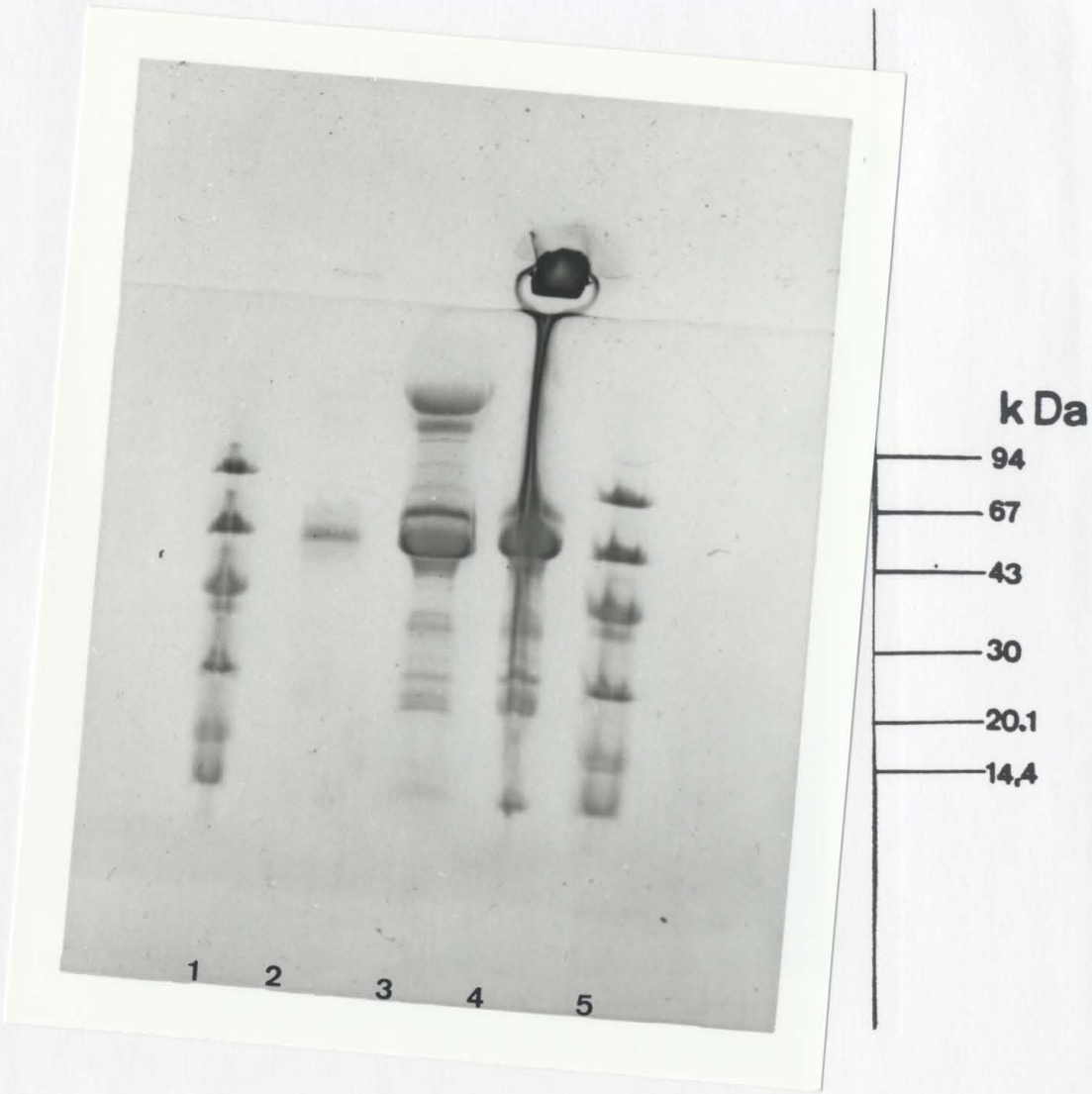


Figure 4.21. SDS-PAGE profiles of HRS, HBF and TBP

Figure 4.21. SDS-PAGE profiles of HRS, HBF and TBP. Electrophoretic profiles are shown of low-molecular-mass-standard mixture (lanes 1 and 5), HBF (lane 4), HRS (lane 3) and TBP (lane 2). Samples were processed for SDS-PAGE as described in Materials and Methods (section II.2.1.). The standard mixture contained phosphorylase b(94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α lactalbumin (14.4 kDa).

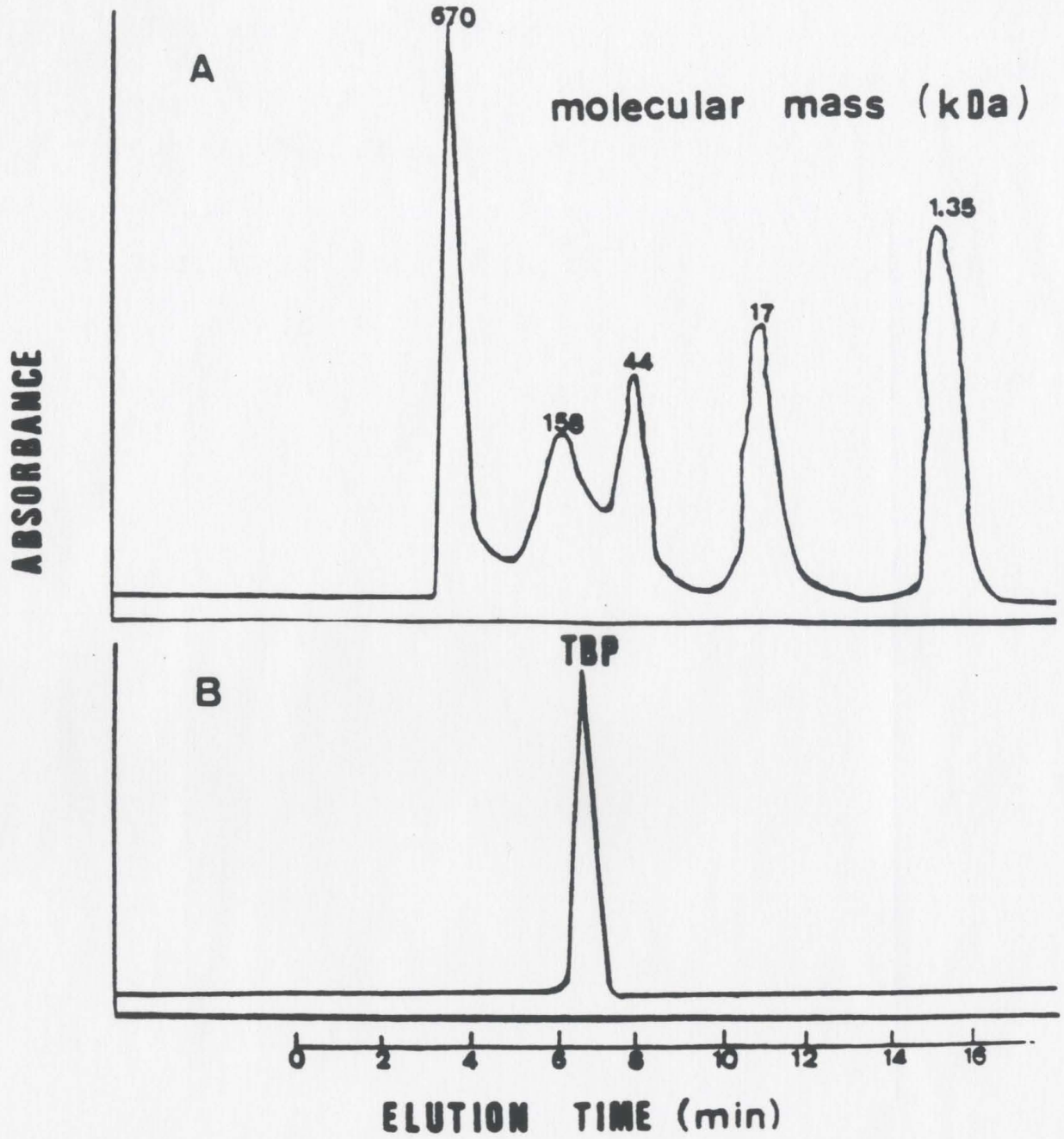


Figure 4.22. Gel filtration HPLC profile of TBP

Figure 4.22. Gel filtration HPLC profile of TBP. Gel filtration HPLC profile of TBP (panel B) is compared to that of molecular-mass-standard-mixture (panel A). The standard contained thyroglobulin (670 kDa), bovine globulin (158 kDa), chicken ovalbumin (44 kDa), bovine myoglobulin (17 kDa) and cyanocobalamin B_{12} (1.35 kDa). HPLC was carried out as described in Materials and Methods (section II.2.2).

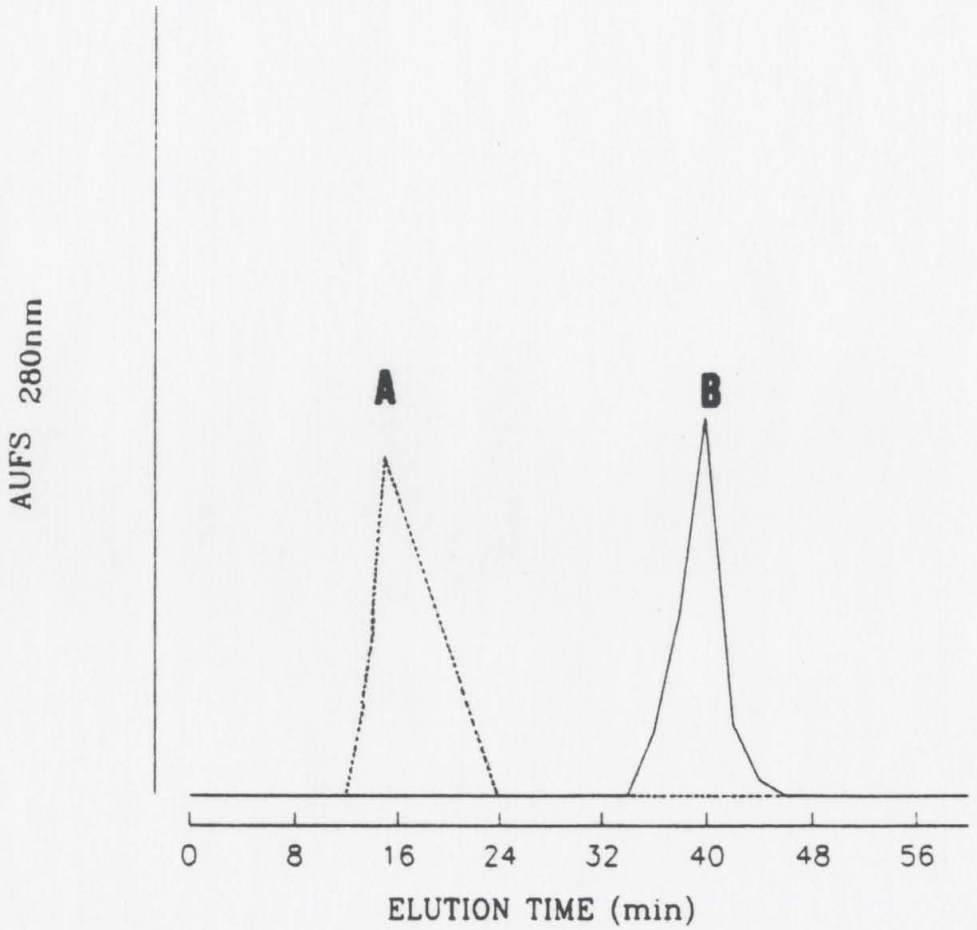


Figure 4.23. Affinity chromatography profiles of the binding of albumin and TBP to Con-A

Figure 4.23. Affinity chromatography profiles of the binding of albumin and TBP to Con-A. Human albumin (A) and TBP (B) were applied to a Con-A affinity column equilibrated with Tris buffer as described in Materials and Methods (section II.2.3.C). This graph was regenerated by digitizing the elution profile with Sigmaplot 5.

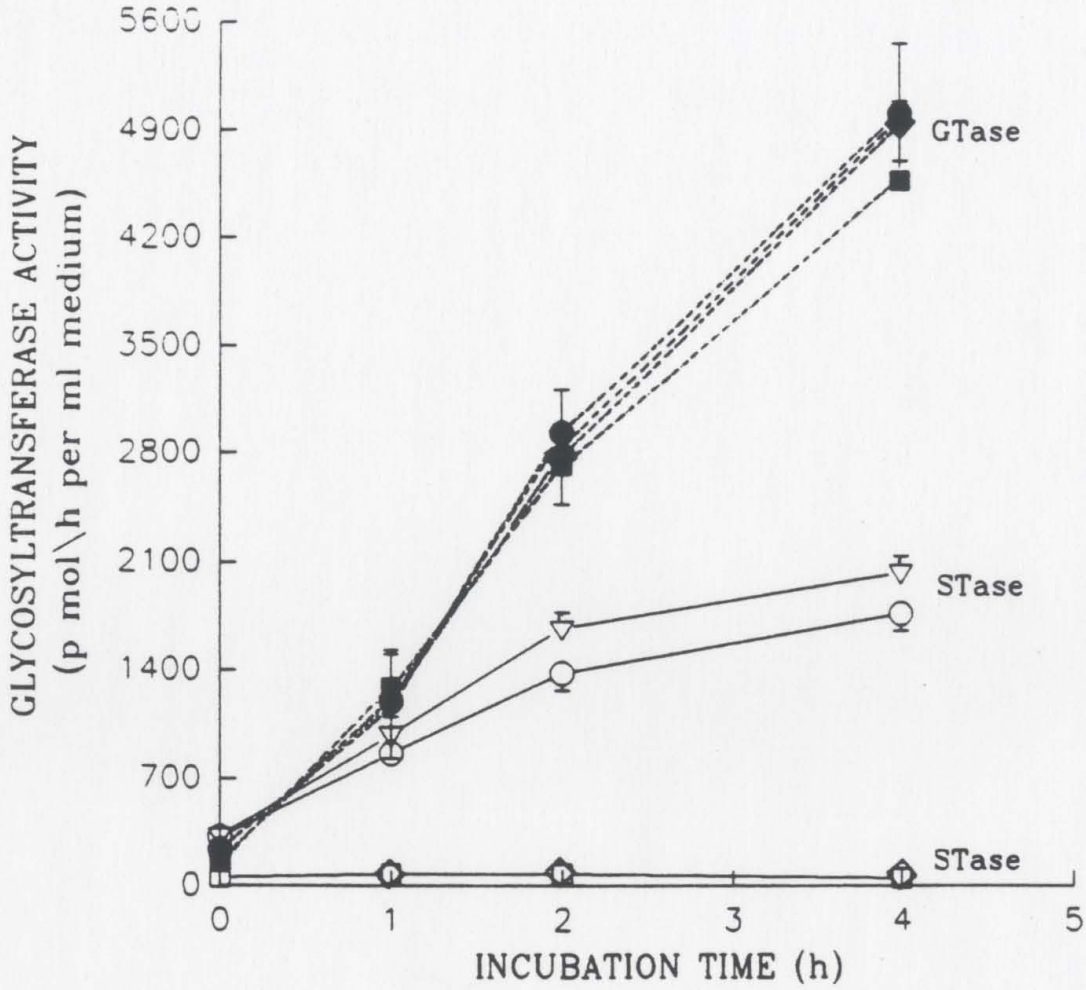


Figure 4.24. Effect of TBP on the STase and GTase activities released during jejunal incubations

Figure 4.24. Effect of TBP on the STase and GTase activities released during jejunal incubations. Jejunal slices were incubated in KRB (□ , ■) or in KRB supplemented with either 500 μg HBF (○ , ●), 250 μg TBP (▽ , ▼) or 500 μg unbound- trypsin fraction (◇ , ◆), medium samples prepared and glycosyltransferase activities measured as described for Figure 4.1. STase (□ , ○, ▽, ◇) and GTase (□ , ○ , ▽ , ◇) activities are expressed as pmol of NeuAc or Gal transferred to DS- or DSG-AGP/h per ml incubation medium. Results represent means ± S.D. from five experiments.

unbound-trypsin fraction (500 μg) was used to supplement KRB during incubations, compared to incubations in KRB [Figure 4.24]. The optimum concentration of TBP required to have an effect on STase was 250 μg per 2 ml medium [Figure 4.25: $P < 0.001$ for incubations containing 125, 250, 500 and 600 μg TBP compared to incubations in KRB]. To achieve a similar effect on STase activity 500 ug of HBF per 2 ml medium was required and in the case of HRS 20% (v/v) corresponding to about 30 mg serum protein was required.

TBP did not have any effect on GTase activity, which remained similar whether incubations were carried out in KRB alone or in KRB + TBP [Figure 4.26; $P > 0.05$ indicating that the means were not significantly different].

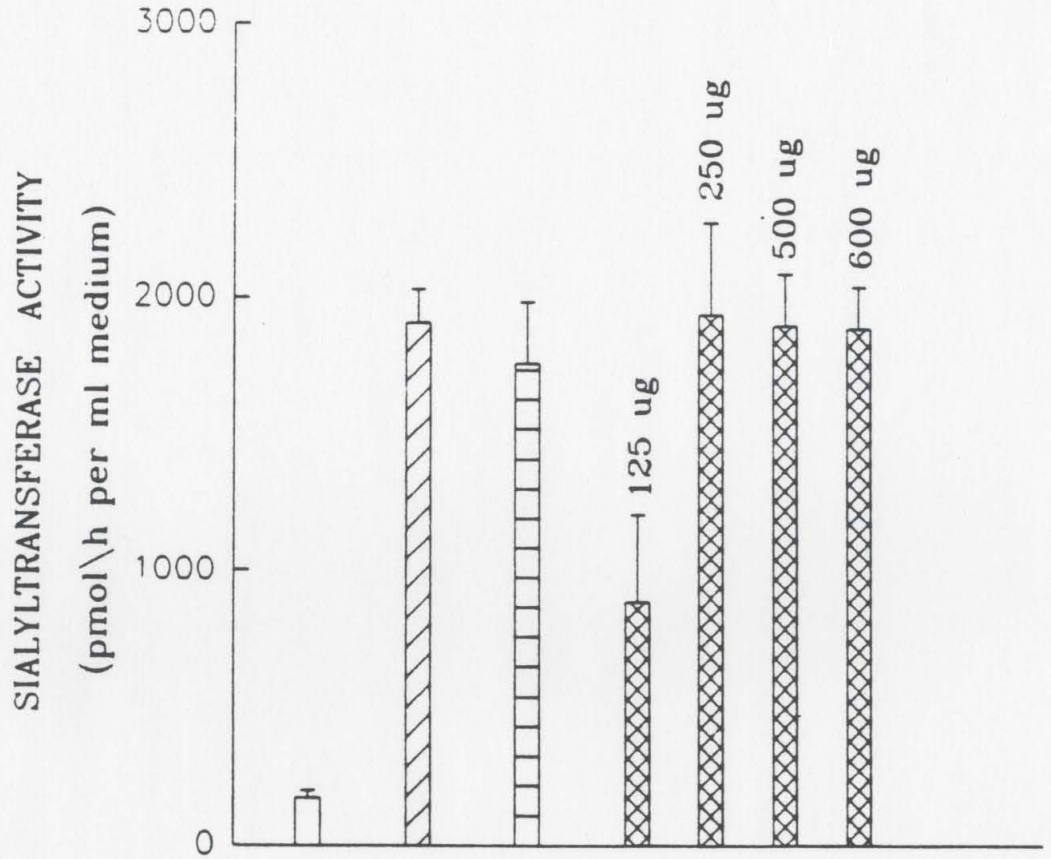


Figure 4.25. Effect of varying concentrations of TBP on STase activity

Figure 4.25. Effect of varying concentrations of TBP on STase activity. Jejunal slices were incubated in KRB (▭) or KRB containing either 500 μg HBF (▤), 20% (v/v) HRS (▨) or 125 to 600 μg TBP (▩) and medium samples prepared at 4 h and STase activity measured as described in Figure 4.1. STase activity is expressed as pmol of NeuAc transferred to DS-AGP/h per ml incubation medium. Results represent means ± S.D. from five experiments.

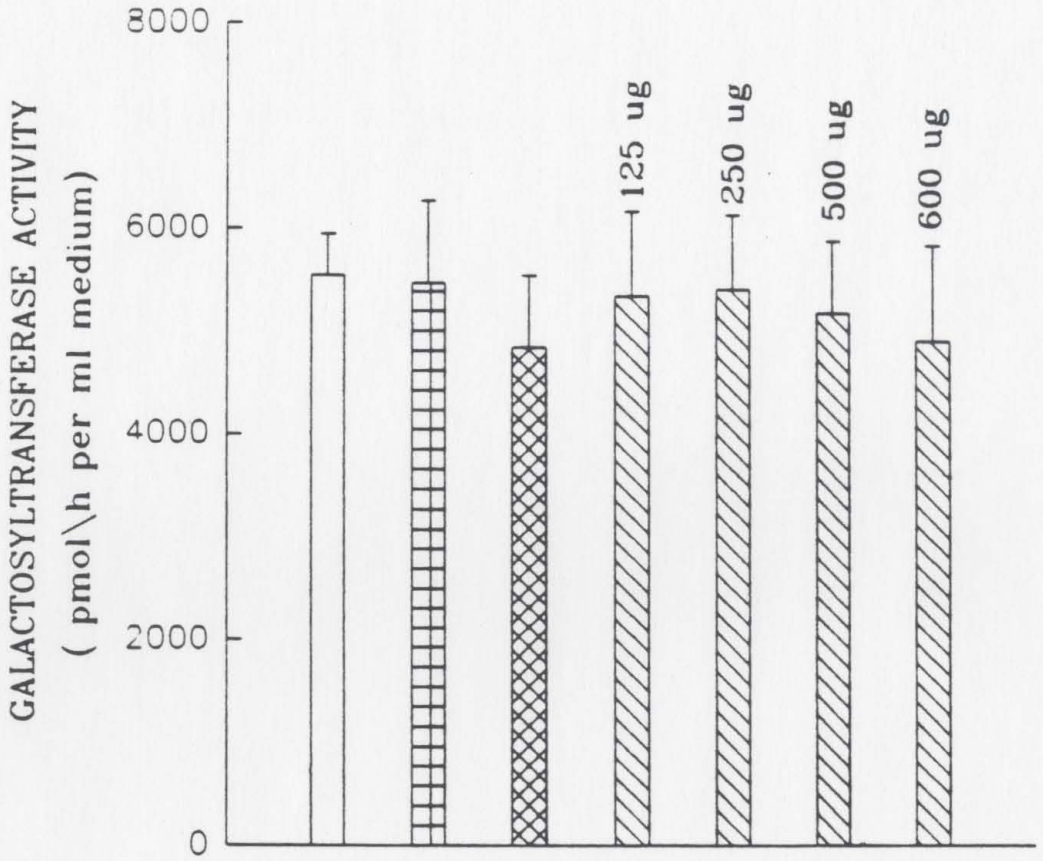


Figure 4.26. Effect of varying concentrations of TBP on GTase activity

Figure 4.26. Effect of varying concentrations of TBP on GTase activity. Jejunal slices were incubated in KRB (▭) or in KRB supplemented with either 500 μg HBF (▩), 20% (v/v) HRS (▤) or 100 to 600 μg TBP (▩) medium samples prepared at 4h and GTase activity measured as described in Figure 4.1. GTase activity is expressed as pmol of Gal transferred to DSG-AGP/h per ml incubation medium. Results represent means ± S.D. from five experiments.

IV.3. Discussion

The relationship between trypsin and plasmin inhibitory activities in the medium and the STase activity released during jejunal slice incubations was studied in the experiments described in this chapter. The effect of HRS and its heparin-binding antiproteolytic proteins, on the levels of STase and GTase activity released during the incubation of jejunal slices was also examined.

It was observed that only trace amounts of STase activity were detected in the medium when incubations were carried out in KRB alone [Figure 4.1]. In order to detect measurable STase activity there was a requirement for either HRS itself or HBF isolated from HRS in the incubation medium. This finding was similar to that observed for HHS in Chapter III. Therefore both HHS and HRS as well as their HBFs were exerting a similar effect on the STase activity released into the medium.

GTase activity, which was also released during the incubation of rat jejunal slices, however was not affected by HBF or HRS [Figures 4.1. and 4.7.]. Unlike STase, there was no difference in the GTase activity detected in the medium when the incubations were carried out in KRB or KRB + HRS. This indicated that the GTase activity released was independent of HRS. The results presented in this chapter indicate that the measurable STase activity released during jejunal incubations was a function of the protease inhibitor/

protease composition of the incubation medium. In all the cases cited above an increase in trypsin and plasmin inhibitory activities was associated with increased STase activity in the incubation medium. GTase activity unlike STase was not influenced by protease activity in the medium.

A number of proteinase inhibitors in blood have been known to bind to heparin including antithrombin, heparin cofactor and protein C inhibitors [Pratt et al., 1992]. Heparin also accelerates the activation of plasminogen by tissue type plasminogen activator (t-PA) [Sprenger and Kluft, 1987; Sane et al., 1993]. While heparin enhanced the inhibitory activities of these proteins, it is also known to decrease inhibition by other proteinase inhibitors. For example heparin has been shown to decrease the rate of inhibition of elastase by A1PI [Frommherz et al., 1991]. In the studies with the jejunal incubations described in this thesis, addition of heparin resulted in inactivation of the proteinase inhibitor(s) responsible for protecting the STase released. This conclusion is based on the fact that TBP a trypsin and plasmin inhibitor, which was effective in protecting STase, was isolated from HBF (Figure 4.24). Also, the addition of heparin resulted in increased trypsin and plasmin activities in the incubation medium along with a decrease in STase activity [Figures 4.9, 4.10 and 4.2].

Glycosyltransferases are membrane bound enzymes which

also occur in the soluble form in tissue fluids. During pathological conditions especially cancer, alterations in levels of STase and GTase have been observed in body fluids. In cancer, elevations in GTase activity have been reported in serum [Podolsky et al., 1977 and 1978], ascitic fluid [Gerber et al., 1979] and pleural effusions [Ram and Mungal, 1984]. Increased serum STase activities have been reported in cancer including in multiple myeloma [Weiser et al., 1982; Baker et al., 1985; 1987; Frithz et al., 1985]. Recent studies have shown that proteolytic enzymes and their inhibitors play a role in malignant conditions [Steven et al., 1992a,b; Wada et al., 1993a,b]. Therefore, it is feasible that the levels of protease inhibitors/ proteases in pathophysiological conditions could affect the levels of enzymes such as STase in tissue fluids.

Protease inhibitors in serum and faeces are used as markers for a number of disease conditions associated with the intestine. Protease inhibitor/ protease complexes are believed to play a role in gastrointestinal disorders such as inflammation and Crohn's disease [Florent et al., 1981; Mizon et al., 1988,1991]. In intestine, severe inflammation is accompanied by marked tissue destruction wherein proteinases and their inhibitors are involved. Serum levels of protease-inhibitors such as A1PI are altered in gastrointestinal disease [Bohe et al., 1986a,b]. Faecal A1PI is considered to

be a marker for intestinal disease and an increase in a glycosylated form of A1PI is considered indicative of the severity of intestinal inflammation [Mizon et al., 1988, 1991]. Therefore in this context, the findings that protease inhibitors might influence STase activity in the gastrointestinal system is interesting.

In the rat, increases in serum STase activity have been observed following colchicine treatment [Ratnam et al., 1981 and 1987], thermal injury [Chu et al., 1988] and turpentine induced inflammation [Kaplan et al., 1983; Fraser et al., 1984]. In all these conditions though serum STase was increased, serum GTase activity remained unchanged. It is therefore apparent that in some conditions GTase and STase activities are regulated differently. However the factors involved in the regulation of these enzymes have not been clearly defined.

In the experiments described in this thesis in which heat-inactivated serum from turpentine treated rats was used to supplement jejunal incubations, there was a marked increase in medium STase activity compared to incubations where heat-inactivated control rat serum was used to supplement incubations [Figure 4.19]. Heat-inactivated serum from turpentine treated rats had higher trypsin and plasmin inhibitory activities compared to heat-inactivated control serum [Figure 4.16]. These results further suggest that the

measurable STase activity released during jejunal incubations is dependent on the concentrations of protease inhibitors in the incubation medium.

During inflammation there is an increase in the levels of certain serum proteins known as the acute phase reactants [Koj, 1974; Kushner and Feldman, 1978]. Protease inhibitors such as A1P1 [Travis and Salvesen, 1983; Heidtman and Travis, 1986], α-2 antiplasmin [Matsuda et al., 1980] and TATI [Ogawa, 1988] are also acute phase proteins whose concentrations in serum are increased during inflammation. Therefore it is feasible that the levels of proteinase inhibitors in extracellular fluids in pathological conditions such as inflammation may govern the levels of enzymes such as STase. Other studies have shown that turpentine induced inflammation in the rat resulted in increased STase and GTase activities in liver [Kaplan et al., 1983; Fraser et al., 1984]. However, in serum, STase activity alone was increased. This increase in serum STase was suggested to be due to the increased activity of a cathepsin-D like proteinase which is thought to selectively cleave the membrane-bound STase [Lammers and Jamieson, 1988,1990]. However, observations from the present work suggest that decreased proteolysis of the soluble STase could also contribute to its increased activity.

The observations in this thesis indicate that during intestinal incubations, the enzyme was released even when

incubations were carried out in KRB alone. However, due to proteases present in the incubation system which destroyed STase, very little activity was detectable in the medium. When trypsin and/or plasmin inhibitors were present in the incubation medium they inactivated the protease and therefore permitted the measurement of STase activity. A proposed mechanism regarding the role of protease inhibitors and proteases on the STase released during jejunal incubations is outlined in Figure 4.27.

Very little is known regarding the factors which govern the levels and activities of STases in tissue fluids. The results presented in this thesis so far suggest that the levels of trypsin and plasmin inhibitors in the incubation medium were important in determining the levels of soluble STase released during jejunal incubations. Increases in medium STase activity were associated with increases in trypsin and plasmin inhibitory activities. GTase, which was also released in the soluble form during jejunal incubations did not exhibit a dependence on trypsin and plasmin inhibitory activity.

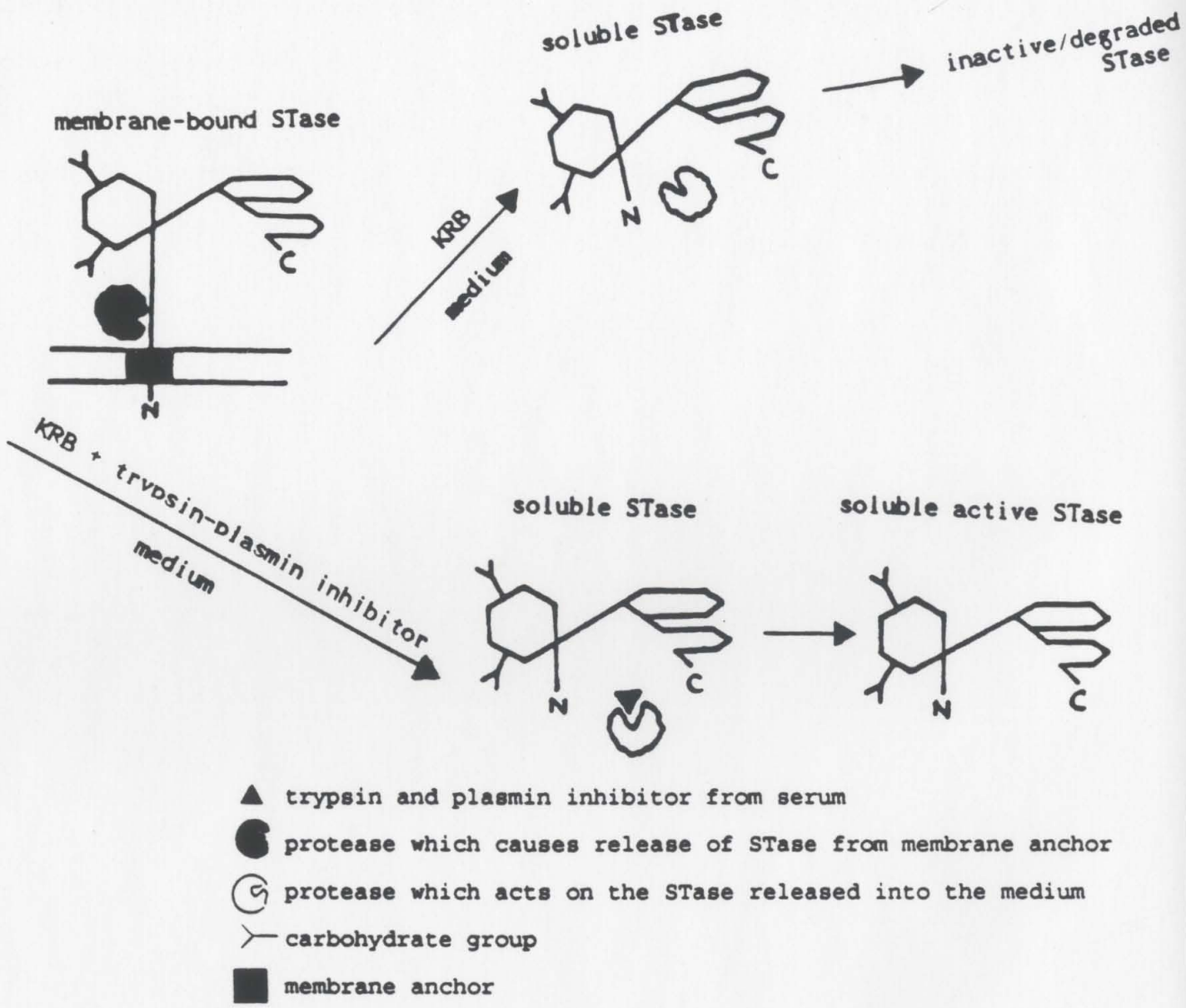


Figure 4.27. Proposed mechanism showing the effect of trypsin and plasmin inhibitors on the STase released during incubation of jejunal slices

Figure 4.27. Proposed mechanism showing the effect of trypsin and plasmin inhibitors on STase released during jejunal incubations. The figure shows the proposed mechanism whereby the membrane-bound enzyme is released from its membrane anchor by proteolytic cleavage. The soluble enzyme is a target for trypsin and plasmin. Trypsin and plasmin inhibitor(s) prevent proteases from destroying STase.

CHAPTER V. THE RELATIONSHIP BETWEEN STASE ACTIVITY AND PROTEASE INHIBITORY ACTIVITIES

V.1. Introduction

The results presented in chapters III and IV showed that the incubation of jejunal slices resulted in the release of soluble STase and GTase into the incubation medium. The STase activity released could only be measured if antiproteases such as HBF, TBP, A1PI or α_2 antiplasmin were present in the incubation medium. This dependence on trypsin and/or plasmin inhibitory activities was specific for STase, as GTase activity was not affected by antiproteases.

The purpose of the following studies was therefore to further investigate the relationship between STase activity and trypsin and plasmin inhibitory activities. This was done by examining

(1a) the effect of trypsin or plasmin on a mixture of commercially available pure STase(s) and GTase,

(1b) the inhibition of these proteases by TBP,

(2) the effect of protease inhibitory activity on serum STase and GTase activities in control and turpentine treated rats and

(3) the effect of protease inhibitory activity of the incubation medium on the STase and GTase activities released during the incubation of hepatocytes.

V.2. Results

V.2.1. Effect of trypsin and plasmin on pure STase and GTase

When either trypsin or plasmin was added in increasing concentrations (0 to 0.0125 mg) to a mixture of pure STase [α2-6(N)] and GTase, it was observed that STase activity was preferentially inhibited compared to GTase activity [Figure 5.1]. TBP when added in increasing concentrations (0 to 0.03 mg) was able to prevent the inhibitory effects of plasmin and trypsin on STase activity, in the presence of 0.01 mg of protease [Figure 5.2].

Similar experiments were carried out using a mixture of pure α2-3 [O] STase and GTase. It was again observed that STase was more prone to proteolytic degradation by either trypsin or plasmin compared to GTase [Figure 5.3]. TBP was able to protect STase activity against the inhibitory effects of plasmin and trypsin [Figure 5.4].

V.2.2. Effect of serum protease inhibitors on STase and GTase activities in serum

The incubation of serum obtained from either control or turpentine treated rats at 37°C resulted in a progressive decrease in its ability to inhibit trypsin or plasmin activity respectively [Figure 5.5]. Both trypsin and plasmin inhibitory activities were higher in serum obtained from turpentine treated rats compared to serum obtained from control rats and

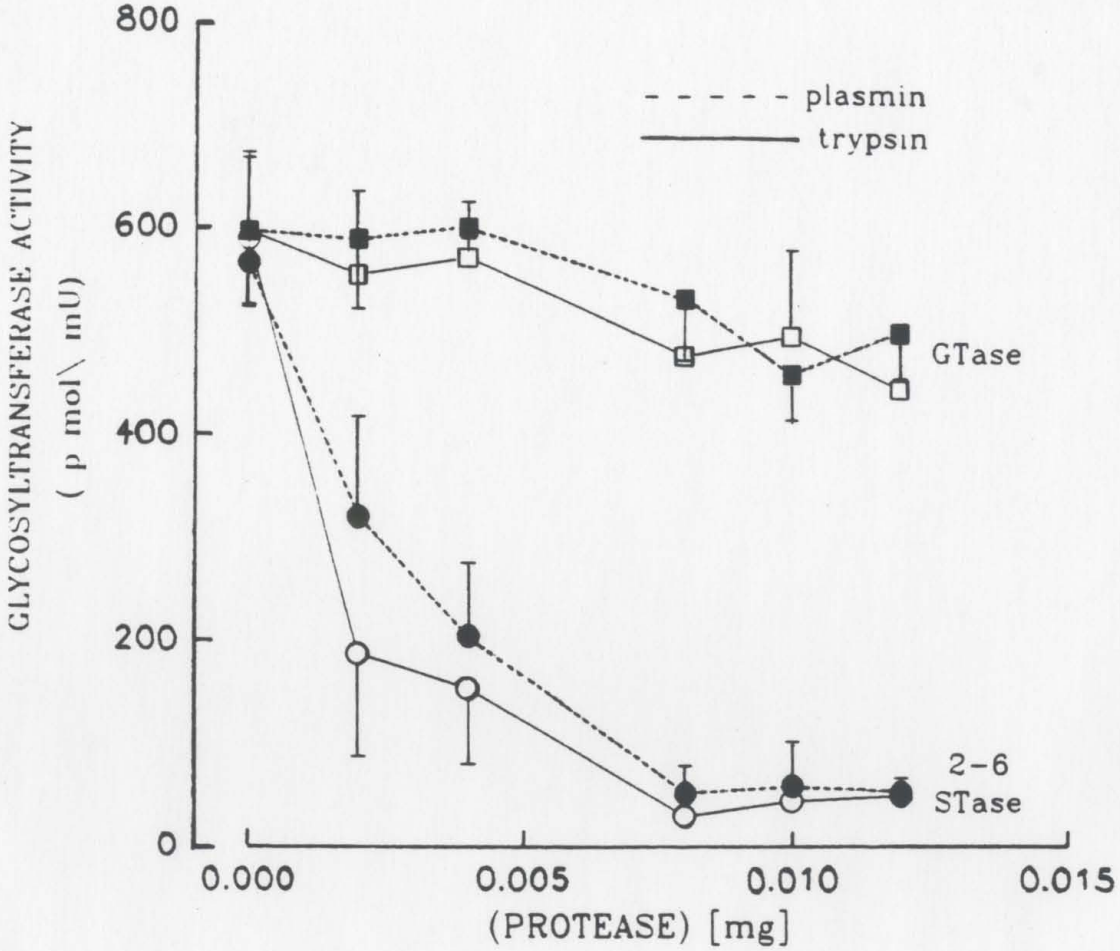


Figure 5.1. Effect of trypsin and plasmin on [α2→6(N)] STase and GTase

Figure 5.1. Effect of trypsin and plasmin on [α2→6(N)] STase and GTase. Trypsin (-□- , -○-) or plasmin (···■··· ,···●···) were added to a mixture of pure STase (○ , ●) and GTase (□ , ■). Samples were processed and assayed for STase and GTase activities as described in Materials and Methods (sections II.10, II.7.1. and II.7.2.). STase and GTase activities are expressed as pmol of NeuAc or Gal transferred per mU enzyme to DS- or DSG-AGP. Results represent means ± S.D. from four experiments.

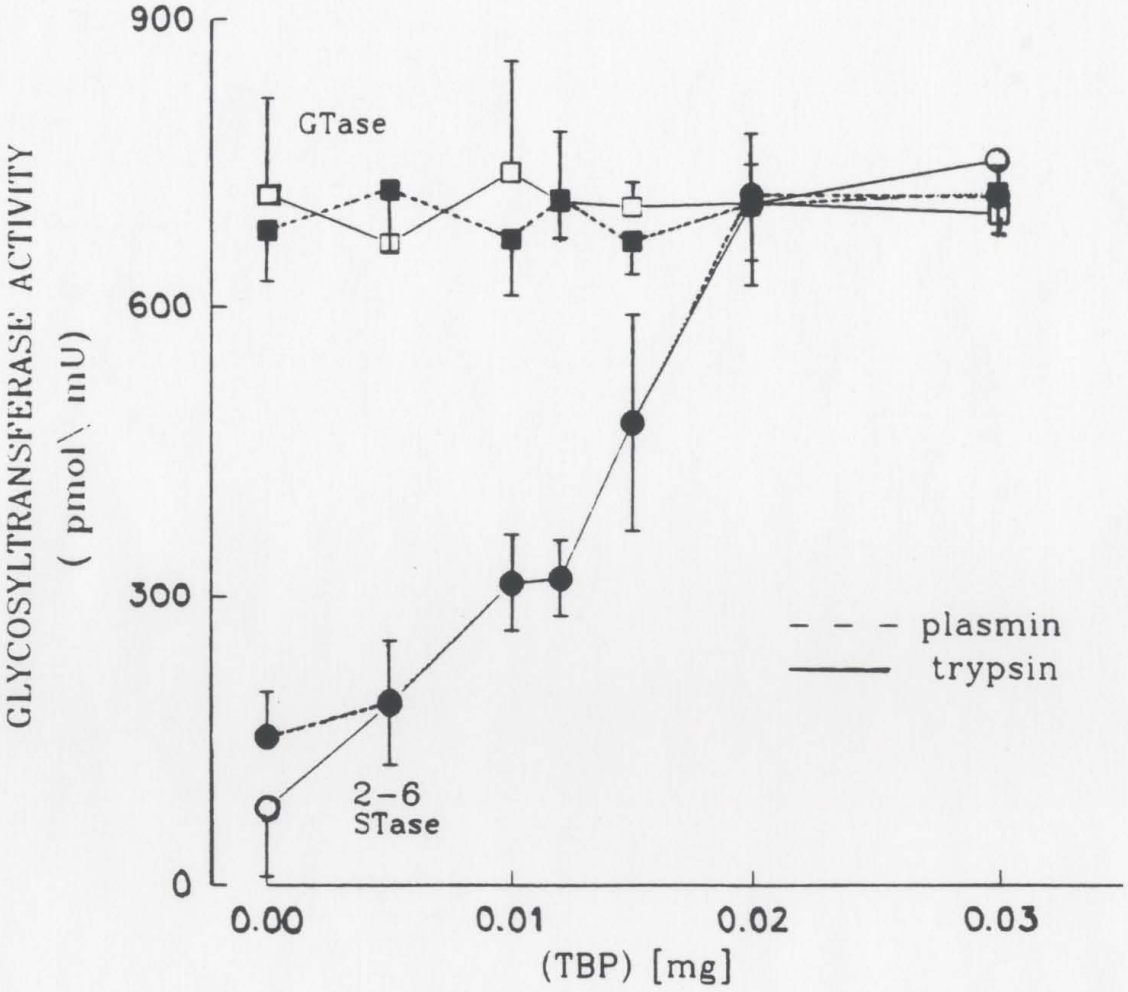


Figure 5.2. Effect of TBP in counteracting the effect of proteases on [α2→6(N)] STase

Figure 5.2. Effect of TBP on counteracting the effect of proteases on [α2→6(N)] STase. 0.01 mg of trypsin (-□- ,-○-) or plasmin (-■- ,-●-) were added to a mixture of pure STase (○ , ●) and GTase (□ , ■), along with increasing concentrations of TBP. Samples were processed and assayed for STase and GTase activities as described in Figure 5.1. STase and GTase activities are expressed as pmol of NeuAc or Gal transferred per mU enzyme to DS- or DSG-AGP respectively. Results represent means ± S.D. from four experiments.

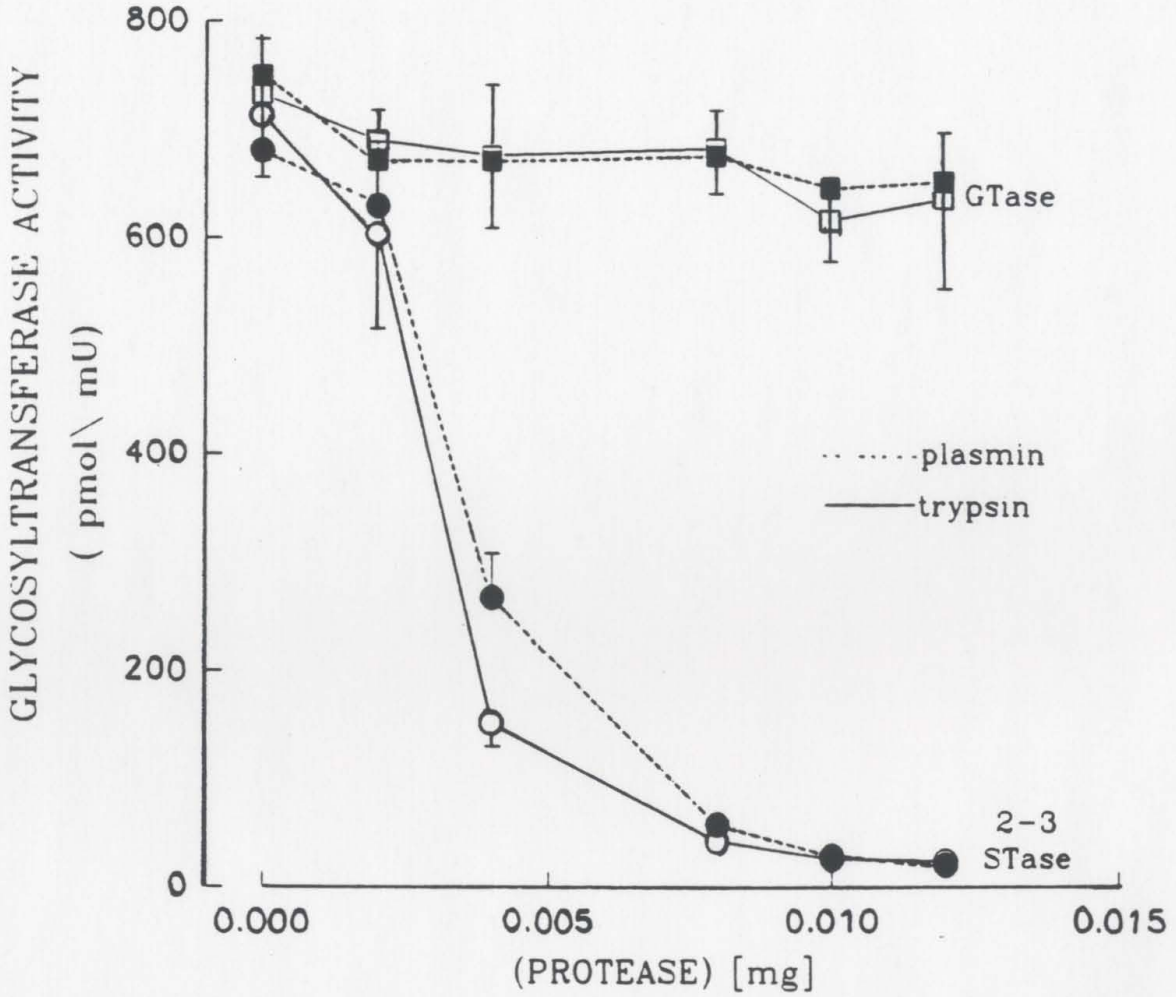


Figure 5.3. Effect of trypsin and plasmin on [α2→3(O)] STase and GTase

Figure 5.3. Effect of trypsin and plasmin on [α2→3(O)] STase and GTase. Trypsin (-□- , -○-) or plasmin (···■··· , ···●···) were added to a mixture of pure STase (○ , ●) and GTase (□ , ■). Samples were processed and assayed for STase and GTase activities as described in Figure 5.1. STase and GTase activities are expressed as pmol of NeuAc or Gal transferred per mU enzyme to DS- or DSG-fetuin respectively. Results represent means ± S.D. from four experiments.

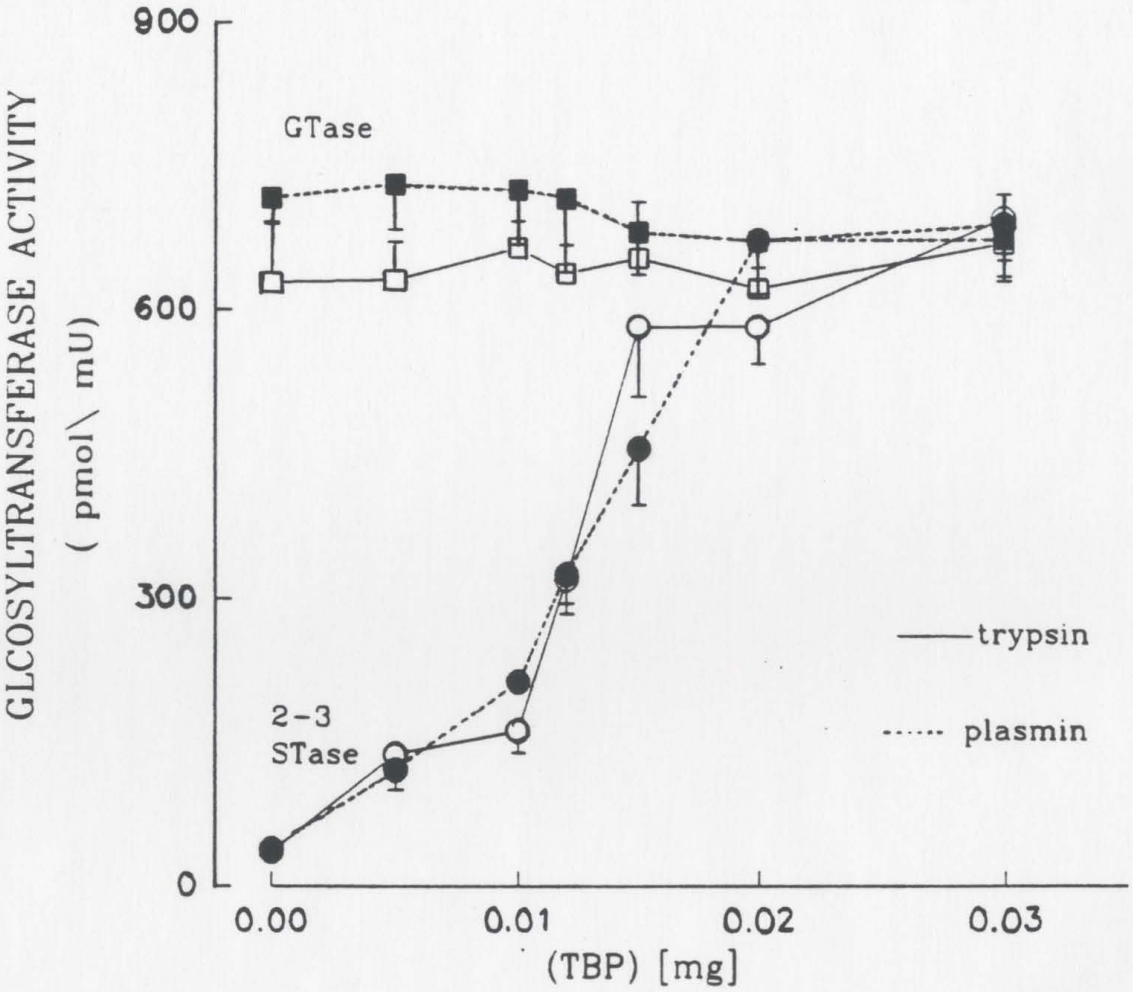


Figure 5.4. Effect of TBP on counteracting the effect of proteases on [α2→3(O)] STase

Figure 5.4. Effect of TBP on counteracting the effect of proteases on [α2→3(O)] STase. 0.01 mg of trypsin (—○— ,—□—) or plasmin (···●··· , ···■···) were added to a mixture of pure STase (○ , ●) and GTase (□ , ■), along with increasing concentrations of TBP. Samples were processed and assayed for STase and GTase activity as described in Figure 5.1. STase and GTase activities are expressed as pmol of NeuAc or Gal transferred per mU enzyme to DS- or DSG-fetuin respectively. Results represent means ± S.D. from four experiments.

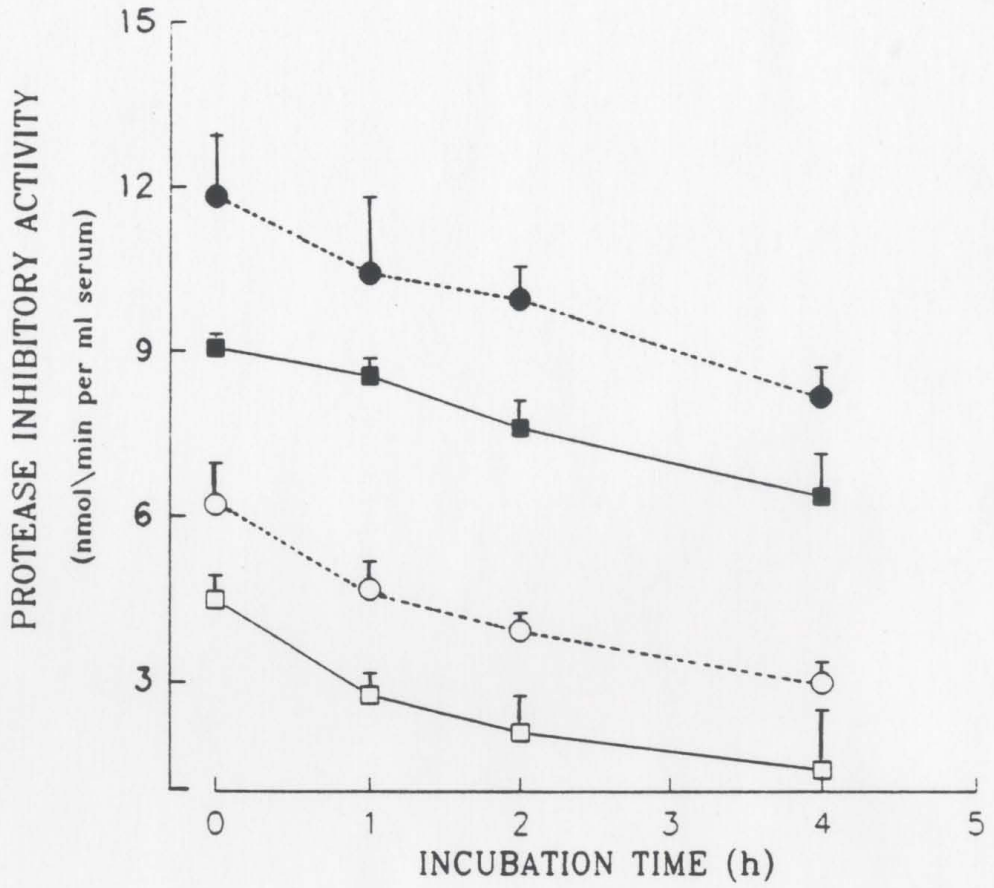


Figure 5.5. Effect of incubation time on trypsin and plasmin inhibitory activities in serum

Figure 5.5. Effect of incubation time on trypsin and plasmin inhibitory activities in serum. Serum samples from turpentine treated (● , ■) and control (○ , □) rats were incubated at 37 °C. At indicated times samples were removed and plasmin (■ , □) and trypsin (● , ○) inhibitory activities measured as described in Materials and Methods (sections II.9. and II.11.2.A. and II.11.3.A). Plasmin and trypsin inhibitory activities were obtained from the differences between activities in the presence and absence of inhibitor and are expressed as nmol\min per ml serum. Results represent means ± S.D. from five experiments.

this difference was maintained throughout the 4 h of incubation [Figure 5.5; $P < 0.01$ for the 0, 1, 2 and 4 h time points between serum from control and turpentine treated rats]. Serum STase activity also remained higher in the turpentine treated rats throughout the 4 hours of incubation [Figure 5.6; $P < 0.01$ for the 0, 1, 2 and 4 h time points between serum samples from control and turpentine treated rats]. In both groups STase activity decreased with incubation time. TBP when added to serum from either turpentine-treated and control rats was able to halt the time dependent decrease in serum STase activity [Figure 5.6]. In the case of STase in serum from turpentine treated rats, $P < 0.01$ for the 1, 2 and 4 h time points between samples incubated with TBP compared to samples without TBP. Likewise, for STase in serum from control rats $P < 0.01$ for the 1, 2 and 4 h time points between samples incubated with TBP compared to samples without TBP. These results indicated that STase activity in serum was unstable and that it could be protected by protease inhibitors such as TBP. In contrast, GTase activity in serum from both control and turpentine treated rats remained similar indicating it was not subject to proteolysis [Figure 5.7; $P > 0.05$ indicating that the means were not significantly different].

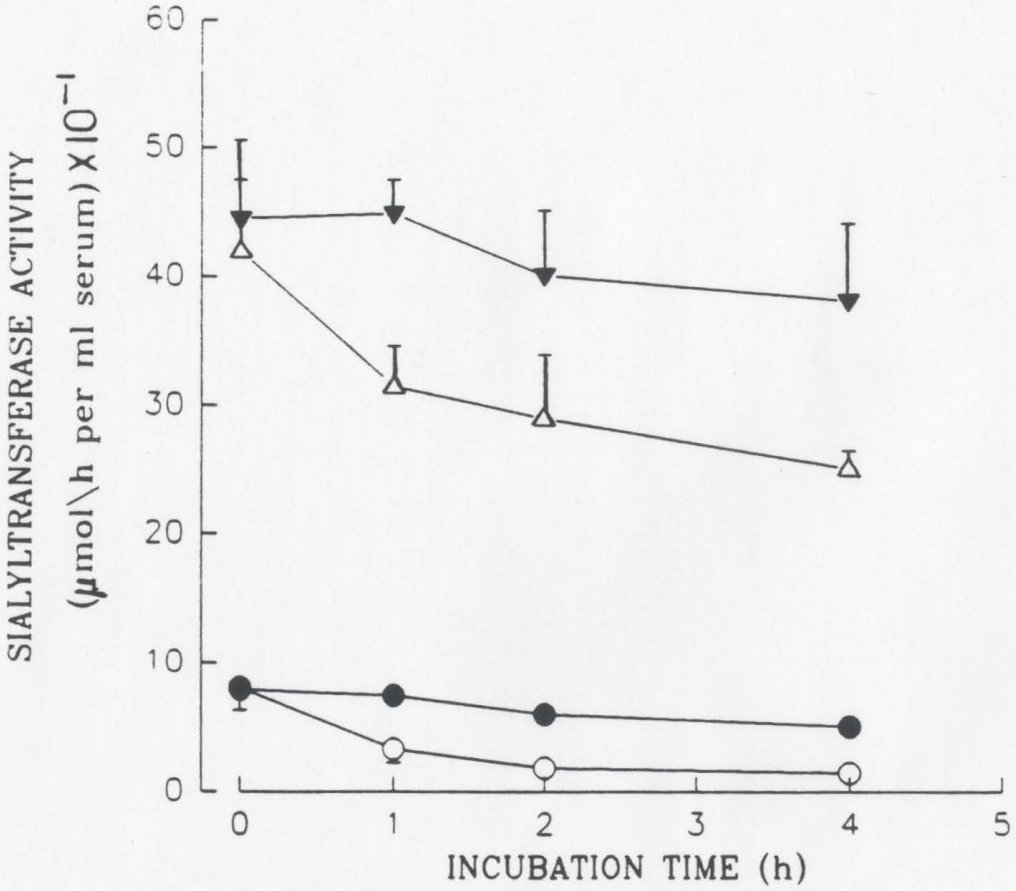


Figure 5.6. Effect of TBP on STase activity in sera of control or turpentine treated rats

Figure. 5.6. Effect of TBP on STase activity in sera of control or turpentine treated rats. Serum from control (○) or turpentine treated (△) rats was incubated at 37 °C. In parallel incubations TBP [125 μg/ml] was added to serum samples from control (●) or turpentine treated rats (▼) and samples were incubated. At indicated times samples were removed and assayed for STase as described in Materials and Methods (sections II.9. and II.7.1.). STase activity is expressed as p mol of NeuAc transferred to DS-AGP/h per ml serum. Results represent means ± S.D. from five experiments.

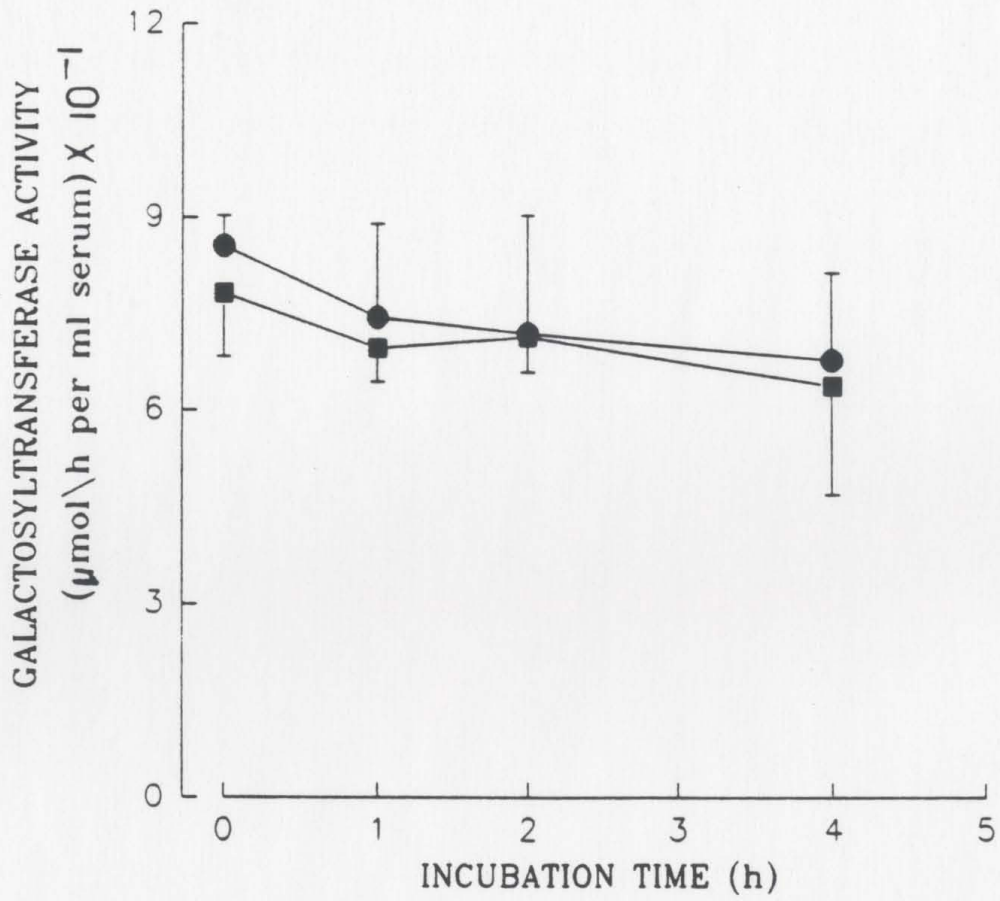


Figure 5.7. Effect of incubation time on GTase activity in sera of control an turpentine treated rats

Figure 5.7. Effect of incubation time on GTase activity in sera of control and turpentine treated rats. Serum from control (●) or turpentine treated (■) rats was incubated at 37 °C. At indicated times samples were removed and assayed for GTase as described in Materials and Methods (sections II.9. and II.7.2.). GTase activity is expressed as p mol of Gal transferred to DSG-AGP/h per ml serum. Results represent means ± S.D. from five experiments.

V.2.3. Effect of the balance between protease and protease inhibitory activities in the incubation medium on the STase and GTase released during the incubation of hepatocytes

Liver is the most likely source of serum glycosyltransferases. Therefore the effect of trypsin and plasmin inhibitors in the incubation medium on the glycosyltransferase activities released during the incubation of hepatocytes was studied. The viability of hepatocytes which was assessed using trypan blue exclusion, indicated that at 0 and 4h, 85% and 75% of the cells were viable.

Trypsin activity was measured in the incubation medium in the absence and presence of protease inhibitors [Figure 5.8]. Trypsin activity was decreased in medium obtained from incubations supplemented with either 500 μg HBF, 250 μg TBP or 20% (v/v) heat-inactivated serum from control rats, compared to incubations without supplement. $P < 0.01$ for the 2 and 4h time points for incubations supplemented with HBF, TBP and heat-inactivated control compared to incubations without antiprotease. Trypsin activity in the medium was lower when heat-inactivated serum from turpentine treated rats was used to supplement hepatocyte incubations compared to similar incubations supplemented with heat-inactivated control serum [$P < 0.01$ for the 2 and 4 h time points].

Similarly plasmin activity was decreased in medium obtained from incubations supplemented with either 500 μg HBF,

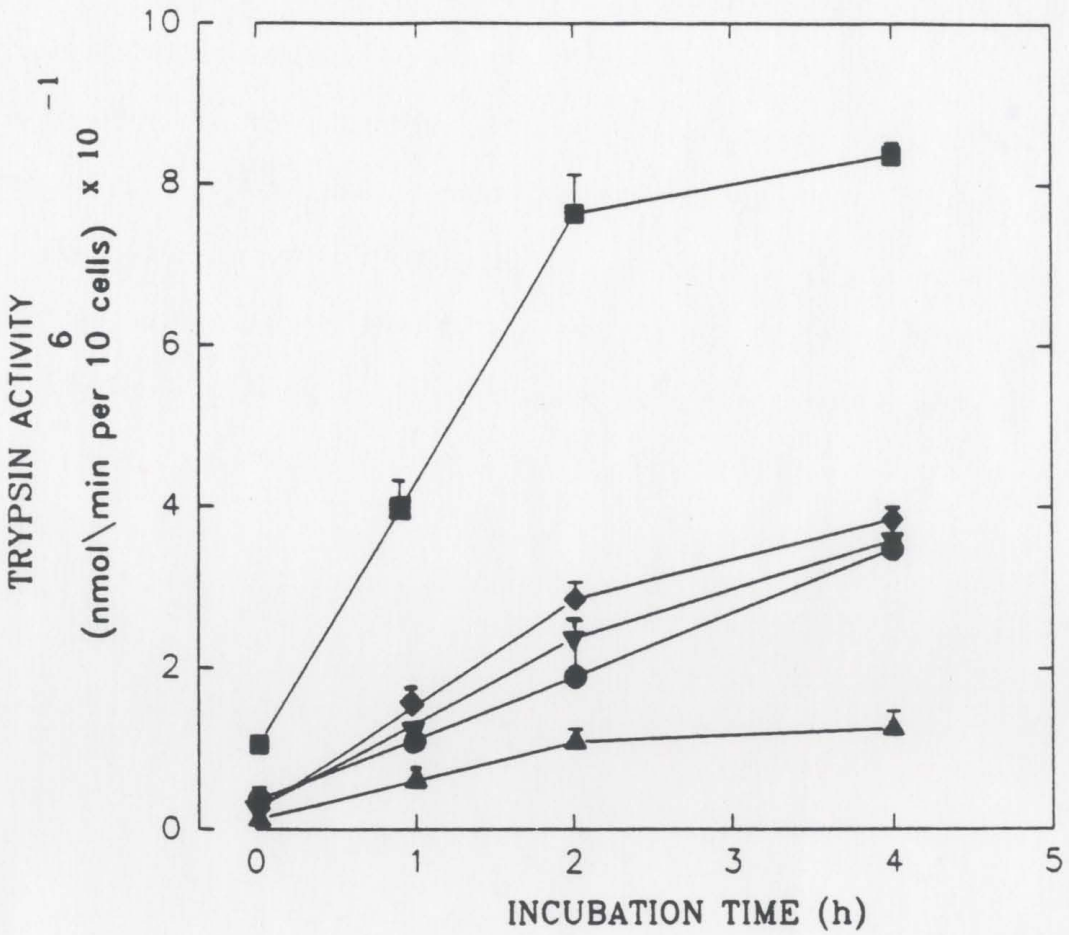


Figure 5.8 Trypsin activity in the medium during the incubation of hepatocytes

Figure 5.8. Trypsin activity in the incubation medium during the incubation of hepatocytes. Hepatocytes were incubated without antiprotease (■), 20% (v/v) heat-inactivated serum from control rats (▼), 20% (v/v) heat-inactivated serum from turpentine treated rats (▲), 500 μg HBF (●) or 250 μg TBP (◆) and at indicated times medium samples were prepared and trypsin activity measured as described in Materials and Methods (sections II.6. and II.11.2.A). Trypsin activity is expressed as n mol of substrate hydrolysed/ min per ml medium. Results represent means ± S.D. from five experiments.

250 μg TBP or 20% (v/v) heat inactivated serum compared to incubations without antiproteases [Figure 5.9]. $P < 0.01$ for the 2 and 4h time points for incubations supplemented with HBF, TBP or heat-inactivated control serum compared to incubations in buffer without antiprotease. Plasmin activity in the medium was lower when heat-inactivated serum from turpentine treated rats was used to supplement hepatocyte incubations compared to incubations containing heat-inactivated control serum [$P < 0.01$ for the 2 and 4h time points].

When HBF (500 μg), TBP (250 μg) and heat-inactivated serum (20%, v/v) from either control or turpentine treated rats,were used to supplement incubations STase activity in the medium was increased compared to incubations without antiprotease [Figure 5.10]. $P < 0.01$ for the 2 and 4 h time points, when incubations were supplemented with either HBF, or TBP, or heat-inactivated serum from control rats, compared to incubations without antiprotease. STase activity in the medium was increased when heat-inactivated serum from turpentine treated rats was used to supplement incubations, compared to incubations supplemented with heat-inactivated serum from control rats [Figure 5.10; $P < 0.01$ for the 2 and 4 h time points].

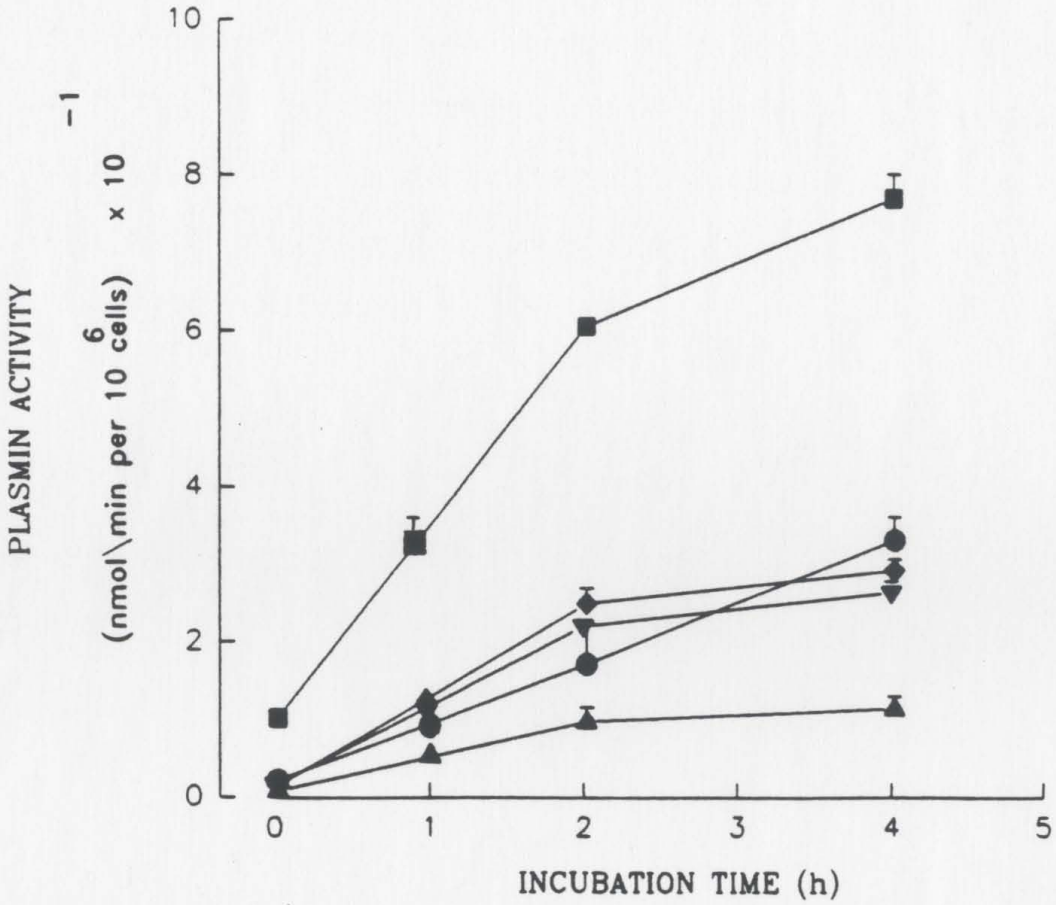


Figure 5.9. Plasmin activity in the medium during the incubation of hepatocytes

Figure 5.9. Plasmin activity in the incubation medium during the incubation of hepatocytes. Hepatocytes were incubated without antiprotease (■), 20% (v/v) heat-inactivated serum from control rats (▼), 20% (v/v) heat-inactivated serum from turpentine treated rats (▲), 500 μg HBF (●) or 250 μg TBP (◆) and at indicated times medium samples prepared as described in Figure 5.8. Plasmin activity was measured as described in Materials and Methods (section 2.11.2.[B]) and is expressed as n mol of substrate hydrolysed/ min per ml medium. Results represent means ± S.D. from five experiments.

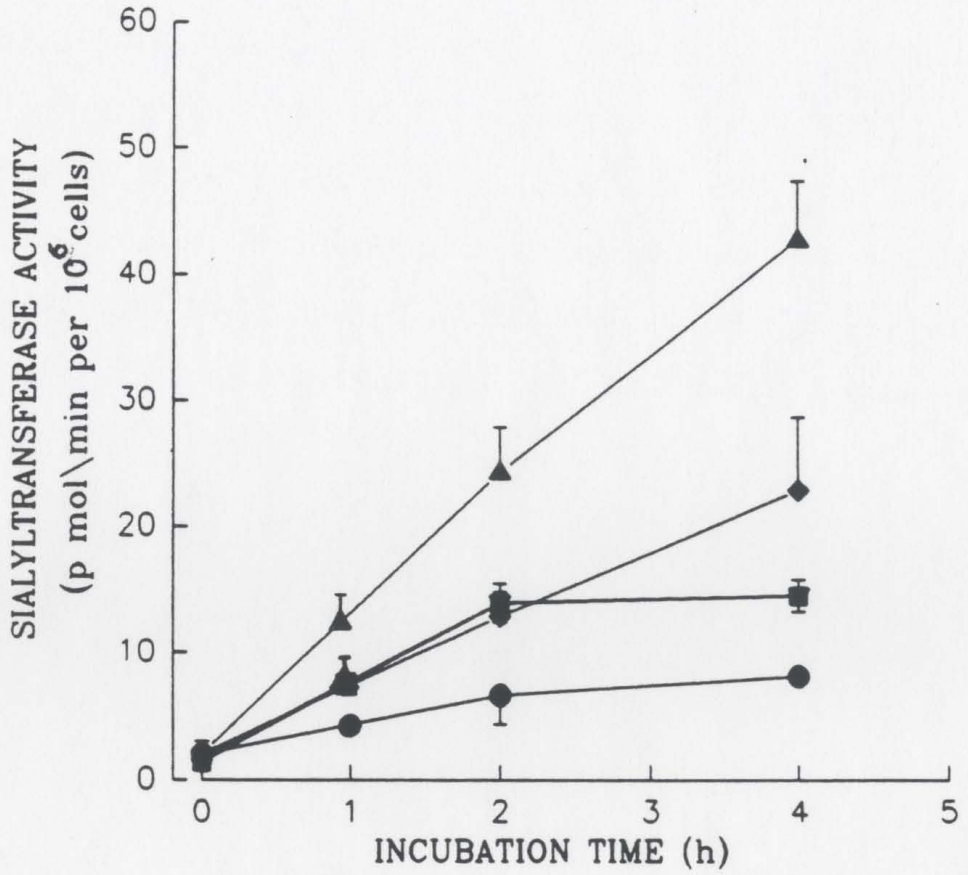


Figure 5.10. Effect of protease inhibitors in the incubation medium on the STase released during the incubation of hepatocytes

Figure 5.10. Effect of protease inhibitors in the incubation medium on the STase released during the incubation of hepatocytes. Hepatocytes were incubated without antiprotease ( ), 20% heat-inactivated serum from control rats (▼), 20% (v/v) heat-inactivated serum from turpentine treated rats (▲), 500 μg HBF (■) or 250 μg TBP (◆) and at indicated times medium samples were prepared as described in Figure 5.8. STase activity was measured as described in Materials and Methods (section II.7.1.). STase activity is expressed as p mol of NeuAc transferred/h per ml medium. Results represent means ± S.D. from five experiments.

When lactosamine was used as an acceptor to assay for STase activity in 4 h medium obtained from hepatocyte incubations in buffer, the results showed that STase was predominantly that which catalysed the α2→6 linkage of NeuAc to Gal indicating the enzyme to be α2→6 NeuAc β1→4Galβ→4 GlcNAc [Figure 5.11].

In the case of GTase, activity remained similar regardless of the supplements used [Figure 5.12; means were not significantly different for the 2 and 4 h time points]. GTase was similar whether incubations were carried out in KRB alone or KRB supplemented with either heat-inactivated control rat serum, HBF, TBP or heat inactivated serum from turpentine treated rats.

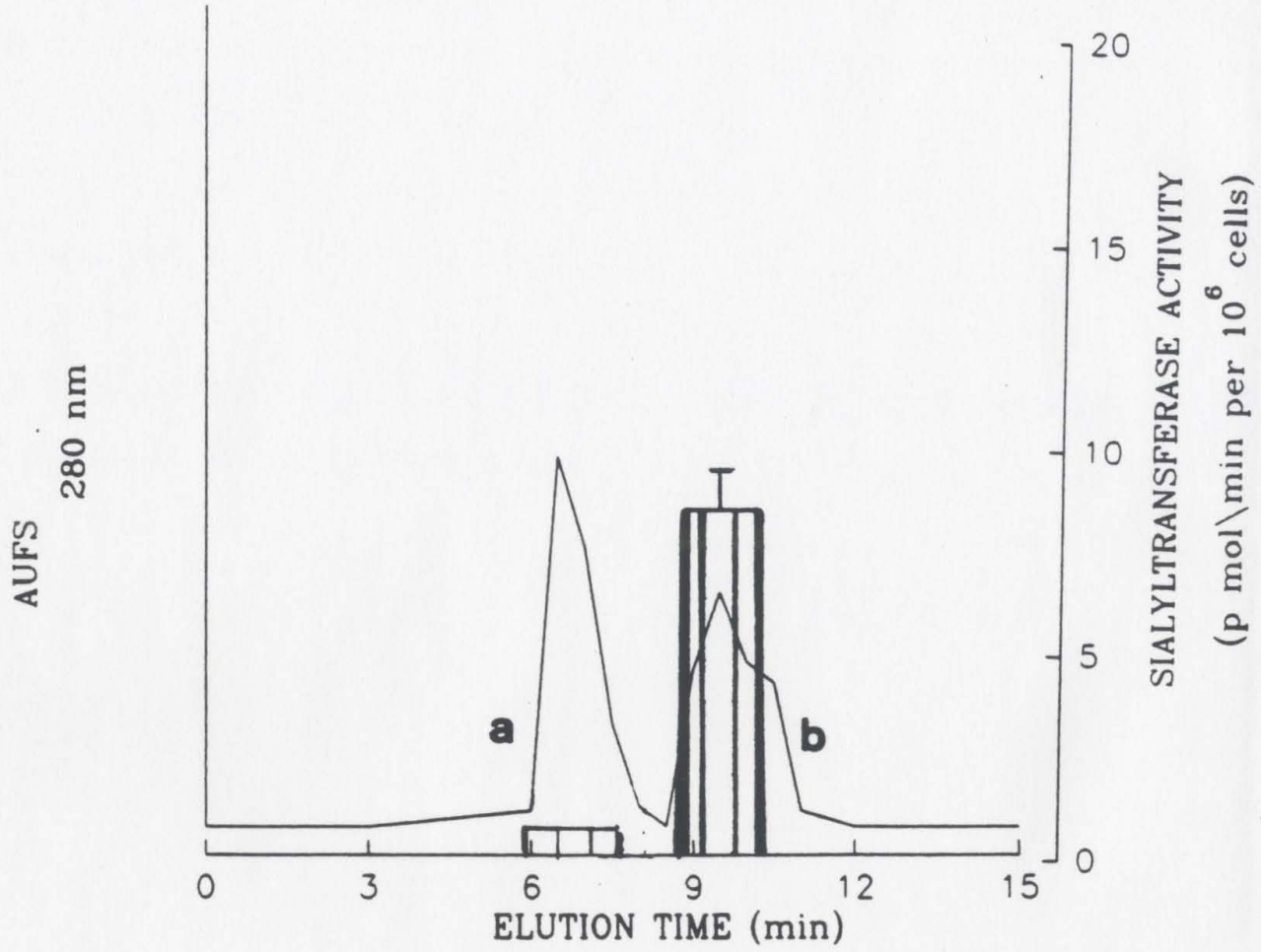


Figure 5.11. HPLC elution profiles of sialyl-lactosamine isomers

Figure 5.11. HPLC elution profiles of sialyl-lactosamine isomers. Medium samples were prepared by incubating hepatocytes in buffer for 4 h as described in Figure 5-10. Samples were assayed for STase using lactosamine as described in Materials and Methods (section II.7.1.B.). Samples were processed and HPLC carried out as described (section II.2.2). Fractions under the peaks for the α2→3 (a) and α2→6 (b) sialyl-lactosamine were collected and counted for radioactivity. STase activity (▥) is expressed as p mol/ min per 10^6 cells. Results represent means ± S.D. from three experiments.

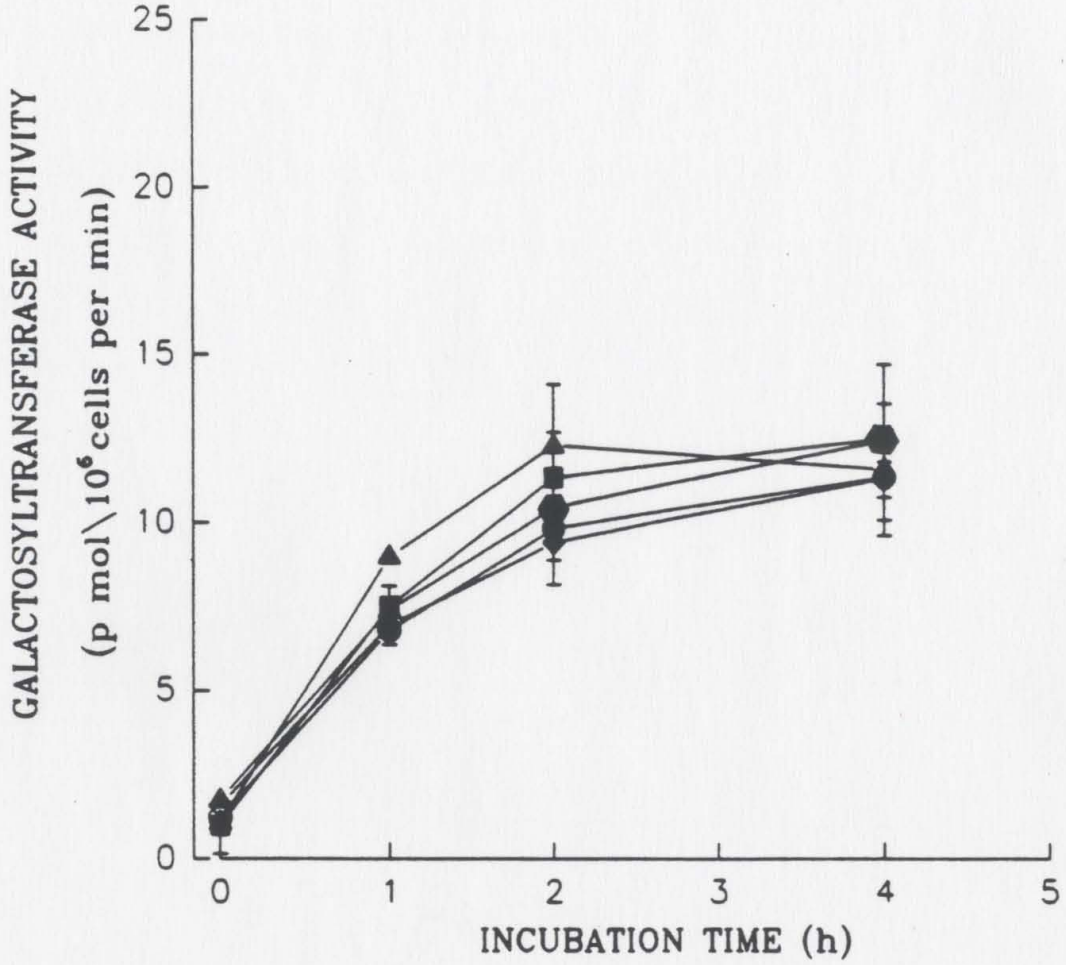


Figure 5.12. Effect of protease inhibitors in the incubation medium on the GTase released during the incubation of hepatocytes

Figure 5.12. Effect of protease inhibitors in the incubation medium on the GTase released during the incubation of hepatocytes. Hepatocytes were incubated without antiprotease (▲), 20% (v/v) heat-inactivated control serum (■), 20% (v/v) heat-inactivated turpentine treated serum (◆), 500 μg HBF (⬢) or 250 μg TBP (○) and at indicated times medium samples were prepared as described in Materials and Methods (section 2.7.2.). GTase activity is expressed as p mol of Gal transferred/h per ml medium. Results represent means ± S.D. from five experiments.

V.3. Discussion

In this chapter the relationship between the balance between proteases /protease inhibitory activity and STase activity was studied in systems other than the jejunal incubations covered in Chapters III and IV. Specifically glycosyltransferases from a commercial source, from serum and from hepatocyte cultures were studied. The results indicate that STase was susceptible to proteolytic inactivation whereas GTase was relatively stable. Protease inhibitors form very tight or even covalent complexes with their target proteases, resulting in inactivation of both the inhibitor and the protease [Travis and Salvesen, 1983]. When a mixture of pure STase(s) or GTase was treated with either trypsin or plasmin it was observed that STase ($\alpha 2\rightarrow 6$[N] or $\alpha 2\rightarrow 3$[O]) was very susceptible to proteolytic inactivation compared to GTase [Figures 5.1 and 5.3]. TBP was able to protect STase from proteolytic inactivation [Figures 5.2 and 5.4]. Thus from these results it can be concluded that STase was more susceptible to the action of trypsin and plasmin than GTase and TBP a protease inhibitor was able to prevent this proteolytic inactivation.

Turpentine induced inflammation in rat is used as a model of the acute phase response. This condition is characterised by an increase in serum STase ($\alpha 2\rightarrow 6$ Gal$\beta 1\rightarrow 4$ GlcNAc) and it has been suggested that the enzyme is an acute phase marker

[Kaplan et al., 1983; Lammers and Jamieson, 1987]. The increase in STase activity in serum is thought to be due to the increased activity of a cathepsin-D like protease in liver which cleaves STase from its membrane-anchor [Lammers and Jamieson, 1988, 1990; McCaffrey and Jamieson, 1993].

The mechanism of release of STase has been elucidated [Weinstein et al., 1987; Paulson and Colley, 1989; Colley et al., 1989; 1992]. The sequence of the enzyme determined from the cDNA showed that the molecule consists of three polypeptide domains [Weinstein et al., 1987; Paulson and Colley 1989]. The protein contained 403 amino acid residues, with the major portion consisting of the catalytic domain [Figure 1.12, page 33]. The catalytic domain was attached to the intralumenal region of the Golgi via a stem region made up of 35 amino acids and this was attached to the membrane domain containing the N-terminus consisting of a nine amino acid cytoplasmic tail and a 17 amino acid signal anchor region [Weinstein et al., 1987; Colley et al., 1989; 1992]. It has been determined that the anchor domain is extremely important for the retention of STase in the Golgi membrane [Colley et al., 1992; Wen et al., 1992]. Therefore, the catalytic unit is oriented inside the Golgi to attach the NeuAc to secretable glycoproteins that are en route through the Golgi network. During the acute phase response it is thought that there is an alteration in the trafficking of lysosomal cathepsin-D, so

that this protease comes in contact with the STase, resulting in proteolytic cleavage of the membrane-bound enzyme which is then released into the medium with other secreted proteins [Lammers and Jamieson, 1988, 1990; McCaffrey and Jamieson, 1993; Jamieson et al., 1993].

Though the mechanism of release of STase from membranes is well worked out, very little is known regarding the factors which control the levels of STase in extracellular fluids. In humans and experimental animals inflammation can arise due to a number of factors including infection, neoplastic disease and chemical agents. Inflammation results in characteristic biochemical and physiological changes known as the acute phase response [Koj, 1974; Kushner and Feldman, 1978; Kaplan et al., 1983]. Among these changes is an increase in the level of serum glycoproteins known as the acute phase reactants. Serum protease inhibitors such as A1PI, TATI, ASTI and α-2 antiplasmin are included in this group of acute phase reactants. The results presented in this thesis with (1) STase released from jejunal slices, (2) pure STases (α2→6 and α2→3), (3) serum STase and (4) STase released from hepatocytes have shown that STase activity is related to the balance between protease inhibitory and protease activities. Protease inhibitors including HBF, TBP, A1PI or α-2 antiplasmin when added to the incubation medium in both jejunal and hepatocyte incubations resulted in higher STase activity being detectable

in the medium. It is therefore probable that serum protease-inhibitors play a role in determining the serum levels of STase in pathological conditions.

Other evidence which supports the hypothesis that soluble STase activity is dependent on trypsin inhibitory activity comes from the work of Kuhlenschmidt and coworkers [1975]. This study involved six paediatric patients with a genetic deficiency of A1PI and associated hepatic cirrhosis. In these patients serum trypsin-inhibitory and STase activities were decreased compared to paediatric controls [Table 5.1]. The authors were unable to explain the significantly lower levels of STase activity observed in these patients. In contrast, serum GTase activity remained similar in patients and controls. Other serum enzymes assayed such as alkaline phosphatase, β-galactosidase, alanine transaminase, aspartate transaminase were either elevated or within the control range. This study provides evidence which supports the hypothesis that STase activity is dependent on protease inhibitory activity. It should be noted that among all the enzymes studied, STase was the only serum enzyme whose activity in serum was decreased.

The release of STase from hepatocytes and liver slices has been studied extensively since liver is considered to be the major source of serum STase [Kaplan et al., 1983; Van Dijk et al., 1986; Woloski et al., 1986]. It was observed that in

Table 5.1. Serum STase and GTase activities in patients with a deficiency of A1PI. Serum STase and GTase activities are shown in individuals with A1PI deficiency and controls. [Adapted from Kuhlenschmidt et al., 1975].

glycosyltransferase activity (μmol/ h per ml serum [x 10^{-1}]

	STase			GTase
acceptor	ds-fetuin	ds-ceruloplasmin	ds-A1PI	ovalbumin
patient I	2.77	1.16	0.70	0.396
patient II	2.39	0.96	0.64	0.334
patient III	1.75	1.02	0.64	0.472
patient IV	2.14	1.08	0.74	0.404
patient V	2.12	1.05	0.69	0.414
patient VI	2.63	1.05	0.67	0.404
mean (I-VI)	**2.30**	**1.05**	**0.67**	**0.404**
Controls				
range	4.04 - 6.64	1.68 - 2.09	1.24 - 1.66	0.26 - 0.59
mean	**6.06**	**1.88**	**1.38**	**0.40**

the presence of dexamethasone an increased release of STase into the incubation medium was observed during experiments with rat hepatocytes [Van Dijk et al., 1986]. The soluble STase activity released into the medium was higher when the incubation medium was supplemented with heat-inactivated serum compared to incubations without the serum supplement. The results presented in this thesis show that the STase activity released into the medium during hepatocyte incubations was higher when the medium was supplemented with antiprotease, (either HBF, TBP, A1PI, α_2 antiplasmin or heat-inactivated serum), compared to incubations without antiprotease. This increase was attributable to the fact that the STase activity released was susceptible to proteolytic degradation and that the protease inhibitors were able to halt this process.

When serum from either turpentine treated or control rats was incubated at 37° C, it was observed that trypsin and plasmin inhibitory activities decreased over the 0 to 4 hours of incubation [Figure 5.5]. Protease inhibitory activity remained higher in turpentine treated serum compared to control serum throughout the 4 hours of incubation [Figure 5.5]. STase activity followed the same pattern as protease inhibitory activity and was higher in serum from turpentine treated rats compared to serum from control rats [Figure 5.6]. Thus higher STase activity was paralleled by higher trypsin

and plasmin inhibitory activities. TBP when added to serum slowed down the decrease in serum STase activity observed during incubation [Figure 5.6]. Serum GTase activity was similar in turpentine treated and control rats, indicating that GTase, unlike STase was not affected by the protease and protease inhibitory activities [Figure 5.7].

Very little is known regarding the factors which control the levels of soluble STase activity in serum and other tissue fluids, as well as the STase released during tissue culture experiments. STase activity in serum is increased in disease conditions including cancer, such as in multiple myeloma [Frithz et al., 1985]. The levels of plasma protease inhibitors are increased during cancer and may influence plasma STase activities [Yoshida et al., 1989; Taccone et al., 1991; Lee et al., 1992].

The function of STase in extracellular fluids is unclear. The levels of CMP-NeuAc in circulation are low, even during the inflammatory response and it is therefore unlikely that the function of STase in serum is to sialylate proteins [Kaplan et al., 1984]. Since serum STase is thought to play a role in inflammation it has been hypothesised that STase is utilized in glycosylation reactions at the site of injury [Jamieson et al., 1993]. However there is no evidence to support this theory at the present time.

STases are key enzymes in the glycosylation of proteins.

NeuAc is usually the terminal sugar on glycan chains and also gives a negative charge to macromolecules, which is thought to be important for the function of the molecule. For example, NeuAc is believed to play an important role in cellular recognition molecules such as the sialyl-Lex present on monocyte, neutrophil and tumour cell surfaces. Sialyl-Lex acts as a ligand for recognition between the family of selectin adhesion receptors on endothelial cells and lymphocytes [Polley et al., 1991; Berg et al., 1992; Majuiri et al., 1992]. The NeuAc in sialyl-Lex is not present in an α2→6 linkage, but in an α2→3 linkage. However the role of serum STase which is the α2→6 enzyme in not well defined and more work is required in order to clearly define the role of STase in serum.

Proteases and their inhibitors are widely distributed in tissues including intestine and liver. Trypsin and plasmin are present in intestine [Boyd et al., 1988; Wakabayasi and Kawaguchi, 1992] and liver [Tanaka et al., 1986; Kitada et al., 1993; Tamanoue et al., 1993]. Trypsin is secreted into the intestine from the pancreas whereas liver synthesizes plasmin and secretes it. The presence of trypsin and/or plasmin inhibitors in intestine [Geboes et al., 1982; Bohe et al., 1986 b, 1987] and liver [Laurell and Ericksson, 1963; Liebermann et al., 1972; Bathurst et al., 1984] have been well documented. Protease inhibitors are synthesised in liver and

secreted into the circulation. Proteases and their inhibitors are thought to play important roles in disease processes including cancer [Goldfarb and Liotta, 1986; Duffy, 1987; Zucker, 1988] and inflammatory diseases [Bohe et al., 1986a,b; Strygler et al., 1990]. Plasma levels of protease-inhibitors and proteinases are altered in diseases such as emphysema [Laurell and Ericksson, 1963; Martin et al., 1973], pancreatitis [Martin et al., 1973; Aroasio and Piantino, 1991; Taccone et al., 1991], inflammation [Matsuda et al., 1980; Jonsson et al., 1982; Travis and Salvesen, 1983; Heidtman and Travis, 1986] and liver diseases [Aoki and Yamanaka, 1978, Trischitta et al., 1991]. Therefore in similar conditions the levels of protease-inhibitors in tissue fluids could affect STase activity.

In conclusion STase activity was related to protease-inhibitory activity in experiments with (1) a mixture of pure STase and GTase, (2) STase in serum from turpentine-treated and control rats and (3) STase activity released during the incubation of hepatocytes. Increased protease inhibitory activity was associated with increased STase activity. This relationship with protease inhibitory activity was not observed in the case of GTase. These studies indicated that soluble STase is more sensitive to proteolytic inactivation by trypsin or plasmin than is soluble GTase.

CHAPTER VI. SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

VI.1. Summary

(1). The incubation of jejunal slices resulted in the release of soluble STase into the incubation medium. However the STase released was susceptible to proteolysis and in order to measure activity there was a requirement for either heat-inactivated serum, A1PI or α_2 antiplasmin in the incubation medium.

(2). The addition of heparin to HHS or HRS supplemented incubations resulted in a decrease in medium STase activity compared to similar incubations without heparin.

(3). HBF isolated from HRS and HHS was determined to be the serum component which was exerting the protective effect on STase. HBF displayed inhibitory activity towards trypsin and plasmin. TBP which also displayed trypsin and plasmin inhibitory activity was isolated from HBF and was able to protect the STase activity released from jejunal slices.

(4). Heat-inactivated serum from turpentine treated rats had higher trypsin and plasmin inhibitory activities than did heat-inactivated serum from control rats. When heat-inactivated serum from turpentine treated rats was used to supplement jejunal and hepatocyte incubations, STase activity released into the medium was increased compared to incubations

supplemented with heat-inactivated control serum.

(5). Trypsin and plasmin, when added to a mixture of pure STase and GTase, preferentially degraded STase activity. TBP was able to prevent the decrease in STase activity caused by proteases.

(6). Serum from turpentine treated rats showed higher STase as well as trypsin and plasmin inhibitory activities compared to control serum. Incubation of sera from control or turpentine treated rats at 37° C lead to a decrease in STase and protease inhibitory activities. TBP was able to protect STase from the action of trypsin and plasmin.

(7) STase activity released into the medium during the incubation of hepatocytes was higher when the incubations were supplemented with either heat-inactivated serum from control rats, HBF or TBP compared to incubations in buffer alone. When the incubations were supplemented with heat-inactivated serum from turpentine treated rats STase activity was increased compared to incubations where heat-inactivated control serum was used.

VI.2. General discussion

VI.2.1. Trypsin and plasmin and their inhibitors

Trypsin and plasmin have been implicated in a number of disease processes. For example, plasmin is thought to play a role in angiogenesis, inflammation and tumour metastasis

[Vassalli et al., 1991]. Trypsin has a function in the onset and development of pancreatitis [Creutzfeldt and Schmidt, 1970; Steer, 1986; Arais et al., 1993]. During pancreatitis trypsin and other pancreatic enzymes are activated within the pancreas causing tissue damage as well as increased levels of trypsin and decreased levels of trypsin inhibitors in plasma. Tumour associated trypsins (TATs) have been isolated from mucinous ovarian cyst fluid and showed some similarities with pancreatic trypsin [Emi et al., 1986; Koivunen et al., 1989, 1990]. TATs are produced by many tumour cell lines of different origins [Ikonen et al., 1990].

In the pancreas the role of trypsin inhibitors such as TATI/PSTI (tumour associated trypsin inhibitor/ pancreatic secretory trypsin inhibitor) is thought to be protection of the gland against destruction by premature activation of trypsin [Pubols et al., 1974]. The main target of A1PI, which is the major protease inhibitor in serum is leucocyte elastase and decreases in serum A1PI are associated with pulmonary and hepatic damage [Erickson, 1984]. Trypsin inhibitors in the intestine are thought to provide the intestinal tissue protection against pancreatic trypsin [Freeman et al., 1990b]. Serum and tissue levels of trypsin inhibitors are increased in malignant conditions and inflammation [Stenman, 1990, 1991; Taccone et al., 1991]. It is believed that these inhibitors protect the tissues from being destroyed by proteases. Severe

infections and tissue destruction also cause increases in serum and urinary levels of trypsin-inhibitors [Lasson et al., 1986; Ogawa et al., 1987, Ogawa, 1988; Stenman et al., 1991].

Trypsin inhibitors including A1PI (α_1 proteinase inhibitor), PSTI/TATI (pancreatic secretory trypsin inhibitor/ tumour associated trypsin inhibitor) and ASTI (acid stable trypsin inhibitor) also inhibit plasmin [Panell et al., 1974; Heidtman and Travis, 1986; Sumi et al., 1987; Turpeinen et al., 1988]. At least three mechanisms have been described which can increase the concentration of trypsin inhibitors in circulation. These include leakage due to tissue damage such as that seen during pancreatitis, increased production and secretion by cells or tumours cells and impaired renal function [Stenman, 1990; Lasson et al., 1986].

It is therefore evident that a number of pathological conditions are associated with alterations in the circulatory levels of trypsin and plasmin inhibitors. The circulatory levels of these inhibitors may in turn affect the levels of circulatory proteins such as STase.

VI.2.2. STase released during jejunal slice incubations

The soluble STase activity released during jejunal incubations was susceptible to proteolytic degradation. Therefore in order to measure STase activity released there was a requirement for either HHS, HRS, TBP, A1PI or α_2

antiplasmin in the incubation medium. All these protease inhibitors inhibited trypsin and plasmin. These observations support the findings of other researchers who reported that HHS when present in the incubation medium caused a release of STase from jejunal slices [Ratnam et al., 1987]. The results in this thesis indicate that the STase was released even in the absence of heat-inactivated serum. However, the STase released was labile and the role of heat-inactivated serum in the release of STase was to prevent the STase released from being destroyed by proteases. Other researchers have also reported that the activity of STase released into the incubation medium was increased when intestinal incubations were supplemented with heat-inactivated fetal calf serum [Kolinska et al., 1990; Hamr et al., 1993]. Therefore the results in this thesis provide an explanation regarding the role of heat-inactivated serum in the release of STase during intestinal incubations. The STase released into the medium was the Galβ1→4GlcNAc STase [Chapter III, this thesis] which supports the observations made by Hamr et al., [1993].

According to the findings in this thesis protease inhibitors had a part in determining the levels of STase in the incubation medium. The implications arising from this observation are that inhibitors of trypsin and /or plasmin in incubation media or body fluids influence the levels of soluble STase. The results indicated that TBP isolated from

HBF was the serum component required in the incubation medium in order to measure the STase released. Other inhibitors of trypsin and plasmin such as A1PI and α_2 antiplasmin were equally effective. Therefore these results imply that rather than one specific inhibitor such as TBP, other inhibitors of trypsin and plasmin in general were also effective in protecting STase.

The intestine is rich in proteases with a number of them originating from the pancreas [Tsukamoto et al., 1986]. Intestinal secretions are also rich in proteases [Geboes et al., 1982]. Therefore the presence of proteinases in the jejunal slice incubations is not surprising. Protease-inhibitors/ proteases are thought to play important roles in the pathophysiology of the intestine. A1PI, ASTI and TATI are produced by the intestinal cells [Geboes et al., 1982; Bohe et al., 1986 a, b]. As discussed in the previous chapter serum levels of proteinase inhibitors including A1PI and TATI are altered during pathological conditions, and the altered balance between proteinase inhibitors/ proteinases may also affect STase.

Trypsin and plasmin inhibitors in serum are increased during the acute phase response [Travis and Salvesen, 1983]. For example thermal injury in the rat was also associated with increased STase activity in intestine and serum [Chu et al.,

1988]. Therefore in this and similar conditions protease inhibitors could influence soluble STase activity in plasma.

VI.2.3. STase released during hepatocyte incubations

The liver is thought to be the major source of serum STase, as a result of which the release of STase from liver slices and hepatocytes has been extensively studied [Kaplan et al., 1983; VanDijk et al., 1986; Lammers and Jamieson, 1988]. In cultures with hepatocytes and liver slices it has been demonstrated that during incubation STase was released into the incubation medium [Kaplan et al., 1983; VanDijk et al., 1986; Lammers and Jamieson, 1988; Harder et al 1990]. The enzyme released from rat liver has been well characterized and it has been shown to be Galβ1→4GlcNAcα2→6 STase, which is similar to the soluble serum enzyme [Kaplan et al., 1983; Lammers and Jamieson, 1988; Harder et al., 1990; this thesis Chapter 5]. It has been shown in experiments with hepatocytes and hepatoma cell lines that glucocorticoids and cytokines caused increased release of STase measured by increased STase activity in the medium [VanDijk et al., 1986; Harder et al., 1990]. This effect of glucocorticoids and cytokines is thought by Wang to be due to increased expression of mRNA for STase [Wang et al., 1989; 1990a].

Serum STase is increased during inflammation in the rat, mouse and guinea pig and the enzyme is considered to be an acute-phase reactant [Lombart et al., 1980; Lammers and

Jamieson, 1986, 1988, 1989, 1990]. This increase in serum STase has been attributed to an increase in the activity of a cathepsin D like protease which cleaves the STase from the membrane. But very little information is available regarding the factors that control the levels of the STase released.

Unlike the jejunal incubations where only trace amounts of STase activity were detected in the medium when the incubations were carried out in KRB alone, appreciable amounts of STase were detected in the medium during the incubation of hepatocytes in KRB. However in both jejunal and hepatocyte incubations addition of heat-inactivated serum or antiproteases to the incubation medium resulted in increased STase activity. Other researchers have reported that the STase activity released during the incubation of hepatocytes was increased when the incubation medium contained HHS [VanDijk et al., 1986]. The results in this thesis suggest that soluble STase activity is protected by trypsin and plasmin inhibitors some of which are present in heat-inactivated rat serum (HRS). This protection from proteolysis is one of the factors involved in the control of STase activity in serum.

Proteinase inhibitors and proteinases are involved in the pathophysiology of liver disease. Proteinases inhibitors including A1PI, ASTI, PSTI and α_2 antiplasmin are acute phase reactants. Increased production of these inhibitors by the

liver during the acute phase response is thought to account for the increased serum concentrations during the acute phase response [Jonsson et al., 1982; Travis and Salvesen, 1983; Heidtman and Travis, 1986]. Increased serum levels of these inhibitors have also been observed in hepatic disease [Trischitta et al., 1991; Lee et al., 1992]. Trypsin inhibitors are also present in bile [Yamamoto et al., 1986] and human hepatoma cells have been shown to release a trypsin inhibitor [McKeehan et al., 1986]. Therefore the levels of proteinase inhibitors in disease conditions could well influence levels of enzymes such as STase in plasma.

VI.2.4. Function of protease inhibitors in incubation systems

Serine proteinases are present in liver and intestine and have been isolated from hepatic membranes [Tanaka et al., 1986; Tamanoue et al., 1993]. The intestine is also rich in proteases [Tsukamoto et al., 1982]. The inclusion of serum proteinase inhibitors in cell/tissue culture systems is believed to curtail proteolytic damage to cells [Tanaka et al., 1986]. The results presented in this thesis indicate that STase released during tissue or cell incubations can also be damaged by proteases, indicating that proteases can destroy proteins released into the incubation medium. Therefore in tissue and cell incubations care should be exercised in the interpretation of results especially if the release of proteins is being researched as these proteins may be the

targets for proteases. This was especially applicable to the jejunal incubation systems.

In the case of jejunal and hepatocyte incubations low levels of STase were observed in buffer and increased levels of STase were observed when the buffer was supplemented with proteinase inhibitors. Thus the low STase activity detected when the incubations were carried out in buffer alone was due to the fact that the STase was being destroyed by proteases and not because of the rate of release.

VI.2.5. STase in serum

Increased STase activity in serum has been observed in disease conditions including cancer and inflammation. Proteinase inhibitors make up at least 10% of the total serum proteins [Travis and Salvesen, 1983]. Changes in the balance between protease inhibitors/ proteases in serum could affect serum levels of STase. Serum STase is considered to be an acute phase marker and during inflammation there is an increased release of STase from the liver [Lammers and Jamieson 1986,1988,1990; Jamieson et al., 1993]. However proteinase inhibitors in blood or serum could also have a bearing on STase activities. In the experiments presented in this thesis serum STase and protease inhibitory activities decreased with time of incubation at 37°C [Chapter V; Figures 5.5 and 5.6]. TBP prevented this decline in STase activity.

Storage of plasma and blood at 22° and 37°C has been shown to increase proteolytic activity [Bode and Norris, 1992; Wallvik et al., 1992]. In the experiments with serum in this thesis, the incubation of serum at 37° C lead to decreases in trypsin and plasmin inhibitory and STase activities [Chapter V; Figures 5.5 and 5.6]. Other studies have also indicated that in serum, decreased trypsin inhibitory activity was accompanied by decreased STase activity [Kuhlenschmidt, et al., 1975]. Therefore, there is strong evidence that the activity of serum STase is closely related to trypsin and/or plasmin inhibitory activities.

GTase was also studied and the results presented in this thesis indicate that GTase was not readily inactivated by trypsin or plasmin and thus its activity did not depend upon the presence of protease inhibitors.

VI.2.6. Protease inhibitors/proteases in serum may represent a means by which activities of other enzymes are controlled

Generally, enzymes are present at higher concentrations in cells than in plasma/ serum or other tissue fluids. Normal plasma levels of enzymes reflect the balance between synthesis and release of enzymes during normal cell turnover and their clearance from circulation. A number of factors contribute to the plasma levels of enzymes. These include the rate of release from cells, the degree of cell damage, induction of

enzyme synthesis, the extent of cell proliferation and the rate of enzyme clearance from plasma [Zilva and Pannal, 1984]. However very little is known regarding the factors that actually control enzyme activities in plasma or serum. As mentioned above, a number of pathological conditions are associated with alterations in the protease inhibitor and protease levels in plasma and other body fluids. Since STase activity in plasma is related to the balance between levels of protease inhibitors and proteases and this observation may signify a means of control of activities of enzymes such as STase in incubation media and body fluids.

VI.3. Future perspectives

The results presented in this thesis indicate that soluble STase activity was directly proportional to the level of trypsin and plasmin inhibitors and inversely proportional to trypsin and plasmin activities. More work is needed to further clarify this relationship. There is a need for studies directed towards measuring levels of STase and proteinase inhibitors in altered metabolic states such as pancreatitis, sepsis, emphysema and trauma where there is an imbalance between protease inhibitors and protease levels in tissue fluids. Pancreatitis is associated with an increase in activation of proteases in pancreatic fluid and blood. Emphysema is another condition where increased tissue proteolysis occurs and the plasma levels of protease

inhibitors are altered.

This work has concentrated on defining the role of proteases and protease inhibitors in stabilising soluble STase activity. In this thesis only serine proteinases and their inhibitors were studied. However blood contains other proteinases and inhibitors including cysteine proteinases. It would be interesting to determine whether other proteinase inhibitors influence levels of serum enzymes such as GTase, alkaline phosphatase and lactate dehydrogenase.

In conclusion, the results of this study show that it is important to consider proteolytic degradation of soluble enzymes in tissue fluids when enzyme activities or concentrations are being studied.

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