



THE PROTEASE-SENSITIVE RELEASE OF
ENZYMES FROM PANCREATIC MICROSOMES

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SUMMARY

This thesis describes the release of enzymes from a rat pancreatic microsomal fraction. During incubation at 37°C α -amylase associated with the microsomal fraction was transferred to the external medium. The addition of proteases, e.g. trypsin and chymotrypsin, to the incubation medium inhibited this transfer. Although the inhibition of release was the result of proteolysis it could be shown that the releasable enzymes were not destroyed. Ribonuclease was similarly released by a process which was inhibited by exogenous proteases.

Explanations for this protease-sensitive enzyme transfer such as de novo synthesis and large-scale vesicle lysis were shown to be untenable, while leakage of vesicle contents and desorption of enzyme from the membrane appeared unlikely.

During incubation, detachment of ribosomes from the microsomal membrane occurred. Addition of Mg^{2+} to the incubation buffer at concentrations in excess of 1 mM inhibited ribosome detachment and enzyme release. The detachment of ribosomes and the transfer of enzyme did not appear to be directly related since ribosomes could be stripped from the vesicles in the presence of Mg^{2+} without enzyme release.

Sonication experiments and the observation that releasable RNAase was protected from digestion by subtilisin BPN' suggested that releasable enzymes were originally

located within the vesicles. This implied that the enzymes crossed the microsomal membrane during transfer. The released α -amylase appeared to have the same molecular form as the secreted enzyme.

The possibility that the release of enzyme was effected by a transport protein in the microsomal membrane is discussed in the light of current knowledge regarding the process of protein secretion.

This thesis contains no material which has been accepted for the award of any other degree or diploma. The studies were carried out in the Department of Biochemistry, University of Adelaide, under the supervision of Professor W.H. Elliott and Dr B.K. May. With the exception of some of the electron microscope work the experiments were done only by myself. To the best of my knowledge and belief this thesis contains no material previously published or written by another person except where due reference is made in the text.

(P.D. Pearce)

ABBREVIATIONS

The following abbreviations have been used in this thesis:

α -amylase	1,4- α -Glucan 4-glucanohydrolase; E.C. No. 3.2.1.1
ATP	Adenosine 5'-triphosphate
DTE	Dithioerithritol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
\underline{g}_{av}	Gravitational field at midpoint of centrifuge tube
\underline{g}_{max}	Gravitational field at base of centrifuge tube
GTP	Guanosine 5'-triphosphate
IgG	Immunoglobulin G
Met-tRNA _f ^{Met}	Formyl-methionine charged transfer RNA
mRNA	Messenger ribonucleic acid
PMSF	Phenylmethyl sulphonyl fluoride
poly(A)	Polyadenylic acid
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
RNAase	Ribonuclease
RNAase A	Ribonucleate 3'pyrimidino-oligonucleotido- hydrolase; E.C. No. 3.1.4.22
SDS	Sodium dodecyl sulphate
TPCK	L-1-Tosylamide-2-phenylethylchloromethyl ketone
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
Triton X-100	p-Isooctylphenoxyethoxyethanol
tRNA	Transfer ribonucleic acid

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Finally I wish to thank my wife Vonette for her support and encouragement during the course of these studies.

CHAPTER ONE

GENERAL INTRODUCTION

GENERAL INTRODUCTION1.1 INTRODUCTION

In both eukaryotic and prokaryotic cells a number of proteins, for example extracellular enzymes, organelle enzymes and some membrane proteins, are transferred across membranes. This raises the problem of how hydrophilic protein molecules such as secretory proteins cross the hydrophobic lipid bilayer of the membrane. Those integral membrane proteins which have substantial hydrophilic regions projecting beyond the extracytoplasmic surface of the membrane (ectoproteins) pose a somewhat similar problem (see the review of J. Rothman and Lenard, 1977).

This thesis describes an investigation of enzyme release from a rat pancreatic microsomal fraction, a previously unreported phenomenon, which may be of relevance to pancreatic enzyme secretion. This introduction will discuss the literature on the synthesis and secretion of proteins from cells. Recently two reviews (Glenn, 1976 and Priest, 1977) have been published describing bacterial extracellular enzyme secretion in some detail, and Palade (1975) has discussed the process of synthesis and secretion of extracellular enzymes in exocrine pancreatic cells. This introduction will be confined to those aspects of the literature relevant to the work described in this thesis with particular emphasis being placed on work reported since the above reviews were written.

1.2 THE SYNTHESIS AND SECRETION OF EXTRACELLULAR ENZYME IN EUKARYOTIC CELLS.

1.2a The Overall Process of Secretion in the Pancreatic Cell.

Much of our current knowledge of extracellular enzyme synthesis and secretion arises from the now classical studies of Palade and his coworkers on mammalian pancreatic exocrine cells. The pancreatic exocrine cell has proved particularly useful in this regard since it is a specialised cell largely devoted to the synthesis of exportable protein.

Several different techniques have been used to study secretion in the pancreatic cell (Palade, 1975) and the generally accepted sequence of events is as follows:

synthesis occurs on polyribosomes attached to the membrane of the endoplasmic reticulum (RER, Fig.1.1) and the nascent peptide is vectorially extruded through the membrane into the cisternal space of the RER. The completed protein moves to the transitional elements of the RER which bud off, enclosing a sample of the luminal contents, to form Golgi vesicles. These vesicles fuse with the condensing vacuoles of the Golgi complex and, as the vacuoles fill with protein, they become mature zymogen granules. The zymogen granules reside in the cytoplasm and, on stimulation of the cell, the secretion products are released by exocytosis. This is brought about by a fusion of the plasmalemma and zymogen granule membranes,

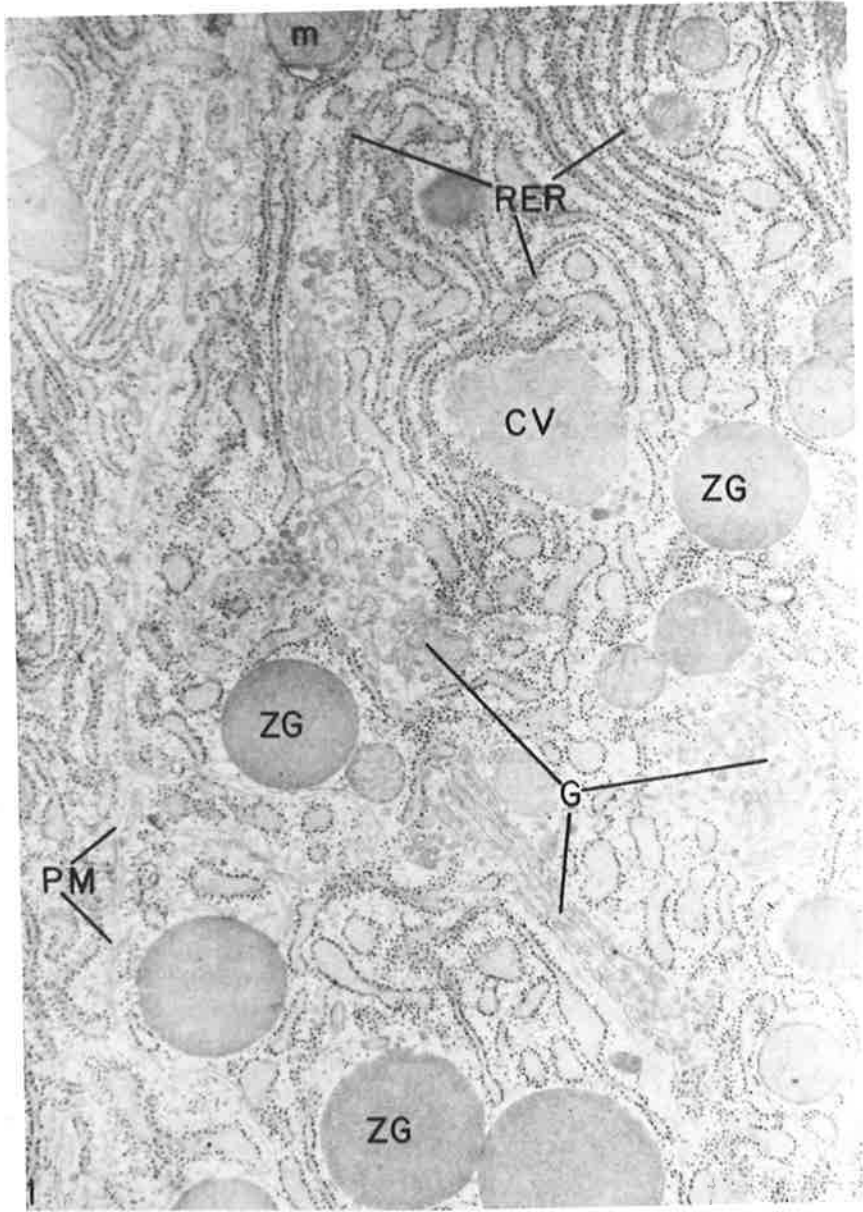
FIGURE 1.1 ELECTRON MICROGRAPH OF AN EXOCRINE PANCREATIC
CELL

Electron micrograph of a thin section through calf
pancreatic cells.

RER = rough endoplasmic reticulum;
CV = condensing vacuoles;
ZG = zymogen granules;
M = mitochondria;
G = Golgi complex;
PM = plasmalemma.

Total magnification: 16,000. Taken from Tartakoff
and Jamieson (1974).

Fig . 1 . 1



followed by fission of the fused membranes. A diagrammatic representation of this process, taken from Palade (1976), is shown in Fig.1.2.

1.2b Vectorial Extrusion of the Nascent Peptide

The first step in the process of secretion is of particular interest, namely, the extrusion of the nascent peptide through the membrane. As many of the early experiments which led to this conclusion are adequately described in the reviews of Palade (1975) and Shore and Tata (1977) they will be discussed in outline only here.

The first evidence that secretory proteins were synthesised on membrane-bound polysomes came from Siekevitz and Palade (1960) who showed that chymotrypsinogen was preferentially synthesised by bound polysomes, and there is now general agreement that, under normal conditions, all proteins to be secreted from the cell are made on bound polysomes (Rolleston, 1974 and Shore and Tata, 1977). The concept of vectorial extrusion of the nascent peptide (i.e concomitant synthesis and transport across the membrane) was introduced by Redman and Sabatini (1966) who proposed that the nascent peptides grew within a channel in the large ribosomal subunit. It was suggested that this channel was, or could be made continuous with the cisternal space through a discontinuity in the membrane, and that transfer of the completed protein was achieved by release of the peptide from the large subunit. The experiments of Blobel and Sabatini (1970), who showed that

30 - 40 residues at the COOH-terminal of the nascent peptide on free ribosomes were protected from proteolysis, and of Sabatini and Blobel (1970), who showed that the entire nascent peptide associated with microsomal vesicles was protected against proteolysis, supported this proposal.

Concomitant synthesis and transport requires a mechanism by which the cell is able to distinguish between polysomes synthesising proteins for intracellular use and those synthesising proteins destined for export. Of the different theories put forward to account for this (see the reviews of Rolleston, 1974, McIntosh and O'Toole, 1976 and Shore and Tata, 1977) the one now favoured is that proposed independently by Blobel and Sabatini (1971) and Milstein et al. (1972). Blobel and Sabatini proposed that mRNA's for secretory proteins contained information which was translated into a sequence at the amino-terminal of the nascent peptide and that this peptide sequence was involved in promoting the binding of polysomes to the membrane. Milstein et al. (1972) observed that detached polysomes synthesised IgG as a precursor protein with an extra sequence at the NH₂-terminal, and proposed that such a sequence would be a simple way of ensuring that secretory proteins were synthesised on bound polysomes.

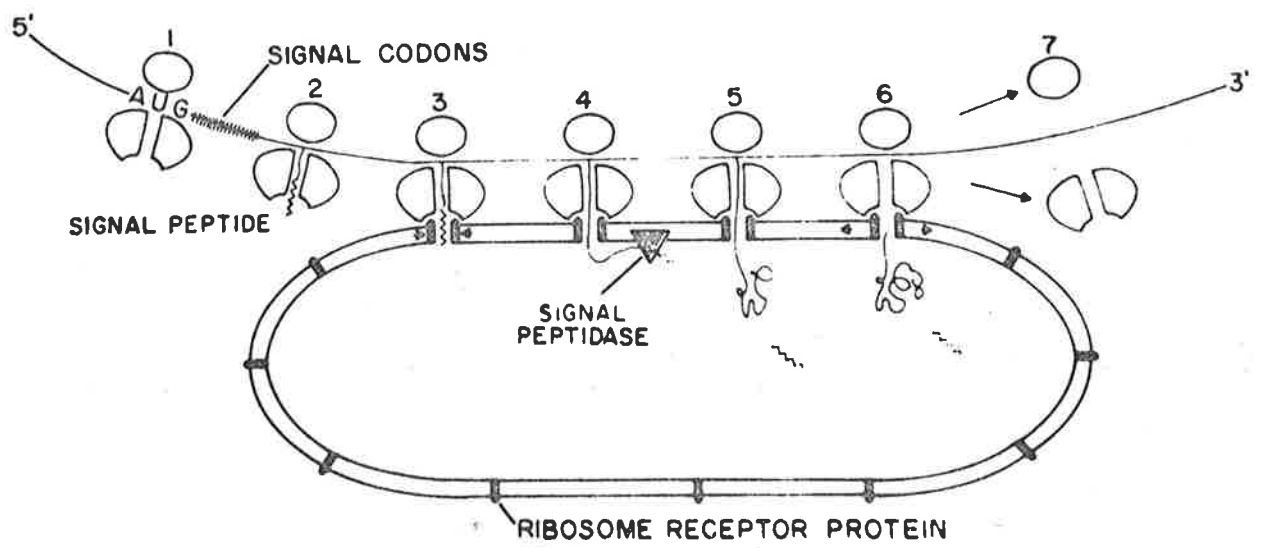
1.2c The Signal Hypothesis.

These proposals were extended by Blobel and Dobberstein (1975a) in their elegant "signal hypothesis" (schematically illustrated in Fig.1.3) which postulated that all mRNA's for secretory proteins contain "signal codons" adjacent to the initiator codon. They proposed

FIGURE 1.3 A SCHEMATIC ILLUSTRATION OF THE SIGNAL
HYPOTHESIS

Taken from Blobel (1976). See text for a detailed explanation of the hypothesis.

Fig. 1.3



that initiation and translation of the signal and the immediately adjacent codons occur on free ribosomes. On emerging from the large ribosomal subunit the signal sequence of the nascent peptide penetrates the membrane of the ER, inducing an association of specific ribosome receptor proteins in the membrane. The result of this association is the formation of a tunnel through the membrane. Interactions between the associated receptor proteins and the large ribosomal subunit lead to the binding of the large subunit to the membrane and stabilisation of the membrane tunnel. The large subunit binds to the membrane in such a way that the newly-formed tunnel in the membrane permits passage of the nascent peptide through the membrane into the intracisternal space. A membrane-bound peptidase - the "signal peptidase" - on the inner surface removes the signal sequence from the nascent peptide after the signal emerges from the tunnel. The processed nascent peptide continues to grow and, after chain termination and release of the ribosome from the membrane, the secretory protein is completely segregated within the lumen of the ER. Detachment of the ribosome from the membrane allows dissociation and diffusion of the receptor proteins thereby eliminating the membrane tunnel.

1.2d Evidence Supporting The Signal Hypothesis.

Evidence supporting the signal hypothesis is the fact that the mRNA's for a number of secretory proteins have now been translated in vitro in the absence of

membranes i.e. under conditions where it might be expected that the proteins would retain their signal sequences. Earlier studies were reviewed by Campbell and Blobel (1976) and all the translation products so far examined, with the exception of ovalbumin (Palmiter et al. 1978) which will be discussed later, have been found to be precursor proteins with largely hydrophobic sequences at the NH₂-terminals. These precursor proteins are called pre-proteins and Table 1.1 (from Habener et al., 1978) lists the amino acid sequences from some pre-proteins. Although the signal sequences all contain an abundance of hydrophobic residues, there are few discernable homologies in their primary structures. The sequences also vary in length from 17 to about 30 residues and there are considerable differences in the amino acids surrounding the cleavage sites.

Further evidence in favour of the signal hypothesis comes from in vitro reconstitution of rough microsomes from heterologous components. Blobel and Dobberstein (1975b) showed that ribosomal subunits from rabbit reticulocytes could function with dog pancreatic microsomes, stripped of ribosomes (obtained by treating rough microsomes with EDTA or puromycin/KCl), in a protein synthesising system from Krebs ascites cells to translate the mRNA for the light chain of immunoglobulin isolated from murine myeloma. As the translation product was protected against proteolysis by exogenous proteases it was assumed that the protein was located within the intravesicular space of the microsome. (It might perhaps be noted that this does not prove the protein is freely soluble within the microsome

TABLE 1.1

Complete and partial amino acid sequences of the amino-terminal extensions of some pre-proteins. Circles and arrows denote sites of cleavage. Taken from Habener et al. (1978). (Pre-proPTH = pre-proparathyroid hormone).

TABLE 1.1

Pre-ProPTH (Bovine)	Met-Met-Ser-Ala-Lys-Asp-Met-Val-Lys-Val-Met-Ile-Val-Met-Leu-Ala-Ile-Cys-Phe-Leu-Ala-Arg-Ser-Asp-	Gly	Lys	-ProPTH
Pre-Proinsulin (Rat)	(X)-Leu-Lys-Met- X -Phe-Leu-Phe-Leu-Leu-Ala-Leu-Leu-Val-Leu-Trp-Glu-Pro-Lys-Pro-Ala-Gln-	Ala	Phe	-Proinsulin
Pre-Placental Lactogen	Met-Pro- X - X - X - X - X -Leu-Leu-Leu- X - X - X -Leu-Leu- X -Leu- X -Pro- X - X - X - X - X - X -	X	Val	-PL
Pre-Growth Hormone (Rat)	Met-Ala-Ala-Asp-Ser-Gln-Thr-Pro-Trp-Leu-Leu-Thr-Phe-Ser-Leu-Leu-Cys-Leu-Leu-Trp-Pro-Gln-Glu-Ala-	Gly	Ala	Leu
Pre-Proalbumin (Rat)	Met-Lys-Trp-Val-Thr-Phe-Leu-Leu-Leu-Leu-Phe-Ile-Ser-Gly-Ser-Ala-Phe-	Ser	Arg	-Proalbumin
IgG L-Chain Precursor (Mouse)	Met-Asp-Met-Arg-Ala-Pro-Ala-Gln-Ile-Phe-Gly-Phe-Leu-Leu-Leu-Leu-Phe-Pro-Gly-Thr-Arg-	Cys	Asp	-M41 L-Chain
Pre-Trypsinogen 2 (Dog)	(X)-Ala-Lys-Leu-Phe-Leu-Phe-Leu-Ala-Leu-Leu-Leu-Ala-Tyr-Val-Ala-	Phe	Pro	-Trypsinogen
Pre-Lipoprotein (<u>E. coli</u>)	Met-Lys-Ala-Thr-Lys-Leu-Val-Leu-Gly-Ala-Val-Ile-Leu-Gly-Ser-Thr-Leu-Leu-Ala-	Gly	Cys	-Lipoprotein
Pre-Lysozyme	Met-Arg-Ser-Leu-Leu-Ile-Leu-Val-Leu-Cys-Phe-Leu-Pro-Leu-Ala-Ala-Leu-	Gly	Lys	-Lysozyme
Pre-Ovomucoid	(X)-Ala-Met-Ala-Gly-Val-Phe-Val-Leu-Phe-Ser-Phe-Val-Leu- X -Gly-Phe-Leu-Pro-Asp-Ala-Ala-Phe-	Gly	Ala	-Ovomucoid
Pre-Promelittin	Met-Lys-Phe-Leu-Val- X -Val-Ala-Leu-Val-Phe-Met-Val-Val-Tyr-Ile- X -Tyr-Ile-Tyr-	Ala	Ala	-Promelittin

since burial within the membrane or attachment to the inner half of the lipid bilayer would also result in the protection of the protein.) The translation product was proteolytically processed to the same size as the authentic light chain of immunoglobulin. In contrast, the translation of rabbit globin mRNA in this system resulted in a product which was sensitive to proteases.

In other reconstitution experiments Dobberstein and Blobel (1977) showed that wheat germ ribosomes translating mRNA for the light chain of murine immunoglobulin could attach to stripped dog pancreatic microsomal membranes to produce a segregated, processed translation product. The wheat germ translation system was also used by Lingappa et al. (1977), who showed that the translation products of bovine pituitary prolactin and growth hormone mRNA's were processed and segregated if dog pancreatic microsomal membranes were present during translation, whereas pre-prolactin and pre-growth hormone were synthesised if membranes were omitted. Shields and Blobel (1977) showed that fish pre-proinsulin was synthesised in a wheat germ system in the absence of pancreatic microsomal membranes. However, with membranes present, proinsulin was produced indicating processing and segregation of pre-proinsulin. In all of these experiments segregation and processing were dependent on cotranslational incubation of the microsomal membranes, since posttranslational incubation did not result in either processing or segregation. The transfer of nascent pre-placental lactogen has also been shown to depend on the cotranslational incubation of membranes with a

Krebs ascites translation system (Birken et al, 1977).

Other evidence in support of the signal hypothesis was cited by Blobel and Dobberstein (1975a) who showed that rough microsomes from murine myeloma produced only processed immunoglobulin when incubated under conditions which allowed chain completion but no reinitiation whereas detached polysomes produced a mixture of processed and unprocessed molecules. These results were interpreted as showing that cleavage of the signal peptide occurs cotranslationally in vivo. While this may be so in the case of murine myeloma, Habener et al. (1976) have identified pre-parathyroid hormone as an early biosynthetic product in pulse-labelled studies using parathyroid slices. In this case the NH₂-terminal peptide, which consisted of 25 amino acids, was cleaved within 1 min of synthesis to yield parathyroid hormone showing that posttranslational cleavage is possible in vivo.

The signal hypothesis proposed that the signal peptidase was located on the cisternal face of the RER, since posttranslational incubation of pre-protein with microsomal membranes did not result in cleavage or in segregation (Blobel and Dobberstein, 1975a). Recently Jackson and Blobel (1977) demonstrated that the protease(s) responsible for cleavage of the signal peptide could be extracted from rough, but not smooth, microsomes by treatment with detergents. Posttranslational addition of this extract reduced the size of pre-prolactin and pre-growth hormone to that of the authentic hormones. Two

small peptides were isolated after the protease treatment of the pre-proteins and it was suggested that these could be the signal peptides. If they are the signal peptides it would indicate that the peptidase acts as an endo-protease.

Blobel (1976) suggested that the signal hypothesis need not apply to secretory proteins only and proposed that membrane proteins which were localised on the luminal side of the ER and proteins which spanned the membrane (i.e. ectoproteins, J. Rothman and Lenard, 1977) might also utilise the signal mechanism. Katz et al. (1977) demonstrated that the membrane glycoprotein G of vesicular stomatitis virus could be synthesised by translation of the mRNA in a wheat germ system supplemented with dog pancreatic microsomal vesicles. The product was core glycosylated and was asymmetrically inserted into the membrane of the vesicles so as to have the same orientation as is found in vivo for native glycoprotein G in RER membrane.

J. Rothman and Lodish (1977) showed that G protein could be synthesised by free ribosomes only if pancreatic microsomal vesicles were added before more than 50 - 55 residues extended beyond the ribosomal subunit. Membranes were not utilised until some 40 residues were found outside the ribosome but correct insertion of G was rapidly lost if more than 50 - 55 residues were exposed. These findings support the theory that the NH₂-terminal peptide is important in directing the binding of ribosomes to membranes and also provide evidence for Blobel's suggestion that transmembrane proteins utilise a signal mechanism.

The experiments of Katz et al. (1977) and Rothman and Lodish (1977) showed that core glycosylation of the nascent chain of the membrane glycoprotein G occurred cotranslationally. The glycoprotein α -lactalbumin is a secretory glycoprotein and Lingappa et al. (1978) demonstrated that the translation of mRNA from rat mammary gland in a wheat germ system resulted in the synthesis of an apparently non-glycosylated α -lactalbumin which contained an NH_2 -terminal sequence of 19 amino acids. When dog pancreatic microsomal membranes were included in the translation system a processed form of α -lactalbumin lacking the NH_2 -terminal sequence was synthesised. The processed protein was core glycosylated, segregated and had an amino acid sequence identical to that of mature α -lactalbumin.

As stated earlier, there is good evidence that in normal cells secretory proteins are synthesised on membrane-bound polysomes. However, it has been reported (Schrieber et al. 1976; McLaughlin and Pitot, 1976) that several minimal deviation hepatomas synthesise albumin only on free polysomes without secreting the end product. These results suggested that hepatomas were deficient in some component(s) necessary for binding the albumin-synthesising polysomes to the membrane of the ER. Strauss et al. (1977) showed that 5123TC hepatoma albumin mRNA directed the in vitro synthesis of a pre-proalbumin with a signal sequence which appeared to be identical to that of normal rat liver pre-proalbumin. Since the correct signal sequence appeared to be present, some other component(s) necessary for binding the polysomes to the membrane must be deficient.

1.2e The Involvement of Membrane Proteins in Ribosome Binding.

There is now a considerable body of evidence which suggests that the signal peptide of the nascent chain is important in facilitating transport of the peptide across the membrane, but, as yet, there is little evidence for the induction of tunnel formation by the signal sequence. The signal hypothesis proposes that tunnel formation is induced by the signal peptide causing an association of membrane ribosome receptor proteins. Subsequent interactions between the associated receptors and the large subunit are said to be responsible for the binding of the ribosomes to the membrane and stabilisation of the tunnel.

There is good evidence that interactions between the nascent peptide and the membrane and between the large subunit and the membrane are important in ribosome binding. Treatment of liver or myeloma rough microsomes with puromycin alone (nascent chain - membrane interactions) or high KCl alone (large subunit-membrane interactions) do not remove bound ribosomes without disrupting subunit structure, whereas puromycin and high KCl in combination allows the non-destructive detachment of functional ribosomes (Adelman et al. 1973 and Harrison et al. 1974). (Detailed discussions of ribosome-membrane interactions are to be found in the reviews of Rolleston (1974), McIntosh and O'Toole (1976) and Shore and Tata (1977) and they will not be repeated here.)

Several studies have shown that limited treatment of rough microsomes with trypsin removes bound ribosomes and destroys the ability of the membrane to bind added ribosomes (Shires et al. 1971, 1973, Shires and Pitot, 1973 a, b; Borgese et al. 1974 and Jothy et al. 1975) suggesting the involvement of protein(s) at the ribosome binding site. Kriebich et al. (1978a,b) showed that rat liver rough microsomes contained two integral membrane proteins, not found in smooth microsomes, which appear to be related to the presence of ribosome binding sites. The proteins, which had molecular weights of 65,000 and 63,000 (designated ribophorins I and II respectively), were shown to be exposed on the surface of the rough microsomes and to be in close proximity to the bound ribosomes. Both proteins were labelled when intact microsomes were incubated with a lactoperoxidase iodination system but only ribophorin I was digested during mild trypsinisation of the intact microsomes. Proteolysis of ribophorin II occurred only when sublytic amounts of detergent were added. Low concentration of glutaraldehyde or a reversible bifunctional reagent were used to crosslink ribosomes to the microsomal membrane. Using glutaraldehyde it was indirectly inferred that the ribosomes were crosslinked to the ribophorins, but using the reversible reagent, it was possible to demonstrate directly an association between the ribophorins and the ribosomes.

1.2f An Alternative View of the Movement of Pancreatic Enzymes.

Although there is a considerable body of evidence

which supports the signal hypothesis, some observations are difficult to reconcile with it. The work of S.S. Rothman described in Rothman (1975) and summarised by Diamond (1978) falls into this category. In his 1975 review Palade considers the transport of secretory enzymes to be vectorial with only the apical membrane of the pancreatic exocrine cell allowing the passage of enzyme, and then only in a cell-to-duct direction. Liebow and Rothman (1974) reported that the apical membrane of rabbit pancreatic cells was apparently permeable in both directions, in vitro, to bovine chymotrypsinogen. A similar bidirectional flux of digestive enzyme across the zymogen granule membrane was reported by Liebow and Rothman (1972), while transport of α -amylase outwards from the cell across the basolateral membrane was reported by Isenman and Rothman (1977).

It is also claimed that the basolateral membrane is permeable to digestive enzyme in the opposite direction i.e. from the extracellular fluid to the cell. Rothman and his coworkers have shown that strips of rabbit pancreas take up chymotrypsinogen via the basolateral membrane (Liebow and Rothman, 1974); that chymotrypsinogen added to the bathing medium of whole rabbit pancreas, in vitro, enters and crosses the cell at a magnitudinally greater rate than albumin and that it can be collected in the ductal secretion (Liebow and Rothman, 1975); that addition of α -amylase to the bathing medium of whole pancreas, in vitro, greatly stimulates total amylase secretion while simultaneously markedly inhibiting ductal

secretion of endogenous α -amylase (Isenman and Rothman, 1975); and that chymotrypsinogen instilled into the intestine or injected into the blood of whole animals can be recovered in pancreatic secretions (Liebow and Rothman, 1975 and Götze and Rothman, 1975).

The reports by Rothman's group that some membranes may be permeable to pancreatic proteins obviously conflicts with Palade's work and he (Palade, 1976) has criticised Rothman's work saying his conclusions are -

"....a good example of the types of problems generated when current cell-fractionation procedures are applied and their results accepted, without being critically assessed and checked by other independent methods of investigation."

While such criticism might be valid in the cell fractionation experiments it does not seem to be relevant when considering the perfused intact pancreas and the whole animal experiments.

The estimate of Götze and Rothman (1975) that 80 - 90% of pancreatic enzymes may be absorbed from the gut for recirculation by the pancreas is somewhat startling, and raises questions as to how, for example, serum proteins and erythrocytes are protected from the effects of digestive enzymes such as proteases, lipases and phospholipases (see the review of Diamond, 1978).

1.2g The Signal Hypothesis and Organelle Enzymes.

With regard to organelle enzymes, Blobel (1976)

has pointed out that if peroxisomal and lysosomal enzymes are synthesised and segregated by rough ER the problem of sorting organelle enzymes from coexisting secretory proteins arises. The attachment of ribosomes to areas where the inner and outer membranes of yeast mitochondria are joined has been reported (Kellems et al. 1975). In this case it is conceivable that the binding of ribosomes is initiated by a signal mechanism which results in transfer of the nascent peptide into the inner compartment of the organelle. However, in the case of chloroplasts, where junctions between cytoplasmic ribosomes and the membrane have not been demonstrated, Blobel (1976) suggests that

"A formula other than the signal mechanism, therefore, may have to be evolved to deal with the problem of transferring proteins synthesised in the cytoplasm across two membranes into the innermost compartment of mitochondria and chloroplasts."

Evidence that the signal mechanism might not operate in chloroplasts was reported by Highfield and Ellis (1978). It was found that the small subunit of the pea chloroplast enzyme ribulose biphosphate carboxylase, which is synthesised in vivo on cytoplasmic polysomes, was synthesised as a precursor protein in a heterologous translation system. (Dobberstein et al. (1977) had earlier reported a similar precursor of the enzyme subunit from Chlamydomonas.) The signal sequence appeared to be rich in acidic, rather than hydrophobic, residues and was longer than the usual signal sequence (around 50 residues compared with the usual 17 - 30 residues). Highfield

and Ellis found that the precursor entered intact chloroplasts with cleavage to the final size, but, unlike other systems, the uptake and cleavage did not require concomitant protein synthesis. It was proposed that the pre-protein combined with a specific carrier in the chloroplast envelope where a protease removed the signal sequence. The cleavage of the peptide triggered a conformational change in the protein leading to transport across the membrane and release into the stroma.

1.2h Ovalbumin - Secretion without a Transient Signal Peptide.

The observation that translation of ovalbumin mRNA in an in vitro system resulted in a product without a transient signal peptide (Palmiter et al. 1978) is difficult to reconcile with the signal hypothesis. The translation product was only one residue longer (an NH₂-terminal methionine) than the secreted ovalbumin. The possibility that cleavage of the signal peptide occurred in vitro was considered and rejected on the grounds that the extra methionine residue was donated by Met-tRNA_f^{Met}. It was considered that the NH₂-terminal sequence possibly served as a signal without being cleaved. However, only 13 of the first 20 residues were hydrophobic compared with the more typical 85 - 90% hydrophobicity found in transient signal sequences, and, on this basis the concept of an uncleaved signal peptide was rejected.

The NH₂-terminal residue of secreted ovalbumin is N-acetylated and it was suggested that the transacetylase directed the nascent chain into a secretory channel during

the process of acetylation. The authors concluded that there were at least two distinct mechanisms of protein secretion in chick oviduct based on the fact that the oviduct secretes lysozyme and ovomucoid, which can be synthesised in vitro as pre-proteins (see Table 1.1), as well as ovalbumin.

The conclusion that ovalbumin mRNA was translated in vitro without a signal sequence was supported by McReynolds et al. (1978) who recently published the nucleotide sequence for the mRNA. The sequence coded for an NH₂-terminal peptide identical to that which was found by Palmiter et al. (1978).

1.2i Conclusion.

The transfer of proteins across membranes by the mechanism proposed in the signal hypothesis seems likely, but, whether this is the only mechanism for transfer remains to be established. It is clear, from the elegant work of Blobel's group, that the transport of proteins across certain membranes only occurs when the protein is presented to the membrane in a nascent form. The evidence that the NH₂-terminal peptide is the signal for distinguishing between exported and intracellular proteins is also compelling. However, the apparent secretion of ovalbumin without the involvement of a signal sequence, and, the transfer of a subunit of ribulose biphosphate carboxylase into intact chloroplasts without concomitant protein synthesis present problems which have yet to be resolved. The conclusions of S.S. Rothman are also difficult to reconcile with the

signal hypothesis. Of the different events proposed by the signal hypothesis, the one least clearly established is the formation of the membrane tunnel. The ribophorins appear to be involved in ribosome binding but whether they are important in tunnel formation has yet to be established. Considerable progress has been made in understanding the process of protein secretion but much still remains to be discovered.

1.3 THE SYNTHESIS AND SECRETION OF EXTRACELLULAR ENZYMES IN BACTERIA.

The organisms used to study bacterial extracellular enzyme synthesis include Bacillus subtilis and B. amyloliquefaciens, which secrete α -amylase, proteases and RNAase, and B. licheniformis which secretes penicillinase. In addition, the alkaline phosphatase of E. coli is a secreted periplasmic enzyme. May and Elliot (1968) proposed that the extracellular enzymes were made on membrane-bound polysomes with the nascent peptides being extruded through the membrane. Sanders and May (1975) reported that B. amyloliquefaciens α -amylase was sensitive to proteolytic digestion during, but not after synthesis by protoplasts, and proposed that the enzyme was emerging from the membrane in a protease-sensitive conformation different from native α -amylase.

Further evidence that secreted proteins are extruded through the membrane during synthesis comes from Smith et al. (1977). Spheroplasts of E. coli were treated with a

labelled, non-penetrating, amino-group reagent and radioactive polysomes were isolated from the membrane-polysome fraction. On completion of translation, in an in vitro system, labelled alkaline phosphatase was identified as a significant translation product. (This is perhaps the best evidence to date in favour of the signal hypothesis.)

The possibility that bacterial extracellular enzymes are synthesised as precursor proteins has been considered by several groups of workers. Yamamoto and Lampen (1975, 1976) showed that the membrane penicillinase of B. licheniformis differed from exopenicillinase in that it had an NH₂-terminal peptide of 25 amino acids with phosphatidylserine as the NH₂-terminal residue. The phospholipopeptide, which appeared to be responsible for the hydrophobic properties of the enzyme, was long enough to span the lipid bilayer of the membrane. The authors pointed out that the segment of mRNA coding for the phospholipopeptide would be hydrophobic, due to a high (80%) purine content, and hence the mRNA would resemble mRNA's which contain poly(A) tracts. They postulated that the hydrophobic region of the mRNA could be important in specifying translation at the membrane. (See the review of Lampen, 1978). This suggestion is based on the observation that eukaryotic mRNA's, with a poly(A) tract at 3' end, remain attached to membranes after removal of ribosomes (Milcarck and Penman, 1974, Lande et al. 1975, and Cardelli et al. 1976). (Attachment of the mRNA to the membrane is not envisaged in the signal hypothesis.)

The alkaline phosphatase of E. coli was synthesised (Inouye and Beckwith, 1977) in a DNA-directed system as a pre-protein which could be processed to approximately the correct size by E. coli outer membrane fragments. The pre-protein was highly hydrophobic and this appeared to be a function of the extra segment.

The lipoprotein of E. coli outer membrane has been synthesised by translation of the mRNA in a cell-free system (Inouye et al. 1977). The mRNA coded for a pre-protein, pre-lipoprotein, which had an NH₂-terminal signal sequence of 20 amino acids the majority of which were hydrophobic.

Incubation of bacteriophage f1 DNA in a coupled transcription-translation system resulted in the production of a precursor protein for f1 coat protein (Chang et al. 1978). The in vitro synthesised pre-coat protein had an NH₂-terminal extension of 23 amino acids and partial sequencing of this extension agreed with the sequence predicted from the nucleotide sequence. The inclusion of E. coli vesicles in the translation system resulted in processing of the nascent protein to mature f1 coat protein. Posttranslational incubation of the vesicles with the pre-protein, however, did not result in processing. After the cotranslational incubation a small peptide was isolated which appeared to be derived from the NH₂-terminal of the nascent pre-coat protein suggesting that cleavage was the result of proteolysis by an endopeptidase.

With regard to bacterial proteins there is evidence that some extracellular and membrane proteins are synthesised as pre-proteins, as predicted by the signal hypothesis, and there is also evidence that some enzymes are extruded through the membrane during synthesis. It remains to be seen, however, whether these observations apply to all secretory and membrane proteins.

1.4 THE AIM OF THIS INVESTIGATION.

The studies reported in this thesis arose from the observation that assayable α -amylase activity increased when a rat pancreatic microsomal fraction was incubated in a simple buffer solution. It was appreciated that this could have had a trivial explanation, such as leakage, but this possibility became less likely as the investigation continued. The possibility that the phenomenon was relevant to the general question of the transport of proteins across membranes was considered sufficient justification for continuing the investigation.

CHAPTER TWO

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 MATERIALS

2.1a Experimental Animals

White male Porton rats weighing between 180 g and 200 g were obtained from the Central Animal House, University of Adelaide. The animals which were maintained on a pellet diet fed ad lib, were starved overnight prior to sacrifice.

2.1b Enzymes and Enzyme Inhibitors

Chymotrypsin and TPCK-trypsin were obtained from Worthington Biochemical Corp.; RNAase A, subtilisin BPN', creatine phosphokinase and phenylmethyl sulphonyl fluoride (PMSF) were purchased from Sigma Chemical Co.

2.1c Chemical Reagents

Puromycin dihydrochloride was obtained from Nutritional Biochemicals Corp.; tetramethylethylenediamine (TEMED) was from Eastman Kodak Co.; acrylamide and N,N'-methylenebisacrylamide were obtained from Merck; sodium deoxycholate was from B.D.H. Chemicals Ltd.; glycogen (oyster) was from Mann Research Chemicals; creatine phosphate, adenosine triphosphate (ATP), guanosine triphosphate (GTP), dithioerithritol (DTE), sodium dodecyl sulphate (SDS), Triton X-100 and β -mercaptoethanol were from Sigma Chemical Co. Other chemicals were analytical grade reagents obtained from Ajax Chemicals Ltd., B.D.H. Chemicals Ltd. and May and Baker Ltd.

2.2 METHODS

2.2a Preparation of Pancreatic Microsomes.

In most experiments a sample of the total microsomal fraction ("microsome") was used. Microsomes were prepared as follows: the pancreas was washed in cold 0.3 M sucrose, 0.2 mM CaCl_2 , pH 6.7, trimmed of mesenteric fat, blotted dry and weighed. It was chopped into small pieces (using scissors) in 9 volumes of the above sucrose solution. The chopped pancreas was homogenised using a motor-driven Potter-Elvehjem homogeniser; three passes of the pestle were made with the motor operating at a speed of approximately 1,000 rpm. The homogenate was centrifuged for 5 min at 600 x g to remove intact cells, cell nuclei and cellular debris. The supernatant was centrifuged for 12 min at 10,000 x g max and the pellet, which consisted of zymogen granules, mitochondria and large fragments of endoplasmic reticulum, was discarded. The post mitochondrial supernatant (7 - 8 ml) was centrifuged at 150,000 x g max using a Beckman Ti50 rotor. All centrifugations were performed at 4°C. The postmicrosomal supernatant was discarded and the surface of the pellet was washed twice with cold sucrose. The pellet was resuspended in 7 - 8 ml of cold STKC buffer (0.3 M sucrose, 50 mM Tris/HCl, 25 mM KCl, 0.2 mM CaCl_2 , pH at 20°C 7.5°C) to give a protein concentration of 5 - 6 mg/ml. A hand-operated Potter-Elvehjem homogeniser was used for the final resuspension.

2.2b Purification of Rough Microsomes

Purified rough microsomes were prepared from the

microsome pellet described above. The pellet was resuspended in 4.0 ml of 1.35 M sucrose. The resuspended microsomes were transferred to a Beckman SW41 centrifuge tube and unlaidd by 2.5 ml of 1.35 M sucrose and 1.5 ml of 2 M sucrose. The microsomes were overlaid with 1.5 ml of 1.2 M sucrose and 2.5 ml of 0.3 M sucrose. The tube was centrifuged at 190,000 x g_{av} for 12 h. using a Beckman SW41 rotor. The rough microsomes banded at the 1.35/2.0 interface. They were collected using a syringe with a bent needle and gently resuspended in STKC buffer at a protein concentration of 5 mg/ml. (Tartakoff and Jamieson, 1974).

2.2c Incubation of Microsomes

The resuspended microsomes were diluted twenty-fold in cold STKC buffer and then incubated at 37°C in a water bath. Aliquots were taken from the diluted microsomes during the course of the incubation for immediate assay of enzyme activity. Other components e.g. proteases or metal ions, were incorporated into the incubation mixture prior to commencing incubation (unless otherwise stated). Low temperature incubations were performed by keeping the microsomes on ice (0°C incubation) or in a 15°C water bath (15°C incubation).

2.2d α -amylase Assays.

α -amylase activity was measured using an insoluble substrate, purchased as Phadebas tablets from Pharmacia Diagnostics. The assay method used was a modification of the method described by the manufacturers. One tablet was

suspended using a magnetic stirrer in 7.0 ml of buffer, pH 7.1, containing 10 mM K_2HPO_4 , 10 mM NaCl and 0.2 mM $CaCl_2$. Aliquots (1.4 ml) were dispensed into clean plastic centrifuge tubes which were pre-incubated at 37°C. After the addition of the sample of microsomes, usually 100 μ l, the contents of the tube were mixed using a vortex mixer and incubated for 1 min. The reaction was stopped by the addition of 0.2 ml of 0.5 M NaOH and the tubes were stored at 0°C until the end of the experiment. The tubes were then centrifuged at 4°C for 10 min in a bench centrifuge. A sample of the supernatant was removed using a Finn pipette, and the absorbance at 620 nm was measured using either a Hitachi 101 or a Varian 657 spectrophotometer.

Under these conditions 50 ng of pig pancreatic α -amylase (obtained from Sigma) produced an absorbance change of 1.0 in 1 min and the assay was linear to an absorbance of 4.0.

2.2e RNAase Assays.

RNAase activity was measured by the method of Kraft and Shortman (1970) using yeast RNA as substrate and a 1 min incubation period. In this procedure 0.2 ml of 1% yeast RNA, 0.2 ml of buffer (50 mM Tris/HCl, 0.24 M NaCl, 50 mM EDTA, pH 7.8) and 0.1 ml of distilled water were pipetted into clean tubes which were pre-incubated at 37°C for 90 sec. On addition of 100 μ l of microsomes, the contents of the tubes were vortex-mixed and then incubated for 1 min. The reaction was stopped by the addition of 0.6 ml of cold EtOH/

HCl/H₂O (80:10:10) and the tubes were stored at 0°C until the end of the experiment. After centrifugation for 30 min at 4°C in a bench centrifuge, 0.5 ml of supernatant was diluted into 2.5 ml of distilled water. The absorbance at 260 nm was measured using a Varian 657 spectrophotometer.

Under these conditions 200 ng of bovine pancreatic RNAase (obtained from Sigma) produced an absorbance change of 0.85 in 1 min.

2.2f PMSF Inactivation of Proteases

PMSF was dissolved in warm 95% ethanol at 50 mg/ml and 1 part of this solution was added to 150 parts of the protein solution (Weber et al., 1972). Inactivation of the proteases occurred within 10 min of the addition of the inhibitor.

2.2g Protease Treatment of Microsomes

Chymotrypsin and TPCK-trypsin were made up as stock solutions at 10 mg/ml in 0.01 M CaCl₂, pH 3 and stored at -20°C. Unless otherwise stated the protease was added to the microsome suspension just before the commencement of incubation. Subtilisin BPN' solutions were prepared fresh as a 10 mg/ml solution in distilled water.

2.2h Removal of Ribosomes from Microsomes

The method of Kreibich et al. (1973) was utilised to remove ribosomes from rough microsomes. Microsomes

were incubated either at 0°C or 37°C in STKC buffer which contained 750 mM KCl, 5 mM Mg²⁺ and 1 mM puromycin.

2.2i Electron Microscopy

Microsomes were diluted from 5 mg protein/ml in STKC buffer to 0.25 mg protein/ml in cold phosphate buffer (10 mM K₂HPO₄, pH 7.05). Carbon-coated copper grids were pre-soaked for 5 mins in chloroform, to render the surface hydrophylic, and blotted and dried thoroughly. One drop of microsome suspension was applied to a grid and allowed to adsorb to the surface for 25 seconds. The grid was then blotted and stained for 20 seconds with one drop of 2% (w/v) uranyl acetate. After blotting and drying, the grids containing negatively stained microsomes were examined at a magnification of 20,000 X in a Siemens Elmiskop 102 electron microscope.

Thin sections of incubated microsomes were prepared as follows: the incubated microsomes were fixed at 0°C for 60 min in STKC buffer, pH 7.5, containing 4% (w/v) glutaraldehyde (EM grade). The fixed microsomes were centrifuged in cellulose nitrate tubes at 8,000 x g for 10 min and the pellet was post-fixed at 0°C overnight in STKC buffer, pH 7.5, containing 1% (w/v) OsO₄. The surface of the post-fixed pellet was washed in 70% (v/v) ethanol. The pellet was then cut into strips in the bottom of the cellulose nitrate tube and dehydrated through an ethanol series. Propylene oxide was used to dissolve the cellulose nitrate and the strips were embedded in TAAB Resin which was polymerised for 48 h at 60°C. Sections were cut on an LKB Ultratome I and they were then stained in 1% (w/v)

alcoholic uranyl acetate at 37°C for 30 min. The stained sections were treated with Reynolds lead citrate (Reynolds, 1963) for 5 min and then examined in a Siemens Elmiskop 102 operated at 60 kV and fitted with a 30 μ gold foil objective aperture. A liquid nitrogen anticontamination device was used to reduce sample contamination.

2.2j Sonication of Microsomes

A sample of pancreatic microsomes was sonicated in bursts (10 sec on, 20 sec off) using a Branson Sonifier, Model B-30, fitted with a microtip. The sonifier was used at maximum power and the total sonication time was 3 min. The microsome suspension was cooled in ice during the sonication procedure.

2.2k Gel Electrophoresis

Slab gels were prepared by the method of Laemmli (1970), which incorporates a stacking gel (3% acrylamide, 0.1% SDS, 0.125 M Tris HCl, pH 6.8) over a separating gel (10% acrylamide, 0.1% SDS, 0.375 M Tris HCl, pH 8.8). The dimensions of the stacking gel were 2 cm x 14 cm x 1.5 mm and those of the separating gel were 10 cm x 14 cm x 1.5 mm. The pH and acrylamide step-gradients thus formed produced good separation of the proteins into tight, distinct bands. Electrophoresis was generally performed at 45 mA for 30 mins to stack the proteins which were then run through the separating gel at 120 V for 2¹/₂ hours, during which time the current dropped steadily to less than 10 mA.

Bands of protein were detected by staining the gel

overnight in 25% isopropanol, 10% acetic acid, 0.05% Coomassie blue and then destaining by gentle shaking in 10% isopropanol, 10% acetic acid for 6 to 8 h. Glycoproteins were detected using the periodic acid - Schiff stain of Zacharius et al. (1969).

2.21 Energy Generating System

The ATP generating system of Ballard et al. (1974) was used in an experiment to detect a transfer of α -amylase into microsomal vesicles. The system was modified in that creatine phosphate and creatine phosphokinase were used. The final concentration of components was ATP, 1 mM; GTP, 0.5 mM; Tris, 60 mM; KCl, 40 mM; MgCl₂, 3.5 mM; DTE, 1 mM; pH 7.5. A concentrated solution of these compounds was made up so that the final concentrations listed above were obtained on diluting the solution 1 to 4 in STKC buffer. Creatine phosphate (14 mg/ml) and creatine phosphokinase (10 μ g/ml) were added immediately after the addition of the concentrated components to the incubated microsomes.

2.2m Preparation of Sepharose-trypsin

Trypsin was coupled to Sepharose 4B beads by the method of Axén and Ernback (1971). The coupling mixture contained 0.08 mM trypsin and a 40% (v/v) suspension of CNBr-activated Sepharose (purchased from Pharmacia (South Seas) Pty. Ltd.) in 25 mM borate buffer (pH 10.2) containing 20 mM CaCl₂. The mixture was gently agitated at 4°C overnight in a shaking water bath. The immobilised protein was washed in a sintered glass filter funnel with 5 volumes of each of

the following buffers: 25 mM borate, pH 10.2, at 4°C; 100 mM borate, pH 8, containing 15 g glycine per litre; 100 mM acetate, pH 4, containing 1 M NaCl; 100 mM borate, pH 8, containing 1 M NaCl; 10 mM acetate, pH 4, containing 100 mM NaCl. The protein preparation was stored at 4°C in 10 mM acetate, pH 4.1, containing 100 mM NaCl and 0.1% sodium azide. Before use, the gel was washed and resuspended in STKC buffer.

2.2n Purification of α -Amylase

The glycogen precipitation method of Schramm and Loyter (1966) was used to purify released and Zymogen granule α -amylase. Released α -amylase was purified from the supernatant obtained after centrifuging 60 min incubated microsomes at 150,000 s g_{av} for 30 min.

Zymogen granules from rat pancreas were isolated by the procedure described by Tartakoff and Jamieson (1974). Chopped pancreas was homogenised with a Potter-Elvehjem homogeniser in 9 volumes of cold 0.3 M sucrose, pH 6.5. The homogenate was centrifuged at 600 x g for 5 min in a bench centrifuge and the resulting supernatant was centrifuged at 1000 x g_{av} for 10 min to produce a pellet of zymogen granules. The surface of the pellet was washed twice with 2.0 ml of cold 0.3 M sucrose, pH 6.5, to remove the overlying layer of mitochondria. The pellet was resuspended in 5 ml 175 mM NaHCO_3 , pH 8.0 and incubated at 4°C for 45 min to release the contents of the granules. Membrane fragments were removed by centrifugation at 150,000 x g_{max} for 60 min. The purification of the granules was performed at 4°C.

Ethanol was added dropwise to the supernatants obtained by the above procedures to give a final concentration of 40% (v/v). The mixture was centrifuged at 10,000 x g for 20 min. To each ml of supernatant reagents were added with mixing in the following order: 0.05 ml 0.2 M phosphate buffer, pH 8.0; 0.05 ml 20% glycogen (w/v); 0.07 ml 96% ethanol. The mixture was shaken for 5 min and then centrifuged at 2,000 x g for 5 min. The pellet was washed twice with 1 ml 40% ethanol containing 0.01 M phosphate pH 8.0. The precipitate was then resuspended in 0.01 M phosphate, pH 7.05, containing 0.01 M NaCl and 0.2 mM CaCl₂ and stored at -20°C. All operations were carried out at 0-2°C.

All experimental results reported in this thesis are the average of at least three separate determinations.

CHAPTER THREE

RESULTS

THE PROTEASE-SENSITIVE RELEASE OF α -AMYLASE FROM
RAT PANCREATIC MICROSOMES.

3.1 INTRODUCTION.

The work described in this thesis started with the observation that when the microsomal fraction, "microsomes", from rat pancreatic cells was incubated in a simple buffer system, α -amylase activity, measured using an insoluble substrate, increased. This was not particularly surprising since leakage of the luminal contents of microsomal vesicles was an obvious possibility. An earlier report had described the loss of contents when such vesicles from rat liver were incubated for 15 min at 30° in distilled water and then rapidly cooled (Glaumann and Dallner, 1968). Later experiments showed that this procedure irreversibly increased the permeability of the membrane to small ions and it was concluded that a cold-induced rupture of the membrane had occurred (Nilsson et al., 1973 and Depierre and Dallner, 1975).

During an investigation of α -amylase secretion by B. amyloliquefaciens, May and Sanders (1975) showed that, while the native α -amylase resisted proteolysis, the enzyme was destroyed by exogenous protease as it emerged from protoplast. Although the analogy was not perfect, it was of interest to see whether perhaps relatively newly synthesised α -amylase, present in the vesicles, was sensitive to proteases. As a preliminary experiment it was decided to test whether the α -amylase appearing during incubation of the microsomes was destroyed by trypsin.

At first it appeared that this was so but it was soon realised that this interpretation was incorrect. This chapter describes the release of α -amylase from pancreatic microsomes and the effects of proteases and other agents on the process.

3.2 THE APPEARANCE OF α -AMYLASE AND THE INHIBITION OF THIS PROCESS BY PROTEASES.

The microsomal fraction (microsomes) used in these experiments was obtained by resuspension of the pellet produced by high speed centrifugation of homogenised rat pancreas postmitochondrial supernatant. (Details of the fractionation procedure are given in Methods.) The majority of the vesicles were rough microsomes, as judged by visual inspection of electron micrographs, although some smooth microsomes and free ribosomes were also present. In a typical experiment the microsomal pellet was resuspended in STKC buffer (0.3 M sucrose, 50 mM Tris/HCl, 25 mM KCl, 0.2 mM CaCl_2 , pH 7.5) by gentle homogenisation, diluted to a protein concentration of 0.2 - 0.3 mg/ml and incubated at 37°C. Aliquots were taken from the microsome suspension during the course of the incubation for immediate assay of α -amylase activity. An insoluble substrate was used for the amylase assays and details of the assay procedure are given in Methods.

When microsome suspensions were incubated at 37°C

an increase in the level of α -amylase activity was observed.¹ The amount of assayable α -amylase increased steadily during the first 20 - 30 min of incubation and then reached a plateau level (Fig.3.1). There was a 250-300% increase in the amount of enzyme over a 60 min period. On some occasions a 5 - 10 min lag period was observed before the amount of α -amylase began to increase; the reason for this delay is not known. (In the early experiments, an incubation time of 10 min was used for the amylase assays. This was reduced to 1 min in order to minimise the error caused by the significant changes in the amounts of α -amylase which occurred in a 10 min period. A 1 min assay was used in all the experiments described in this thesis.)

When chymotrypsin, at a final concentration of 100 $\mu\text{g/ml}$, was added to the microsome suspension before commencing the incubation (Fig.3.1) only a small increase (25 - 30%) in the amount of assayable α -amylase was observed. Chymotrypsin, at a final concentration of 10 $\mu\text{g/ml}$, had a similar effect, but, at a final concentration of 1 $\mu\text{g/ml}$, the effect was less noticeable.

The increase in the level of α -amylase was also inhibited when trypsin (final concentration, 100 $\mu\text{g/ml}$) was

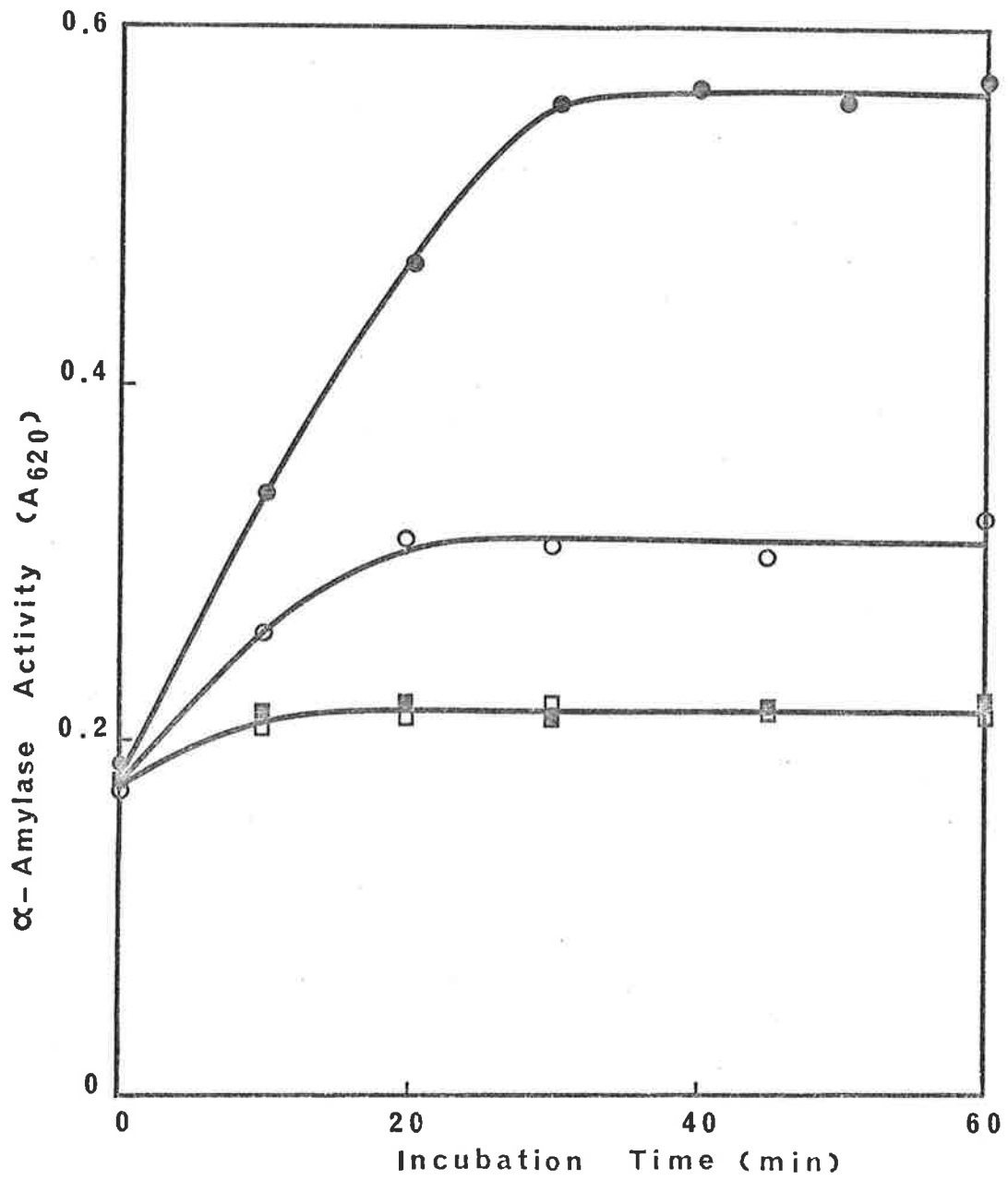
¹ Although it had not at that stage been established, it became apparent from experiments described later that this was due to an increase in the amount of assayable enzyme, rather than a change in the activity of a constant amount of enzyme. This increase, therefore, will be referred to as an increase in amount.

FIGURE 3.1 THE EFFECT OF CHYMOTRYPSIN ON THE
APPEARANCE OF α -AMYLASE IN INCUBATED
RAT PANCREATIC MICROSOMAL SUSPENSIONS

Microsome suspensions were incubated at 37°C in STKC buffer (●) or STKC buffer containing chymotrypsin at a final concentration of 1 $\mu\text{g/ml}$ (○), 10 $\mu\text{g/ml}$ (□) or 100 $\mu\text{g/ml}$ (■).

Samples were taken from the microsome suspensions, at the times indicated, for immediate assaying of α -amylase activity. (Assays were performed using an insoluble substrate and the details are given in Methods).

Fig. 3.1



substituted for chymotrypsin in the incubation buffer (Fig.3.2), although trypsin was somewhat less effective than chymotrypsin, on a weight basis. This was more noticeable at lower protease concentrations and can be seen by comparing the effect of chymotrypsin and trypsin (10 $\mu\text{g/ml}$) in Fig.'s 3.1 and 3.2 respectively. The difference between the proteases could be due, in part, to the fact that the pancreas contains a trypsin inhibitor which is specific for trypsin (the secretory pancreatic trypsin inhibitor, Kazal et al. 1948). Subtilisin BPN', the alkaline protease from B. amyloliquefaciens, at a final concentration of 1 $\mu\text{g/ml}$, also inhibited the increase in the amount of α -amylase (Fig.3.3).

It will be noticed that there is a measurable amount of α -amylase at time zero. Some of this initially assayable enzyme is likely to be non-specifically adsorbed to the membrane of the vesicles. It is also probable that some of it is due to appearance of α -amylase during the course of the assay. However, it is not known if these explanations account for all the assayable enzyme observed at the start of the incubation.

3.3 THE EFFECT OF CYCHOHEXIMIDE ON THE APPEARANCE OF α -AMYLASE.

The protease-sensitive increase in the levels of α -amylase could be explained if the increase was the result of de novo synthesis, and the proteases inhibited

FIGURE 3.2 THE EFFECT OF TRYPSIN ON THE APPEARANCE
OF α -AMYLASE IN MICROSOMAL SUSPENSIONS.

Microsome suspensions were incubated at 37°C in STKC buffer (●), STKC buffer containing trypsin at 10 μ g/ml (Δ) or 100 μ g/ml (\blacktriangle) or STKC buffer containing chymotrypsin at 100 μ g/ml (\blacksquare). (All concentrations are final concentrations).

Fig. 3.2

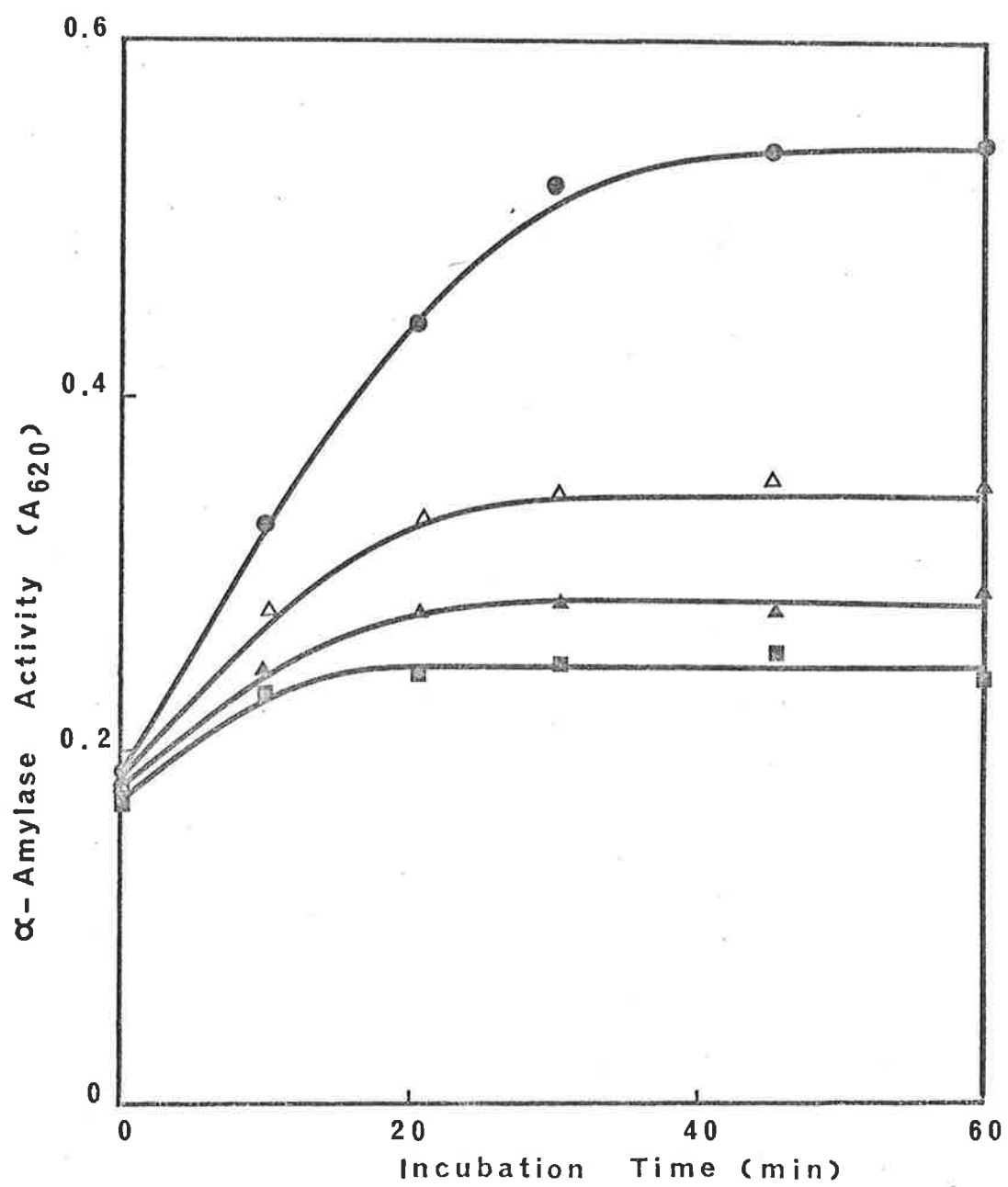
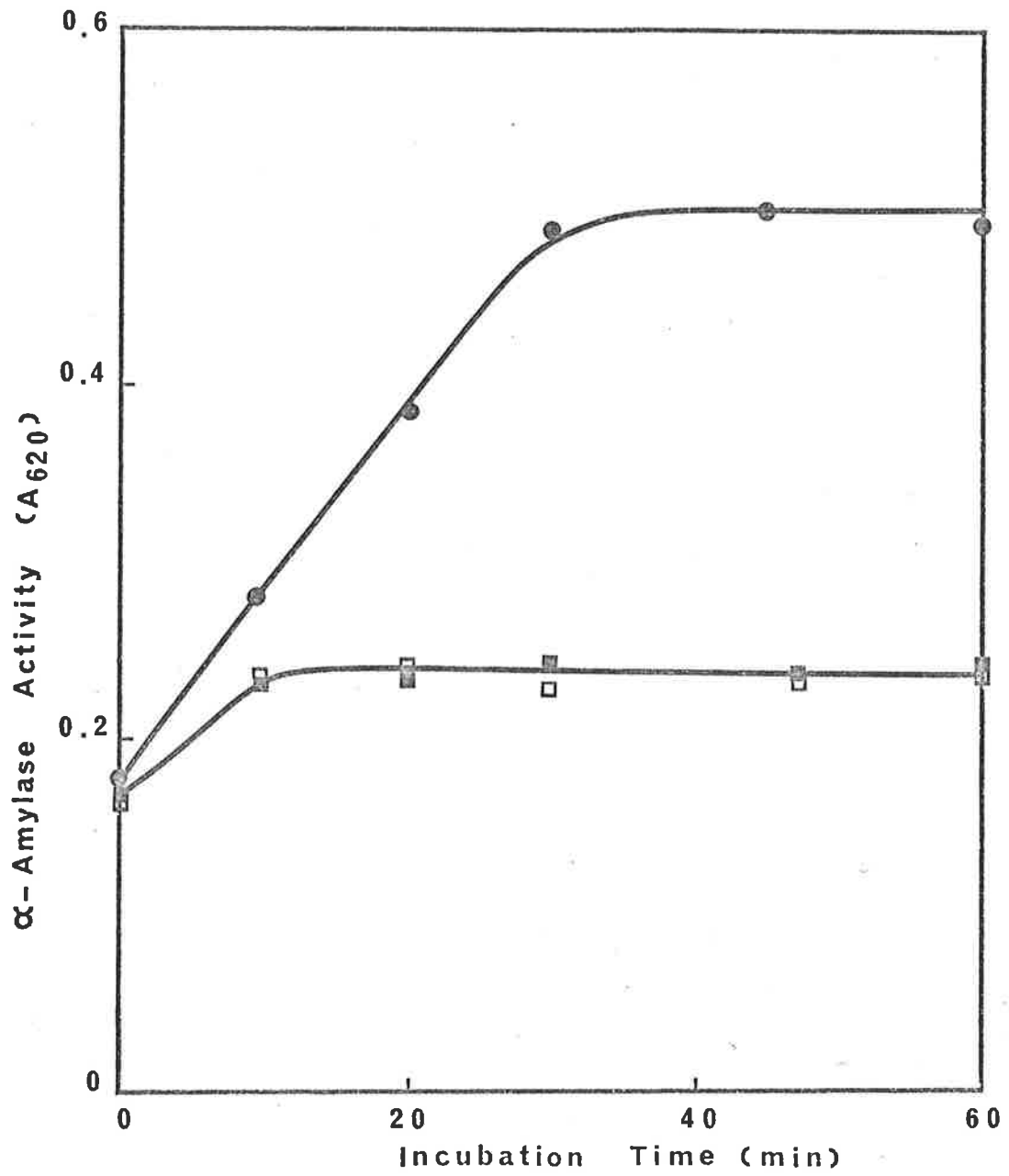


FIGURE 3.3 THE EFFECT OF SUBTILISIN BPN' ON THE
APPEARANCE OF α -AMYLASE IN MICROSOMAL
SUSPENSIONS

Microsome suspensions were incubated at 37°C in STKC buffer (●), STKC buffer containing chymotrypsin at 100 μ g/ml (■) or subtilisin BPN' at 1 mg/ml (□). (All concentrations are final concentrations).

Fig. 3.3



synthesis directly or destroyed the nascent peptides or both. The possibility of de novo synthesis seemed unlikely since there was no source of tRNA, soluble protein synthesis factors or amino acids, and there was no source of energy present. However, it was tested by incubating microsomes in the presence of cycloheximide. An aqueous solution of the inhibitor was added, at a final concentration of 100 µg/ml, to resuspended microsomes before commencing a 60 min incubation. Cycloheximide had no effect on the appearance of α-amylase (Fig.3.4) thereby eliminating protein synthesis as an explanation.

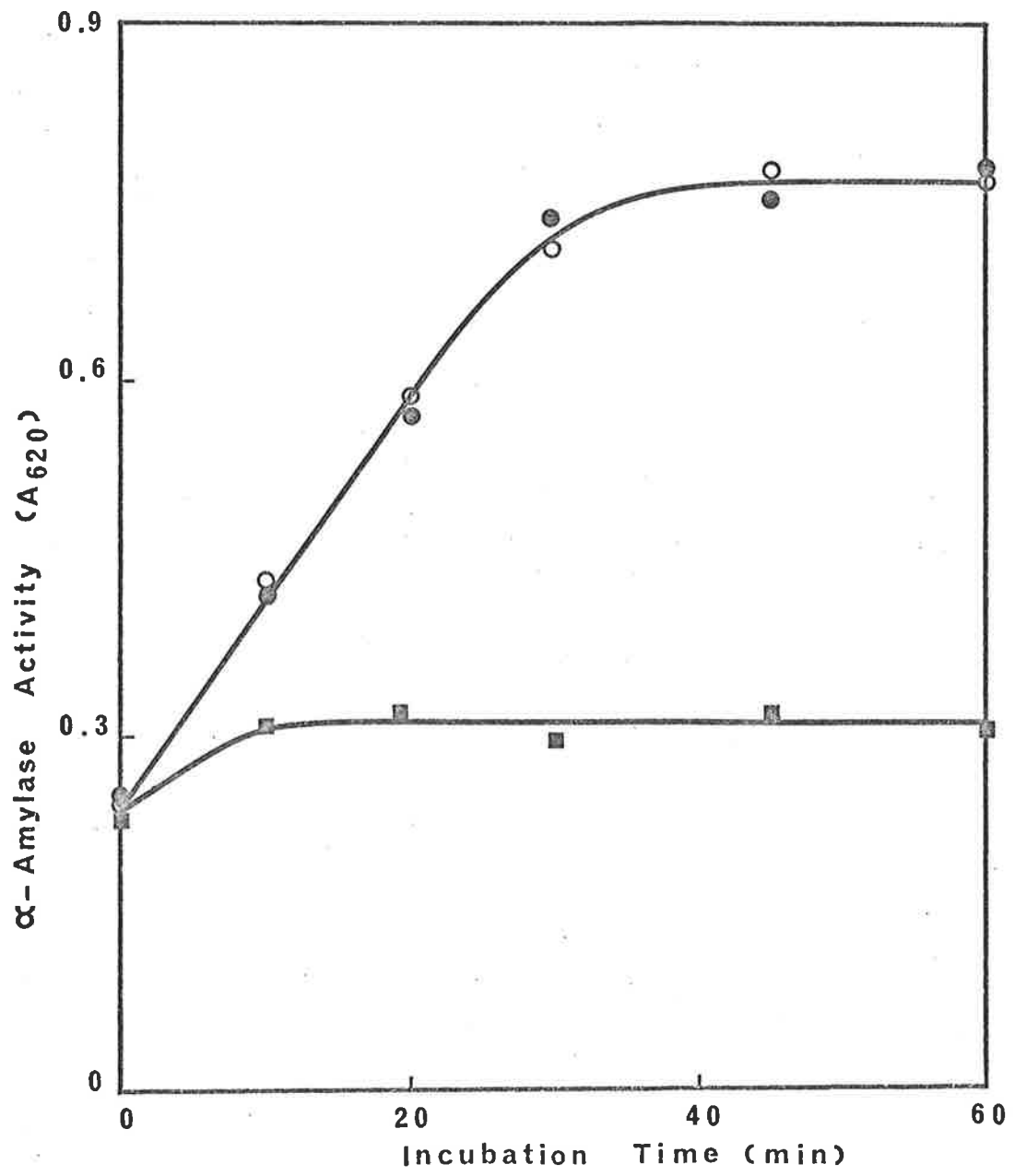
3.4 THE EFFECT OF PROTEASES ON α-AMYLASE.

Assuming that the increased levels of α-amylase activity were due to increased amounts of enzyme, the results of the previous experiment implied the existence of a pre-formed pool of enzyme, not initially accessible to the insoluble substrate, which became increasingly accessible as the incubation progressed. The protease-sensitivity of the increase could be explained if the pre-formed enzyme was sensitive to proteases either after, or as it became accessible. (It should be noted that Fisher and Stein (1960) reported the resistance of mammalian pancreatic α-amylases to digestion by trypsin and chymotrypsin provided that enzyme had a least 1 Ca²⁺ per mole of enzyme. Removal of the Ca²⁺ by dialysis against EDTA or by electro dialysis led to loss of enzymatic activity and rendered the protein sensitive to proteases.)

FIGURE 3.4 THE EFFECT OF CYCLOHEXIMIDE ON THE
APPEARANCE OF α -AMYLASE IN MICROSOMAL
SUSPENSIONS

Microsome suspensions were incubated at 37°C in STKC buffer (●), STKC buffer containing cycloheximide (○) or STKC buffer containing chymotrypsin (■). Cycloheximide and chymotrypsin were used at final concentrations of 100 μ g/ml.

Fig. 3.4



The possibility of a protease-sensitive amylase (the one initially favoured to explain the phenomenon) was tested in two ways. In the first experiment, microsome suspensions were incubated for 60 min and then trypsin or chymotrypsin (final concentration, 100 $\mu\text{g/ml}$) was added. The incubation was continued for a further 30 min and during this time the amount of α -amylase remained constant (Fig.3.5). This eliminated the possibility that α -amylase was destroyed after it became accessible to its substrate.

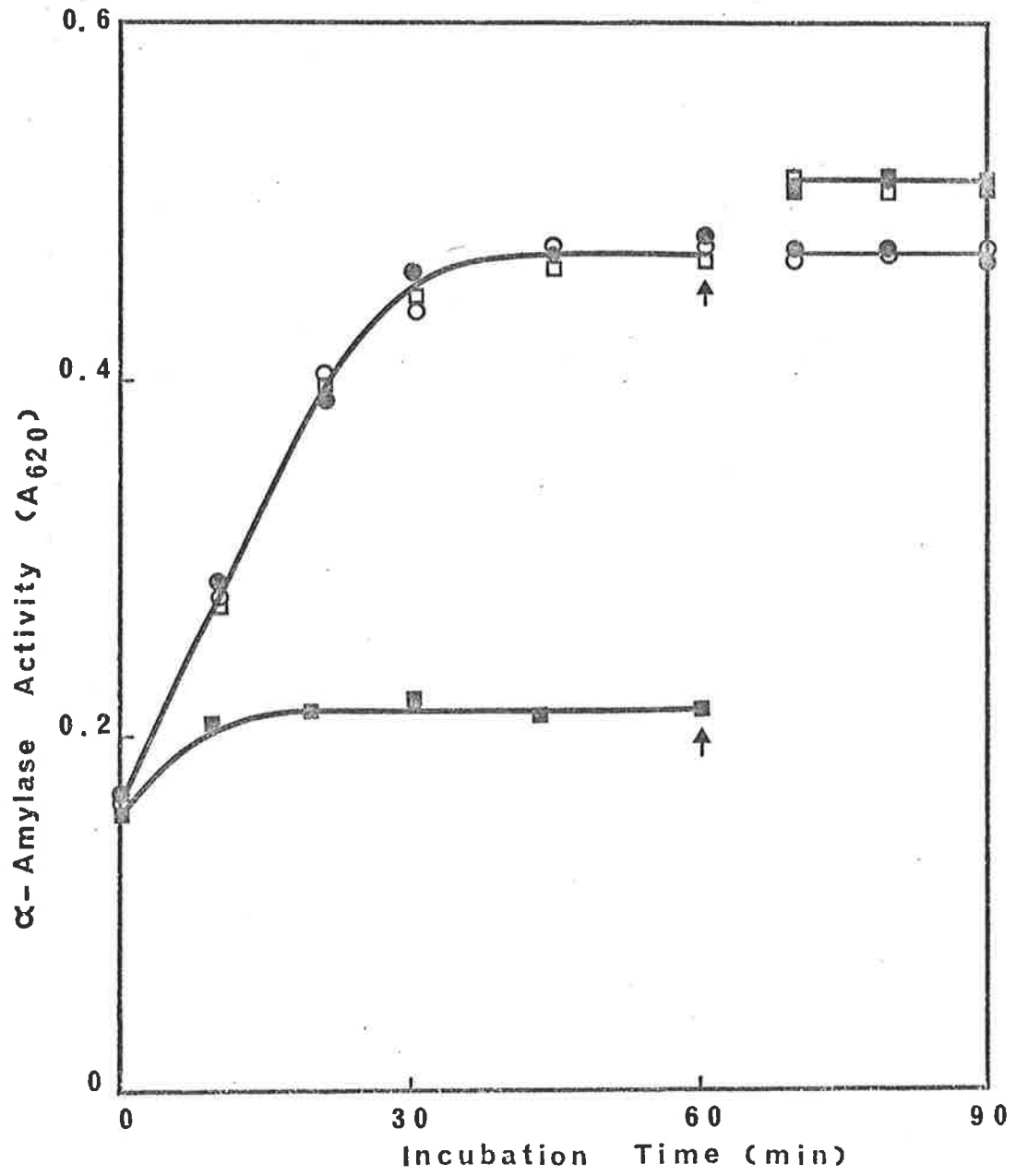
To show that α -amylase was not destroyed during the process of become accessible to its substrate it was necessary to show that incubation of microsomes in the presence of protease had no effect on total amylase levels (i.e. accessible and non-accessible enzyme). This was demonstrated by incubating microsomes with and without chymotrypsin (final concentration, 100 $\mu\text{g/ml}$) for 60 min and then adding deoxycholate (final concentration, 0.5% (w/v)) to dissolve the microsomal membranes. The incubation was continued for a further 30 min and α -amylase levels monitored. Addition of detergent to the microsomes without added protease led to a small increase in assayable α -amylase (Fig.3.5). However, detergent addition to the chymotrypsin treated vesicles brought the level of assayable α -amylase up to that which was found in the control microsomes (no protease) showing that α -amylase had not been destroyed. After the initial rise, caused by the detergent addition, enzyme levels remained constant confirming the result of the previous experiment that α -amylase resisted digestion. (This experiment, incidentally,

FIGURE 3.5 THE RESISTANCE OF PANCREATIC α -AMYLASE
TO DIGESTION BY TRYPSIN AND CHYMOTRYPSIN

Microsome suspensions were incubated at 37°C in STKC buffer for 60 min. Chymotrypsin (●), trypsin (○) or deoxycholate (□) was then added and the incubation continued for a further 30 min. A sample of microsomes was also incubated at 37°C in STKC buffer with chymotrypsin (■) for 60 min. Deoxycholate was then added and the incubation continued for a further 30 min. The proteases and deoxycholate were used at final concentrations of 100 μ g/ml and 0.5% (w/v) respectively. (The arrow indicates the time of protease or detergent addition).

Separate experiments showed that release of α -amylase upon addition of DOC to microsomes was instantaneous.

Fig. 3.5



further confirms that synthesis of new α -amylase is not occurring.) A separate experiment showed that deoxycholate, at the concentration used above, did not inhibit chymotrypsin.

3.5 TRANSFER OF α -AMYLASE FROM THE MICROSOMAL FRACTION TO THE SOLUBLE FRACTION.

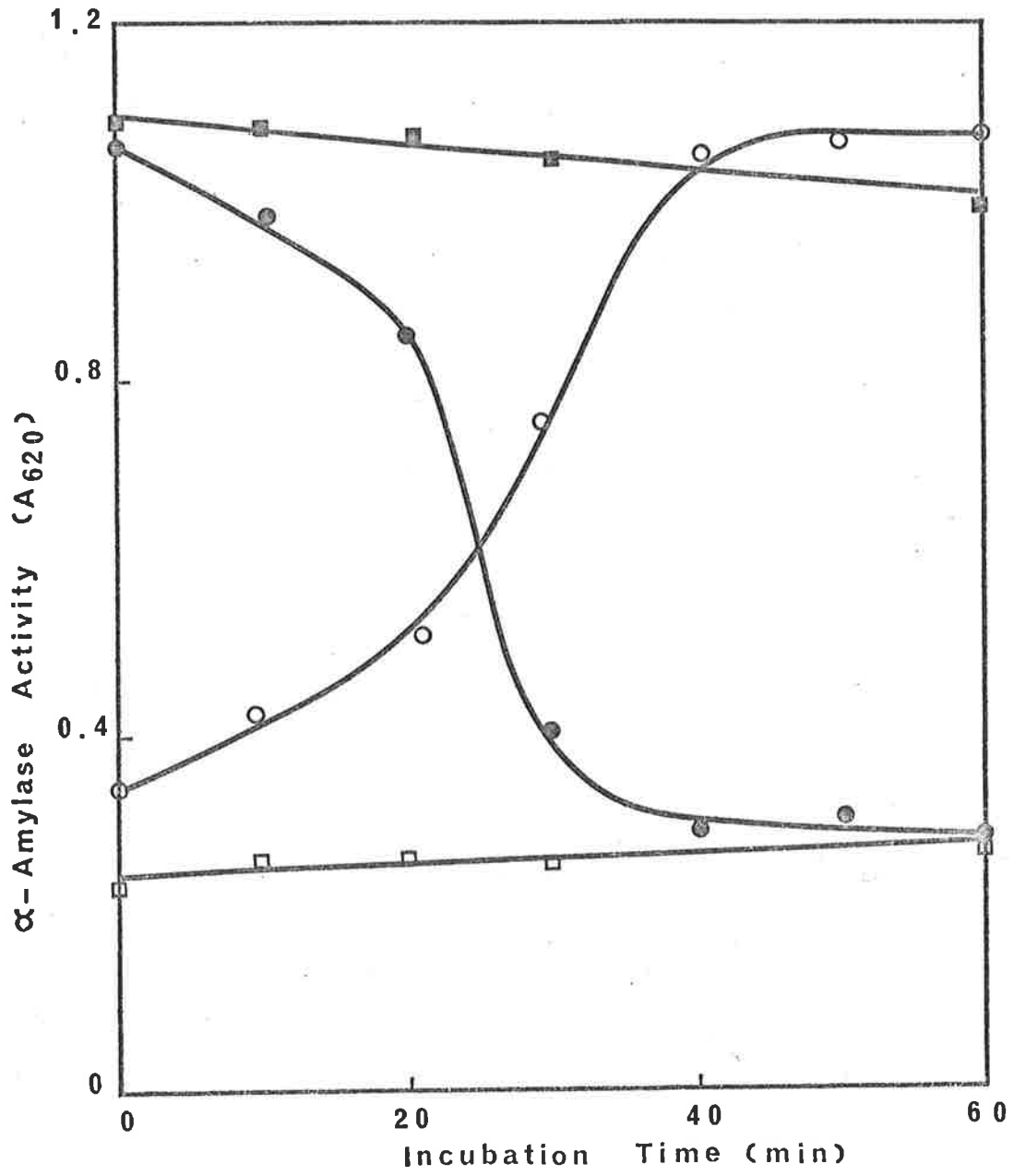
The next question investigated was whether the appearance of α -amylase activity represented a transfer of enzyme from some form of association with the microsomal fraction to the incubation medium. Microsomes were incubated at 37°C for times ranging from 0 min to 60 min and a microsomal pellet was separated from the soluble fraction by centrifugation at $150,000 \times g_{\max}$ for 30 min at 0°C. The supernatants were decanted and the pellets resuspended in STKC buffer containing 0.5% (w/v) deoxycholate to dissolve the membranes; detergent was also added to the supernatants to allow for its effect on enzyme activity. The results are shown in Fig.3.6.

Before the start of the incubation, most of the α -amylase was associated with the microsomal pellet, but, after 60 min incubation at 37°C, the majority of the amylase activity was found in the soluble fraction. The total amount of α -amylase (sum of pellet and supernatant activities) remained constant throughout the course of the incubation showing that no net synthesis had occurred, further confirming the result of the cycloheximide experiment. When chymotrypsin was incorporated in the incubation medium (Fig.3.6), at a final concentration of 100 $\mu\text{g/ml}$, most of

FIGURE 3.6 THE TRANSFER OF α -AMYLASE FROM THE
MICROSOMAL FRACTION TO THE SOLUBLE
FRACTION

Microsome suspensions were incubated at 37°C in STKC buffer (○), (●) or STKC buffer containing chymotrypsin (final concentration, 100 μ g/ml) (□), (■). At the times indicated the suspensions were separated into sedimentable and soluble fractions. The pellets (●), (■) were resuspended in STKC buffer containing 0.5% (w/v) deoxycholate and detergent was also added to the supernatant fractions (○), (□) (final concentration, 0.5% (w/v)). The α -amylase activity of each sample was then measured.

Fig. 3.6



the α -amylase remained associated with the pellet during incubation. Again, the total amount of α -amylase remained constant. This confirmed the result of the previous experiment which showed that the enzyme was not destroyed as it became accessible to the substrate.

The increase in assayable α -amylase during the course of the incubation was, thus, the result of a transfer of enzyme associated in some way with the microsomal fraction, to the soluble fraction i.e. the external medium. Little of the enzyme was initially accessible to the insoluble substrate which suggested that the enzyme was not located on the cytoplasmic surface of the vesicle membrane (unless one postulated that it was inactive in the adsorbed form). This experiment showed that chymotrypsin inhibited the transfer of vesicle-associated α -amylase to the medium, and it also showed that the protease did not destroy the enzyme during the transfer.

3.6 THE EFFECT OF OTHER PROTEINS ON α -AMYLASE RELEASE.

The experiment described above showed that proteases inhibited the release² of α -amylase from the microsomes. Modification of membrane proteins by the proteases seemed a possible explanation, but a non-specific adsorption of protein to the membrane could also be responsible for the inhibition e.g. by physically blocking holes in the membranes.

² At this stage the term "release" is used for convenience but is to be taken only in the sense that enzyme associated with the membranes becomes solubilised in the medium.

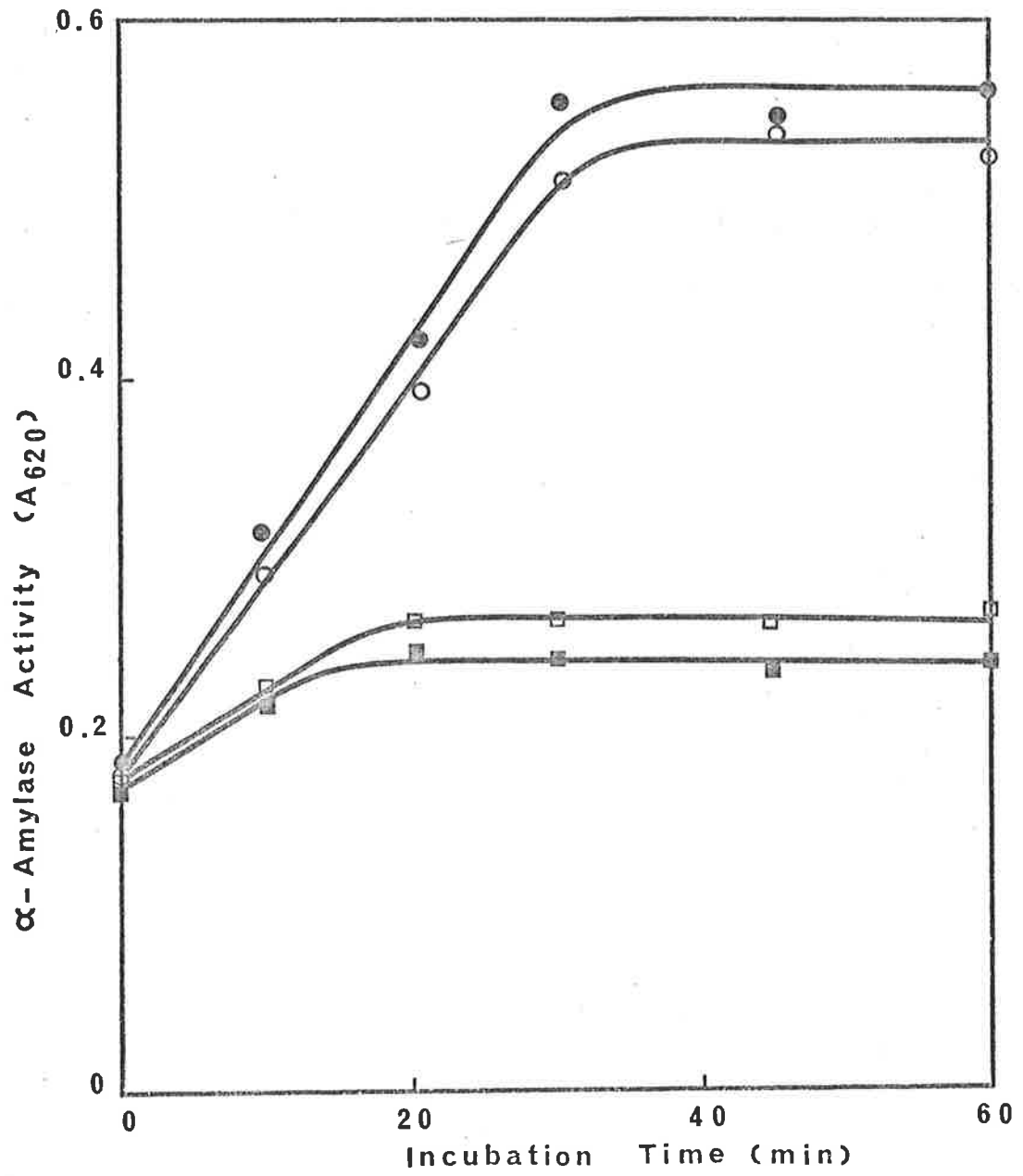
To see whether the effect was due to the proteolytic activity of proteases, microsomes were incubated with chymotrypsin which had been inactivated by prior treatment with phenylmethyl sulphonyl fluoride (PMSF, see Methods). (PMSF inhibits by forming a covalent bond with the serine residue at the active site of serine enzymes.) The inclusion of PMSF-inactivated chymotrypsin (final concentration, 10 $\mu\text{g/ml}$) in the incubation buffer had very little effect on the release of α -amylase (Fig.3.7) suggesting that chymotrypsin inhibited release as a result of its proteolytic activity.

It could be argued that the PMSF-treatment of the protease affected its ability to bind to the membrane and hence the experiment described above does not prove that chymotrypsin activity is involved. The alternative to chymotrypsin inhibiting by virtue of its proteolytic ability is that the enzyme molecules adsorb to the vesicles and in some way block the transfer of α -amylase to the medium. Attachment of the protease to Sepharose beads would be expected to reduce the number of molecules which could so adsorb and, if the inhibition was due to non-specific binding, it might be expected that α -amylase would be released under these conditions. However, when microsomes were incubated with Sepharose-trypsin beads equivalent in activity to 100 $\mu\text{g/ml}$ of free trypsin (Fig.3.7), release of α -amylase was inhibited and the degree of inhibition was only slightly less than that obtained with free trypsin. This reduction was possibly due to the reduced mobility of the trypsin. Separate tests showed

FIGURE 3.7 THE EFFECTS OF PMSF-INACTIVATED CHYMOTRYPSIN
AND SEPHAROSE-TRYPSIN ON α -AMYLASE RELEASE

Microsome suspensions were incubated at 37°C in STKC buffer (●), or STKC buffer containing PMSF-inactivated chymotrypsin (○), Sepharose-trypsin (□) or chymotrypsin (■). The proteins were used at final concentrations of 10 μ g/ml and the Sepharose-trypsin was used at a concentration equivalent in activity to 100 μ g/ml of free trypsin.

Fig. 3.7



that the conditions used in this experiment resulted in less than 0.5 $\mu\text{g/ml}$ of trypsin being removed from the beads and free trypsin at that concentration had very little effect on α -amylase release. This experiment supported the conclusion that proteolytic digestion is the reason for inhibition of release by proteases.

It was conceivable that inhibition of release was a general property of pancreatic enzymes, although it was not obvious how the inhibition might be so effected. This possibility was tested by incubating microsomes in the presence of bovine pancreatic RNAase A at a final concentration of 100 $\mu\text{g/ml}$. Normal release of α -amylase was observed (Fig.3.8).

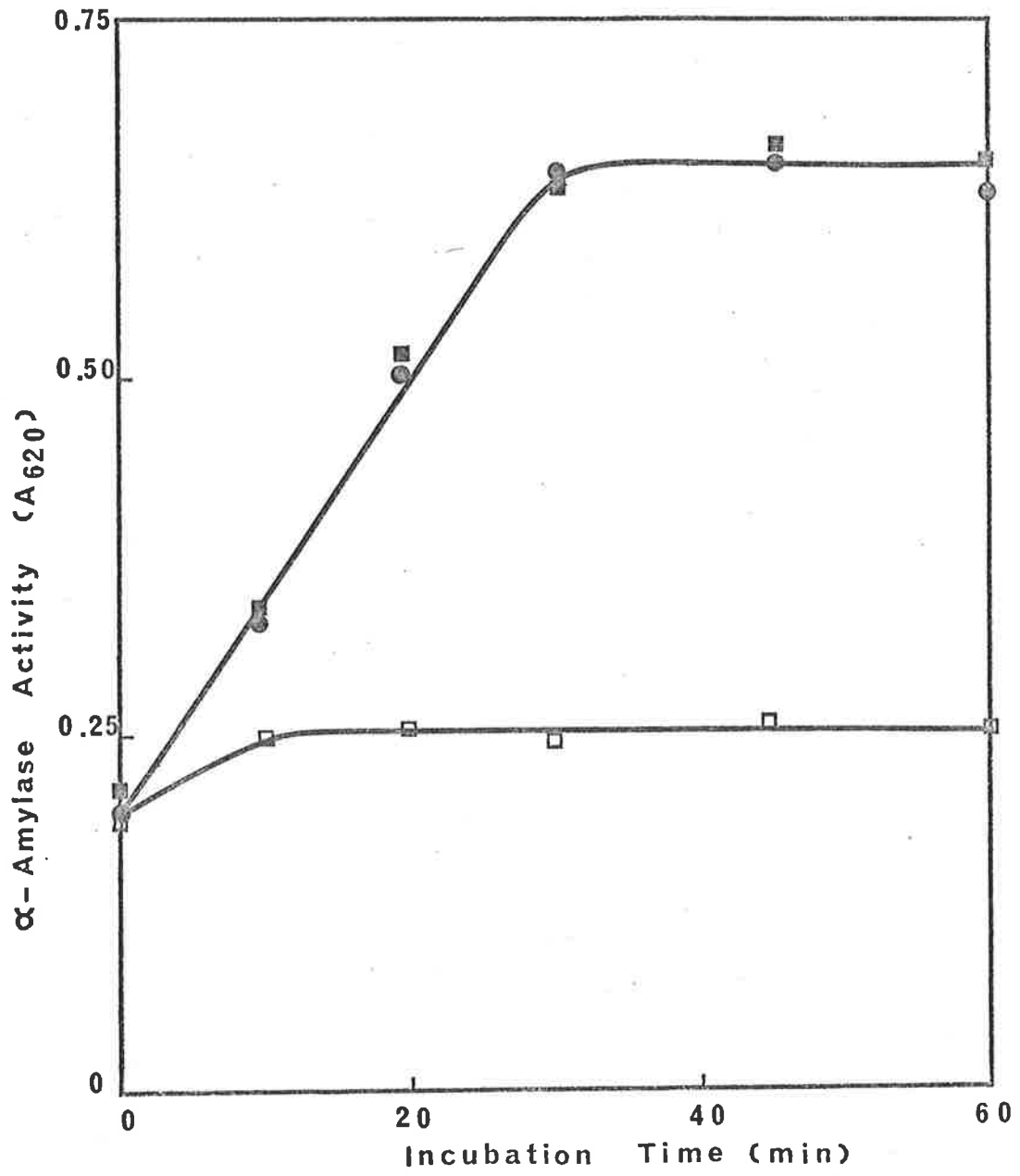
The results of the three experiments described above appear to have eliminated the possibility that proteases inhibit the release of α -amylase by adsorption to the microsomal membrane, and demonstrate that the inhibition is the result of proteolysis. This conclusion is reinforced by the fact that trypsin, chymotrypsin and bacterial protease all inhibit in the same apparent way.

(It might be mentioned here that proteases have an inhibitory effect at levels lower than those used in these experiments, as is shown in Fig.'s 3.1 and 3.2. Time has not allowed a full investigation of this point but an honours student in this department, Ms. L. Duncan, has shown that chymotrypsin affects release when present at concentrations greater than 0.1 $\mu\text{g/ml}$. These experiments

FIGURE 3.8 THE EFFECT OF BOVINE PANCREATIC RNAase
A ON α -AMYLASE RELEASE.

Microsome suspensions were incubated at 37°C in STKC buffer (●) or STKC buffer containing bovine pancreatic RNAase A (■) or chymotrypsin (○) at final concentrations of 100 μ g/ml.

Fig. 3.8



are not reported in full as they were not performed by this candidate.)

3.7 THE ORIGIN OF THE RELEASED α -AMYLASE.

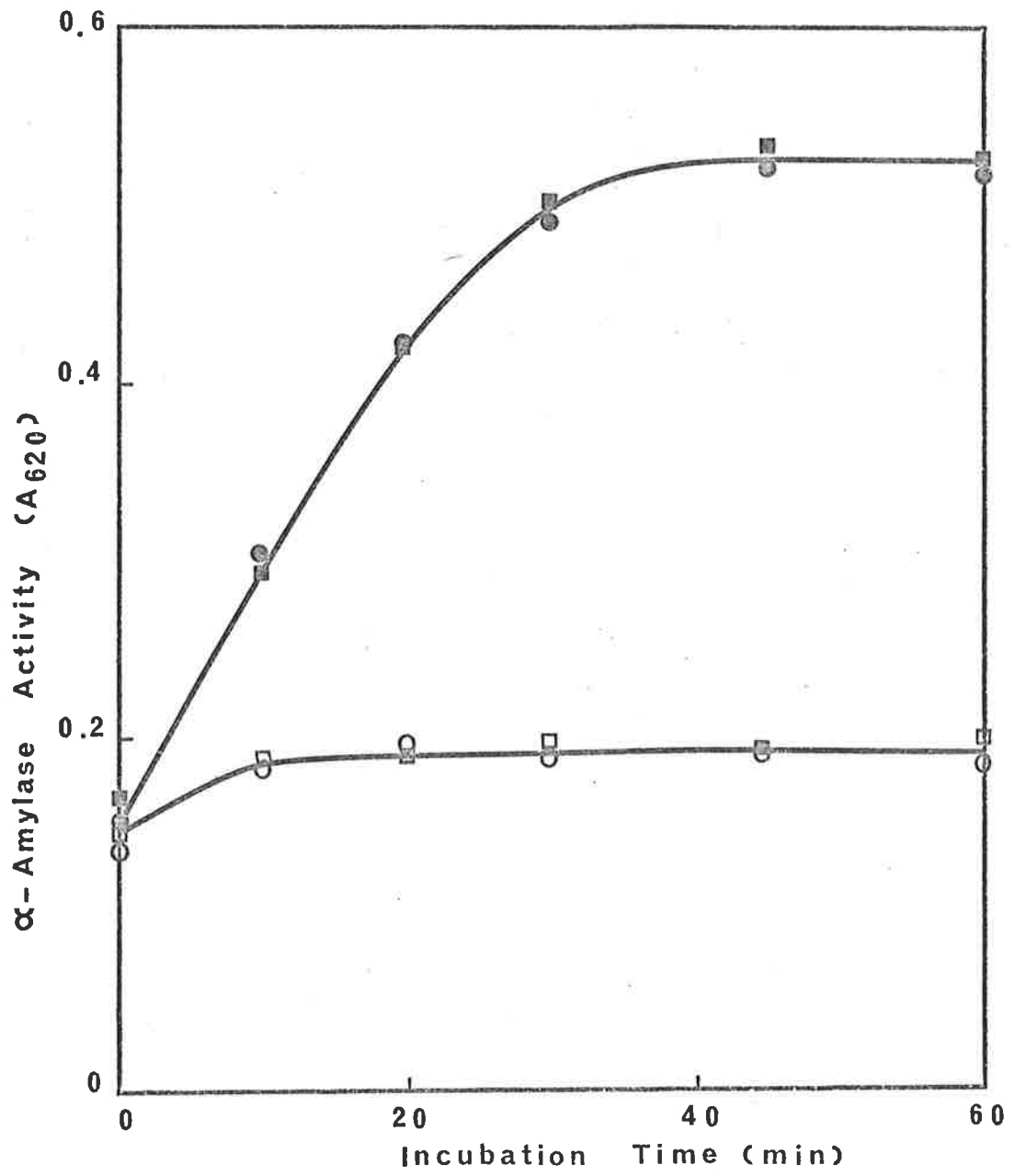
The observation that little α -amylase was accessible to the insoluble substrate prior to incubation suggested the releasable enzyme might be sequestered with the microsome. It was, however, possible that during the homogenisation procedure enzyme had become bound to the cytoplasmic surface of the microsomal membrane and that during the course of the incubation it dissociated from the membrane. If the latter suggestion was correct, then the α -amylase was either not active or was inaccessible to the substrate while it remained bound to the membrane, and desorption of the enzyme from the membrane was inhibited by proteolysis, which seems unlikely.

If the α -amylase was adsorbed to the cytoplasmic side of the membrane it might be possible to remove it using washing procedures. This was tested by resuspending the microsome pellet in STKC and storing a sample at 0°C (control sample) while the remainder were centrifuged at $150,000 \times g_{\max}$ for 30 min. After a second resuspension and centrifugation the pellet was resuspended, diluted and the microsomes incubated. The release of α -amylase from the washed microsomes was very similar to that observed in the control sample which suggested the amylase was either very tightly bound to the outer surface of the membrane or it was located within the microsome (Fig.3.9).

FIGURE 3.9 THE EFFECT OF WASHING ON THE RELEASE
OF α -AMYLASE

The pellet of microsomes obtained after centrifugation of the postmitochondrial supernatant was resuspended in STKC buffer. A portion of the microsome suspension (unwashed microsomes) was stored at 0°C while the balance was washed twice by recentrifugation and resuspension in STKC buffer. The washed (■), (□) and unwashed (●), (○) microsomes were incubated at 37°C in STKC buffer (●), (■) or STKC containing chymotrypsin at a final concentration of 100 μ g/ml (○), (□).

Fig. 3.9



In an attempt to resolve this question sonication was used to disrupt the microsomal membrane: if the α -amylase was initially adsorbed to the membrane, either specifically or non-specifically, the enzyme might be expected to remain associated (or re-associate) with the membrane fraction after sonication and centrifugation. However, if it had been free in the lumen of the microsome then it might be found in the supernatant after centrifugation. Microsomes were sonicated in short bursts for a total of 3 min (see Methods), centrifuged at $150,000 \times g_{\max}$ for 60 min, and the pellets and supernatants separated. The pellets were redissolved in STKC containing 0.5% (w/v) deoxycholate and assayed for α -amylase activity. Deoxycholate was also added to the supernatants which were then assayed for enzyme activity. It was found that the bulk of the α -amylase in sonicated microsomes was located in the soluble fraction (Table 3.1) suggesting that the lumen of the microsome had been the source of the enzyme.

In this experiment the assumption was made that enzyme which was associated with the membrane would not remain permanently dislodged from the membrane after sonication. While this seems reasonable the possibility that α -amylase might be removed by sonication cannot be eliminated. On the basis of the two experiments just described it is not possible to totally preclude the membrane as the source of the releasable α -amylase. However, experiments which will be described in Chapter 4 provide further evidence for the luminal origin of the

TABLE 3.1

THE DISTRIBUTION OF α -AMYLASE IN SONICATED AND
UNSONICATED MICROSOMES

	<u>α-AMYLASE ACTIVITY (A_{620})</u>	
	Pellet	Soluble
Unsonicated microsomes	0.72	0.21
Sonicated microsomes	0.16	0.81

(A sample of microsomes was sonicated in bursts for a total of 3 min. The sonicated suspension and a sample of unsonicated microsomes were then separated into pellet and soluble fractions by centrifugation. The pellets were dissolved in STKC buffer containing 0.5% (w/v) deoxycholate and detergent was also added to the soluble fractions (final concentration, 0.5% (w/v)). The α -amylase activity of each sample was then measured.)

released enzyme.

3.8 TEMPERATURE SENSITIVITY OF α -AMYLASE RELEASE.

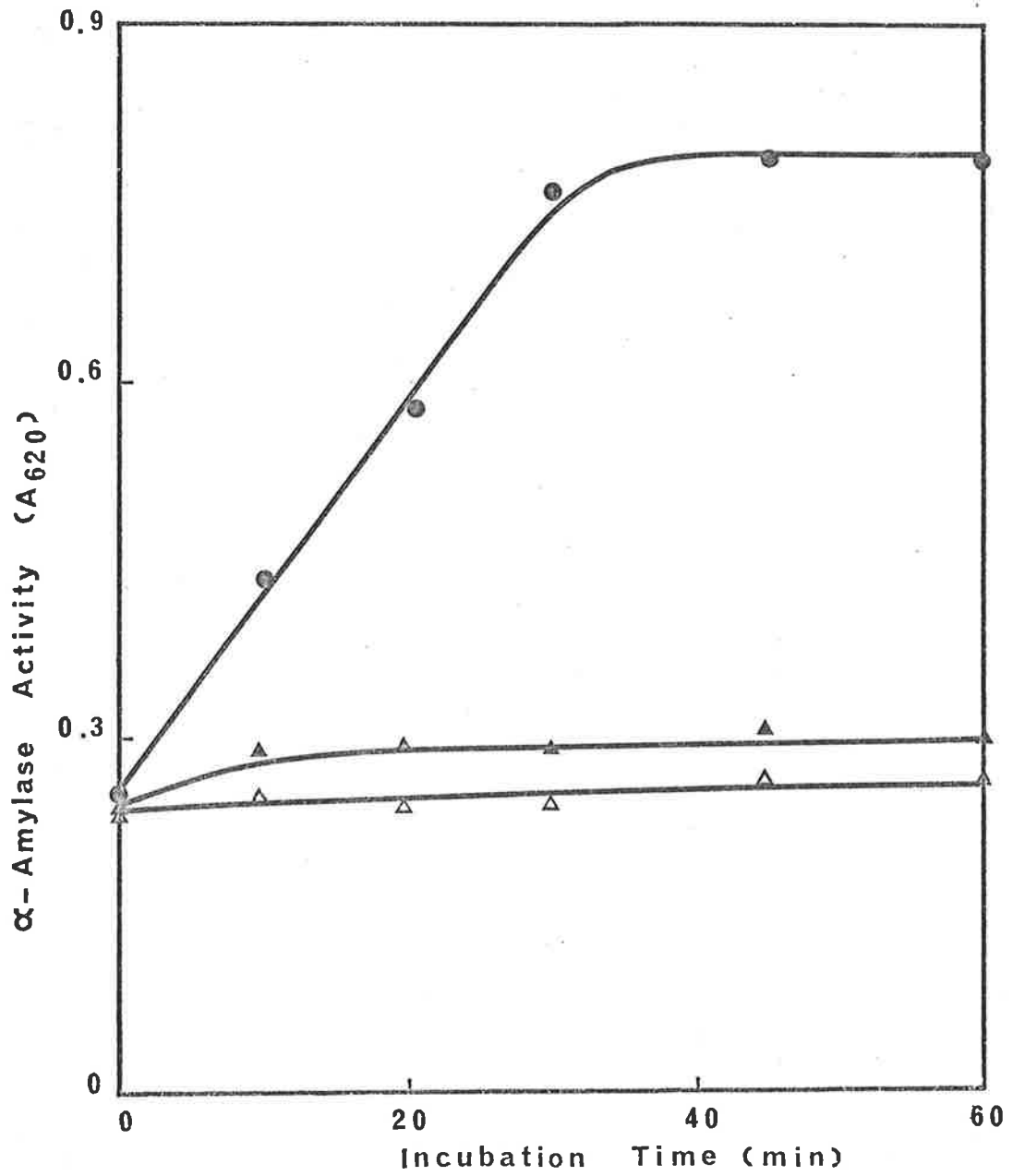
The results of the experiments described in the preceding sections suggested that a transfer of α -amylase from the lumen of the microsome to the incubating medium occurred during incubation and that the process was inhibited by protease treatment of the microsome. If this was correct the enzyme was crossing the microsomal membrane. A possible explanation for the transfer would be leakage or diffusion through either a natural pore or an artefactual hole in the membrane. If the release of α -amylase was effected by leakage the process might be expected to continue at a similar rate at lower temperatures, e.g. 0°C, since diffusion rates are related to absolute temperature. However, if the transfer was the result of some membrane-related function, perhaps a carrier protein, release would be inhibited by incubation at lower temperatures.

This was tested by incubating microsomes at 0°C and 15°C for 60 min and comparing the release with that obtained with microsomes incubated at 37°C. The amount of α -amylase released at 0°C was less than 3% of that released at 37°C (Fig.3.10) and similarly, little enzyme was released at 15°C. The possibility that low temperature may occlude membrane holes by a change in the physical state of the lipids cannot be excluded but it is not obvious how such a sealing effect might occur.

FIGURE 3.10 THE EFFECT OF INCUBATION TEMPERATURE
ON THE RELEASE OF α -AMYLASE,

Microsome suspensions were incubated in STKC buffer
at 0°C (Δ), 15°C (\blacktriangle) or 37°C (\bullet).

Fig. 3.10



The observation that release does not occur at low temperatures militates against the possibility of leakage through holes in the membrane. Such an explanation is also extremely difficult to reconcile with the inhibitory effects of proteases when it is remembered that they inhibit release as a result of proteolysis.

3.9 THE EFFECT OF BRIEF PROTEOLYTIC TREATMENT ON α -AMYLASE RELEASE.

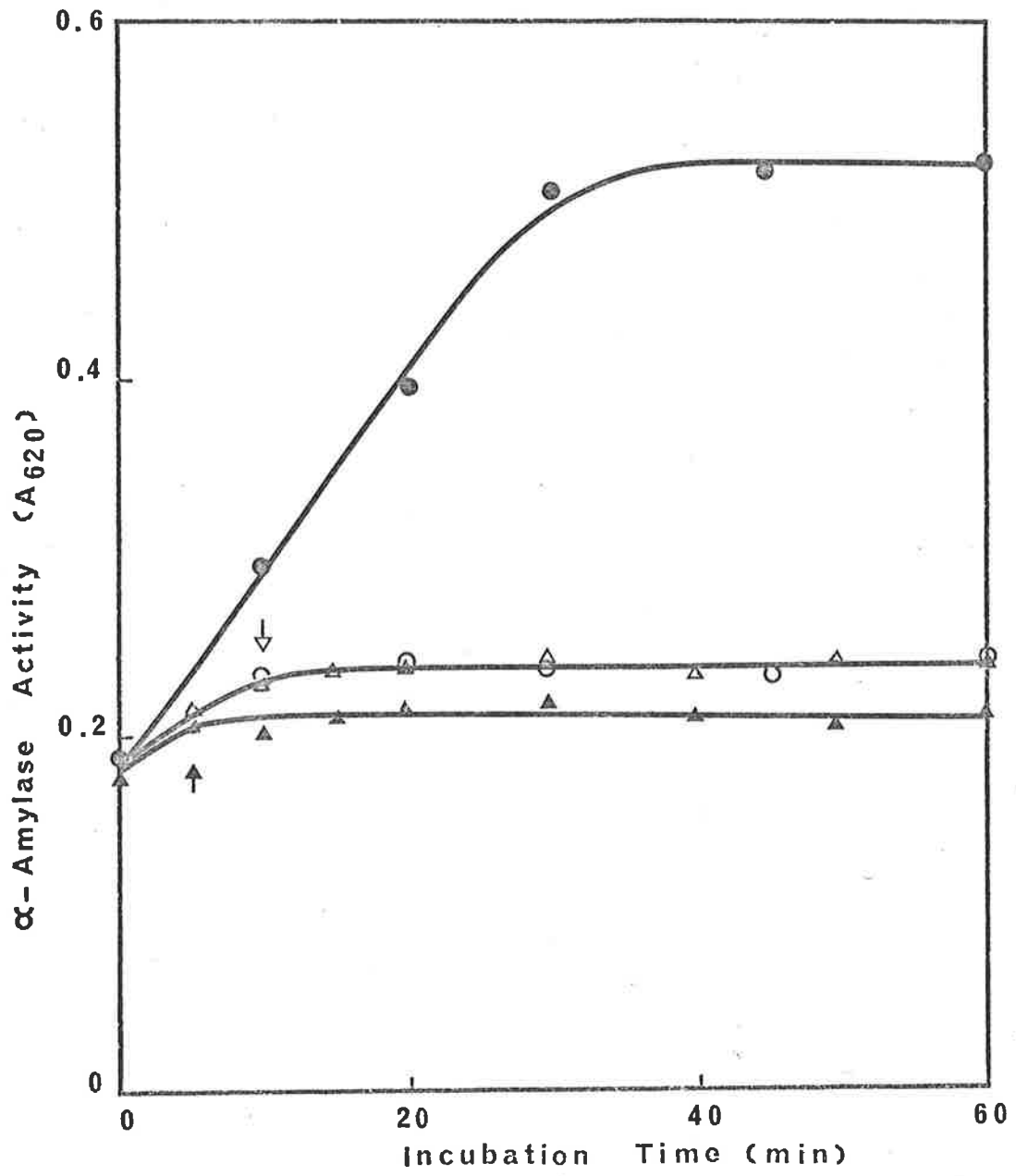
A question of interest concerning the protease-sensitive release of α -amylase was whether brief protease treatment permanently affect release of microsomal enzymes.

This was investigated by incubating microsomes for 5 or 10 min at 37°C in the presence of chymotrypsin (100 μ g/ml), adding a 500-fold excess (on a molar basis) of PMSF to inactivate the protease and then continuing the incubation for a further 50 or 55 min. At the protease concentration used in this experiment, 5 min proteolysis caused a marked inhibition of α -amylase release (Fig.3.11) which did not recover on inactivation of the protease. A similar result was found with the 10 min proteolysis showing that the protease effect was permanent. It might be argued that the PMSF inhibited release of enzyme, as well as inactivating proteases, but a control experiment, in which PMSF was added to microsomes which had been incubated for 10 min, showed that PMSF did not affect release. (Fig.3.11).

FIGURE 3.11 THE EFFECT OF BRIEF PROTEOLYSIS ON
 α -AMYLASE RELEASE IN MICROSOMAL
SUSPENSIONS

Microsome suspensions were incubated at 37°C in STKC buffer (●) or STKC buffer containing chymotrypsin (○) to which PMSF was added 5 min (▲) or 10 min (Δ) after commencing the incubation. Chymotrypsin and PMSF were used at final concentrations of 100 μ g/ml and 2 mM respectively. (The arrow indicates the time of PMSF addition.)

Fig. 3.11



3.10 THE EFFECT OF ADDING PROTEASE TO MICROSOMES DURING INCUBATION.

It was possible that the protease-sensitive release of α -amylase was caused by an enzyme, perhaps a phospholipase, which attacked the membrane and allowed the luminal contents to leak out. If the enzyme was destroyed by proteases the presence of exogenous proteases would cause the observed inhibition of α -amylase release. If this was correct it might be expected that the damage caused by the enzyme puncturing the membrane would be permanent and that release would not be inhibited if protease was added after the membrane damage was done, say, 10 min after beginning the incubation. This was investigated by incubating microsomes for 10 or 15 min before adding chymotrypsin (final concentration, 100 μ g/ml) and it was found that on addition of the protease the release of α -amylase was rapidly inhibited (Fig.3.12). Assuming that a punctured vesicle would not be able to reseal itself, this experiment appears to rule out the existence of a protease-sensitive, membrane-puncturing enzyme.

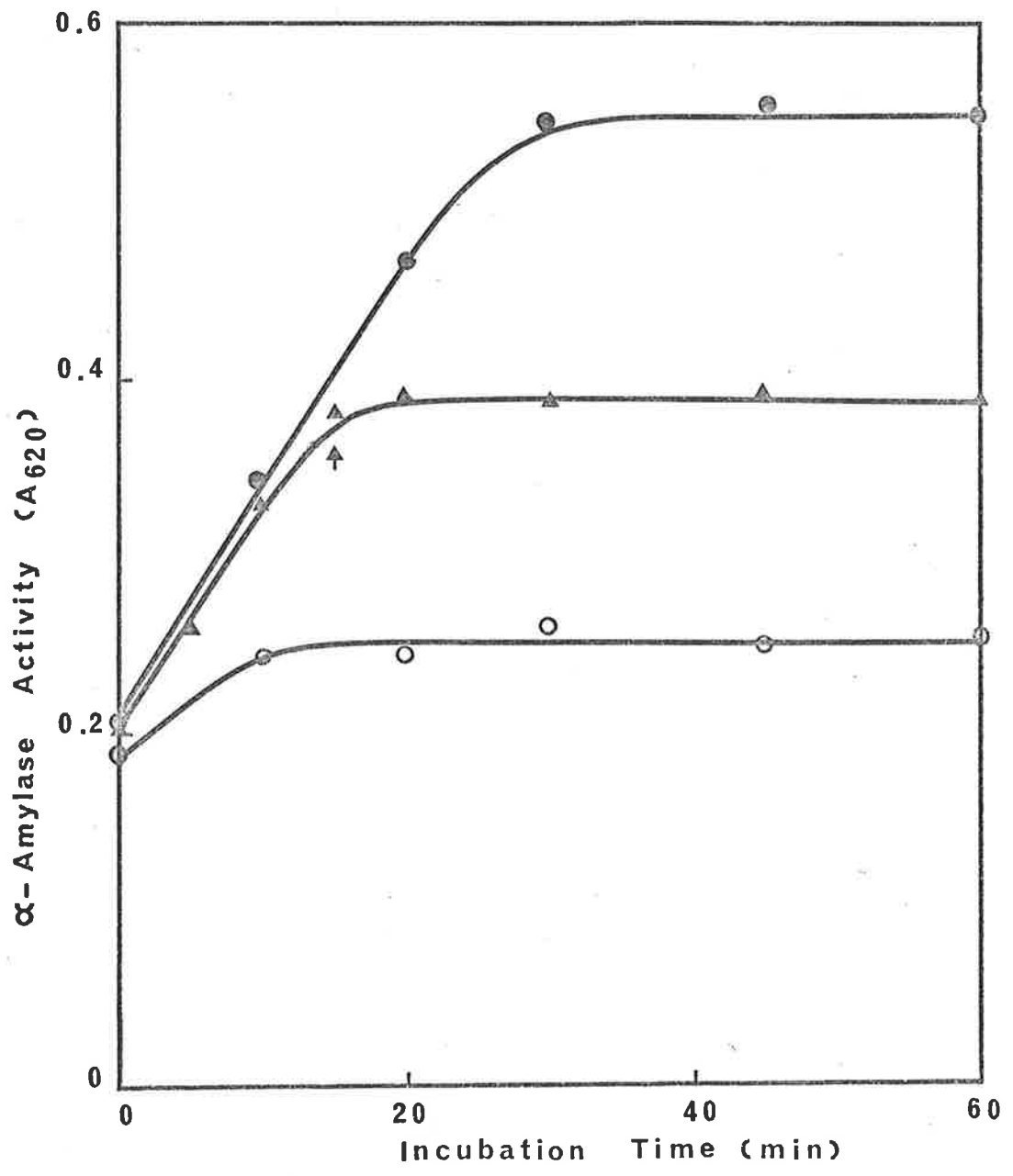
3.11 ELECTRON MICROSCOPE STUDIES OF MICROSOMAL VESICLES.

Although the results of some of the earlier experiments militated against the possibility of leakage through holes in the membrane, another, less likely, possible explanation for the appearance of α -amylase was that the microsomes lysed during the incubation and that the presence of exogenous proteases somehow prevented lysis.

FIGURE 3.12 THE EFFECT ON α -AMYLASE RELEASE OF
CHYMOTRYPSIN ADDITION 15 MIN AFTER
COMMENCING THE INCUBATION.

Microsome suspensions were incubated at 37°C in STKC buffer (●) to which chymotrypsin (final concentration, 100 μ g/ml) was added before (○) or 15 min (▲) after the incubation. (The arrow indicates the time of chymotrypsin addition).

Fig. 3.12



This was tested by examining electron micrographs of microsomes at different stages during incubation: both negative staining and thin sections were used in this investigation.

Micrographs of unincubated, negatively stained microsomes showed that the microsome preparation consisted of a high percentage of rough microsomes together with some smooth microsomes and free ribosomes. The microsomes appeared to be intact and were correctly orientated i.e. the ribosomes were on the outer surface of the vesicles (Fig.3.13a). After incubation at 37°C for 20 min many of the ribosomes had disappeared (Fig.3.13b) while after 60 min only smooth microsomes were observed. These still appeared to be intact (Fig.3.14a). Micrographs of microsomes incubated with chymotrypsin at 100 µg/ml for 60 min before negative staining also showed apparently intact smooth microsomes and a complete absence of ribosomes (Fig.3.14b). These results were confirmed by electron micrographs of thin sections of glutaraldehyde fixed, osmium tetroxide postfixed, unincubated, 60 min incubated and 60 min protease incubated microsomes (Fig.'s 3.15a, b and c respectively).

Two important points emerged from these experiments. Firstly, visual inspection of the micrographs showed that large scale lysis of vesicles did not occur and, secondly, they demonstrated that the ribosomes detached from the membranes and disintegrated during the course of the incubation. (A separate test showed that detachment did

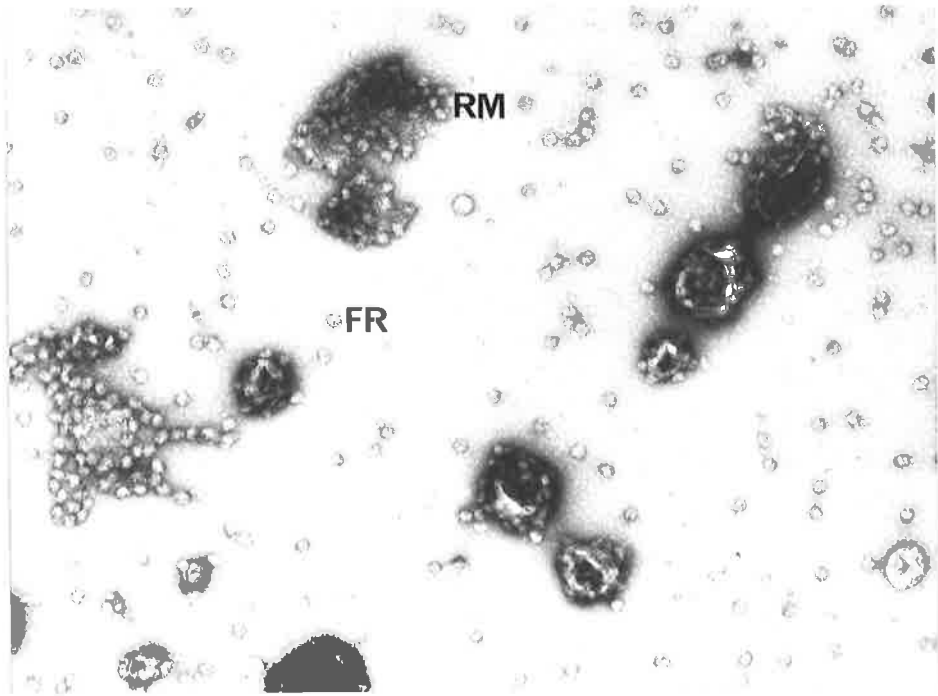
FIGURE 3.13 ELECTRON MICROGRAPH OF UNINCUBATED AND
20 MIN INCUBATED MICROSOMES

- (a) A sample of unincubated microsomes was negatively stained with uranyl acetate.
Total magnification: 60,000

- (b) Microsome suspensions were incubated at 37°C in STKC buffer. A sample was taken after 20 min incubation and negatively stained with uranyl acetate.
Total magnification: 60,000

RM = rough microsomes;
SM = smooth microsomes;
FR = free ribosomes.

a



b

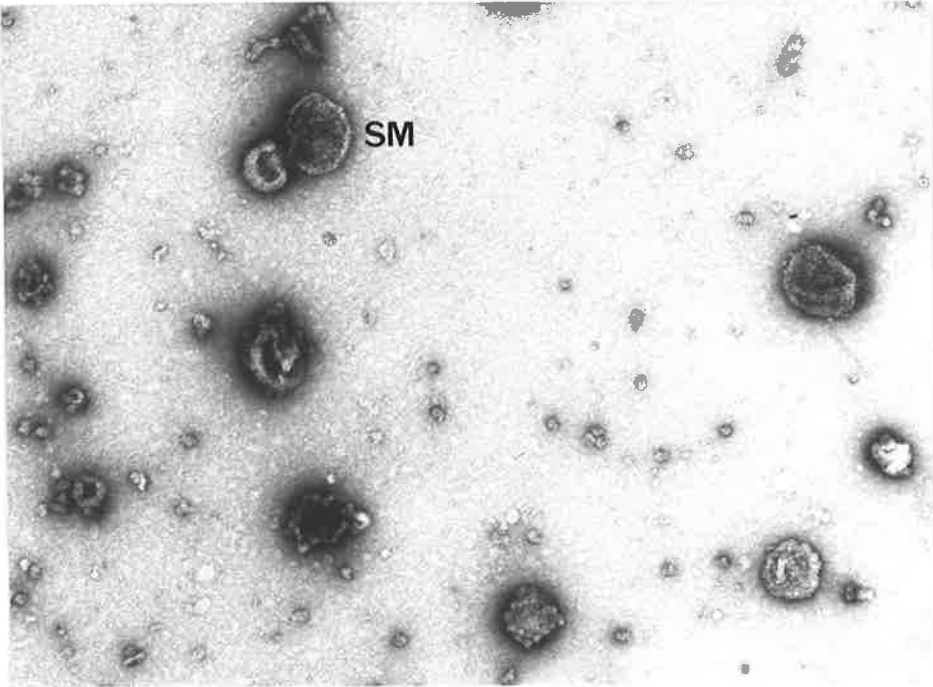
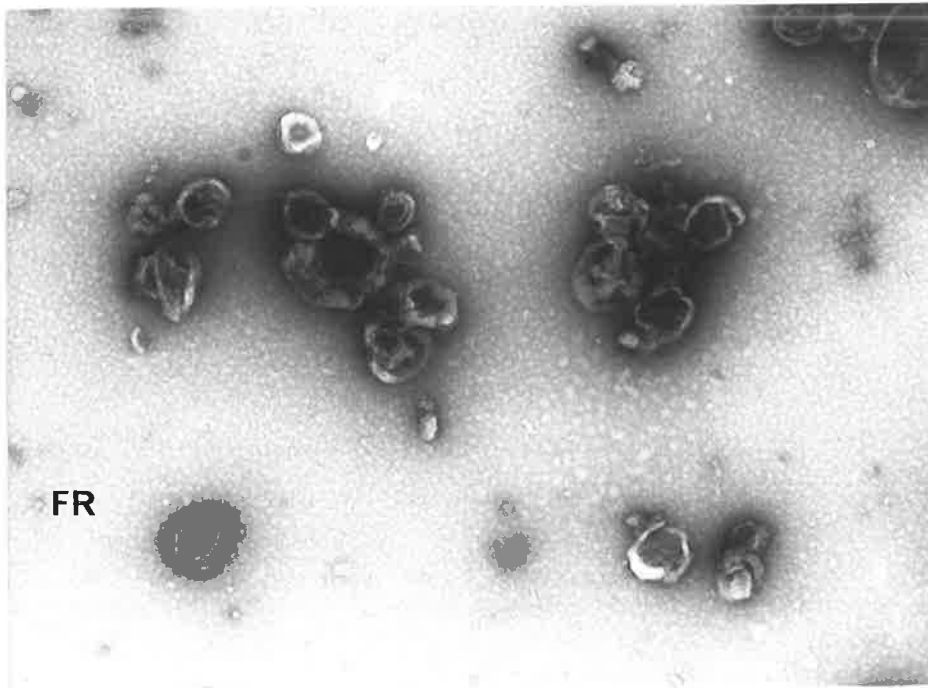


FIGURE 3.14 ELECTRON MICROGRAPHS OF MICROSOMES
AFTER 60 MIN INCUBATION

- (a) Microsome suspensions were incubated at 37°C in STKC buffer. A sample was taken after 60 min incubation and negatively stained with uranyl acetate.
Total magnification: 60,000
- (b) Microsome suspensions were incubated at 37°C in STKC buffer containing chymotrypsin (final concentration, 100 µg/ml). A sample was taken after 60 min incubation and negatively stained with uranyl acetate.
Total magnification: 60,000

SM= smooth microsomes;
FR = free ribosomes.

a



b

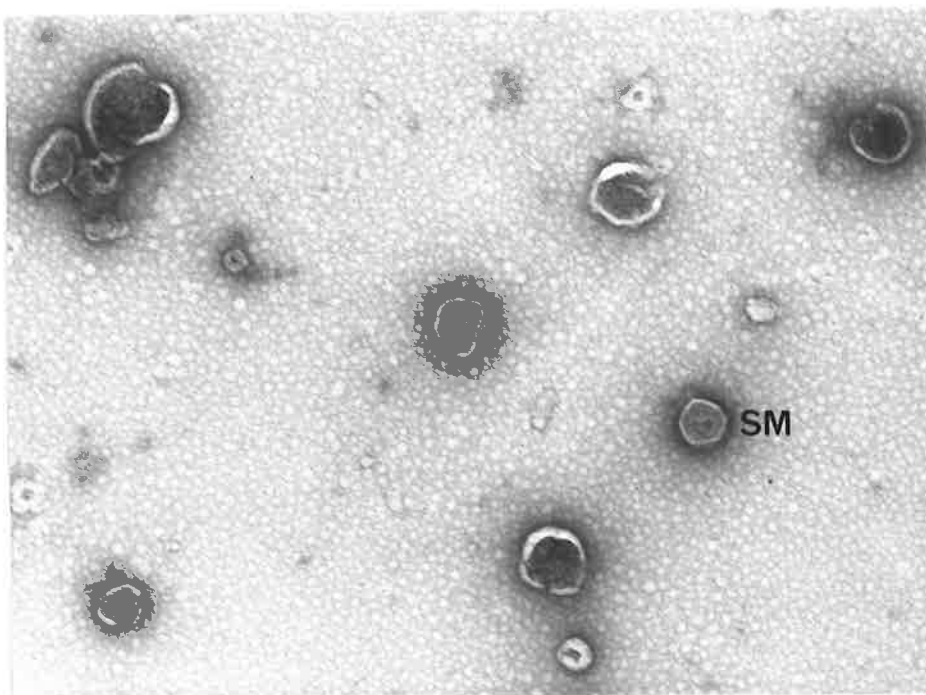
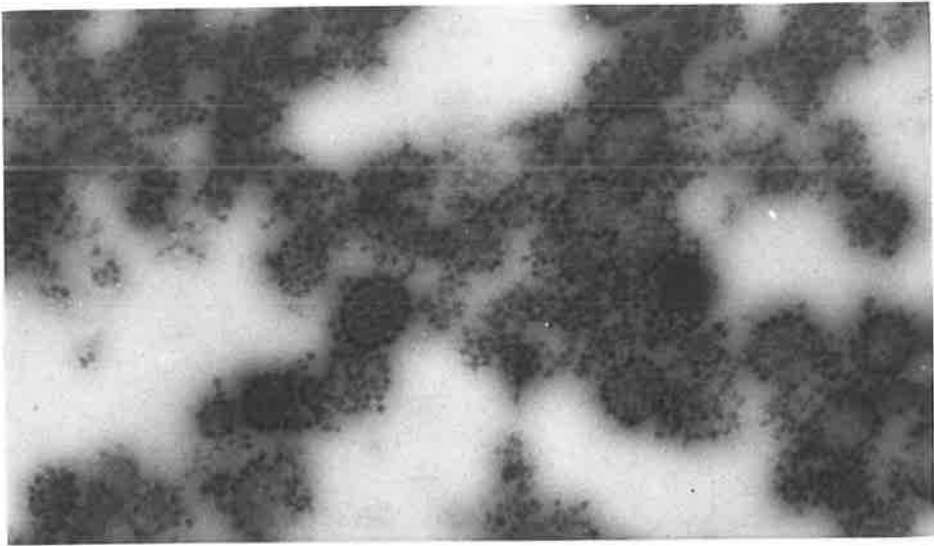


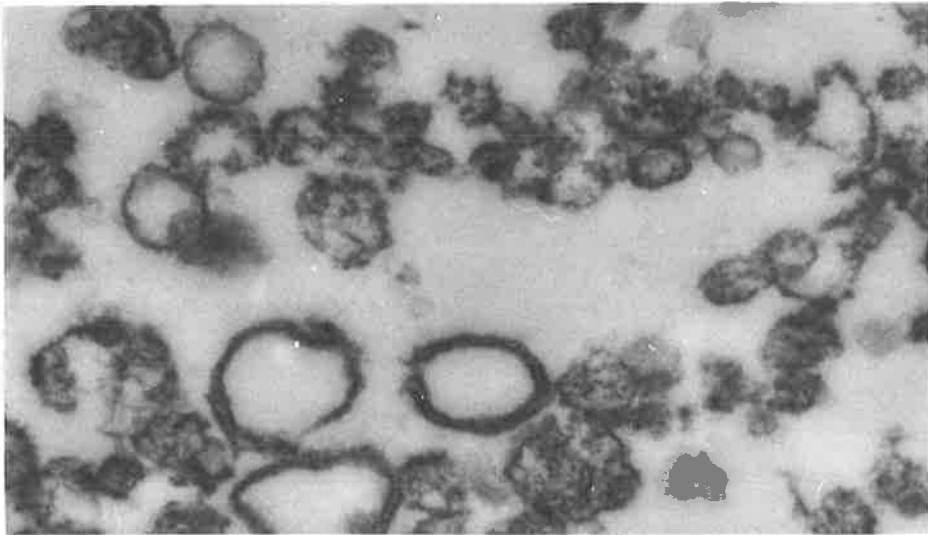
FIGURE 3.15 ELECTRON MICROGRAPHS OF UNINCUBATED,
60 MIN INCUBATED AND 60 MIN PROTEASE
INCUBATED MICROSOMES

- (a) A thin section of glutaraldehyde fixed OsO_4 post-fixed unincubated microsomes.
Total magnification: 60,000
- (b) A thin section of microsomes which were incubated in STKC buffer at 37°C for 60 min before being glutaraldehyde fixed and OsO_4 post-fixed.
Total magnification: 60,000
- (c) A thin section of microsomes which were incubated in STKC buffer containing chymotrypsin (final concentration, $100 \mu\text{g/ml}$) at 37°C for 60 min before being glutaraldehyde fixed and OsO_4 post-fixed.
Total magnification: 60,000

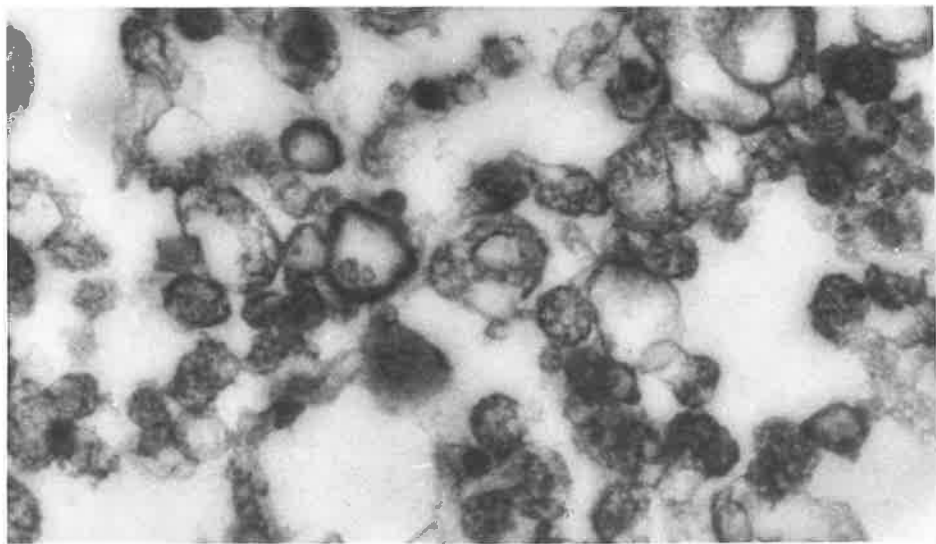
a



b



c



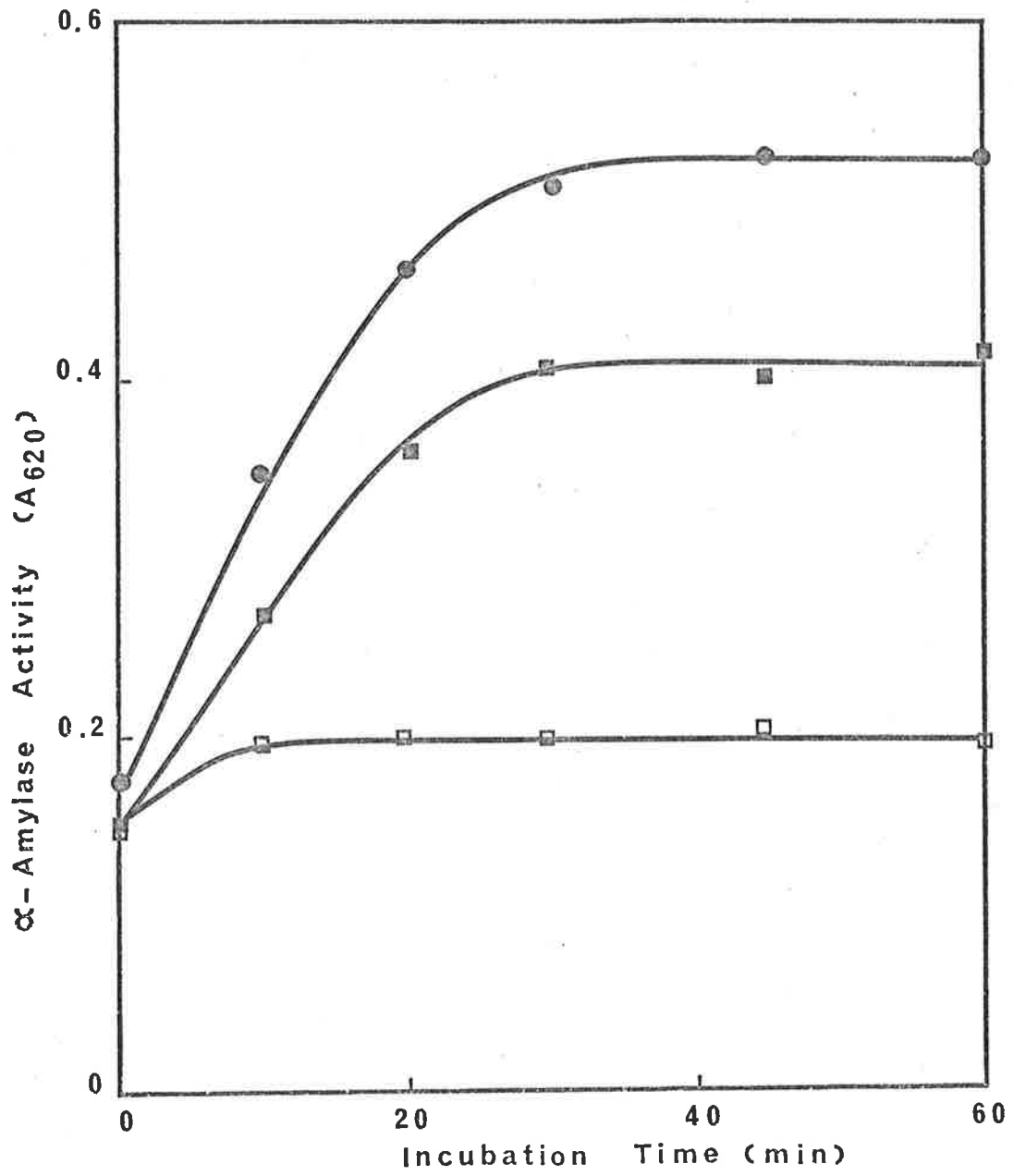
not occur if the microsomes were kept at 0°C; result not shown.) The detachment of ribosomes was not surprising since Mg^{2+} was not included in the incubation medium and it is well known that Mg^{2+} is required for ribosome stability (Chao, 1957). The possible relationship between the detachment of ribosomes and enzyme release will be discussed in Chapter 5.

3.12 THE RELEASE OF α -AMYLASE BY PURIFIED ROUGH MICROSOMES.

In all of the above experiments the microsomal fraction used was a crude preparation in that it contained a mixture of rough and smooth microsomes together with free microsomes. It was of interest, therefore, to see whether purified rough microsomes released α -amylase. Rough microsomes were isolated by centrifugation of resuspended microsomes on a step gradient (see Methods). This gave a clean preparation of rough microsomes as judged by visual inspection of electron micrographs.

The purified rough microsomes were resuspended in STKC buffer and incubated with and without chymotrypsin (final concentration, 100 μ g/ml) for 60 min. Protease-sensitive release of α -amylase was observed (Fig.3.16). (The "smooth microsome" fraction obtained using the above procedure was found to be contaminated with rough microsomes and, therefore, was not tested for α -amylase release.)

Fig. 3.16



3.13 THE MOLECULAR FORM OF THE RELEASED α -AMYLASE

As described in Chapter 1, it is known that nearly all secretory proteins can be synthesised in vitro with an NH_2 -terminal "signal peptide" which, in vivo, is usually removed before synthesis is completed. The mRNA for dog pancreatic α -amylase has been translated in vitro (McDonald et al., 1977) as a pre-protein which differed from normal α -amylase in that it had a higher molecular weight and it was not precipitable by glycogen. This result implied that the pre-protein differed conformationally from secreted α -amylase.

The α -amylase released from microsomes during incubation was active and it was of interest to see whether it differed in size from the secreted enzyme. This was tested by purifying both zymogen granule α -amylase and the released enzyme by glycogen precipitation (see Methods). Samples of each enzyme were electrophoresed on Laemlli slab gels and no detectable difference in size was observed. (It should be noted it is unlikely that a difference in molecular weight of less than 1,200 would be detected using this procedure.)

To see whether the released enzyme was glycosylated, microsomes were incubated in STKC buffer for 60 min and the suspension was then centrifuged for 30 min at $150,000 \times g_{\text{max}}$. The soluble supernatant proteins were precipitated, by adding an equal volume of 10% trichloroacetic acid, and pelleted by centrifugation. The pellet was redissolved in a small volume of dilute NaOH and, after boiling with

SDS reagents, the samples were electrophoresed on Laemlli slab gels. A sample of the purified zymogen granule α -amylase was used as a marker. Half of the slab gel was stained with Coomassie blue to locate proteins and the other half was treated with periodic acid - Schiff reagent (Zacharius et al., 1969) to detect glycoproteins. The bands corresponding to α -amylase gave a weakly positive reaction with the carbohydrate stain. This suggested that the released enzyme might be a glycoprotein, or, at least contained some glycosylated α -amylase. (Time has not permitted further investigation of this point which is of some interest.)

3.14 SUMMARY

The experiments described in this chapter demonstrated that incubation of rat pancreatic microsomes led to an increase in assayable α -amylase activity. This increase was the result of a transfer of α -amylase from the microsomal fraction to the external medium. The presence of exogenous proteases such as trypsin and chymotrypsin inhibited the transfer (or release). The released α -amylase was resistant to proteolysis and it was shown that the protease-sensitivity of the release was not due to proteolysis of the enzyme during its transfer to the external medium. The observation that inactivated chymotrypsin had no effect on release and the fact that immobilised Sepharose-trypsin inhibited release suggested that the proteases exerted their effect on release by virtue of their proteolytic activity.



Trivial explanations of the phenomenon such as de novo synthesis of enzyme, non-specific leakage of vesicle contents, vesicle lysis or desorption of enzyme from the cytoplasmic surface of the microsomal membrane are not tenable. The non-occurrence of de novo synthesis was demonstrated (i) by showing that cycloheximide had no effect on release and (ii) in the transfer experiment where it was shown that the total amount of α -amylase in the microsomal suspensions did not increase. The possibility that leakage occurred seemed unlikely since release was not observed during incubation at low temperatures and it is difficult to reconcile leakage with the inhibitory effects of proteolytic activity. A study of electron micrographs revealed that lysis did not occur on a large scale and, again, it is difficult to see how lysis could be inhibited by proteolysis.

The fact that little enzyme was initially accessible to the insoluble substrate, together with the observation that washed microsomes released α -amylase, suggested that the releasable enzyme was not located on the cytoplasmic surface of the membrane. If it can be assumed that sonication did not permanently dislodge proteins from the membrane the sonication experiments showed that the releasable α -amylase was located in the lumen of the vesicle. This implied that the enzyme crossed the membrane during release.

CHAPTER FOUR

RESULTS

THE PROTEASE-SENSITIVE RELEASE OF
RIBONUCLEASE FROM MICROSOMES.

4.1 INTRODUCTION

The experiments described in the previous chapter demonstrated that α -amylase was transferred, most probably from the interior of the microsome, to the external medium during incubation. This transfer, or release, was inhibited by the addition of proteases to the incubating medium. It was obviously of interest to determine whether this phenomenon applied to other pancreatic secretory proteins or if it was restricted to α -amylase only. With this in mind a series of experiments similar to those just described (Chapter 3) was performed, and in this second series ribonuclease (RNAase) activity was estimated instead of α -amylase activity.

4.2 RIBONUCLEASE RELEASE FROM PANCREATIC MICROSOMES
AND THE INHIBITION OF THIS PROCESS BY CHYMOTRYPSIN.

The release of RNAase from pancreatic microsomes was demonstrated by incubating microsomes at 37°C and removing aliquots for RNAase assay during the course of the incubation. Yeast RNA (with a high molecular weight) was used as a substrate for the RNAase assays to prevent the possible entry of substrate into the lumen of the microsome. When microsomes were incubated in STKC buffer RNAase was released in a manner very similar to that of

the release of α -amylase i.e. release was observed for the first 20 - 30 min of incubation and then a plateau level was reached (Fig.4.1). The inclusion of chymotrypsin, at a final concentration of 100 $\mu\text{g/ml}$, in the incubating medium had an inhibitory effect on RNAase release (Fig.4.1).

Pancreatic RNAase is not itself destroyed by chymotrypsin; this was demonstrated in two experiments. Trypsin or chymotrypsin (final concentration, 100 $\mu\text{g/ml}$) was added to a suspension of microsomes, which had been incubated at 37°C for 60 min, and the incubation was continued for a further 30 min. The amount of RNAase remained constant after addition of the protease showing that the released RNAase resisted proteolytic digestion (Fig.4.2).

The second experiment was to demonstrate directly that the effect of chymotrypsin on measured RNAase release, from microsomes, was not due to destruction of the enzyme as it was released. To do this it was necessary to show that the total RNAase in the system did not change under any condition of incubation. Microsomes, with and without added chymotrypsin (final concentration, 100 $\mu\text{g/ml}$), were incubated for 60 min and then Triton X-100 was added (final concentration, 0.5% (w/v)) to dissolve the microsomal membrane. Incubation was continued for a further 30 min and RNAase levels measured. There was a small increase in assayable RNAase on addition of the detergent to the microsomes which did not have added protease (Fig.4.2).

FIGURE 4.1 THE EFFECT OF CHYMOTRYPSIN ON THE
RELEASE OF α -AMYLASE IN MICROSOMAL
SUSPENSIONS.

Microsome suspensions were incubated at 37°C in STKC buffer (●) or STKC buffer containing chymotrypsin at a final concentration of 100 μ g/ml (○).

Samples were taken from the microsome suspensions, at the times indicated, and the RNAase activity was immediately measured using yeast RNA as a substrate. (See Method for details of the assay procedure).

Fig. 4.1

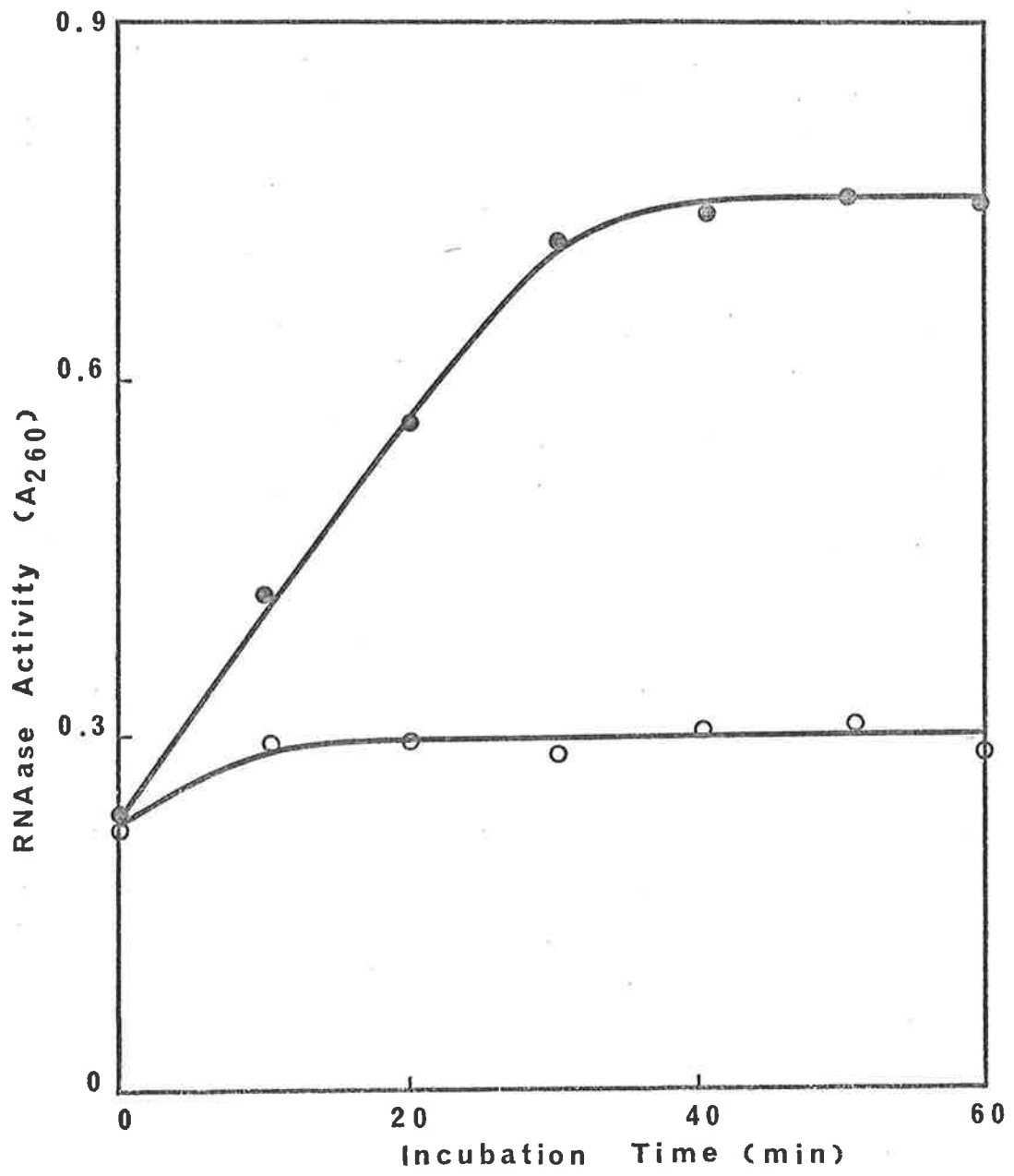
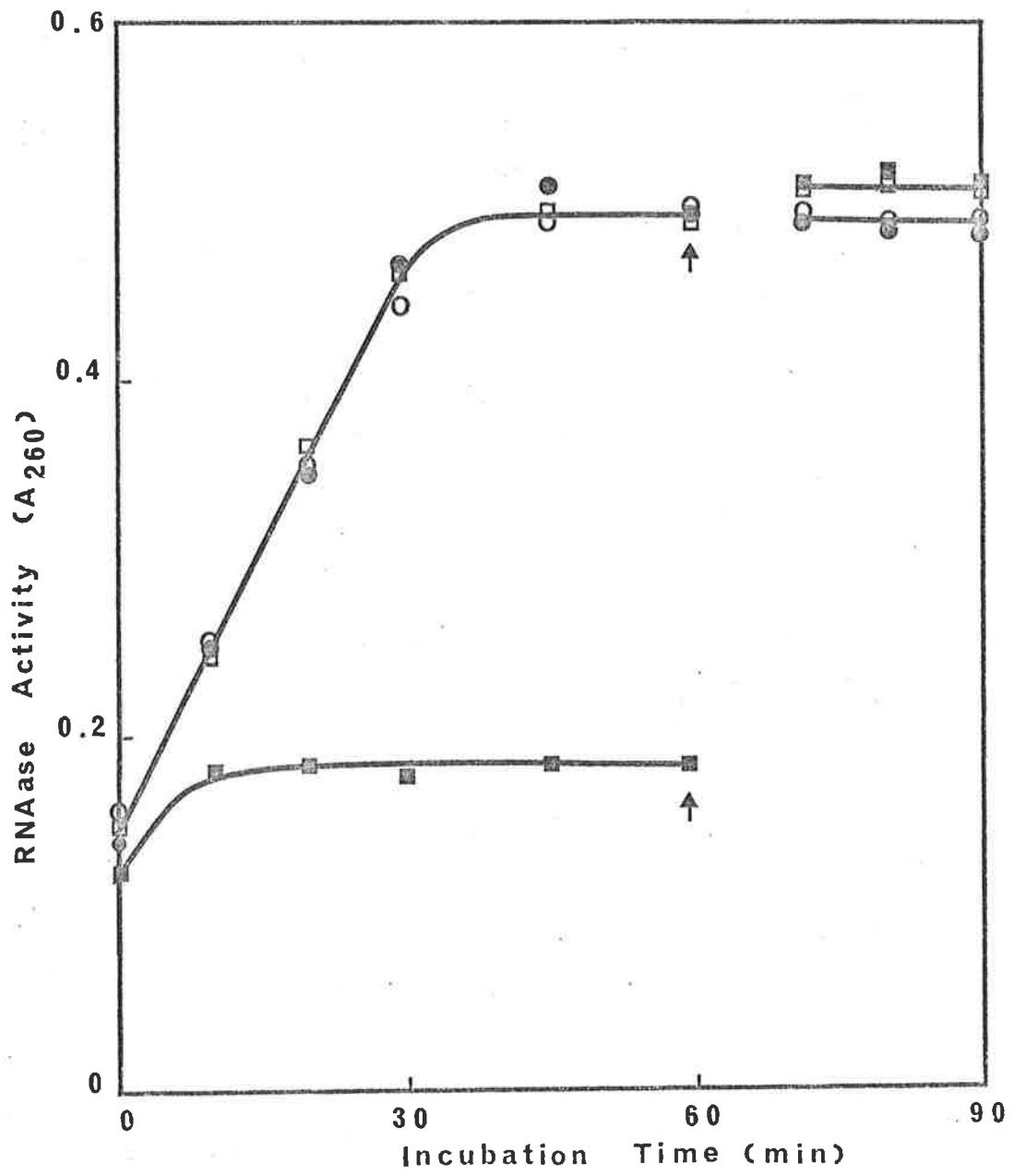


FIGURE 4.2 THE RESISTANCE OF PANCREATIC RNAase TO
DIGESTION BY TRYPSIN AND CHYMOTRYPSIN

Microsome suspensions were incubated at 37°C in STKC buffer for 60 min. Chymotrypsin (●), trypsin (○) or Triton X-100 (□) was then added and the incubation continued for a further 30 min. A sample of microsomes was also incubated at 37°C in STKC buffer containing chymotrypsin for 60 min (■). Triton X-100 was then added and the incubation continued for a further 30 min. The proteases and detergent were used at final concentrations of 100 µg/ml and 0.5% (w/v) respectively. (The arrow indicates the time of protease or detergent addition.)

Fig. 4.2



However, the addition of the detergent to the protease-treated vesicles brought the level of RNAase activity up to that which was found in microsomes from which protease had been omitted. This showed that RNAase had not been destroyed. After the initial rise caused by the addition of the Triton X-100, RNAase levels remained constant showing that RNAase resisted digestion and confirming the result of the previous experiment. A separate test showed that Triton X-100, at the concentration used in this experiment, did not inhibit chymotrypsin.

4.3 TRANSFER OF RNAase FROM THE MICROSOMAL FRACTION TO THE SOLUBLE FRACTION.

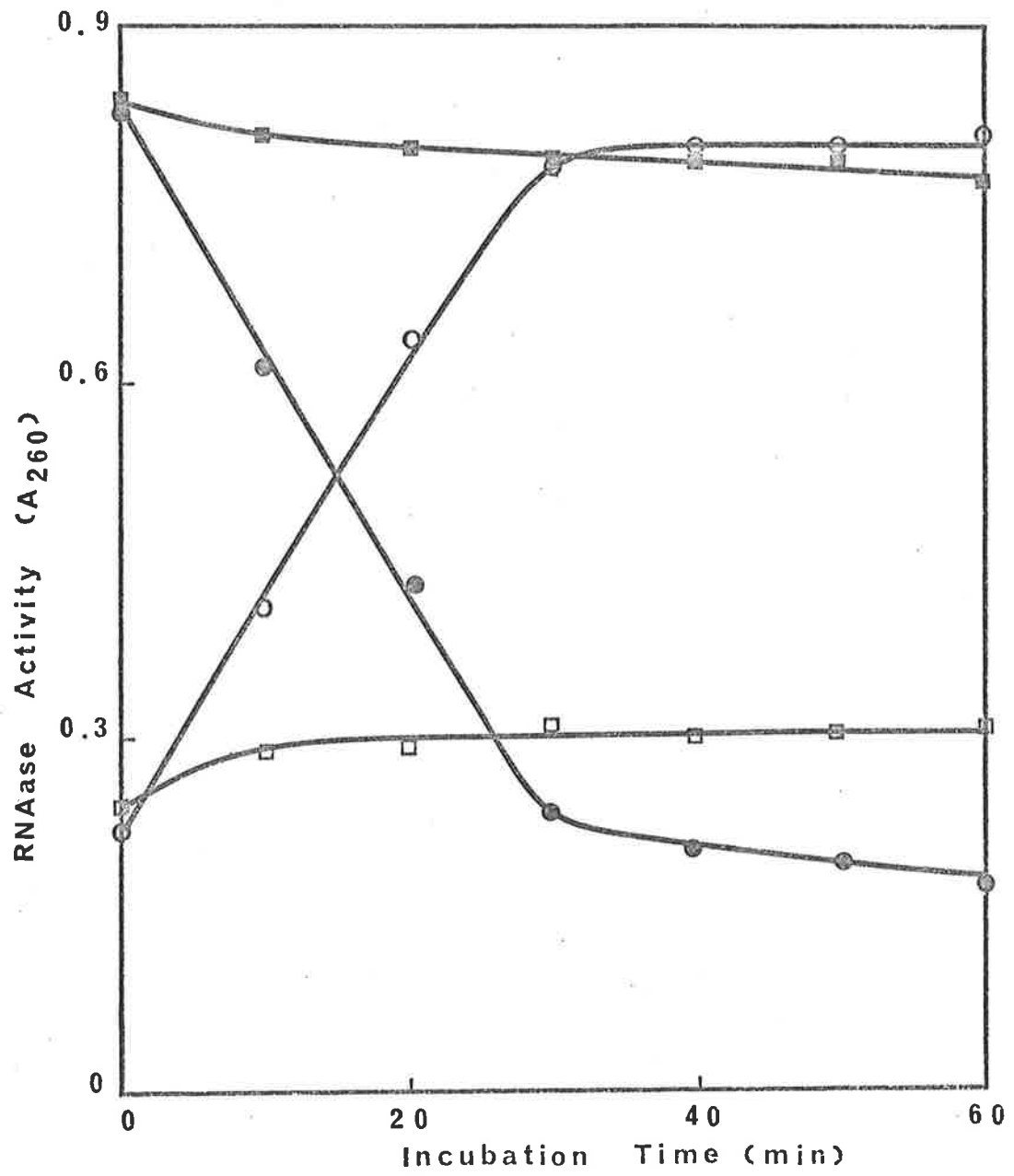
The experiment described in the first part of this chapter, showed that the inclusion of chymotrypsin in the incubating medium inhibited the observed increase in the amount of assayable RNAase. It seemed likely that, like α -amylase, the RNAase was transferred from the microsome fraction to the medium during the course of the incubation. This was tested by incubating microsomes at 37°C, with and without chymotrypsin (final concentration, 100 μ g/ml), for times up to 60 min before centrifugation at 150,000 \times g_{max} for 30 min. The supernatants were decanted and the pellets of microsomes were resuspended in STKC buffer containing 0.5% (w/v) Triton X-100 to dissolve the membranes. Detergent was also added to the supernatants at the same concentration, to control for the effects of this on enzyme activity, and the soluble and pellet fractions were assayed for RNAase activity.

As was found in the α -amylase experiments, most of the RNAase was associated with the microsome pellet before commencement of the incubation, and during the course of the incubation the enzyme was transferred from the sedimentible fraction to the soluble fraction (Fig.4.3). When chymotrypsin was included in the incubating buffer, little RNAase was transferred to the soluble fraction and most of the enzyme remained associated with the microsomes (Fig.4.3). In both cases the total amount of RNAase (i.e. supernatant plus pellet activities) remained constant during the incubation, showing that de novo synthesis of RNAase did not occur when protease was absent, and that RNAase was not destroyed by chymotrypsin during release of enzyme.

4.4 THE ORIGIN OF THE RELEASED RNAase.

The results of the washing and sonication experiments, described in Chapter 3, suggested that the releasable α -amylase was originally located in the lumen of the microsome, although the evidence for this was not conclusive. Blobel and Dobberstein (1975a) propose that the best evidence that a protein is sequestered within a microsome is protection of the protein from exogenous proteases. This type of evidence could not be obtained with active α -amylase since it is unusually resistant to digestion by most of the commonly available proteases. RNAase, however, which in its native form is resistant to trypsin and chymotrypsin, is susceptible to attack by subtilisin BPN' (the alkaline protease from B. amyloliquefaciens).

Fig.4.3



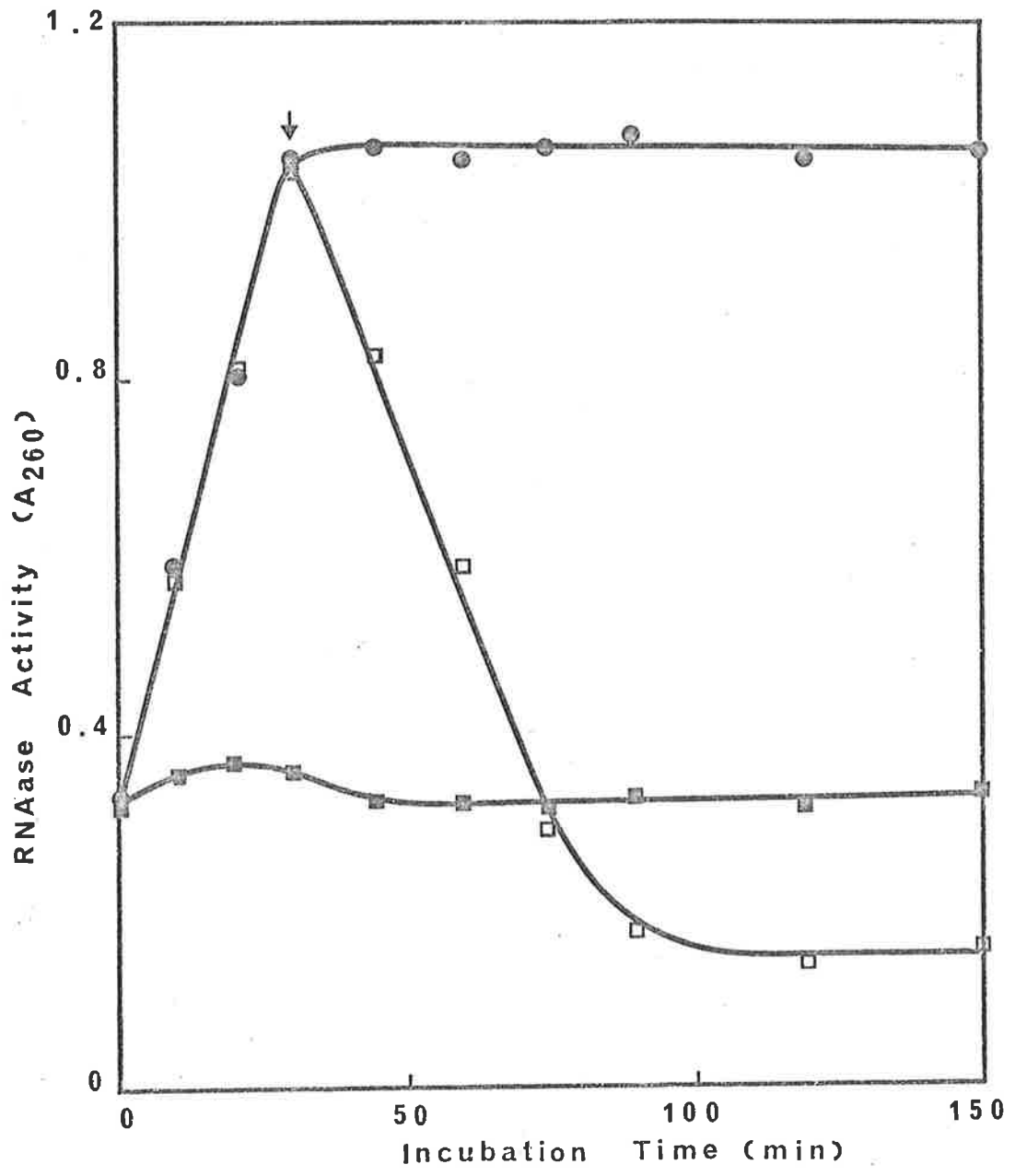
To see whether the releasable RNAase associated with microsomes was protected from proteolysis, the following experiment was performed. Two samples of microsomes with no added protease and one sample containing subtilisin BPN', at a final concentration of 1 $\mu\text{g/ml}$, were incubated for 30 min, and then subtilisin was added to one of the two samples originally lacking protease. The incubation was continued for a further 2 hr and PMSF (final concentration, 2 mM) was then added to all three samples to inactivate the protease. To ensure inactivation, the samples were incubated with PMSF for 15 min and then Triton X-100 (final concentration, 0.5% (w/v)) was added to dissolve the membrane of the microsome and release all RNAase. Aliquots were taken from each sample after the PMSF-detergent treatment for RNAase assay. These values therefore gave the total RNAase remaining in each sample.

The sample of microsomes which did not have protease added during the first incubation period released RNAase for 30 min, and then reached a level which remained constant for the rest of the experiment (Fig.4.4). After the PMSF and detergent treatments, the total amount of RNAase present was sufficient to produce an absorbance change of 0.95 in 1 min (not shown in Fig.4.4), and this was taken as being 100% of the RNAase. The assayable RNAase in the sample of microsomes which was incubated for 30 min before the addition of the subtilisin BPN' increased for the first 30 min, as did the first sample, but then steadily decreased after the protease addition (Fig.4.4).

FIGURE 4.4 THE EFFECT OF SUBTILISIN BPN' ON THE
RELEASE OF RNAase AND ITS EFFECT ON
RELEASED RNAase

Microsome suspensions were incubated ^{without subtilisin} at 37°C in STKC buffer (●) to which subtilisin BPN' was added before (■) or 30 min after (□) commencing the incubation. Protease was used at a final concentration of 1 mg/ml. (The arrow indicates the time of protease addition).

Fig. 4.4



The total RNAase at the end of the experiment was less than 15% of the original total (the absorbance change was 0.12 in 1 min). Thus, when RNAase was released for 30 min, subtilisin added after this time was able to inactivate more than 85% of the enzyme.

In the final sample, which was incubated for 150 min with subtilisin, little RNAase release was detected during the entire incubation period. However, after the PMSF and detergent treatments, the total RNAase produced an absorbance change of 0.72 in 1 min (not shown in figure) i.e. over 75% of the RNAase was present even after exposure of the microsomes to protease for 150 min.

The observation, that greater than 85% of the total RNAase was destroyed when subtilisin BPN' was added 30 min after starting the incubation, shows that the released enzyme was susceptible to digestion by the protease, but, when the protease was added before the incubation commenced, less than 25% of the RNAase was destroyed. Two possible explanations exist for this latter observation: either the releasable enzyme is associated with the cytoplasmic surface of the membrane in a form which is resistant to proteolytic digestion or, alternatively, it is located within the microsome, and the membrane of the vesicle confers protection on the RNAase. The latter explanation seems the more likely, taking into account the washing and sonication experiments described earlier, especially when it is remembered that the alternative

requires that desorption is prevented by proteolysis. It is not possible to determine if the releasable enzyme is attached to the luminal side of the microsomal membrane or whether it is part of the soluble luminal contents of the microsome. The enzyme could also be buried within the membrane. If it can be assumed that sonication would not promote detachment of the enzyme from the membrane, then the only origin for the releasable enzyme is the soluble contents of the lumen. It seems reasonable to assume from this that the release of enzyme during incubation involves a transfer across the microsomal membrane from the lumen to the medium.

4.5 SUMMARY

The experiments described in this chapter demonstrated that RNAase, as well as α -amylase, is released from pancreatic microsomes during incubation and, although the release of other secretory enzymes was not examined, it seems reasonable to postulate that release is a general phenomenon which applies to all pancreatic secretory proteins. As with α -amylase, enzyme was transferred during incubation from the microsomes to the soluble fraction by a process which was inhibited by the proteases.

The fact that the releasable RNAase was protected from digestion by subtilisin BPN' suggests that it, and presumably α -amylase as well, was originally located in the lumen of the microsome. If this was so then the release of RNAase and α -amylase observed in these experiments involved the transfer of enzymes across a

membrane without concomitant protein synthesis.

CHAPTER FIVE

RESULTS

CHARACTERISTICS OF ENZYME RELEASE.

5.1 INTRODUCTION.

The experiments described in the previous chapters have shown that enzymes are transferred, apparently from within the microsome, to the external medium. The question therefore arises as to how such a transfer is achieved. From the results of earlier experiments it seemed unlikely that the transfer had a trivial explanation (see Chapter 3). However, it had been noted that both enzyme release and ribosome detachment occurred during incubation at 37°C, while neither occurred at 0°C. This raised the possibility that enzyme release and ribosome detachment were related. The experiments to be described in this chapter were designed to test this possibility while further examining the characteristics of enzyme release.

5.2 THE EFFECTS OF Mg²⁺ ON ENZYME RELEASE.

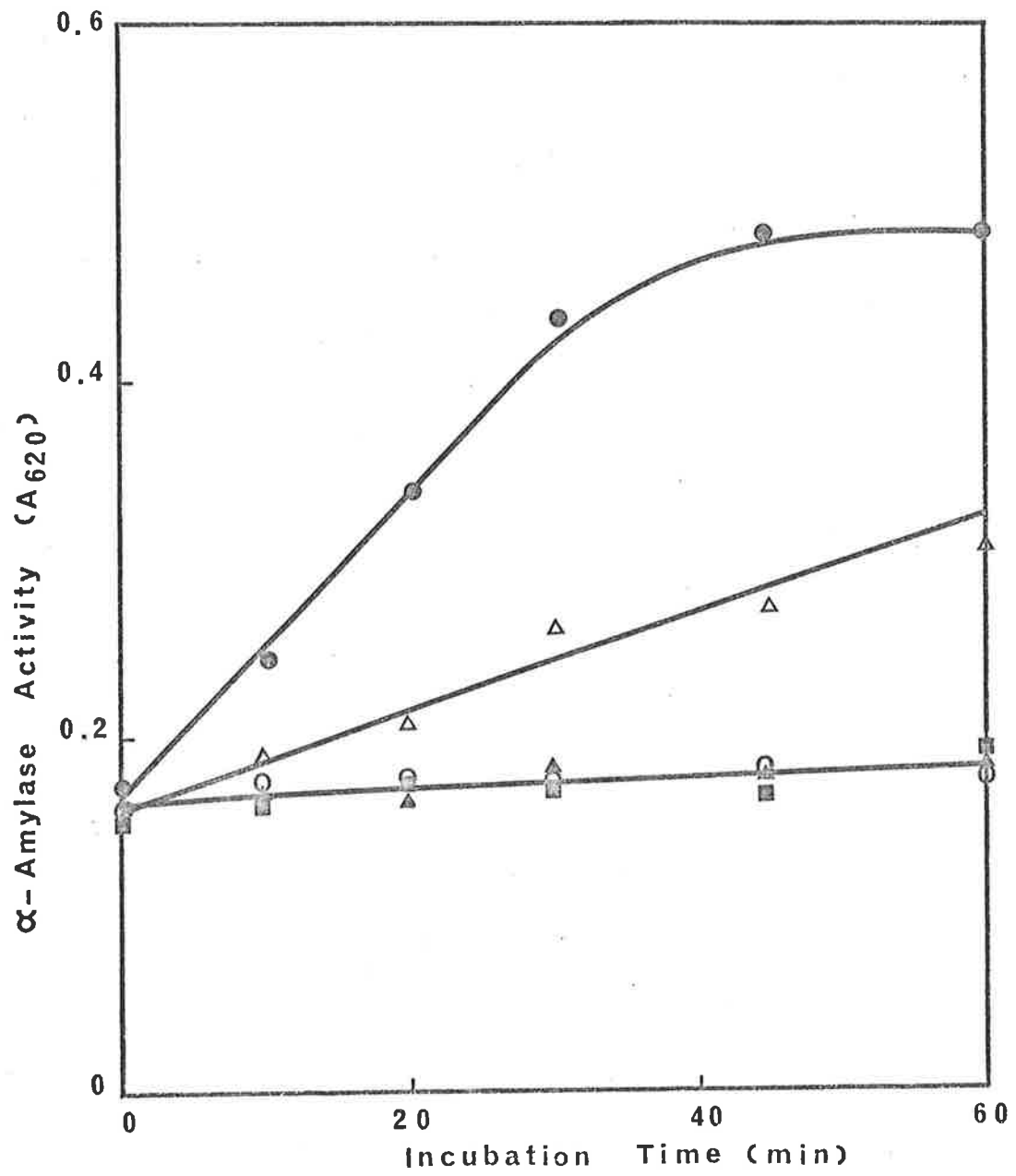
It was of interest to determine whether release occurred under conditions where the ribosomes remained attached to the membrane. This was tested by including different amounts of Mg²⁺ in the incubating buffer as it has been shown that this metal ion is required to stabilise ribosomes (Chao, 1957) and to maintain the attachment of ribosomes to membranes. It was found that the release of α -amylase was almost completely abolished when Mg²⁺ was added to the incubation buffer at final concentrations in excess of 1 mM (Fig.5.1). Enzyme release was also

FIGURE 5.1 THE EFFECT OF Mg^{2+} ON THE RELEASE OF
 α -AMYLASE IN MICROSOMAL SUSPENSIONS

Microsome suspensions were incubated at 37°C in STKC buffer (●) or STKC buffer containing 0.33 mM (Δ), 1.0 mM (▲), 5 mM (○) or 10 mM- $MgCl_2$ (■).

Samples were taken from the microsome suspensions, at the times indicated, and immediately assayed for α -amylase activity using an insoluble substrate. (See Methods for details).

Fig. 5.1



inhibited when 0.33 mM Mg^{2+} was present; the rate of release in this case however was approximately half the rate observed in the absence of the metal ion.

Samples of these incubated microsomes were negatively stained after completion of the incubation and then subjected to electron microscopic examination. Visual inspection indicated little apparent detachment of ribosomes occurred when 5 or 10 mM Mg^{2+} (Fig.5.2) was present, but almost complete detachment occurred at 0.33 mM. The fact that enzyme release was inhibited and ribosome detachment occurred in the presence of 0.33 mM Mg^{2+} suggests that release and detachment are not directly related.

5.3 DETACHMENT OF RIBOSOMES IN THE PRESENCE OF 5 mM Mg^{2+} .

To obtain further evidence on the above question, microsomes were incubated with 5 mM Mg^{2+} under conditions which are known to promote detachment of ribosomes.

Adelman et al. (1973) demonstrated that more than 85% of bound ribosomes were removed from rat microsomes when incubated in buffers containing puromycin and a high concentration of KCl. This technique was used to detach ribosomes in the presence of Mg^{2+} from pancreatic rough microsomes. Two experiments were performed in which microsomes were incubated at either 0°C or 37°C.

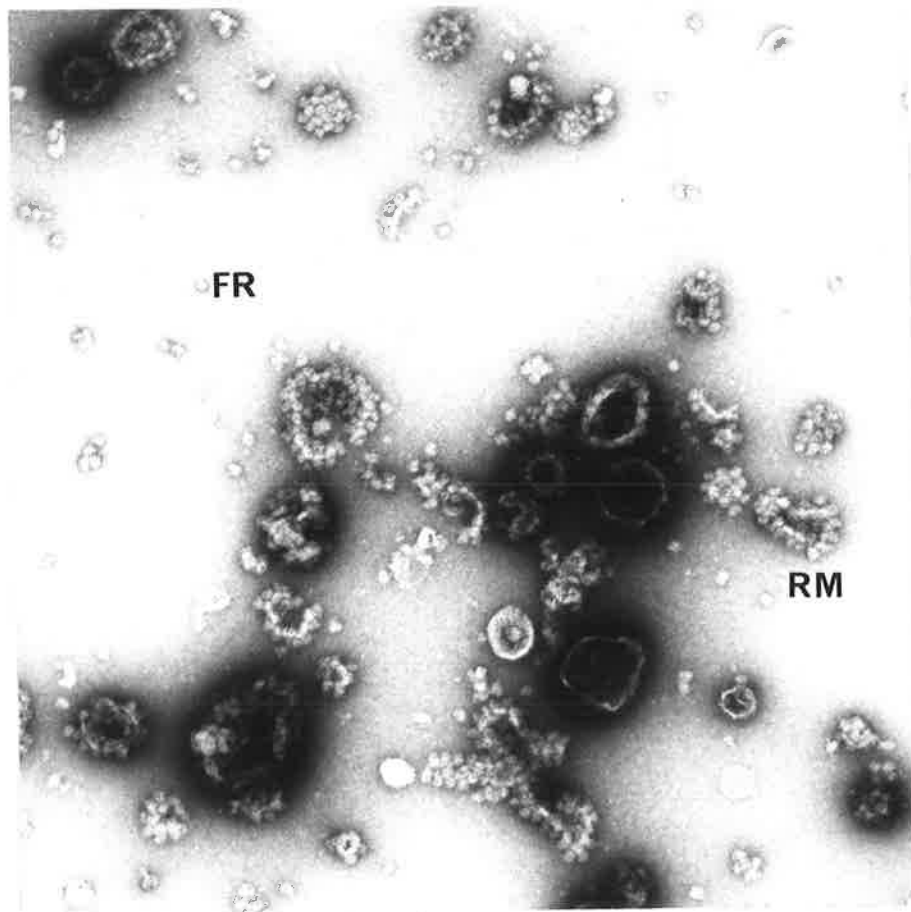
As mentioned earlier, little ribosome detachment and little enzyme release were observed during incubation at 0°C. In the first experiment, microsomes were incubated at 0°C in STKC containing 1 mM puromycin, 1 mM puromycin/

FIGURE 5.2 ELECTRON MICROGRAPH OF MICROSOMES INCUBATED
FOR 60 MIN IN Mg²⁺ CONTAINING BUFFER.

Microsome suspensions were incubated in STKC buffer containing 5 mM MgCl₂. A sample was taken after 60 min incubation and negatively stained with uranyl acetate. Total magnification: 60,000.

RM = rough microsomes;
FR = free ribosomes.

Fig. 5.2



750 mM KCl or 1 mM puromycin/750 mM KCl/5 mM MgCl₂. Electron microscopic examination of samples negatively stained after 60 min incubation at 0°C, indicated that puromycin/KCl/Mg²⁺ had caused considerable detachment of ribosomes and that puromycin/KCl had caused almost complete ribosome detachment. However, little enzyme was released during incubation with any of the combinations used (Fig.5.3). The fact that puromycin/KCl causes almost complete detachment of ribosomes without release of enzyme suggests that detachment and release are not related, but it could be argued that puromycin and/or KCl inhibit release themselves.

In the second experiment, microsomes were incubated at 37°C for 60 min in STKC buffer containing 750 mM KCl, 1 mM puromycin/750 mM KCl, 750 mM KCl/5 mM MgCl₂ or 1 mM puromycin/750 mM KCl/5 mM MgCl₂. Electron microscopic examination of samples negatively stained after 60 min incubation, showed that ribosome detachment was complete if Mg²⁺ had been omitted from the incubating buffer. If Mg²⁺ was included in the buffer, considerable, but not complete, detachment was observed. Release of α-amylase at 37°C was observed only if Mg²⁺ was absent (Fig.5.4). The presence of high KCl or high KCl and puromycin appeared to reduce the amount of α-amylase released, but the reason for such a reduction is now known.

(A quantitative estimation of ribosome detachment from electron micrographs is difficult as the size of the ribosomes and the variability of the staining procedure

FIGURE 5.3 THE EFFECT OF RIBOSOME DETACHMENT AT
LOW TEMPERATURES ON α -AMYLASE RELEASE

Microsome suspensions were incubated at 0°C in STKC buffer (○) or STKC buffer containing puromycin (Δ), puromycin and KCl (▲) or puromycin, KCl and MgCl₂ (■). Microsomes were also incubated in STKC buffer at 37°C (●). Puromycin, KCl and MgCl₂ were used at final concentrations of 1 mM, 750 mM and 5 mM respectively.

Fig. 5.3

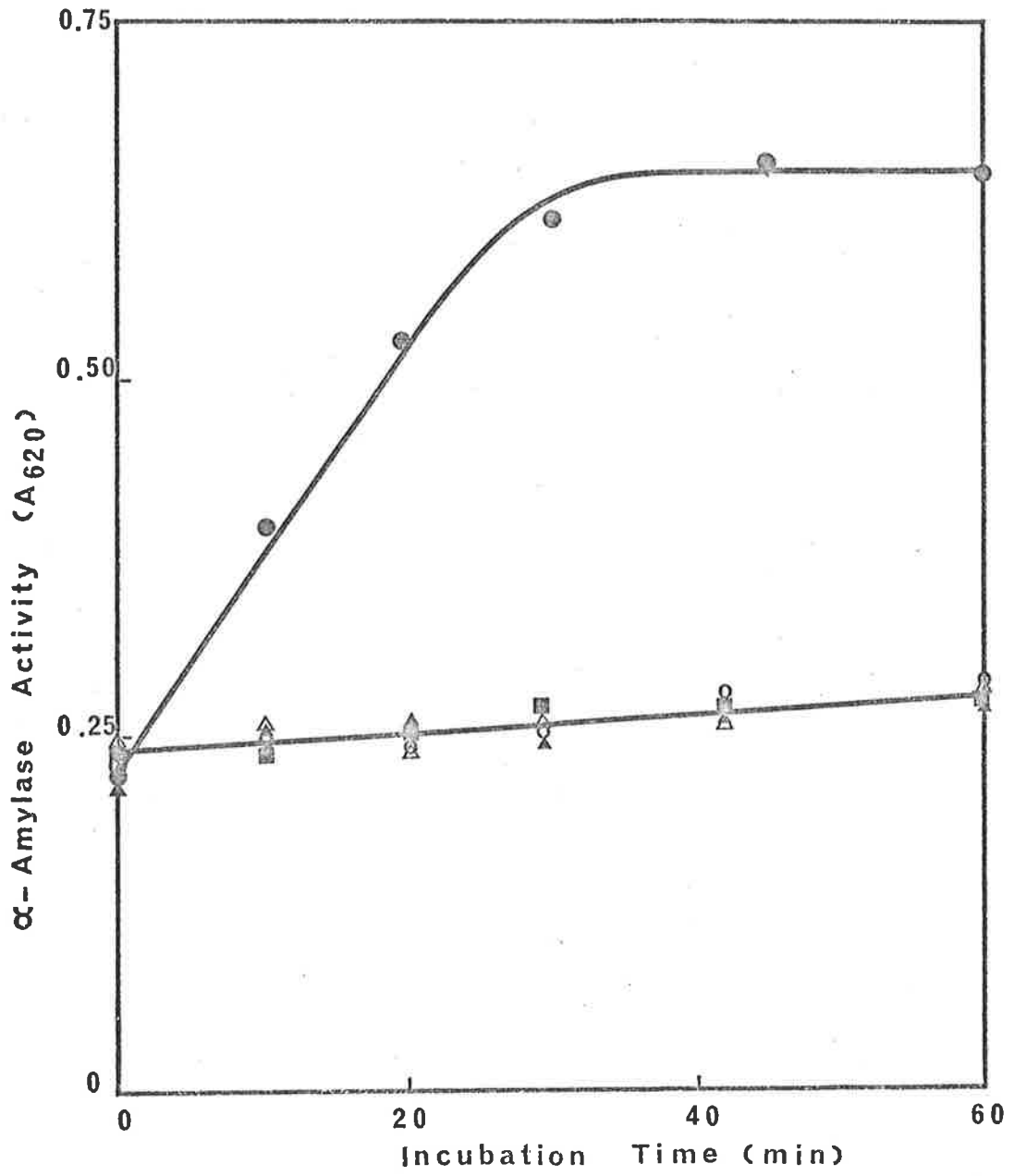
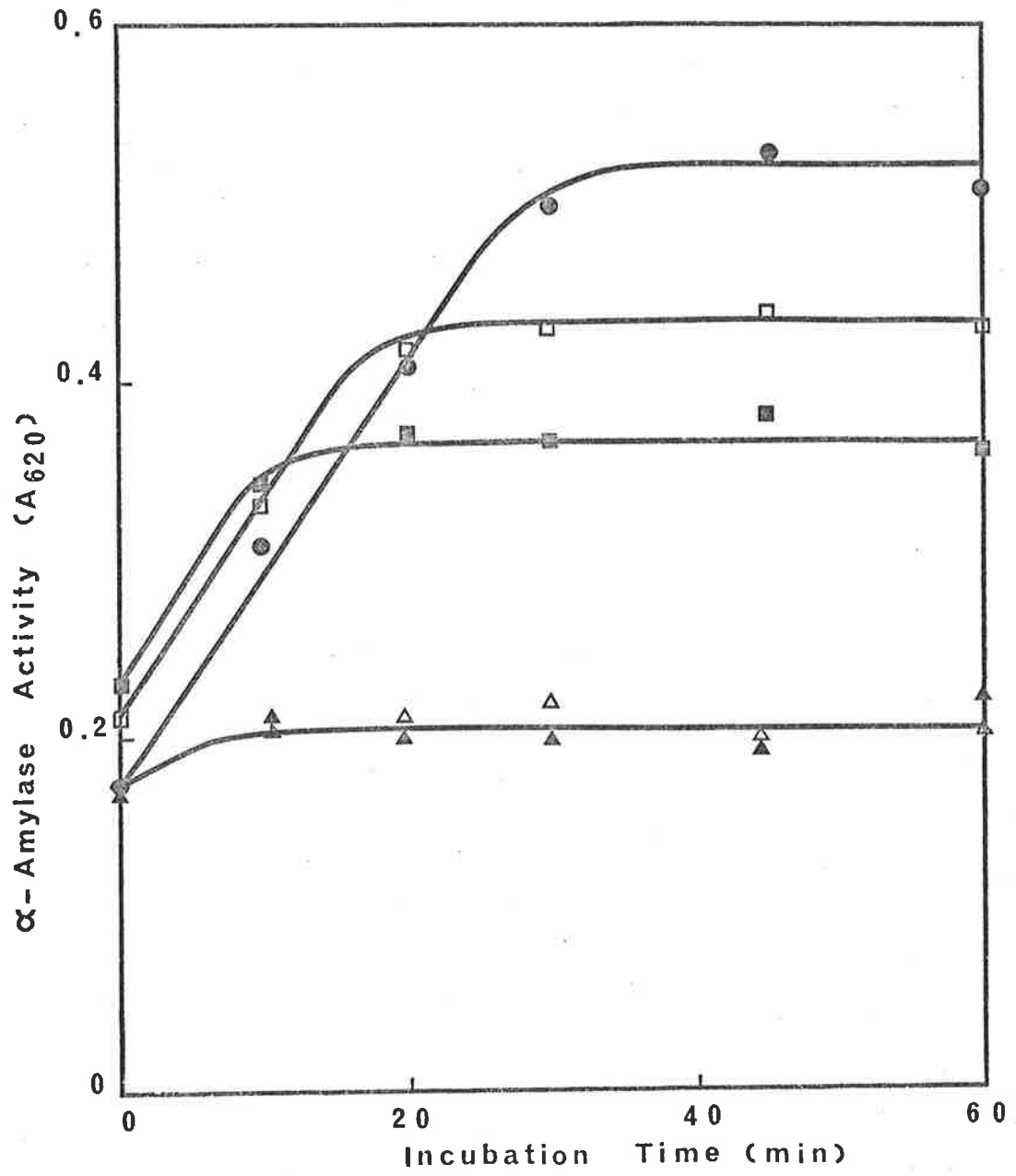


FIGURE 5.4 THE EFFECT OF RIBOSOME DETACHMENT AT 37°C
ON α -AMYLASE RELEASE IN THE PRESENCE OF
Mg²⁺.

Microsome suspensions were incubated at 37°C in STKC buffer (●) or STKC buffer containing KCl (□), KCl and puromycin (■), MgCl₂ and KCl (Δ) or MgCl₂, KCl and puromycin (▲). MgCl₂, KCl and puromycin were used at final concentrations of 5 mM, 750 mM and 1 mM respectively.

Fig. 5.4



make accurate estimates of bound ribosome populations almost impossible. Accurate estimates of free ribosome number are also difficult as the subunits dissociate and disintegrate in the absence of Mg^{2+} .)

The observation that ribosome detachment can occur without a release of enzyme implies that Mg^{2+} inhibits the outward transfer of enzyme from the microsomes in some way other than by stabilising ribosome binding. This does not necessarily mean, however, that the two processes are unrelated, as ribosome detachment may be a pre-requisite for enzyme release. If it had been possible to demonstrate that release occurred while ribosomes remained attached to the membrane it could be said that release and detachment were unrelated. However, such a demonstration has not been possible.

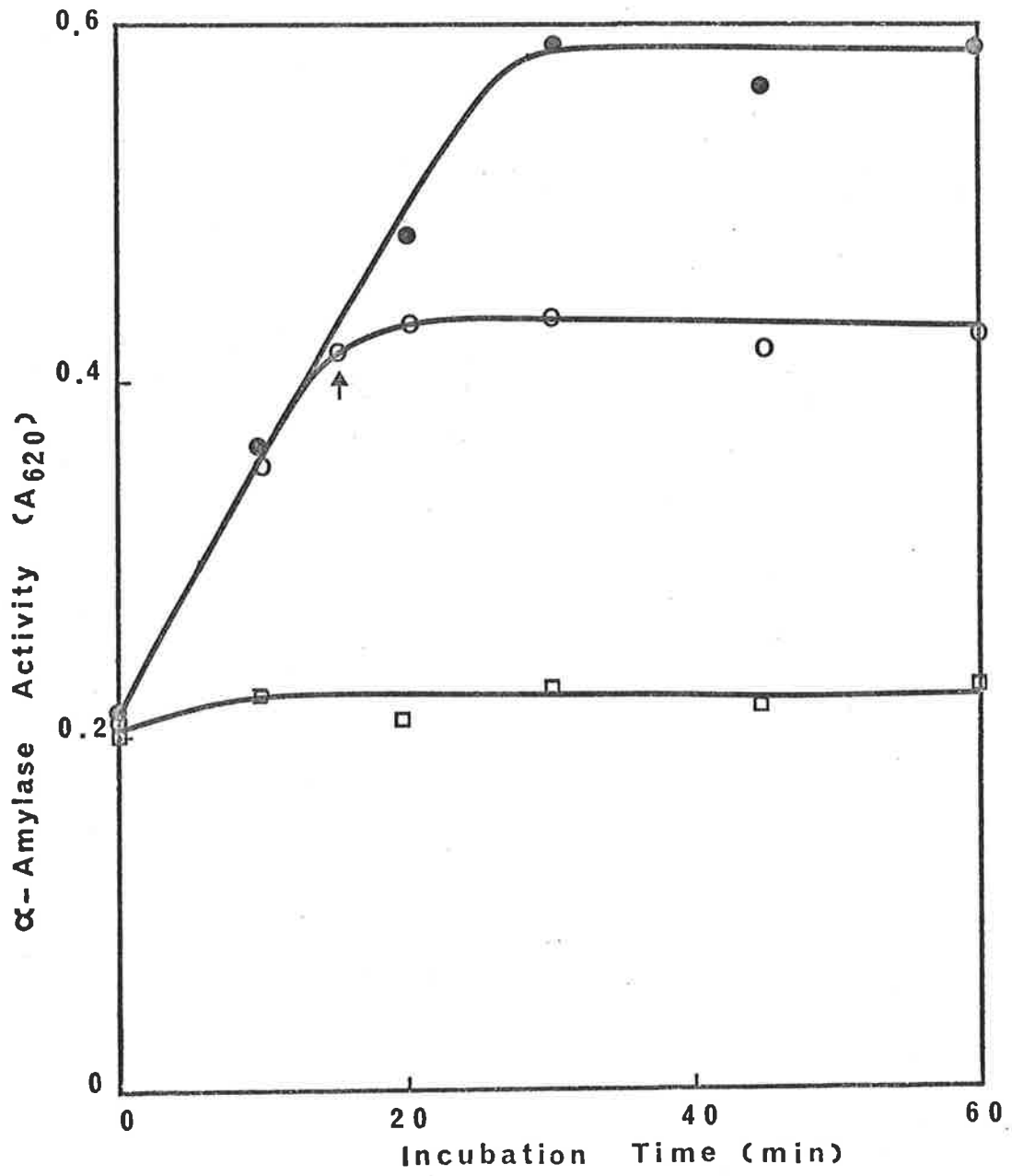
5.4 THE EFFECT OF ADDING Mg^{2+} DURING THE INCUBATION.

It seemed probable that stabilisation of ribosome binding was not the primary cause of the inhibitory effects of Mg^{2+} on the release of α -amylase. However, it was of interest to examine the effect of the metal ion on release when it was added after commencing the incubation. With this in mind, microsomes were incubated in STKC buffer for 15 min and the release process was established; then Mg^{2+} was added (final concentration, 5 mM) and the incubation continued for a further 45 min. The addition of Mg^{2+} to microsome suspension brought about a rapid and almost complete inhibition of enzyme release (Fig.5.5).

FIGURE 5.5 THE EFFECT OF Mg^{2+} ON α -AMYLASE RELEASE
WHEN ADDED DURING THE INCUBATION

Microsome suspensions were incubated at 37°C in STKC buffer (●) to which $MgCl_2$, at a final concentration of 5 mM, was added either before (□) or 15 min after (○) the incubation commenced. (The arrow indicates the time of Mg^{2+} addition).

Fig. 5.5



5.5 THE EFFECT OF Ca^{2+} ON ENZYME RELEASE.

The observation that Mg^{2+} inhibited the transfer of enzymes from microsomes raised the possibility that other metal ions would similarly inhibit release. This possibility was tested by incubating microsomes in STKC buffer containing different amounts of Ca^{2+} . The metal ion was tested at concentrations from 0.2 mM to 20 mM. The addition of Ca^{2+} at these concentrations had no effect on enzyme release (Fig.5.6) and demonstrates that the metal ion has no inhibitory effect on enzyme transfer. Shortage of time has not allowed an exhaustive study of metal ion effects on enzyme release.

5.6 A POSSIBLE MECHANISM FOR ENZYME RELEASE.

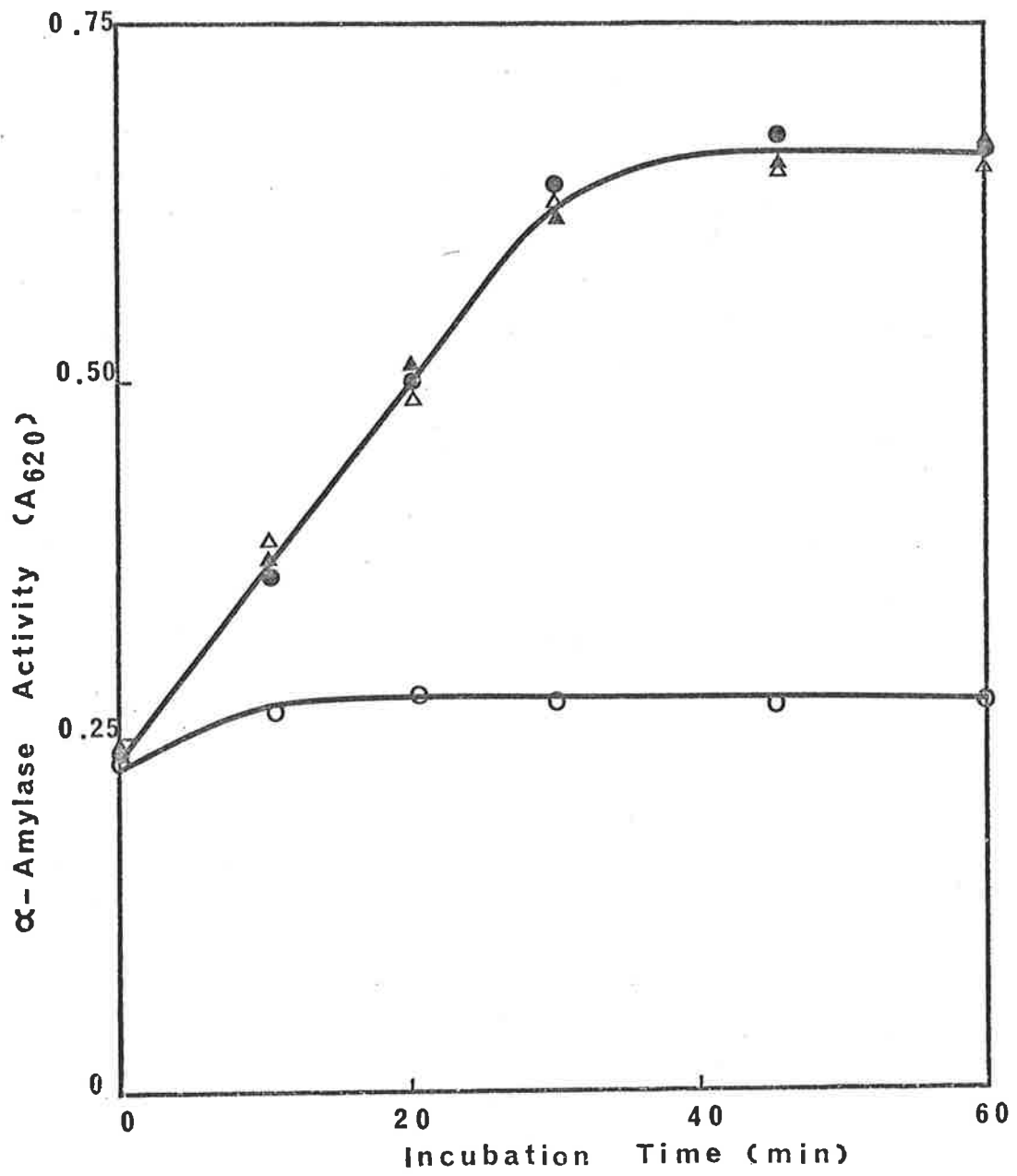
The transfer of enzyme to the external medium does not appear to have a trivial explanation such as non-specific leakage or lysis of the vesicles. Such explanations are difficult to reconcile with the observation that proteases inhibit release, especially when it is remembered that they exert their effect by virtue of their proteolytic ability (see Chapter 3). As discussed in the previous chapters, it seems reasonable to assume that the observed release involves the passage of proteins across membranes. If this is correct the question now arises as to how the process occurs.

At this stage speculative hypotheses are all that is possible as there is insufficient evidence to resolve this question. One possible explanation, which fits the

FIGURE 5.6 THE EFFECT OF Ca^{2+} ON α -AMYLASE RELEASE

Microsome suspensions were incubated at 37°C in STKC buffer, which contains 0.2 mM CaCl_2 (●) or STKC buffer containing added CaCl_2 at a final concentration of 2 mM (▲) or 20 mM (△). Microsomes were also incubated in STKC buffer containing MgCl_2 at a final concentration of 5 mM (○).

Fig. 5.6



data, is that a transport protein (a protein permease) in the membrane is responsible for transferring the protein across the membrane. This proposal, which conflicts with the signal hypothesis concept of concomitant synthesis and transport, would explain both the transfer of enzyme and the protease-sensitivity of the release. Full discussion of this transport protein proposal and its implications is reserved for Chapter 6, which will deal with the question of whether such an hypothesis can be reconciled with the established facts of the signal hypothesis.

The outward enzyme release observed in these experiments could be the result of an unphysiological activity of a transport protein normally involved in transferring proteins in an inward direction. If this were so, it might be possible to promote a transfer of enzyme into vesicles in vitro. Release of enzyme is down a concentration gradient whereas transport into microsomal vesicles would be against a concentration gradient and would require an energy source in some form.

The possibility of transfer into vesicles was tested by incubating microsomes for 30 min, to allow enzymes to be released, and then adding a creatine phosphate-creatine phosphokinase ATP-generating system (see Methods). Incubation was continued for a further two hours and α -amylase activity was measured during the 2.5 hr incubation period. There was no detectable difference in α -amylase levels between the control

microsomes and the microsomes to which the energy-generating system was added (Fig.5.7). A small steady decrease in the level of α -amylase activity was observed after the addition of the energy-generating system. The reason for this decrease is not known.

The fact that transport of α -amylase into the vesicles was not detected does not disprove the transport protein hypothesis as it may well be that inward transfer of proteins requires the presence of a leader sequence or, alternatively, there may be a requirement for bound ribosomes. It is also possible that transfer might not be detected using the method described above, as the intravesicular volume of the microsomes is very small in comparison to the bulk of the external medium. Further investigations using radioactive tracer enzymes are planned for the future.

As mentioned earlier, the transport protein proposal will be discussed fully in Chapter 6.

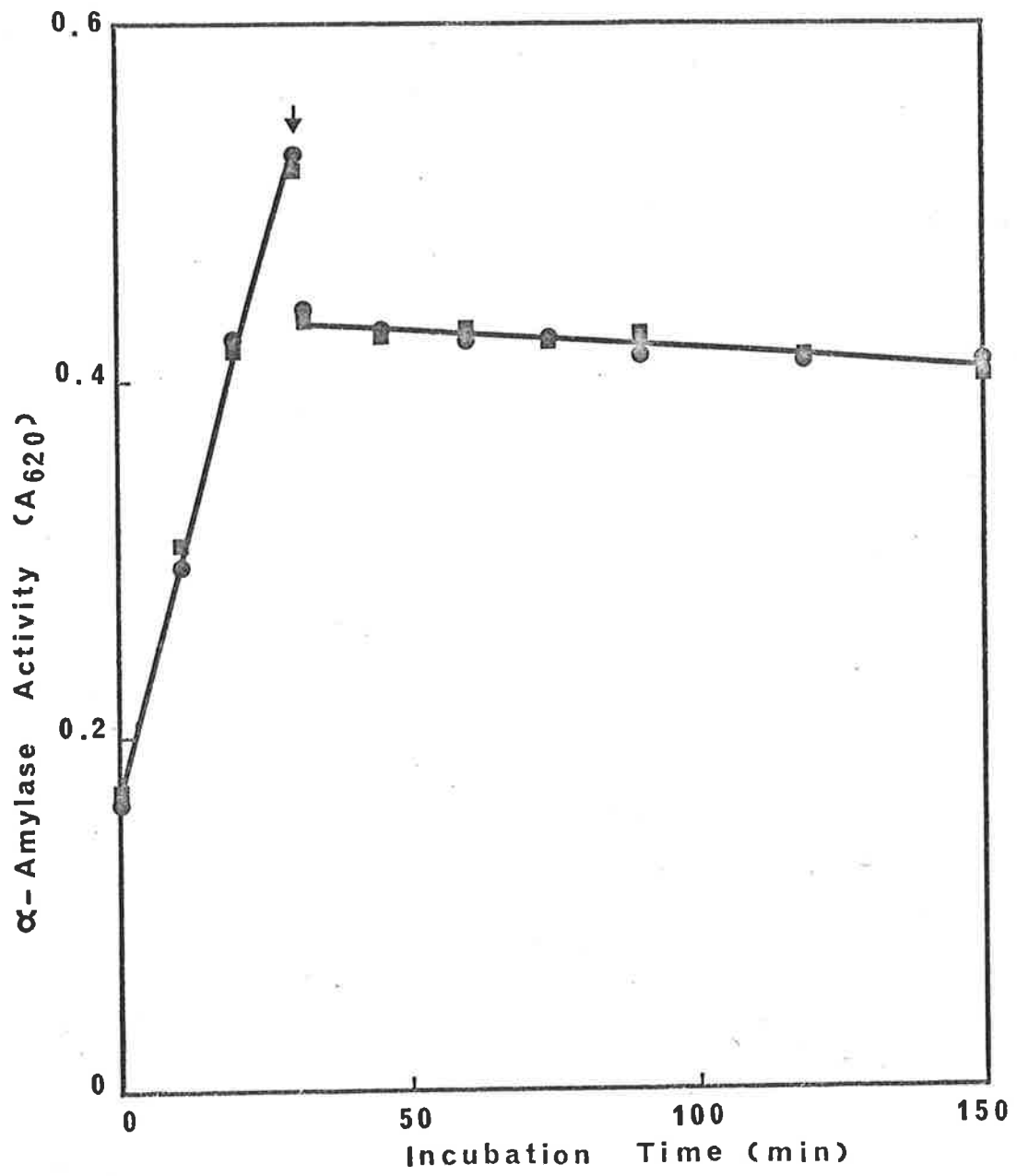
5.7 SUMMARY.

The experiments described in this chapter showed that Mg^{2+} , at concentrations in excess of 1 mM, almost completely abolished the release of α -amylase from microsomes. The addition of Mg^{2+} during the incubation led to a rapid and almost complete inhibition of enzyme release. There does not appear to be a direct relationship between the detachment of ribosomes and enzyme release, since ribosomes can be removed in the presence of Mg^{2+} without the

FIGURE 5.7 THE EFFECT OF THE ADDITION OF AN ENERGY
GENERATING SYSTEM ON α -AMYLASE LEVELS
IN AN INCUBATED MICROSOMAL SUSPENSION

Microsome suspensions were incubated at 37°C in STKC buffer (●) or in STKC buffer to which an energy generating system was added 30 min after commencing the incubation (■). The energy generating system consisted of creatine phosphate, creatine phosphokinase and ATP (see methods). (The arrow indicates the time of addition. The sharp drop in α -amylase levels on addition of the energy generating system is a dilution effect).

Fig. 5.7



occurrence of enzyme release. Ca^{2+} does not have the same inhibitory effect on release that Mg^{2+} does. The possibility that α -amylase could be transported into vesicles by active transport was investigated but transport was not detected.

CHAPTER SIX

GENERAL DISCUSSION

GENERAL DISCUSSION.

6.1 INTRODUCTION.

The experiments which have been described in this thesis show that a transfer of enzyme, apparently through the microsomal membrane to the external medium occurs when rat pancreatic microsomes are incubated. Important questions which need to be asked in relation to this process are:

- (i) Is there a trivial explanation for the observed enzyme release?
- (ii) What is the origin of the releasable enzyme?
- (iii) Assuming that the enzyme is located within the vesicle, how does enzyme cross the membrane?
- (iv) Does the process of release occur in vivo?
- (v) What, if any, relationship does this release have to protein secretion in vivo?

6.2 IS THERE A TRIVIAL EXPLANATION FOR RELEASE?

As mentioned in the introduction to Chapter 3, the fact that the level of α -amylase activity, measured using an insoluble substrate, increased when the microsomes were incubated was not particularly surprising since a number of trivial explanations could be invoked to account for the phenomenon. Such explanations include leakage of enzyme from within the vesicle as a result of damage to the membrane, desorption of enzyme non-specifically

adsorbed to the microsomal membrane, large scale lysis of the vesicles and de novo synthesis of enzymes.

A number of the experiments were designed to test these possibilities. The fact that cycloheximide had no effect on enzyme release indicated that de novo synthesis was not responsible for the increased α -amylase levels. An examination of electron micrographs of microsomes incubated for different times under different conditions, suggested that vesicle lysis did not occur on a large scale. It should also be noted that, since essentially the entire amount of α -amylase associated with the vesicle becomes soluble in the medium, then, virtually total lysis of vesicles would be required to account for this observation. Enzyme release was not observed at low temperatures which indicated that leakage of enzyme from the vesicles through "ordinary" physical holes in the membrane did not occur. The fact that both washed and unwashed microsomes released similar amounts of enzyme, and the observation that releasable RNAase was protected against digestion by subtilisin BPN' suggested that the releasable enzyme was not located on the cytoplasmic (outer) side of the membrane and hence desorption seems an unlikely explanation.

In addition to this direct evidence against trivial explanations is the fact that trypsin and chymotrypsin inhibited the release of enzymes by a process which did not result in inactivation of the enzymes either during or after their release. Furthermore, the proteases appeared to inhibit release by acting specifically as proteases. It

is difficult, therefore, to reconcile the explanations suggested above with the inhibitory effects of trypsin and chymotrypsin. Indeed, it might not be unreasonable to suggest that incubation in the presence of proteases would enhance such processes as leakage, desorption and lysis of microsomal vesicles.

The observation that release does not occur in the presence of Mg^{2+} also militates against the possibility that the observed increase in levels of enzyme activity has a trivial explanation such as leakage. However, this is not a strong argument since it could be envisaged that Mg^{2+} exerted a stabilising effect on the phospholipid bilayers.

6.3 WHAT IS THE ORIGIN OF THE RELEASED ENZYME?

As already indicated, the possibilities for the original location of the releasable enzyme include attachment to the cytoplasmic (outer) side of the membrane, burial within the membrane, attachment to the luminal (inner) side of the membrane, or it could be freely soluble as a part of the luminal contents of the vesicles. The possibility that the releasable enzyme is attached to the cytoplasmic surface of the membrane requires that RNAase is attached to the membrane in such a way that it is inactive, that it is resistant to proteolysis, and finally, that its release from the surface is prevented by proteolysis. This would apply regardless of whether the attachment was specific or non-specific. It is not apparent why attachment to the membrane should confer protection against proteolysis

and it therefore seems unlikely that the releasable enzyme comes from the outer surface of the membrane.

The usual criterion that a polypeptide is sequestered within a vesicle is protection of the polypeptide against proteolysis by exogenous proteases (Blobel and Dobberstein, 1975a). Such protection is usually regarded as evidence that the polypeptide is a part of the soluble lumen contents. However, as discussed earlier, burial of the protein within the lipid bilayer or attachment to the luminal surface of the membrane would confer protection equally well. It does not seem to be possible to unequivocally establish, either by sonication or by the use of low concentrations of detergents, that a protein is part of the luminal contents. With regard to sonication it could be argued that the high frequency vibrations dislodged loosely attached secretory proteins from the membrane, and it has been suggested that the low concentrations of detergents, used by Kreibich et al. (1973) to release luminal contents, could also remove loosely bound secretory proteins from the membrane (McIntosh and O'Toole, 1976).

If it can be assumed that secretory proteins, which might be loosely attached to the membrane, are not permanently dislodged by sonication, then the fact that α -amylase is found in the soluble fraction after sonication of microsomes, and the observation that releasable RNAase is protected from proteolysis by subtilisin BPN' suggest that the releasable enzymes are a part of the soluble contents of the lumen. This implies that the enzyme

crosses the vesicle membrane during release.

6.4 HOW DO THE ENZYMES CROSS THE MEMBRANE?

Trivial explanations for the observed release of enzyme have already been discussed and they do not seem to be appropriate to the phenomenon. Any explanation for the release must account for

(i) transport of the enzymes across the membrane

and

(ii) inhibition of the process by proteases.

At this stage there is insufficient evidence to resolve the question of how the enzymes cross the membrane and speculative hypotheses are all that is possible. However, one obvious possibility is to propose that the enzymes traverse the membrane by a process which is dependent on the presence of one or more membrane proteins readily destroyed by proteases i.e. that a carrier or transport protein is responsible for the transfer of the enzymes.

6.5 DOES RELEASE OCCUR IN VIVO?

It was observed in all of the experiments that the direction of release was outwards from the vesicle, whereas the process of secretion as envisaged by Palade (1975) requires transport in the opposite direction. When Mg^{2+} was included in the incubation medium at physiological concentrations, the release of enzyme was inhibited. This suggests that transfer of the enzyme outwards from the vesicle would not occur in vivo and does not conflict with

the broad outline of Palade's proposed secretory process.

6.6 IS THE OBSERVED RELEASE RELATED TO ENZYME SECRETION IN VIVO?

The speculative proposal that enzyme release is effected by a carrier or transport protein poses many problems. If release does not occur in vivo and if release is effected by a transport protein (or protein permease) the question which must be asked is whether the transport protein is involved in the process of secretion.

Assuming that the protein exists, and that it is involved in secretion, the possibility exists that the outward release observed in the experiments described in this thesis is the result of non-physiological activity of the transport system which, in vivo, is involved in the inward vectorial transport of completed proteins into the ER. A second possibility is that movement of proteins across the ER membrane is freely reversible as postulated by Rothman. Both of these possibilities will now be discussed.

In considering whether the observed release is the reverse of the normal secretory process in the pancreas, there is the clear implication that proteins are transported as finished proteins rather than by extrusion of the nascent peptide, as originally proposed by Redman and Sabatini (1966) and now incorporated into the signal hypothesis. The evidence for the signal hypothesis is unequivocal in

several important aspects, namely, that pancreatic and most other secretory protein mRNA's translated in vitro have a "signal" peptide at the NH₂-terminal, and that segregation and cleavage of the translation product only occurs in vitro if membranes are present during translation. Therefore, if the release phenomenon is to be considered as being related to secretion, the question which must be answered is whether transport of completed proteins can be incorporated into the signal hypothesis in place of nascent peptide extrusion.

This is not impossible because there is no direct evidence which proves that nascent peptide is extruded through the membrane in pancreatic microsomes. It may be that the hydrophobic "signal peptide" functions only as a signal that a secretory protein is being synthesised and not as the "leader sequence" which guides the nascent polypeptide through the membrane. If this were so, it could be envisaged that the "signal peptide" interacts with the transport protein in such a way that binding of ribosomes to the membrane is promoted. When the ribosome binds to the membrane it does so in such a way that the large subunit is immediately adjacent to (or possibly directly above) the transport protein. This allows the nascent peptide to remain in close association with the transport protein during synthesis. The transfer of the protein across the membrane would occur after the completion of synthesis and could, perhaps, be initiated by detachment of the ribosomes from the membrane.

Although the sequence of events outlined is highly speculative it seems appropriate to examine it in light of our current knowledge of the secretion process. A number of experiments described in Chapter 1 showed that segregation and cleavage of secretory proteins occurred only when vesicles were incubated cotranslationally in vitro. Posttranslational incubation of vesicles with pre-proteins results in neither segregation nor cleavage. This has been interpreted as evidence that synthesis and transport occur concomitantly. It should be pointed out, however, that signal sequences are usually hydrophobic and that they are, therefore, likely to be buried within the completed pre-protein molecule. If the NH₂-terminal extension was the signal for the transport protein, and the peptide was not fully accessible or was in the wrong conformation, segregation might not be expected to occur. (Blobel and Dobberstein (1975b) suggested that burial of the signal in a completed protein could explain the observation that cleavage does not occur posttranslationally. However, a recent report by Jackson and Blobel (1977) provides evidence that the peptidase is located on the luminal side of the vesicle membrane.)

In this regard the experiments of Highfield and Ellis, described in Chapter 1, are interesting. They showed that the precursor of the small subunit of ribulose biphosphate carboxylase was transferred into intact chloroplasts without concomitant protein synthesis. Only processed enzyme was found in the chloroplasts. They proposed that the transfer was effected by a carrier in the

chloroplast envelope. The NH_2 -terminal peptide of the precursor appeared to be very acidic and, therefore, hydrophilic. It might, therefore, be expected to be on, or very close to, the surface of the completed pre-protein where it could act as a signal and interact with its carrier in the envelope.

Much of the evidence for vectorial extrusion of nascent peptides in eukaryotic cells comes from the type of experiment where it is shown that the nascent polypeptide is protected from proteolysis by exogenous proteases e.g. Blobel and Sabatini (1970). However, if the bulk of putative transport protein and its associated nascent polypeptide were located deep in the membrane directly beneath, or very close to, the large subunit, protection might be conferred on the nascent polypeptide.

Both the signal hypothesis and the carrier protein proposal envisage that the role of the NH_2 -terminal extension is to

- (i) act as a signal that a secretory protein is being synthesised and
- (ii) interact with a membrane protein(s) so as to ensure binding of the ribosome to the membrane.

Once binding has occurred it could be maintained by interactions between membrane proteins and the large subunit, and/or, interactions between the nascent peptide and the membrane. (As mentioned in Chapter 1, there is evidence that both types of interaction occur). There may not be any further requirement for the signal sequence once ribosome

binding has occurred.

The timing of the cleavage of the signal appears to be something of an open question. The signal hypothesis proposes that cleavage occurs during synthesis based on two observations. Firstly, only processed proteins were found to be associated with vesicles and secondly, detached polysomes from murine myeloma synthesised a mixture of processed and unprocessed molecules in an in vitro system (Blobel and Dobberstein, 1975a,b). The latter observation, in particular, suggests cleavage occurs during synthesis. However, Habener et al. (1976) reported that pre-proparathyroid hormone could be detected in intact parathyroid cells. The pre-protein was converted to parathyroid hormone within minutes suggesting that pre-proteins could be synthesised in vivo and that posttranslational cleavage is possible. Similarly, Patzelt et al. (1978) have reported that pre-proinsulin can be detected in intact cells. They found that most of the pre-protein was rapidly converted to proinsulin but some appeared to escape processing altogether.

Patzelt et al. suggested that pre-proparathyroid hormone (115 residues) and pre-proinsulin (109 residues) were observed in the intact cells because the polypeptide chain was not long enough to be cleaved during synthesis. In this regard it should be noted that precursors of larger secretory proteins, e.g. growth hormone (MW 20,000), have not been detected in intact cells (Schmeckpeper et al. 1975,

Sussman et al. 1976, Boime et al. 1977).

An attractive proposition, with regard to the timing of cleavage, is that hydrolysis occurs as the polypeptide is carried across the membrane. Cleavage at this time might ensure the release of the completed polypeptide from its transport protein into the lumen of the endoplasmic reticulum. The retention of the signal until after completion of synthesis would also mean that both ends of the nascent chain are firmly anchored, and, therefore, are not free to drift into the cytoplasm.

In order to explain the observed release of enzyme it must be assumed that the transport protein is capable of transferring processed α -amylase, since the released enzyme appears to be identical to the zymogen granule enzyme.

The possibility that a transport protein existed was tested by incubating microsomes in the presence of released α -amylase and an energy source, and then looking for an uptake of enzyme (Chapter 5). This was not detected and it may well be that inward transport occurs only if the protein has an accessible signal sequence so as to allow recognition by the putative transport protein. (It is possible that the only time a hydrophobic signal is accessible is during the early stages of synthesis.) Further investigations are being carried out, using radioactively labelled enzymes, in an attempt to detect inward transfer of enzymes into vesicles.

Assuming that a transport protein exists, and that it is responsible for the observed enzyme release, it seems possible that the transfer of enzyme in an outward direction could be achieved by facilitated diffusion. Such a process would not be energy dependent as transport would be down a concentration gradient i.e. from the relatively small volume of the lumen to the bulk of the external medium. It is possible that the transport protein is the membrane ribosome receptor complex.

The release of enzyme, reported here, is difficult to relate to S.S. Rothman's proposal that proteins can reversibly and freely cross the ER membranes. The fact that Mg^{2+} , at physiological concentrations, almost completely inhibits release suggests that the phenomenon does not occur in vivo. If this is so, the transfer of enzyme observed here does not seem to be related to the transport observed by Rothman (1975).

Clearly this discussion is extremely speculative, since there is no direct evidence that enzyme release is related to the "signal mechanism" for enzyme secretion. One piece of evidence which is difficult to reconcile with the concept of transport of completed proteins is the data of Smith et al. (1977) who showed that E. coli alkaline phosphatase was extruded through the membrane during synthesis. However, it may be that the mechanism of secretion differs in bacteria. In this regard it should be mentioned that Lampen (1978) has suggested that the mRNA for B. licheniformis has an affinity for the membrane (see Chapter 1). (This proposal is not unlike proposals

that the poly(A) tract at the 3' end of eukaryotic mRNA is important in specifying translation by bound polysomes, Milcarek and Penman (1974) and Cardelli et al. (1976).

6.7 CONCLUSION.

There is increasing evidence that more than one mechanism exists whereby proteins can cross membranes e.g. ovalbumin in the chick oviduct has no transient signal peptide (Palmiter et al. 1978), a chloroplast subunit enzyme is transported without concomitant protein synthesis (Highfield and Ellis, 1978) and the nascent peptide of E. coli alkaline phosphatase appears to be extruded vectorially through the membrane (Smith et al. 1977). In the case of the pancreatic microsomal membrane a considerable body of evidence has been established which shows that there is an intimate relationship between synthesis and secretion. However, as yet, there is no direct evidence which proves conclusively that the nascent peptide is extruded through the membrane.

Much work is required to establish whether the enzyme release, observed in the experiments described in this thesis, is related to the physiological transfer of proteins across membranes. In this regard, further investigations by other workers in this department are proceeding.

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