THE RAT PANCREATIC MICROSOME ENZYME RELEASE PHENOMENON

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ABBREVIATIONS

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STATEMENT

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. The experiments were performed only by myself and to the best of my knowledge this thesis contains no material previously published or written by another person except where due reference is made in the text.

(LINDA TABE)

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Dad, Mum and Ferne
for their unwavering confidence,
support and thoughtfulness.

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SUMMARY

THE RAT PANCREATIC MICROSOME ENZYME RELEASE PHENOMENON

It has been reported that active secretory enzymes are released into the suspending medium when rat pancreatic microsomes are incubated at 37°C in cation-depleted medium. The enzyme release process, which seemed to involve the transfer of fully-formed proteins across intact microsomal membranes, was inhibited by incubation of the vesicles in the presence of active proteases (Pearce *et al.*, 1978). The work described in this thesis was directed towards defining the possible relationship between the pancreatic microsome enzyme release phenomenon and the physiological process of secretory protein translocation across the ER membrane *in vivo*.

1. It was demonstrated that intra-microsomal amylase was protected from attack by added subtilisin, although amylase released from the vesicles by incubation at 37°C was readily degraded by the protease. Similarly, intra-microsomal amylase was inaccessible to membrane surface-specific labelling with ^{125}I, whereas released, extra-vesicular enzyme was strongly labelled.

Brief incubation of microsomes in the presence of a protease was found to retard the subsequent release of amylase from the vesicles when they were incubated at 37°C, after their removal from contact with the hydrolase. These results supported the previous suggestions (Pearce *et al.*, 1978), that the enzyme release phenomenon involved the passage of secretory proteins across intact microsomal

membranes in a manner dependent on the integrity of a membrane-associated protein.

2. Amylase release from rat pancreatic microsomes was inhibited by several protease inhibitors which have been reported to inhibit the activity of the signal peptidase enzyme. Inhibitors which did not affect signal peptidase had no inhibitory effect on amylase release.

3. The amylase release process was blocked by denatured ovalbumin and denatured or native BSA, but not by other mature, processed secretory proteins, or by the denatured or native forms of a cytoplasmic protein. The denatured form of catalase, an enzyme which *in vivo*, is transported across the peroxisomal membrane without proteolytic processing, also inhibited the release of amylase from rat pancreatic microsomes.

4. The treatment of rat microsomes with protease and/or high salt was found to inhibit the amylase release phenomenon. This inhibition was partly reversed by the re-addition of the salt extract to the salt-washed membranes. The active constituent of the extract could be destroyed either by proteolysis or by treatment with NEM.

5. The release of amylase from rat pancreatic microsomes was found to be synchronized with the disintegration of the ribosomes in the vesicle suspensions, during incubation at 37°C in the absence of Mg^{2+}. An increase in the sensitivity of the amylase release process to various inhibitors correlated temporally with the complete disappearance of the membrane-bound ribosomes.

6. The similarities between the experimentally-determined properties of the rat pancreatic microsome enzyme release phenomenon, and the published properties of the transport of secretory proteins into the ER *in vivo* are discussed in the light of current theories of trans-membrane protein translocation.

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Biological membranes are the vitally important barriers which separate specialized intra-cellular compartments and maintain the integrity of living cells in the face of hostile external environments. These complex structures are permeable to a selected few of the molecules on either side of the barrier, thus allowing the controlled passage of various substances between compartments, or into or out of the cell. The way in which this traffic is monitored is just beginning to be understood.

Perhaps the process most difficult to imagine is the passage across the hydrophobic lipid bilayer of many large, water-soluble, globular proteins. Nonetheless, the transmembrane movement of proteins; sometimes the very efficient transfer of large quantities of protein, is known to occur in numerous situations.

The enormous pool of data, which has accumulated in the past decade, on the trans-membrane translocation of proteins, indicates that a significant proportion of the information specifying the potential of a particular protein for transport across a particular membrane resides within the primary and secondary structure of the protein itself. Except in the case of biosynthetic transfer, where ribosomes are involved, the complement of the information specifying protein transport generally resides within the membrane, for example, in the form of membrane-integrated receptor/translocator proteins. Therefore, simplistically, when a transport-competent protein

interacts with the correct membrane, translocation of the protein across, or into the lipid bilayer will ensue.

The work presented in this thesis involves the investigation of the *in vitro* trans-membrane movement of proteins, by an unknown mechanism. In order to provide a frame of reference in which the possible physiological significance of this work could be assessed, a detailed review of current information on various, cellular, protein translocation mechanisms was deemed necessary. It was felt that the length of this discussion was warranted by the recent, rapid progress in this field, and by the lack of a current review dealing with the mechanistic details of trans-membrane protein translocation. (Since this chapter was written, a review of the "Mechanisms for the Incorporation of Proteins in Membranes and Organelles" has been published by Sabatini *et al.*, (1982)).

1.2 TRANSFER OF TOXIN MOLECULES ACROSS MEMBRANES

One example of "non-biosynthetic" protein translocation across membranes is the entry, into sensitive cells, of toxin molecules. Toxins produced by a number of bacteria, and some plants, must cross the cell membrane and gain access to the cytoplasm in order to produce their cytotoxic effects. Most known toxins have a similar mode of entry, as exemplified by the well-characterized diphtheria toxin (Pappenheimer, 1977).

This toxin is secreted by *Corynebacterium diphtheriae* as a single polypeptide chain of molecular weight 62,000

daltons, which is subsequently split, by specific proteolytic cleavage and thiol reduction, into an A fragment (M_r 21,000) and a B fragment (M_r 40,000). The A fragment contains the enzyme activity which catalyses the ADP-ribosylation of elongation factor 2, thereby halting protein synthesis in the intoxicated cell, but fragment A alone is non-toxic to intact cells. Fragment B enables the toxin molecule to bind to the surface of sensitive cells and subsequently ensures that fragment A reaches the cytoplasm.

A recent model of diphtheria toxin penetration predicts the following sequence of events (Kagan *et al.*, 1981). The uncleaved toxin molecule binds to a surface receptor on the plasma membrane of a sensitive cell, then the receptor-toxin complex is internalized by endocytosis and delivered to an intra-cellular lysosome where the cleavage between fragment A and fragment B occurs. At the low, intra-lysosomal pH, fragment B spontaneously integrates into the lysosomal membrane. A pore is thereby formed in the lipid bilayer, through which fragment A is thought to pass, possibly in the form of an extended polypeptide chain, which refolds on entering the neutral environment of the cell cytosol. It is suggested that the pH gradient across the lysosomal membrane may provide the driving force for the translocation of fragment A through the channel (Kagan *et al.*, 1981).

In the case of diphtheria toxin, "membrane information" in the form of cell surface receptors is required for the toxin molecule to be delivered to the lysosome, where

environmental factors trigger the integration of fragment B into the lysosomal membrane. Once proteolysis and reduction have occurred, in an environment of low pH, membrane integration is specified solely by the specialized structure of fragment B, ultimately leading to the complete transmembrane translocation of fragment A.

The more complex cholera toxin consists of five 'B' subunits and a disulphide-linked dimer of 'A' subunits, one of which penetrates to the inner lipid layer of the cell membrane, where it stimulates membrane-associated adenylate cyclase. At an unknown stage of this process, reduction of the disulphide bond between the two A subunits is thought to occur (Waksman *et al.*, 1980, Wisnieski and Bramhall, 1981). In both of the described instances of toxin penetration, initial contact with the appropriate membrane is dependent on membrane-integrated receptors on the cell surface, while subsequent penetration of the lipid bilayer results from the action of environmental factors on the specially-structured toxin proteins. It is assumed that a significant change in conformation of the appropriate subunits accompanies penetration of the membrane, as previously water-soluble, globular proteins must enter an extremely hydrophobic milieu. In the case of subunit A of diphtheria toxin, the protein may have to completely unfold in order to pass through the membrane, although, as yet, there is no experimental evidence for this mechanism (Kagan *et al.*, 1981).

1.3 VECTORIAL TRANSFER OF PROTEINS INTO MITOCHONDRIA

The bulk of mitochondrial proteins are encoded by nuclear genes and translated, as precursors, on free polysomes in the cell cytoplasm (Schatz, 1979). It has been demonstrated conclusively that the transfer of these precursors from the cytosol, to their correct locations in the mitochondria, is a post-translational event (most refs. cited in this section). The import of mitochondrial proteins thus poses a problem similar to that posed by the entry of toxins into cells; a globular, water-soluble protein must cross one, or two, membrane barriers in order to assume its physiological role.

The sequence of events that leads to the eventual, correct localization of mitochondrial proteins is now well established. Translation of the mRNA occurs on free polysomes giving rise to a cytoplasmic pool of precursor proteins which is detectable by *in vivo* pulse-labelling (Mori *et al.*, 1981, Maccecchini *et al.*, 1979, Mihara and Blobel, 1980, Nelson and Schatz, 1979, Raymond and Shore, 1979, 1981, Brambl, 1980). The precursors move from the cytoplasm, where they are relatively stable, into the mitochondria with highly variable rates, ranging from a cytoplasmic half-life of two minutes for carbamyl phosphate synthase (Raymond and Shore, 1981) and ornithine transcarbamoylase (Mori *et al.*, 1981) to indefinite storage in the cytoplasm in the case of subunits IV to VII of cytochrome c oxidase, in dormant fungal spores (Brambl, 1980).

Different precursor proteins are directed to different micro-environments within the mitochondrion. Cytochrome c peroxidase and apo-cytochrome c reside in the inter-membrane space, the latter being ionically associated with the external surface of the inner membrane (Maccecchini *et al.*, 1979a, Zimmerman *et al.*, 1981). These proteins are translocated across the outer mitochondrial membrane only. Subunits of cytochrome c oxidase (Mihara and Blobel, 1980), and the ADP/ATP carrier protein (Zimmermann and Neupert, 1980), are translocated across the outer membrane, then inserted into the inner membrane where they exist as integral membrane proteins, while ornithine transcarbamoylase (Mori *et al.*, 1981), carbamyl phosphate synthase (Raymond and Shore, 1979) and aspartate aminotransferase (Sonderegger *et al.*, 1980) are translocated completely across both membranes, to a final destination, free in the mitochondrial matrix.

The transport of these large, globular proteins through the mitochondrial envelope is accompanied by structural and conformational changes, generally including the proteolytic removal of a 'signal sequence' of M_r 2,000 to 6,000 daltons. An extension of the well-known 'Signal Hypothesis', which describes the co-translational translocation of secretory proteins into the ER, has been proposed as a model for the molecular mechanism of mitochondrial protein import (Fig. 1.1 and Blobel, 1980). Salient features of the model include the recognition of integral membrane receptor proteins, in the outer mito-

FIGURE 1.1

The Signal Hypothesis

Schematic models for:

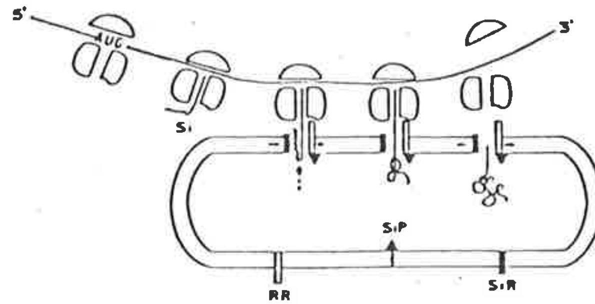
1. (top) co-translational translocation of proteins across one membrane; eg. transfer of secretory proteins into ER.

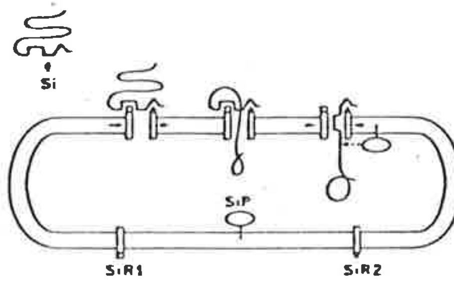
2. (middle) post-translational translocation of proteins across one membrane; eg. transfer of proteins into peroxisomes.

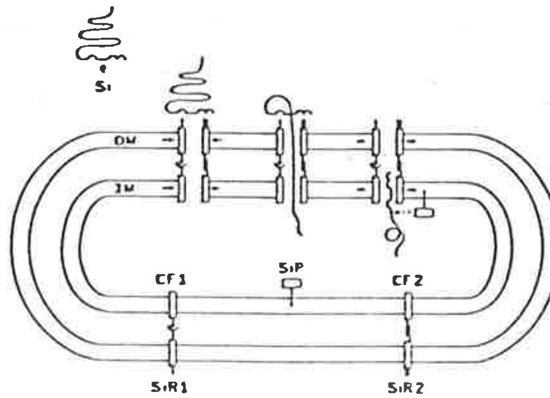
3. (bottom) post-translational translocation of proteins into mitochondria.

SiR = signal receptor, RR = ribosome receptor, CF = coupling factor, SiP = signal peptidase, Si = signal sequence, OM = outer membrane, IM = inner membrane.

Taken from Blobel (1980).







1. The Signal Hypothesis

chondrial membrane, by two distinct domains in the signal sequence of a precursor. This binding to the organelle surface triggers the formation of a hydrophilic pore in the outer membrane, which, in the case of the import of inner membrane proteins and matrix proteins, is contiguous with a similar pore in the inner mitochondrial membrane (see Fig. 1.1).

After pore formation, the protein passes through the discontinuity in the membrane(s) to its specified destination, with or without concomitant proteolytic processing, and the pore disaggregates, restoring the integrity of the envelope. It is proposed, by Blobel, that three different types of 'signal' exist within the imported protein structure to direct this process. Proteins of the inter-membrane space carry a signal addressed to a pore which breaches only the outer mitochondrial membrane. Soluble matrix proteins carry a signal addressed to a different pore which forms a contiguous tunnel through both inner and outer membranes and the inter-membrane space. Integral proteins of the inner membrane carry a similar signal which initiates transport through the same tunnel. Transfer of these proteins is aborted, as they cross the inner membrane, by an internal 'stop-transfer' sequence, which causes disaggregation of the pore, leaving the proteins inserted in the lipid bilayer.

Experimental evidence supports some aspects of this model. The existence of transient signal sequences in mitochondrial proteins is well documented, however little

is known about their primary structure or their location within the total amino acid sequence of the protein, although they are often presumed to be N-terminal (Neupert and Schatz, 1981). The fallibility of this presumption is illustrated by the finding of a possible, carboxy-terminal, uncleaved signal sequence in apo-cytochrome c (Matsuura *et al.*, 1981). The involvement, in protein import, of externally-exposed, integral membrane proteins of the mitochondrial envelope is indicated by the finding that *in vitro* uptake of apo-cytochrome c is prevented by mild trypsinization of isolated rat liver mitochondria (Matsuura *et al.*, 1981).

The entry of apo-cytochrome c into mitochondria is not accompanied by proteolytic cleavage, therefore, the apo-enzyme, which can be obtained in large amounts from the intra-mitochondrial holo-enzyme by the chemical removal of heme, is functionally and structurally equivalent to the cytoplasmic precursor form of the protein. Competition experiments in which excess, unlabelled apo-cytochrome c prevented the uptake, by isolated mitochondria, of *in vitro*-synthesized, labelled cytochrome c, support the idea that the enzyme is imported via a limited number of receptor channels in the outer membrane. Similar experiments showed that a 10,000-fold excess of apo-cytochrome c, relative to precursor, did not inhibit the internalization, by mitochondria, of *in vitro*-synthesized precursors of ADP/ATP carrier protein or of subunit 9 of the oligomycin-sensitive ATPase (Zimmermann *et al.*, 1981). Both these proteins are inserted

into the inner mitochondrial membrane; without proteolytic processing in the case of the adenine nucleotide transporter, and with proteolytic removal of a signal sequence in the case of the ATPase subunit. This provides strong support for the contention that there are separate receptors in the outer membrane for pre-proteins bound for different intra-mitochondrial destinations.

These results also illustrate the different types of covalent modification, and associated conformational changes, that can accompany protein translocation. These modifications may supply at least some of the energy required for translocation, while ensuring that transport is irreversible (Schatz, 1979, Neupert and Schatz, 1981). The differences in conformation between cytoplasmic and mitochondrial forms of the same protein are probably minimal in the case of the matrix enzymes, where both forms are water-soluble and differ only in the presence or absence of a relatively short signal sequence, and in terms of oligomerization. For example, precursor and mature ornithine transcarbamoylase (OTCase) have been shown to give rise to similar peptides, on partial proteolysis, as well as being immunologically closely-related (Conboy and Rosenberg, 1981). There are, however, differences in the ways in which mature and precursor OTCase interact with ionic detergents (Kraus *et al*, 1981). It has been demonstrated by the same workers, that mature OTCase does not compete with pre-OTCase for uptake by isolated mitochondria, confirming the importance of the signal sequence in translocation.

The conformational differences between cytoplasmic apo-cytochrome c and holo-cytochrome c of the intermembrane space, are presumably conferred by the binding of the heme prosthetic group and attachment to the surface of the inner mitochondrial membrane. It has been reported that holo-cytochrome c will not compete with apo-cytochrome c for uptake by mitochondria, proving that the receptor protein in the mitochondrial envelope can distinguish between the two forms (Korb and Neupert, 1978). Antigenic differences were also found between the two proteins, implying conformational dissimilarities, as the amino acid sequence is the same in both, due to the lack of proteolytic processing.

Perhaps the most obvious differences in conformation exist between the water-soluble cytoplasmic precursors and the mature, membrane-integrated forms of the mitochondrial inner membrane proteins. A recent study compared the *in vitro*-synthesized, water-soluble form of the ADP/ATP carrier protein with the corresponding inner membrane protein, which is inserted without proteolytic cleavage. Not surprisingly, it was found that the protein was highly hydrophobic in both forms, and that the cytoplasmic form was only able to exist in aqueous solution by forming oligomers of M_r 120,000 daltons (tetramer) or large aggregates exceeding M_r 500,000 daltons (Zimmermann and Neupert, 1980). Subunit V of cytochrome c oxidase is also thought to form aggregates in the cytoplasm (Neupert & Schatz, 1981). These observations would seem to indicate that the

conformational differences between the intra-mitochondrial and extra-mitochondrial forms of the inner membrane proteins may not be as drastic as previously supposed. That some differences do exist, however, is indicated by the fact that the cytoplasmic, but not the intra-mitochondrial form of the ADP/ATP carrier, will bind to hydroxyapatite in the presence of triton X-100 (Zimmermann and Neupert, 1980).

In summary, a large number of water-soluble, globular proteins are imported post-translationally into mitochondria. It has been deemed necessary to invoke a significant difference in conformation between intra- and extra-mitochondrial forms of the same protein in order to explain the unidirectionality of vectorial transfer. Why this should be so is difficult to see, however, if the signal receptors specifying transport are only present on the outside of the envelope, as suggested by Blobel's modified signal hypothesis (Fig. 1.1, Blobel, 1980). Conformational changes in the transported protein may contribute to the energy required for protein translocation, but energy in the form of ATP or a proton gradient is also required, at least for translocation across the mitochondrial inner membrane (Nelson and Schatz, 1979; Zimmermann *et al.*, 1981). Changes in conformation obviously do accompany protein transport and may be brought about by proteolytic processing, insertion into a lipid bilayer, binding of prosthetic groups, oligomerization, or combinations of these; however, the very fact that cytoplasmic precursors are identified by

cross-reaction with antibodies raised to the mature, mitochondrial proteins must indicate that the two structures are closely related.

The model illustrated in Figure 1.1 implies that the proteins unfold during translocation so that they pass through the membrane as extended chains of amino acids. As yet, there has been no experimental evidence to support such a mechanism, but superficially, it seems energetically wasteful for the covalent, ionic and hydrophobic interactions maintaining the secondary structure of a protein to be formed, then disrupted and re-formed during trans-membrane transport. Alternatively, the existence of similar, folded structures on both sides of the membrane suggests that the globular conformation of a protein may be preserved during its energy-dependent transport across the mitochondrial envelope via an integral membrane receptor/translocator protein.

1.4 VECTORIAL TRANSFER OF PROTEINS INTO OTHER ORGANELLES

Post-translational import of cytoplasmically-synthesized, water-soluble proteins into chloroplasts, peroxisomes and glyoxysomes occurs in a manner very similar to the uptake of proteins by mitochondria. Proteins destined for an intra-lysosomal location will not be considered here as they are firstly translocated into the ER, in the same way as secretory proteins, which are discussed in section 1.6.

Several of the proteins involved in photosynthesis can be synthesized *in vitro* as larger precursors which

are taken up by chloroplasts, in the absence of protein synthesis, seemingly via an envelope carrier protein (Highfield and Ellis, 1978; Apel and Kloppstech, 1978; Nelson *et al.*, 1980; Schmidt *et al.*, 1981). Transfer is ATP-dependent and, in the case of the small subunit of ribulose bisphosphate carboxylase, proteolytic removal of the large, acidic, N-terminal signal sequence is thought to be accompanied by a conformational change in the protein subunit,which may contribute to the mechanism of translocation (Grossman *et al.*, 1980; Highfield and Ellis, 1978). In all known instances of protein import by chloroplasts, uptake is closely followed by covalent modification of the precursor by proteolytic removal of a signal sequence, by a soluble, stromal peptidase (Dobberstein *et al.*, 1977).

Similarly, two peroxisomal enzymes of rat liver, uricase and catalase are synthesized in the cell cytoplasm, on free polysomes, giving soluble, globular products which must be transported post-translationally into peroxisomes, presumably via a membrane receptor/ translocator protein (Goldman and Blobel, 1978). It has been demonstrated that the primary translation products of catalase and uricase mRNAs have the same molecular weights as the mature forms of the enzymes (Robbi and Lazarow, 1982), so it seems that catalase and uricase may cross the peroxisomal membrane with little or no structural modification. Unlike secretory proteins, nascent catalase and uricase are not transported into microsomes, *in vitro*, which is interesting in view of

the fact that peroxisomes are formed as budding outgrowths of the ER. Presumably receptor/translocator proteins which recognise completed uricase and catalase, or some uncleaved, signal region within their structures, is active in the peroxisomal membrane but not in the ER membrane.

Glyoxysomal proteins of plants are also imported via a post-translational, cytosolic route, with proteolytic processing in the case of malate dehydrogenase (Walk and Hock, 1978), and without processing in the case of malate synthase (Kruse *et al.*, 1981). The covalent modification, by proteolysis, of malate dehydrogenase might be expected to cause conformational differences between the intra- and extra-glyoxysomal forms of the enzyme, however a mono-specific antibody against mature, glyoxysomal malate dehydrogenase (MDH) precipitates pre-MDH while failing to interact with the mature MDH isozymes of the cytosol and of mitochondria (Walk and Hock, 1978). Thus the mature and precursor MDH must demonstrate considerable structural similarity, indicating that passage across the glyoxysomal membrane may involve little change in conformation.

The internalization of malate synthase does not involve proteolytic cleavage but conformational changes might be expected to result from the insertion of the water-soluble precursor into the glyoxysomal membrane, malate synthase being an integral membrane protein. In comparison to the mitochondrial ADP/ATP carrier protein, however, the cytoplasmic form of malate synthase

aggregates to form octamers or a large, 100S complex, while the monomers have a high affinity for amphipathic lipids, as would be expected for the membrane-integrated form. Although it contains no transient signal sequence, immuno-precipitable malate synthase is imported by glyoxysomes only, when *in vitro*-synthesized radioactively-labelled enzyme is incubated with a mixture of plant cell organelles. This observation suggests the existence of a glyoxysomal surface receptor which recognises all or part of the completed malate synthase protein, leading to its specific internalization by glyoxysomes; although the authors fail to comment on radioactive label which appeared in mitochondria and chloroplasts, in the same experiment (Kruse *et al.*, 1981). The energy requirements for internalization of proteins by glyoxysomes and peroxisomes have not been investigated.

In conclusion, current research is beginning to reveal the way in which the specific import of a large number of water-soluble cytoplasmic proteins, by a multiplicity of sub-cellular organelles, is orchestrated. It appears that specific receptors in an organelle envelope recognize all or part of the globular, cyto-plasmic precursor proteins destined for that particular organelle, and translocation then occurs, across one or more membranes, with or without detectable covalent modification of the transported protein. The trans-membrane trans-location is energy-dependent in most cases, and is thought to involve integral membrane translocator

proteins, although the actual molecular mechanism of transport is unknown.

1.5 THEORETICAL MECHANISMS OF "BIOSYNTHETIC TRANSFER" OF PROTEINS ACROSS MEMBRANES

In specialized, mammalian secretory cells, large quantities of protein are rapidly and efficiently transported from the aqueous, cytosolic compartment, where protein synthesis occurs, through the lipid bilayer of the ER membrane, into the ER lumen, from whence they are exported from the cell. Protein import by the ER differs from protein import by other organelles in that it is more tightly coupled to protein synthesis. Thus, secretory proteins addressed to the ER are synthesized by ribosomes bound to the ER membrane and are transported directly into the cisternal space rather than being first released into the cytoplasm.

In parallel with observations reported by Milstein *et al.*, (1972), the Signal Hypothesis was advanced by Blobel and Dobberstein (1975a&b) to describe secretory protein translocation into the ER. It was subsequently extended to encompass the insertion of integral membrane proteins and transport of proteins into other sub-cellular organelles, as discussed in sections 1.3 and 1.4 (Blobel and Dobberstein, 1975a, 1975b; Blobel, 1979; Lingappa *et al.*, 1980; Blobel, 1980). Details of the main points of the signal hypothesis and early supportive evidence have appeared in numerous reviews (Kreil, 1981; Emr *et al.*, 1980; Davis and Tai, 1980), so only a brief description of the hypothesis and the more recent evidence

relating to the actual mechanism of protein translocation will be considered here.

The translocation of secretory proteins across the ER membrane is similar to the transport of proteins into other organelles in that, in both situations, the information specifying trans-membrane translocation is distributed between the transported protein and the membrane. In the former case, however, the protein synthetic "machinery" also contributes to the mechanism of translocation. The signal hypothesis neatly defines the separate roles of these components (see Figure 1.1). The "protein information" consists of the signal sequence, a stretch of 15 to 30 predominantly hydrophobic amino acids, which is usually at the amino-terminus of the protein, so that it is the first part of the nascent polypeptide to emerge from the ribosome at the beginning of translation. This sequence rapidly binds to a receptor on the surface of the ER membrane, thus initiating the aggregation of integral membrane protein subunits to form a hydrophilic tunnel through the hydrophobic lipid bilayer. The ribosome binds to the cytoplasmically-exposed domain of this pore and translation proceeds, with the newly-polymerized peptide passing directly from the ribosome into the trans-membrane pore as an extended chain of amino acids.

The signal sequence is removed by the action of a specific peptidase associated with the cisternal surface of the ER membrane as soon as the cleavage site enters the lumen, thus proteolytic processing is co-translational.

In the ER lumen, the secretory protein assumes its native conformation, then begins its journey through the membrane-bounded "pipe-line" and ultimately out of the cell (Palade, 1975). In the case of the insertion of integral membrane proteins, additional "protein information", in the form of an internal "stop-transfer" sequence, halts translocation and causes disaggregation of the pore, leaving the newly-synthesized protein inserted in the lipid bilayer. Repeated, internal signal and stop-transfer sequences could presumably lead to complex orientations of integral membrane proteins relative to the membrane boundaries (Blobel, 1980; Sabatini *et al.*, 1982). Although originally addressed to the problem of protein translocation in eukaryotes, the signal hypothesis is also thought to provide an adequate description of the secretion of proteins across the plasma membrane of prokaryotes (Davis and Tai, 1980).

A flood of reports of short-lived, amino-terminal, hydrophobic signal sequences in secretory proteins promptly confirmed the main premis, but due to the complexity of the *in vivo* and *in vitro* systems used to study the secretory process, examination of the finer points of the signal hypothesis has proved problematical. In recent years, many reported exceptions to various mechanistic details of the signal model have spawned a plethora of alternative theories of transmembrane protein translocation.

The signal hypothesis has been challenged on three

related issues. Firstly, it is argued that the translocated protein may pass directly through the hydrophobic environment of the lipid bilayer, rather than through a hydrophilic discontinuity in the membrane, produced by a trans-membrane protein pore. Secondly, it has been proposed that the secondary structure of a polypeptide may play an active role in its own translocation across a membrane, rather than the protein being maintained as an extended chain of amino acids during transport. Lastly, the obligatory co-translational nature of protein translocation into the ER, and across the bacterial plasma membrane, has been questioned. Before considering the experimental evidence pertaining to these issues, the main alternatives to the signal hypothesis will be summarized.

A more speculative aspect of the signal hypothesis is highlighted by the lack of evidence for the existence of a trans-membrane protein pore; in fact many researchers dispute the involvement of any type of membrane transport protein in secretion. Based largely on thermodynamic considerations, several theories have been advanced which propose that the translocated protein partitions directly into the lipid bilayer.

The Trigger Hypothesis proposes that the completed, water-soluble precursor of the phage M13 coat protein inserts spontaneously and post-translationally into the *E. coli* plasma membrane with an accompanying change in conformation from a hydrophilic to a lipid-stable form, closely followed by the proteolytic removal of a 23-amino

acid signal sequence (Fig. 1.2.a; Wickner, 1979). Integration, and correct orientation of the precursor within the bilayer requires a trans-membrane electrochemical gradient but is independent of "membrane information" in the form of receptor/translocator proteins, the only membrane protein involved being the leader peptidase which processes the pro-coat after its correct insertion (Date *et al.*, 1980a, 1980b; Watts *et al.*, 1981). It should be noted that, if it is not inserted within one to two minutes of synthesis, the cytoplasmic pro-coat protein rapidly loses its competence for membrane integration, due to denaturation (Goodman *et al.*, 1981). This phenomenon is thought to explain the failure of other workers to observe post-translational translocation of M13 pro-coat protein into *E. coli* plasma membrane vesicles (Chang *et al.*, 1978, 1979).

A translocation model very similar to the trigger hypothesis has been proposed to account for the post-translational transport of the periplasmic leucine binding protein, and the plasmid-encoded β-lactamase, across the *E. coli* plasma membrane (Daniels *et al.*, 1981). The essential difference between the two models is that, in the latter, proteolytic removal of the signal sequence triggers a second conformational change leading to complete transfer of the processed proteins across the membrane, to a final location in aqueous solution in the periplasmic space (Fig. 1.2.b).

Similar schemes involve *co*-translational protein transport in both prokaryotes and eukaryotes. Two

FIGURE 1.2.a

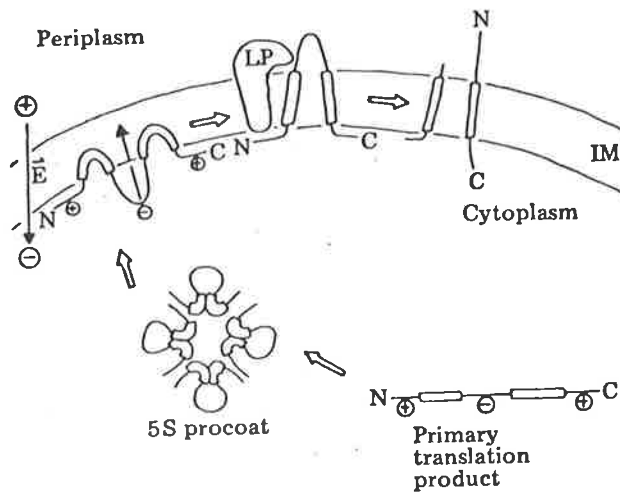
The Trigger Hypothesis

Schematic model for the post-translational insertion of the bacteriophage M13 procoat protein into the inner membrane (IM) of *E. coli*. It is proposed that the procoat molecules form a water-soluble complex in the cytoplasm before encountering and inserting, with the aid of an electrochemical gradient, into the plasma membrane, where the signal peptide is cleaved by the leader peptidase (LP).
Taken from Date *et al.*, (1980).

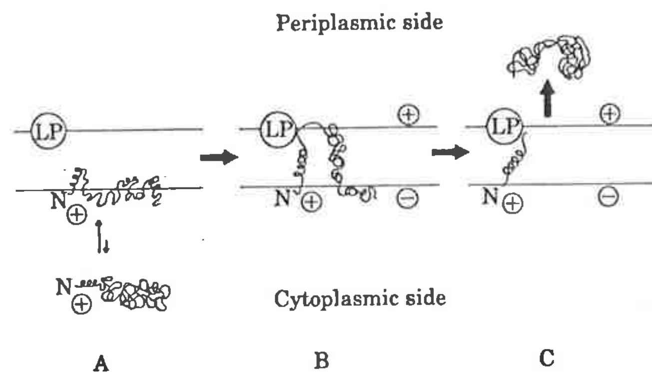
FIGURE 1.2.b

Daniels *et al.*

Schematic model for the post-translational transport of periplasmic proteins across the plasma membrane of *E. coli*. It is proposed that the periplasmic proteins are synthesized as water-soluble precursors which integrate into the plasma membrane and are correctly orientated relative to the leader peptidase (LP) enzyme by a membrane potential. Following proteolytic processing, the mature proteins are thought to refold into a water-soluble conformation on the other side of the membrane.
Taken from Daniels *et al.*, (1981).



2.a. The Trigger Hypothesis



2.b. Daniels *et al.*

different groups have suggested that prokaryotic signal sequences partition into the lipid in the form of a loop, with the amino-terminal, charged residues of the signal remaining on the cytoplasmic side of the membrane (DiRienzo *et al.*, 1978; Engelman and Steitz, 1981). In the "Helical Hairpin Hypothesis", which also refers to eukaryotic proteins, continuing translation feeds the amino acid chain of the mature protein through the lipid bilayer in the form of an α-helix (Fig. 1.3.a). This is energetically feasibly by virtue of the fact that, for every amino acid that enters the bilayer on the cytoplasmic side, another passes out of the other side of the membrane, into the external, aqueous environment (Engelman and Steitz, 1981). In both models, the only membrane protein involved in translocation is the signal peptidase, and proteolytic processing is mandatory for release of the secreted protein into the periplasm.

In eukaryotic systems, an analagous, co-translational partitioning of the entire, secreted amino acid sequence into the lipid bilayer provides the basis for both the "Direct Transfer Model" (von Heijne and Blomberg, 1979), and the "β-transorption hypothesis" (Steiner *et al.*,1980; Chan *et al.*, 1979). These modesl differ from the situation in prokaryotes in that, following signal sequence insertion, the proximity of the eukaryotic ribosome to the membrane leads to an ionic association of the ribosome with an integral ribosome-binding protein of the ER membrane (Fig. 1.3.b). Continued translation feeds the amino acid

FIGURE 1.3.a

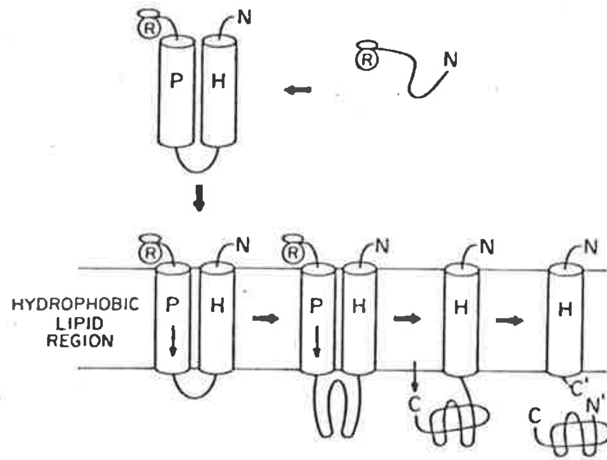
The helical hairpin hypothesis

Schematic model for the co-translational secretion of proteins. It is proposed that translation by cytoplasmic ribosomes results in the synthesis of the initial segment of a secretory protein which spontaneously partitions into the membrane in the form of a hairpin containing two helices, one of which is the hydrophobic (H) signal sequence. The rest of the secreted protein sequence forms a polar helix (P) as it passes through the membrane, as protein synthesis continues. Cleavage of the leader peptide results in the release of the mature protein on the extra-cytoplasmic side of the membrane.
Taken from Engelman & Steitz (1981).

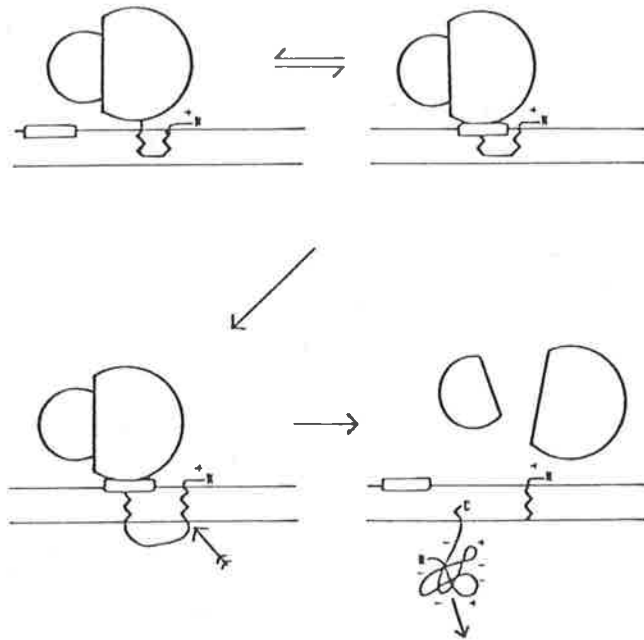
FIGURE 1.3.b

The direct transfer model

Schematic model for the co-translational secretion of proteins. After the initiation of translation by a cytoplasmic ribosome, it is proposed that the initial segment of a secretory protein partitions into the membrane as a loop containing two α-helices. The ribosome binds to an integral membrane protein and continued translation results in extrusion of the secretory protein (in the form of an α-helix), through the membrane. The mature protein is released on the extra-cytoplasmic side of the membrane after cleavage of the signal region, and the ribosome dissociates from its binding site.
Taken from von Heijne (1980a).



3.a. The Helical Hairpin Hypothesis



3.b. The Direct Transfer Model

chain through the lipid bilayer, to the ER lumen, where the protein folds into its native form, following proteolytic removal of the amino-terminal signal sequence, which presumably remains in the membrane. The β-transorption hypothesis invokes a specialized secondary structure of the signal sequence to explain its spontaneous integration into the lipid, while the direct transfer model predicts an α-helical conformation for both the signal sequence and the "mature" amino acid sequence during its passage through the hydrophobic, membrane core. Both models require the existence of a membrane-associated signal peptidase and a membrane-associated ribosome-binding protein (see Fig. 1.3).

The gap between the aforementioned theories and the signal hypothesis is bridged by Garnier *et al.*, (1980) and B.M. Austen (1979) who have independently proposed that translocation is initiated by the spontaneous integration of a specially-structured signal sequence into the lipid bilayer, followed by formation of a hydrophilic core, through which the rest of the secreted protein passes. The membrane proteins involved in this process are the signal peptidase, and one or more transmembrane, pore-forming proteins with cytoplasmically-exposed ribosome-binding sites. The essential difference between these ideas and the signal hypothesis is that, in the latter theory, initial contact between the secretory protein and the membrane consists of binding of the signal sequence to a receptor protein on the membrane with the consequence of pore-formation. Thus the entire trans-

located protein, including the signal sequence, passes through a hydrophilic discontinuity in the membrane, as an extended chain of amino acids, without ever coming into direct contact with the hydrophobic environment of the lipid bilayer (Walter and Blobel, 1981a&b).

In considering the experimental data pertaining to the transport of newly-synthesized proteins across, or into, membranes, it is useful to place the theoretical mechanisms of translocation on a continuum, with the trigger hypothesis at one end and the signal hypothesis at the other. The former theory assigns the information specifying membrane integration almost exclusively to the structure of the translocated protein itself, with minimal contribution from the accepting membrane or from other factors, such as the protein-synthetic machinery. At the other end of the spectrum, the signal hypothesis minimises the contribution, to transport, of the structure of the translocated protein. According to this theory, the bulk of the information specifying transport resides in the membrane in the form of a protein complex which recognizes both ribosome and signal sequence, and forms a pore through which the secreted protein is extruded. Beyond the initial recognition and binding of the signal sequence, the structure of the translocated protein plays no part in transport, except where specific, internal "stop-transfer" sequences halt the process. Theories falling between these extremes propose a varying distribution of "transfer-information" between the secondary

structure of the translocated protein and integral membrane proteins.

Other parameters which vary along the continuum are, the extent of contact between the translocated protein and the lipid bilayer, and the timing of translocation relative to translation. The degree of contact between secretory protein and membrane lipid ranges from complete integration, through sequential integration and partial integration, to complete segregation of one from the other. The actual process of trans-membrane translocation is seen either as a discrete event, occurring immediately following release of the completed protein from the polysome, or as a sequential, co-translational transfer of the growing polypeptide chain. The main points of these theories are illustrated in figs. 1.1 to 1.4. As previously stated, the passage of proteins across membranes is thought to be very similar in prokaryotes and eukaryotes. Subtle differences do exist, however, and because of these; and in view of the complexity and bulk of the recent experimental data on secretion, protein transport across the plasma membrane of prokaryotes, and the analagous process of protein transport across the ER membrane of eukaryotes, will be considered in separate sections.

1.6 TRANSLOCATION OF NEWLY-SYNTHESIZED PROTEINS ACROSS THE PLASMA MEMBRANE OF PROKARYOTES

In the absence of sub-cellular organelles, prokaryotes secrete proteins directly across the plasma membrane to

final destinations in the outer membrane, in the periplasmic space, or in the plasma membrane itself. Early studies on prokaryote secretion revealed both similarities and differences between this process and the translocation of secretory proteins across the ER membrane in eukaryotes. The major difference was highlighted by the finding that premature chain termination by puromycin, in a medium of low ionic strength, is sufficient to release bound prokaryotic ribosomes from the plasma membrane (Smith *et al.*, 1978,1979), whereas eukaryotic ribosomes bound to microsomes can only be removed by puromycin and high salt (Adelman *et al.*, 1973).

These results confirm the existence of an ionic bond between the eukaryotic ribosome and the ER membrane, while the association between the prokaryote ribosome and the plasma membrane seems to be stabilized by the nascent peptide alone. It was not possible to exclude the possibility that the membrane-ribosome association is actually the same, *in vivo*, in both cases, but that one of the *in vitro* systems fails to accurately reflect the *in vivo* situation (Davis and Tai, 1980).

On the other hand, the similarity of the mechanism of prokaryote secretion to that of eukaryote secretion was established by the discovery of transient, signal peptides in all three classes of extra-cytoplasmic, prokaryotic proteins (Inouye *et al.*, 1977; Inouye and Beckwith, 1977; Sugimoto *et al.*, 1977). These sequences, and known eukaryotic signal sequences, were found to be

similar in length, distribution of charged and hydrophobic amino acids, and the nature of the carboxy-terminal amino acid residue, further validating the comparison between prokaryotic and eukaryotic systems (Kreil, 1981).

The vital role of the signal sequence in secretion was emphasised when the characterization of a group of secretion-defective mutants revealed that the genetic lesions preventing the correct localization of the periplasmic maltose-binding protein, and of the phage lambda-receptor of the outer membrane, fall exclusively within the signal sequences of those proteins (Bassford and Beckwith, 1979; Emr *et al.*, 1980; Bedouelle *et al.*, 1980). The implication of these findings; that a signal sequence is the only information, within a translocated protein, required to specify trans-membrane transport, was tested directly by using gene fusion techniques to add a signal sequence to a normally untransported protein.

This beautifully straight-forward approach revealed that the fusion of the DNA encoding the amino-terminal 39 amino acids of the λ-receptor protein, (including the entire 24-amino acid signal sequence) with the gene coding for the cytoplasmic enzyme, β-galactosidase, produces a hybrid protein which binds transiently to the plasma membrane, then remains in the cytoplasm (Moreno *et al.*, 1980). Inclusion in the fusion-product of approximately 5/11 of the λ-receptor sequence produces a hybrid protein which is almost equally distributed between the cytoplasm, the inner membrane and the outer

membrane, while pre-fixing the β-galactosidase sequence with roughly 7/11 of the λ-receptor gene leads to 80% of the hybrid proteins being correctly localized in the outer membrane (Emr *et al.*, 1980). These surprising results clearly indicate the involvement of more than just the signal sequence in trans-membrane translocation.

A possible explanation, compatible with the signal hypothesis, involves proposing that a "stop-transfer" sequence exists between 5/11 and 7/11 of the way along the λ-receptor protein, leading to its insertion in the plasma membrane, as an integral membrane protein, which is subsequently transported to the outer membrane by vesicles budding from the plasma membrane and fusing with the outer membrane (Emr *et al.*, 1980). If this is true, a hybrid protein could be correctly localized in the outer membrane without the β-galactosidase amino acid sequence actually having passed through a lipid bilayer. This hypothesis is consistent with the results of similar experiments in which varying amounts of the DNA encoding the amino-terminal section of the periplasmic maltose-binding protein were fused with the β-galactosidase gene. Even the inclusion in the fusion product of nearly all of the maltose binding protein sequence, which presumably would not include a stop-transfer sequence, could not direct the hybrid protein into the periplasm. In these strains the hybrid proteins became "stuck" in the plasma membrane (Bassford *et al.*, 1979).

Although the correct, detailed interpretation of the

results of the gene fusion experiments is still in doubt, it is obvious that simple addition of a signal sequence to a protein is not sufficient to cause its translocation across a membrane. On the contrary, it seems that the "mature" amino acid sequence of a secretory protein will pass through the plasma membrane, whereas the amino acid sequence of a non-secretory protein will not. It is not known whether this is due to blocks of hydrophobic amino acids which cannot be translocated through a hydrophilic pore, or to blocks of hydrophilic amino acids which cannot pass through the membrane lipid, or to incorrect folding of all or part of the protein, thus forming a "translocation-incompetent" structure.

The question of whether the transported protein passes through a protein pore or directly through the membrane lipid has been addressed by a number of investigators. Powerful support for the idea of translocation through the lipid comes from the finding that the newly-synthesized M13 pro-coat protein can be correctly inserted into liposomes containing only one protein component, the purified leader peptidase (Watts *et al.*, 1981). Energetic considerations also favour passage of a folded protein chain through the lipid (Engelman and Steitz, 1981), however there is a growing body of evidence pointing to the involvement of integral membrane proteins in extra-cytoplasmic protein transport across the prokaryotic plasma membrane.

When inverted *E. coli* inner membrane vesicles are

added to an *in vitro* protein-synthesizing system programmed with mRNA coding for the secreted proteins, diphtheria toxin or alkaline phosphatase, the proteins are co-translationally processed and segregated within the vesicles. If the inverted vesicles are subjected to mild proteolysis before addition to the system, segregation and processing are not observed, indicating that membrane proteins exposed on the cytoplasmic surface of the *E. coli* inner membrane are involved in secretory protein translocation. The connection of these proteins with the secretory process is strengthened by the observation that complexing ribosomes to the membrane prior to proteolysis prevents inactivation of the translocation capability of the vesicles (Smith, 1980). Proteolysis of the opposite (normally periplasmic) surface of the membrane, before vesicle formation, does not affect segregation or processing, implying that neither an essential pore-forming protein, nor the signal peptidase enzyme are exposed on the periplasmic surface of the plasma membrane, as suggested in the signal hypothesis (Chang *et al.*, 1978, 1979). The prokaryotic signal peptidase therefore seems to be exposed only on the cytoplasmic side of the plasma membrane, or to be completely buried within the lipid bilayer.

Further evidence for a membrane protein component in the secretory apparatus comes from the analysis of secretion-defective mutants in which the signal sequences of the exported proteins are unimpaired. Two mutants which have been characterized, fail to export a

number of apparently normal periplasmic and outer membrane proteins (Wanner *et al.*, 1979; Oliver and Beckwith, 1981). In one strain, the mutation has been found to map in, or very near, a cluster of genes involved in cell envelope biosynthesis, suggesting that the gene product affected may be a plasma membrane protein (Oliver and Beckwith, 1981). Significantly, both of the mutants fail to export a specific sub-set of extra-cytoplasmic proteins while others are secreted in normal, or increased, amounts. Thus, it seems that there may be different, integral membrane transport proteins which recognise and export different, extra-cytoplasmic proteins rather than there being one, general mediator of protein translocation. Alternatively, some ecto-proteins could be transported via a receptor/ translocator protein while others pass directly through the lipid bilayer.

These notions are supported by the observation that the previously-mentioned fusion strains of *E. coli* with large amounts of the maltose binding protein/ β-galactosidase hybrid protein "stuck" in the plasma membrane (p.27), accumulate a number of secretory protein precursors in the cytoplasm. In these strains, some proteins are exported normally while precursors to the wild-type periplasmic maltose binding protein and alkaline phosphatase, and the outer membrane λ-receptor protein and *ompF* and *ompA* proteins remain in the cytoplasm, indicating that the membrane receptors for this class of exported proteins had been saturated by the hybrid

proteins (Ito *et al.*, 1981). The fact that this untransported sub-set contains both periplasmic and outer membrane proteins implies that there is not simply a different transport protein for different extra-cellular locations. Inhibition of insertion of plasma membrane proteins was presumably not observed, which may mean that these proteins can be integrated directly into the lipid bilayer, as observed by Wickner *et al.*, (1981), in the case of M13 coat protein.

Despite the existence of convincing evidence for integral membrane protein involvement in protein export by bacteria, there is still no information on the nature of the membrane components involved. In particular, the existence of a finite number of signal sequence receptors on the cytoplasmic surface of the plasma membrane is inferred. It is not known, however, whether they are simply surface receptors which recognise secretory proteins, which are subsequently translocated through the hydrophobic lipid bilayer, or if they represent the exposed portions of trans-membrane, hydrophilic pores, through which the secreted polypeptides are extruded.

Studies aimed at determining the exact timing of translocation relative to protein synthesis, and the conformation of the secretory proteins during their passage across the membrane, may ultimately distinguish between the alternatives of extrusion of an extended chain through a pore and transport of a completed, folded protein through the lipid. W.P. Smith *et al.*, (1977,1979,1980) have demonstrated that several bacterial proteins are

co-translationally extruded through the plasma membrane. The generality of the mechanism is indicated by the finding that different, secreted polypeptides, in protoplasts of several different organisms, are available to external, non-penetrating label whilst still attached to ribosomes via tRNA, on the inside of the membrane.

Further evidence for the involvement of ribosomes in the translocation of proteins is provided by the discovery of suppressor mutations, mapping in a ribosomal gene cluster, which restore the export of a λ-receptor protein containing a mutant signal sequence (Emr *et al.*, 1981). The exclusiveness of the co-translational transfer mechanism is challenged, however, by the previously mentioned, post-translational insertion of M13 pro-coat protein into the plasma membrane and by the post-translational translocation, across the membrane, of β - lactamase and leucine binding protein (Wickner, 1979; Daniels *et al.*, 1981).

Recently other workers have found that a number of *E. coli* periplasmic and outer membrane proteins can be processed, and by inference, translocated, either post-translationally or co-translationally (Josefsson and Randall, 1980, 1981). Most of the proteins examined must reach a minimum of 80% of their total length before processing will occur. Since other evidence indicates that the signal peptidase enzyme seems to be localized on the cytoplasmic side of the plasma membrane or within the lipid bilayer, (see p.29), this observation indicates

that the proteins are at least 80% complete before crossing the membrane.

Support for this proposal comes from the study of secretion of β-lactamase by *Salmonella typhimurium*. It was reported by Koshland and Botstein (1980) that wild type β-lactamase is synthesized as a full-length precursor which is post-translationally exported and processed, presumably crossing the membrane in a folded conformation. The essential nature of the whole sequence is illustrated by the fact that chain termination mutants lacking as little as twenty one amino acids from the carboxy-terminus of the protein are not transported out of the cytoplasm. On the other hand, an amber mutant of *E. coli* maltose binding protein, which lacks the carboxy-terminal two thirds of the protein, is processed and at least partially translocated through the plasma membrane, as it is found to be membrane-bound, but sensitive to external proteases (Ito and Beckwith, 1981). In this case, translocation can at least be initiated by the amino terminal region alone, but the carboxy-terminus seems to be necessary for release of the protein on the other side of the membrane.

In summary, both co-translational and post-translational modes of translocation across the prokaryote plasma membrane are observed. Although the signal sequence is certainly vital for transport of a protein, several lines of evidence point to active involvement, in the mechanism of translocation, of the rest of the amino acid sequence of at least some proteins. Existing

experimental data have failed to determine conclusively whether the proteins pass directly through the lipid bilayer or through a protein-bounded, hydrophilic discontinuity in the bilayer, however the essential role in translocation of an integral membrane receptor protein is strongly indicated. It therefore appears that, in some cases at least, a parallel may exist between the transport of proteins across the prokaryote plasma membrane and the transport of proteins across, not only the eukaryote ER membrane, but also across the envelopes of other eukaryotic, sub-cellular organelles. In other words, prokaryotic exo-proteins seem to be able to cross the plasma membrane as extended chains of amino acids, as completed, folded structures, or in the form of intermediates between these two extremes.

1.7 TRANSLOCATION OF NEWLY-SYNTHESIZED PROTEINS ACROSS THE ER MEMBRANE OF EUKARYOTES

Two powerful advantages of studying secretion in prokaryotes rather than in eukaryotes are illustrated in section 1.6. They are, the greater amenability of prokaryotes to genetic manipulation, and the easy accessability of both surfaces of the membrane barrier in bacterial systems. Nonetheless, some very informative studies have been conducted *in vivo* in eukaryote cells, and using *in vitro* systems constructed from eukaryotic cellular components. Many recent reports pertain to the question of whether secreted proteins pass through the lipid or through a protein pore during trans-membrane translocation. In particular, the integral membrane

proteins involved in secretion have been investigated.

The membrane-associated proteins predicted by the various transport theories to function in translocation of secretory proteins are ribosome binding proteins, the signal peptidase enzyme, signal sequence receptors and pore-forming proteins. Ribosome binding proteins have been studied more extensively than any of the other components, and many estimates of the number and molecular weights of membrane proteins involved in ribosome binding have been published (Jothy *et al.*, 1975; Fujita *et al.*, 1977; Kreibich *et al.*, 1978a,1978b; Sharma *et al.*, 1978; Aulinskas and Scott-Burden, 1979; Yamaguchi *et al.*, 1981). The most widely accepted appear to be the ribophorins which co-purify with ribosomes in detergent-solubilized rough microsomes (Kreibich *et al.*, 1978a,1978b). The ribophorins are found in the membranes of rough microsomes but not of smooth microsomes, confirming their role in ribosome binding and in secretion; although a recent, puzzling report demonstrates that smooth microsomes lacking the ribophorins can process and segregate pre-human placental lactogen and pre-human chorionic gonadotropin, *in vitro*, just as efficiently as ribophorin-containing rough microsomes (Bielinska *et al.*, 1979).

The fact that signal sequences are removed from translocated proteins necessitates the existence of a signal peptidase enzyme. Little is known about the precise localization of the enzyme or its spatial relationship to the other protein components of the

translocation machinery, however its latency in intact microsomes suggests a lumenal or intra-membrane location. The peptidase activity is thought to be closely associated with the receptor/translocator proteins as processing and translocation of secretory proteins are tightly coupled (Blobel and Dobberstein, 1975a; Walter and Blobel, 1981b). Eukaryotic signal peptides vary significantly in length and amino acid sequence, thus it is difficult to see how microsomal signal peptidase can recognise and correctly cleave both homologous pre-proteins and heterologous pre-proteins from a wide variety of sources (Shields and Blobel, 1977; Brennan *et al.*, 1980; Lane *et al.*, 1980; Lane, 1981). Comparison of a large number of signal peptidase cleavage sites reveals no common amino acid sequence, giving rise to the proposal that the peptidase recognises a conformational determinant rather than the primary sequence (Kreil, 1981). The suggestion that the signal peptidase recognises a folded structure is supported by the observation that amino acid substitutions at positions 15 and 41 in the mature amino acid sequence of pheasant lysozyme can apparently alter the cleavage site of the pre-protein (Jollès *et al.*, 1979).

Although it is well-established that the role of the signal peptide is to bind the nascent secretory protein to the ER surface, and to initiate translocation of the peptide across the membrane, the reason for its subsequent proteolytic removal is obscure. It has been suggested by proponents of the signal hypothesis that

proteolytic processing may be necessary to activate the translocated protein, or to ensure that translocation is irreversible, while most of the other transport theories imply that processing must occur before the mature protein can be released from the membrane (see Figs. 1.2 & 1.3). The demonstration of trans-membrane translocation of unprocessed proteins (Lin *et al.*, 1978; Hortin and Boime, 1980) and of the existence of enzymically active pre-proteins (Ferenci and Randall, 1979; Haugen and Heath, 1979; Brown and Wold, 1981) militates against these ideas. The signal peptidase enzyme is described in more detail in Chapter 4.

The existence of signal sequence receptor proteins in the ER membrane is also well-documented. The results of competition experiments show that saturable receptors on the surface of rough microsomes bind nascent peptide chains of ovalbumin (Lingappa *et al.*, 1979), several polypeptide hormones (Majzoub *et al.*, 1980; Rosenblatt, 1980) and of the membrane glycoprotein "G" of vesicular stomatitis virus (Lingappa *et al.*, 1978). Ovalbumin, pre-prolactin and the precursor to the viral membrane protein all compete for the same ER membrane surface receptors supporting the idea that secretory proteins and integral membrane proteins initially share a common mechanism of translocation (Lingappa *et al.*, 1978,1979).

Completed, *in vitro*-synthesized pre-proinsulin and pre-placental lactogen, and the nascent forms of these pre-proteins, all compete for binding sites on rough microsomes proving that these receptors can recognize

the signal peptide in at least two fully-folded proteins as well as in nascent polypeptides (Prehn *et al.*, 1980,1981). Although the competition between nascent peptide chains and fully-synthesized pre-proteins indicates that post-translational binding of these precursors to signal receptors on the ER membrane occurs with the correct specificity, the absence of subsequent post-translational translocation shows that this binding is not fully functional. Post-translational binding of the precursors is not hindered by the presence of membrane-bound ribosomes, indicating that the signal sequence receptor site and the ribosome binding site are discrete entities, although they could conceivably reside in different parts of the same protein.

In the first study to approach the problem of characterizing the membrane proteins involved in the actual translocation process, it was reported that removal of a membrane-surface protein by extraction of stripped rough microsomes, in a medium of high salt, abolished the ability of the membranes to co-translationally segregate and process secretory proteins. This capability could be reconstituted by re-addition of the salt extract to the inactivated microsomes (Warren and Dobberstein, 1978). Subsequent work revealed that the salt-extractable factor was generated by the action of endogenous proteases on the microsomal membranes, a process that could be duplicated, with greater efficiency, using added trypsin, clostripain or elastase. The necessity for a high salt medium to remove the factor

from the membrane argued for the existence of an ionic bond as well as a peptide bond in the original attachment of the factor to the membrane. Fractionation of the salt extract showed the active constituent to be a protein, of molecular weight 60,000 daltons, which was thought to represent the cytoplasmically-exposed domain of a larger, integral membrane protein (Meyer and Dobberstein, 1980a,1980b).

In a parallel study, Blobel and co-workers found that high salt alone did <u>not</u> deplete microsomal membranes of their translocation capacity. They reported, however, that treatment of the microsomes with a low concentration of trypsin, in a medium of low salt, liberated a protein factor essential to secretory protein segregation by the vesicles (Walter *et al.*, 1979). It was later reported that high salt, although it did not completely deplete the translocation activity of rough microsomes, produced an extract which was quantitatively superior to the tryptic/low salt extract in reconstituting the segregating and processing activity of trypsin-treated microsomal membranes (Jackson *et al.*, 1980). In agreement with Meyer and Dobberstein, it was suggested that endogenous proteases generated a protein fragment, consisting of the cytoplasmically-exposed domain of a larger membrane protein, and that this domain remained associated with the membrane by virtue of an ionic bond which could be disrupted by high salt concentrations. The functionally-similar, trypsin-releasable and salt-releasable factors were found to both contain a sulphydral

group essential to their activity, and it was proposed that the salt factor consisted of the trypsin factor plus an extra domain which accounted for the ability to ionically associate with the microsomal membrane (Jackson *et al.*, 1980).

In contrast to the earlier work, salt extraction of rough microsomes, in the absence of protease, was subsequently found to inactivate the membranes with regard to translocation and processing of pre-secretory proteins. Fractionation of the salt extract revealed that the active constitutent was a complex of 6 proteins of molecular weights 72,000, 68,000, 54,000, 19,000, 14,000 and 9,000 daltons. Although walter-soluble, this complex was found to have some hydrophobic properties in keeping with its proposed role as the cytoplasmically-exposed portion of an integral membrane protein (Walter and Blobel, 1980).

Three recent publications describe the further characterization of the salt-extractable protein complex or "signal recognition protein" (SRP) (Walter *et al.*,1981; Walter and Blobel, 1981a, 1981b). It is demonstrated that SRP binds to ribosomes with relatively low affinity and to polysomes synthesizing preprolactin with high affinity. This SRP/polysome complex then binds to the salt-extracted microsomes and translocation and processing of the prolactin molecules occur. The inability of polysomes synthesizing preprolactin to bind to salt-extracted microsomes in the absence of SRP demonstrates convincingly that the inter-

action between the nascent pre-secretory protein and the membrane is mediated by a receptor protein, rather than being a simple insertion of the signal sequence into the lipid bilayer. It does not preclude, however, an interaction of the signal sequence with both a receptor protein and the membrane lipid.

The role of SRP in *in vitro* protein translocation, is described by Walter *et al.*, as follows. In the absence of membranes, SRP binds to both the ribosome and the signal sequence, as soon as it emerges from the ribosome, in polysomes translating the mRNA coding for a secretory protein. This binding arrests translation, freezing the protein-synthesizing apparatus in the correct conformation for interaction with the microsomal membrane. When an appropriate membrane is encountered, polysome binding occurs and translation proceeds with the secretory protein being co-translationally extruded into the membrane, with concomitant proteolytic processing. Binding to the membrane is thought to be mediated by an integral membrane SRP-receptor protein. Evidence for the existence of the membrane-embedded protein includes the demonstration that SRP will not bind polysomes to phospholipid vesicles or to trypsin-treated, salt-extracted microsomes, indicating coincidentally that SRP and the previously-reported, trypsin-generated factor are unrelated. This contradicts the earlier contention that the protease-generated and high salt-generated factors are functionally equivalent (Jackson *et al.*, 1980).

A model, reproduced from Walter and Blobel (1981b),

for the function of SRP in the translocation of secretory proteins across the ER membrane *in vivo*, is presented in Figure 1.4. This scheme maximises the efficiency of secretory protein transfer into the ER, expiaining why miscompartmentalization of completed pre-proteins is rarely observed *in vivo*. The experimental data support most of the details of the mechanism up to the point of contact between the ribosome/SRP/signal complex and the membrane. Although the presence of an SRP-receptor protein is indicated, there is no evidence regarding the nature of that protein, or the way in which secretory protein translocation actually occurs. Specifically, although the existence of a signal-receptor protein has been established beyond reasonable doubt, there is as yet no evidence for the existence of a trans-membrane, hydrophilic, protein-transport pore in the ER membrane.

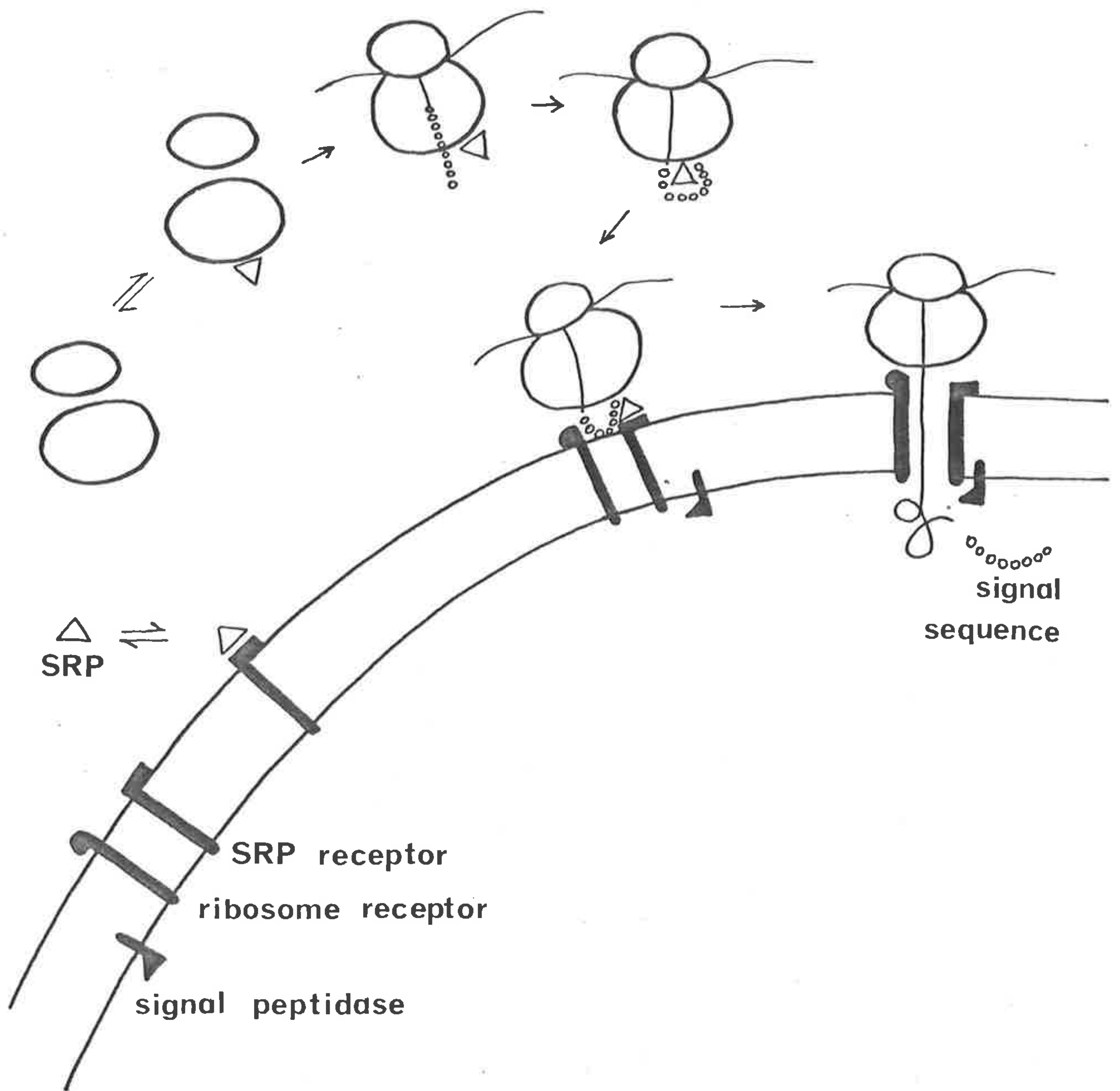
Very little information is available concerning the way in which secretory proteins are transferred across the ER membrane following recognition and binding of the signal peptide. A number of reports stress the fact that microsomal membranes must be present during the early stages of translation *in vitro* in order for segregation and processing of the newly-synthesized protein to occur, implying that translocation is strictly co-translational (Jackson and Blobel, 1977; Lingappa *et al.*, 1977; Boime *et al.*, 1977; Kamine and Buchanan, 1978; Brennan *et al.*, (1980)). One elegant study demonstrated that microsomes must be added before the nascent peptide chain of vesicular

FIGURE 1.4

The proposed role of the signal recognition protein in secretion

Schematic model for the mechanism of trans-membrane translocation of secretory proteins. The signal recognition protein (SRP) is proposed to bind to the signal sequence of a nascent secretory protein, halting translation until the appropriate membrane is encountered. The signal/SRP complex and the ribosome bind to separate membrane-integrated receptors with the consequent formation of a hydrophilic, trans-membrane pore through which the secretory protein is co-translationally extruded. The signal sequence is cleaved by the signal peptidase which is associated with the pore complex.

Taken from Walter & Blobel (1981b).


SRP
SRP receptor
ribosome receptor
signal peptidase
signal
sequence

stomatitis virus G-protein has exceeded 80 amino acids in length, in an *in vitro* translation system, in order for correct insertion of the protein into the membrane to occur. It was also shown that core-glycosylation occurred well before the completion of the polypeptide chain. Since glycosylation was thought to occur only in the ER lumen, these results were interpreted to mean that polysome binding occurs co-translationally and the nascent peptide is extruded through the membrane, into the ER lumen during its synthesis (Rothman and Lodish, 1977). A recent investigation of the trans-membrane location of the enzymes involved in ecto-protein glycosylation questions this conclusion.

Snider *et al.*, (1980) found that a number of the enzymes involved in the synthesis of the oligosaccharide-lipid precursor of asparagine-linked oligosaccharides are sensitive to added, exogenous proteases in suspensions of intact microsomes. Permeabilization of the microsomes with detergent did not increase the sensitivity to proteolysis of these enzymes implying that they are predominantly exposed on the cytoplasmic side of the ER membrane. Thus it is possible that glycosylation of a nascent polypeptide occurs after the binding of the peptide to the membrane, but immediately before the translocation of the protein across the bilayer.

Similarly, the observation of proteolytic removal of the signal sequence before the completion of a secretory polypeptide has been cited as evidence for

co-translational extrusion of the peptide chain through the membrane. The fact that the signal peptidase is resistant to exogenous proteases added to intact microsomes was thought to indicate a lumenal location of the processing enzyme; therefore, access of the nascent peptide chain to the enzyme would imply penetration of the nascent peptide into the ER lumen (Walter *et al.*, 1979). This reasoning may need to be reassessed in view of the recent demonstration that the prokaryotic signal peptidase does not appear to be exposed on the extra-cytoplasmic surface of the bacterial plasma membrane (Smith *et al.*, 1980). Extrapolation to the eukaryotic analogy would mean that the eukaryotic peptidase is similarly not exposed on the extra-cytoplasmic, or cisternal, face of the ER membrane. Taken together, these reports suggest that the signal peptidase is exposed on neither surface of the ER membrane and is, in consequence, completely buried in the lipid bilayer.

Given the revised locations of the enzymes involved, the reports of co-translational proteolytic processing and glycosylation of secretory or membrane proteins may simply mean that the nascent chains are in contact with enzymes buried in the ER membrane, rather than the data constituting proof of the actual spanning of the membrane by nascent peptide chains, as has been demonstrated for some bacterial proteins (see Section 1.6). In other words, it is still not possible to eliminate the suggestion that eukaryotic secretory proteins bind

co-translationally to the ER membrane and form completed, folded structures on the cytoplasmic face of the bilayer, or within the bilayer itself, immediately before, or during translocation.

On the contrary, the possibility of post-translational transport of completed, secretory proteins across the ER membrane is supported by the finding, *in vivo*, of full-length precursors to a number of polypeptides including preproinsulin (Patzelt *et al.*, 1978), pre-pro-parathyroid hormone (Habener *et al.*, 1980), and preprolactin (Maurer and McKean, 1978). It is uncertain, however, whether these precursors represent normal, biosynthetic intermediates, or if they are merely small amounts of miscompartmentalized protein or protein which is co-translationally translocated but escapes subsequent proteolytic processing. In the case of preproPTH it was proved that the completed precursor is associated with the cytoplasmic face of the ER membrane, but it was not determined whether or not this population of molecules is subsequently translocated through the membrane (Habener *et al.*, 1980).

Other *in vivo* pulse-labelling studies have failed to find pre-proteins in intact cells (Schmeckpeper *et al.*, 1975; Sussman *et al.*, 1976), however very short-lived intermediates would be extremely difficult to detect. There have been no demonstrations of post-translational translocation of eukaryotic proteins into the lumen intact micro-somes, *in vitro*, but, as previously mentioned, similar studies failed to detect the post-translational integration

of M13 procoat protein into *E. coli* vesicles because the completed precursor rapidly denatures in the absence of membranes (Chang *et al.*, 1978,1979; Goodman *et al.*, 1981). It is possible that analagous, unstable, translocation-competent forms of fully-synthesized, eukaryotic, secretory pre-proteins have so far eluded detection because membranes were not added soon enough after their completion in *in vitro* translation systems. It is also possible that, rather than being completed before translocation, eukaryotic secretory proteins may begin to assume folded, secondary structures while in the process of passage across the membrane.

There is no significant sequence homology between the signal peptides of most secreted or membrane-integrated proteins yet these diverse primary structures are recognized by the same membrane receptors. This has been taken as evidence that signal sequences assume a common secondary structure which interacts with the ER membrane, initiating translocation (Majzoub *et al.*, 1980; Chan *et al.*, 1979). The tentative localization of an uncleaved signal sequence at residues 234 to 253 in the ovalbumin amino acid sequence must mean that more than half the polypeptide chain is synthesized before the initiation of translocation across the ER membrane. It has been demonstrated, however, that nascent ovalbumin competes for the same membrane receptors as nascent preprolactin which has a normal, amino-terminal, cleavable signal sequence. Thus the internal ovalbumin signal can presumably form a secondary structure functionally identical

to that of amino-terminal signal sequences (Lingappa *et al.*, 1979).

After initiation of translocation by the internal signal sequence, the partially-completed ovalbumin polypeptide must pass through the membrane, possibly in the form of some sort of folded structure. It should be mentioned that the internal localization of a signal sequence in ovalbumin has been challenged by Meek *et al.*, (1980) who find that nascent ovalbumin and nascent ovomucoid, which has an amino-terminal signal sequence, can bind to microsomes equally early, with a chain length of less than 100 residues.

The different translocation theories described in section 1.5 vary in their predictions of the secondary structure adopted by the amino acid chain of the mature secretory protein during its passage across the ER membrane (see Figs, Ch.1). As mentioned in section 1.6, the signal hypothesis suggests that the translocated protein is completely devoid of secondary structure in that it is transferred through a hydrophilic pore as a "passive", extended chain of amino acids. The only restriction placed on the primary structure of a translocated protein, therefore, is that it should not contain a block of amino acids constituting a signal for disaggregation of the trans-membrane pore. A so-called stop-transfer sequence would presumably have to interact with the hydrophilic pore, then, following pore subunit dispersal, form a stable interaction with the hydrophobic lipid bilayer.

Alternative theories of translocation maintain that the whole amino acid chain of the secreted protein is able to interact transiently with the lipid core of the membrane. The entry of hydrophilic sections of a protein into the hydrophobic milieu is thought to be achieved by virtue of the secondary structure of the translocated polypeptide (see figs. 1.2 and 1.3). For example, the direct transfer model predicts that proteins pass directly through the lipid bilayer in the form of an α-helix. Circumstantial evidence for this contention emerges from the observation that in secretory protein amino acid sequences, and in the translocated or integrated segments of membrane proteins, the distribution of charged amino acids relates to the periodicity of an α-helix. Thus, in these sequences, an α-helical conformation would mean that positively charged amino acids are generally juxtaposed on negatively charged residues, resulting in an overall charge neutralization which would be expected to minimize the ionic resistance to entry of the protein into a hydrophobic environment (von Heijne (1980b)).

Physico-chemical analysis in terms of the direct transfer model of protein translocation, has been used to correctly predict the trans-membrane orientation of an impressive number of both prokaryotic and eukaryotic secreted and membrane-bound polypeptides, thus lending support to the idea of a direct interaction between secreted proteins and membrane lipids (von Heijne (1980a)). This type of reasoning, reinforced by the lack

of evidence for a hydrophilic discontinuity in the ER membrane, argues against the extrusion of secreted peptides through a protein pore. On the other hand, the unequivocal evidence for the involvement of signal sequence receptor proteins in translocation across the ER membrane argues against the interaction of secreted proteins with the lipid alone. An obvious solution to this impassé is to propose that translocated polypeptides interact with both protein and lipid on their way through the membrane.

1.8 TRANSLOCATION MECHANISMS: A SUMMARY

Basically, all the examples of trans-membrane transport described in this review embody the same problem: how does a hydrophilic protein molecule traverse a hydrophobic lipid bilayer? The key to this apparent dilemma lies in appreciating that both the polypeptide to be translocated, and the membrane barrier, are complex mixtures of hydrophobic and hydrophilic elements, which are able to interact. This concept is probably most clearly illustrated by the passage of toxin molecules across, or into, the membranes of target cells. In such instances it is undisputed that proteins which are initially water-soluble become integrated into, or transferred through, a lipid bilayer. The specificity of this process is determined by the interaction of toxin subunits with membrane receptors. Following this binding step, parts of the specially-structured toxin complex assemble spontaneously into the membrane lipid, sometimes

resulting in complete trans-membrane translocation of other parts of the complex (Kagan *et al.*, 1981).

There is an obvious parallel between this mechanism and what is known about the import of cytoplasmic precursor proteins by eukaryotic, sub-cellular organelles. In striking similarity to toxin molecules, mitochondrial and glyoxysomal preproteins have been shown to possess hydrophobic properties, which lead to the aggregation of the proteins to form water-soluble complexes in the hydrophilic environment of the cytoplasm (Zimmerman and Neupert, 1980; Neupert and Schatz, 1981; Kruse *et al.*, 1981). It is also known that the specificity of transfer into organelles is determined by the interaction of parts of the translocated proteins with membrane receptor proteins.

Extrapolation to complete the analogy leads to the proposal that, following the binding step, the precursors of mitochondrial, chloroplast, peroxisomal and glyoxysomal proteins interact with both membrane proteins and the lipid bilayer in such a way that transfer across the organelle envelope ensues. The mechanism of such a translocation presumably involves interactions between the hydrophilic elements of the specially-structured, transported protein and those of the organelle envelope, and similar interactions between the hydrophobic elements of the protein and those of the envelope. Since the inner mitochondrial membrane is composed of 75% protein, this process may be envisaged as the translocated protein "shouldering" its way through a mixture of lipid molecules

and hydrophobic segments of membrane proteins, including a receptor/translocator protein, rather than the peptide dissolving in a sea of lipid (Waksman, 1980). Alternatively, translocation may involve only protein-protein interactions within the hydrophobic membrane core.

This basic model for the transport of toxins and eukaryotic organelle pre-proteins is also directly applicable to the post-translational transport of leucine binding protein and β-lactamase across the prokaryote plasma membrane (Daniels *et al.*, 1981). It follows that essentially the same mechanism may be employed by proteins such as alkaline phosphatase which can be processed and translocated either post-translationally or co-translationally (Josefsson and Randall, 1981; Smith, 1977). This reasoning provides the missing link in the spectrum of transport events ranging from the spontaneous membrane-integration of toxin molecules to the apparently co-translational transfer of proteins across the prokaryotic plasma membrane or across the eukaryotic ER membrane.

Since the co-translational and post-translational modes of protein transfer across membranes must obviously obey the same thermodynamic laws, it can be postulated that the only real difference between the two mechanisms is a matter of efficiency. Thus, in the eukaryotic cell, proteins synthesized in the cytoplasm are addressed to various organelles via specially structured signal sequences. Precursors destined for the "house-keeping" organelles are completed on free

polysomes, released in the cytoplasm, and usually within a few minutes of synthesis, bind to the appropriate envelope receptors and enter the organelles. Proteins destined for export from the cell are also synthesized in the cytoplasm, however, in this case the protein structure has evolved such that the signal sequences bind co-translationally to their receptors, leading to association of the polysomes with the ER membrane. The fact that post-translational transfer of proteins into microsomes is not observed indicates that if this co-translational binding does not occur, a translocation-competent protein structure is not formed. In the situation where co-translational binding does occur however, the subsequent transfer of the protein across the membrane could either be co-translational or it could occur immediately after completion of the protein on the membrane surface. In the latter case, secretory proteins would be discharged into the ER lumen, with maximum efficiency, by a mechanism very similar to that employed in other organelles.

Currently, very little is known about the molecular mechanism of trans-membrane translocation of proteins, although a number of theoretical mechanisms have been proposed (see section 1.5). The most well-documented of these is the signal hypothesis, which predicts that secretory proteins are extruded, in a strictly co-translational manner, as extended chains of amino acids, through a protein-bounded hydrophilic pore in the ER membrane. The available experimental data on protein

transport into the ER, when taken in context with information from other protein translocating systems, may equally well support such a co-translational mechanism or a rapid, post-translational mechanism. It is the aim of many current investigations to distinguish between these possibilities.

1.9 THE PANCREATIC MICROSOME ENZYME RELEASE PHENOMENON

It has been discovered in this laboratory that the incubation of rat pancreatic microsomes, in buffered sucrose, at 37°C, causes the appearance of active α-amylase and RNAse in the suspending medium (Pearce *et al.*, 1978). It was demonstrated that this phenomenon involves the transfer of fully-formed enzymes from the particulate to the soluble fraction of the suspension, rather than being a consequence of *de novo* protein synthesis. The evidence that the release of enzymes from the microsomes involves trans-membrane translocation of the proteins hinges on the demonstration that before incubation, RNAse activity is latent, and the enzyme is protected from attack by proteases added to the suspension. After incubation of the microsomes at 37°C however, RNAse activity is detected in the medium and is sensitive to added protease. Disruption of unincubated vesicles with detergent also releases RNAse and exposes it to external proteolytic attack. Since protection from proteolysis is regarded as the only rigorous criterion for segregation of proteins within microsomes (Blobel and Dobberstein, 1975 a), these results indicate that incubation at 37°C

causes the transfer of RNAse from inside the microsomes, across an intact membrane, to the suspending medium.

Examination of the vesicles using electron microscopy confirmed that the microsomes are intact both before and after incubation at 37°C for 60 minutes, ruling out large-scale vesicle lysis as an explanation of release. When maintained at 2°C, the microsomes do not release appreciable amounts of enzyme, although significant leakage would be expected at this temperature if the mechanism of release was simply diffusion of proteins through holes in damaged membranes. Most significantly, it was found that brief proteolysis of the microsomes abolishes the release of α-amylase and RNAse. It was demonstrated, in the latter case, that the enzyme remains sequestered within the microsomes rather than being released, then destroyed by the exogenous protease. Taken together, these results imply that fully formed, active α-amylase and RNAse pass through the microsomal membrane by a mechanism which depends on the integrity of membrane proteins exposed on the external surface of the vesicles (Pearce *et al.*, 1978).

This suggestion conflicts with the published, hypothetical descriptions of *in vivo* transport of secretory proteins across the ER membrane on two counts. Firstly, the release phenomenon is characterized by the apparent, post-translational transport of complete, globular proteins across the microsomal membrane whereas transfer into the ER *in vivo* is thought to involve the co-translational extrusion, across the membrane, of folded

or extended chains of amino acids. Secondly, since the microsome lumen corresponds to the ER lumen, the *in vitro*, trans-membrane transfer of enzymes is in the opposite direction to the physiological process of secretion. It has been demonstrated, however, that the *in vitro* release of rat pancreatic microsome content enzymes is inhibited by physiological concentrations of magnesium ions, therefore this reverse transport across the ER membrane would not be expected to occur *in vivo* (Pearce *et al.*, 1978).

We have hypothesized, in consequence, that the *in vitro* enzyme release phenomenon may be due to the unphysiological activity of a transport system in the rat pancreatic microsomal membranes which is normally involved in the vectorial transfer of secretory proteins <u>into</u> the ER *in vivo*. If this is the case, the observation that fully formed, active enzymes can traverse the ER membrane *in vitro*, in the absence of magnesium, may have implications for the details of the physiological mechanism of protein translocation. Specifically, the admittedly artefactual, post-translational trans-membrane translocation of proteins *in vitro* may argue against a strictly co-translational mechanism of protein transport across the ER membrane *in vivo*, as suggested by the signal hypothesis.

1.10 AIM OF THE INVESTIGATION

The primary goal of the work presented in this thesis was to clarify the relationship, if any, between the

in vitro pancreatic microsome enzyme release phenomenon and the *in vivo* process of protein secretion.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Experimental animals

White, male Porton rats weighting between 250 gms and 300 gms were obtained from the Central Animal House, University of Adelaide. The animals, which were maintained on a pellet diet fed *ad libitum*, were starved overnight prior to sacrifice by chloroform anaesthesis followed by exsanguination.

2.1.2 Enzymes and Proteins

Chymotrypsin, papain (type IV), subtilisin BPN', ovalbumin, pancreatic RNAse A, hen egg lysozyme, bovine catalase and bovine serum albumin (BSA) were products of Sigma Chemical Co. TPCK-trypsin was obtained from Worthington Biochemical Corp.; *Staphlacoccus aureus* protein A was purchased from Pharmacia Diagnostics, and pronase was from Calbiochem-Behring Corp. Haemoglobin was purified from chicken blood and kindly donated by Dave Bird of the Biochemistry Department, University of Adelaide.

2.1.3 Enzyme Inhibitors

N-α-tosyl-L-lysine chloromethyl ketone (TLCK) and L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) were purchased from Cyclo Chemical Corp. and Worthington Biochemical Corp. Leupeptin, chymostatin, elastatinal, antipain and phosphoramidon were from the Peptide Research Foundation, Japan. 1,10 Phenanthroline,

benzamidine HCl, phenylmethyl sulphonyl fluoride (PMSF) and p-hydroxymercuribenzoate (pOHMB) were from Sigma Chemical Co., while N-ethyl maleimide was obtained from Calbiochem-Behring Corp.

2.1.4 Antibody

Anti-rat pancreatic α-amylase antibody was generously supplied by Dr. R.J. MacDonald from the Department of Biochemistry and Biophysics, University of California, San Francisco.

2.1.5 Chemical Reagents

Sodium ^{125}I-iodide was purchased from Amersham; Phadebas α-amylase test tablets were from Pharmacia Diagnostics; sodium deoxycholate (DOC), sodium dodecyl sulphate (SDS), guanosine triphosphate (GTP), β-mercaptoethanol (BME) and dithiothreitol (DTT) were from Sigma Chemical Co.; puromycin dihydrochloride was obtained from Sigma Chemical Co. and from Nutritional Biochemicals Corp.; ethylenediaminetetra-acetic acid (EDTA) was obtained from Sigma Chemical Co. and B.D.H. Chemicals Ltd.; acrylamide and N,N'-methylenebis-acrylamide were from Merck, and were re-crystallized before use; tetramethylenediamine (TEMED) was from Eastman Kodak Co. and 1,3,4,6-tetra-chloro-3α,6α diphenylchloroglycoluril (marketed as "Iodogen") was purchased from Pearce Chemical Co. All other chemicals were analytical grade reagents obtained from Ajax Chemicals Ltd., B.D.H. Chemicals Ltd. and May and Baker Ltd.

2.2 METHODS

2.2.1 Preparation of pancreatic microsomes

A total microsomal fraction was used in all the experiments described in this thesis. The following isolation procedure was carried out as rapidly as possible, with the tissue fractions kept at 2°C at all times. All glassware was kept free of contaminating detergent.

The pancreas was removed, rinsed in cold 0.3 M sucrose/10 mM KCl/0.2 mM $CaCl_2$ (SKC), then chopped into small pieces (using scissors) in 10 mls (approximately 9 volumes) of fresh SKC solution. The chopped tissue was homogenized using a motor-driven Brendler homogenizer with a clearance of approximately 0.15 mm. A series of 3 (10 second) strokes was made with the motor operating at approximately 1,000 rpm. The homogenate was centrifuged at 600 g_{av} to remove nuclei and cellular debris. The post-nuclear supernatant was centrifuged at 10,000 g_{av} for 30 mins. The post-microsomal supernatant was discarded and the surface of the microsome pellet was rinsed with .3 M sucrose/ 50 mM Tris HCl/25 mM KCL/0.2 mM $CaCl_2$, pH 7.5 at 20°C (STKC buffer). The vesicles were resuspended in 5 to 6 mls of cold STKC buffer, by one, gentle stroke in the homogenizer used in the first step of the preparation. The resultant microsome suspension, containing approximately 5 mg total protein/ml, was diluted in STKC buffer to a total protein concentration of .2 to .3 mg/ ml for incubations involving amylase determinations.

2.2.2 Incubation of microsomes

Freshly-prepared microsomes suspended in STKC buffer at a concentration of .2 - .3 mg total protein/ml were incubated at 37°C. Unless otherwise specified, each incubation was started by transferring a tube containing 2 mls of a microsome suspension from an ice bath at 2°C to a water bath at 37°C. The initial lag phase observed in most amylase release profiles was therefore at least partly due to the equilibration of the microsome suspension to 37°C. In some experiments the initial rates of amylase release from microsomes were determined as accurately as possible (sections 7.2, and 7.4). In order to eliminate any variation introduced by the equilibration of vesicle suspensions at the incubation temperature, these incubations were initiated by injecting 100 μl of a concentrated microsome suspension (5 mg total protein/ml) at 2°C, into 1.9 mls of STKC buffer pre-incubated at the appropriate temperature (usually 37°C). In this way, the dilution necessitated by the amylase assay method was achieved while ensuring that the temperature of the microsome suspension reached 37°C very quickly after the beginning of the measured incubation period.

50 μl aliquots were taken from microsome suspensions during the course of incubation, for immediate assay of amylase activity. Unless otherwise stated, potential effectors of the release phenomenon (e.g. protease inhibitors, cations, denatured proteins) were added to

vesicle suspensions immediately prior to commencing incubation.

2.2.3 Preparation of microsomes in the presence of protease inhibitors

In order to preserve the integrity of any protease-sensitive amylase precursor which may have been present in the pancreatic cells, microsomes were isolated, as described in section 2.2.1, from a chopped pancreas to which a mixture of protease inhibitors was added, immediately after removal of the tissue from the rat, and before the homogenization step. The inhibitor cocktail contained benzamidine HCl (.05 M), PMSF (.1 M), TLCK (.05 M) and 1,10-phenanthroline (.02M) in DMSO. 50 μl of the mixture was added for every ml of pancreas/SKC suspension resulting in final inhibitor concentrations of 2.5 mM (benzamidine and TLCK), 1 mM (1,10-phenanthroline) and 5 mM (PMSF). As soon as it was sedimented from the post-mitochondrial supernatant, the microsome pellet was denatured and dissolved in gel loading buffer, in preparation for polyacrylamide gel electrophoresis, as described in section 2.2.7.

2.2.4 Isolation of stripped pancreatic microsomes

In some of the experiments described in chapter 7, membrane-bound ribosomes were removed from rough microsomes by incubation of crude microsome fractions in the presence of puromycin/KCl or lithium chloride. Following

the degranulation procedure, ribosome-free vesicles were purified on a sucrose "sandwich" gradient as used by Scheele *et al.*, (1978).

Approximately 5-6 mls of a microsome suspension was introduced into a sandwich gradient, all layers of which contained 50 mM Tris HCl/25 mM KCL/.2 mM $CaCl_2$, pH 7.5, and, in some cases 5 mM $MgCl_2$. The composition of the gradient, from bottom to top, was; 1.5 mls of 2.25 M sucrose, 1 ml of 1.3 M sucrose, 6 mls of microsome suspension adjusted to 1.25 M sucrose, 1 ml of 1.2 M sucrose, and $\sim$2.5 mls of 0.3 M sucrose (to top of tube). The gradient was spun overnight (16-18 hours) at 190,000 g_{av} at $2^{o}C$, with a Beckman SW41 rotor, in a Beckman L5-50 or L8 centrifuge.

Smooth, stripped microsomes were recovered, by aspiration of a minimum volume ($\sim$1 ml), from the 0.3 M/1.2 M interface, rough microsomes were collected from the 1.3 M/2.25 M interface, and free ribosomes formed a pellet at the bottom of the tube. The microsome fractions were diluted with STKC to a protein concentration appropriate for amylase assay. This technique resulted in complete, distinct separation of stripped vesicles from rough microsomes and free ribosomes, as assessed by EM examination.

2.2.5 α-Amylase Assay

Amylase activity was measured using an insoluble substrate purchased from Pharmacia Diagnostics as Phadebas amylase test tablets. The assay method employed

was a modification of the method suggested by the manufacturer. Each tablet was suspended, by a magnetic stirrer, in 7.25 mls of 100 mM K_2HPO_4/ 10 mM NaCl/0.2 mM $CaCl_2$, pH 7.1, then 1.45 ml aliquots were dispensed into disposable plastic centrifuge tubes and pre-incubated at 37°C. After the addition of a 50 μl microsome sample, the assay suspension was mixed briefly on a Vortex mixer then returned to the water bath and incubated at 37°C for exactly 60 seconds. The reaction was stopped by the addition of 0.2 mls of .5 N NaOH and the tubes were stored on ice until the end of the experiment. After sedimentation of the insoluble substrate by a 10 min centrifugation at 1,000 g_{av}, in a bench centrifuge, a 1 ml sample of the supernatant was removed, using a Finn pipette. The absorbance of the samples at 620 nm (A_{620}) was measured in either a Hitachi 101 or Varian 657 spectrophotometer.

Under these conditions 50 ng of pig pancreatic α-amylase (Sigma) produced an absorbance increase of 1.0 in 1 min and the assay was linear to an absorbance of 4.0 (Pearce, 1978). The background A_{620} resulting from the incubation of substrate in the absence of microsomes was of the order of 0.01.

2.2.6 Iodination of microsome samples using chloroglycoluril

The method used to iodinate rat pancreatic microsome suspensions with chloroglycoluril was very similar to

the procedure used by Markwell and Fox (1978) to iodinate cultured cells and encapsulated viruses. The optimum ratio of chloroglycoluril to protein in a sample was reported to be 1:10 where membrane surface-specific labelling was desired. 0.5 mg samples of microsomal protein were to be labelled, therefore, disposable soda glass scintillation vials were each plated with 50 μgs of chloroglycoluril. The following procedure was found to give maximum stability and adherence of the chloroglycoluril film to the walls of the reaction vessels. 0.5 ml of a 100 μg/ml solution of chloroglycoluril in chloroform was pipetted into each of 3 glass vials. The chloroform was evaporated by incubation in a commercial clothes dryer on "medium" setting, for approximately 30 mins, leaving a stable coating of chloroglycoluril on the inside of the vessels.

Microsomes were incubated as described in section 3.4, then all samples were cooled slowly to $2^{o}C$. A 1 ml (0.5 mg protein) aliquot of each suspension was incubated in a plated vial, at $2^{o}C$ for 10 mins, in the presence of 5 μl (500 μCi) of sodium ^{125}I-iodide (13-17 mCi/μg). At the end of the 10 min incubation, each sample was decanted from the reaction vessel into a 10 ml, polycarbonate Ti50 centrifuge tube (Nalge) and spun at 100,000 g_{av} for 30 mins. After centrifugation the supernatants were decanted and a 400 μl sample of each was transferred to an eppendorf tube. Each microsome pellet was resuspended in 1 ml of

immunoprecipitation buffer (IP buffer: 10 mM Tris HCl pH 7.4, 150 mM NaCl, 0.5% triton X-100, 0.1% DOC) and 400 μls of each sample were transferred to eppendorf tubes for immunoprecipitation.

The supernatant fractions were adjusted to 150 mM NaCl, 0.5% triton X-100, 0.1% DOC, and 1 mg of purified anti-amylase antibody (in 50 μl of GDW) was added to each supernatant and microsome pellet sample. The tubes were incubated at room temperature for 60 mins, then at $4^{o}C$ overnight. The precipitates were sedimented by spinning for 5 mins in an eppendorf centrifuge and the pellets were washed 3 times in IP buffer. After the final rinse, each pellet was dissolved in 20 μl 4% SDS, 30 μl 2X gel loading buffer (see section 2.2.7) + 5 μl BME. The samples were heated at $100^{o}C$ for 5 mins then loaded immediately onto an SDS-10% polyacrylamide slab gel.

2.2.7 SDS-Polyacrylamide gel electrophoresis

Slab gels were prepared by the method of U.K. 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 pH and acrylamide step gradients thus formed produced good separation of the proteins into distinct bands. The immunoprecipitated, ^{125}I-labelled samples described in section 3.3 were electrophoresed on a gel whose dimensions were: stacking gel, 2 cm x 14 cm x 1.5 mm and separating gel, 10 cm x 14 cm x 1.5 mm. The

samples which were prepared in the presence of protease inhibitors (section 4.4) were electrophoresed on a longer gel in order to completely separate any full-length pre-amylase (M^r 57,500 - 59,000) present, from the mature enzyme (M^r 56,000). The dimensions of the stacking gel were 6 cm x 20 cm x 1.5 mm and those of the separating gel were 32 cm x 20 cm x 1.5 mm. Because of the extra distance travelled by proteins in these gels, the resolution of the bands was generally not as good as that achieved with the shorter gels.

In the case of the shorter gels, electrophoresis was performed at 45 mA for 30 mins to stack the proteins which were then run through the separating gel at 120V for 2 to 3 hours, during which time the current dropped steadily to below 10 mA. Samples on the longer gels were stacked at 45-50 mA (250 - 300V) for 40 mins then run through the separating gel at 250V for 8-9 hours.

Protein bands were detected by staining overnight in 25% isopropanol, 10% acetic acid, 0.05% Coomassie blue, then destaining in 10% isopropanol, 10% acetic acid for 8 to 10 hrs., with gentle shaking. In order to detect radioactively-labelled bands, gels were vacuum and heat dried onto Whatman 3 mm paper in a gel drying apparatus from Hoefer Scientific Instruments Ltd., before being exposed to X-ray film.

2.2.8 Transfer of proteins to nitrocellulose

Microsome samples were electrophoresed on an SDS-

polyacrylamide slab gel as described in section 2.2.7. Proteins were transferred from the unstained gel to a nitrocellulose sheet as described by Towbin *et al.*, (1979). The nitrocellulose (Millipore, 0.45 µm pore size) was wetted briefly with GDW and laid on a Scotchbrite pad. The gel was trimmed to a segment 10 cm x 15 cm which contained the microsome samples with the exception of the very high M^r proteins (>150,000 daltons) and the very low M^r proteins (>20,000 daltons). The gel was carefully laid on the nitrocellulose and all air bubbles were excluded thus ensuring a complete, even contact between the surfaces. Another Scotchbrite pad was placed on top of the gel and the whole assembly was sandwiched between two rigid, plastic grids around each of which had been wound several turns of nichrome wire, which acted as electrodes. Four large rubber bands were passed around the outside, to maintain a firm contact between the gel and the nitrocellulose, and the apparatus was submerged in electrode buffer (25 mM Tris/192 mM glycine/20% (v/v) methanol, pH 8.3).

The transfer was carried out by applying a voltage gradient of 6V/cm for 1 hr. with the nitrocellulose facing the anode. The apparatus was then dismantled and additional protein binding sites on the nitro-cellulose were blocked by soaking in 100 mls of 3% BSA/ 0.9% NaCl/10 mM Tris HCl pH 7.4, at 40°C for 1 hr. The gel was stained with Coomassie blue as described in section 2.2.7 but no remaining protein was detected.

The nitrocellulose was rinsed twice with 0.9% NaCl/10 mM Tris HCl pH 7.4, then sealed in a plastic bag into which was introduced 3.5 mg of anti-amylase antibody in 4.5 mls of 3% BSA/0.9% NaCl/10 mM Tris HCl 7.4. This was incubated at room temperature for 1 hr., then at 4°C overnight.

The nitrocellulose was removed from the bag and washed in 5 x 100 mls of 0.9% NaCl/10 mM Tris HCl pH 7.4 over a period of 30 mins., then resealed in a clean bag with 4 mls of BSA/saline/Tris containing $\sim 0.5 \times 10^6$ cpm of *Staphlacoccus aureus* protein A (Pharmacia) which had been labelled with ^{125}I by I. Borthwick, (Biochemistry Dept., University of Adelaide), using chloroglycoluril as an iodinating agent (Markwell and Fox, 1978). The bag was incubated at 37°C for 2½ hrs. then the nitrocellulose was removed and washed with 5 x 200 mls of 1 M NaCl/10 mM Tris HCl/0.4% sarkosyl, pH 7.4 over a period of 30 mins. Finally, the nitrocellulose was rinsed briefly with GDW, blotted and dried and exposed to X-ray film for 3 hrs.

2.2.9 Purification of rat pancreatic α-amylase

Rat amylase was purified by the method of Vandermeers and Christophe (1968) for use as a molecular weight marker on acrylamide gels and as an antigen for the preparation of a specific antibody. A purified antibody was later obtained from Dr. R. MacDonald (see section 2.1.4) and was used in preference to the antibody raised, in a goat, to the purified amylase.

Pancreata were removed from 15 rats (average weight 250 gms, starved overnight) and homogenized finely in 20 mls of 0.2 M Tris HCl, pH 8.2 by several strokes in a motor-driven Brendler tissue homogenizer. The homogenate was frozen ($-20^{o}C$ for 1 hr), then thawed, to release enzyme from membrane vesicles, and the cell debris and nuclei were removed by spinning at 900 g_{av} for 20 mins in a bench centrifuge, at $4^{o}C$. The supernatants were decanted and stored on ice while the pellets were resuspended in a total of 10 mls of 13 mM Tris HCl pH 8.2. The suspension was spun at 900 g_{av} for 20 mins at $4^{o}C$ and the supernatant fractions were combined with the stored supernatants (total volume ∿25 mls) and centrifuged at 100,000 g_{av} for 60 mins.

The resultant supernatant was loaded onto a Sephadex G-25 column (3 cm x 53 cm) which was linked in series with a DEAE-cellulose (positively charged) column (2 cm x 22 cm). The column buffer was 13 mM Tris HCl pH 8.2 and the flow rate was initially 50 mls/hr which was reduced to 15 mls/hr when the protein entered the DEAE column. The A_{280} of the eluent was monitored. A sample of each fraction was assayed for amylase activity and the most active fractions were pooled. The volume was reduced to 25 mls by placing the pooled fractions in dialysis tubing which was covered with dry Sephadex G-25 and incubated at $4^{o}C$ for 24 hrs.

The reduced fraction was passed through a Sephadex G-100 column (3 cm x 95 cm) and a single main peak was detected by A_{280} monitoring of the eluent. Amylase

activity was assayed and samples of the most active fractions were run on an SDS-polyacrylamide slab gel. Most fractions contained a minor, lower molecular weight contaminant as well as amylase. The amylase-peak fractions were pooled and passed through a Biogel P100 column (3 cm x 60 cm) equilibrated with 13 mM Tris HCl/0.1 M NaCl, pH 8.2. The running speed was 25 mls/hr and 5 ml fractions were collected. One A_{280} peak was detected and was found to contain amylase activity. Electrophoresis of samples of the most active fractions showed only one protein band of M^r 56,000 daltons.

2.2.10 Denaturation of Proteins

10 mgs of protein (ovalbumin, BSA, globin, amylase, lysozyme, RNAse or catalase) were dissolved in 200 µl 0.25 M Tris HCl pH 7.5 to which was added 240 mgs re-crystallized urea, 12 µl 1M DTT and GDW to a total volume of 0.5 ml. The solution was incubated at 37°C for 60 mins then at 100°C for 15 mins. 0.5 ml of GDW was added and the solution was cooled to 2°C on ice. 100 µl of 50% trichloroacetic acid (TCA) was added and the protein was precipitated at 2°C for 10 mins. The pellet resulting from centrifugation at 1,000 g_{av} for 10 mins at 4°C was dissolved in 200 µl of .2N NaOH and 800 µl STKC buffer. The preparations were stored at -20°C between experiments.

Denatured ovalbumin and denatured BSA were digested with trypsin as follows. 20 mgs of each protein were denatured as described above, TCA precipitated, neutralized

and re-dissolved in 2 mls each of STKC buffer. 200 μg of TPCK-trypsin (Worthington) were added every 6 hrs during 24 hr incubation at 37°C. Proteolysis was terminated by the addition of PMSF to a final concentration of 2 mM. The tryptic fragments were precipitated with 5% TCA, spun down, neutralized with 0.4 ml of 0.2 N NaOH and re-dissolved in 1.6 ml STKC buffer. The solutions were stored at -20°C.

2.2.11 Electron Microscopy

Microsome samples were suspended at a density of 0.2 - 0.3 mg total protein/ml in cold STKC buffer. Carbon-coated copper grids were soaked in chloroform for 5 mins to render the surface hydrophilic, then blotted and dried thoroughly. One drop of microsome suspension was applied to a grid and allowed to adsorb to the surface for 30 secs following which the grid was blotted and stained for 10 secs with one drop of fresh 2% (w/v) uranyl acetate. After blotting and drying, the grids containing negatively-stained microsomes, were examined at a magnification of 20,000X in a Siemens Elmiskop 102 electron microscope.

CHAPTER 3

RESULTS

PROTEASE-SENSITIVE AMYLASE RELEASE FROM MICROSOMES INVOLVES THE PASSAGE OF PROTEIN ACROSS AN APPARENTLY INTACT MEMBRANE.

3.1 INTRODUCTION

Preliminary characterization of the pancreatic microsome enzyme release phenomenon revealed that incubation of rat pancreatic microsomes at 37°C, in the absence of Mg^{2+}, results in the co-ordinate release of the secretory enzymes normally contained within these vesicles (Tabe, 1978). The release process could be followed by assaying for the appearance of any one of these enzymes in the suspending buffer, the most obvious choice being α-amylase because of its relative abundance and the availability of a simple assay for its activity.

The assay involves incubation of microsomes with an insoluble substrate for the amylase enzyme (section 2.2.5). As it has been demonstrated that *de novo* synthesis does not occur in this system, (Pearce *et al.*, 1978), the increase in apparent amylase activity, with incubation time, reflects transfer of the enzyme from the intra-microsomal to the extra-microsomal compartment. This has been confirmed by separating the microsomes from the medium, by centrifugation, and showing that an increase in amylase activity in the medium correlates with a depletion of the intra-microsomal enzyme (Pearce *et al.*, 1978). By inference therefore, this transfer of amylase from an intra-microsomal to an extra-microsomal location seemed to involve the passage of an active enzyme across an intact

membrane. Latent amylase activity was preserved, but the transfer was abolished by the addition of active proteases to microsome suspensions, leading to the hypothesis that the release of enzymes across the membrane may be mediated by an integral membrane transport protein (Pearce *et al.*, 1978). These suggestions were supported by the experiments described in this chapter.

3.2 RELEASE OF AMYLASE FROM MICROSOMES

The microsomal fraction used in experiments described in this thesis was obtained by resuspension, in buffered sucrose, of the pellet produced by high speed centrifugation of a post-mitochondrial supernatant derived from homogenized rat pancreas (for details of fractionation procedure, see section 2.2.1). Up to 90% of the vesicles were intact rough microsomes, as judged by inspection of electron micrographs of sectioned pellets (Pearce, 1978). The remainder of the vesicles were apparently smooth microsomes, with large numbers of free ribosomes also present.

In a typical experiment, the microsomal pellet was resuspended, by gentle homogenization, in STKC buffer (0.3 M sucrose, 50 mM TrisHCl pH 7.5, 25 mM KCl, 0.2 mM $CaCl_2$), diluted to an appropriate protein concentration, (usually .2-.3 mg total microsomal protein/ml) and incubated at 37°C. At regular time intervals during the course of the incubation, aliquots were taken from the suspension and assayed immediately for amylase activity by the method described in section 2.2.5.

A certain "background" level of amylase was always detected in the zero time assay (see, for example, fig. 3.1). If microsomes were re-sedimented immediately after their first resuspension, without any incubation, this background amylase was found largely in the supernatant fraction. Homogenization of the second pellet generated a microsome suspension with a similar background level and a correspondingly diminished total amylase content (results not shown). It therefore seemed that the basal level of free amylase in fresh microsome suspensions arose mainly from damage of some vesicles during resuspension, by homogenization, of microsomal pellets.

By virtue of the nature of the assay procedure, each consecutive assay, during the incubation of a microsome suspension, represented a cumulative estimate of the amylase activity in the extra-microsomal medium. A typical release profile incorporated a short, initial lag period followed by a rapid release phase leading to a plateau level of enzyme activity which was maintained during subsequent incubation. The total amylase enzyme activity present in a suspension was established by disrupting the membranes with 0.1% w/v deoxycholate (DOC). Samples were incubated at 37°C for 10 minutes after the addition of the detergent to allow the release of all the enzyme before the final assay (see, for example, fig. 3.1).

The rate of enzyme release, the zero time amylase level and the percentage of the microsome content amylase

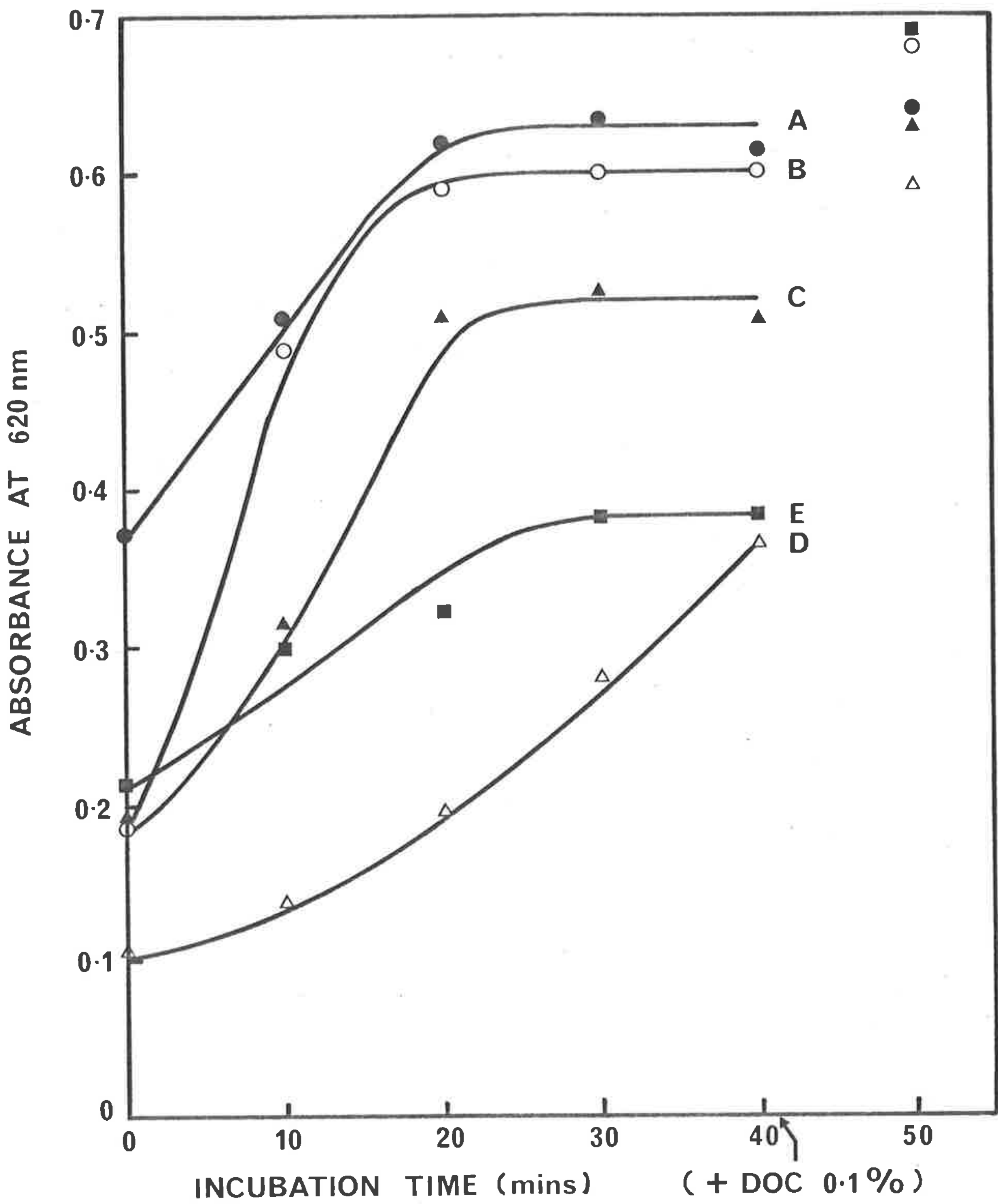
FIGURE 3.1

Incubation of microsomes at 37°C.

Five different preparations of rat pancreatic microsomes, suspended at a concentration of 0.2 - 0.3 mg total microsomal protein/ml in STKC buffer, were incubated at 37°C. At 10 minute intervals, 50 µl aliquots were removed and assayed immediately for α-amylase activity (for procedural details, see sections 2.2.2 and 2.2.5).

The total amylase activity present in each suspension was estimated by adding the detergent deoxycholate (DOC) to the microsomes after 40 mins incubation. A final amylase assay was performed 10 mins later, after release of all remaining intra-vesicular enzyme by disruption of the membranes.

FIG 3.1.



which was released (i.e. the plateau level relative to the DOC-releasable total) all varied noticeably between microsome preparations. Figure 3.1 compares the profiles of amylase release from several preparations of microsomes which were isolated on different occasions, from different animals. In by far the majority of cases, virtually all of the originally latent, microsome-associated amylase activity was released into the medium on incubation of suspensions of vesicles at 37°C (for example; fig. 3.1, profiles A, B and C). Less frequently, slower, incomplete enzyme release was observed (for example; fig. 3.1, profiles D and E).

Although the primary cause of these fluctuations is unknown, it was thought that variations in physiological parameters such as the age and nutritional status of the animals used in the studies may contribute to the observed differences. These factors were standardized where possible, so that male rats of 250 to 300 gms body weight were normally killed after a 15 to 20 hour fast.

Although it is possible that physiological parameters may affect the subsequent phenomenon of enzyme release from isolated rat pancreatic microsomes, it must be emphasized that this reverse transport of active secretory proteins across the ER membrane would not be expected to occur *in vivo*. This statement is based on the observation that amylase release from microsomes is prevented by physiological concentrations of Mg^{2+} ions (Pearce *et al.*, 1978). It was found that the release phenomenon was also prevented by Zn^{2+} (fig. 3.2), Mn^{2+} (fig. 3.3) and Ca^{2+}

FIGURE 3.2

The effects of Zn^{2+} on amylase release

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

(●) no additions

(○) 0.1 μM $ZnCl_2$

(▲) 1.0 μM $ZnCl_2$

(△) 10 μM $ZnCl_2$

(□) 100 μM $ZnCl_2$

FIG 3.2.

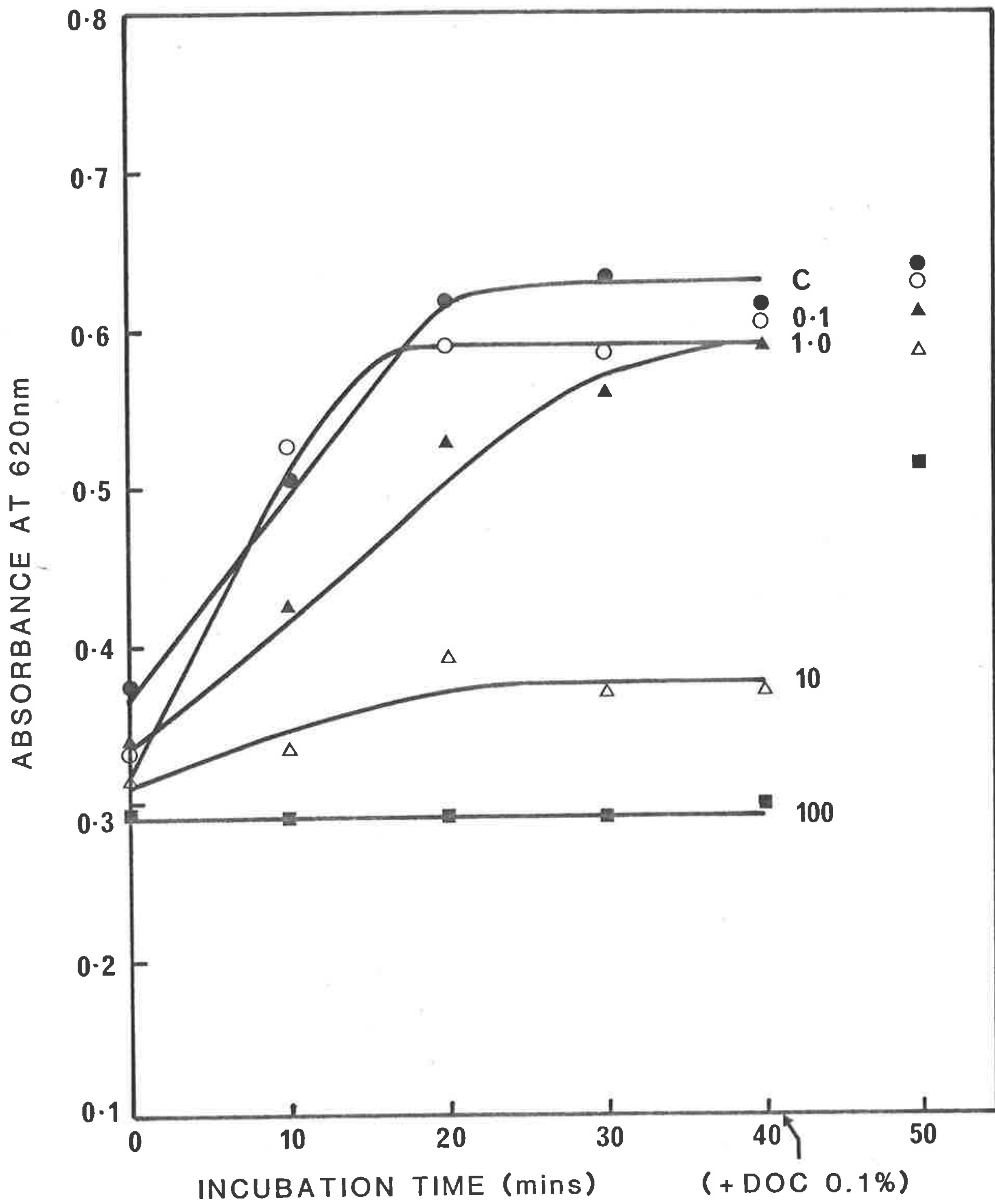


FIGURE 3.3

The effects of Mn^{2+} on amylase release

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

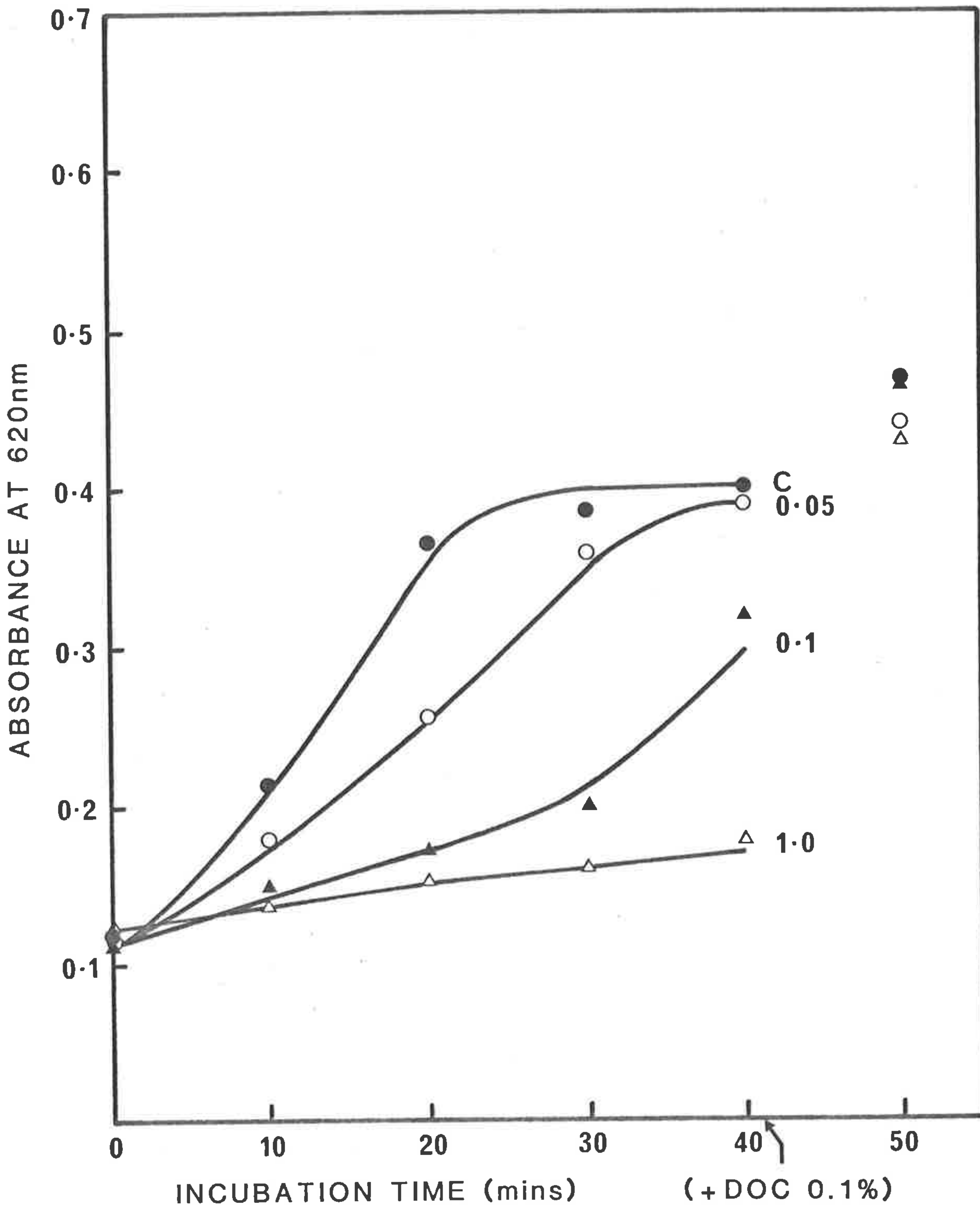
(●) no additions

(○) 0.05 mM $MnCl_2$

(▲) 0.1 mM $MnCl_2$

(△) 1.0 mM $MnCl_2$

FIG 3.3.



(fig. 3.4) ions at concentrations exceeding 100 μM, 1 mM and 2 mM respectively. In all the experiments described in this thesis, microsomes were suspended in STKC buffer which contained 0.2 mM $CaCl_2$ which stabilized the amylase enzyme activity without significantly retarding the enzyme release phenomenon (see fig. 3.4).

3.3 PROTECTION OF INTRA-MICROSOMAL AMYLASE FROM PROTEOLYSIS

It was pointed out in section 1.9 that the rat pancreatic microsome enzyme release phenomenon represents a radical departure from the currently accepted ideas on protein translocation across the ER membrane. In investigating the release phenomenon, it was therefore initially sought to confirm that release of secretory enzymes from the rat microsomes actually entailed passage of the proteins across the membrane rather than just desorption from an extra-vesicular location.

As stated in section 1.9, it had been demonstrated that microsome-associated RNAse was protected from proteolysis, but that released RNAse, in the suspending medium, was readily degraded. This may be regarded as evidence that RNAse is originally sequestered within intact membrane vesicles from which it is subsequently released. At the time when this experiment was performed, it was thought that such a demonstration would be difficult in the case of α-amylase, due to the intrinsic resistance of that enzyme to many proteases (Pearce *et al.*, 1978). As amylase release is the preferred parameter of study,

FIGURE 3.4

The effects of Ca^{2+} on amylase release

Rat pancreatic microsomes were incubated at 37°C in STK buffer (0.3 M sucrose; 50 mM Tris HCl pH 7.5, 25 mM KCl) containing:

(●) no additions

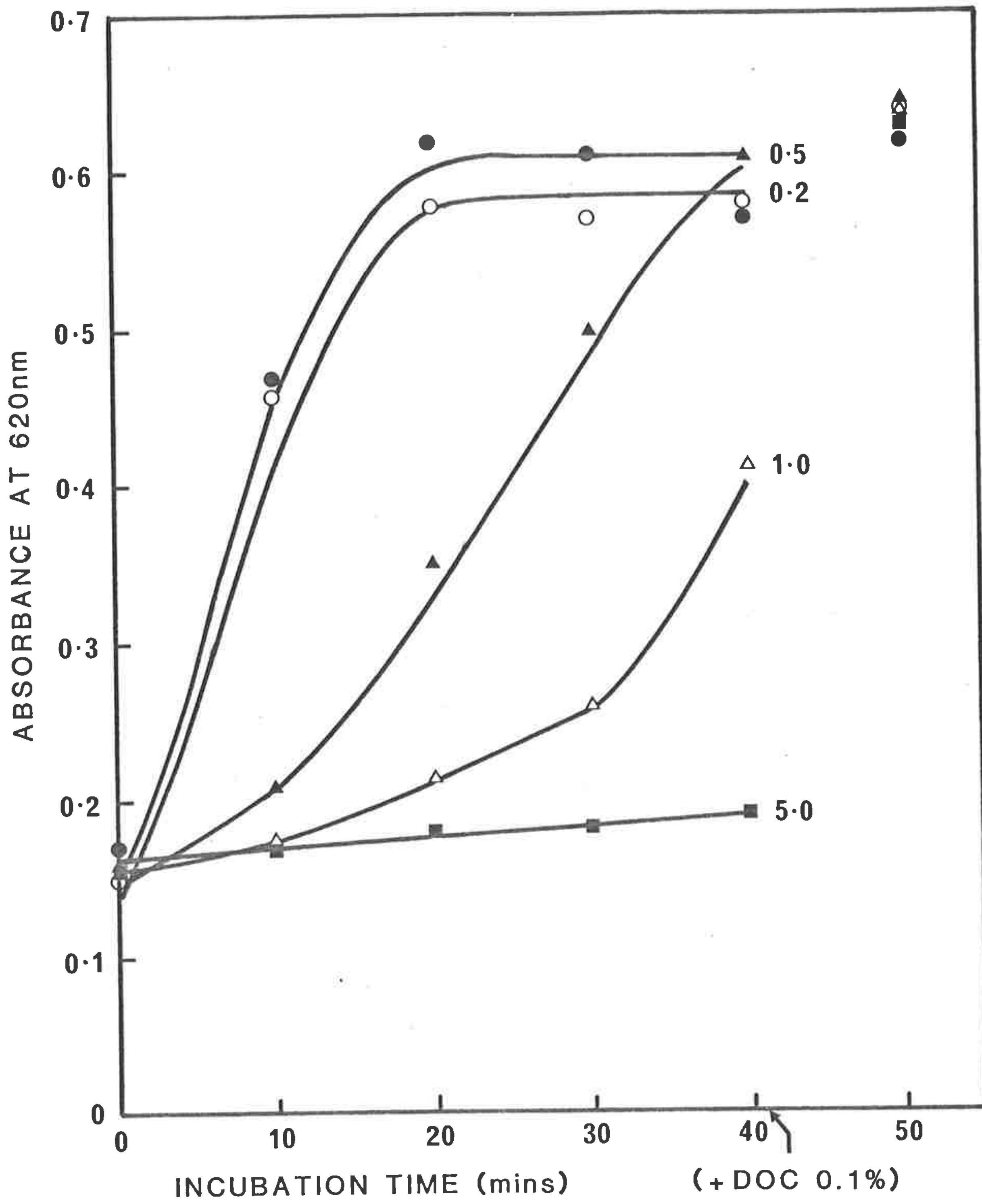
(○) 0.2 mM $CaCl_2$

(▲) 0.5 mM $CaCl_2$

(△) 1.0 mM $CaCl_2$

(■) 2.0 mM $CaCl_2$

FIG 3.4.



it was considered important to confirm that this enzyme does actually cross an intact membrane during its release from microsomes at 37°C.

A solution of amylase was prepared by incubating microsomes (3 mg total protein/ml in STKC) at 37°C for 40 mins., then removing the depleted vesicles by centrifugation at 100,000 g_{av} for 30 mins., at 2°C. The resultant post-microsomal supernatant (containing approximately 0.2-0.4 mg amylase/ml) was divided into 0.5 ml aliquots each of which was incubated with one of a number of proteases, at a concentration of 2 mg/ml, for 30 mins. at 37°C (Table 3.1). The amylase activity was resistant to degradation by chymotrypsin, trypsin, pronase and papain, but sensitive to the bacterial hydrolase, subtilisin BPN'. This protease was therefore used to investigate the trans-membrane orientation of α-amylase before and after incubation of microsome suspensions at 37°C.

Freshly prepared microsomes were suspended in STKC at a concentration of approximately 3 mg total protein/ml and 0.5 ml aliquots were incubated in the absence of protease, or in the presence of 2.5 mg/ml subtilisin added at 0 mins., 30 mins. or 40 mins. after the beginning of incubation at 37°C. After a total incubation time of 70 mins., protease was inactivated by the addition of PMSF to a final concentration of 100 μg/ml, and the total amylase present was estimated by disrupting the microsomal membranes with DOC. The results presented in figure 3.5 show that the control, extra-vesicular amylase

TABLE 3.1

The protease sensitivity of rat pancreatic α-amylase

Protease	Amylase Activity at 0 mins (A_{620} units)	Amylase activity at 30 mins (A_{620} units)
None	.670	.690
Trypsin	"	.590
Pronase	"	.620
Papain	"	.550
Subtilisin BPN'	"	.095

0.5 ml aliquots of a post-microsomal supernatant containing ∿0.2 - 0.4 mg/ml amylase were incubated at 37°C, for 30 mins, in the presence of each of the listed proteases (final concentration of each protease = 2 mg/ml). 50 μl samples were assayed for amylase activity at 0 mins and 30 mins.

FIGURE 3.5

The protection of intra-microsomal amylase from exogenous protease

Rat pancreatic microsomes, suspended in STKC buffer at a concentration of ∿3 mg total microsomal protein/ml, were incubated at 37°C. One suspension (●), was incubated for 40 mins, at which time subtilisin was added to give a final protease concentration of 2.5 mg/ml. Another suspension (O) received the same amount of subtilisin at 30 mins, while in a third suspension (Δ), subtilisin was added immediately before the beginning of the 37° incubation. Amylase activity was assayed at 10 min intervals in all 3 suspensions.

At 70 mins, PMSF was added to all suspensions (final concentration = 100 μg/ml), immediately followed by the addition of DOC (final concentration 0.1%). Total amylase activity in each suspension was assayed 10 mins later.

In this experiment 5 μl aliquots were taken for assay of amylase activity, as the original microsome suspensions were more concentrated than in other experiments.

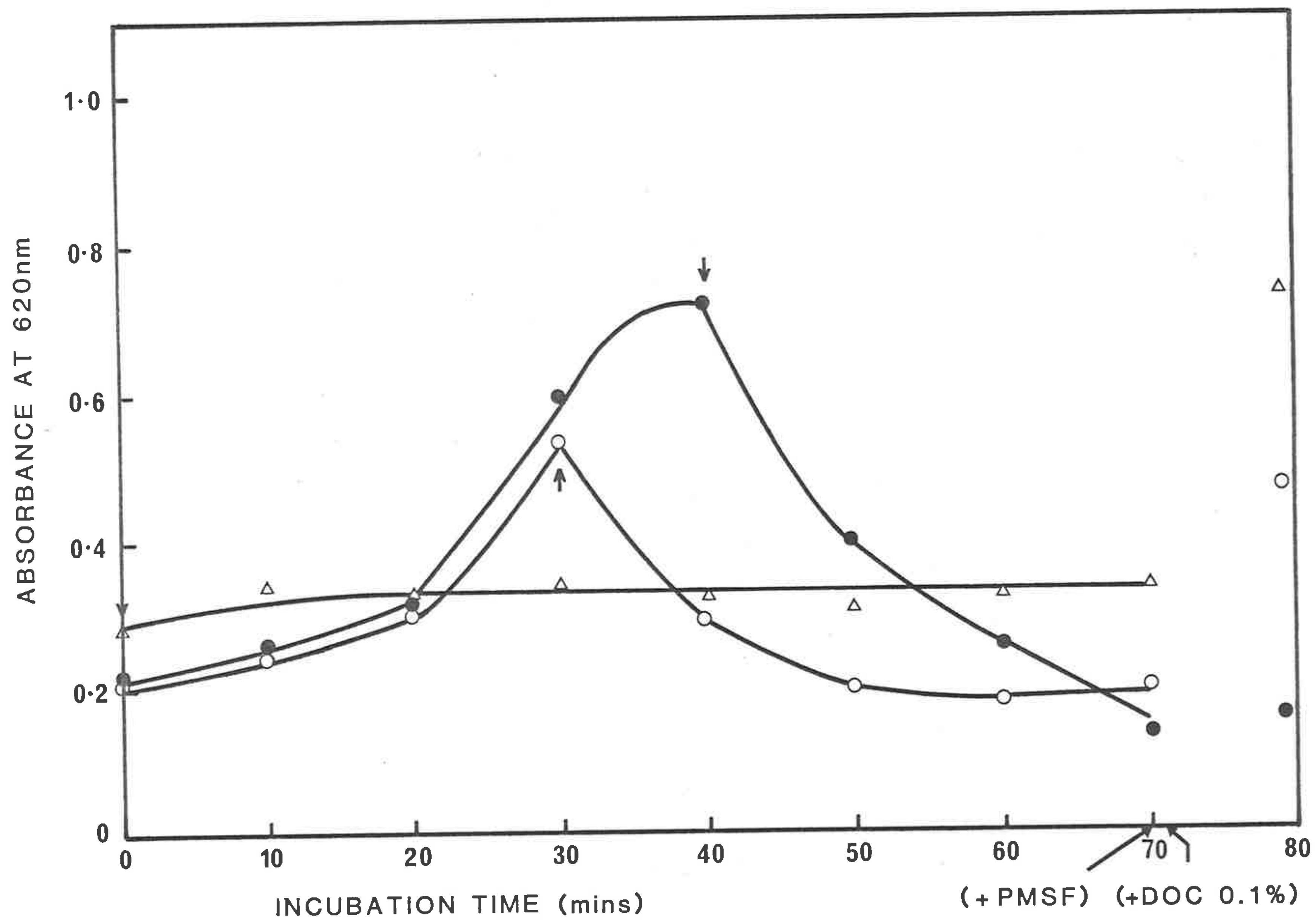


FIG 3.5

concentration reached a plateau at 40 mins. after the beginning of incubation, which represented release of virtually all of the microsome content enzyme.

If subtilisin was added to such a system at 40 mins., the released amylase was rapidly inactivated. Subsequent addition of PMSF and detergent revealed no latent enzyme activity. If subtilisin was added at 30 mins., amylase release was abruptly halted and the amylase activity in the medium declined to a basal level. If this suspension was then treated with PMSF and DOC, a considerable quantity of amylase was released, implying that addition of the protease at 30 mins. stopped the release process and sealed the remaining intra-microsomal amylase in a protease-resistant compartment, while degrading the released enzyme. It can be further inferred that the protease could not enter the microsomes, thus testifying to the integrity of the vesicle membranes during incubation at 37°C.

When subtilisin was added at zero time, little or no amylase release was observed during the subsequent incubation, however detergent-disruption of the microsomes, after addition of PMSF, revealed a total enzyme content only slightly lower than that of the control suspension. Therefore, before release, amylase was sequestered from proteolytic attack in intact membrane vesicles. During incubation at 37°C, it became sensitive to exogenous protease, thereby implying that amylase was released from the intra-microsomal compartment, through the vesicle membrane.

A puzzling feature of these results is the fact that the background amylase appears to be protease resistant. If the protease-treated microsomes were sedimented by centrifugation, over 60% of the extra-microsomal amylase was found in the supernatant fraction, (results not shown) thus it would appear that the resistant amylase is in free solution. In this case, subtilisin-resistance would presumably be conferred by some structural feature of the amylase enzyme. For example, the original background amylase could be derived from lysis of contaminating condensing vacuoles or secretory granules, and therefore could differ from "releasable", intra-microsomal enzyme in the degree of post-translational modification of the protein structure. Alternatively, the amylase background could be associated with the surface of the microsomes in such a way that the enzyme is protected from attack by subtilisin, but available to the insoluble substrate, and easily dislodged by high speed centrifugation, which seems improbable. The behaviour of the zero time, "background" amylase cannot be satisfactorily explained on current data, however this aspect will be further investigated in this laboratory.

3.4 PROTECTION OF INTRA-MICROSOMAL AMYLASE FROM IODINATION

Recently, experiments wherein a single protease is used as a probe to determine the transmembrane orientation of a protein have been criticized on the grounds that exposed portions of the protein under investigation may not contain cleavage sites for that particular protease

(Coleman and Bell, 1980). Since α-amylase was resistant to all available proteases, except subtilisin, an alternative approach was adopted to confirm the initial, intra-vesicular location of amylase in rat pancreatic microsome suspensions.

Markwell and Fox (1978) have investigated the use of the iodinating reagent, 1,3,4,6-tetra-chloro-3α,6α diphenylglycoluril (chloroglycoluril) as a membrane surface-specific label in a number of well-characterized systems including enveloped viruses, erythrocytes and cultured cell lines. They established conditions under which proteins exposed on the surface of the membranes were iodinated, whereas proteins on the inner face of the lipid bilayer were not.

Chloroglycoluril was plated onto the surface of glass vials by evaporation of a chloroform solution of the compound. Because this substance is not water-soluble, cells or viruses in aqueous suspension could be introduced into the plated reaction vessels without dislodging the chloroglycoluril from the walls of the tubes. If a low concentration of ^{125}I-sodium iodide was added to such a system, the generation of molecular ^{125}I by the chloroglycoluril resulted in membrane surface-specific labelling of cellular or viral proteins. Using the same experimental conditions with rat pancreatic microsome suspensions, it was possible to demonstrate that α-amylase was inaccessible to chloroglycoluril-generated ^{125}I before release, but became available for iodination during incubation at 37°C.

Glass vials were "plated" with chloroglycoluril, as

described in section 2.2.6. Aliquots of microsomes were incubated for 30 mins at:-

(1) $2^{o}C$,

(2) $37^{o}C$,

(3) $37^{o}C$ in the presence of chymotrypsin.

All suspensions were then cooled slowly to $2^{o}C$. 1 ml samples (containing 500 μgs total protein) were taken from each suspension and added to separate, chloroglycoluril-plated vials, in which they were incubated at $2^{o}C$ for 10 mins in the presence of a concentration of ^{125}I shown by Markwell and Fox to give surface-specific labelling of membranes. The reaction was terminated by pouring the microsome suspensions out of the plated tubes. Microsomes were sedimented by centrifugation at 100,000 g_{av} for 30 mins and anti-α-amylase immunoprecipitates of the resultant pellets and supernatants were electrophoresed on a polyacrylamide/SDS gel (for procedural details see sections 2.2.6 and 2.2.7).

Protein bands were detected by Coomassie blue staining, while ^{125}I-labelled bands were detected by autoradiography of the dried gel. Figure 3.6 shows that α-amylase present in the supernatants was strongly labelled with ^{125}I, however, α-amylase present in the microsomal pellet after incubation at $2^{o}C$, or at $37^{o}C$ in the presence of protease, contained no detectable ^{125}I. The lower molecular weight bands on the gel were labelled in parallel with amylase and were immuno-precipitable by the specific anitbody. Whether they represent amylase degradation products, other cross-reacting material or non-specifically

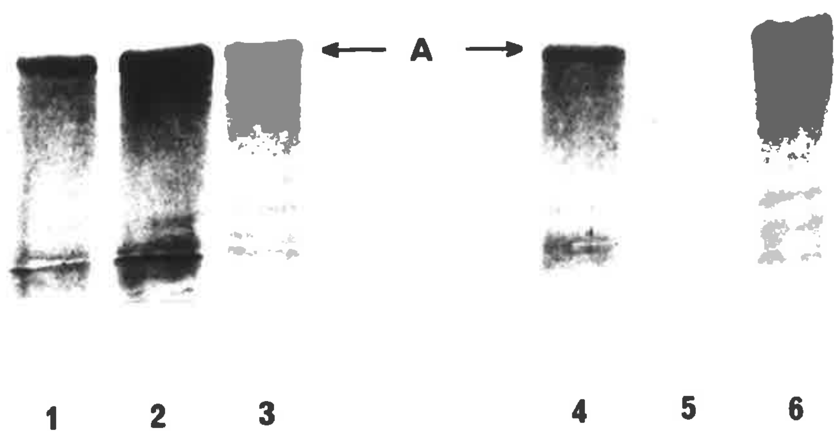
FIGURE 3.6

The protection of intra-microsomal amylase from iodination

Aliquots of rat pancreatic microsomes (0.5 mg total mic. protein/ml STKC) were incubated and subjected to membrane surface-specific iodination, using chloro-glycoluril, as described in the text. After separation into pellet (intra-microsomal) and supernatant (extra-microsomal) fractions, immuno-precipitated α-amylase was electrophoresed as shown.

Top panel: Coomassie Blue protein-staining.
Bottom panel: Autoradiography (^{125}I).

Track No.	Sample	
1	microsomes incubated at 2°C	: s/n
2	microsomes incubated at 37°C	: s/n
3	microsomes incubated at 37°C in the presence of 10 μg/ml chymotrypsin	: s/n
4	microsomes incubated at 2°C	: pellet
5	microsomes incubated at 37°C	: pellet
6	microsomes incubated at 37°C + chymotrypsin	: pellet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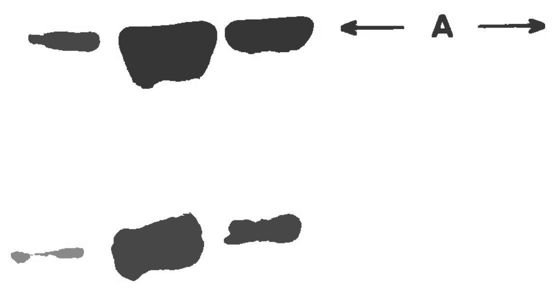

A

precipitated material is not known.

The complete absence of ^{125}I in amylase found in microsome pellets reaffirms the conclusions drawn from the protease protection experiment described in section 3.3. The inaccessibility of the amylase to surface-specific labelling of microsomes bespeaks an intra-vesicular location of the enzyme, although it is impossible to say whether the enzyme is free in the lumen of the microsomes or associated with the inner face of the membrane. Since iodination of proteins occurs only at tyrosine residues, a third possibility; that parts of the protein containing no tyrosine are exposed on the membrane surface, cannot be excluded. The absence of ^{125}I-labelling of microsomal amylase also indicates that the microsomal membranes in these preparations form intact permeability barriers, as Markwell and Fox (1978) found that internal, nucleo-capsid proteins were labelled in addition to the surface proteins, if the membranes of enveloped viruses were damaged.

Thus, the finding that microsome-associated amylase is inaccessible to ^{125}I, whereas enzyme released by incubation at 37°C is strongly labelled, indicates that the amylase travels across intact microsomal membranes during the release phenomenon. The fact that amylase became accessible to ^{125}I after being released from freshly-prepared vesicles by detergent-disruption of the membranes (results not shown) is also consistent with this interpretation.

Amylase within protease-treated microsomes was unlabelled (fig. 3.6) demonstrating that these membranes

were intact; yet the protease had modified the vesicles such that the release of significant quantities of contained enzyme did not occur. Some amylase release from these microsomes was observed, as a relatively low concentration of protease was used in this experiment. The strong labelling by ^{125}I of this small amount of released amylase provides a control for the effect of the protease on the labelling procedure (fig. 3.6).

As well as indicating that microsome-associated amylase is sequestered in intact membrane vesicles, the results presented in sections 3.3 and 3.4 demonstrate that the release of amylase from the vesicles is prevented by the proteases chymotrypsin and subtilisin (figs. 3.5 and 3.6). Pearce *et al.*, (1978) have hypothesized that the mechanism of this effect may be the degradation of a membrane-integrated, amylase-transport protein which is exposed on the outside of the rat pancreatic microsomes. This suggestion is examined in section 3.5.

3.5 INHIBITION OF AMYLASE RELEASE BY PROTEOLYSIS OF THE MICROSOMES

It had previously been demonstrated that release of amylase at 37°C from rat pancreatic microsomes was very sensitive to the presence of added, active proteases in the suspending medium. For example, concentrations of chymotrypsin as low as 2 μg/ml completely abolished the release of amylase from microsomes suspended in STKC buffer at a density of 0.2 to 0.3 mg total microsomal protein/ml (Tabe, 1978). If the observed inhibition of

release was due to the destruction of an integral membrane transport protein, as hypothesized by Pearce *et al.*, (1978), the continued presence of the protease should not be required to give continued inhibition of release after the initial destruction of the transport protein.

In order to verify this point, two aliquots of microsomes, containing 5 mg/ml total microsomal protein, were incubated at 2°C, for 14 mins., one (A) in the presence of 1 mg/ml chymotrypsin, and one (B) in the absence of protease. A high concentration of protease was used to compensate for the high concentration of microsomal protein and the diminished activity of the protease at 2°C. After incubation, PMSF was added to both suspensions, to a final concentration of 0.25 mg/ml (thereby inactivating the protease in sample A), and the microsomes were sedimented by centrifugation at 100,000 g_{av} for 30 mins. at 2°C.

The supernatants were discarded and the microsome pellets were resuspended in fresh STKC, producing suspensions A and B, both of which contained latent amylase activity, although the A vesicles had been modified by proteolysis at 2°C while the B vesicles had not.

The release of amylase from both samples, during subsequent incubation at 37°C was assessed as described in section 2.2. In order to determine whether any residual, active protease or any other diffusable inhibitor remained in the protease-treated microsomes, aliquots of A and B were mixed, and amylase release from the mixture was

followed as above. The results appear in figure 3.7.

The control microsomes (B), released virtually 100% of their content amylase, while the protease-treated microsomes (A) released less than 40% of their content enzyme. The remaining amylase in these microsomes was releasable by DOC-disruption of the membranes. The profile of amylase release from the mixed suspension of A and B microsomes corresponded almost exactly to the sum of the separate curves A and B indicating that there was no inhibition of amylase release from control microsomes by the addition of protease-treated vesicles.

These results show that the inhibitory effect of active protease on amylase release from microsomes is indeed not dependent on the continued presence of the degradative enzyme. Brief contact between the protease and the microsomes was sufficient to produce a permanent inhibition of the release phenomenon, a finding which is consistent with the proposal that the protease attacks a protein on the surface of the vesicle membrane. PMSF, which was used to inactivate the protease, had no effect on amylase release at the concentration used here, as was evident from the lack of inhibition of release from control microsomes, to which PMSF had also been added.

3.6 SUMMARY AND DISCUSSION

In assembling the results described in this chapter, it can be stated that the amylase present in fresh, rat pancreatic microsomes is protected from degradation by the bacterial protease, subtilisin BPN'. Treatment of the

FIGURE 3.7

The effects on amylase release of brief proteolysis of the microsomes

Rat pancreatic microsomes (5 mg total mic. protein/ml STKC) were incubated at 2°C for 14 mins in the absence (B) or in the presence (A) of 1 mg/ml chymotrypsin. After addition of PMSF (final conc. 0.25 mg/ml) to each suspension, the microsomes were sedimented, and then resuspended in fresh STKC at a concentration of 0.25 mg total mic. protein/ml.

The following suspensions were incubated at 37°C:

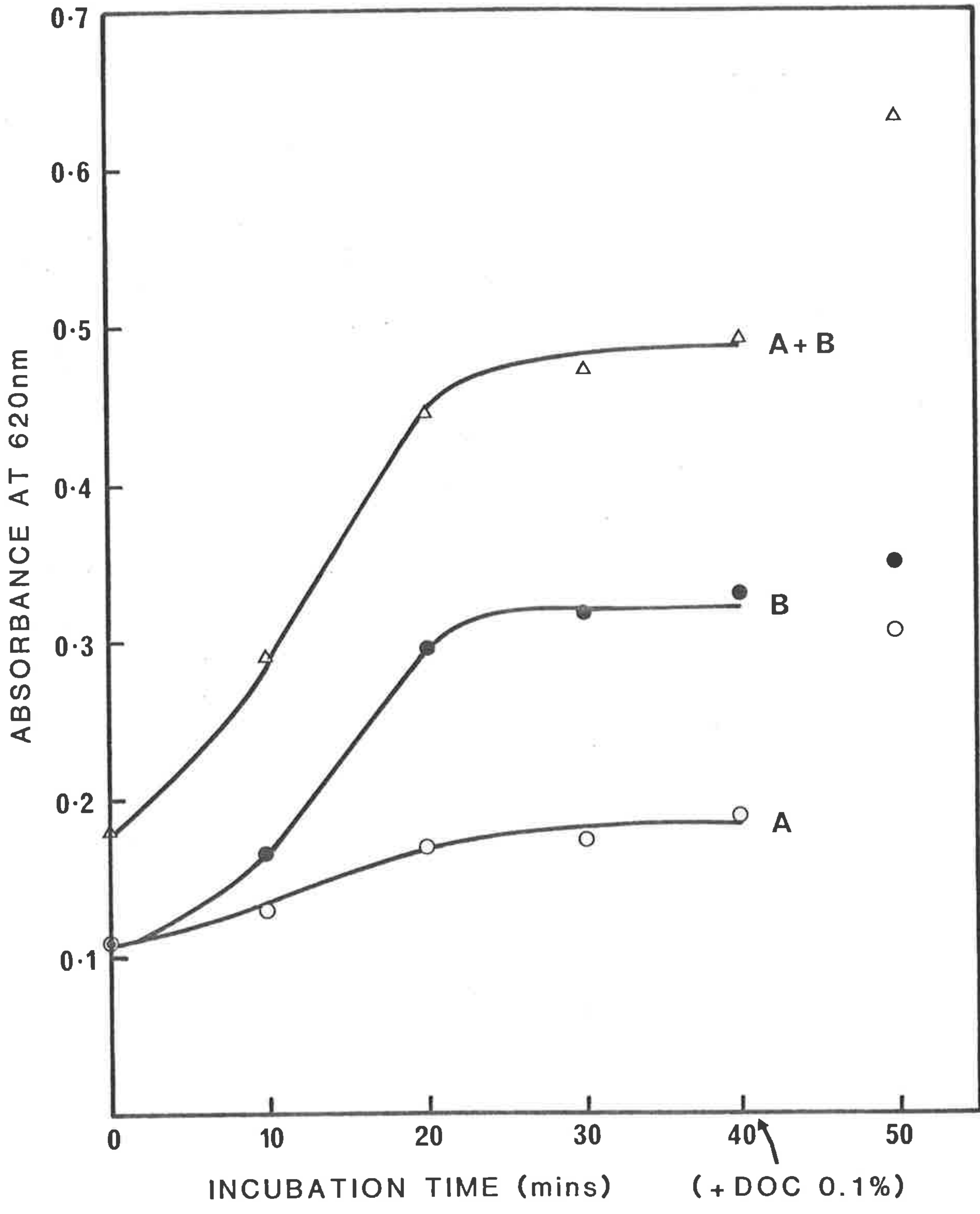
(○) 2 mls suspension A

(●) 2 mls suspension B

(△) 1 ml suspension A
+ 1 ml suspension B

50 μl aliquots were taken at 10 min intervals for immediate assay of amylase activity.

FIG 3.7.



microsomes with either chymotrypsin or subtilisin seals the amylase inside the vesicles. In this situation, the amylase is protected from attack by either protease, indicating that it is enclosed by intact, microsomal membranes. Upon disruption of the membranes with detergent, active amylase is released into the suspending medium where it can be readily degraded by subtilisin, while maintaining its intrinsic resistance to chymotrypsin. Similarly, as it appears in the medium, amylase released from control microsomes by incubation at 37°C becomes sensitive to degradation by subtilisin.

These observations support the suggestion that amylase is transferred across the intact microsomal membrane during its release from the vesicles. This proposal is further verified by the finding that microsome-associated amylase is protected from an extra-microsomal iodinating agent before release of the enzyme from the vesicles, but is readily iodinated after its release into the suspending medium, by incubation of the microsomes at 37°C. The amylase inside protease-treated membranes was also shown to be resistant to iodination.

These results, considered together with the finding that lasting inhibition of amylase release could be achieved by brief proteolysis of the microsomes, point to the passage of α-amylase across the microsomal membrane via the agency of an integral membrane protein which is exposed to external proteolytic attack. This conclusion reaffirms the suggestions put forward by Pearce *et al.*, (1978) based on studies of the release of RNAse and amylase from rat

pancreatic microsomes. This confirmation was considered necessary in view of the superficial improbability of the proposal that fully-formed enzymes could traverse the intact microsomal membrane in the opposite direction to the physiological process of secretion. Experiments described in the following chapters were aimed at elucidating the relationship between this *in vitro* release phenomenon and the *in vivo* process of secretion.

It should be strongly emphasized that previous work (Pearce, 1978) eliminated the possibility that amylase release from microsomes was caused by the action of a phospholipase or protease on the microsomal membranes.

It was found that the addition of trypsin to microsomes half-way through the release of intra-microsomal amylase immediately halted the amylase release process. Continued release of amylase would be expected if the amylase were leaking through "holes" in the membrane made by a contaminating degradative enzyme.

CHAPTER 4

RESULTS

THE EFFECTS OF INHIBITORS OF SIGNAL PEPTIDASE ACTIVITY ON THE RELEASE OF AMYLASE FROM RAT PANCREATIC MICROSOMES

4.1 INTRODUCTION

Although the membrane proteins participating in secretory protein translocation across the ER membrane have yet to be fully characterized, the existence of at least four functionally distinct components can be inferred from experimental data (for details see section 1.7). At least in terms of the signal hypothesis, the translocator mechanism is thought to consist of a signal peptidase enzyme, a signal peptide binding site, a ribosome binding site and one or more integral membrane, pore-forming proteins. In comparing the mechanism through which amylase escapes from rat pancreatic microsomes *in vitro*, with the mechanism mediating translocation of secretory proteins into the ER *in vivo*, it is useful to consider the components of the latter system separately.

In investigating the mechanism of the transport of secretory proteins into the ER, two main approaches have been used. Many studies have examined the structures of the translocated proteins themselves, (for references see chapter 1), while others have aimed to identify and characterize the membrane-associated components of the translocation machinery (see section 1.7). The latter approach, which has generally taken the form of searching for the structure associated with an observable function, has been obstructed by the complexity of the protein composition of the ER membrane. One of the most easily

observable features of the translocation process is the proteolytic processing of secretory proteins which accompanies their transport into microsomes *in vitro*. Studies on the enzyme responsible for this covalent modification have been undertaken by several workers, with the result that signal peptidases from both the prokaryote plasma membrane and eukaryotic microsomal membranes have been characterized with respect to their sensitivity to a number of known protease inhibitors (Strauss *et al.*, 1979, Mumford *et al.*, 1979, Sussman *et al.*, 1976, Gayda *et al.*, 1979).

The spatial relationship between the processing enzyme and the other inferred components of the translocator apparatus, such as pore-forming proteins, ribosome binding site and signal receptor protein, is unknown. The tight coupling which seems to exist between the processing and segregation of secretory proteins, by microsomes, in *in vitro* translation systems, suggests that the signal peptidase may be closely associated with, if not an integral part of a complex formed by the other components. As a first step to comparing the putative, membrane transport protein involved in the pancreatic microsome enzyme release phenomenon, with the protein complex mediating secretory protein transport into the ER, the responses of each system, to a number of protease inhibitors, were examined.

The effects, on amylase release, of adding a variety of inhibitors to rat pancreatic microsome suspensions, were compared with the published effects of the same inhibitors

on signal peptidase activity in several different situations. It was deemed valid to draw on data from both prokaryotic and eukaryotic experimental systems because of the documented similarities between secretion across the bacterial plasma membrane and secretion across the mammalian ER membrane (see section 1.6). It is known that the bacterial signal peptidase enzyme will correctly mature cloned, eukaryotic secretory proteins such as proinsulin (Talmadge *et al.*, 1980), further demonstrating the similarity between the enzymes from prokaryotic and eukaryotic sources. It is obvious, however, that the comparison between the effectors of the release phenomenon and the effectors of the eukaryotic signal peptidase will be the more valid.

4.2 THE EFFECTS OF INHIBITORS OF EUKARYOTIC SIGNAL PEPTIDASE ACTIVITY ON AMYLASE RELEASE FROM RAT PANCREATIC MICROSOMES

4.2.1 Inhibitors of the signal peptidases of dog pancreatic microsomes and of ascites membranes

Rough microsome preparations derived from dog pancreas and ascites lysates have been found to correctly cleave human pre-placental lactogen (pre-PL) to its mature form, *in vitro* (Strauss *et al.*, 1979). These workers have reported that the processing activity in both these membranes, is completely inhibited by high concentrations of chymostatin, a peptide of microbial origin, which is known to inhibit chymotrypsin and similar endopeptidases. Leupeptin, elastatinal and antipain, related peptides with

different specificities had no effect on processing of pre-PL when added to microsomes at the same concentration as chymostatin (600 μg/ml). Figure 4.1 demonstrates that chymostatin, when added to rat pancreatic microsome suspensions at a final concentration of 600 μg/ml, significantly inhibited amylase release from the vesicles, whereas leupeptin, elastatinal and antipain had little or no effect over that produced by the solvent, DMSO, alone.

4.2.2 Inhibitors of DOC-solubilized signal peptidase from dog pancreatic microsomes

Extraction of dog pancreatic microsomal membranes with the detergent, deoxycholate (DOC) was found, by Strauss *et al.*, (1979), to result in solubilization of the signal peptidase activity, which could then be assayed using synthetic peptide substrates. In this way, a site-specific endopeptidase could be distinguished from contaminating aminopeptidase and "chymotrypsin-like" activities. The DOC-solubilized endopeptidase was found to be significantly inhibited by 1,10-phenanthroline (100 μg/ml), as well as chymostatin (600 μg/ml). The peptidase was inhibited to a lesser extent by both phenylmethylsulphonyl fluoride (PMSF) and L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), each at a concentration of 100 μg/ml (Strauss *et al.*, 1979). TPCK (100 μg/ml) had also been independently reported to give inefficient inhibition of pre-growth hormone processing in rat pituitary tumor cells (Sussman *et al.*, 1976).

In the rat pancreatic microsome system, PMSF, at a

FIGURE 4.1

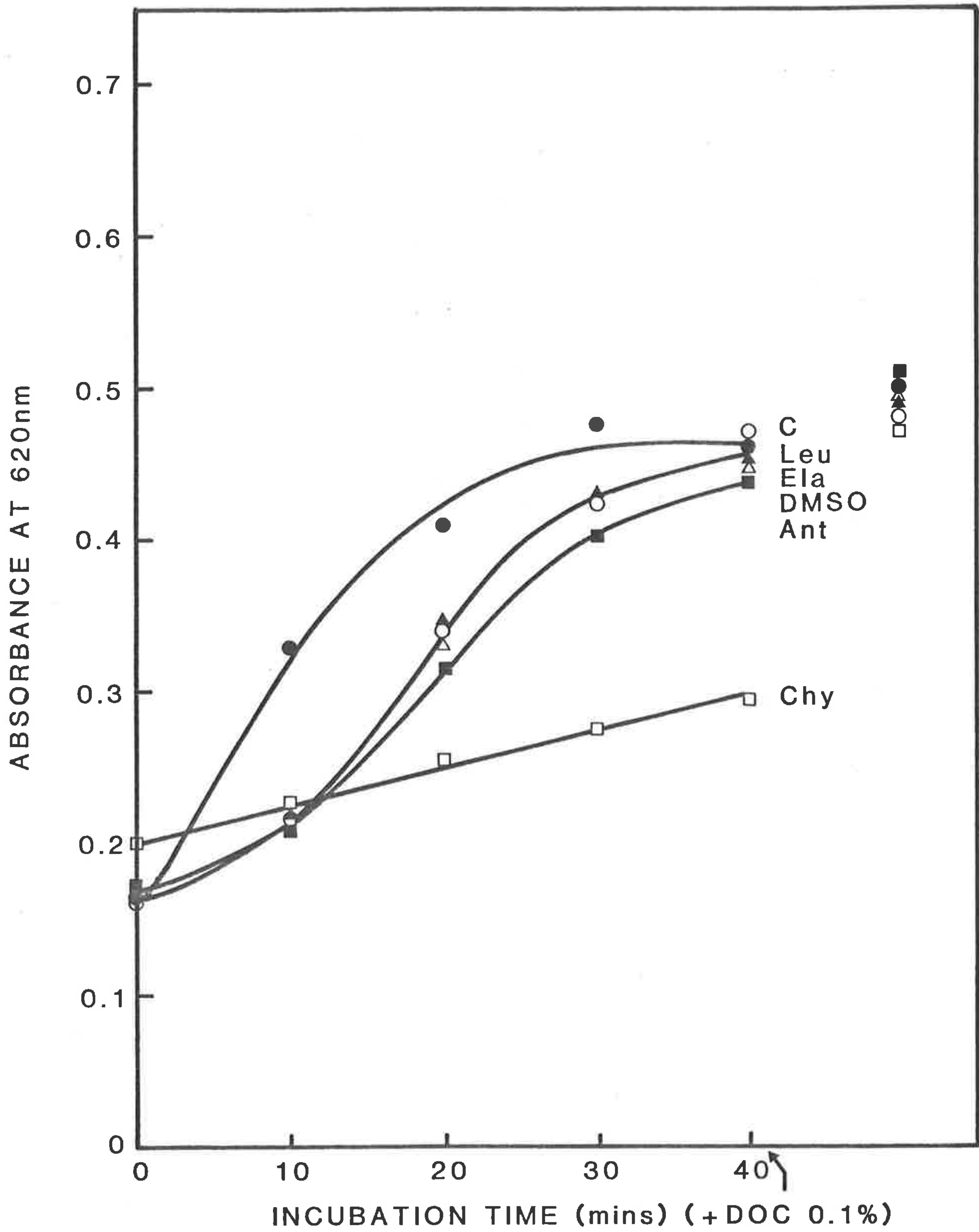
The effects on amylase release of microbial peptide protease inhibitors

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

- (●) no additions
- (△) 6% v/v DMSO
- (○) 600 μg/ml leupeptin and 6% DMSO
- (▲) 600 μg/ml elastatinal and 6% DMSO
- (■) 600 μg/ml antipain and 6% DMSO
- (□) 600 μg/ml chymostatin and 6% DMSO

(All concentrations are final concentrations. Each inhibitor was added to a (2 ml) microsome suspension as an aliquot (120 μl) of a 10 mg/ml solution of the inhibitor in DMSO).

FIG 4.1.



concentration of 500 μg/ml, inhibited amylase release by approximately 30% (fig. 4.2). TPCK (440 μg/ml) also diminished the release of amylase from microsomes, but this was found to be a composite effect (see fig. 4.3). The relatively low total amount of amylase liberated from TPCK-treated microsomes by DOC-disruption of the membranes at the end of the 37° incubation, revealed that TPCK inhibited the activity of the amylase enzyme itself. Despite this lower total, TPCK-treated microsomes released only 60% of their content amylase during a 40 min. incubation at 37°C, compared to 100% release from control vesicles. TPCK therefore appears to affect not only amylase activity, but also the actual release process, although a small part of this inhibition seems to be attributable to the ethanol in which the inhibitor was dissolved (see fig. 4.3). The low solubility of both TPCK and PMSF in an aqueous medium, as evidenced by the white precipitate which formed when either inhibitor was added to microsome suspensions, may be partly to blame for the inefficient inhibition of the release phenomenon by these compounds.

Amylase release from rat pancreatic microsomes was almost completely inhibited by 20 mM 1,10-Phenanthroline (fig. 4.4). By comparison, the concentration of this inhibitor which gave a similar level of inhibition of hydrolysis of synthetic substrate by DOC-extracted dog microsomes, was 0.5 mM. Since the mode of action of 1,10-phenanthroline involves the chelation of cations rather than actual binding to the active site of a protease, this

FIGURE 4.2

The effect of PMSF on amylase release

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

(●) no additions

(○) 500 μg/ml PMSF, final concentration

(20 μl of a 50 mg/ml solution of PMSF in ethanol was added to a 2 ml microsome suspension to give the appropriate final concentration of inhibitor. The final concentration of 1% v/v ethanol in the microsome suspension, had no effect on enzyme release; for result see fig. 4.6).

FIG 4.2.

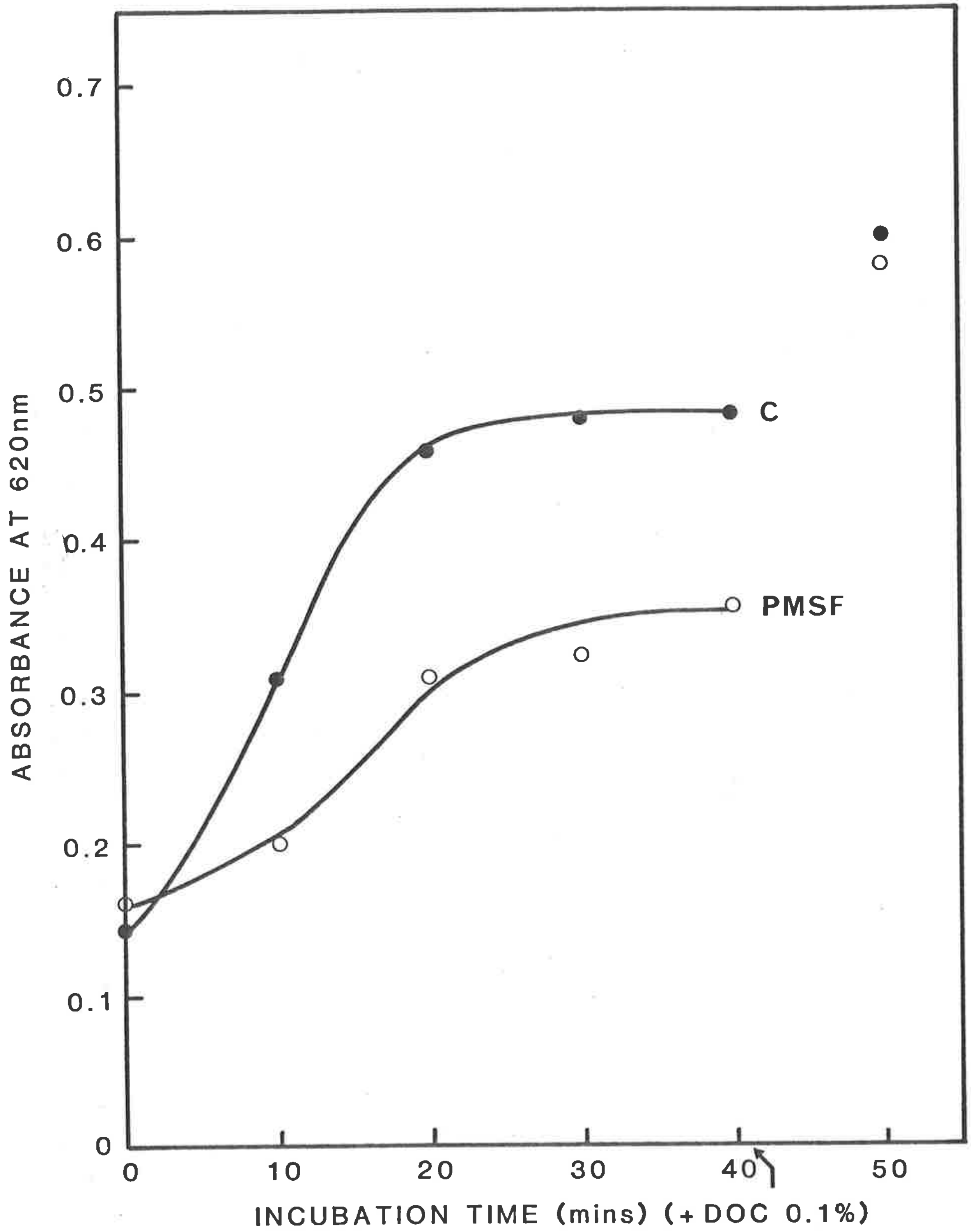


FIGURE 4.3

The effect of TPCK on amylase release

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing: (●) no additions

(○) 4% v/v ethanol

(▲) 2.5 mM TPCK and 4% ethanol

(△) 5.0 mM TPCK and 4% ethanol

(All concentrations are final concentrations. Aliquots of a 125 mM solution of TPCK in ethanol were added to microsomes to give the appropriate final concentration of inhibitor).

FIG 4.3.

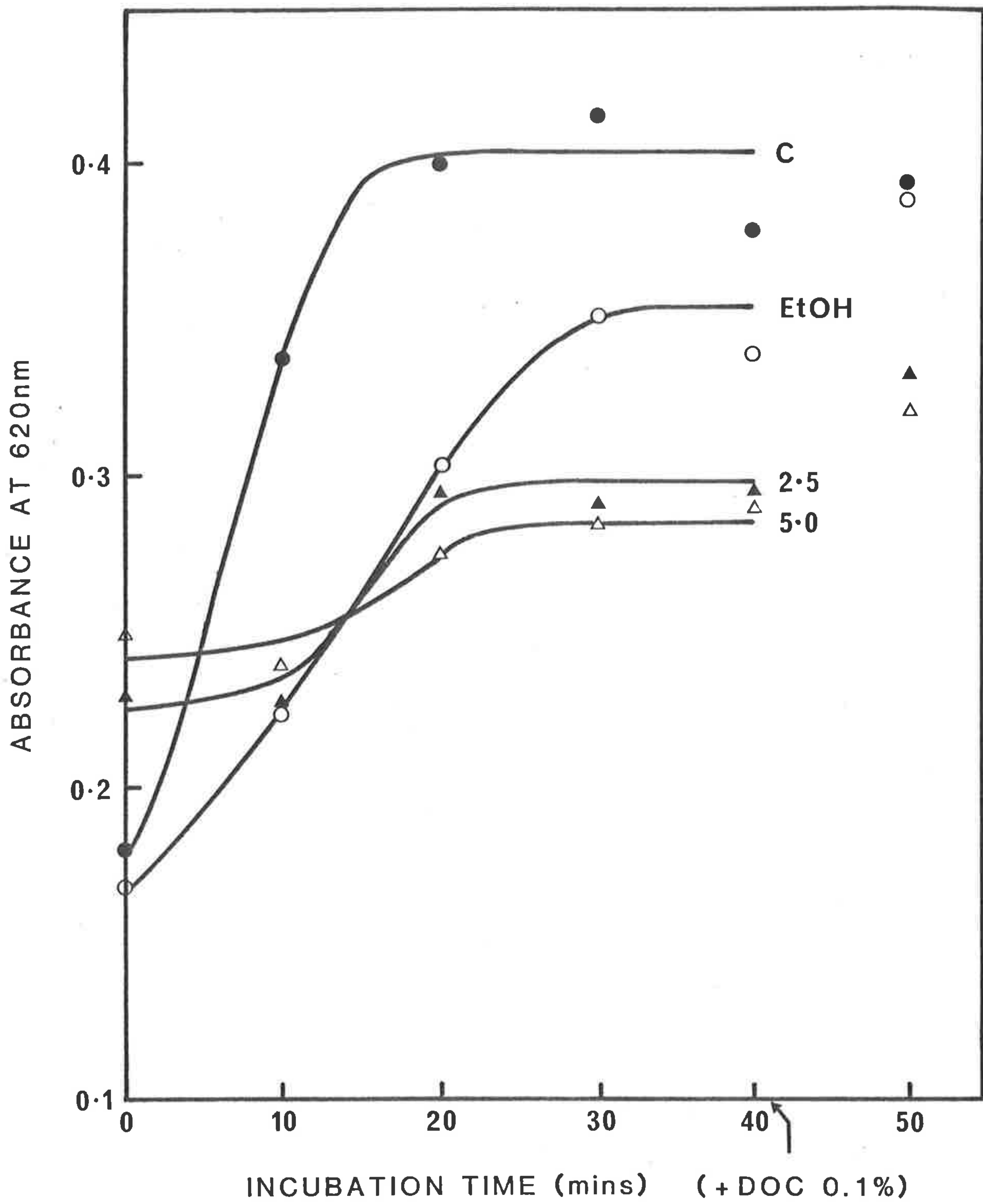


FIGURE 4.4

The effect of 1,10-phenanthroline on amylase release

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

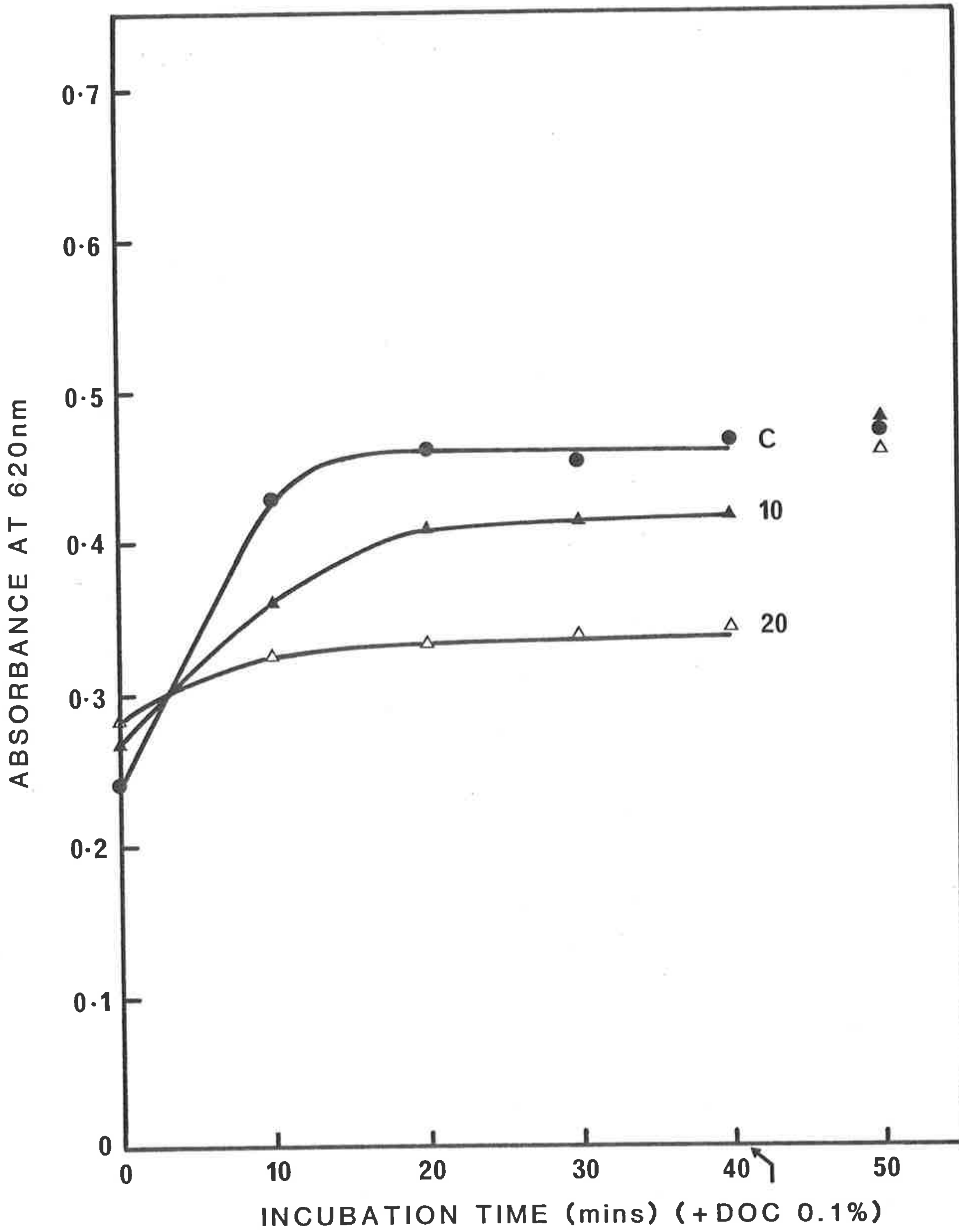
(●) no additions

(▲) 10 mM 1,10-phenanthroline

(△) 20 mM 1,10-phenanthroline

(Aliquots of a 2M solution of 1,10-phenanthroline in ethanol were added to microsome suspensions to give the appropriate final concentration of inhibitor. The resultant final concentrations of ethanol in the microsome suspensions (0.5% and 1.0% v/v) had no effect on enzyme release: see fig. 4.6).

FIG 4.4.



difference may be explicable in terms of different ionic environments in the two systems. Thus, 1,10-phenanthroline inhibits both signal peptidase activity extracted from dog pancreatic microsomes and amylase release from rat pancreatic microsomes.

4.2.3 Inhibitors of OBG-solubilized signal peptidase from dog pancreatic microsomes

The signal processing endopeptidase of dog pancreatic membranes was further characterized by Mumford *et al.*, (1979) who studied enzyme extracted from dog microsomes with the detergent octyl β-glucopyranoside (OBG). OBG-solubilized peptidase, assayed using a synthetic, fluorogenic substrate, was found to be strongly inhibited by 0.5 mM 1,10-phenanthroline, 2 μg/ml phosphoramidon (a microbial inhibitor of thermolysin), and a number of synthetic inhibitors of thermolysin, implying that the signal peptidase is a zinc metallo-endopeptidase. The enzyme was insensitive to elastatinal, leupeptin and anti-pain, as in intact dog microsomes. However, in contrast to the previously published properties of the DOC-solubilized peptidase, the OBG-solubilized enzyme was insensitive to TPCK and PMSF. For an unknown reason, pre-PL processing by the OBG extract was also insensitive to phosphoramidon in the presence of DOC (Mumford *et al.*, 1979).

In relation to these reported observations, it is interesting that phosphoramidon did not produce appreciable inhibition of amylase release from rat pancreatic micro-

somes, at any of the concentrations tested (fig. 4.5). This result therefore contrasts with the effect of phosphoramidon on OBG-solubilized signal peptidase, but is in accord with the lack of effect of phosphoramidon on the same peptidase in the presence of DOC. Furthermore, Mumford *et al.*, report that processing enzymes similar to the endopeptidase from dog pancreatic microsomes were found in rat parotid gland membranes, purified RER and SER from rat liver, rat lacrymal gland membranes, murine macrophages and porcine brain microsomes. Phosphoramidon, and the synthetic thermolysin inhibitors, did not inhibit processing of human pre-PL by either these intact microsome preparations, or by OBG-extracts of the membranes (Mumford *et al.*, 1979).

4.3 THE EFFECTS OF INHIBITORS OF PROKARYOTIC SIGNAL PEPTIDASE ACTIVITY ON AMYLASE RELEASE FROM RAT PANCREATIC MICROSOMES

It has been proposed by Gayda *et al.*, (1979), that the prokaryotic signal peptidase may resemble trypsin, on the basis of their finding that a number of compounds inhibit both trypsin activity and the processing of the precursor of an outer membrane protein of *E. coli*. Specific trypsin inhibitors such as N-α-tosyl-L-lysine chloromethyl ketone (TLCK) and benzamidine inhibited the proteolytic conversion of the 42 kDal precursor protein (M1) to its mature form (M2, 40 kDal), in *E. coli* minicells containing the structural gene for the M1 and M2 proteins. The same processing reaction was inhibited by a number of

FIGURE 4.5

The effect of phosphoramidon on amylase release

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

(●) no additions

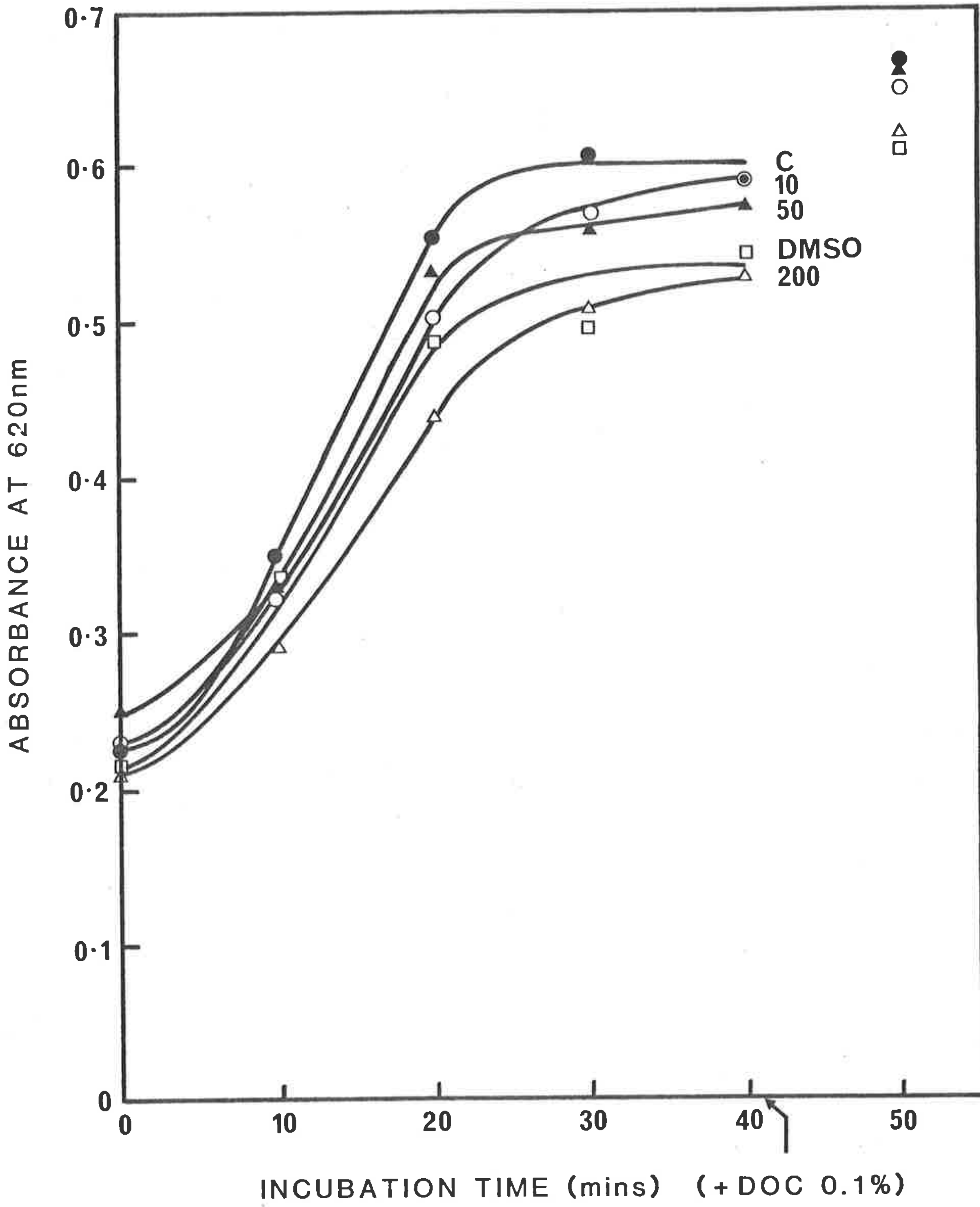
(○) 10 µg/ml phosphoramidon and 2% DMSO

(▲) 50 µg/ml phosphoramidon and 2% DMSO

(△) 200 µg/ml phosphoramidon and 2% DMSO

(□) 2% v/v DMSO

FIG 4.5.



local anaesthetics, including procaine HCl, which was also found to competitively inhibit the activity of pure trypsin.

In the absence of inhibitors, M1 is found in significant amounts in the outer membrane of minicells along with the processed M2 protein. This implies that the sequence of events in the biosynthesis of M2, in minicells, is synthesis and translocation of the precursor to the outer membrane, followed rapidly by proteolytic processing of M1 to M2. The presence of trypsin inhibitors or local anaesthetics allowed accumulation of M1 in the outer membrane, indicating that they inhibited the cleavage activity of the bacterial signal peptidase, but not the translocation of the precursor protein across the plasma membrane (Gayda *et al.*, 1979). The differential inhibition of exo-enzyme production in prokaryotes, by procaine has been noted elsewhere (Fishman *et al.*, 1980, Lazdunski *et al.*, 1979).

When added to rat pancreatic microsome suspensions, TLCK, benzamidine and procaine HCl all inhibited the appearance of amylase in the medium (fig. 4.6). After incubation of microsomes in the presence of inhibitors, amylase could be released from within the vesicles by DOC-disruption of the membranes, proving that the amylase had not simply been released and inactivated by the inhibitors. This point was verified by establishing, using polyacrylamide gel electrophoresis, that amylase remains associated with microsomes after incubation with TLCK (results not shown). Although no information was

FIGURE 4.6

The effects of trypsin inhibitors on amylase release

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

(●) no additions

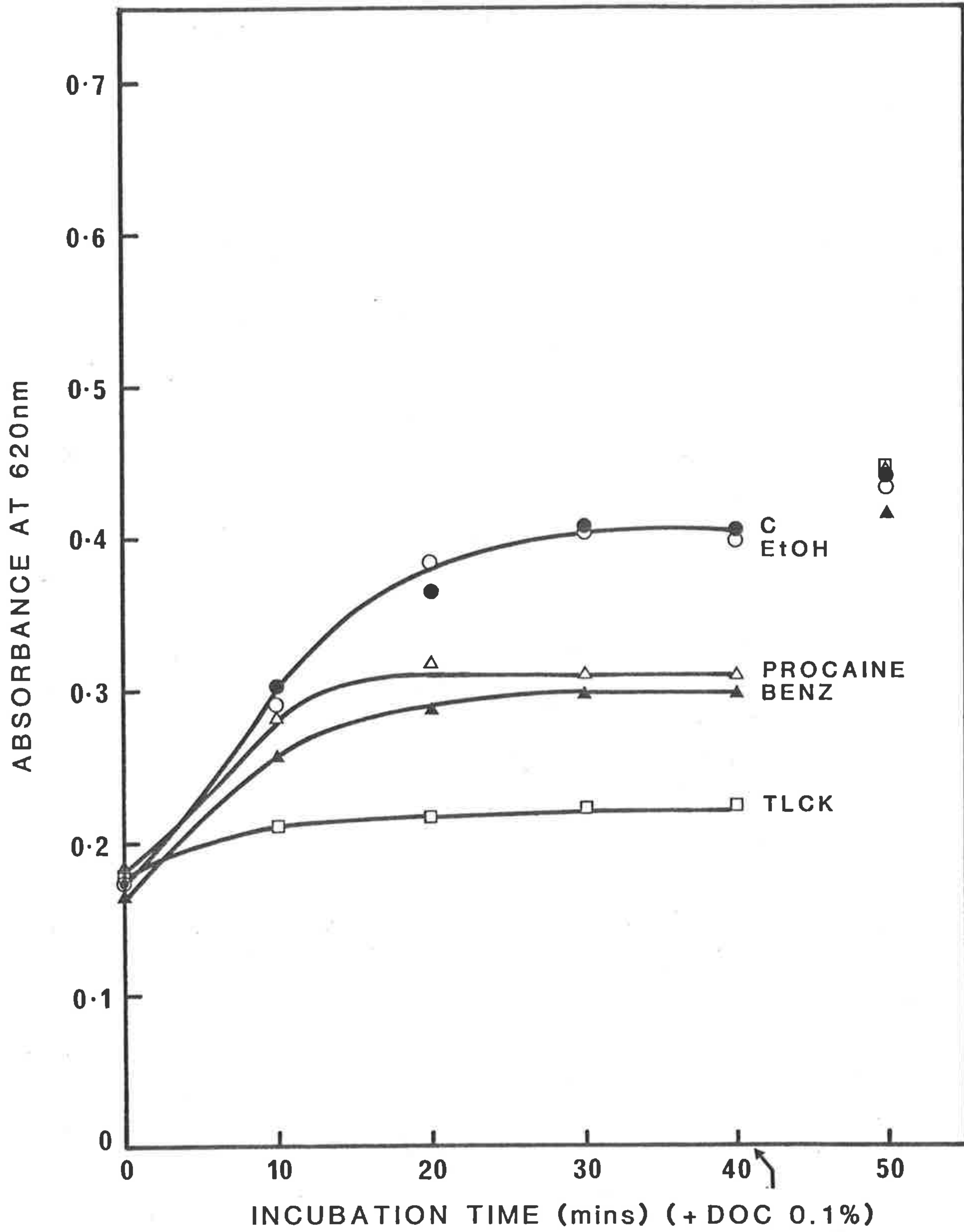
(○) 2% v/v ethanol

(△) 1% w/v procaine HCl

(▲) 20 mM benzamidine HCl

(□) 5 mM TLCK and 2% ethanol

FIG 4.6.



available on their effects on signal peptidase activity, two naturally-occurring trypsin inhibitors, traysylol and soybean trypsin inhibitor, were also tested on rat microsomes and were found to result in a diminuition of amylase release (figure 4.7).

It was concluded from the work described in the preceding three sections, and from consideration of published data, that some inhibitors of trypsin, as well as some inhibitors of chymotrypsin and thermolysin, will depress both signal peptidase activity and the release of amylase from rat pancreatic microsomes. This discovery prompted attempts to clarify the possible role of signal peptidase activity in the pancreatic microsome enzyme release phenomenon.

4.4 THE MOLECULAR FORM OF INTRA-MICROSOMAL AMYLASE

The finding that the pancreatic microsome enzyme release phenomenon was depressed by signal peptidase inhibitors seemed to infer that signal peptidase activity was somehow a part of the mechanism of amylase release. It had been previously established, however, that enzyme release occurred normally in the presence of cycloheximide thus ruling out the possibility that *de novo* synthesis of amylase was involved in the release phenomenon (Pearce *et al.*, 1978). Therefore, if removal of a signal peptide occurs during amylase release, the substrate for the peptidase must be fully-formed pre-amylase.

The proposal of the existence of a full-length pre-protein in microsomes constitutes an obvious contradiction

FIGURE 4.7

The effects on amylase release, of traysylol and soybean trypsin inhibitor

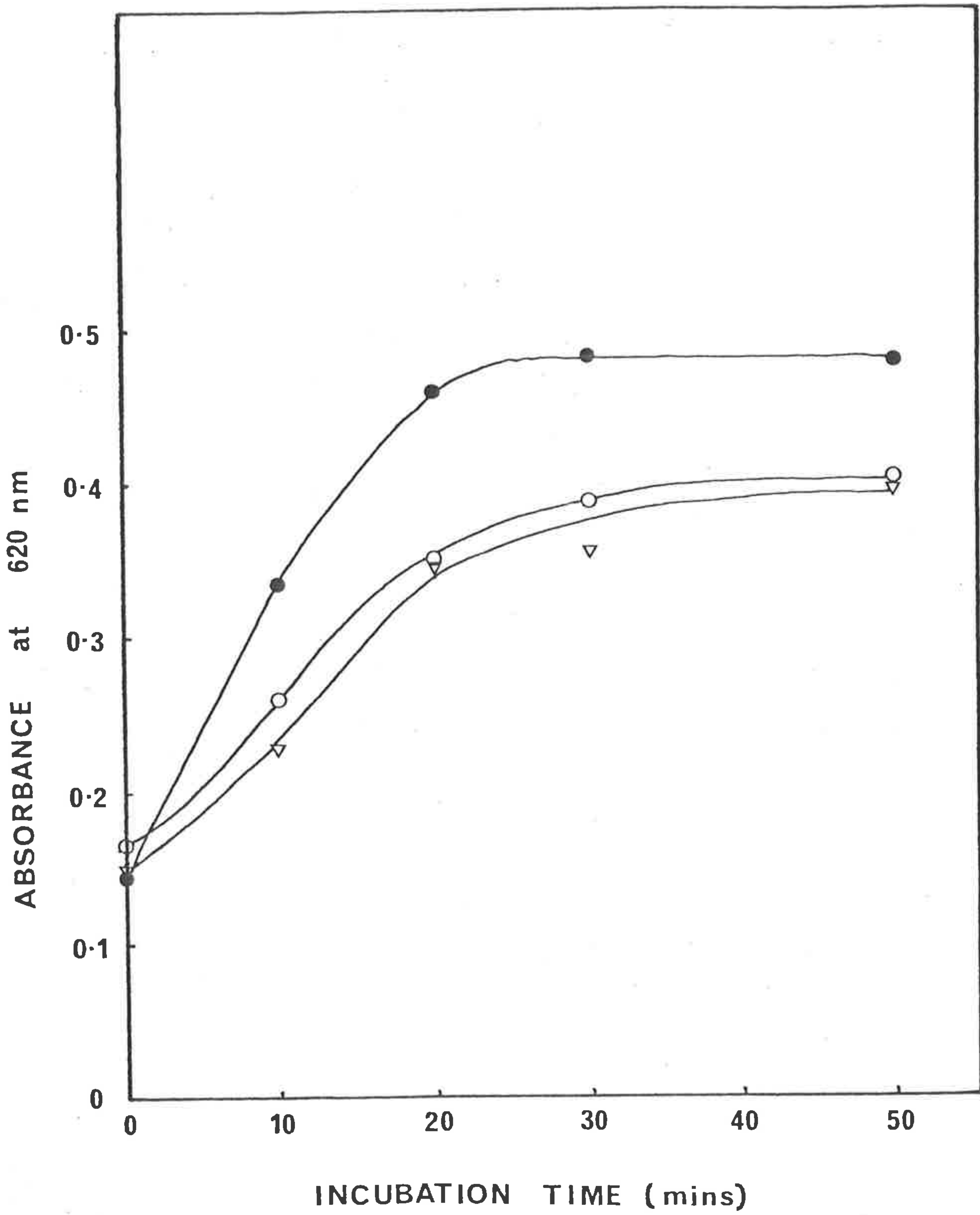
Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

(●) no additions

(○) 100 Kallikrein inactivator units/ml traysylol

(△) 0.5 mg/ml soybean trypsin inhibitor.

FIG 4.7.



of the signal hypothesis which states that signal peptide removal is co-translational. This would mean that intra-microsomal amylase would have been processed during its co-translational translocation into the ER *in vivo*. Precedents for suggesting that full-length pre-amylase may exist in microsomes have been established, however, by the discovery, *in vivo*, of full-length pre-proinsulin (Patzelt *et al.*, 1978), pre-proparathyroid hormone (Habener, 1980), and pre-prolactin (Maurer and McKean, 1978).

Mammalian pancreatic pre-amylase synthesized by *in vitro* translation of mRNA, in the absence of microsomal membranes, has been found to contain an amino-terminal signal peptide of 1500 to 3000 daltons molecular weight (MacDonald *et al.*, 1977, Brown and Wold, 1981). Pre-amylase, which is enzymically active (Brown and Wold, 1981), should therefore be distinguishable from mature amylase by this difference in molecular weight. If signal peptidase activity was involved in the mechanism of amylase release from microsomes, it could be argued that before release, intra-microsomal amylase would be in the form of full-length pre-protein, which would somehow be processed to give mature amylase during transfer across the microsomal membrane. This postulate was tested by comparing the molecular weights of intra-microsomal and released, extra-microsomal amylase.

Intra-microsomal amylase was prepared by isolating microsomes from rat pancreas in the presence of a protease inhibitor "cocktail", similar to that used by Nelson and

Schatz (1979) to preserve short-lived, cytoplasmic precursors to mitochondrial proteins, during cell fractionation (see section 2.2.3. Three different preparations were electrophoresed on an SDS-polyacrylamide gel (see section 2.2.7) along with "released" amylase contained in samples of the supernatant fraction from microsomes which had been incubated at 37°C for 60 mins., in the absence of inhibitors, then centrifuged at 100,000 g_{av} for 30 mins. Rat pancreatic α-amylase purified by the method described in section 2.2.9 was used as a reference. After separation on a gel, the microsomal proteins were transferred to nitro-cellulose by the method of Towbin *et al.*, (1979) (see section 2.2.8 for details of procedure). Amylase bands were detected by probing the surface of the nitrocellulose firstly with anti-amylase immunoglobulin, then with ^{125}I-labelled *Staphlacoccus aureus* protein A.

As shown in figure 4.8, both intra-microsomal amylase and released amylase co-migrated exactly with purified α-amylase. A molecular weight difference between pre-amylase and the mature enzyme, of the order of 1500 daltons, would have been resolved by this gel system, as indicated by the separation between purified, mature α-amylase (M^r 56,000) and the bovine liver catalase monomer (M^r 58,000) used as a molecular weight marker. It was concluded that the actual processing activity of the signal peptidase enzyme was not involved in the release of mature amylase across the microsomal membrane, thus implying that signal peptidase inhibitors affect release in some other way, as discussed in section 4.5.

FIGURE 4.8

A comparison of the molecular weights of intra-microsomal amylase and released amylase

Rat pancreatic microsome samples were electrophoresed on an SDS-polyacrylamide slab gel as described in section 2.2.7. Proteins contained in a central section of the gel were transferred to nitro-cellulose, as described in section 2.2.8. Amylase bands were detected by probing the nitrocellulose with amylase antibody followed by ^{125}I-labelled *Staphlacoccus aureus* protein A. Bands were visualized by autoradiography (4 hrs exposure).

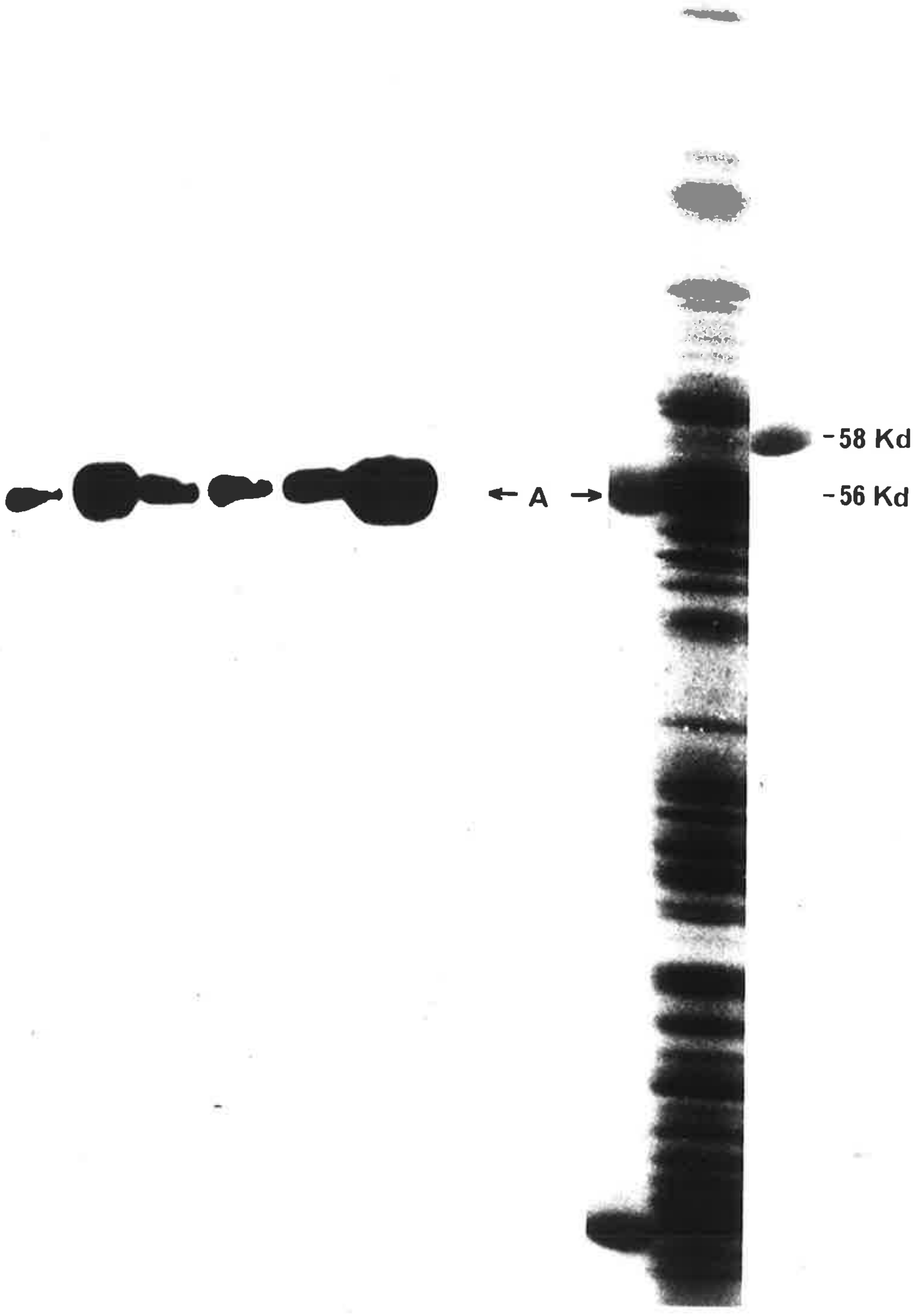
The samples shown are (from left):

1. released amylase (37° supernatant)
2. total microsomal protein, preparation A
3. released amylase (37° supernatant)
4. total microsomal protein, preparation B
5. total microsomal protein, preparation C (20 μl)
6. total microsomal protein, preparation C (40 μl)

A portion trimmed from the original polyacrylamide gel and stained with Coomassie blue, is shown in the right-hand panel.

The samples are (from left):

1. purified rat pancreatic α-amylase (see section 2.2.9)
2. total microsomal protein, preparation A
3. bovine liver catalase, and trypsin (Sigma).



← A →
-58 Kd
-56 Kd

4.5 SUMMARY AND DISCUSSION

A strong correspondence has been demonstrated between those protease inhibitors which have been reported to inhibit signal peptidase activity, in a variety of situations, and those compounds which inhibit amylase release from rat pancreatic microsomes. The most convincing comparison can be made between the reported effects on eukaryotic signal peptidase activity, and the effects on amylase release, of a number of peptide protease inhibitors of microbial origin. Chymostatin, a tripeptide inhibitor specific for chymotrypsin, completely abolished both processing of human pre-PL, by dog pancreatic microsomes (Strauss *et al.*, 1979), and amylase release from rat pancreatic microsomes, at a concentration of 600 μg/ml. Leupeptin, elastatinal and antipain, at the same concentration, had no effect on either system. Phosphoramidon, although it inhibited hydrolysis of a synthetic substrate by OBG-solubilized signal peptidase from dog microsomes, inhibited neither pre-PL processing by mammalian microsomes from a number of different sources (Mumford *et al.*, 1979), nor amylase release from rat pancreatic microsomes.

TPCK and PMSF were both reported to inhibit dog microsomal signal peptidase, with varying efficiencies in different situations (Mumford *et al.*, 1979, Strauss *et al.*, 1979). The same compounds were found to be relatively inefficient inhibitors of the amylase release phenomenon. 1,10-phenanthroline inhibited amylase release as well as dog microsomal signal peptidase activity (Strauss *et al.*,

1979), although a 40 to 100 fold greater concentration of inhibitor was necessary to achieve a comparable effect, in the former system. Although the trypsin inhibitor, TLCK had no apparent effect on OBG-solubilized dog microsomal signal peptidase (Mumford *et al.*, 1979), TLCK, benzamidine and the local anaesthetic, procaine reportedly inhibited prokaryotic signal peptidase activity (Gayda *et al.*, 1979). These 3 compounds were all found to depress the amylase release phenomenon.

The proposed function of mammalian signal peptidase *in vivo* is the co-translational removal of the signal peptide from a growing, secretory peptide chain during its passage across the ER membrane. Translation has been shown not to contribute to the amylase release phenomenon, thus, if signal peptidase were active in the rat pancreatic microsome suspensions described in this thesis, it would have to act on a fully-formed pre-protein which traverses the microsomal membrane in the opposite direction to the physiological process of secretion. The proven absence of full-length pre-amylase in the rat microsomes therefore argues against the involvement of signal peptidase activity in the release phenomenon.

Although the exact way in which signal peptidase inhibitors produce their effects on amylase release is unknown, it is possible to speculate on the mechanism of inhibition. As stated in section 4.1, the tight coupling of processing and translocation in normal secretion, implies that the signal peptidase enzyme is closely associated with the membrane proteins involved in secretory protein

translocation. Other evidence (see section 1.7) suggests that the signal peptidase enzyme may be completely buried within the ER membrane. Taken together, these statements imply that the signal peptidase in rat microsomal membranes may be an integral membrane protein which forms part of a complex containing the enzyme itself, a ribosome binding site, a signal receptor site and translocator/pore-forming proteins. Pre-proteins passing through this mechanism, into the ER *in vivo*, would be exposed to the active site of the peptidase, resulting in proteolytic processing of the translocated proteins (for schematic illustration see fig. 4.9).

The results presented in chapter 3 demonstrate that active -amylase is apparently released from intact rat pancreatic microsomes through the agency of a microsomal membrane protein. In this chapter it is demonstrated that this amylase-releasing mechanism is sensitive to inhibitors of signal peptidase activity. The absence of a signal peptide substrate within the amylase protein seems to rule out cleavage by the signal peptidase enzyme as part of the amylase release mechanism, yet a correlation between the signal peptidase and the putative amylase-releasing protein is indicated.

This reasoning raises the interesting possibility that, in a non-physiological ionic environment (ie., in the absence of Mg^{2+}), at 37°C, fully-formed secretory proteins escape from rat microsomes via the translocator mechanism responsible for the transport of the same secretory proteins <u>into</u> the ER *in vivo*. In the light of this hypothesis, a possible mode of action of the signal

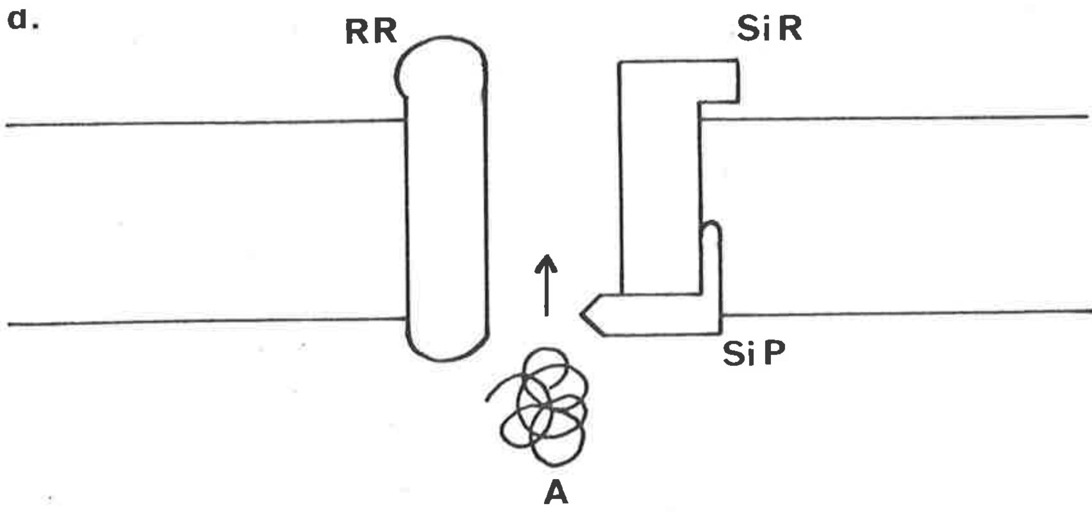
FIGURE 4.9

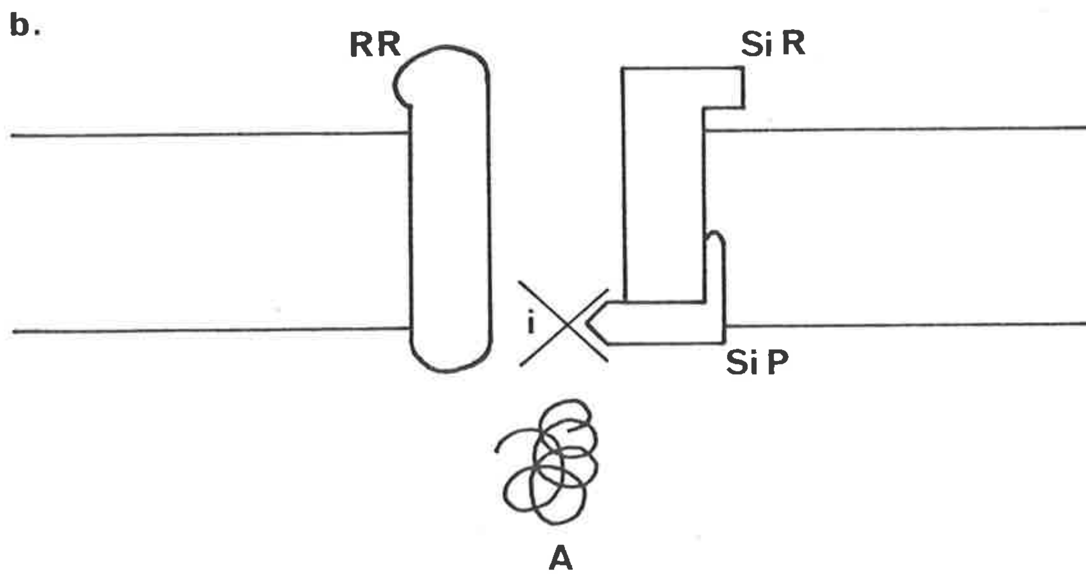
A possible mechanism for the inhibition of amylase release from rat pancreatic microsomes by signal peptidase inhibitors

A schematic model for the *in vitro* escape of intra-microsomal amylase through the mechanism proposed to mediate the inward translocation of secretory proteins into the ER, *in vivo*. To allow convenient comparison, the mechanism is represented essentially as the pore structure drawn by Walter and Blobel (1981b), in which the signal peptidase is shown to be exposed on the lumenal surface of the ER membrane.

a) Incubation of rat pancreatic microsomes in Mg-free buffer results in release of amylase (A) by backwards translocation of the completed protein through the secretory pore. The signal peptidase enzyme (SiP) is seen as an integral part of a complex formed by several membrane proteins, including a ribosome receptor (RR) and a signal sequence receptor (SiR).

b) Inhibitors of signal peptidase activity (i) may bind to the active site of the enzyme thus either causing a conformational change in the translocator complex, or sterically blocking the reverse passage of amylase through the transport mechanism.


a.
RR
SiR
SiP
A


b.
RR
SiR
i
SiP
A

peptidase inhibitors in the rat pancreatic microsome system could be the binding of the inhibitors to the active site of the signal peptidase, thus physically blocking the reverse passage of α-amylase through the translocator mechanism or secretory pore, as illustrated schematically in figure 4.9. Alternatively, binding of the inhibitors, or chelation of essential ions, as in the case of 1,10-phenanthroline, could cause conformational changes in the signal peptidase, or the entire translocator complex, thereby blocking *in vitro* amylase release through the mechanism. Signal peptidase inhibitors are therefore proposed to affect amylase translocation by virtue of the proximity of the peptidase enzyme and the translocator mechanism.

In relation to these ideas, it is noted that workers who have investigated the effects of various inhibitors on the activity of mammalian signal peptidase, did not determine whether or not translocation of pre-PL was inhibited along with proteolytic processing of the precursor (Strauss *et al.*, 1979, Mumford *et al.*, 1979). On the other hand, in the report of the effects of trypsin inhibitors on processing of the precursor to an outer membrane protein of *E. coli*, it was established that processing was inhibited, but translocation of the precursor across the plasma membrane was not; although pre-protein insertion into the outer membrane did not appear to occur correctly (Gayda *et al.*, 1979). Thus, the trypsin inhibitors block processing, but not translocation of a membrane protein in *E. coli*, while the same

inhibitors block reverse translocation of processed amylase across the rat pancreatic ER membrane *in vitro*. This differential effect may be explained by any one of the obvious dissimilarities between the two systems.

On the other hand, this anomaly may point to a different, speculative interpretation of the data presented in this chapter. As previously stated, α-amylase does not appear to act as a substrate for the signal peptidase enzyme during the release process. However, it is possible that the escape of mature amylase from within the microsomes may initially be blocked by nascent peptide chains occupying the secretory "pores" in the ER membrane at the time of the disruption of the pancreatic cells.

Cleavage of these peptides might allow their escape from the membrane, thus leaving the translocator mechanism open for the release of intra-microsomal amylase. In this case, the addition of signal peptidase inhibitors to rat pancreatic microsomes would presumably prevent amylase release from the vesicles due to the actual inhibition of signal peptidase enzyme activity. This hypothesis may provide a more acceptable explanation of the effect of 1,10-phenanthroline on the enzyme release phenomenon, as well as reconciling the differential effects of TLCK on protein translocation in the *E. coli* and pancreatic microsome systems.

In any case, as previously mentioned, the most valid comparisons made in this chapter are those drawn between effectors of mammalian signal peptidases and effectors of the rat pancreatic microsome enzyme release phenomenon.

The near-perfect correspondence of these effectors provides a strong conceptual link between the microsomal membrane protein proposed to mediate amylase release and the signal peptidase which can be assumed to be present in the same membrane. It must be emphasized that the evidence for this link is only correlative. It was felt, however, that the results presented in this chapter demanded further investigation of a possible relationship between the rat pancreatic microsome enzyme release phenomenon and the physiological process of secretory protein translocation across the ER membrane.

CHAPTER 5

RESULTS

INHIBITION OF THE PANCREATIC MICROSOME ENZYME RELEASE PHENOMENON BY DENATURED PROTEINS

5.1 INTRODUCTION

Because a signal peptide is the key to the translocator mechanism in the ER membrane, it can be inferred that the translocator machinery must include a component which specifically recognises signal peptides. The experimental evidence for the existence of such signal peptide receptor sites on the microsomal membrane consists mainly of demonstrations that nascent, transported proteins will compete for saturable binding sites on the membrane surface. It has been shown that nascent pre-prolactin and the nascent form of a viral membrane glycoprotein compete for binding sites on dog pancreatic microsomes. This result has been interpreted as supporting the hypothesis that membrane proteins and secretory proteins enter the ER membrane in a fundamentally similar manner (Lingappa *et al.*, 1978).

The same experimental approach has been used to investigate the relationship between the export of "normal" secretory proteins, containing transient, amino-terminal signal sequences, and the secretion of ovalbumin, which does not undergo cleavage during its translocation into microsomes. The very fact that ovalbumin is secreted argues for the functional equivalent of a signal sequence in the ovalbumin protein structure. The absence of proteolytic processing accompanying secretion must therefore mean that the signal region is conserved in the mature protein.

This was confirmed experimentally by Lingappa *et al.*, (1979), who found that denatured, but not native ovalbumin competitively inhibited the co-translational processing and segregation of preprolactin by dog pancreatic microsomes.

Although the exact location of the signal region within the ovalbumin amino acid sequence is uncertain (see Meek *et al.*, 1980), it appears that the signal is exposed, and able to interact with the membrane binding site, in the denatured protein (Ov_D), but is buried within the folded structure of native ovalbumin (Ov_N). The specificity of the inhibition of preprolactin processing by Ov_D was demonstrated by the lack of effect, on the same system, of denatured bovine serum albumin (BSA_D). Ov_D therefore constitutes a specific probe for the signal receptor site on the ER membrane. For this reason, the effects of Ov_D on amylase release from rat pancreatic microsomes were assessed in the hope of determining whether or not the rat microsomal membrane protein(s) seemingly involved in the release phenomenon were in any way related to the signal peptide binding sites on dog microsomal membranes.

5.2 AMYLASE RELEASE FROM RAT PANCREATIC MICROSOMES IN THE PRESENCE OF OVALBUMIN

Purified chicken ovalbumin (Sigma) was denatured by heating in the presence of urea and dithiothreitol (DTT), according to the method of Lingappa *et al.*, (1979) (for details see section 2.2.10). The protein was TCA-

precipitated then neutralized and redissolved in STKC buffer. Rat pancreatic microsomes suspended in STKC buffer at a concentration of approximately .25 mg total protein/ml (≃1.8 A_{260} units/ml) were incubated in the presence of various concentrations of Ov_D or Ov_N.

The addition of 1 mg/ml, Ov_D resulted in 80% inhibition of amylase release from the microsomes (fig. 5.1). Thus the sensitivity of the rat pancreatic microsome enzyme release phenomenon to Ov_D seems to be even greater than that of preprolactin processing by dog microsomes. In the latter system, which contained a comparable concentration of membranes, 8 mg/ml Ov_D produced roughly 50% inhibition of preprolactin processing. Ov_D (100μg/ml) inhibited amylase release from rat microsomes by 60% whereas Ov_N, at the same concentration had only a very slight inhibitory effect on enzyme release (fig. 5.1).

5.3 AMYLASE RELEASE FROM RAT PANCREATIC MICROSOMES IN THE PRESENCE OF OTHER PROTEINS

In order to assess the specificity of the effect of denatured ovalbumin on the release phenomenon, a number of other proteins, in either their native or denatured forms, were added to microsome suspensions. All the proteins were denatured by the method used for ovalbumin, thus providing controls for the effects of adding residual urea, DTT or acid to the microsomes (see section 2.2.10).

The cytoplasmic protein, globin produced no observable inhibition of amylase release, in either its native or denatured configuration (fig. 5.2). Denatured, mature,

FIGURE 5.1

The effects of denatured ovalbumin on amylase release

Rat pancreatic microsomes (0.2 - 0.3 mg total microsomal protein/ml) were incubated at 37°C in STKC buffer containing:

(●) no additions

(▲) 0.1 mg/ml native ovalbumin

(△) 0.02 mg/ml denatured ovalbumin (OV_D)

(○) 0.1 mg/ml OV_D

(□) 1.0 mg/ml OV_D

All concentrations are final concentrations.

FIG 5.1.

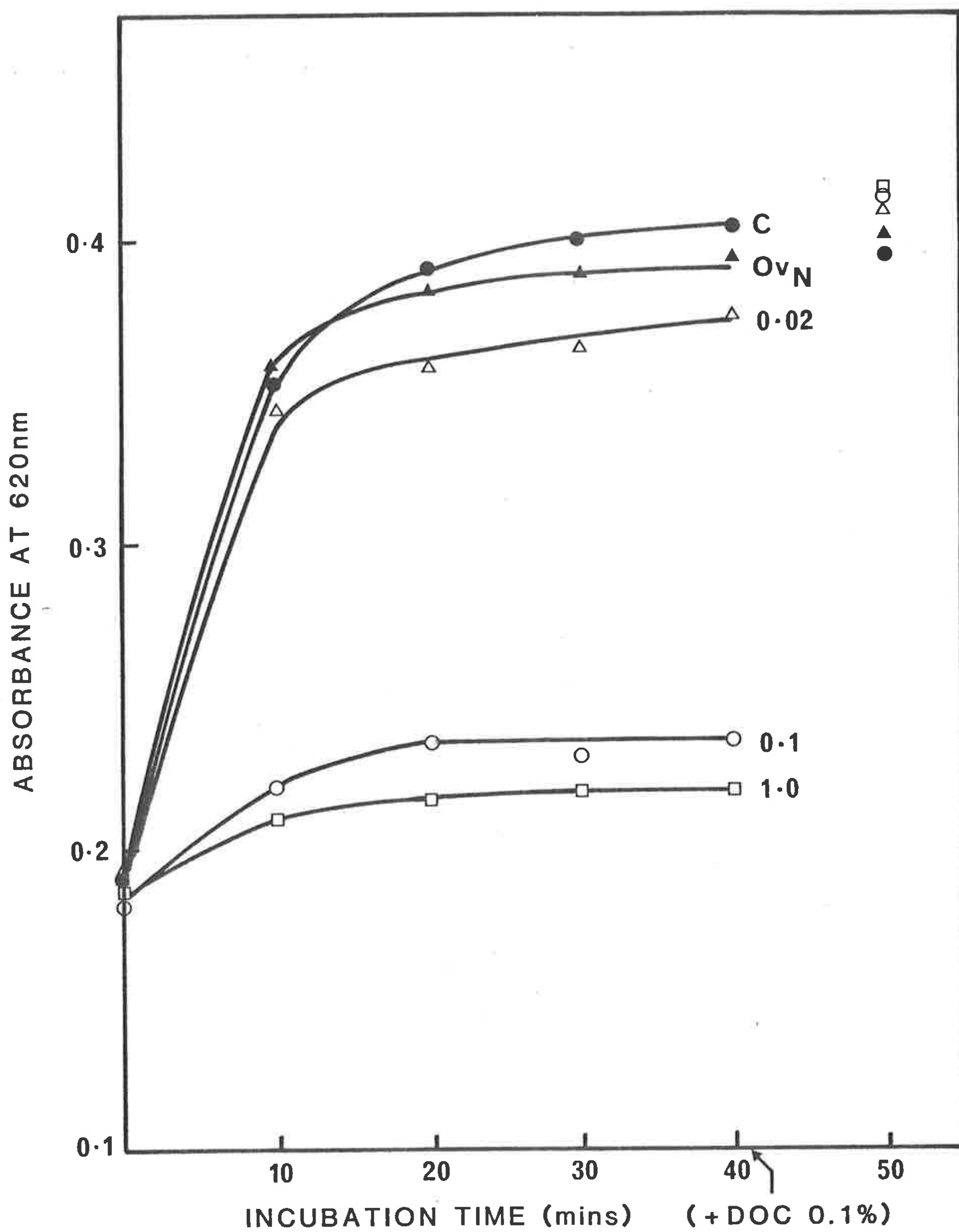


FIGURE 5.2

The effects of globin on amylase release

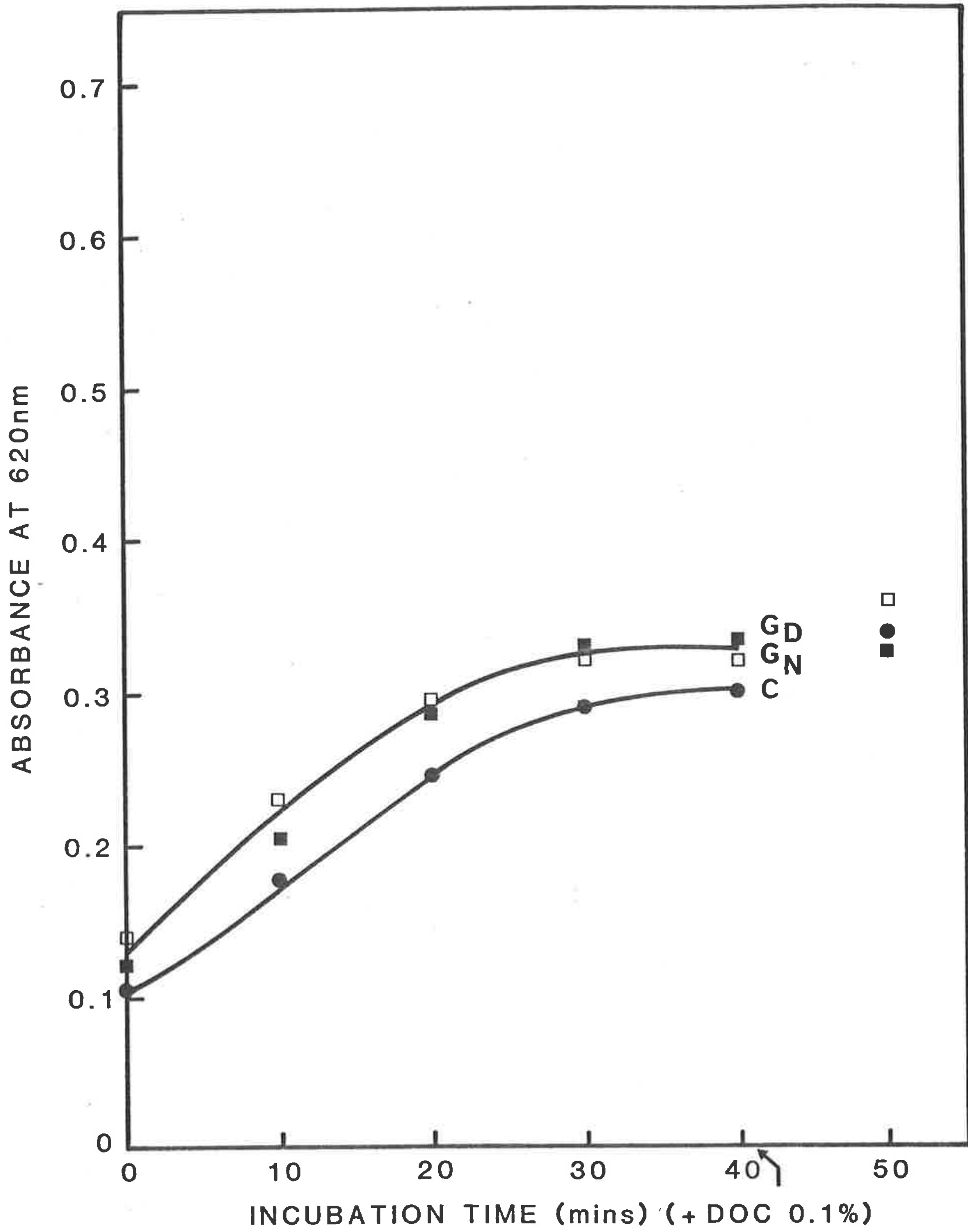
Rat pancreatic microsomes were incubated at $37^{o}C$ in STKC buffer containing:

(●) no additions

(■) 100 μg/ml denatured globin (G_D)

(□) 100 μg/ml native globin (G_N)

FIG 5.2.



secretory proteins, which no longer contained signal sequences, also failed to inhibit amylase release from microsomes. The results of adding denatured α-amylase, RNAse or hen egg lysozyme, each at a concentration of 100 μg/ml, to rat pancreatic microsome suspensions, are shown in figure 5.3. The slight stimulation of release observed in all these cases is possibly due to the large amounts of added protein protecting the putative translocator mechanism from endogenous proteases.

In contrast to its published lack of effect on preprolactin processing by dog pancreatic microsomes (Lingappa *et al.*, 1979), BSA_D inhibited amylase release from rat pancreatic microsomes to the same extent as Ov_D (fig. 5.4). Native BSA (BSA_N) also produced significant inhibition of enzyme release, as opposed to the slight effect of Ov_N on the same system. If both ovalbumin and BSA were denatured and digested with a low concentration of trypsin, as described by Lingappa *et al.*, (1979), (see section 2.2.11), the resultant solutions of tryptic fragments were also inhibitory to the release phenomenon (fig. 5.5). Amylase release was depressed to the same extent by both fragmented ovalbumin (Ov_f) and the intact, denatured protein, whereas the inhibitory effect of BSA_D was slightly attenuated by tryptic dissection of the protein. Thus, it seemed that the inhibitory activity of both Ov_D and BSA_D was preserved in the fragmented molecules, rather than inhibition of the amylase release phenomenon being a property of each protein as a whole.

Following its biosynthesis in the cytoplasm, *in vivo*,

FIGURE 5.3

The effects on amylase release of denatured, mature secretory proteins

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

(●) no additions

(■) 100 μg/ml denatured RNAse

(○) 100 μg/ml denatured amylase

(□) 100 μg/ml denatured lysozyme

(△) 100 μg/ml denatured catalase

FIG 5.3.

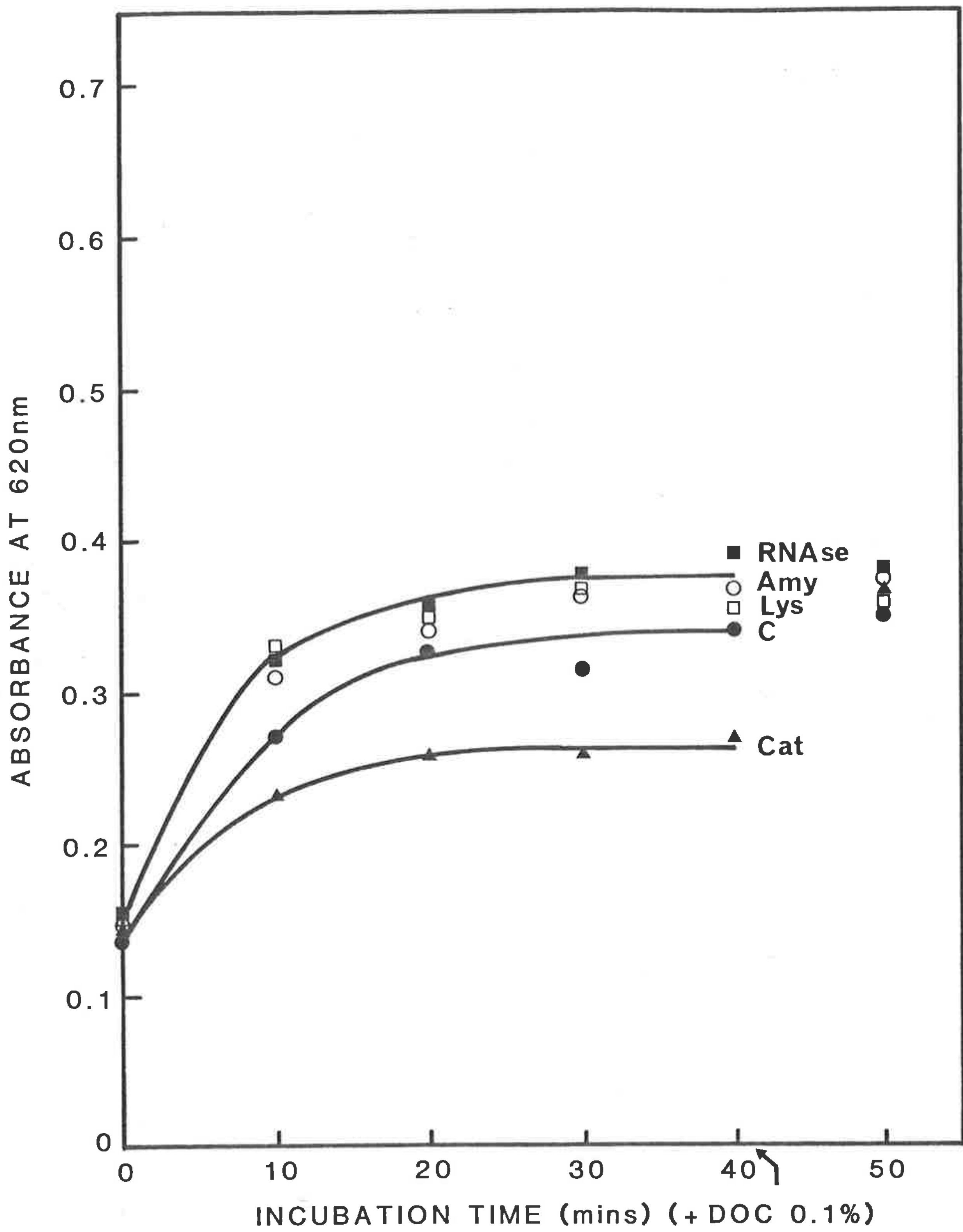


FIGURE 5.4

The effects of ovalbumin and BSA on amylase release

Rat pancreatic microsomes were incubated at $37^{o}C$ in STKC buffer containing:

(●) no additions

(□) 100 μg/ml native ovalbumin (OV_N)

(▲) 100 μg/ml denatured ovalbumin (OV_D)

(△) 100 μg/ml native BSA (BSA_N)

(○) 100 μg/ml denatured BSA (BSA_D)

FIG 5.4.

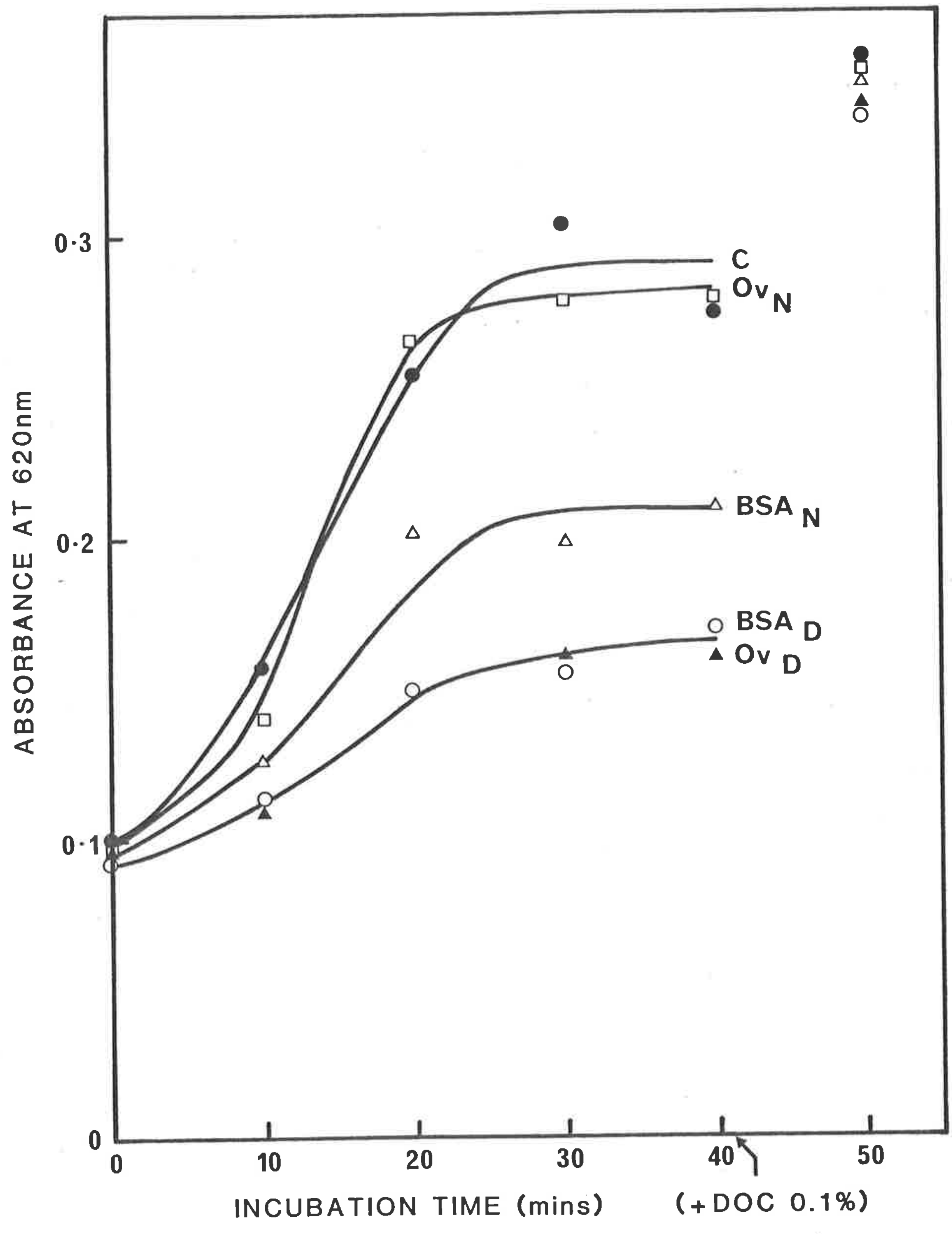


FIGURE 5.5

The effects on amylase release of tryptic fragments of ovalbumin and BSA

Rat pancreatic microsomes were incubated at $37^{o}C$ in STKC Buffer containing:

(●) no additions

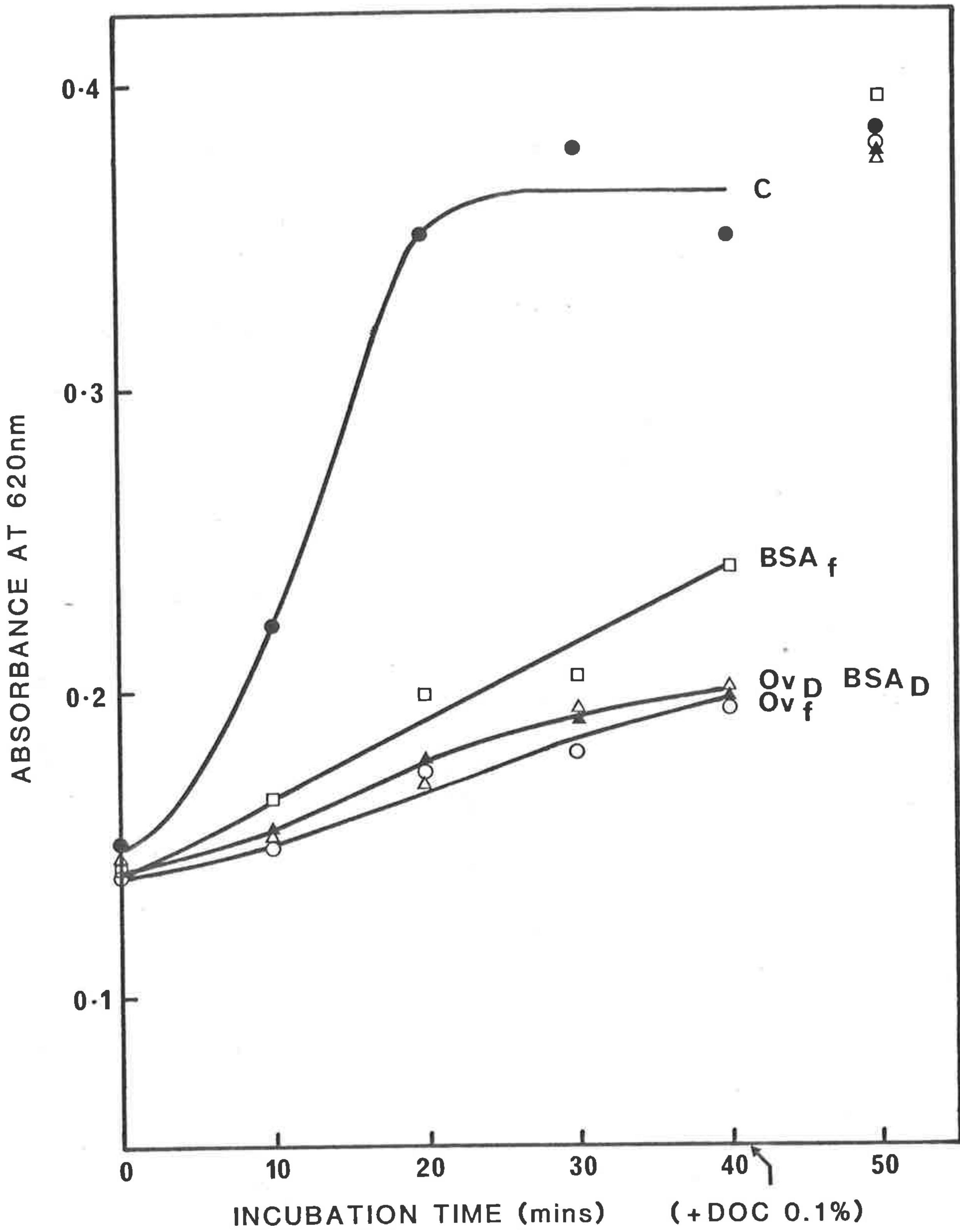
(△) 100 μg/ml OV_D

(○) ∿100 μg/ml tryptic fragments of OV_D (OV_f)

(▲) 100 μg/ml BSA_D

(□) ∿100 μg/ml tryptic fragments of BSA_D (BSA_f)

FIG 5.5.



the peroxisomal protein, catalase appears to be translocated across the peroxisomal membrane without proteolytic processing (Goldman and Blobel, 1978, Robbi and Lazarow, 1982). It is interesting, therefore, that denatured, but not native catalase inhibited the release of amylase from rat pancreatic microsomes (fig. 5.6). Denatured catalase produced 20% and 60% inhibition of amylase release, at concentrations of 100 μg/ml and 200 μg/ml respectively. This is relatively inefficient compared to the 60% decrease in amylase release caused by incubation of microsomes in the presence of 100 μg/ml Ov_D. The implications of these findings are discussed in section 5.4.

5.4 SUMMARY AND DISCUSSION

Denatured, but not native ovalbumin was found to substantially inhibit the rat pancreatic microsome enzyme release phenomenon. It has been reported that denatured ovalbumin, by virtue of its uncleaved signal region, constitutes a specific probe for the signal peptide receptor site present on the surface of dog pancreatic microsomal membranes (Lingappa *et al.*, 1979). Considering these statements together, it can be hypothesized that Ov_D inhibits amylase release from rat pancreatic microsomes by binding specifically to a signal peptide receptor site on the surface of the vesicles. Consistent with this interpretation is the finding that Ov_D, in which the signal region is exposed (i.e. able to competitively inhibit the interaction of preprolactin with dog microsomal membranes),

FIGURE 5.6

The effects of catalase on amylase release

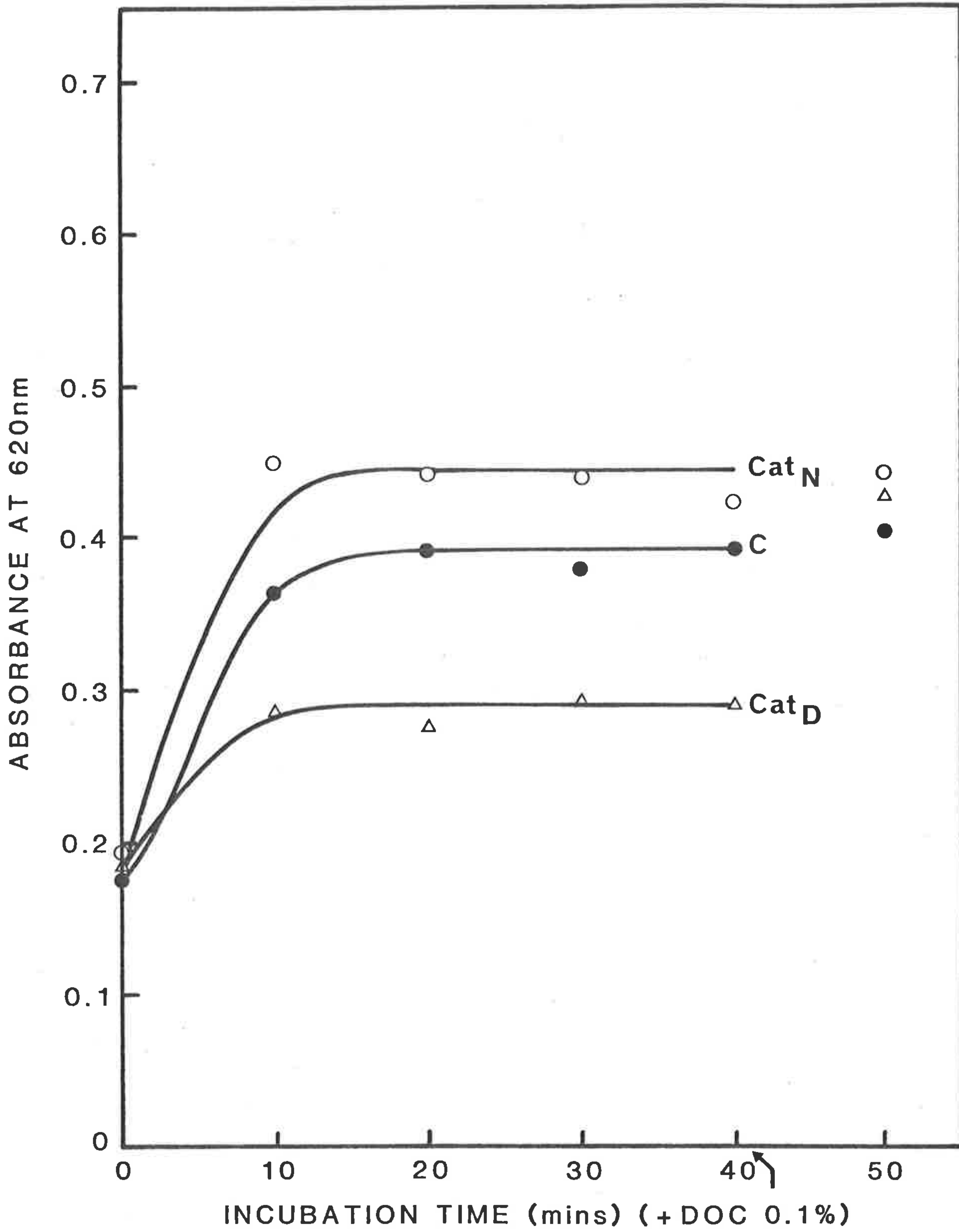
Rat pancreatic microsomes were incubated at $37^{o}C$ in STKC buffer containing:

(●) no additions

(○) 200 μg/ml native bovine liver catalase

(△) 200 μg/ml denatured catalase

FIG 5.6.



inhibited amylase release from rat microsomes, whereas Ov_N, in which the signal activity is masked, was unable to inhibit the release phenomenon. Furthermore, proteins which do not contain a signal sequence, such as a cytoplasmic protein, and several mature, processed, secretory proteins, did not inhibit the amylase release phenomenon.

The highly hydrophobic, secreted protein, BSA did inhibit the escape of amylase from rat microsomes, although it reportedly displayed no signal activity in the *in vitro* assay system containing nascent preprolactin and dog pancreatic microsomes (Lingappa *et al.*, 1979). The reason for this anomaly possibly lies in the differences between the experimental systems to which the BSA was added. In the dog microsome system, inhibition of pre-prolactin processing presumably arises from an introduced protein competing with the authentic signal sequence of preprolactin for binding to signal receptor sites on the membranes. Therefore, unless the introduced protein contained its own signal sequence, or a functionally identical region, the preprolactin sequence would bind preferentially to the receptor site, and inhibition of secretory protein processing would not be observed.

It has been demonstrated that, in rat pancreatic microsome suspensions, neither the presence of full-length pre-proteins (see section 4.4) nor *de novo* synthesis contribute to the enzyme release phenomenon (Pearce *et al.*, 1978). It is therefore unlikely that peptide chains containing signal sequences would be important in this

system. In the absence of authentic ligands, signal peptide binding sites on the microsomal membranes would be susceptible to non-specific interactions with added, hydrophobic molecules. Thus, added BSA may bind, by virtue of its hydrophobicity, to the signal receptors on rat pancreatic microsomes, although it was reportedly unable to compete with authentic signal peptides for the receptors on dog microsomes in an *in vitro* system synthesizing prolactin (Lingappa *et al.*, 1979). The same argument can be used to explain the slight inhibition of amylase release from rat microsomes by native ovalbumin. The absence of competing signal peptides in the rat pancreatic microsome suspensions could also explain why amylase release from these vesicles appears to be more sensitive to the presence of denatured ovalbumin than preprolactin processing by dog microsomes (see section 5.2).

These considerations culminate in the suggestion that Ov_D inhibits the amylase release phenomenon by binding specifically to signal receptor sites on the microsomal membranes, while BSA_D inhibits amylase release by binding non-specifically to the same sites. A comparison of the patterns of inhibition of the release phenomenon produced by the different forms of ovalbumin and BSA, does reveal slight differences in the modes of action of the two proteins. Ov_D and Ov_f both inhibited release strongly, while Ov_N had little effect. These results point to the active inhibitor being a relatively small part of the ovalbumin protein sequence (i.e. contained within one or more

tryptic fragments), which is normally buried within the tertiary structure of the molecule. On the other hand, BSA_N and BSA_f (tryptic fragments of BSA_D) inhibited the release phenomenon only slightly less strongly than BSA_D. Thus the inhibitory activity of BSA is not masked to a significant extent in the native protein, and is slightly sensitive to degradation by trypsin. It is possible to explain this pattern in terms of a single active peptide which is partly buried in the native protein structure and which contains trypsin cleavage site(s) near its ends. In view of the known hydrophobicity of the BSA protein, however, a more likely explanation of the mode of action of BSA on the release phenomenon, is that the inhibitory activity of BSA is dispersed among many hydrophobic regions throughout the molecule, some of which are exposed on the surface of the native protein.

The proposal that Ov_D inhibits amylase release from rat pancreatic microsomes by binding to signal peptide binding sites on the surface of the vesicles, points to another link between *in vivo* secretion and the *in vitro* enzyme release phenomenon. To recapitulate, it was concluded from past work (Pearce *et al.*, 1978) and from the data presented in chapter 3, that amylase escapes from intact, rat pancreatic microsomes via a membrane protein, or protein complex. Work described in chapter 4 revealed that the passage of amylase through this mechanism is blocked by a number of protease inhibitors which have been reported to inhibit signal peptidase activity. In this chapter it has been demonstrated that amylase release

from microsomes is blocked by the highly hydrophobic protein, BSA and by denatured ovalbumin, but not by several other proteins, including native ovalbumin. It is consequently hypothesized that the transfer of amylase through the putative translocation mechanism mentioned above, is stopped by the binding of a signal sequence, or a similar, hydrophobic peptide, to a signal peptide receptor site on the membrane. In summary, the protein complex which appears to mediate the release of amylase from rat pancreatic microsomes has properties in common with both the signal peptidase and signal receptor components of the translocator mechanism which is proposed to operate in *in vivo* secretion.

In this context, it is highly suggestive that the denatured form of catalase, a protein which is reportedly transferred across a membrane without proteolytic removal of a signal sequence, weakly inhibited the amylase release phenomenon. The native catalase enzyme had no effect on release, implying that the active part of the molecule may be buried within the folded structure of the protein. Although it is premature to use the rat pancreatic microsome system predictively, these observations suggest that an uncleaved signal addressed to the translocation mechanism in the peroxisomal membrane may cross-react, to a certain extent, with the analagous mechanism in the ER membrane. This proposed homology seems reasonable in view of the fact that peroxisomes bud directly from the ER. The possible interaction of catalase with the ER membrane translocator complex could not be functional however, as catalase cannot

be transferred into dog pancreatic microsomes *in vitro* (Goldman and Blobel, 1978).

CHAPTER 6

RESULTS

THE EFFECTS OF NEM AND SALT WASHING ON THE RAT PANCREATIC MICROSOME ENZYME RELEASE PHENOMENON

6.1 INTRODUCTION

Recent reports describe an investigation of the nature of the signal peptide binding component of the secretory protein translocating apparatus in dog pancreatic microsomal membranes (Walter *et al.*, 1981; Walter and Blobel, 1981a&b). This study represents the convergence of two experimental approaches to the characterization of this part of the ER membrane translocator mechanism. The first approach, which is outlined in section 5.1, has consisted of the accumulation of evidence for the existence of specific signal peptide binding sites on the surface of dog pancreatic microsomes. The second approach, which is described in detail in section 1.7, consists of studies on the peripheral membrane protein factors which are essential to the translocation and processing of secretory proteins, and which can be removed from the surface of dog pancreatic microsomal membranes by partial proteolysis and/or high salt extraction of the vesicles.

By combining these two concepts, Walter, Ibrahimi and Blobel have defined the "signal recognition protein" or "SRP", which is proposed to recognize and bind to the signal peptide of a nascent secretory protein, as it emerges from the ribosome, at the beginning of protein synthesis. This SRP-signal peptide-polysome complex then binds to its own receptor protein in the ER membrane, thus initiating the assembly of the entire protein translocating mechanism. Continued protein synthesis hypothetically results in co-translational extrusion of

the secretory protein through the membrane into the ER lumen (Walter *et al.*, 1981, Walter and Blobel, 1981).

The bond between the SRP and its membrane-integrated receptor is proposed to be primarily an ionic interaction as extraction of the microsomal membranes with .75 M or .5 M KOAc depletes the vesicles of their translocating activity (Walter and Blobel, 1980; Walter *et al.*, 1981). This finding contrasts with previous reports in which .5 M KCl did not inactivate microsomes, although treatment with low concentrations of trypsin in an environment of low ionic strength did generate an SRP-like factor (Walter *et al.*, 1979). Other salt-extractable factors were found to be protease-generated (Meyer and Dobberstein, 1980a&b), implying that there is a peptide linkage as well as an ionic interaction between such factors and the membrane-integrated transport mechanism.

The relationship between the solubilized factors described by different workers is unclear, however the peptides seem to be functionally similar. In summarizing these reports it can be said that an essential component of the secretory protein translocator mechanism can be removed from the microsomal membrane by an undefined combination of protease action and/or extraction in high salt. This component can be inactivated by treatment with N-ethyl maleimide (NEM), indicating that it contains a sulphydryl group essential to its function (Jackson *et al.*, 1980; Meyer and

Dobberstein, 1980b). The SRP can recombine with salt-extracted membranes and polysomes synthesizing secretory peptides, to form a functional, membrane-bound polysome complex which, with continuing translation, results in processing and segregation, of the mature secretory proteins within the vesicles (Walter *et al.*, 1981; Walter and Blobel, 1981a&b).

In order to extend the comparison between the rat pancreatic microsome enzyme release phenomenon and the published properties of the translocation of secretory proteins into dog pancreatic microsomes, the effects on the amylase release phenomenon of salt extraction and NEM treatment of the rat microsomes, were investigated.

6.2 PROTEASE AND HIGH SALT TREATMENT OF RAT PANCREATIC MICROSOMES

The effects on the rat microsome system of protease treatment and high salt extraction combined, were initially investigated. Microsomes were subjected to mild proteolysis at $2^{o}C$, using either trypsin or elastase. After the inactivation of the protease by the addition of PMSF, the suspensions were supplemented with an equal volume of STKC/1MKCl, resulting in a final KCl concentration of 0.525 M. The vesicles were sedimented by centrifugation at 100,000 g_{av} for 30 mins, and the supernatants were tested for their ability to restore amylase-releasing activity to either the microsomes from which they were derived, or to other

preparations of protease-treated, salt-washed microsomes. A representative experiment is described below.

The microsomal pellet derived from one homogenized rat pancreas (wet weight approx. 1 gm) was resuspended in 6 mls of STKC buffer (approx. 5 mg total microsomal protein/ml). This suspension was divided into two aliquots of 3 mls each. To the control sample (A), PMSF was added to a final concentration of 100 μg/ml, while to the other suspension (B), trypsin was added to a final concentration of 10 μg/ml. Both samples were incubated at 2°C for 30 mins, then PMSF was added to suspension B (final concentration 100 μg/ml). 3 mls of STKC were added to A and 3 mls of STKC/1 M KCl were added to B, following which both samples were incubated for a further 10 mins at 2°C, then centrifuged at 100,000 g_{av} for 30 mins.

The supernatants were decanted and B was reserved as the protease/salt extract. The surface of each microsome pellet was rinsed by gently rolling 2 x 5 mls of STKC buffer around the centrifuge tube. This solution was discarded and each pellet was resuspended in 3 mls of fresh STKC. The following diluted suspensions were incubated at 37°C and in each case the amylase activity in the medium was assayed at 10 minute intervals by the method described in section 2.2.5.

(1) Control microsomes (400 μls A microsomes + 1.6 mls STKC);

(2) Control microsomes + protease/salt extract (400 μls

A microsomes + 400 μls supernatant B + 1.2 mls STKC);

(3) Protease/salt-treated microsomes (400 μls B microsomes + 1.6 mls STKC);

(4) Protease/salt-treated microsomes + protease/salt extract (400 μls B microsomes + 400 μls supernatant B + 1.2 mls STKC).

The contribution of the amylase in the added protease/salt extract to the apparent amylase activity in suspensions (2) and (4) was estimated by assaying an appropriately diluted aliquot of the extract alone. The resultant value of 0.200 A_{620} units was substracted from each amylase estimation in profiles (2) and (4), as illustrated in figure 6.1. The percentage of the total intra-vesicular amylase (i.e. the percentage of that which is latent at zero time, in the absence of detergent) which was actually released by each microsome suspension during the 37°C incubation was calculated (fig. 6.1). By comparing these values, and the graphs of amylase release, it can be seen firstly that mild proteolysis of microsomes followed by washing in a medium of high salt was sufficient to drastically reduce the extent of amylase release from these vesicles during subsequent incubation at 37°C.

If the inactivated microsomes (B) were incubated in the presence of protease/salt extract, amylase release was stimulated by an amount approximately equal to 75% of the level of release from protease/salt-treated microsomes alone. Stimulation of the initial rate, and to a lesser extent, the final plateau level of amylase

FIGURE 6.1

The effects on amylase release of protease and high salt treatment of the microsomes

Untreated rat pancreatic microsomes (c) were incubated at 37°C in STKC buffer containing:

(●) no additions

(△) 400 μl protease/salt extract (PSE).

Microsomes which had been protease-treated and washed in high salt medium as described in the text (RM_i), were incubated at 37°C in STKC buffer containing:

(○) no additions

(▲) 400 μl protease/salt extract (PSE)*.

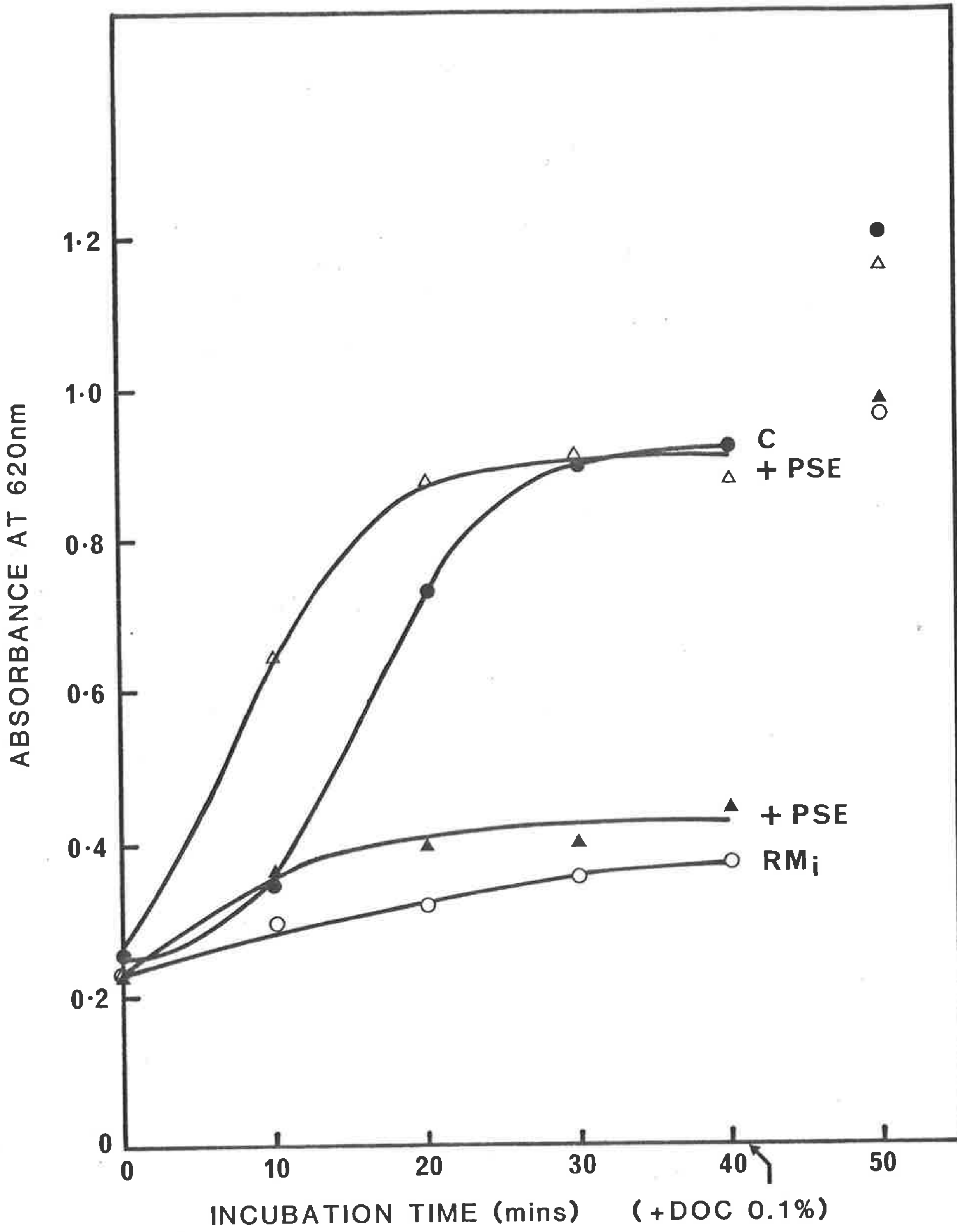
*This amount of protease/salt extract was found to contribute 0.200 A_{620} units to the amylase content of the microsome suspensions. This value was therefore subtracted from each amylase determination in these incubations, resulting in the release curves shown.

The %age of the intra-microsomal released by each suspension was calculated:

$$\% \text{ amylase released} = \frac{\text{amylase released } (A_{620} \text{ at 40 mins} - A_{620} \text{ at 0 mins})}{\text{total intra-microsomal amylase } (A_{620} \text{ after DOC} - A_{620} \text{ at 0 mins})} \times 100$$

microsome suspension		% amylase released
C	(●)	70
C + PSE	(△)	75
RM_i	(○)	20
RM_i + PSE	(▲)	34

FIG 6.1.



release from control microsomes (A), by protease/ salt extract, was also observed.

These data represent the most successful of many attempts to reconstitute enzyme release from protease and high salt-treated rat pancreatic microsomes. The addition of protease/salt extracts produced a level of stimulation of amylase release from protease/salt-treated microsomes which varied between 0 and 100% of the level of release from the inactivated microsomes alone (results not shown). The actual amount of amylase released from reconstituted vesicles was always very low compared to the amount of enzyme released from control microsomes, for example see fig. 6.1. In this experiment, although the addition of protease/salt extract almost doubled the extent of amylase release from protease/salt-treated vesicles, this stimulated level of enzyme release was only equivalent to roughly one third of the amount of amylase released from control microsomes. This is the highest level of reconstitution which was achieved by adding a protease/ salt extract to protease/salt-treated microsomes. In several cases the low levels of reconstitution were thought to be due to residual protease activity, which was detected by hide powder assay, in the protease/salt extract (results not shown). In order to eliminate this problem, microsomes were treated with high salt alone (see next section).

6.3 KCl-EXTRACTION OF RAT PANCREATIC MICROSOMES

Microsomes isolated from a homogenized rat pancreas, as described in section 2.2.1, were resuspended in 3 mls of STKC buffer. The microsome suspension was divided into two aliquots of 1.5 ml, one of which (A) was supplemented with 1.5 mls of STKC buffer to give 3 mls of a control suspension containing approximately 5 mg total microsomal protein/ml. The other aliquot (B) was supplemented with 1.5 ml STKC/1 M KCl to yield microsomes suspended in STKC/.5 M KCl. Both suspensions were incubated at $2^{o}C$ for 30 mins, after which the vesicles were sedimented by centrifugation at 100,000 g_{av} for 30 minutes.

The supernatants were decanted and supernatant B, which constituted the microsomal salt extract, was retained. The surface of each microsome pellet was rinsed, then the pellets were each resuspended in 3 mls of fresh STKC buffer at $2^{o}C$. These microsomes were diluted and incubated at $37^{o}C$ as follows.

(1) Control microsomes (200 µl A microsomes + 1.8 ml STKC);

(2) Control microsomes + salt extract (200 µl A microsomes + 200 µl salt extract (SE) + 1.6 ml STKC);

(3) Control microsomes + KCl (200 µl A microsomes + 200 µl STKC/.5 M KCl + 1.6 ml STKC);

(4) Salt-washed microsomes (200 µl B microsomes + 1.8 ml STKC);

(5) Salt-washed microsomes + salt extract (200 μl B microsomes + 200 μl SE + 1.6 ml STKC);

(6) Salt-washed microsomes + KCl (200 μl B microsomes + 200 μl STKC/.5 M KCl + 1.6 ml STKC).

A profile of amylase release was plotted for each suspension, and the percentage of the total intra-vesicular amylase released by each sample was calculated (fig. 6.2). It was found that treatment of the vesicles with high salt caused slight inhibition of enzyme release from rat pancreatic microsomes. A noticeable amount of the microsome-associated amylase was lost from KCl-treated vesicles during the high salt wash, thus the graph of amylase release from salt-washed microsomes is lower than the graph of amylase release from control microsomes, which were washed in STKC buffer only. Comparison of the relative amounts of amylase released from each suspension however, reveals that the salt-washed microsomes released 65% of their intra-vesicular amylase, which is only slightly less than the 73% released by control microsomes (fig. 6.2).

When salt extract (which contained 0.5 M KCl) was added to salt-washed microsomes, amylase release was increased to 80% of the intra-vesicular enzyme. The initial rate of amylase release was also significantly increased. This effect contrasts sharply with the effect of adding an equivalent volume of 0.5 M KCl alone, to salt-washed microsomes (final concentration 0.125 M KCl, including KCl in STKC buffer). In this

FIGURE 6.2

The effect on amylase release of high salt-washing the microsomes

Untreated rat pancreatic microsomes (C) were incubated at 37°C in STKC buffer containing:

(●) no additions

(△) 200 µl salt extract (SE)*

(□) 200 µl STKC buffer containing 0.525 M KCl*

Microsomes which had been washed in high salt medium as described in the text (RMi) were incubated at 37°C in STKC buffer containing:

(○) no additions

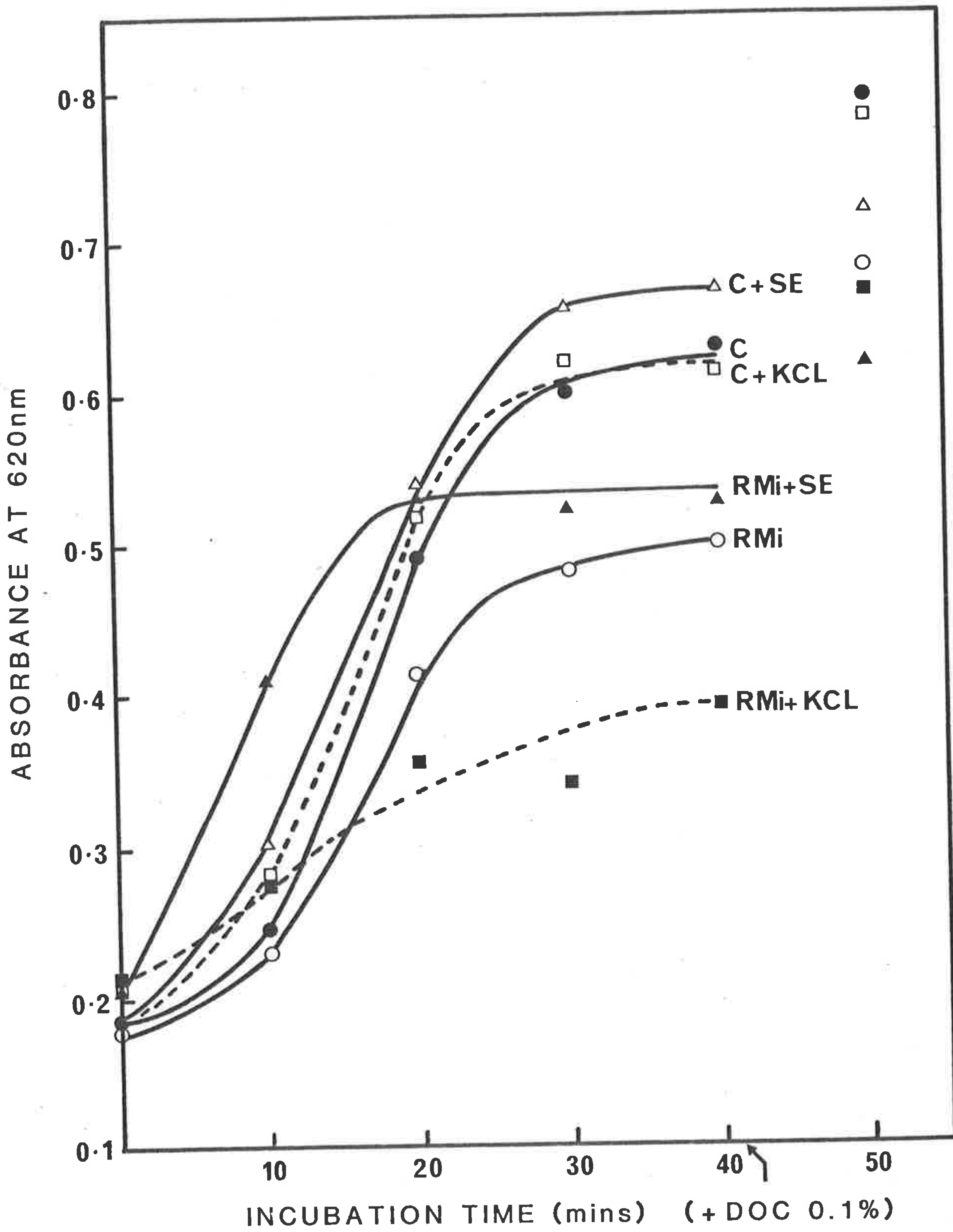
(▲) 200 µl salt extract (SE)*

(■) 200 µl STKC buffer containing 0.525 M KCl*

*All these microsome suspensions contained a final KCl concentration of 0.125 M.

Microsome suspension		% amylase released
C	(●)	73
C + SE	(△)	88
C + KCl	(□)	74
RM_i	(○)	65
RM_i + SE	(▲)	80
RM_i + KCl	(■)	40

FIG 6.2.



latter case, the amylase released during incubation of the microsome suspension at $37^{o}C$ for 30 mins, was equal to only 40% of the total, intra-vesicular enzyme. On the other hand, the addition of KCl alone, to control microsomes, stimulated amylase release slightly, as did the addition of salt extract (fig. 6.2).

The mechanism and significance of the differential effect of 0.125 M KCl on high salt-washed microsomes, as opposed to control microsomes is unknown. It is clear, however, that the course of amylase release from salt-washed microsomes + salt extract should be compared with amylase release from salt-washed vesicles + an equivalent volume of 0.5 M KCl, rather than with enzyme release from salt-washed vesicles alone. Within this frame of reference (i.e. in the presence of 0.125 M KCl), salt extract can be seen to stimulate the release of amylase from salt-washed vesicles by an amount equivalent to 100% of the initial level of enzyme release from these vesicles.

From the results presented in sections 6.2 and 6.3, it is concluded that both protease-treatment followed by a high salt wash, and high salt extraction alone, can significantly inhibit the release of amylase from rat pancreatic microsomes. The finding that release of enzyme from the inactivated microsomes could be reconstituted by re-addition of the extracts to the treated vesicles, suggested that a factor which was active in the release process, could be removed from the membranes, then replaced. To investigate the nature of

this factor, a further experiment was performed (see next section).

6.4 PROTEASE-OR NEM-TREATMENT OF SALT EXTRACT

As a first step towards characterizing the active constituent of the salt extract, the sensitivities of the extract to a protease, and to NEM, were assessed. A microsome pellet derived from one rat pancreas (approx. wet weight 1 gm) was resuspended in 6 mls STKC/0.5 M KCl. The suspension was incubated at 2°C for 30 mins then centrifuged at 100,000 g_{av} for 30 mins. The supernatant (salt extract) was decanted and the pellet of salt-washed microsomes was carefully rinsed with 2 x 5 mls of STKC buffer, then resuspended in 6 mls of fresh STKC. The salt extract was divided into aliquots which were treated as described below.

(1) Control: approximately 4 mls of salt extract were maintained at 2°C for 30 mins.

(2) Protease: a) Protease treatment: to 0.5 ml of salt extract, trypsin was added, to a final protease concentration of 20 μg/ml. The extract was incubated in the presence of the active hydrolase at 37°C for 10 mins. A molar excess of the inhibitor TLCK was then added (final concentration 1 mM) and the solution was cooled to 2°C.

b) Protease/control: 0.5 ml of salt extract was supplemented with TLCK to a final inhibitor concentration of 1 mM. Trypsin was then added (final concentration 20 μg/ml), and the extract

was incubated at 37°C for 10 mins, then cooled to 2°C.

(3) NEM: a) NEM/37°C: 0.4 ml of salt extract was supplemented with a fresh solution of NEM to a final inhibitor concentration of 5 mM, and incubated at 37°C for 10 mins. A molar excess of β-mercaptoethanol (βME) was then added (final concentration 12.8 mM) to destroy the unreacted maleimide, and the solution was cooled to 2°C.

b) NEM/2°C: procedure as for a) except the solution was maintained at 2°C instead of being incubated at 37°C.

c) NEM/control: procedure as for b), except βME was added before NEM.

The rates of amylase release from salt-washed microsomes in the presence of 75 mM KCl alone, or in the presence of each of the above extracts (final concentration of KCl in these microsome suspension = 75 mM), were determined (figs. 6.3 and 6.4). The percentage of the intra-vesicular amylase released by each suspension is also displayed. Salt-washed microsomes released 28% of their content enzyme in the presence of 75 mM KCl, whereas salt-washed vesicles supplemented with the unmodified salt extract (1) released 38% of their amylase at a significantly accelerated rate. A comparable level of enzyme was released at the same high rate from vesicles incubated in the presence of the protease/control extract (2(b)), however, treatment of the salt extract with active protease (extract 2(a)) completely abolished the

FIGURE 6.3

The effects of proteolysis on the activity of the salt extract

Rat pancreatic microsomes which had been washed in high salt medium as described in the text (RM_i), were incubated at 37°C in STKC buffer containing:

- (●) 100 µl STKC buffer containing 0.525 M KCl
- (○) 100 µl untreated salt extract (SE)
- (▲) 100 µl protease/control salt extract (see text)
- (△) 100 µl protease-treated salt extract (see text)

All microsome suspensions contained 200 µl salt-washed microsomes in a total volume of 2 mls. The final concentration of KCl in each suspension was 75 mM.

Microsome suspension		% amylase released
RM_i + KCl	(●)	28
RM_i + SE	(○)	38
RM_i + SE/PC	(▲)	27
RM_i + SE/P	(△)	39

FIG 6.3.

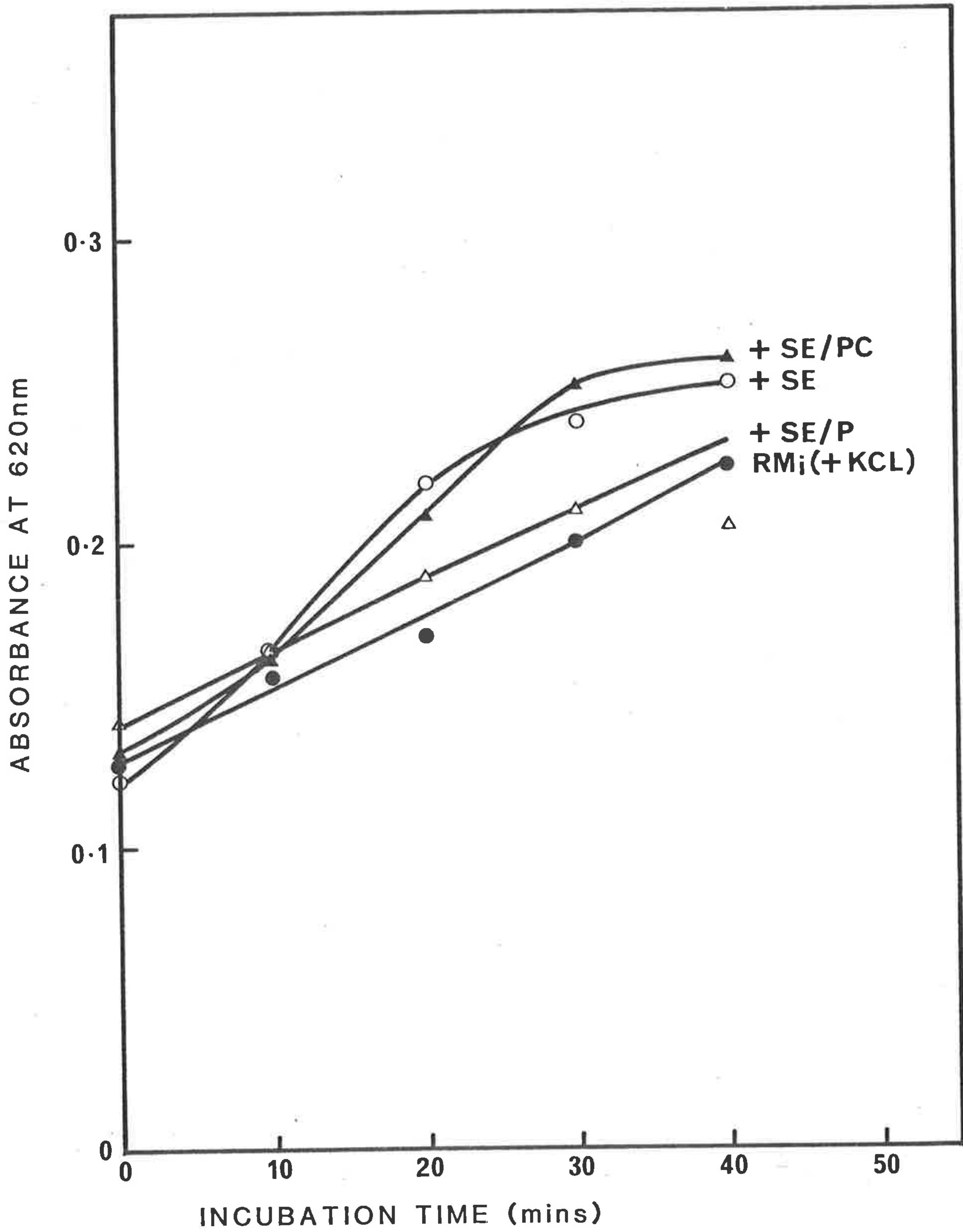


FIGURE 6.4

The effect of NEM-treatment on the activity of the salt extract

Rat pancreatic microsomes which had been washed in high salt medium as described in the text (RM_i), were incubated at 37°C in STKC buffer containing:

(●) 100 µl STKC containing 0.525 M KCl

(○) 100 µl untreated salt extract (SE)

(▲) 100 µl NEM/control salt extract (see text)

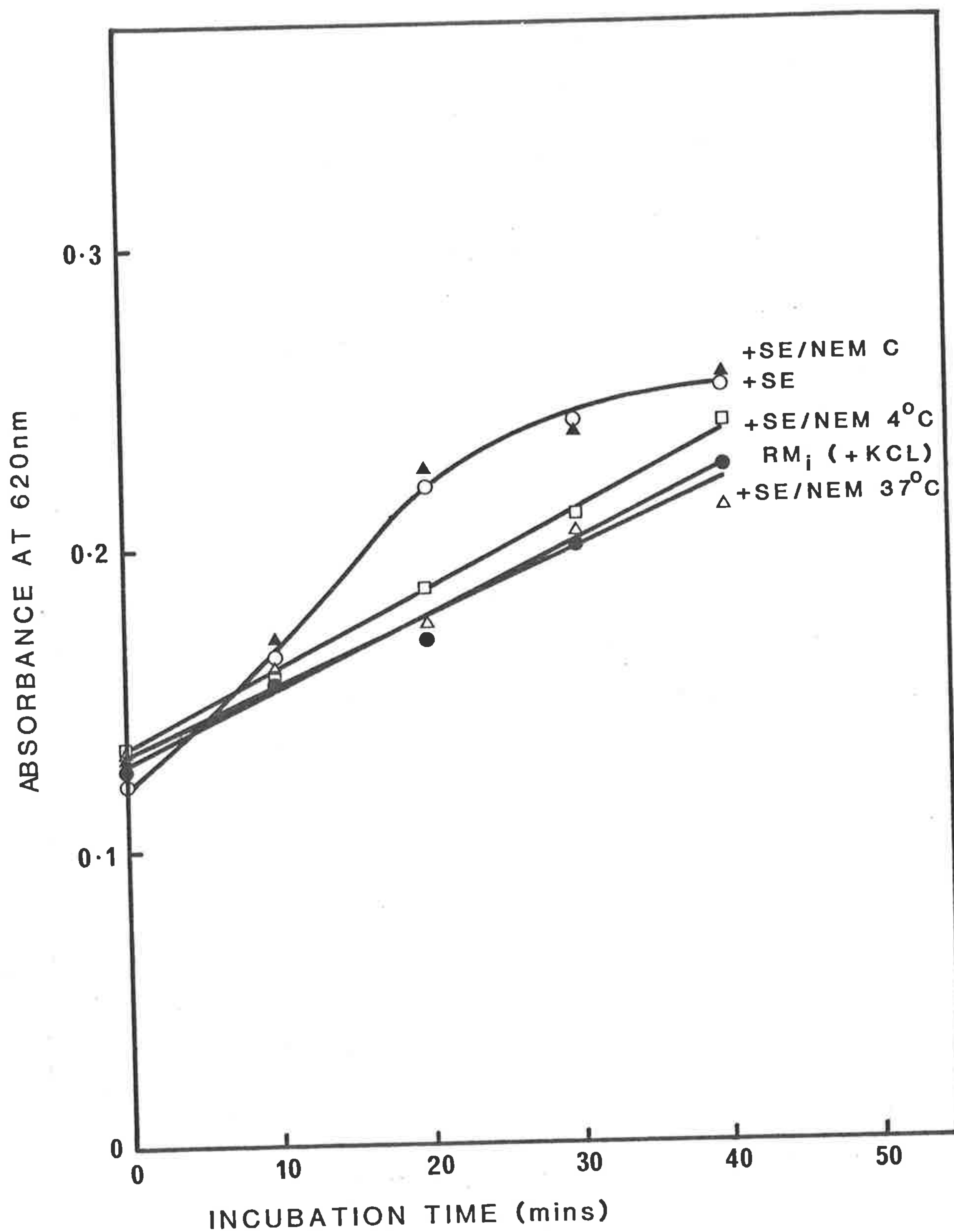
(△) 100 µl salt extract which had been treated with NEM at 37°C (see text)

(□) 100 µl salt extract which had been treated with NEM at 2°C (see text)

All microsome suspensions contained 200 µl salt-washed microsomes in a total volume of 2 mls. The final concentration of KCl in each suspension was 75 mM.

Microsome suspension	% amylase released
RM_i + KCl	28
RM_i + SE	38
RM_i + SE/NEM C	37
RM_i + SE/NEM 37°	25
RM_i + SE/NEM 2°	32

FIG 6.4.



stimulatory properties of the extract (see fig. 6.3).

Similarly, enzyme release from salt-washed vesicles was stimulated by the NEM/control salt extract (3(c)), whereas treatment of the extract with 5 mM NEM at 37°C (extract 3(a)) completely destroyed the activating properties of the extract. NEM treatment of salt extract at 2°C had a parallel, but slightly less extreme effect (fig. 6.4). The maintenance of the original, low rate of amylase release from the salt-washed microsomes to which the inactivated extracts (2(a), 3(b), 3(a)) were added, as well as the stimulatory effects of the control extracts (2(b) and 3(c)) signified that the modified salt extracts contained no diffusable inhibitors of amylase release such as active protease or NEM molecules.

The most logical interpretation of these findings involves postulating that a protein which has an unknown role in the amylase release phenomenon, can be removed from rat pancreatic microsomes by washing the vesicles in a medium of high ionic strength. This protein seems to contain a sulphydryl group which is essential to its activity and which is vulnerable to attack by NEM.

6.5 INHIBITION OF AMYLASE RELEASE FROM RAT PANCREATIC MICROSOMES BY NEM

The suggestion that a salt-extractable, NEM-sensitive protein was apparently involved, in some way, in the mechanism of amylase release from rat pancreatic microsomes, represented the culmination of two concurrent

lines of investigation. The first approach; that of the study of the effects of high salt washing of the microsomes on the enzyme release phenomenon, was described in sections 6.3 and 6.4. The second approach, which comprised a study of the effects of NEM on the release of amylase from fresh rat pancreatic microsomes (i.e. not salt-washed) is described below.

Freshly prepared rat pancreatic microsomes (method, section 2.2.1) were incubated at 37°C in the presence of a range of concentrations of NEM (Fig. 6.5). It was seen that NEM concentrations in excess of 50 μM caused significant inhibition of the release phenomenon. The kinetics of the inhibition were remarkable in that NEM-treated microsomes released amylase at the same rate as control samples for approximately 10 to 15 mins,after which enzyme release quite suddenly ceased (see fig. 6.5).

The inhibition was dependent on the concentration of NEM up to a level of between 2 and 5 mM, beyond which maximal inhibition was observed, with amylase release being abruptly and completely halted after 10 mins of incubation. Even in the presence of 10 or 20 mM NEM, a normal, control rate of amylase release from microsomes occurred during the initial 10 minute period. If microsome suspensions were transferred from 2°C to 37°C at zero time, then supplemented with 5 mM NEM at 2 mins or 5 mins after the beginning of incubation, complete inhibition of amylase was still not observed until a total of 10 mins of incubation had elapsed

FIGURE 6.5

The inhibition of amylase release from microsomes by NEM

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

(●) no additions

(○) 0.1 mM NEM

(▲) 2 mM NEM

(■) 5 mM NEM

(△) 10 mM NEM

(□) 20 mM NEM

A stock solution of 0.2 M NEM in glass distilled water, was prepared seconds before the addition of aliquots of the solution to microsome suspensions, resulting in the listed final concentrations of inhibitor. The microsome suspensions were transferred from 2°C to 37°C immediately after the addition of NEM.

FIG 6.5.

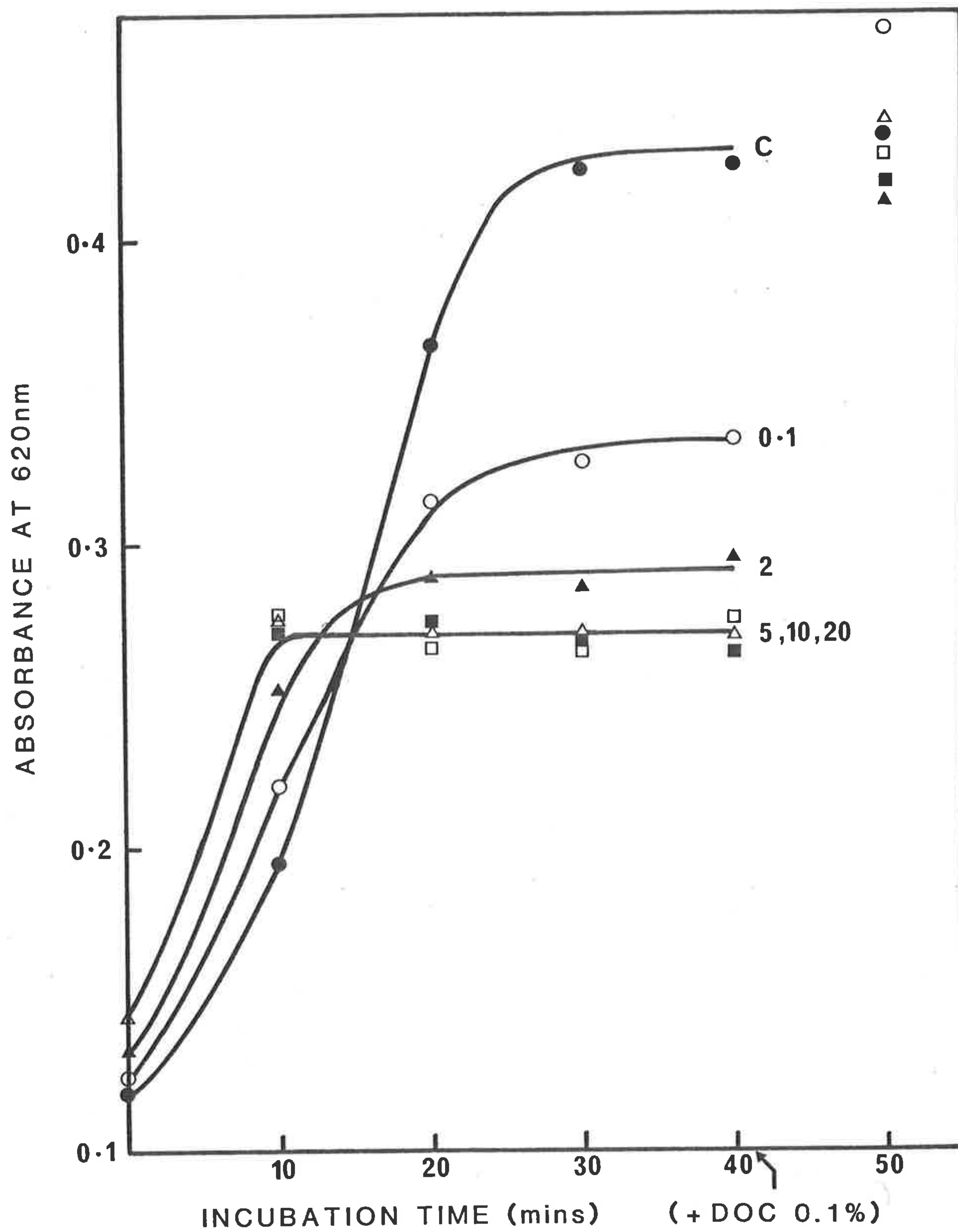


FIGURE 6.6

Addition of NEM to microsomes at 0, 2 & 5 mins after the beginning of incubation

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

(●) no additions

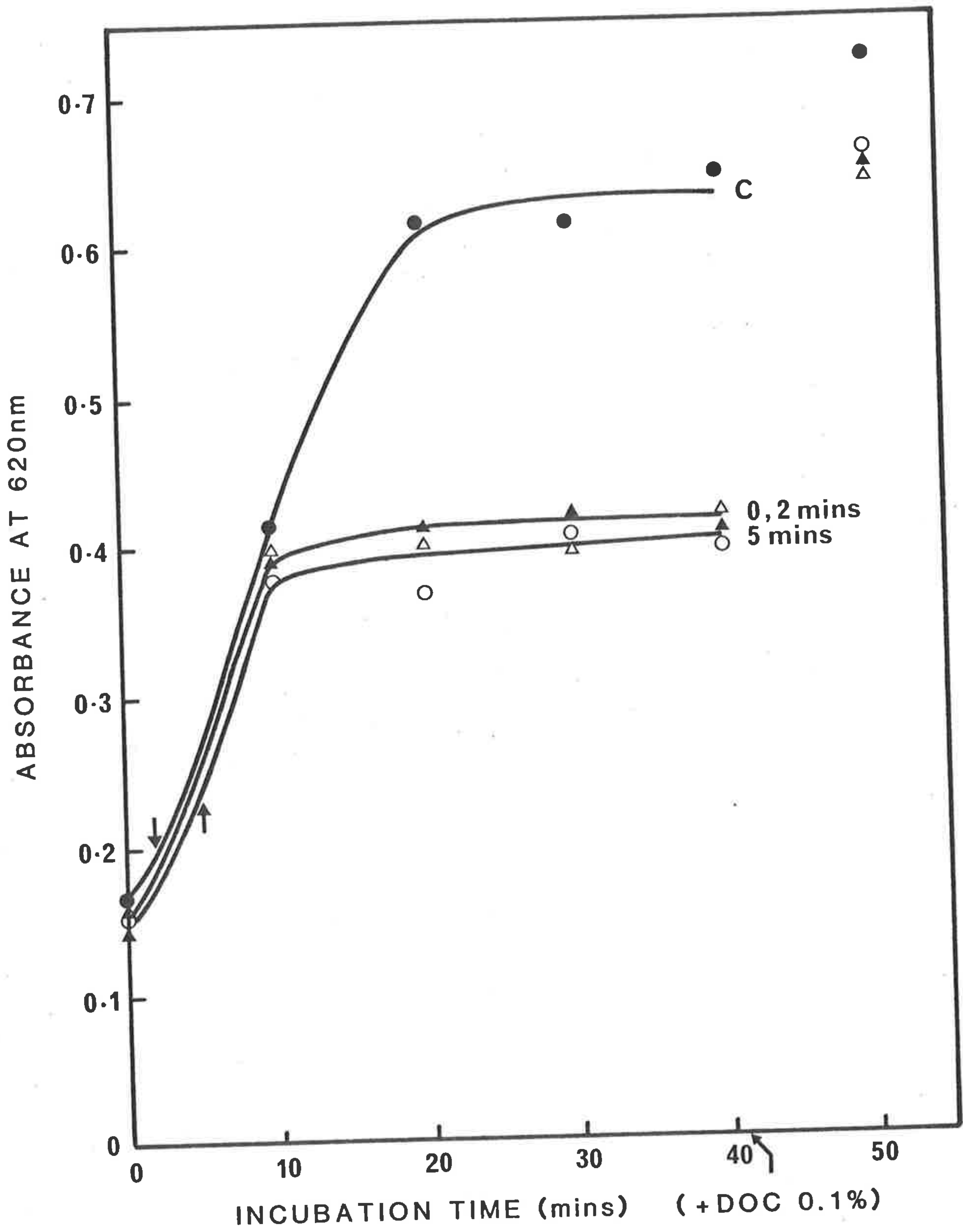
(▲) 5 mM NEM, added at zero time

(△) 5 mM NEM, added at 2 mins

(○) 5 mM NEM, added at 5 mins

Incubation was initiated by transferring 2 mls of a microsome suspension from an ice bath at 2°C to a water bath at 37°C. NEM was added at the indicated times after the beginning of incubation.

FIG 6.6.



(fig 6.6). If NEM was added to the microsomes at 10 mins or 15 mins after the beginning of incubation amylase release was halted immediately (fig. 6.7). A similar effect was observed with the sulphydryl reagent p-hydroxymercuribenzoate (pOHMB) as illustrated by figure 6.8.

It was confirmed that the initial rate of amylase release from NEM-treated microsomes was indeed equivalent to the rate of enzyme release from control rat pancreatic microsomes by assaying the level of amylase activity in the suspensions at 2 minute time intervals rather than at 10 minute time intervals as was routinely practised (fig. 6.9). These incubations were initiated by introducing 100 µl aliquots of a freshly-prepared, concentrated microsome suspension at $2^{o}C$ (5 mg total microsomal protein/ml), into 1.9 mls of STKC buffer pre-equilibrated at $37^{o}C$. In this way, any lag in enzyme release arising from the equilibration of the microsome suspension to $37^{o}C$ was minimized. Under these conditions, the cut-off point of amylase release in the presence of 5 mM NEM, could be accurately located at 8 mins after the beginning of incubation (fig. 6.9).

Thus it appeared that the release of amylase from rat pancreatic microsomes became sensitive to sulphydryl reagents after an 8 to 10 minute incubation period. As illustrated in figure 6.10, pre-incubation of vesicles at 2° or $28^{o}C$, for 20 mins, in the presence of NEM, did not alter the timing of the onset of NEM-induced inhibition of amylase release when the microsome suspension was

FIGURE 6.7

Addition of NEM to microsomes at 0, 10 & 15 mins after the beginning of incubation

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

(●) no additions

(○) 5 mM NEM added at zero time

(▲) 5 mM NEM added at 10 mins

(△) 5 mM NEM added at 15 mins

NEM was added at the indicates times after the beginning of incubation.

FIG 6.7.

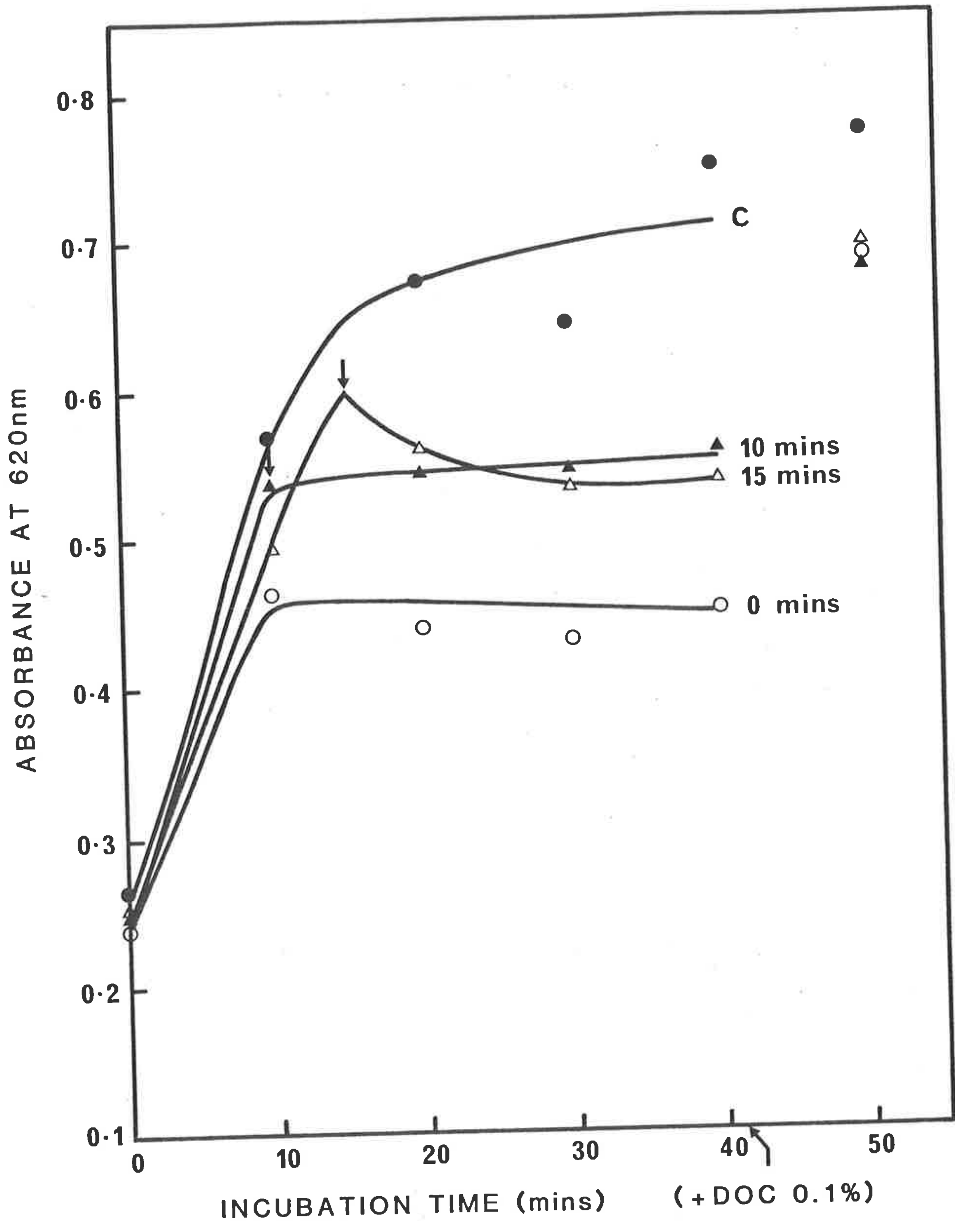


FIGURE 6.8

The effects on amylase release of pOHMB

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

(●) no additions

(O) 20 μM pOHMB added at zero time

(Δ) 20 μM pOHMB added at 15 mins after the beginning of incubation

FIG 6.8.

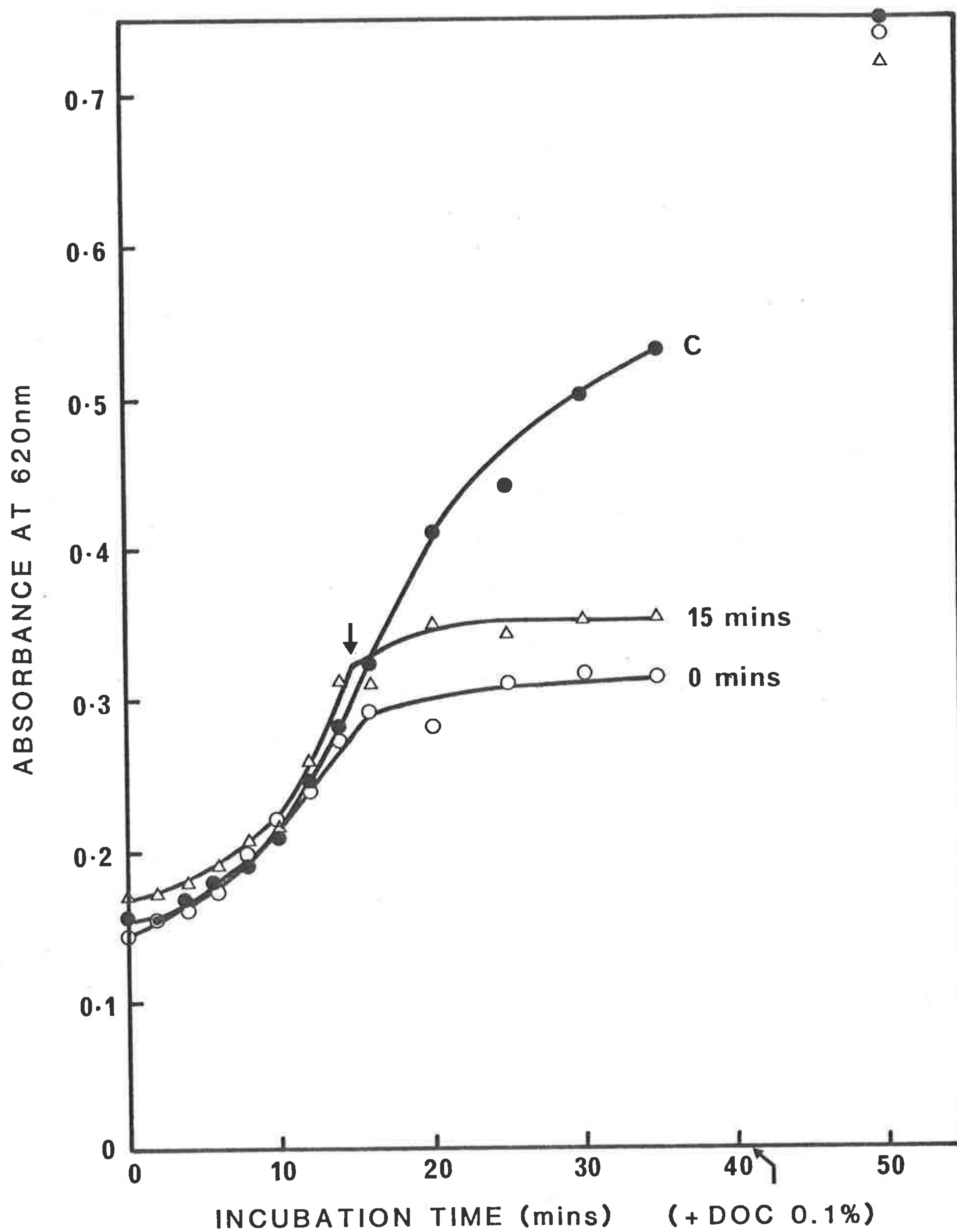


FIGURE 6.9

Initial rates of amylase release in the presence of NEM

Rat pancreatic microsomes incubated at 37°C in STKC buffer containing:

(●) no additions

(○) 5 mM NEM added at zero time

(△) 5 mM NEM added at 5 mins after the beginning of incubation

In each case, incubation was initiated by injecting a 100 μl aliquot of a concentrated microsome suspension into 1.9 mls of STKC buffer pre-equilibrated at 37°C. Samples were analysed for amylase activity at 2 minute intervals.

FIG 6.9.

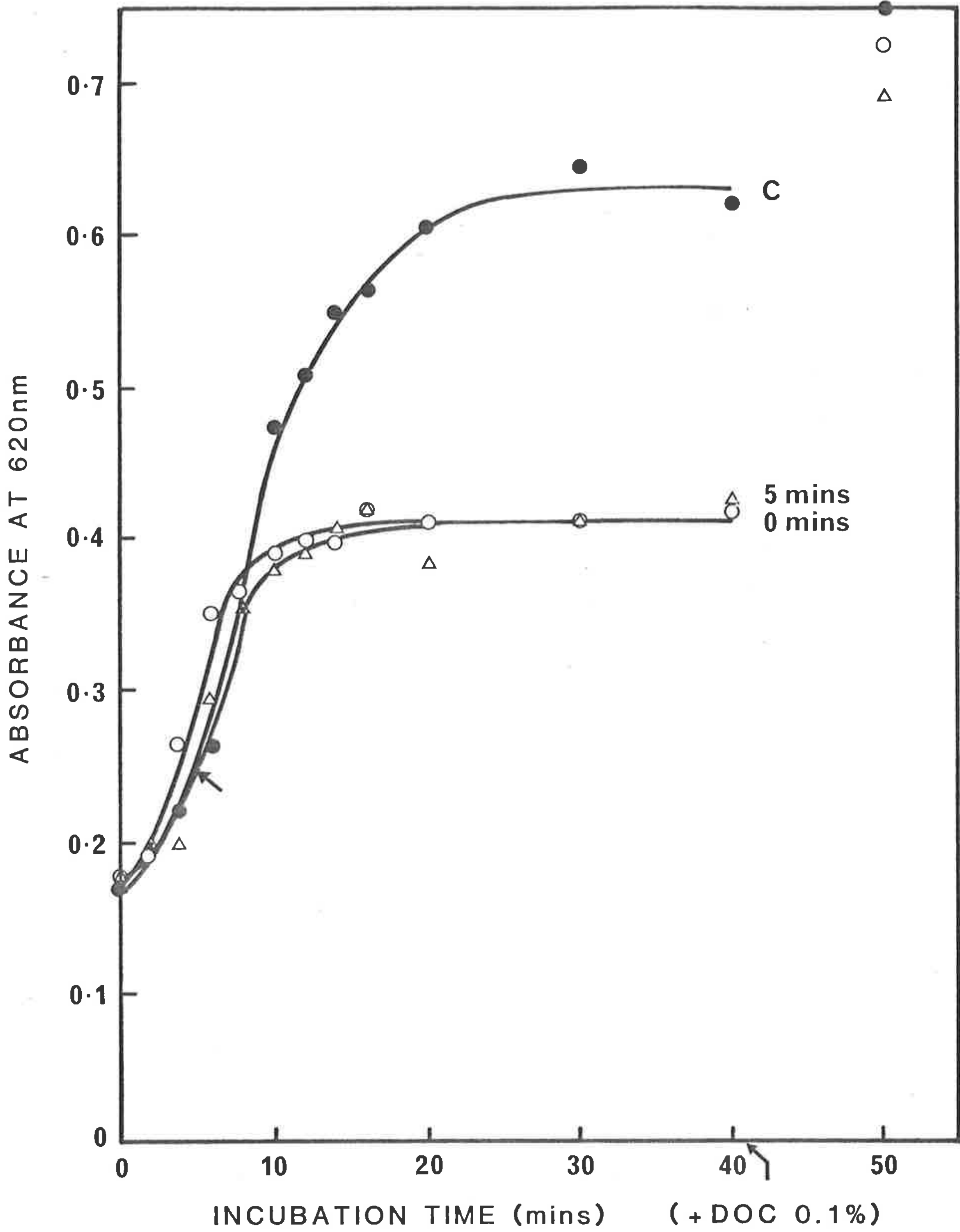


FIGURE 6.10

Pre-incubation of microsomes at 2°, 28° & $30^{o}C$, in the presence of NEM

Rat pancreatic microsomes were incubated in STKC buffer, for 20 mins at:

(●) $2^{o}C$

(▲) $2^{o}C$, in the presence of 5 mM NEM

(O) $28^{o}C$, in the presence of 5 mM NEM

(△) $30^{o}C$, in the presence of 5 mM NEM

NEM was added at zero time in all cases. At 20 mins after the beginning of pre-incubation, all suspensions were transferred to $37^{o}C$.

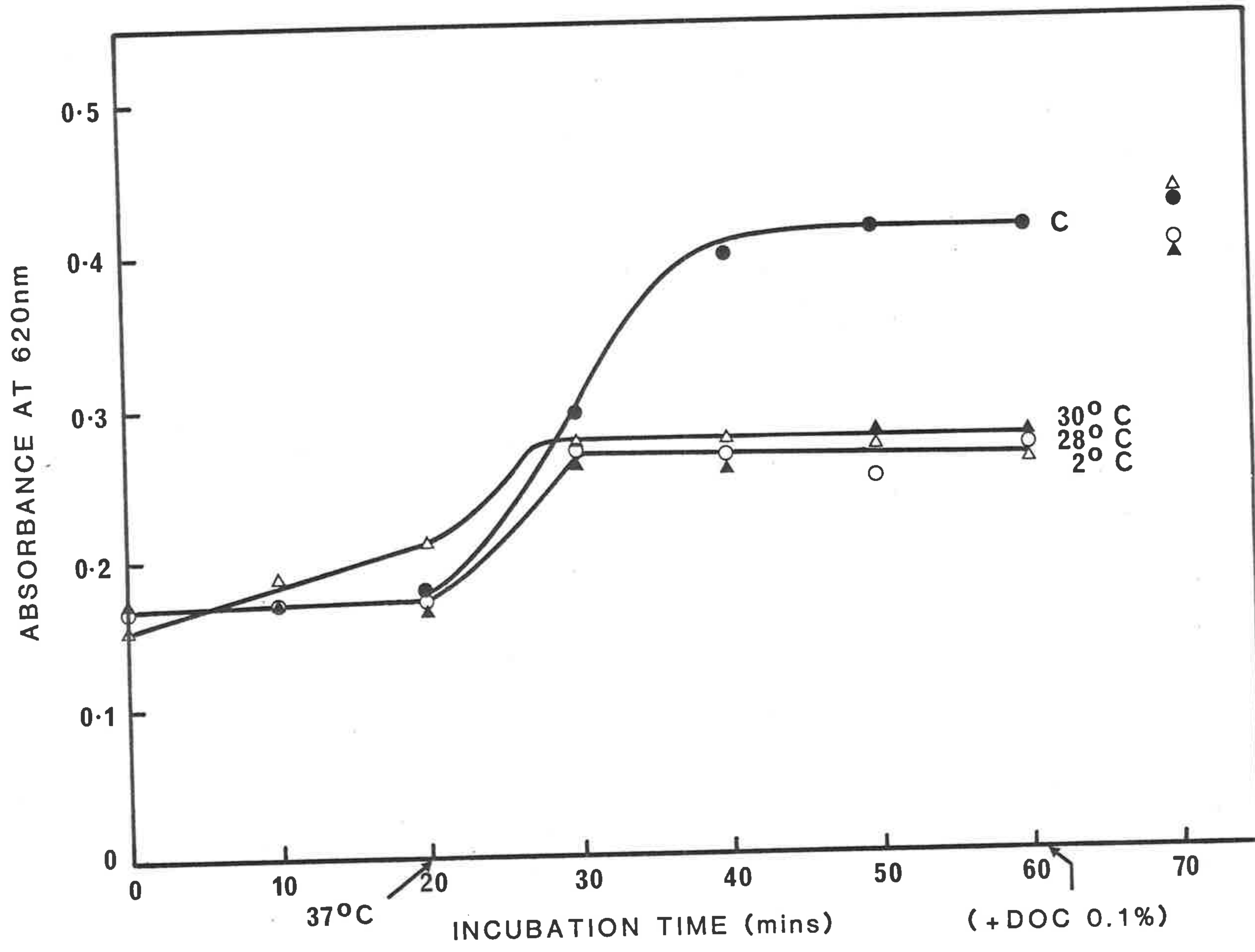


FIG 6.10.

transferred to 37°C. No release of enzyme occurred during the 2° or 28° incubations. If vesicles were pre-incubated at 30°C in the presence of NEM, some amylase was released over a 20 minute period. When these microsomes were transferred to 37°C, cessation of amylase release occurred slightly sooner than in the previous instance, but because of the amylase released during the 30°C incubation, the level of extra-microsomal enzyme present in both suspensions at the onset of NEM-induced inhibition, was the same. This same amylase concentration was observed in control microsomes incubated at 37°C in the presence of 5 mM NEM (see fig. 6.10 & 6.9).

It therefore seemed that there was a relationship between the time and temperature of incubation, the actual stage of the microsome enzyme release process, and the timing of NEM-induced inhibition of amylase release. To determine whether or not NEM exerted its effect by interacting with a product of the release process, for example extra-microsomal amylase, a sample of microsomes was incubated at 37°C for 40 mins to completely release the intra-vesicular enzymes. This suspension was then cooled to 2°C and mixed with control microsomes which had been maintained at 2°C since their isolation. During subsequent incubation at 37°C, 5 mM NEM halted enzyme release from the control vesicles after 10 mins, whether or not they had been mixed with the "exhausted" suspension (fig. 6.11). It was therefore concluded that NEM-induced inhibition is not

FIGURE 6.11

Effects of release products on NEM inhibition of amylase release

Rat pancreatic microsomes were incubated at $37^{\circ}C$ in STKC buffer for 40 mins, and then cooled to $2^{\circ}C$ (Exhausted microsomes). Control microsomes were maintained at $2^{\circ}C$ for 40 mins. The following mixtures were incubated at $37^{\circ}C$:

(●) Control microsomes (0.3 mg total mic. protein/ml)

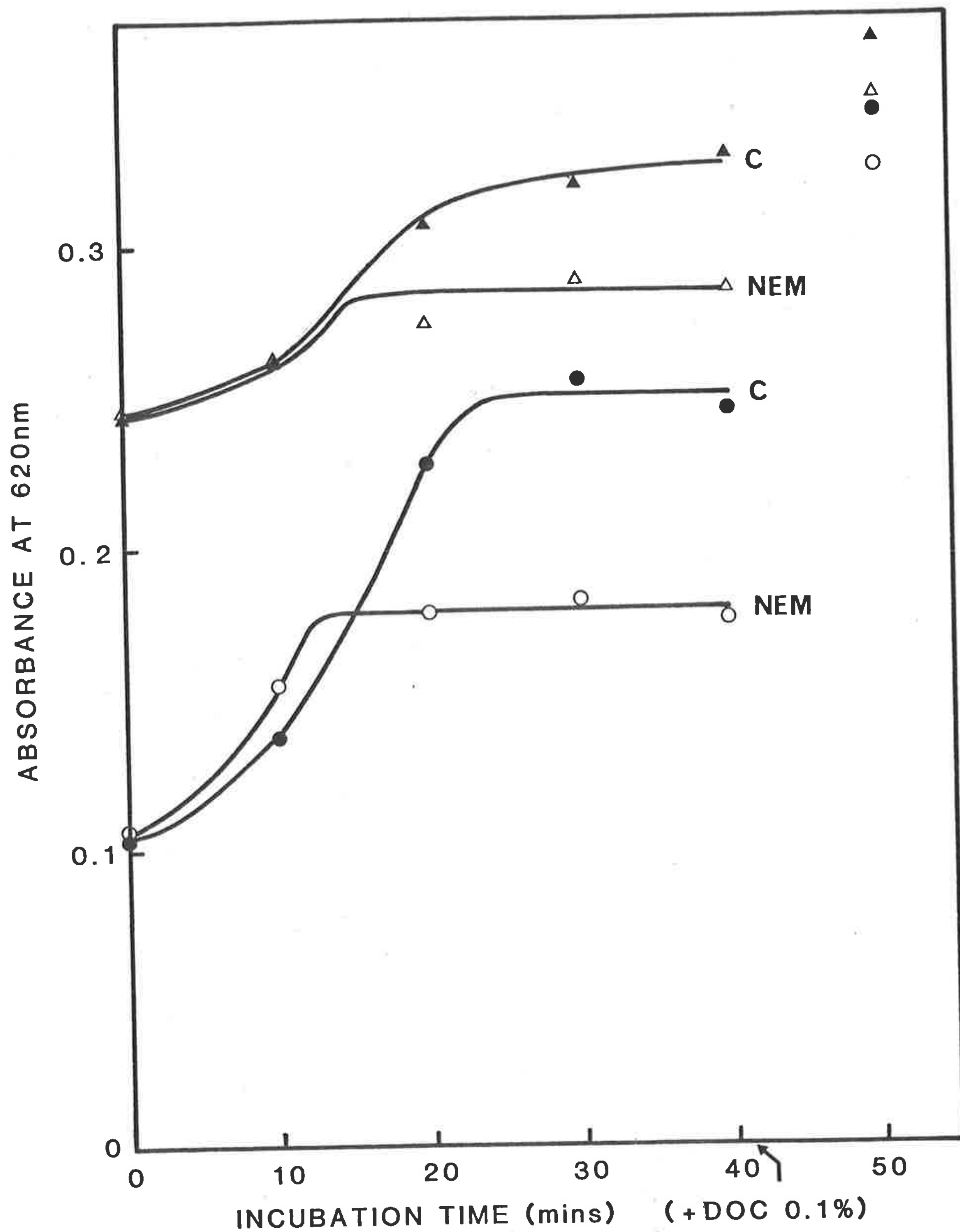
(O) Control microsomes + 5 mM NEM

(▲) Control microsomes (0.15 mg/ml) + Exhausted microsomes (0.3 mg/ml)

(△) Control microsomes + Exhausted microsomes + 5 mM NEM.

All concentrations are final concentrations.

FIG 6.11.



dependent on a certain concentration of some release product in the extra-microsomal medium.

The results presented in this section lead to the proposition that some kind of change in the microsomes themselves, which accompanies the amylase release process, renders that process sensitive to inhibition by NEM, after approximately 10 mins incubation at 37°C. The initial insensitivity of the release phenomenon to NEM was illustrated by maintaining freshly prepared vesicles at 2°C in the presence of 5 mM NEM for 20 mins. The NEM was then quenched by addition of a molar excess of βmercapto-ethanol and the microsomes were transferred to 37°C, resulting in a "normal" rate of amylase release (fig. 6.12). This finding confirms that a brief exposure of microsomes to NEM prior to an inferred change in the membranes, which occurred after about 10 mins incubation at 37°C, had no inhibitory effect on subsequent amylase release from the vesicles.

6.6 SUMMARY AND DISCUSSION

The response of the rat pancreatic microsome enzyme release phenomenon to salt extraction of the microsomes establishes yet another area of correspondence between the release phenomenon and the published properties of the physiological trans-membrane translocation of secretory proteins. In the latter case it has been reported by two different research groups, that a protein which contains an NEM-sensitive sulphydryl group, and

FIGURE 6.12

Pre-incubation of microsomes at 2°C in the presence of NEM

Rat pancreatic microsomes were maintained at 2°C for 20 mins in the presence of:

(● ▲) no additions

(○) 5 mM NEM

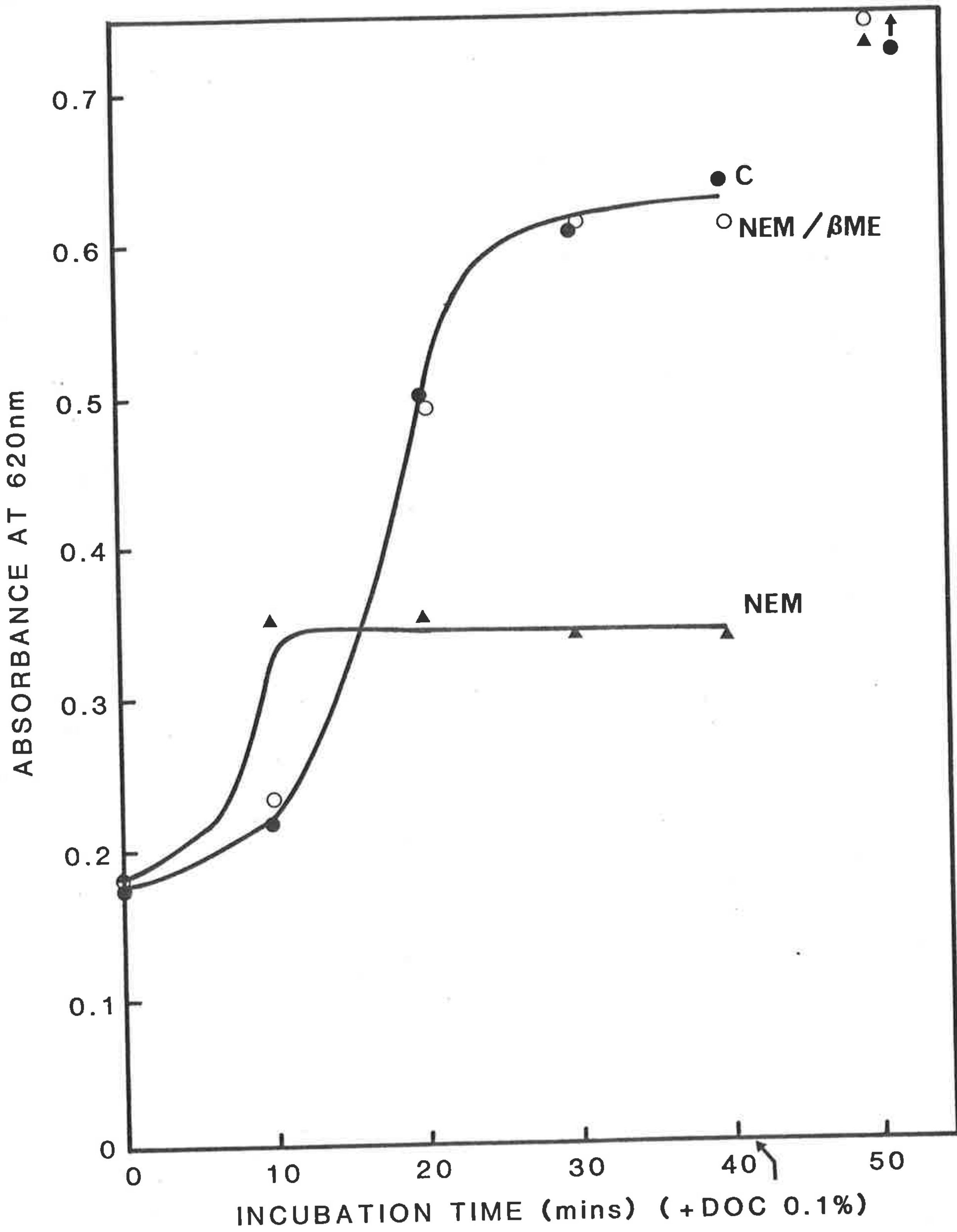
At 20 mins, βME (final concentration, 12.8 mM) was added to the suspension containing NEM. The suspensions were incubated at 37° in the presence of:

(●) no additions

(▲) 5 mM NEM (fresh solution)

(○) no further additions.

FIG 6.12.



which is essential to the translocation process, can be removed from dog pancreatic microsomes by proteolysis and/or salt extraction (for refs. see sections 6.1 and 1.7).

The data reported in this chapter reveal that salt extraction, with or without prior proteolysis, will diminish the rate and extent of amylase release from rat pancreatic microsomes. Re-addition of the extracts to the vesicles was found to re-activate the release process to some extent, indicating that the effect of the high salt wash was reversible. The active constituent of the salt extract was characterized insofar as it was found to be destroyed by proteolysis of the extract, or treatment of the extract with NEM. By these criteria, it can be postulated that the inhibitory effect on amylase release from rat pancreatic microsomes, of salt-washing the vesicles, can be partially reversed by the addition of a protein containing an NEM-sensitive sulphydryl group.

The parallel between this statement and the reported effects of salt extraction on secretory protein translocation across the dog pancreatic microsomal membrane is obvious. In both cases the inhibition of the apparent trans-membrane translocation of secretory proteins, by salt extraction of the microsomes, is reversible by the addition of an NEM-sensitive protein. This correlation suggests a similarity between the two transport mechanisms despite the fact that one mechanism seems to transfer nascent peptides <u>into</u> dog microsomes,

while the other appears to transfer active, fully-formed enzymes out of rat microsomes.

In the case of secretory protein transport into dog microsomes, the mode of action of the extractable protein factor has been well defined. Walter and Blobel (1980) have purified the active constituent of the salt extract and demonstrated that this signal recognition protein (SRP) binds to polysomes synthesising secretory proteins, and to a membrane-integrated receptor protein in salt-washed dog pancreatic microsomes. The formation of this polysome-membrane complex results in the synthesis, translocation into vesicles, and proteolytic processing of bovine pre-prolactin. The data indicate that SRP binds to the signal peptide of the nascent pre-protein, to the ribosome, and to an SRP-receptor protein in the microsomal membrane, thereby assembling the pore mechanism proposed by Blobel and co-workers to mediate secretory protein transfer into the ER (Walter *et al.*, 1981).

In contrast, the data presented in this chapter are insufficient to allow any proposal regarding the mechanism of the observed stimulation of amylase release from salt-washed rat pancreatic microsomes by the re-addition of salt extract containing NEM-sensitive protein(s). In rat pancreatic microsome suspensions, membrane-bound polysomes are initially detectable by EM, however as incubation at 37°C proceeds (in the absence of Mg^{2+}), the ribosomes disappear (see chapter 7). All the components of the disintegrated polysomes presumably

remain in the suspending medium, although the macromolecular structure collapses in the absence of stabilizing cations. It therefore seems unlikely that the salt-extracted factor of rat microsomes interacts with polysomes and salt-washed rat membranes (in the absence of Mg^{2+}) in the same way as the SRP complex is thought to behave in a translation system containing dog pancreatic microsomes (and Mg^{2+}).

Results described in section 6.5 show that NEM inhibits the release of amylase from fresh rough microsomes (i.e. not salt-washed) as well as abolishing the stimulatory activity of the salt-extracted factor described in sections 6.3 and 6.4. It is interesting to compare the relative sensitivities to NEM of the salt-extracted factor and of amylase release from intact microsomes.

Both the stimulatory activity of the extracted factor and the amylase-releasing capability of intact microsomes are inactivated by treatment with NEM for 10 mins at 37°C, indicating that both contain a sulphydryl group important to their role in amylase transport. Since the salt-extracted factor is derived from intact microsomes, it seems reasonable to propose that the important sulphydryl group in intact microsomes and the important sulphydryl group in the extracted factor are one and the same. In other words, it is possible that the sulphydryl group involved in amylase release from intact microsomes is located on a peripheral membrane protein which can be removed from the membranes by salt extraction.

It was found that the salt-extracted factor was inactivated by treatment with NEM at 2°C (fig. 6.4) while the enzyme-releasing ability of intact microsomes was unaffected by pre-incubation of the vesicles with NEM at 2°C (fig. 6.12). Thus the sulphydryl group seems to be exposed to attack by NEM in the salt-extracted protein, but masked from the inhibitor in fresh rough microsomes, prior to their incubation at 37°C. The observed, initial delay in the NEM-induced inhibition of amylase release from microsomes, at 37°C, may therefore be due to some kind of conformational change in the membranes, leading to the unmasking of the sulphydryl group on the salt-extractable moiety. This point is re-examined, in a different context, in chapter 7.

CHAPTER 7

RESULTS

STUDIES ON A POSSIBLE RELATIONSHIP BETWEEN RIBOSOME DISINTEGRATION AND AMYLASE RELEASE FROM RAT PANCREATIC MICROSOMES

7.1 INTRODUCTION

It has been tentatively proposed that α-amylase can be released from rat pancreatic microsomes via the agency of an integral membrane translocator protein (Pearce, 1978, Pearce *et al.*, 1978, Tabe, 1978, Chapter 3). Results presented in chapters 4 to 6 reveal a number of correlations between the experimentally-determined characteristics of this enzyme release phenomenon and the published properties of the physiological translocation of secretory proteins across the ER membrane. Specifically, the putative amylase-translocating mechanism has been found to have certain properties in common with the signal peptidase and signal recognition components of the *in vivo* secretory protein translocating mechanism.

Other components which are thought to participate in secretory protein translocation across the ER membrane *in vivo* are membrane-bound ribosomes (Blobel and Dobberstein, 1975b, von Heijne, 1979, Steiner *et al.*, 1980, Garnier *et al.*, 1980, Austen, 1979). Therefore, in order to further the comparison between the rat pancreatic microsome enzyme release phenomenon and the physiological translocation of proteins into the ER, attention was focussed on the involvement of membrane-bound ribosomes in each of the two mechanisms.

In vivo, membrane-bound ribosomes have been identified as the sites of secretory protein synthesis and trans-

location across the ER membrane (Redman and Sabatini, 1966, Blobel and Sabatini, 1971). It is well established that functionally-bound eukaryotic ribosomes can be removed from microsomal membranes by a combination of puromycin treatment and washing in a medium of high ionic strength, but not by either treatment alone (Adelman *et al.*, 1973). The implication of this finding is that ribosomes are attached to the membrane surface by an ionic bond between the ribosome and the membrane, as well as being linked through the nascent peptide chain. The demonstration that partial proteolysis of the microsome surface inhibited ribosome binding implicated a membrane protein in the binding process (Shires *et al.*, 1971, Borgese *et al.*, 1974). More recent studies have produced several estimates of the number and the molecular weights of microsomal membrane proteins involved in ribosome binding (Jothy *et al.*, 1975, Fujita *et al.*, 1977, Kreibich *et al.*, 1978a & b, Sharma *et al.*, 1978, Aulinskas and Scott-Burden, 1979, Yamaguchi *et al.*, 1981).

The identification of membrane-bound ribosomes as the effectors of secretory protein synthesis has led to the idea that the ribosome-binding site on the ER membrane is a component of a mechanism which co-translationally translocates the exported proteins across the lipid bilayer. Some theories of secretion see this binding site as the only membrane-associated protein, in addition to the signal peptidase, which is required for the translocation of exported proteins through the ER membrane (see section 1.5). The signal hypothesis on the other hand, suggests that the ribosome binding site resides on one or more

pore-forming proteins which combine with the previously-mentioned SRP and signal peptidase components to form a hydrophilic pore in the ER membrane, underneath the bound ribosome (Walter and Blobel, 1981b). The presence of the bound ribosome is thought to stabilize the pore structure, the components of which hypothetically disperse in the plane of the lipid bilayer following ribosome detachment, at the completion of translation (Walter and Blobel, 1981b, Blobel and Dobberstein, 1975a & b).

The membrane vesicles in the rat pancreatic rough microsome fraction which was used in all the experiments described in this thesis, were shown by electron microscopy of sectioned microsome pellets, to be covered with bound ribosomes immediately after the isolation and resuspension of the vesicles in STKC buffer, at 2°C (Pearce, 1978). As this medium contained no Mg^{2+}, which is known to be essential for integrity of the ribosome structure, the membrane-bound ribosomes, and the many free ribosomes in the suspensions, gradually disintegrated over a period of several hours at 2°C. This process was much more rapid at 37°C and was accompanied by the release of amylase and other secreted enzymes from the membrane vesicles, which themselves appeared to remain intact (Pearce, 1978). Experiments described in this chapter were designed to examine the possible relationship between the disintegration of membrane-bound ribosomes and amylase release from the microsomes.

7.2 CORRELATIVE EVIDENCE FOR A RELATIONSHIP BETWEEN AMYLASE RELEASE FROM RAT PANCREATIC MICROSOMES AND THE DISINTEGRATION OF MEMBRANE-BOUND RIBOSOMES

7.2.1 Introduction

Soon after the transfer of a suspension of freshly-isolated rat pancreatic microsomes (in STKC buffer lacking Mg^{2+}) from $2^{o}C$ to $37^{o}C$, the numerous free and membrane-bound ribosomes disintegrate. This phenomenon can be visualized by electron microscopy of negatively-stained samples of microsomes taken from a suspension at intervals during incubation. Ribosome disintegration is therefore easy to observe but difficult to quantitate. Since it was considered unlikely that free ribosomes were involved in the trans-membrane, amylase release process, a comparative measure of the extent of disintegration of membrane-bound ribosomes was sought. In the absence of a more reliable indicator, the data presented in this chapter were generated by estimating the number of ribosomes which had disappeared from the vesicle surfaces relative to the number of membrane-associated ribosomes which were initially present in the freshly-prepared suspensions (expressed as % degranulation). Electron micrographs of representative samples are displayed (fig. 7.2).

Since only intact ribosomes could be recognized by electron microscopy, it could not be determined by direct observation, whether any part of the ribosomes remained associated with the microsomal membranes following ribosome disintegration. The term "degranulation" therefore refers to the apparent, complete detachment of membrane-bound

ribosomes from the microsomes during incubation in the absence of Mg^{2+}, although the possibility that some ribosomal components remain bound to the membranes cannot be excluded on the basis of the electron microscopic data alone (see section 7.3).

7.2.2 Comparative rates of amylase release and microsomal membrane degranulation

As a first step in the investigation of the possible connection between ribosome disintegration and amylase release from rat pancreatic microsomes, a comparison was made between the progression of each phenomenon in a single microsome suspension incubated at 37°C. Unless otherwise specified, incubations described in this section were initiated by transferring tubes containing 2 mls of a dilute microsome suspension (0.25 mg total microsomal protein/ml) from an ice bath at 2°C to a water bath at 37°C. The rate of release of amylase from the vesicles was determined as in sections 2.2.2 and 2.2.5. Seconds after the removal of each aliquot of the microsome suspension for assay of amylase activity, a duplicate sample was removed from the suspension and adsorbed onto a carbon-coated grid. Excess liquid was removed, by careful blotting, and the sample was stained for 15 seconds with 2% w/v uranyl acetate (see section 2.2.11).

Figure 7.1 shows extra-microsomal amylase activity plotted against time of incubation, in four separate experiments (*a,b,c,d*). In each experiment the membrane vesicles were almost all covered with bound ribosomes at the beginning of the incubation period. Because a crude

FIGURE 7.1

Amylase release and membrane degranulation in four different microsome preparations

Four different preparations of rat pancreatic microsomes were incubated at 37°C in STKC buffer:

(△) *a*

(○) *b*

(●) *c*

(▲) *d*

At 3 minute intervals, duplicate 50 μl samples were taken from each suspension. One of these aliquots was assayed for amylase activity while the other was negatively stained with uranyl acetate and examined under an electron microscope. The point at which 100% degranulation of membranes was first observed is marked with an arrow on each amylase release profile.

FIG 7.1.

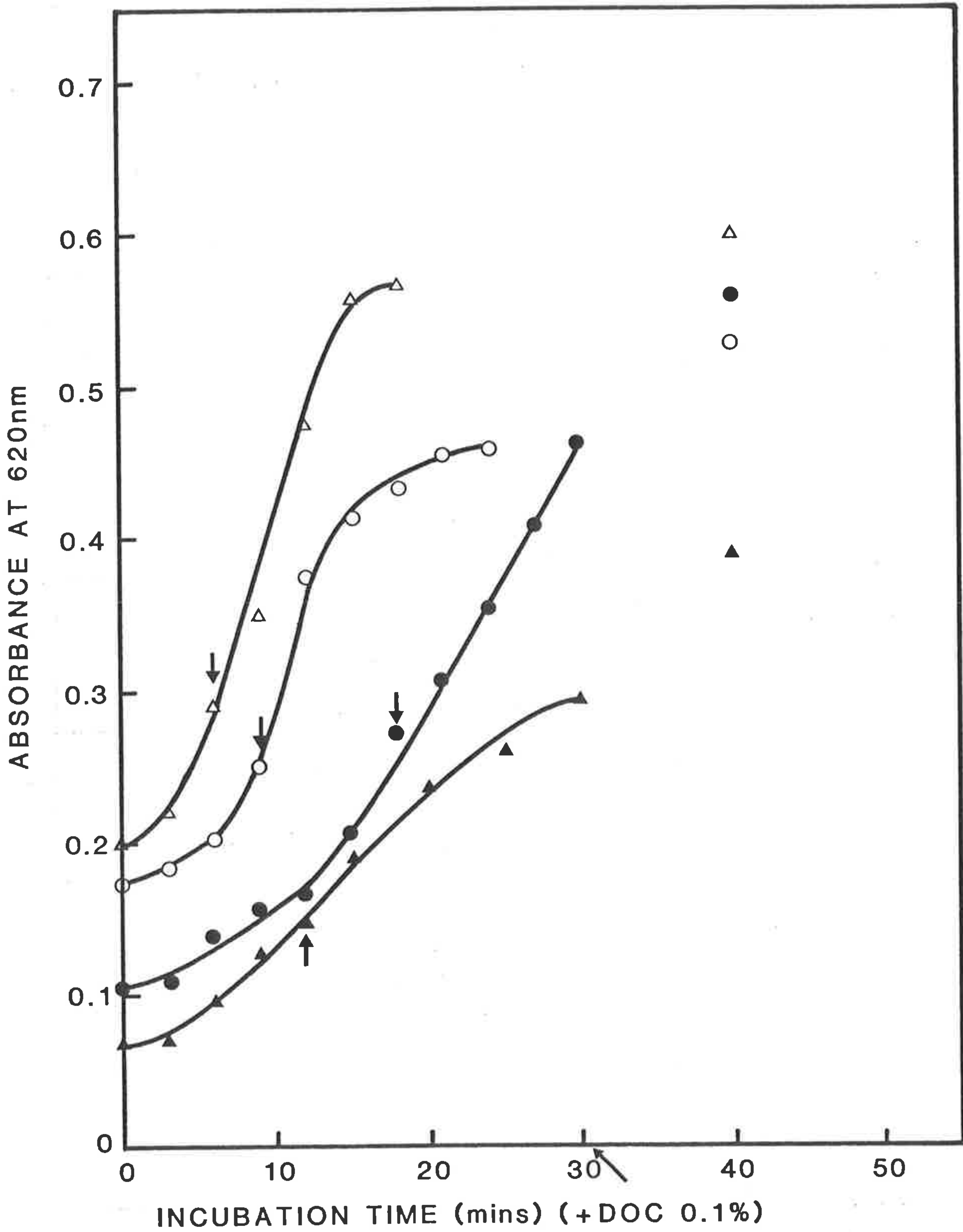


FIGURE 7.2

The disintegration of ribosomes which results from incubation of microsomes at 37°C in Mg^{2+} free buffer

Rat pancreatic microsomes were incubated at 37°C in STKC buffer. The profile of α-amylase release from these vesicles is shown in fig. 7.1.*a*. 50 μl aliquots taken from the suspension at 3 minute intervals were negatively stained with uranyl acetate and examined under an electron microscope (for details see section 2.2.11).

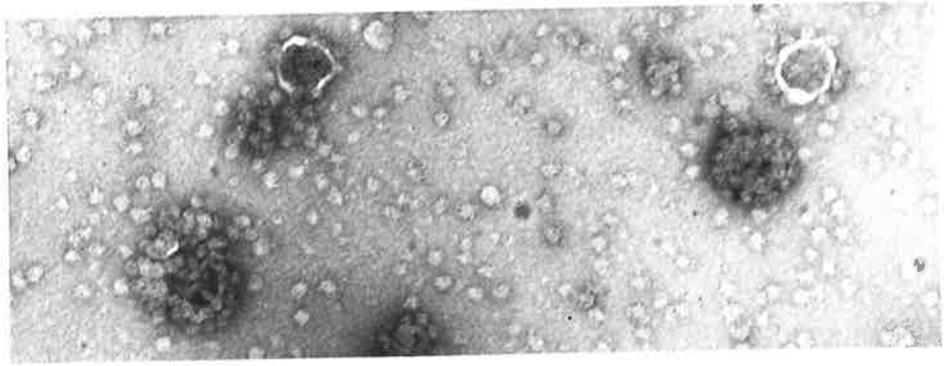
The samples shown were taken at:

(A) zero time

(B) 3 mins

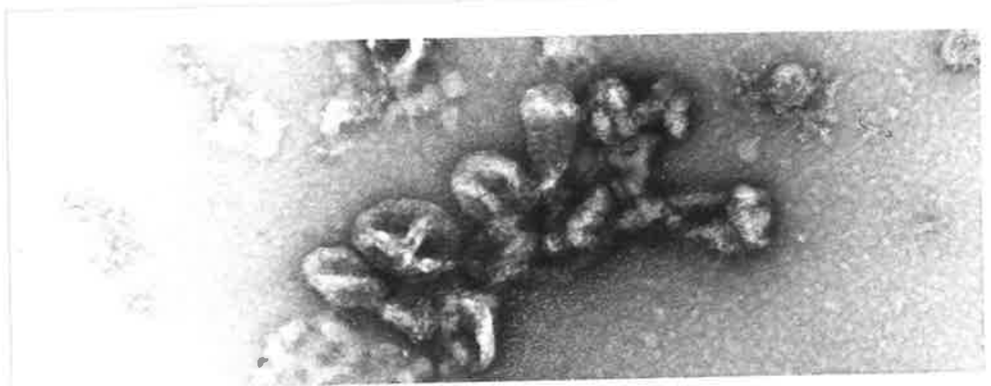
(C) 6 mins, after the transfer of the microsome suspension from 2°C to 37°C. Total magnification 80,000X.

A.



B.

C.



microsome fraction was used in all the work described in this thesis, some smooth vesicles, which were probably derived from the pancreatic cellular smooth ER or golgi membranes, were present at zero time. However, the majority (approximately 90%) of each preparation consisted of rough microsomes (see fig. 7.2.A).

Electron micrographs presented in figure 7.2 illustrate the progression of ribosome disintegration in microsome suspension *a* following its transfer from 2°C to 37°C. After examining similar grids, subjective estimates were made of the degree of degranulation of the negatively-stained microsome samples taken at the time of each amylase determination in experiments *a*,*b*,*c* and *d*. The point at which 100% degranulation of the membranes was first observed was marked on each graph (fig. 7.1).

Although the rates of amylase appearance in the suspensions differed significantly between the different microsome preparations, 100% degranulation was observed at the same stage of the release process in each case. This point corresponded to the beginning of the rapid, linear phase of amylase release. Thus, variations between microsome preparations, the causes of which are unknown, apparently affected the rates of enzyme release and membrane degranulation in a co-ordinate manner.

7.2.3 <u>The effects of incubation temperature on the rates of amylase release and microsomal membrane degranulation</u>

In most of the experiments described in this thesis, incubation of microsomes was initiated by transferring a suspension of vesicles (0.2 - 0.3 mg total microsomal

protein/ml) from an ice bath at 2°C, to a water bath at 37°C (see section 7.2.2). This procedure resulted in an initial delay in the appearance of amylase activity in the extra-microsomal medium (see fig. 7.3). This initial lag phase could be avoided by injecting a 100 μl aliquot of a fresh, concentrated microsome suspension, at 2°C (∿5 mg/total microsomal protein/ml), into 1.9 mls of STKC buffer pre-equilibrated at 37°C (as described in section 6.5). In this way, the dilution of the original vesicle suspension necessitated by the sensitivity of the amylase assay method was achieved, while ensuring that the microsomes reached a temperature of 37°C within seconds of the beginning of the measured incubation period.

Injection of rat pancreatic microsomes into pre-incubated buffer was found to accelerate both the initial rate of amylase release into the medium, and the rate of ribosome disintegration. Figure 7.3 shows that 100% membrane degranulation was observed 4 minutes after microsomes were introduced into buffer pre-incubated at 37°C, while transfer of a dilute suspension of the same microsome preparation from 2°C to 37°C, at zero time, resulted in complete degranulation after 10 minutes of incubation.

In view of the finding that a more rapid transition to 37°C hastened both α-amylase release and membrane degranulation, it is not surprising that the two phenomena responded similarly to changes in the temperature at which microsome suspensions were incubated. 100 μl aliquots of a concentrated microsome preparation were introduced into tubes containing 1.9 mls of STKC buffer pre-incubated at 37°, 35°, 31°, 29° or 28°C respectively. Amylase release

FIGURE 7.3

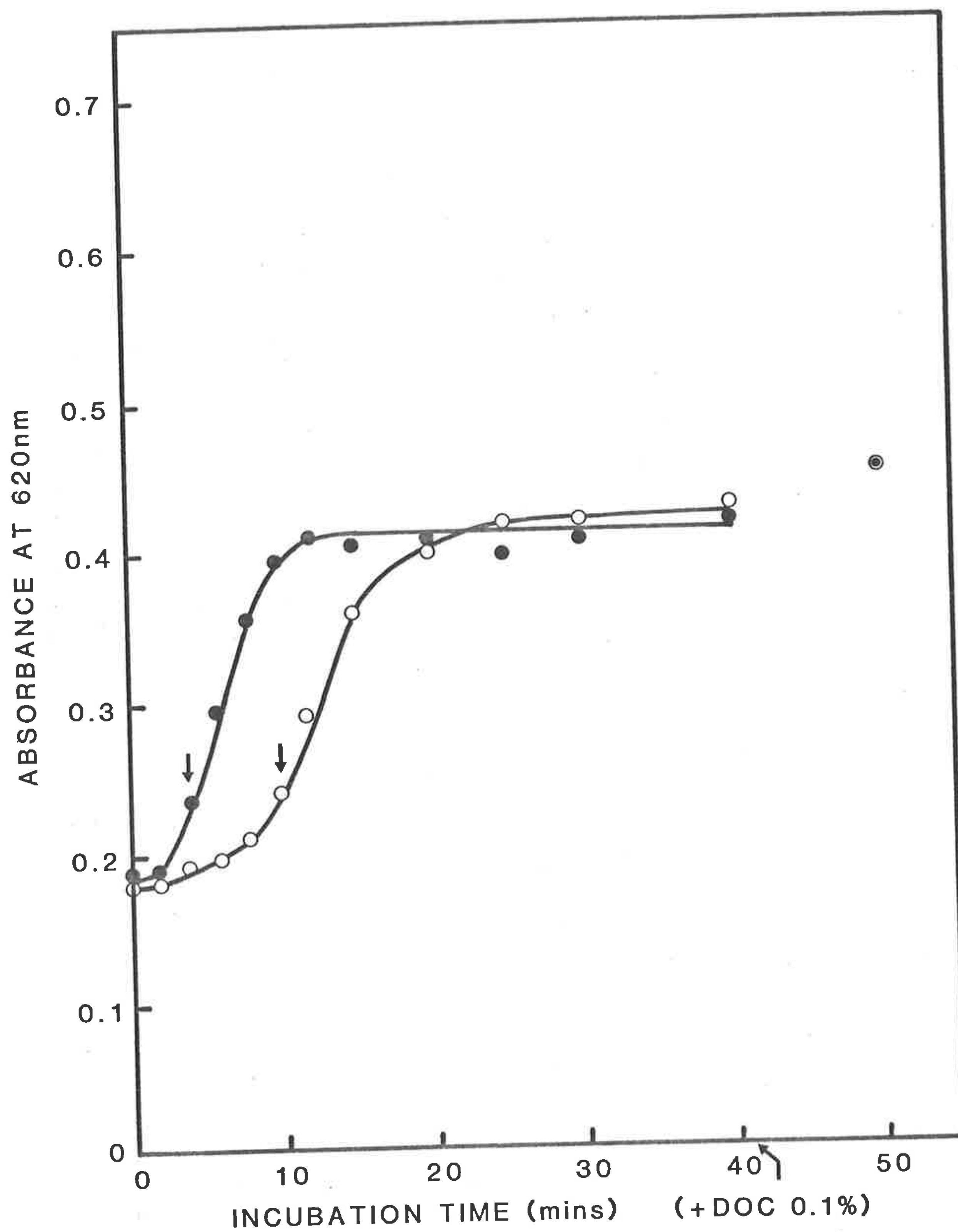
Amylase release and ribosome disintegration in microsome suspensions transferred from 2°C to 37°C either rapidly or gradually

Membrane degranulation and the appearance of amylase activity in the extra-vesicular medium were assessed in two separate suspensions of the same preparation of rat pancreatic microsomes.

In one case (o), incubation at 37°C was initiated by transferring 2 mls of a dilute suspension of the vesicles (0.2 - 0.3 mg total microsomal protein/ml STKC buffer) from an ice bath at 2°C to a water bath at 37°C.

In the other case (●), a 100μl aliquot of a concentrated suspension of the microsomes (∿5 mg total microsomal protein/ml STKC) was innoculated into 1.9 mls of STKC buffer pre-equilibrated at 37°C. The point at which 100% degranulation of membranes was first observed is marked with an arrow on each amylase release profile.

FIG 7.3.



at each temperature was plotted, and the extent of degranulation of membranes in each suspension was estimated after 30 mins. incubation.

Inspection of figure 7.4 reveals that the course of amylase release was altered by changes in incubation temperatures of the vesicles. Decreased incubation temperature resulted in the appearance of an initial lag period before the establishment of the rapid phase of enzyme release, the rate of which was roughly proportional to the incubation temperature. Thus, 100% of the intra-vesicular amylase was released within 10 to 12 minutes by microsomes incubated at 37°C, while 100% release was attained only after 40 mins. incubation at 29°C. A further decrease in the incubation temperature to 28°C, essentially prevented the rat pancreatic microsome enzyme release phenomenon (fig. 7.4).

A similarly well-defined effect of incubation temperature on membrane degranulation was observed. Microsomes incubated at 37°, 35°, 31° or 29°C were completely degranulated following a 30 min. incubation period (as for fig. 7.2.C), whereas the degree of ribosome disintegration and detachment from vesicles incubated at 28°C for 30 minutes was barely detectable (as for fig. 7.2.A). This latter effect was comparable to the negligible rates of amylase release and ribosome disintegration observed in microsome suspensions maintained at 2°C for 30 mins. (results not shown). It was therefore evident that changes in the temperature at which microsome suspensions were incubated had similar effects on both amylase release from the vesicles and membrane degranulation.

FIGURE 7.4

Amylase release and membrane degranulation in microsome suspensions incubated at a range of temperatures

Rat pancreatic microsomes suspended in STKC buffer were incubated at:

(●) 37°C

(○) 35°C

(△) 31°C

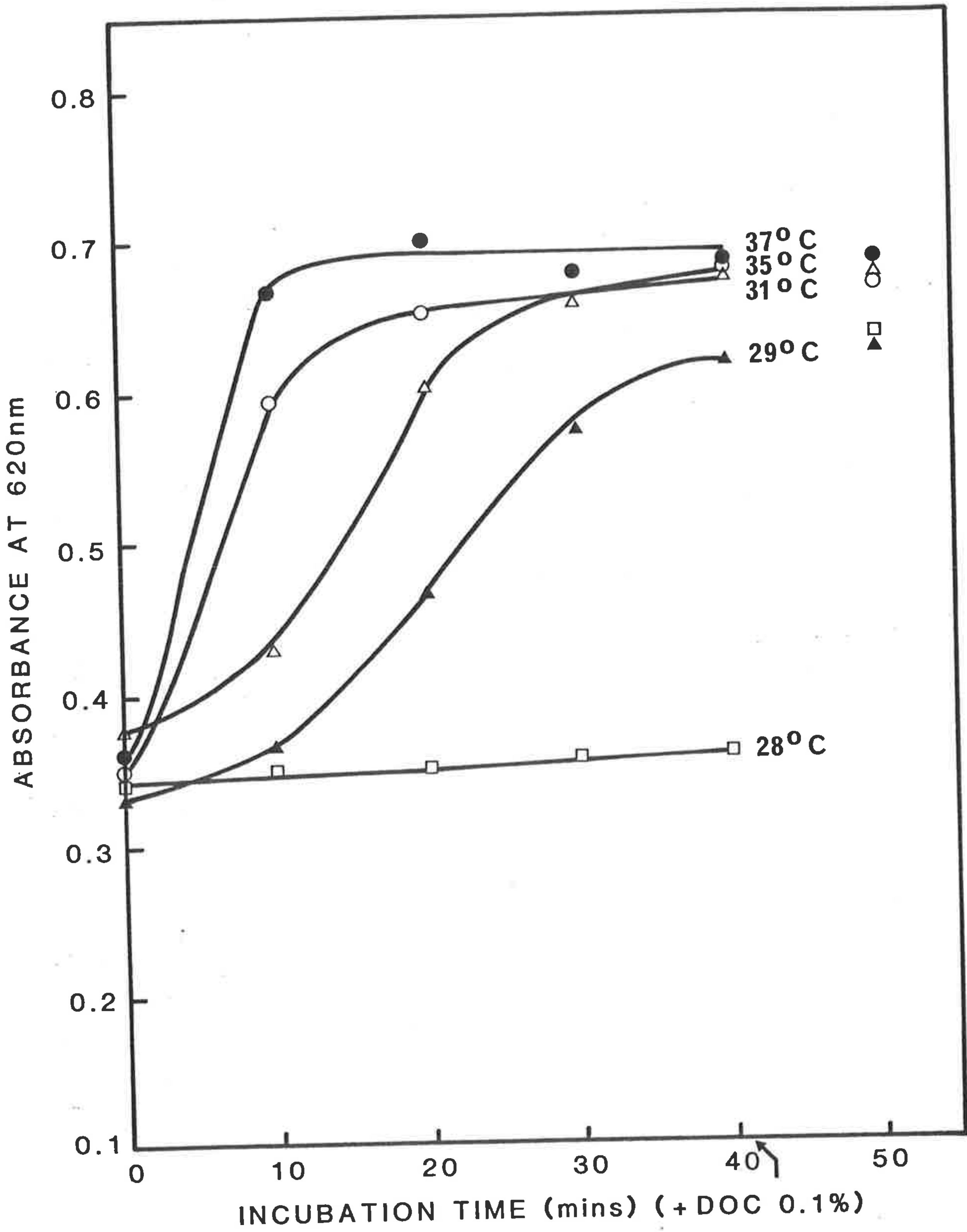
(▲) 29°C

(□) 28°C

The appearance of amylase activity in the extra-microsomal medium was assayed. Samples taken from each suspension, after 30 mins incubation, were negatively stained and examined under an electron microscope and the degree of membrane degranulation was estimated.

Incubation temperature (°C)	37	35	31	29	28
Membrane degranulation after 30 mins (%)	100	100	100	100	<10

FIG 7.4.



7.2.4 The effects of inhibitors on the rates of amylase release and microsomal membrane degranulation

The relationship between enzyme release and membrane degranulation was further investigated by ascertaining the effects on each process of a number of different inhibitors. As detailed in previous chapters, the rat pancreatic microsome enzyme release phenomenon has been found to be inhibited by proteases, some cations, signal peptidase inhibitors, sulphydryl poisons, and denatured ovalbumin. Representatives of each of these categories were found to inhibit amylase release independently of any effect of the compounds on ribosome disintegration. Thus membrane degranulation occurred "normally" despite the inhibition of amylase release from microsomes by trypsin, 1,10-phenanthroline, NEM and denatured ovalbumin (table 7.1).

In the case of the inhibition of the enzyme release phenomenon by cations; Ca^{2+}, Mn^{2+} and Zn^{2+} all prevented amylase release without affecting the progress of membrane degranulation. However, Mg^{2+} prevented both the disintegration and detachment of membrane-bound ribosomes, and the escape of amylase from microsomes at 37°C (table 7.1). The possible meaning of this result is examined in the next section. In summarizing the effects of inhibitors on amylase release and membrane degranulation, it can be said that amylase release is invaribaly accompanied by membrane degranulation although most inhibitors were found to prevent the former process without affecting the latter.

TABLE 7.1

The effects of inhibitors on
amylase release and membrane degranulation

Inhibitor	% of intra-vesicular amylase released after 40 mins incubation	% degranulation of membranes after 40 mins incubation
none	100	100
100 µg/ml trypsin	10	100
20 mM 1,10-phenanthroline	25	100
5 mM NEM	40	100
100 µg/ml OV_D	20	100
5 mM Ca^{2+}	5	100
1.0 mM Mn^{2+}	10	100
10 µM Zn^{2+}	15	100
5 mM Mg^{2+}	5	<10

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing one of the listed inhibitors. In each case, the amount of intra-microsomal amylase which was released during 40 mins incubation was calculated.

$$\text{\% intra-vesicular amylase released} = \frac{(A_{620} \text{ at 40 mins} - A_{620} \text{ at 0 mins})}{(A_{620} \text{ after DOC} - A_{620} \text{ at 0 mins})} \times 100$$

% membrane degranulation, after 40 mins incubation, was estimated by inspection of negatively-stained samples under an electron microscope.

7.3 REMOVAL OF RIBOSOMES FROM RAT PANCREATIC MICROSOMAL MEMBRANES

7.3.1 Introduction

It was stressed in section 7.2.1, that the visualization of microsomes did not afford sufficient resolution to determine whether or not ribosome disintegration (in samples incubated in the absence of Mg^{2+}) resulted in complete detachment of all ribosomal components from the membranes. This point was considered important to the stated aim of this work which was to compare the involvement of ribosomes in the mechanism of amylase release from rat pancreatic microsomes with the role of ribosomes in secretory protein translocation *in vivo*.

As outlined in section 7.1, eukaryotic ribosomes synthesizing secretory proteins must be bound to receptor sites on the ER membrane for protein translocation across the lipid bilayer to ensue. Thus, *in vivo*, the attachment of a ribosome to the ER membrane activates the protein translocation mechanism. The results presented in section 7.2 indicate that the reverse applies to the rat pancreatic microsome enzyme release phenomenon. In the latter situation, the apparent detachment of ribosomes from the microsomal membranes correlates temporally with the beginning of amylase release from the vesicles.

Membrane degranulation, as assessed by electron microscopy, is complete several minutes before all the amylase has been released from microsomes, thus the later part of enzyme release occurs through apparently ribosome-free membranes. This observation obviously conflicts with the

signal hypothesis in which it is proposed that the secretory protein-translocating pore does not exist in the absence of a membrane-bound ribosome.

As previously mentioned, electron microscopy was inadequate for following the fate of individual ribosomal components after the disintegration of the ribosome super-structure in microsome suspensions incubated at 37°C, in the absence of Mg^{2+}. It was therefore impossible to say whether the apparently ribosome-free microsomes were devoid of all ribosomal proteins, or whether some small part of the ribosomes remained bound to the membranes, possibly maintaining the integrity of trans-membrane pores. The experiments described in this section were undertaken in order to determine the effects on the release phenomenon of complete removal of intact ribosomes from the microsomal membranes.

7.3.2 Degranulation of microsomes with puromycin/KCl

It was reported by Pearce (1978) that incubation of rat pancreatic microsomes at 37°C in STKC buffer containing Mg^{2+}, puromycin, and a high concentration of KCl, resulted in complete detachment of intact ribosomes from the microsomal membranes. Amylase was not released from the vesicles under these conditions. To interpret this result it is necessary to recall that Mg^{2+} alone (i.e. without puromycin/KCl), inhibits both amylase release and ribosome detachment in rat pancreatic microsome suspensions incubated at 37°C (see section 7.2.3).

It is possibel that Mg^{2+} inhibits the apparent transport of α-amylase out of the vesicles (as do Ca^{2+}, Mn^{2+}

and Zn^{2+}) independently of its effect on membrane degranulation. Alternatively, there may be a causal relationship between the two activities of Mg^{2+}. That is, Mg^{2+} may effect inhibition of the release phenomenon by binding ribosomes to the microsomal membranes, thereby blocking the release of amylase through a translocator mechanism underneath the bound ribosome.

In the context of these alternatives, there are two possible interpretations of the result reported by Pearce (see above). If a membrane-bound ribosome was required to maintain the integrity of a transport pore, as suggested in the signal hypothesis, removal of membrane-bound ribosomes from microsome surfaces would result in the collapse of such a pore. Therefore, the finding that amylase is not released from microsomes from which the bound ribosomes have been removed, may constitute support for this hypothesis.

On the other hand, it can be proposed that, following the removal of membrane-bound ribosomes, the putative translocator protein remains potentially operative, but release of amylase through the mechanism is blocked by direct inhibition of its activity by Mg^{2+}. To distinguish between these possibilities, it was sought to study, in a medium which did not contain Mg^{2+}, the release of amylase from microsomes which were completely free of ribosomes.

Attempts were made to strip the ribosomes from the surface of fresh, rough microsomes while conserving the endogenous, intra-vesicular enzymes. In order to be sure of removing all ribosomal components from the membranes, microsomes were generally degranulated with puromycin and

high KCl in the presence of Mg^{2+}, so that the ribosomes remained intact (Rolleston, 1972, Adelman *et al.*, 1973, Borgese *et al.*, 1974). It should be noted that in none of the published methods for removing membrane-bound ribosomes from (mostly rat liver) microsomes, was the fate of the endogenous, intra-microsomal, secretory proteins predetermined. The results of treating fresh, crude preparations of rat pancreatic microsomes with puromycin and KCl are shown in table 7.2.

Microsome suspensions were initially incubated at 2°C for 60 mins, and then at 25°C for 15 mins, in the presence of 0.5 mM puromycin, 0.5 M KCl and 2.5 mM Mg^{2+}, as suggested by Fielder *et al.*, (1978). The extent of membrane degranulation was assessed by examining negatively stained samples taken immediately after the stripping incubations. In most cases, ribosome removal was incomplete, but varying amounts of completely stripped vesicles could be separated from detached ribosomes and partly degranulated microsomes, by centrifugation in a sucrose step gradient for approximately 16 hours, as described in section 2.2.4. Thus, in table 7.2, a low "% degranulation" means that most microsomes retained some of their membrane-bound ribosomes, resulting in a low yield of completely degranulated vesicles after fractionation of the suspension on a sucrose gradient.

Table 7.2 shows that several experiments employing the same stripping procedure produced a broad spectrum of results. Application of the protocol of Fielder *et al.*, (1978) led to the recovery, from the 1.2/1.25 M sucrose interface, of varying yields of degranulated microsomes

TABLE 7.2 DEGRANULATION OF RAT PANCREATIC MICROSOMES USING PUROMYCIN/KCl

Stripping treatment		% Degranulation after stripping treatment, or control incubation	% of intra-microsomal amylase released during stripping incubations	Estimated leakage of intra-microsomal amylase during purification of vesicles*	% of intra-microsomal amylase released by purified microsomes
0.5 mM puromycin/ 0.5 M KCl/2.5 mM $MgCl_2$ 60 mins @ $2^{\circ}C$, 15 mins @ $25^{\circ}C$					
Expt. No. 1	C	<10	<10	low	60
	Exp.	60	<10	low	30
2	C	<10	<10	low	30
	Exp.	50	<10	low	20
3	C	<10	<10	low	90
	Exp.	90	<10	low	45
4	C	<10	<10	low	<10
	Exp.	90	<10	low	<10
0.5 mM puromycin/0.5 M KCl 60 mins @ $2^{\circ}C$, 15 mins @ $25^{\circ}C$					
	C	20	10	low	50
	Exp.	60	15	low	30
0.5 mM puromycin/ 0.5 M KCl/5 mM $MgCl_2$ 30 mins @ $2^{\circ}C$, 20 mins @ $20^{\circ}C$, 10 mins @ $37^{\circ}C$					
	C	100	<10	total	-
	Exp.	100	<10	total	-

For experimental details, refer to text.

*Amount of intra-microsomal amylase/mg microsomal protein was determined before (x) and after (y) purification of vesicles on surcrose gradients. Leakage = x - y.

which were completely free of ribosomes as assessed by electron microscopy, and which contained latent α-amylase activity. When these vesicles were homogenized gently in fresh STKC buffer, and then appropriately diluted and incubated at 37°C, in the absence of Mg^{2+}, they released little of their intra-microsomal enzyme. Omission of Mg^{2+} from the original stripping incubation gave a similar result (see table 7.2).

Control microsomes which were incubated in the absence of puromycin/KCl and then purified on separate sucrose step gradients, retained most of their membrane-associated ribosomes, and were recovered from the 1.3/2.2 M sucrose interface. These vesicles, which initially contained latent amylase activity, also released significantly less than their total intra-microsomal enzyme during subsequent incubation at 37°C, in the absence of Mg^{2+} (see table 7.2).

The reasons for the highly variable effects of applying this ribosome stripping procedure to rat pancreatic microsomes are unknown. Attempts were made to increase the extent of ribosome removal and improve the amylase-releasing capability of the degranulated vesicles, by methodically altering aspects of the stripping protocol, such as time and temperature of incubation, and concentration of puromycin, KCl or Mg^{2+}. Protease inhibitors were also included in the sucrose gradients, and the length of the centrifugation period was varied. The effects of these manipulations were obscured by the intrinsic variability of the system (results not shown), thus it was not possible to optimize the membrane degranulation procedure.

One obvious trend was an increase in the efficiency of degranulation and a corresponding decrease in the integrity of the microsomal membranes which accompanied an increase in incubation temperature. For example, incubation of rat pancreatic microsomes in the presence of 0.5 mM puromycin/0.5 M KCl/5 mM Mg^{2+} at $20^{o}C$ for 20 mins and then at $37^{o}C$ for 10 mins, as suggested by Kreibich *et al.*, (1978), consistently resulted in 100% degranulation of the membranes (table 7.2). However, the stripped vesicles contained no latent amylase activity following purification on a sucrose gradient.

In this experiment, control microsomes were incubated at $20^{o}C$ for 20 mins then at $37^{o}C$ for 10 mins, in STKC buffer containing 5 mM Mg^{2+}, but not puromycin/KCl. These vesicles retained their membrane-bound ribosomes, but were also essentially devoid of intra-microsomal enzyme after recovery from a sucrose gradient. No amylase release occurred during the control or stripping incubations (table 7.2, also Pearce, 1978), thus it seemed that the intra-vesicular enzymes leaked from the pre-incubated microsomes during their purification on a sucrose gradient, at $2^{o}C$. Since both the stripped and control microsomes were devoid of intra-microsomal amylase after purification, it was tentatively concluded that the brief, $37^{o}C$ incubation affected the integrity of the vesicles during subsequent manipulations, rather than the loss of amylase being a consequence of the stripping procedure.

The reason for attempting to degranulate microsomes using puromycin/KCl was to maintain the integrity of the ribosome structure, thus precluding the possibility of any

ribosomal proteins remaining bound to the membranes following apparent degranulation. Despite this aim, methods of microsome "stripping" involving ribosome disassembly were investigated when it became apparent that the puromycin/KCl procedure was inappropriate. Similarly unsatisfactory results, from the viewpoint of amylase release studies, were obtained by degranulating membranes using lithium chloride (Scott-Burden and Hawtrey, 1969), EDTA (Blobel and Dobberstein, 1975), RNAse and EDTA (Shires *et al.*, 1971) or sodium pyrophosphate (Kruppa and Sabatini, 1977) (results not shown).

The only conclusions which could be drawn from this work were that those treatments which were consistently effective in detaching membrane-bound ribosomes from rat pancreatic microsomes also either inhibited amylase release from the purified, stripped vesicles or eroded the integrity of the microsomal membranes.

7.4 THE SENSITIVITY OF ENZYME RELEASE TO INHIBITORS

Due to the failure of attempts to isolate completely ribosome-free microsomes, the question of whether or not ribosomal components were necessary to maintain the integrity of a trans-membrane, amylase transport channel could not be answered. The occasional observation of amylase release from apparently "stripped" vesicles argues against such an idea, but the inconsistency of the results preclude a firm conclusion (see table 7.2). An alternative approach was used to answer a related question concerning the spatial relationship between membrane-bound ribosomes and the mechanism mediating amylase release across the microsomal

membrane.

It has been proposed that the sites of ecto-protein translocation across the prokaryotic plasma membrane lie underneath membrane-bound ribosomes. The evidence for this hypothesis consists of the demonstration that the translocation mechanism is protected from proteolytic attack when ribosomes are cross-linked to the surface of inverted *E. coli* plasma membrane vesicles (Smith, 1980). In this context, preliminary experiments with stripped rat pancreatic microsomes had shown that amylase release from at least partially degranulated vesicles, appeared to be more sensitive to inhibition by proteolysis of the membranes than was enzyme release from fresh rough microsomes (Tabe, 1978).

As demonstrated in figures 7.1 and 7.3, incubation of fresh rough microsomes apparently results in complete membrane degranulation several minutes before the completion of intra-vesicular amylase release. The latter part of the phenomenon of enzyme release from rat pancreatic microsomes incubated at 37°C, in the absence of Mg^{2+}, therefore involves the escape of amylase through seemingly ribosome-free membranes. This latter phase of the phenomenon approximates to release of amylase from stripped vesicles (in the presence of solubilized ribosomal components). In order to substantiate the earlier inference that the target of protease attack was more exposed in degranulated microsomes (Tabe, 1978), a comparison was made between the sensitivity to protease of the early and late phases of amylase release from crude, (initially) rough microsomes.

Figure 7.5 shows that the addition of a low concentration of trypsin (25 μg/ml) to a microsome suspension at the beginning of incubation at 37°C resulted in delayed inhibition of amylase release from the vesicles. If the same amount of protease was added to a similar suspension after 8 minutes of incubation, complete, immediate cessation of amylase release was observed. In both cases, amylase activity was retained within the microsomes after protease treatment, as revealed by disruption of the membranes with detergent (fig. 7.5).

This effect is strongly reminiscent of the pattern of inhibition of the amylase release phenomenon by NEM. It was demonstrated (in section 6.5) that the initial stage of amylase release from microsomes was virtually completely insensitive to NEM, whereas strong inhibition ensued after approximately 10 minutes of incubation at 37°C. A similar pattern emerged when either the signal peptidase inhibitor, 1,10-phenanthroline (fig. 7.6) or low concentrations of denatured ovalbumin (fig. 7.7) were added to microsomes. In each case, the effect of adding the inhibitor half-way through the amylase release process was clearly more immediate than adding the inhibitor at zero time in the incubation.

Conversely, if 0.25 mM Mg^{2+} was added to a microsome suspension after 9 minutes of incubation at 37°C, its inhibitory effect on amylase release was less than or equal to the effect of adding Mg^{2+} at zero time (fig. 7.8). Thus, in summary, it was found that during the first few minutes of incubation of microsome suspensions, while membrane-associated ribosomes were beginning to disintegrate,

FIGURE 7.5

The effects on amylase release of adding trypsin to microsomes at zero time, or after 8 mins incubation at 37°C

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

(●) no additions

(○) 25 μg/ml trypsin, added at 0 mins in the incubation period

(▲) 25 μg/ml trypsin, added at 8 mins in the incubation period

(In the experiments described in figs. 7.5-7.8 it was aimed to compare the effects of adding inhibitors to microsome suspensions either before the beginning of amylase release and membrane degranualtion, or during amylase release, and after 100% degranulation of the membranes. It had been demonstrated that 100% degranulation preceded the half-way point of amylase release (see fig. 7.1), therefore it was aimed to add inhibitors at a time when half of the amylase had been released to the suspending medium. Because the rates of amylase release from different preparations of microsomes varied slightly, inhibitors were added at different times in different experiments).

FIG 7.5.

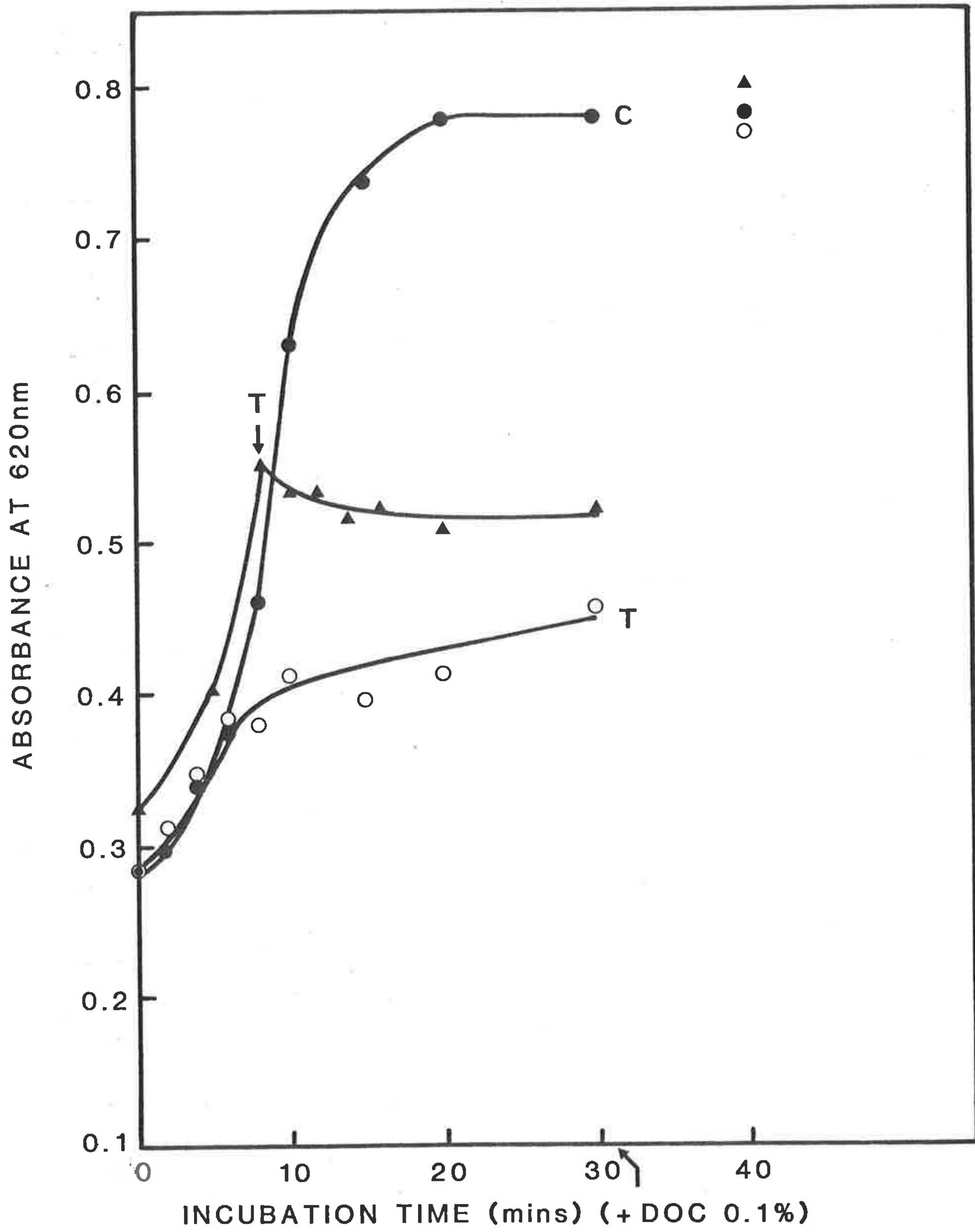


FIGURE 7.6

The effects on amylase release of adding 1,10-phenanthroline to microsomes at zero time, or after 10 mins incubation at 37°C

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

(●) no additions

(○) 15 mM 1,10-phenanthroline, added at 0 mins in the incubation period

(△) 15 mM 1,10-phenanthroline, added at 10 mins in the incubation period.

FIG 7.6.

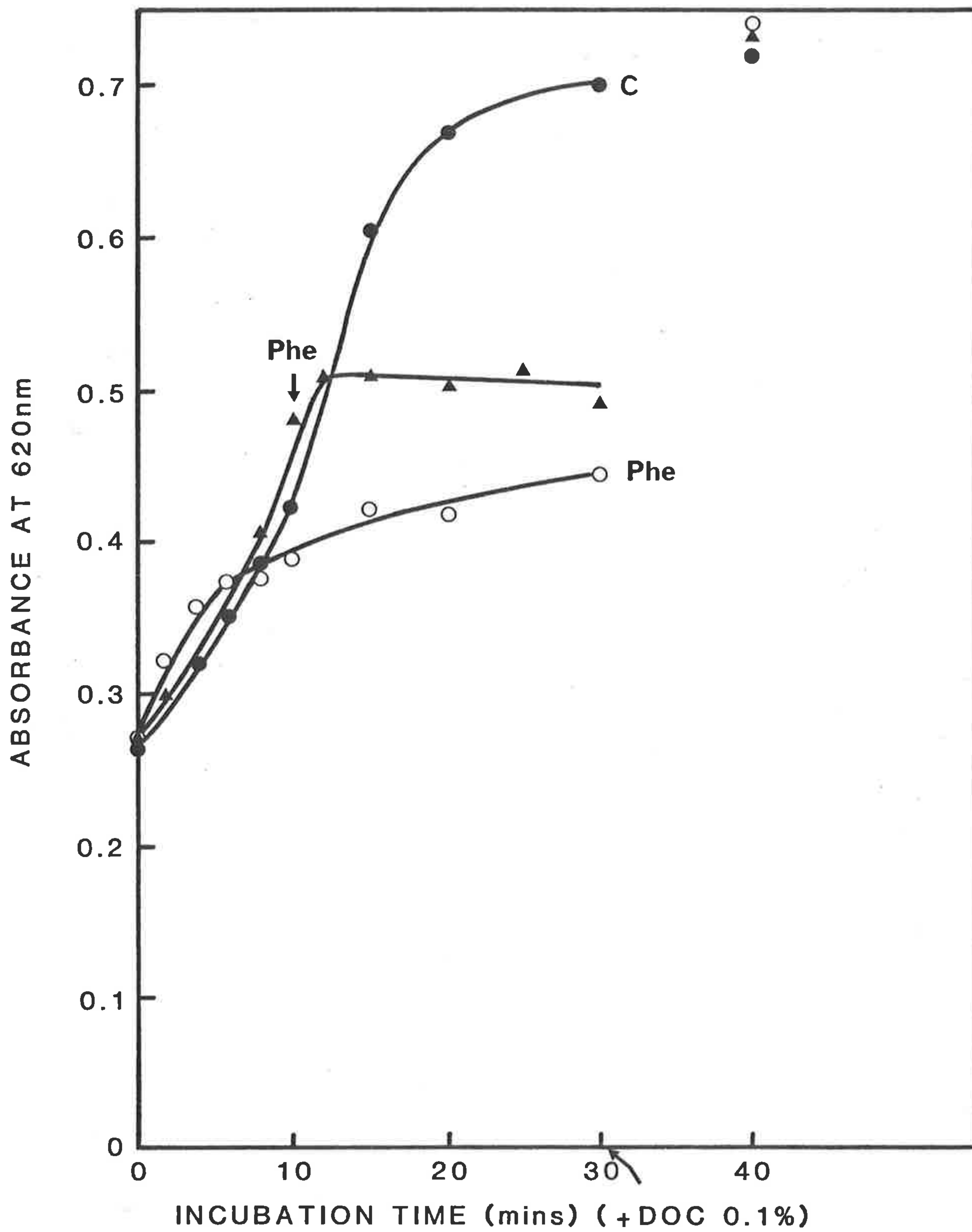


FIGURE 7.7

The effects on amylase release of adding denatured ovalbumin to microsomes at zero time, or after 8 mins incubation at 37°C

Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

- (●) no additions
- (○) 30 μg/ml denatured ovalbumin, added at 0 mins in the incubation period
- (▲) 30 μg/ml denatured ovalbumin, added at 8 mins in the incubation period.

FIG 7.7.

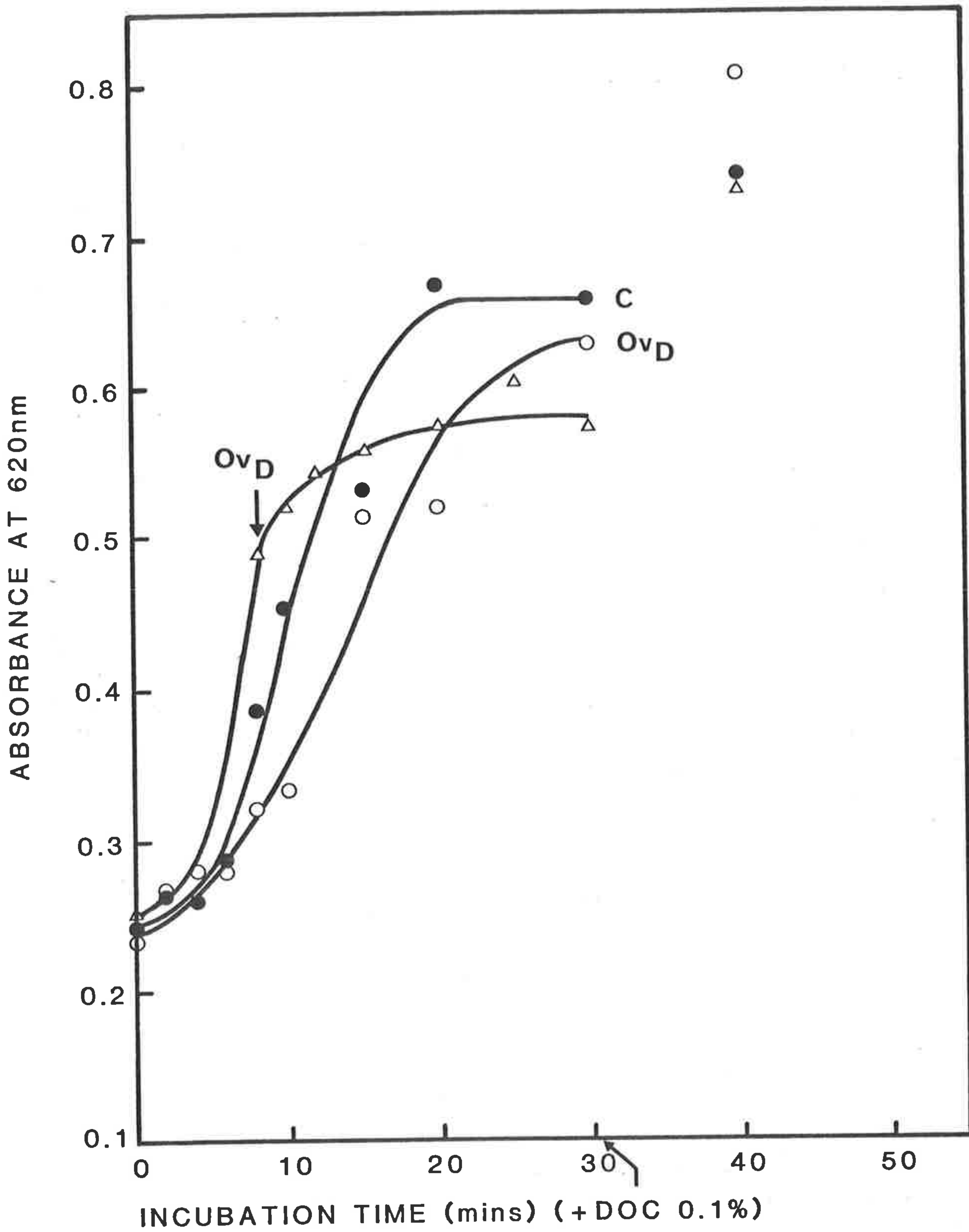


FIGURE 7.8

The effects on amylase release of adding magnesium ions to microsomes at zero time, or after 9 mins incubation at 37°C

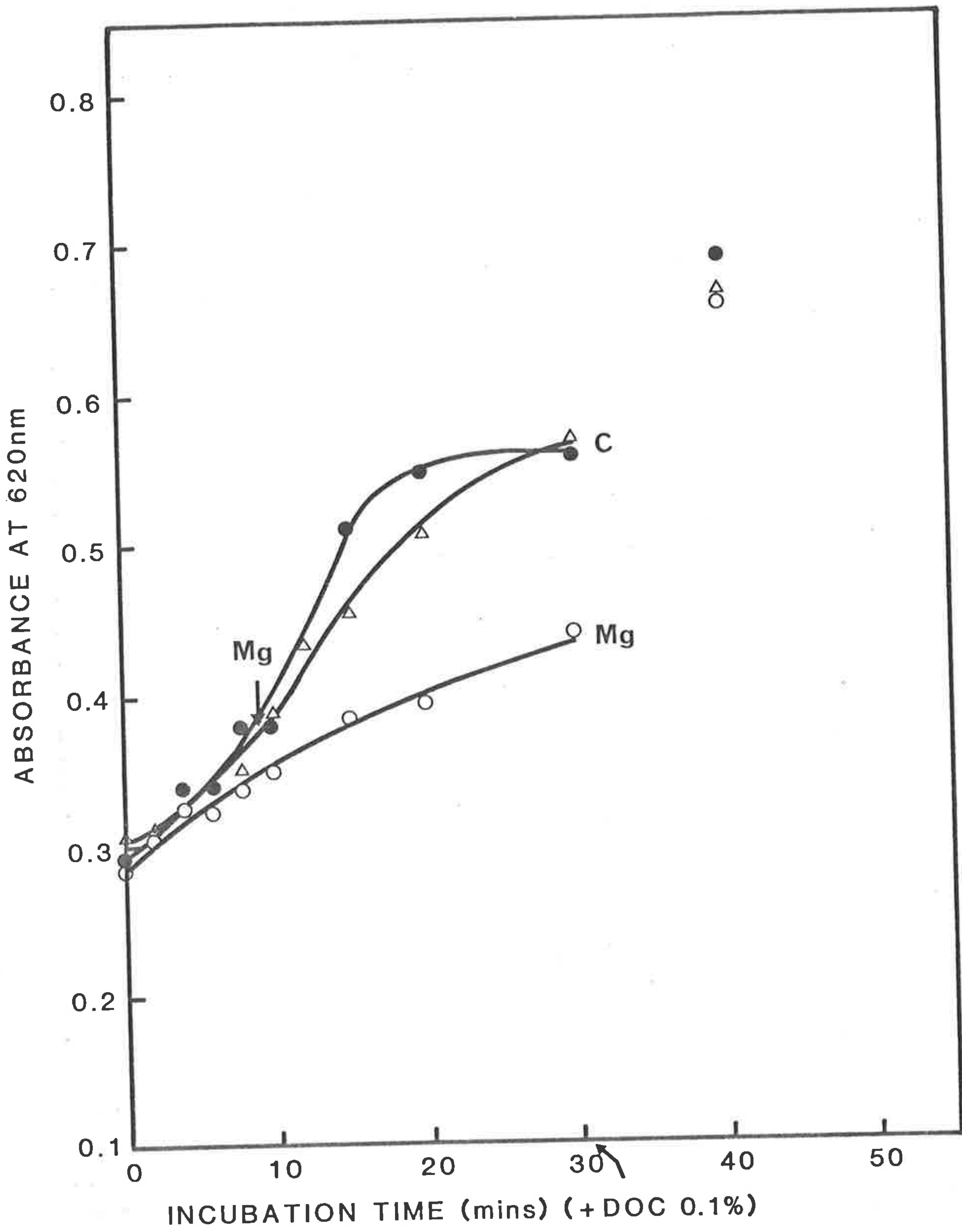
Rat pancreatic microsomes were incubated at 37°C in STKC buffer containing:

(●) no additions

(O) 0.25 mM $MgCl_2$, added at 0 mins in the incubation period

(Δ) 0.25 mM $MgCl_2$, added at 9 mins in the incubation period.

FIG 7.8.



the release of amylase from the vesicles was relatively insensitive to a number of inhibitors. In contrast, the same inhibitors quickly halted the later, rapid phase of enzyme release, which coincides with the complete decomposition of ribosome structure, as assessed by electron microscopy. It appeared, on the basis of this temporal correlation, that the targets of trypsin, NEM, 1,10-phenanthroline and denatured ovalbumin may be initially protected from the inhibitors by membrane-associated ribosomes. This was not true in the case of inhibition of amylase release by a low concentration of Mg^{2+}.

The effects on the release phenomenon, of NEM and 1,10-phenanthroline, differ in one respect from the effects of trypsin and denatured ovalbumin. Even in the presence of very high concentrations of the former inhibitors, delayed inhibition of amylase release from microsomes persists (figs. 4.4 and 6.5), whereas high concentrations of either proteases (fig. 3.5 and Pearce, 1978) or denatured ovalbumin (fig. 5.1) result in immediate inhibition of amylase release. The significance of this observation is not known.

7.5 SUMMARY AND DISCUSSION

It has been reported that the release of secretory proteins from rat pancreatic microsomes during incubation at 37°C, in the absence of Mg^{2+}, is accompanied by the disintegration and apparent detachment of membrane-bound ribosomes from the surfaces of the vesicles (Pearce, 1978, Pearce *et al.*, 1978). The experiments described in this chapter address the question of whether or not there is an

association between the two processes. Results presented in section 7.2 provide correlative evidence for a link between the two phenomena.

It was found that the complete disappearance of bound ribosomes from the microsome surfaces was always synchronized with the beginning of the rapid, linear phase of amylase release from the vesicles. The rates of enzyme release and ribosome disintegration responded in parallel to intrinsic variations between different microsome preparations, and to changes in the temperature at which microsome suspensions were incubated.

Since visualization of samples by electron microscopy was used to assess membrane degranulation, the fate of individual ribosomal proteins could not be determined after the disintegration of the macro-molecular structure of the ribosomes. Therefore, although it appeared that degranulation was complete after 5 to 10 mins of incubation at 37°C, the possibility that one or more ribosomal components remained bound to the membranes throughout the entire amylase release process could not be eliminated on the strength of the electron microscopic data.

Amylase release was invaribly accompanied by ribosome disintegration, but several inhibitors of the enzyme release phenomenon were found to prevent enzyme escape from microsomes without affecting the process of membrane degranulation. The exception to this rule was Mg^{2+} which prevented both amylase release and the disintegration and detachment of membrane-bound ribosomes. The possibility that Mg^{2+} stopped enzyme release by binding ribosomes to a translocator protein in the membrane was precluded by

the report that intact ribosomes could be removed from the microsome surfaces, in the presence of Mg^{2+}, at 37°C (by puromycin/KCl), without amylase being released from the vesicles (Pearce, 1978).

Two possible interpretations of this finding are outlined in section 7.3.2. On one hand, the result could be taken as support for the proposal that amylase escapes from the microsomes through trans-membrane "pores", the integrity of which depends on bound ribosomal components. Alternatively, the removal of membrane-bound ribosomes could leave the putative amylase translocator potentially operative, but with amylase release prevented by a direct inhibition of the mechanism by Mg^{2+}.

To distinguish between these possibilities, it was hoped to determine whether amylase release would occur in microsome suspensions containing neither ribosomes nor Mg^{2+}. To this end, attempts were made to strip intact ribosomes from microsomes, while preserving the latency of the intra-vesicular amylase. In the experience of this author, this aim represents a precedent in that none of the published methods of microsome degranulation concern themselves with the fate of the endogenous, intra-vesicular secretory proteins during the stripping procedures.

The goal of studying enzyme release from completely ribosome-free microsomes was not achieved. For unknown reasons, the results of applying the microsome stripping procedures which have been commonly used on liver rough microsomes, to rat pancreatic microsomes were extremely variable (see section 7.3.2). This lack of reproducibility made it very difficult to draw any conclusions from the

work, but some observations can be made.

Microsomes which contained latent amylase activity were isolated, and found by electron microscopy to be completely free of ribosomes. Since the ribosomes had been removed as intact entities, in the presence of Mg^{2+}, microscopic visualization was considered an adequate criterion for judging the degree of degranulation of these membranes (any contaminating ribosomes would be intact and therefore visible under the electron microscope). In most cases, subsequent incubation of these purified, stripped microsomes at 37°C, in the absence of Mg^{2+}, resulted in the release of some intra-vesicular amylase, although release of more than 50% of the contained enzyme was never observed (table 7.2). A contributing reason for the low levels of amylase release may be the removal from the membranes of the salt-extractable factor described in chapter 6. It was shown in section 6.3 that incubation of microsomes at 2°C, for relatively short time periods, in the presence of concentrations of KCl comparable to those used in the stripping procedures, significantly impaired the capability of the salt-treated vesicles to release amylase.

Another factor contributing to the low level of amylase release from the stripped vesicles appears to be independent of the actual degranulation procedure, as low release was also observed from control microsomes which had been incubated in the absence of puromycin/KCl and similarly purified on sucrose gradients (table 7.2). This effect is possibly due to endogenous, pancreatic proteases attacking microsomal membranes during the 16-hour centrifugation.

Despite the low release, the finding that some enzyme was released through completely ribosome-free membranes seems to militate against the idea of an amylase-transport pore whose integrity depends on the presence of ribosomal components. This inference is compatible with the observation that membrane degranulation (by ribosome disintegration) is apparently complete several minutes before the completion of amylase release from fresh, rough microsomes incubated at 37°C, in the absence of Mg^{2+}. Thus the beginning of amylase release occurs while the macro-molecular structure of the ribosomes in the suspension is decomposing, whereas most of the subsequent, rapid phase of enzyme release occurs from essentially "stripped" vesicles (see fig. 7.1).

This system therefore offers a chance to examine some of the characteristics of amylase release in the absence of ribosomes (but in the presence of solubilized ribosomal components). Preliminary investigation of enzyme release from partially degranulated microsomes had indicated that amylase release from stripped vesicles was more sensitive to inhibition by proteases than amylase release from fresh, rough microsomes (Tabe, 1978). The same trend was evident when a low concentration of protease was added to suspensions of rough microsomes at either zero time, or after 8 minutes of incubation at 37°C (fig. 7.5). In the former case, delayed inhibition of amylase release was observed, whereas addition of protease to microsomes which had been incubated for several minutes at 37°C (by which time membrane degranulation would have been complete), resulted in immediate inhibition of amylase release.

While it is appreciated that correlations do not constitute proof; the temporal co-incidence of membrane degranulation and the onset of increased sensitivity of amylase release to protease, may suggest that membrane-associated ribosomes initially shield the amylase releasing mechanism from protease added to the suspending medium. This proposal also draws support from the previously-mentioned report of the increased sensitivity to protease, of amylase release from partially-degranulated rat pancreatic microsomes (Tabe, 1978). The inference that the target of protease attack may lie under membrane-bound ribosomes is compatible with the proposal that the physiological, secretory protein-translocating mechanism (which seems to lie under membrane-bound ribosomes), mediates α-amylase release from rat pancreatic microsomes.

The site of inhibition by denatured ovalbumin also seems to be shielded from the inhibitor during the early stages of amylase release. It is possible that the disintegrating ribosomes initially hinder the binding of the ovalbumin to signal peptide receptor sites on the rat microsomal membranes, thereby blocking the passage of amylase through an associated translocator protein. It has been reported, however, that membrane-bound ribosomes do not seem to impede the binding of fully-formed pre-proteins to the appropriate receptors on dog pancreatic microsomes (Prehn *et al.*, 1980, 1981).

Amylase release from rat pancreatic microsomes incubated at 37°C is initially unaffected by the presence of NEM, but becomes sensitive to the inhibitor at approximately the time when complete membrane degranulation is first observed

(see section 6.5). This finding raises the possibility that membrane-associated ribosomes may initially protect an amylase translocator protein from inactivation by the sulphydryl reagent. Jackson *et al.*, (1980) have reported that membrane-bound ribosomes do not seem to affect the inhibition, by NEM, of protein translocation into dog pancreatic microsomes.

Interpretation of the 1,10-phenanthroline data is also problematical in terms of the hypothesis that membrane-associated microsomes shield the amylase release mechanism in the rat microsomal membranes from inhibitors. It is difficult to see how the presence of ribosomes could block the action of an ion chelator such as 1,10-phenanthroline.

Therefore, in summary, the sequence of events following the transfer of a suspension of rat pancreatic microsomes from $2^{o}C$ to $37^{o}C$ begins with the disintegration and apparent detachment of membrane-bound ribosomes, and the release of intra-vesicular enzymes. Amylase release and ribosome disintegration are always very closely synchronized in the absence of added inhibitors. The data were insufficient to determine whether ribosome detachment caused amylase release, although ribosomes could be removed from membranes without causing amylase release at $37^{o}C$, in the presence of Mg^{2+} (Pearce, 1978), or at $4^{o}C$ in the absence of Mg^{2+} (table 7.2).

At approximately the time when complete membrane degranulation is first observed, the process of amylase release from the microsomes becomes very sensitive to inhibition by trypsin, denatured ovalbumin, NEM and 1,10-

phenanthroline. Thus, the increase in sensitivity of enzyme release to inhibitors immediately follows complete ribosome disintegration, and occurs at a time when approximately 1/4 to 1/3 of the intra-vesicular amylase has been released (as indicated by the onset of inhibition of amylase release from microsome suspensions to which inhibitors were added at zero time; for results see figs. 6.5, 7.5, 7.6 and 7.7).

The data were insufficient to delineate any relationship between the beginning of amylase release, the disintegration of membrane-bound ribosomes, and the increase in the sensitivity of the release process to inhibitors. The temporal correlation between the two phenomena could imply that the increase in sensitivity of amylase release to inhibitors is a direct consequence of the disintegration of membrane-bound ribosomes. In other words the targets attacked by protease, denatured ovalbumin, NEM and 1,10-phenanthroline could originally be shielded underneath membrane-associated ribosomes. Unfortunately, stripped microsomes could not be used to test this hypothesis.

Alternatively, whether or not the putative amylase translocator protein initially lies underneath membrane-bound ribosomes, the increase in sensitivity of amylase release to inhibitors may be completely independent of membrane degranulation. It is possible that some kind of change occurs in the microsomes themselves during the first few minutes of incubation at 37°C. A change could occur in the conformation of a trans-membrane, amylase-transport protein, although amylase release is seen to occur both before and after the inferred change, and is equally sensitive

to inhibition by Mg^{2+} in each phase (see fig. 7.8 and control curves in figs. 7.5, 7.6 and 7.7). The second phase of amylase release more closely resembles physiological translocation of secretory proteins in that both are inhibited by NEM, whereas the initial phase of release is apparently resistant to the inhibitor (see section 6.5).

Few conclusions can be drawn from the data with regard to the involvement of membrane-bound ribosomes in the rat pancreatic microsome enzyme release phenomenon. Correlative evidence suggests, but does not prove, that there is some connection between the phenomenon of amylase release from microsomes and the apparent degranulation of the membranes. It can merely be stated that the disintegration of the ribosomes coincides with the beginning of the apparent trans-membrane translocation of amylase out of microsomes, thus representing the converse of the *in vivo* situation where the binding of ribosomes to the ER membrane triggers inward translocation of newly-synthesized proteins.

CHAPTER 8

CONCLUDING DISCUSSION

8.1 THE RAT PANCREATIC MICROSOME ENZYME RELEASE PHENOMENON

The work described in this thesis follows the discovery, in our laboratory of an unusual phenomenon which has been labelled "The Rat Pancreatic Microsome Enzyme Release Phenomenon". It was found that active secretory enzymes were released from apparently intact rat pancreatic microsomes, possibly through a trans-membrane protein permease, when vesicles were incubated at 37°C in Mg^{2+}-free buffer.

The pancreatic ER membrane is known to be selectively permeable to secretory proteins *in vivo*. Therefore, having ruled out all the obvious, trivial explanations for the release phenomenon, it was tentatively suggested that the protease-sensitive transfer of α-amylase and RNAse out of rat microsomes *in vitro*, may represent the non-physiological activity of the transport mechanism which, *in vivo*, mediates the inward transfer of (nascent) secretory proteins across the same membrane. The aim of the work presented here was to further evaluate this speculative hypothesis.

8.2 THE CHARACTERISTICS OF AMYLASE RELEASE

It was initially established by the rigorous criteria of loss of protection from proteolysis, and loss of protection from membrane surface-specific labelling, that amylase was indeed released from inside microsomes, across an intact membrane during incubation of the vesicles at 37°C. The inaccessibility to ^{125}I of the amylase contained within fresh pancreatic microsomes, and in vesicles

incubated in the presence of protease at 37°C, indicated that the membranes of both these preparations constituted effective permeability barriers. Furthermore, if the protease, subtilisin was added to microsomes during their incubation at 37°C, on-going amylase release was immediately halted, and the remaining intra-microsomal amylase was protected from inactivation, although the released, extra-vesicular enzyme was rapidly degraded by the added hydrolase (see section 3.3).

It can be inferred from this result that the large amount of added protease (M_r 28,000) could not enter the membrane vesicles through the same mechanism which mediated the escape of the amylase (M_r 56,000). Similarly, it had been found that incubation of "emptied" microsomes in the presence of extra-microsomal amylase and an ATP-generating system did not result in transfer of amylase into the vesicles (Pearce, 1978).

The driving force for the apparently undirectional transfer of enzymes out of microsomes may be the steep gradient which would initially exist between the intra-microsomal and extra-microsomal concentrations of the proteins. In this context, it is noted that unsuccessful attempts have been made to assess the specificity of the transport process. Detergents were used to permeabilize the rat microsomal membranes, with the aim of introducing into the vesicles, proteins which are not normally transported across membranes, for example the cytoplasmic protein globin.

It was hoped to re-seal the membranes by removal of the detergent (by dialysis or filtration) and then determine

whether the foreign proteins would be released during subsequent incubation at 37°C in Mg^{2+}-free buffer. This work did not yield useful results since satisfactory entrapment of enzymes within microsomal vesicles was never achieved (Briggs, 1980).

The data presented in chapter 3 therefore reinforced and extended the previous indications that the rat pancreatic microsome enzyme release phenomenon involved the outward translocation of the secretory proteins contained within the vesicles, rather than a general permeabilization of the microsomal membrane. Further evidence was also presented for the involvement in amylase release, of a protein exposed on the surface of the membranes.

This leads to the question of whether the apparently protein-mediated transfer of secretory proteins out of rat pancreatic microsomes *in vitro*, bears any relationship to the inward translocation of (nascent) secretory proteins across the same membrane, *in vivo*. The latter process is mediated by a relatively well-characterized protein complex consisting of a signal peptidase enzyme, a signal sequence receptor, a ribosome receptor and possibly, two or more pore-forming proteins. Thus the question was approached by comparing the published properties of the apparently co-translational translocation of secretory proteins through this complex with the experimentally-determined characteristics of amylase release.

The results, which are presented and discussed in detail in chapters 4 to 7, revealed a remarkable correspondence between the responses of each transport mechanism to a number of manipulations. Briefly, it was found that

several reported inhibitors of signal peptidase activity depressed the release of amylase from rat pancreatic microsomes, as did denatured ovalbumin, which has been reported to constitute a specific probe for the signal sequence receptors on dog pancreatic microsomes (Lingappa *et al.*, 1979). These interactions were specific in that protease inhibitors which did not affect signal peptidase activity, and other denatured proteins (with the exception of the highly hydrophobic BSA), had no inhibitory effect on the release phenomenon.

These correlations suggest, but do not prove, that the secretory translocator mechanism may be involved in amylase release from rat microsomes. Speculative suggestions were made in chapters 4 and 5 regarding the ways in which these effectors could influence amylase release if it occurred through the secretory "pore". Figure 4.9 shows a schematic model for the reverse passage of α-amylase through the secretory translocator mechanism which is drawn essentially as represented by Walter and Blobel (1981b). It can be imagined that the binding, to the signal receptor, of a denatured protein containing a signal sequence, might block protein release through the mechanism, as might the binding of an inhibitor to the signal peptidase.

Compelling, but again correlative, evidence for the identity of the amylase release mechanism and the physiological secretory protein translocator was provided by the finding that amylase release was inhibited by proteolysis and/or high salt treatment of rat microsomal membranes. As reported in the case of physiological secretion, this inhibition could be partly reversed by re-addition, to the

membranes, of the salt extract, the active constituent of which appeared to be an NEM-sensitive protein. The data were insufficient to provide any insight into the mechanism of this effect in the rat pancreatic microsome suspensions.

On the crucial question of whether membrane-bound ribosomes are implicated in the amylase release phenomenon, the evidence is unsatisfactory in that the investigations were plagued by variability, the source of which could not be pinpointed. With this serious reservation, there were nonetheless suggestions that the disintegration of ribosomes may be associated with the onset of enzyme release.

8.3 SUGGESTIONS REGARDING THE RELEVANCE OF THE RELEASE PHENOMENON TO SECRETION

The correlative lines of evidence listed in section 8.2 culminate in the proposal that the mechanism mediating amylase release from rat pancreatic microsomes *in vitro*, is closely related to the mechanism which functions in secretion *in vivo*, and which is known to be present in the microsomal membrane. There are two obvious discrepancies between the two systems.

The major difference lies in the direction of secretory protein transport in the two situations. *In vivo*, newly-synthesized proteins are translocated from the cytoplasm, across the ER membrane, into the ER lumen. The observed transfer of amylase from a protease-resistant space in the microsomes, to the suspending medium, therefore represents protein transport across the same membrane, in the opposite direction to secretion.

However, physiological concentrations of Mg^{2+} ions

prevent the *in vitro* release phenomenon (Pearce, 1978), therefore this reverse passage of mature amylase across the ER membrane would not be expected to occur *in vivo*. This important finding distinguishes the suggestions put forward in this discussion from the theory advanced by S.S. Rothman and colleagues (reviewed by Diamond, 1978), who propose that the ER membrane is bi-directionally permeable to secretory enzymes *in vivo*. A similar scheme has been advanced by Waksman *et al*., (1980).

The non-physiological direction of apparent amylase transport emphasizes the artefactual nature of the rat pancreatic microsome enzyme release phenomenon, and thus qualifies the tentative conclusions presented in the rest of this discussion. It should also be stated that the release phenomenon described in this thesis may be unique to the rat pancreas. Pancreatic microsomes from two different strains of rats were found to release enzymes in a repeatable, protease-sensitive manner, when incubated at 37°C in the absence of added Mg^{2+}. A similar, protease-sensitive release of intra-vesicular proteins was not observed from microsomes isolated from rat liver (Tabe, 1978). Variable results were obtained from 37°-incubation of crude pancreatic microsome preparations from other species (guinea pig, pigeon, chicken, rabbit & dog), but there was no clear, repeatable, protease-sensitive release of amylase, as observed in the rat.

The release phenomenon therefore might be the result of a physiological peculiarity of the rat pancreas which allows the reverse passage across the ER membrane, of fully-formed secretory proteins, when microsomes are

incubated at physiological temperatures, in cation-depleted medium. Despite this qualification, the fact remains that if amylase release does occur through the physiological secretory protein translocator mechanism, the release process retains many of the properties of *in vivo* secretion, as listed in section 8.2.

It must also be recalled that the said properties of physiological secretion have been largely elucidated from the study of *in vitro* translation systems containing stripped pancreatic microsomes. These microsomes were commonly stripped by treatment with EDTA (∿10 mM) and stored at -80°C. Both EDTA-treatment and freezing have been found to result in leakage (as opposed to protease-sensitive release) of the endogenous secretory proteins of rat pancreatic microsomes (results not shown).

A comparison of the degree of protection from proteolysis of intra-microsomal proteins in dog and rat microsomes is informative. Translation of secretory protein mRNA in the presence of stripped dog pancreatic microsomes has routinely resulted in incomplete proteolytic processing (50-75%) and protection from exogenous proteolysis (10-43%) of the newly-synthesized polypeptide chains (Scheele *et al.*, 1980). It was reported by Scheele *et al.*, that 30% of processed, (and therefore apparently intra-microsomal) α-amylase in the dog pancreatic microsomes was protected from proteolysis when the vesicles were incubated for 60 minutes at 0°C, in the presence of 50 μg/ml each of chymotrypsin and trypsin, without added membrane stabilizer (tetracaine). The degree of protection of the processed enzyme from proteolysis fell to 0% when microsomes

were incubated with the same proteases at 22°C for 10 minutes or more.

By comparison, incubation of the rat pancreatic microsomes used in these studies, in the presence of 2 mg/ml subtilisin BPN', at 37°C for 70 minutes, resulted in protection of 90-100% of the endogenous, intra-vesicular amylase, from proteolysis (fig. 3.5). Incubation of rat microsomes in the presence of 100 µg/ml chymotrypsin at 37°C for 60 minutes also resulted in virtually 100% "protection" of intra-microsomal amylase (Pearce *et al.*, 1978); although it was demonstrated that released, extra-microsomal amylase was naturally very resistant to degradation by chymotrypsin under similar incubation conditions (see table 3.1).

It was found by Scheele *et al.*, (1980) that incubation of dog microsomes containing segregated, processed dog α-amylase, at 22°C, for 90 minutes, in a translation system which contained 0.95 - 1.45 mM Mg^{2+}, did not result in "redistribution" (of secretory proteins) "across the microsomal membrane" as "postulated ..... by Tabe *et al.*" (1980). Incubation of rat pancreatic microsomes at temperatures below 28°C (see fig. 7.4) or at 37°C in the presence of Mg^{2+} concentrations exceeding 1 mM (Pearce, 1978), similarly did not result in redistribution of intra-microsomal amylase. It would be interesting to observe the effects of incubating EDTA-treated dog pancreatic microsomes containing mature, *in vitro*-synthesized amylase, at 37°C in Mg^{2+}-free buffer.

Thus, although the rat pancreatic microsome enzyme release phenomenon involves the reverse translocation of

fully-formed amylase across the microsomal membrane, it may, in some ways, be less far removed from the *in vivo* situation than *in vitro* translation systems containing stripped dog pancreatic microsomes which fail to protect intra-vesicular enzymes from external proteases except in the presence of a membrane-modifying agent (Scheele *et al.*, 1980). It is noteworthy that EDTA treatment of microsomes has been discontinued in some recent studies (Walter & Blobel, 1980, Walter *et al.*, 1981, Walter & Blobel, 1981a & b).

As previously stated, the reverse transport of amylase out of rat pancreatic microsomes shares many properties with protein transport into dog pancreatic microsomes *in vitro*, and into the ER *in vivo*. In this context, the post-translational nature of the amylase release phenomenon is interesting; in fact, it becomes very suggestive when taken in context with the examples of the post-translational translocation of proteins across membranes *in vivo*, which were presented in chapter 1 and summarized in section 1.8.

It was pointed out that, in many situations, fully-synthesized, initially water-soluble proteins are translocated across or into biological membranes. Toxin molecules integrate into, and partially traverse the plasma membranes of both prokaryotic and eukaryotic cells, while the post-translational transfer of proteins into eukaryotic organelles is also well-documented. In all these cases, the interaction between the protein and the membrane appears to depend on the existence of integral membrane receptor proteins which may or may not play a part in the subsequent translocation of the transported protein across the membrane.

Post-translational transport of newly-synthesized periplasmic proteins across the bacterial plasma membrane has also been demonstrated (Josefsson & Randall, 1980, 1981, Smith *et al.*, 1977, 1978, 1979, 1980, Koshland & Bottstein, 1980). On the other hand, some bacterial proteins have been proposed to span the plasma membrane co-translationally, as demonstrated by the accessibility of the nascent peptide chains to external, non-penetrating label (Smith *et al.*, 1977, 1978, 1979). Thus, both post-translational and co-translational transfer of periplasmic proteins seem to occur across the prokaryotic plasma membrane.

In some cases, it even appears that individual proteins may follow either a co-translational or post-translational route across the membrane (Josefsson & Randall, 1981, Smith, 1977, 1978, 1979, Sabatini *et al.*, 1982). When considered with the obvious fact that both co-translational and post-translational translocation of proteins across membranes must obey the same thermodynamic laws, this finding militates against the often-expressed notion that the two modes of trans-membrane protein transport are fundamentally different (please see section 1.8).

It is possible to argue that the only real difference between co- and post-translational protein transfer across biological membranes may be the efficiency of each process. For example, in the case of secretory protein export in prokaryotes and eukaryotes, large quantities of potentially destructive enzymes must be transferred across the plasma and ER membranes respectively. In eukaryotes,

evolution appears to have tailored this process so that maximum efficiency of transfer is achieved. The polysomes synthesizing secretory proteins are directed, by the signal sequence in the protruding nascent peptide chain, to receptors on the cytoplasmic face of the ER membrane. The co-translational binding of the polysomes to the receptors ensures that the secretory enzymes are conducted immediately through the ER membrane, and thence efficiently out of the cell, thus precluding the possibility of the active, degradative enzymes being released in the cell cytoplasm.

There is little experimental data relating to the actual mechanism of protein transport across the ER membrane, however the process is thought to be analagous to protein transfer across the bacterial plasma membrane, where it has been demonstrated that exported proteins may cross the lipid bilayer either co-translationally or post-translationally. Thus, by analogy, the possibility is raised, of post-translational protein transport across the ER membrane.

The work reported in this thesis may therefore be significant in that it demonstrates the post-translational transfer of an active secretory enzyme across the rat pancreatic microsomal membrane, *in vitro*. Although the observed trans-membrane movement of αamylase is in the opposite direction to that of physiological secretion, it has been pointed out that the amylase transfer process appears to share many properties with the secretory mechanism. The results reported here may therefore have implications for the mechanism of secretory protein

transport into the ER *in vivo.*

Taken in context with the preceding discussion, the amylase release phenomenon may support the suggestion that there is no real distinction between what has been defined as co-translational and post-translational trans-membrane protein transport. This idea is reinforced by the aforementioned report that individual prokaryotic proteins appear to traverse the plasma membrane either co-translationally or post-translationally.

Thus, it may be envisaged that a secretory protein could fold into a "translocation competent" structure either during or immediately after its synthesis in the cytoplasmic compartment of a eukaryotic cell. The factors determining which mode of transport is utilized by a given protein in a given situation might include, the length of the polypeptide required to fold into a "translocation-competent" conformation, local fluctuations in the dynamic membrane structure, the local ionic environment, and many other possible parameters. It remains to be elucidated, whether the transported proteins interact with both integral membrane proteins and lipids, or with proteins alone, as they cross the membrane barrier.

The signal hypothesis suggests that the secretory proteins interact only with integral membrane proteins during the passage of the former across the membrane, through a hydrophilic pore mechanism. It is proposed that secretory proteins are extruded co-translationally through the pore complex, as illustrated schematically in figure 1.4. In terms of this model, amylase release from rat microsomes must presumably be explained by proposing that

fully-formed amylase can pass backwards through the pore, as illustrated in figure 4.9. Assuming that the amylase release phenomenon is relevant to the *in vivo* situation, this may be taken to indicate that the physiological process of secretion involves the inward transfer of fully-formed proteins through a pore.

The possibility of a hydrophilic discontinuity, in the ER membrane, of a size which would accommodate globular proteins, seems unlikely. It seems more probable that post-translational translocation across the ER membrane could be explained by a scheme involving interactions between the transported protein and the membrane lipid, essentially as suggested by Waksman *et al.*, (1980), to explain the trans-membrane movement of some mitochondrial proteins.

An alternative interpretation of all the date pertaining to trans-membrane protein translocation is that the actual process of translocation is exclusively post-translational. This has been convincingly demonstrated in the case of protein import into eukaryotic organelles, and in specific cases of protein transfer across the prokaryotic plasma membrane. The main impediment to this interpretation is the data of Smith *et al.*, (1977, 1978, 1979) who have shown that nascent periplasmic proteins in *E. coli*, are accessible to external, non-penetrating label.

In explaining these observations, it is possible to speculate that the periplasmic proteins are fed into a translocator/pore mechanism in the membrane such that the actual process of translocation does not occur until after the completion of protein synthesis, although an external,

water-soluble labelling compound can diffuse into the pore and attach to the growing peptide chain.

In the case of protein transfer across the eukaryotic ER membrane, there is incontrovertible evidence that the binding of polysomes synthesizing secretory proteins, to the membrane surface, occurs co-translationally. There is, however, no data which excludes the possibility of a secretory protein folding up in an intra-membranous pore or pocket, under a membrane-associated ribosome. As explained in section 1.7, the demonstration of co-translational proteolytic processing and co-translational glycosylation of nascent secretory proteins and nascent membrane proteins, may simply indicate that the nascent peptides are in contact with enzymes buried within the lipid bilayer, rather than proving that the peptides have passed through the membrane.

Thus, secretory proteins could be translocated across the ER membrane immediately after their completion by membrane-bound ribosomes, via a mechanism virtually identical to the mechanisms which mediate the import of fully-formed proteins by other eukaryotic organelles.

8.4 CONCLUSION

It is recognized that the enzyme release phenomenon described in this thesis is inherently improbable. The proposal that fully-formed secretory proteins can move backwards through the rat pancreatic microsomal membrane, possibly via the "forwards" secretion mechanism, conflicts with current ideas. On the other hand, the data presented in this thesis, and data reported by others (Pearce, 1978,

Tabe, 1978, Pearce *et al.*, 1978), leave little doubt that α-amylase (and ribonuclease) is released from rat pancreatic microsomes with characteristics which are hardly to be expected from a non-specific leakage.

The weakness in proposing that amylase release is mediated by the secretory protein translocator mechanism lies in the fact that the suggestion is based on correlative evidence of similarities between the two systems. Obviously, more work is required to substantiate (or reject) these ideas. Areas which demand further investigation include the nature of the salt-extractable protein factor described in chapter 6, and the mechanism by which it stimulates the enzyme release process. Clarification is also needed regarding the involvement of membrane-bound ribosomes in the release phenomenon.

In prescribing further work, however, the considerable effort required to yield meaningful results must be weighed against the potential value of those results. In the case of the rat pancreatic microsome enzyme release phenomenon, all extrapolations to the physiological process of secretion suffer from the serious qualification that the *in vitro* amylase transport across the microsomal membrane is in the opposite direction to the *in vivo* translocation of secretory proteins across the ER membrane. It may therefore be wisest to simply note that the *in vitro* release of fully-formed α-amylase across intact microsomal membranes does occur, under certain conditions, and must be explained by any theory which purports to constitute a comprehensive description of trans-membrane protein translocation. It is possible that the rat pancreatic

microsome enzyme release phenomenon may prove to be a valuable experimental approach to the problem of trans-membrane protein translocation, in the light of future developments in this rapidly advancing field of study.

ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
amylase) α-amylase)	1,4-α glucan 4-glucanohydrolase; E.C. No. 3.2.1.1.
ATP	Adenosine 5'-triphosphate
A_{280}) A_{620})	Absorbance at 280 or 620 nanometers
βME	β-mercapto ethanol
BSA	Bovine serum albumin
BSA_D	Denatured BSA
BSA_N	Native BSA
BSA_f	Tryptic fragments of denatured BSA
Chloroglycoluril	1,3,4,6-tetra-chloro-3α,6α diphenyl-glycoluril
DNA	Deoxy-ribonucleic acid
DMSO	Dimethyl sulphoxide
DOC	Deoxycholic acid, sodium salt
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
EM	Electron Microscopy
ER	Endoplasmic Reticulum
g_{av}	Gravitational field at mid-point of centrifuge tube
GDW	Glass distilled water
Kd	Kilo-dalton
MDH	Malate de-hydrogenase
M_r	Molecular weight
NEM	N-ethyl maleimide
N-terminal	Amino-terminal
OBG	Octyl β-glucopyranoside

OTC-ase	Ornithine transcarbamoylase
OV_D	Denatured ovalbumin
OV_f	Tryptic fragments of denatured ovalbumin
OV_N	Native ovalbumin
PMSF	Phenylmethyl sulphonyl fluoride
pOHMB	p-hydroxy mercuribenzoate
pre-PL	pre-placental lactogen
RNAse	Ribonucleate 3' pyrimidino-oligonucleotido-hydrolase E.C. No. 3.1.4.22.
SDS	Sodium dodecyl sulphate
SKC	0.3 M sucrose; 10 mM potassium chloride; 0.2 mM calcium chloride
SRP	Signal recognition protein
STKC	0.3 M sucrose; 50 mM Tris HCl, pH 7.5; 25 mM potassium chloride; 0.2 mM calcium
TCA	Trichloro-acetic acid
TLCK	Tosyl-l-lysine chloromethyl ketone
TPCK	L-l-tosylamide phenylethylchloromethyl ketone
Triton X-100	p-Iso-octylphenoxypolyethoxyethanol

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