EXTRACELLULAR ENZYME FORMATION BY BACILLUS AMYLOLIQUEFACIENS

A Thesis

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1. Exponentially growing Bacillus amyloliquefaciens cells, harvested by a rapid cooling technique, yields polyribosome-containing cell extracts active in protein synthesis. The requirements of the endogenous mRNA-directed cell-free system are similar to those from other Bacillus species with respect to dependence on ribosomes, soluble fraction and an energy generating system. Extracellular protease may be an important factor in determining the activity of resulting cell extracts.

2. Clean cytoplasmic membrane fragments were isolated by the osmotic lysis of B. amyloliquefaciens protoplasts, Although the fragments are seemingly capable of protein synthesis, electron microscopy has failed to detect the presence of associated ribosomes. Initial studies indicate that the fragments are apparently incapable of synthesising active extracellular enzymes.

3. Total cell wall removal is not required for the loss of exoenzyme-forming ability as previously indicated. Lysozyme addition results in only a slight impairment of total cellular protein synthesis but causes a rapid cessation of extracellular enzyme appearance in the supernatant at a time long before protoplast detection. Electron micrographs

reveal the release of small vesicles, mesosomal in nature, immediately after lysozyme addition and also suggest that a cell wall mucopeptide layer lying near the cytoplasmic membrane may be initially removed.

4. A study of protease formation by B. amyloliquefaciens revealed that synthesis and secretion of this exoenzyme occurred for 80 min. in the presence of rifampicin or actinomycin D. Labelling studies demonstrated that this protease production involves de novo synthesis of the enzyme. It is proposed that harvested cells contain an accumulated pool of protease mRNA capable of supporting protease synthesis for this time period. The mRNA is not stable in the sense of being translated more times than normal, for when this pool is exhausted, further protease synthesis shows normal sensitivity to rifampicin and actinomycin D. It is further postulated that for extracellular protease synthesis, mRNA migrates from the gene to membrane translational extrusion sites and that the accumulated mRNA represents that in transit to these sites.

5. Further in vitro protein synthesis studies established that B. amyloliquefaciens extracts are less active than E. coli extracts, when endogenous mRNA-directed activity

alone is measured. The two systems were found to differ greatly in their ability to translate polyuridylic acid. The establishment of an E. coli cell-free system capable of translating natural MS2 phage RNA enabled preliminary translational studies to be carried out with purified RNA from B. amyloliquefaciens. Significant ^{14}C-amino acid incorporation into protein was achieved with this RNA, suggesting it is 'messenger-like' in nature.

6. Protein synthesis inhibitors specific for the 50S ribosomal subunit apparently inhibit the synthesis of both protease and α-amylase to a greater degree than the synthesis of intracellular proteins. The fact that 30S-subunit-specific inhibitors do not show such a differential effect, except in the case of two inhibitors both of which are capable of acting at the initiation level, suggests that extracellular enzymes are synthesised on peripherally located ribosomes with the 50S subunit built into the membrane and that a unique initiation mechanism may exist.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University.

The work in Chapter V was to a considerable extent carried out with myself as an equal collaborator with one other person. Apart from this, the remainder of the work was carried out by myself except where otherwise stated.

To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except when due reference is made in the text.

Signed:

JAMES L. MCINNES

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CHAPTER I

INTRODUCTION

INTRODUCTION

This thesis is concerned with the secretion of extracellular enzymes. A study of this phenomenon has been made using the organism Bacillus amyloliquefaciens. This organism was previously described as an unclassified strain of Bacillus subtilis; however, it has since been identified as B. amyloliquefaciens by Welker and Campbell (1967) on the basis of DNA base composition.

B. amyloliquefaciens secretes large amounts of α-amylase, protease and ribonuclease into the external medium and so provides a favourable system for study. The work was particularly directed at determining the site of extracellular enzyme synthesis and elucidating the mechanism of secretion.

The introduction deals firstly with the secretion of proteins and enzymes by mammals and bacteria and finally with general properties of bacterial extracellular enzymes.

A. GENERAL INTRODUCTION TO EXTRACELLULAR ENZYMES

The production of extracellular proteins is a widespread phenomenon and many different types of cells secrete large amounts of proteins into the external medium. These include serum proteins, which constitute the majority of the proteins synthesised by the liver, antibodies and some hormones. A large number of proteins with enzymic properties are also secreted into the external medium by some mammalian, plant and bacterial systems. In mammals, perhaps the best known extracellular enzymes are the α-amylase, protease and ribonuclease produced by the exocrine pancreas cells, whereas in plants these same enzymes are synthesised by the aleurone-cell layer of germinating barley seeds. Microorganisms also produce a wide variety of extracellular enzymes and the subject of bacterial enzyme secretion has been reviewed by Pollock (1962) and Lampen (1965).

Apart from the physiological interest of extracellular enzymes, the mechanism by which a cell can secrete large amounts of potentially lethal enzymes such as proteases and ribonucleases without either the protein synthesising capacity of the cell being affected or the cell itself undergoing lysis, poses an interesting biochemical problem. Some cells, such as the pancreatic cell, overcome this problem by utilising secretory granules;

the extracellular proteins contained within the granules are released from the cell by a process similar to reverse pinocytosis, which entails fusion of the granules with the cell membrane such that the contents of the granules are discharged to the outside of the cell (Palade et al., 1962). However, the mechanism of secretion of these proteins through the endoplasmic reticular membrane prior to their enclosure within the secretory granules is the main problem, and because of its relevance to bacterial extracellular enzyme secretion, the process will now be discussed in some detail.

B. MECHANISM OF SECRETION OF MAMMALIAN SECRETORY PROTEINS

(i) SITE OF SYNTHESIS OF SECRETORY PROTEINS

The ribosomes of mammalian cells are known to occur in two morphologically and biochemically distinguishable states. One class of ribosomes exists apparently free in the cytoplasm, while another class is closely associated with the endoplasmic reticulum. Membrane structures studded with ribosomes were noted long ago by electron microscopy and denoted as rough endoplasmic reticulum (Palade, 1955).

Siekevitz and Palade (1960) were responsible for the main advances in the under-standing of the mechanism of mammalian protein secretion. They suggested that secretory proteins were synthesised on the ribosomes that

were attached to the endoplasmic reticulum rather than on the free ribosomes, which were engaged in the synthesis of non-exportable proteins. Based on electron microscopic evidence, Birbeck and Mercer (1961) postulated similar roles for the two classes of ribosomes.

Early biochemical support for this hypothesis was provided by studies on the pancreas. Siekevitz and Palade (1960) showed that radioactive α-chymotrypsinogen, a secretory protein, was bound to attached ribosomes of the pancreas rather than to free ribosomes, at 1 min. after an intravenous injection of a radioactive amino acid into a guinea pig. Caro and Palade (1964), using a radio-autographic technique, demonstrated that protein synthesised by membrane-bound ribosomes in pancreatic acinar cells was first located within the vesicles of the rough endoplasmic reticulum, then within smooth vesicles and finally was condensed into discrete packets of protein in the Golgi vesicles before being discharged from the cell.

Since these initial studies, numerous reports have appeared in the literature dealing with the isolation, properties and the functional significance of these two ribosomal classes from different eucaryotic cell types. Campbell (1970) and Tata (1971) have presented review articles on the possible functional significance of these two different ribosomal populations. The proportion of

ribosomes associated with membranes varies widely in different types of cells. This proportion can be related to the degree of specialisation of these cells for exporting products (Goldberg and Green, 1964; Kimmel, 1969). Cells which are specialised for the synthesis of protein destined to be exported, like the pancreas and liver, have a well developed endoplasmic reticulum and numerous membrane-bound ribosomes. Similarly, the majority of ribosomes in the plasma cell, which is the site of immunoglobulin synthesis, are associated with membranes. The opposite extreme is found in rapidly growing embryonic cells and de-differentiated tumour cells. Very little endoplasmic reticulum is found in these cells and the great majority of ribosomes are free.

Many biochemical studies suggest that both the endoplasmic reticulum-associated ribosomes and the free ribosomes are engaged in the synthesis of specific proteins. It has been clearly shown that the secretory proteins of the pancreas and liver are synthesised by ribosomes attached to the endoplasmic reticulum membrane. Siekevitz and Palade (1966) established that α-amylase was synthesised <u>in vivo</u> on such a membrane-ribosome complex. Guinea pigs were injected with ^{14}C-leucine and the pancreas removed, homogenised with a low concentration of deoxycholate and the subcellular components fractionated on a sucrose gradient. Radioactive α-amylase with the

highest specific activity (that is, newly synthesised enzyme) was associated with the pellet which consisted of membrane with attached ribosomes.

Redman (1968) demonstrated that secretory proteins of the liver are synthesised by membrane-bound ribosomes rather than free ribosomes. This author found that after labelling rat liver *in vivo* with ^{14}C-leucine, more than 90% of the labelled nascent serum albumin protein was associated with membrane-bound ribosomes; this result was confirmed by Takagi *et al.* (1970) who presented both *in vivo* and *in vitro* experiments showing that serum albumin is synthesised on bound polysomes of liver cells.

Further studies, both *in vitro* and *in vivo* have confirmed that in rat liver, while the bulk of the serum albumin is synthesised on membrane-associated ribosomes, a retained, non-exportable liver protein such as ferritin, is preferentially synthesised on the free ribosomes (Ganoza and Williams, 1969; Hicks *et al.*, 1969; Redman, 1969). In the case of immunoglobulin secretion, Sherr and Uhr (1970), using subcellular fractionation procedures, have separated free from bound polyribosomes of an immunoglobulin-producing mouse myeloma cell labelled *in vitro* with ^{3}H-leucine. Nascent polypeptides released from polyribosomes were then precipitated with antiserum specific for mouse immunoglobulin. The results indicated that immunoglobulin, virtually the sole protein secreted

by plasma cells, is synthesised preferentially by bound polyribosomes. This result has been recently confirmed by Pryme et al. (1973).

Ueonoyama and Ono (1972a) studied albumin synthesis in 5123D hepatoma cells and found the protein to be synthesised by the free polyribosomal fraction; this result, however, is still compatible with those above, since the albumin in this case is not exported but is retained in the hepatoma cells.

The occurrence of bound ribosomes in those cells, which lack, in general, an obvious secretory activity, suggests that the attachment of ribosomes to membranes may not be exclusively related to the synthesis of proteins to be exported. Indeed, some exceptions to the general concept have been reported (Rolleston, 1972). Conceivably, the structural proteins of membranes or the degradative enzymes in lysozymes may be the products of this special class of polyribosomes. Tata (1971), in particular, has suggested that the attachment may be topographically to segregate different populations of ribosomes presumably synthesising different classes of proteins, a function which may be critical during rapid developmental or adaptional changes. Hence, although the functional significance of free and membrane-bound ribosomes in all eucaryotic cell types has not been unequivocally determined,

there appears to be no serious argument against the uniqueness of bound-polyribosomal populations in their ability to produce exported proteins, at least in the parenchymal cells of the pancreas and liver (Campbell, 1970).

(ii) SIMULTANEOUS SYNTHESIS AND SECRETION OF PROTEINS

The rough endoplasmic reticulum breaks down after cell fractionation to give closed vesicles (that is, microsomes) with externally attached ribosomes. Redman et al. (1966) first reported that α-amylase, synthesised in vitro, was released into the interior of microsomes. These workers prepared from pigeon pancreas, a microsomal fraction which could incorporate labelled amino acids into α-amylase; a tryptic digestion of isolated labelled enzyme and separation of the peptides showed that the label was distributed throughout the protein thus confirming that in vitro synthesis had occurred. After different periods of labelling, the microsomal system was fractionated. Initially, most of the total α-amylase radioactivity was associated with membrane-bound ribosomes which were thus identified as the site of synthesis. However, in addition to synthesising the enzyme, the microsomal system was capable of transporting a large fraction of enzyme produced on the ribosomes to a deoxycholate-soluble fraction, believed to represent the contents of the microsomes. Hence, it was concluded that newly synthesised α-amylase had been

preferentially transferred from its site of synthesis at the ribosomes into the microsomal cavity (the microsomal equivalent of the cisternal space *in vivo*).

In addition to a specific protein, it was also claimed that nascent proteins labelled *in vitro* are also vectorially transferred to the interior of microsomes upon their release with puromycin. Redman and Sabatini (1966) incubated *in vitro* a guinea pig liver microsomal system with ^{14}C-leucine and hence labelled nascent proteins still attached to membrane-bound ribosomes. Upon treatment with puromycin, up to 80% of the labelled nascent proteins were released from ribosomes into the fraction solubilised by deoxycholate treatment and believed to represent the microsomal contents. The discharge of nascent proteins into the microsomal cavity appeared to be a direct consequence of their release from ribosomes; streptovitacin A, which does not liberate incomplete peptides as does puromycin, did not give apparent release of nascent protein into the cavity. Similarly, Redman (1967) reported that in a rat liver microsomal system, puromycin caused the vectorial transfer of labelled nascent protein into the cavity of the microsomes. These results suggest, that from the onset of protein synthesis, the growing peptide chain is directed towards the cisternal space of the endoplasmic reticulum into which it diffuses upon release from attached ribosomes.

The vectorial release of nascent immunoglobulin peptides has been studied by Bevan (1971) in vitro, who examined a microsomal preparation from a mouse plasmacytoma that secretes immunoglobulin. Nascent chains were released with puromycin and characterized with specific antiserum against the immunoglobulin product of the tumour. The results were in accord with the previous studies on liver and pancreas, demonstrating that the immunoglobulin chains remain associated with the microsomal vesicles after release from the ribosomes.

In the studies described so far, the tissues examined synthesise most of their proteins for export from the cell. Andrews and Tata (1971) compared the vectorial release of nascent protein from microsomes of secretory (rat liver) and non-secretory tissues (rat cerebral cortex and skeletal muscle). Using the technique described by Redman and Sabatini (1966) of post-incubation with puromycin (followed by treatment with deoxycholate) to follow the vectorial release of nascent chains, it was found that whereas most of the newly synthesised polypeptides discharged from bound polysomes of liver were released into the vesicular lumen of the membrane, those from brain and muscle were released directly into the supernatant. This result is compatible with the relative lack of secretory function of these two tissues and , in this respect, bound ribosomes from non-secretory tissues behave as free

ribosomes from secretory and non-secretory tissues.

Two reports questioning the validity of microsomal subfractionation procedures employing deoxycholate to determine the locality of the puromycin-mediated release of radio-active proteins synthesised *in vitro*, have appeared recently. Sauer and Burrow (1972) examined the puromycin-mediated release of labelled nascent proteins from rat liver microsomes labelled with ^{14}C-leucine. When the microsomes were fractionated by sodium deoxycholate, the distribution of the radioactivity among the microsomal subfractions indicated that the puromycin-mediated release could be directed either toward the microsomal membrane or toward the intravesicular space. An unequivocal interpretation was not possible because of subfraction contamination and protein binding phenomena. However, the distribution of radioactivity following gel filtration of deoxycholate-solubilised microsomal membranes, or dispersion of the labelled microsomes by sonication, suggested that the direction of the puromycin-mediated release is toward the microsomal membrane rather than toward the intravesicular lumen. The results of Burke and Redman (1973) suggested that the appearance of acid-insoluble radioactivity in a detergent soluble fraction may not be sufficient evidence for vectorial transport and that other explanations need to be carefully excluded.

(iii) MODEL FOR SECRETION OF PROTEINS THROUGH THE ENDOPLASMIC RETICULAR MEMBRANE

Studies on the attachment of ribosomes to the microsomal membrane (Sabatini, et al., 1966) and the puromycin-mediated release of nascent peptides from the attached ribosomes (Redman and Sabatini, 1966) led to a proposed model for the synthesis and secretion of proteins through the endoplasmic reticular membrane.

Sabatini et al. (1966) demonstrated that EDTA-treatment of isolated guinea pig liver microsomes caused a step-wise release of the attached ribosomal subunits. The small subunit was preferentially lost first, leaving the large subunit still intact on the membrane surface. Even at high concentrations of EDTA, a considerable proportion of large subunits remained attached and these subunits contained most of the newly synthesised protein. It was concluded that ribosomes were attached to the endoplasmic reticular membrane by the large subunit and that the growing peptide chain may also contribute to the attachment of this subunit. These binding studies were also substantiated by electron micrographs.

On the basis of these observations, Redman and Sabatini (1966) proposed a model for the synthesis and secretion of proteins. In this model, the membrane-bound large ribosomal subunit is assumed to contain a central channel which is continuous with the cisternal space through

a discontinuity in the membrane. The peptides being synthesised are assumed to grow within the central channel, such that the unidirectional character of peptide chain release is explained by structural restrictions.

This model has gained further support from experiments bearing on the location of nascent polypeptides within hepatic ribosomes and the relationship of the nascent chains of bound ribosomes to the microsomal membrane (Blobel and Sabatini, 1970; Sabatini and Blobel, 1970). These workers have studied the controlled proteolytic digestion of nascent polypeptide chains labelled in vitro in rat liver cell fractions. The susceptibility of labelled nascent chains to digestion by added proteases was taken as an index of their accessibility to the enzymes. Results indicated that in rough microsomes, virtually the entire polypeptide chain was resistant to proteolytic digestion. It was proposed that the growing end of the nascent polypeptide (carboxy-terminal segment) is shielded by virtue of its location within the proposed channel through the interior of the large ribosomal subunit; the structural restrictions of the binding of the large subunit to the microsomal membrane confers protection to the amino-terminal remainder of the nascent polypeptide.

The conclusion that ribosomes are attached to membranes through the large subunit (60S) has been supported by several observations. Baglioni et al. (1971)

showed that, in intact plasmacytoma cells, 60S subunits can attach to membranes independently of small ribosomal subunits (40S) and in the absence of protein synthesis. This inferred that ribosomes do not attach to membranes in the form of polyribosomes. Rolleston (1972) provided direct evidence from in vitro binding studies that the 60S ribosomal subunit binds much more efficiently to rough endoplasmic reticulum membranes from mouse liver than the 40S subunit; in these same studies, the 40S subunits were shown to bind more efficiently to membranes in the presence of 60S subunits. Attardi et al. (1969) and Rosbash and Penman (1971) studied the membrane-bound ribosomes in HeLa cells. Treatment with EDTA released essentially all the small subunits leaving a significant amount of the larger subunits attached to the membrane. Thus, this fraction of membrane-bound ribosomes is apparently attached to the membrane via the large subunit, in agreement with the EDTA dissociation behaviour of the ribosomes of the rough endoplasmic reticulum from rat liver (Sabatini et al., 1966).

The role played by the nascent polypeptide chains in the binding of ribosomes to membranes has also been the subject of numerous investigations. Originally, the retention of nascent polypeptide chains was studied by treating rough microsomes with EDTA or trypsin and it was thought that the nascent chains may anchor the ribosome

to the membrane (Sabatini et al., 1966; Chefurka and Hayashi, 1966). However, the findings that pretreatment of tissue with puromycin did not decrease the number of bound ribosomes (Blobel and Potter, 1967; Andrews and Tata, 1971; Rosbash and Penman, 1971) and that 60S subunits could attach to membranes in the absence of protein synthesis in intact cells (Baglioni et al., 1971) suggested that nascent peptides are not required to attach ribosomes to membranes. More recently, Adelman et al. (1973) have shown in an elaborate study, that it is possible to identify a role for the nascent polypeptide chain in binding, by establishing that puromycin-dependent ribosome release from rat liver rough microsomes only occurs in a medium of high ionic strength. These workers concluded that ribosomes are bound to membranes via two types of interactions; a direct one between the membrane and the large ribosomal subunit (labile at a high KCl concentration) and an indirect one in which the nascent chain anchors the ribosome to the membrane (puromycin labile).

Studies utilising a disulphide-interchange enzyme activity as an indirect measurement of polyribosome binding to endoplasmic reticulum have been reported and suggest that steroid hormones may play an important role in the binding process (James et al., 1969; Sunshine et al., 1971; Williams and Rabin, 1971). A possible role for specific binding proteins in the attachment process has also been

suggested from the recent studies of Fridlender and Wettstein (1970) who showed that two ribosomal proteins present in free ribosomes were missing in the membrane-bound particles of chick embryos. Similarly, the report of Burka and Bulova (1971) on the different electrophoretic ribosomal protein patterns from membrane-bound and free ribosomes from rabbit reticulocytes has given further support for this possibility.

C. MECHANISM OF PROTEIN SECRETION BY BACTERIA

The mechanism of secretion in bacterial extracellular enzymes is not as well understood as that for the secretion of mammalian extracellular enzymes. A considerable amount of evidence exists which associates protein synthesis (though not necessarily that of extracellular enzymes) with the bacterial cytoplasmic membrane. An attractive hypothesis therefore is that, as in the animal cell, secreted bacterial enzymes are formed by membrane-bound polysomes and transferred by a vectorial process into or through the cytoplasmic membrane. Indeed, this view is now supported by recent studies reported in the literature and from results to be reported in this thesis.

(i) EVIDENCE THAT THE CELL MEMBRANE IS A SITE OF PROTEIN SYNTHESIS IN BACTERIA

Evidence for the cytoplasmic membrane being a site of protein synthesis in bacteria has come essentially from the detection of membrane-bound polysomes in cell lysates and from both *in vitro* and *in vivo* experiments showing that these membrane fractions possess appreciable protein synthesising activities. Studies of this nature have been reviewed by Hendler (1965, 1968).

Early evidence linking membrane sites to protein synthesis came from the work of Schlessinger (1963) and Schlessinger *et al.* (1965) who found that if lysates of *Bacillus megaterium* were prepared in the presence of 0.01 M Mg^{2+} and 0.01 M Na^{+}, between 33 to 50% of the cellular RNA was membrane-bound. The RNA could not be released from the membrane by repeated washes but was released by the removal of Mg^{2+} or the use of deoxycholate. This polysome membrane fraction was shown to readily incorporate *in vitro* radioactive amino acids into protein.

A similar pattern of attachment of ribosomes to membranes was found with systems other than *B. megaterium*. Thus, Tani and Hendler (1964) and Hendler and Tani (1964) showed that membrane-bound ribosomes existed in *Escherichia coli* and that the extent of binding of active ribosomes was dependent on the severity of the cell-disruption technique; spheroplasts, pulse-labelled with radioactive

amino acids, were either gently disrupted by osmotic shock or vigorously disrupted with a tissue press and the ribosome and membrane fractions were then examined for the presence of nascent protein. Gentle disruption gave a membrane fraction consistently more radioactive than the free ribosome fraction but the reverse was true upon vigorous disruption. More recently, Scharff et al. (1972) have isolated membrane-envelope fragments from E. coli by extremely gentle techniques in order to preserve their structural integrity as much as possible. These membrane fragments, containing a high proportion of associated RNA, attained extremely high levels of incorporation of radioactive amino acids into protein.

Moore and Umbreit (1965) found approximately 12% of the cellular RNA was membrane-bound in preparations of Streptococcus faecalis lysed in the presence of 0.01 M Mg^{2+} and 0.12 M K^{+}. A linear correlation was shown to exist between the ability of fractions to incorporate labelled amino acids with their phospholipid content, suggesting that a membrane constituent is a limiting factor in protein synthesis.

The localisation of ribosomes at membrane sites in the above-mentioned bacteria was further demonstrated by studies involving the incorporation of labelled precursors into RNA (Suit, 1962; Yudkin and Davis, 1965; Abrams et al., 1964). In addition, the presence has been

established at the cell membrane of the main components necessary for protein synthesis; viz: amino-activating enzymes, transfer RNA and rapidly labelled RNA which is believed to function partly as a precursor to ribosomes and partly as messenger RNA (Abrams et al., 1964; Yudkin and Davis, 1965; Bubela and Holdsworth, 1966).

Electron micrographs supporting the existence of membrane-associated ribosomes have been presented from studies with different bacterial cells. Schlessinger et al. (1965), in studies with B. megaterium, presented electron micrographs of sectioned cell 'ghosts' and membrane fragments confirming that polysomes were attached to the membrane. Fitz-James (1964) studied the same organism and presented similar evidence. Hendler et al. (1964) presented electron micrographs of a membrane fraction from lysed spheroplasts of E. coli showing ribosomes associated with the inner membrane surface of the cytoplasmic membrane.

Electron microscopy has also revealed the presence of polysome clusters at the membrane of Bacillus cereus (Pfister and Lundgren, 1964), S. faecalis (Abrams et al., 1964) and of helically arranged polysomes attached to the membrane of Rhodospeudomonas palustris (Tauschel and Drews, 1969). More recently, Scharff et al. (1972) have isolated E. coli membrane-envelope fragments in the absence of lysozyme and have presented electron micrographs showing membrane-attached ribosomal clusters. The presence of these

clusters argues against the artifactual attachment of ribosomes to membranes by the use of lysozyme as suggested by Patterson et al. (1970).

The proportion and size distribution of the ribosomes in the membrane fraction of bacterial cell lysates appear to be a function of the growth phase in which the cells are harvested (Moore et al., 1966) and the composition of the lysing buffer especially with respect to the cations (Schlessinger et al., 1965; Coleman, 1969c). In cell-free extracts derived from S. faecalis, Moore et al. (1966) found that protein synthesis directed by endogenous mRNA increased as the culture aged; this increased activity was accompanied by an increase in the percentage of membrane-bound ribosomes. The ionic conditions influencing the binding of polyribosomes to membranes of Bacillus amyloliquefaciens have been investigated by Coleman (1969c) who reported that divalent cations (Mg^{2+}) favour attachment (see also Schlessinger et al., 1965) whereas monovalent cations (K^{+}) depress the binding. Coleman found that under conditions known to preserve maximal cell-free protein synthesis, less than 10% of the B. amyloliquefaciens ribosomes were membrane-bound, and suggested that one must treat with caution claims that the majority of bacterial protein synthesis occurs on the membrane as suggested by Hendler (1968).

Very few studies on the nature of attachment of

polysomes to bacterial cell membranes have been carried out and these have been mainly indirect in their approach. Aronson (1966), in a detailed *in vitro* study of the nature of attachment of polysomes to isolated *B. megaterium* membranes, presented evidence that the presence of nascent polypeptides is a critical factor in the establishment of association with the bacterial membrane. This conclusion was based primarily on the finding that treatment of the polysomes with pronase reduced their binding capacity.

Cundliffe (1970), using the 'Sarkosyl M band' technique of Tremblay *et al.* (1969), isolated membrane fractions from *B. megaterium* containing attached polyribosomal material. The selective retention of 50S ribosomal subunits in the M bands obtained from protoplasts incubated previously with actinomycin D suggests that the bacterial cell membrane may selectively bind the larger ribosomal subunit. Similarly, Moore and Umbreit (1965) presented evidence that in *S. faecalis*, the ribosomes appear to be bound to membranes through the 50S subunit. These findings are in direct agreement with the observations in eucaryotic cells for the binding of ribosomes to membranes through the large subunit (Sabatini *et al.*, 1966; Attardi *et al.*, 1969; Rosbash and Penman, 1971; Baglioni *et al.*, 1971; Rolleston, 1972).

A possible role for specific binding proteins in the bacterial ribosome-membrane attachment process has

been suggested from the studies of Brown and Abrams (1970) and Scheinbuks *et al*. (1972) who found that the membrane and cytoplasmic ribosomes isolated from *S. faecalis* and *Azotobacter vinelandii*, respectively, were not completely identical with respect to their protein components. Brown and Abrams (1970) showed that an extra protein existed in the cytoplasmic 50S subunit whereas Scheinbuks *et al*. (1972) reported the presence of an additional protein associated with the membrane-bound ribosomes.

Despite the volume of evidence on bacterial membrane-bound ribosomes, it is perhaps true to say that the subject is not in a very satisfactory state. It is difficult to assess the significance of much of the work because of the possibility of much of the reported associations being fortuitous. Moreover, it is still not possible to confidently formulate any rational role for the reported membrane-bound ribosomes, although clearly this does not exclude roles such as membrane synthesis and secretion. So far as the author is aware, no one has produced convincing evidence from electron micrographs for the bacterial membrane in sectioned cells having ribosomes attached as in the endoplasmic reticulum. This does not exclude the possibility that several trivial reasons for the failure could exist, but perhaps some degree of caution is warranted until unequivocal proof of biologically meaningful membrane-bound ribosomes in bacteria is produced.

(ii) MODELS FOR PROTEIN SECRETION IN BACTERIA

Despite the fact that there is no evidence for the synthesis of specific proteins by membrane-bound ribosomes in bacteria, an attractive hypothesis is that such complexes are involved in the synthesis of extracellular enzymes.

Lampen (1965) originally proposed a mechanism of extracellular enzyme secretion for penicillinase in which enzyme synthesis was viewed as a facet of membrane formation, possibly taking place on the mesosome during membrane growth. It was suggested that the enzyme synthesised at the mesosome was inserted into and bound to the membrane at its point of growth in the mesosome and that release of the enzyme from the mesosomal invagination was dependent upon this structure becoming the external membrane of the cell. Beaton (1968) implicated the mesosome of *Staphylococcus aureus* in penicillinase secretion when it was found that the mesosome was structurally altered upon release of the enzyme.

Ghosh *et al*. (1968) also found that in response to a penicillinase inducer, cells of *Bacillus licheniformis* synthesised structures composed of tubules and vesicles which were enclosed by an internal invagination of the cell membrane. These structures were morphologically similar to mesosomes and appeared to be the cellular location of the cell-bound penicillinase (Sargent *et al*., 1968).

Subsequent work, however, (Sargent et al., 1969) showed that these structures (previously termed 'secretory apparatus') were not essential for the actual synthesis of penicillinase.

More recent work (Sargent and Lampen, 1970a) has now shown that a culture of B. licheniformis contains at least three active forms of penicillinase; the hydrophilic exoenzyme and two cell-bound lipophilic forms, one apparently in clusters of periplasmic vesicles (this material is readily released from the cell) and the other as a more stable resident in the general plasma membrane. The bound penicillinases can be readily extracted in a state that is clearly different than that of the exoenzyme and can be converted, under certain conditions, to forms resembling the exoenzyme by a variety of physical criteria (Sargent and Lampen, 1970a,b). It is inferred that these cell-bound forms represent conformational variants of the exoenzyme.

These findings have been incorporated into the following model for penicillinase secretion. It is proposed that as the polypeptide chain is synthesised by membrane-bound ribosomes and inserted into the cell membrane at special growing points, a large portion of the new enzyme passes directly through the membrane, assuming the most stable form, at some stage in its passage. The rest of the new polypeptide is incorporated into the two membrane sites as lipophilic forms complementary in structure to adjacent

lipophilic proteins. Although these membrane-bound forms can, under appropriate conditions, be converted to the hydrophilic enzyme and so be released from the membrane, the available evidence indicates that they are not obligatory intermediates in the secretion of exopenicillinase (Lampen, 1972).

In addition, Bettinger and Lampen (1971) have provided evidence that the penicillinase of B. licheniformis is extruded from the membrane in an incompletely folded form. The cell-bound and exocellular penicillinases are resistant to trypsin and chymotrypsin; however, using protoplasts stripped of part of their membrane-bound enzyme, the production of penicillinase sensitive to these proteases was detected. This implied that the proteases most likely acted at the outer surface of the membrane, degrading the emerging polypeptide before it could assume an active conformation. Similar evidence for an incompletely folded membrane form of a secreted proteinase from a strain of Sarcina was presented by Bissell et al. (1971).

In this laboratory, Elliott and associates have carried out studies on bacterial extracellular enzyme secretion with the organism Bacillus amyloliquefaciens which secretes large amounts of α-amylase, protease and ribonuclease into the external medium. It has been established (Coleman and Elliott, 1965; May and Elliott, 1968a) that for all three enzymes, inhibition of protein

synthesis stops secretion instantly. There are only small amounts of extracellular enzymes associated with cell lysates (Coleman and Elliott, 1962; May and Elliott, 1968a) and this might well represent enzyme simply adsorbed. Indeed, Smeaton and Elliott (1967a) presented evidence suggesting that extracellular enzymes have never existed as such inside the cell. When extracellular ribonuclease is produced, an intracellular protein appears at the same time which specifically and completely inhibits the activity of the cell's own extracellular ribonuclease if the two are placed in contact. This inhibitor protein has been isolated and found to combine tightly and immediately with B. amyloliquefaciens ribonuclease. Since the enzyme-inhibitor complex is very stable and can only be separated by drastic conditions (Hartley, 1970), it is hard to imagine that the enzyme could ever have been in contact with the cytoplasm. Whilst it is conceivable that a biological mechanism could exist whereby the complex is pulled apart on secretion, it is very unlikely especially since no inhibitory proteins for α-amylase and protease exist. The enzyme and inhibitor are the products of distinct genes and hence could not therefore represent secreted and non-secreted portions, respectively, of a precursor zymogen.

The hypothesis has therefore been proposed (May and Elliott, 1968a) that B. amyloliquefaciens extracellular enzymes never exist as such inside the cell but rather are

extruded as nascent polypeptide chains to take up their tertiary structure outside the cell membrane. This mechanism would account for the immunity of the cell to the ribonuclease and would be analogous to secretion of enzymes through the pancreatic cell endothelial reticular membrane proposed by Redman (1967). This proposal of a specific membrane site of protein synthesis for extracellular enzymes is supported by the report of Glew and Heath (1971) who have shown that the synthesis of extracellular alkaline phosphatase by Micrococcus sodonensis was more sensitive to certain inhibitors of protein synthesis than was general cell protein formation. Further support for this proposed model of extracellular enzyme secretion in B. amyloliquefaciens will be seen from results to be reported in this thesis.

D. PROPERTIES OF BACTERIAL EXTRACELLULAR ENZYMES

The secretion of specific enzymes into the culture medium by various microorganisms has been known for many years (Pollock, 1962). Most of the enzymes secreted are concerned with the degradation of large molecular weight substances which cannot penetrate the cell. Presumably, the function of extracellular enzymes, (for example, proteases, α-amylases and ribonucleases) is to degrade such external molecules to products small enough to enter the cell and

be utilised. Penicillinase is exceptional in attacking external penicillin which can readily penetrate the cell, but this probably constitutes a protective device to destroy the antibiotic before it reaches the cell.

Most of the organisms known to produce extracellular enzymes are Gram-positive bacteria and fungi and it is noteworthy that exoprotein production is fairly rare in Gram-negative bacteria. Explanations for this observation are based on the fact that Gram-negative bacteria have very complex cell walls containing substantial amounts of lipopolymers and one effect of the latter is likely to be reduced permeability to water soluble macromolecules. However, the convincing evidence of a true exocellular penicillinase in E. coli 0:127 (Tanko et al., 1970) should lead to a reappraisal of the 'permeability barrier' hypothesis in Gram-negative bacteria.

In general, bacterial extracellular enzymes are proteins with a molecular weight not exceeding 80,000. For example, in Bacillus species, most of the well-documented alkaline proteases (for example, subtilisin BPN', Novo and Carlsberg) have molecular weights in the range 27,000 - 30,000, whilst the neutral proteases have molecular weights in the range 40,000 - 45,000. In comparison, most of the α-amylases have molecular weights of about 50,000 whilst the extracellular ribonuclease tends to be a much smaller molecule. In particular, the extracellular ribonuclease,

barnase, produced by B. amyloliquefaciens, is made up of a single polypeptide (110 amino acid residues) of molecular weight 12,380 (Hartley and Barker, 1972).

A curious and striking difference is observed between bacterial and mammalian extracellular enzymes in that the former, with very few exceptions, are comparatively free of any cyst(e)ine in their molecules while the latter are known to possess disulphide bonds (Pollock and Richmond, 1962). For example, the pancreatic extracellular ribonuclease contains two disulphide bridges even though its molecular weight of 13,000 is very nearly the same as that of the B. amyloliquefaciens enzyme which is completely devoid of cyst(e)ine. Pollock and Richmond (1962) suggested that small extracellular enzyme molecules lacking disulphide bonds would be expected to lack rigidity, a feature possibly helpful in penetrating the cell-wall barrier.

Although lacking disulphide bridges, bacterial exoenzymes do not necessarily lack a strong tertiary structure and indeed, a large number require a divalent cation, notably calcium, for activation or stabilisation. It has been suggested that this ion may replace cystine bridges in conferring the correct conformation on the proteins for activity (Pollock, 1962). Many proteases and α-amylases, in particular, have Ca^{2+} associated with their molecules; the B. subtilis protease, such as BPN', and α-amylase require Ca^{2+} for stability, immunity to protease

digestion and also catalytic activity. While native B. subtilis α-amylase is resistant to proteolytic attack and in fact co-exists in the culture medium with protease, it is rapidly digested by protease after removal of bound Ca^{2+} from the α-amylase molecule (Stein and Fischer, 1958). Similarly, Ca^{2+} play an essential role in preventing the autodigestion of the extracellular proteinase of a Sarcina strain (Bissell et al., 1971). Hsiu et al. (1964) proposed that Ca^{2+}, bound inside the α-amylase molecule, formed tight metal-chelate intramolecular cross-links similar in function to disulphide bridges and removal of these ions allowed disorganisation of the conformation of the enzyme. However, the requirement for Ca^{2+} is certainly not a universal property of extracellular enzymes; it seems that neither penicillinases nor most B. subtilis ribonucleases require this ion (Pollock, 1962).

CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

A. MATERIALS

1. Bacterial strain

The organism used in these studies was previously described as an unclassified strain of Bacillus subtilis; however, it has since been identified as Bacillus amyloliquefaciens (Welker and Campbell, 1967).

2. Liquid growth medium

The culture medium contained 34 mM $(NH_4)_2HPO_4$, 1 mM $MgSO_4$, 5 mM KCl, 4.25 mM sodium citrate, 0.125 mM $CaCl_2$, 0.0125 mM $ZnSO_4$, 0.5 mM $FeCl_3$, 0.5% (w/v) Bacto Casamino acids, 0.05% (w/v) Bacto yeast extract, trace metal solution (0.25 ml/l) and 1% (w/v) maltose. The final pH was adjusted to 7.3 with H_3PO_4. The trace metal solution contained 0.5 mg $CoCl_2.6H_20$, 0.5 mg ammonium molybdate, 5.0 mg $MnCl_2.4H_20$ and 0.01 mg $CuSO_4.5H_20$ dissolved in 1 l. of water.

3. Washed-cell suspending medium

The suspending medium was the same as the liquid growth medium but $FeCl_3$ and yeast extract were omitted, thereby limiting cell growth.

4. Tris-HCl buffers

Crystalline tris was obtained from Sigma Chemical Co., as 'Trizma Base, Reagent Grade'. Solutions of tris were adjusted to the required pH value by the addition of HCl.

5. Radioisotopes

All radioactive compounds were obtained from Schwarz/Mann, Orangeburg, New York, except $^{32}PO_4^{3-}$ (carrier-free) which was obtained from the Australian Atomic Energy Commission.

6. Enzymes

(a) Lysozyme was three-times crystallised from egg white and was supplied by the Sigma Chemical Co.

(b) Deoxyribonuclease from bovine pancreas was obtained free of ribonuclease and was supplied by the Sigma Chemical Co.

(c) Ribonuclease A (bovine pancreas, type II-A) was supplied by the Sigma Chemical Co.

7. Reagents for Cell-free Amino Acid Incorporating System

Adenosine triphosphate (ATP; disodium salt), guanosine triphosphate (GTP; disodium salt), pyruvate kinase (rabbit skeletal, type II-A in 2.1 M $(NH_4)_2SO_4$), and poly-uridylic acid (poly-U) were products of Sigma Chemical Co. Phosphoenolpyruvate was prepared as the dipotassium salt (Clark and Kirby, 1966). L-^{12}C-amino acids and 2-mercapto-ethanol were products of Schwarz/Mann.

8. Sucrose

Ribonuclease-free sucrose was purchased from

Schwarz/Mann, Orangeburg, New York.

9. Scintillation Fluid.

Scintillation fluid contained 3 g of 2,5 diphenyl-oxazole (PPO) and 0.3 g of 1,4-bis-[2-(4 methyl-phenyloxazolyl)] benzene (POPOP) per litre of toluene. PPO and POPOP were supplied by the Packard Instrument Co., Melbourne.

10. Anti-microbial Agents.

Actinomycin D was obtained from Merck, Sharp and Dohme; rifampicin from Schwarz/Mann; chloramphenicol from Parke Davis; tetracycline HCl from Lederle Laboratories; streptomycin sulphate from Sigma; kanamycin sulphate from Bristol Laboratories; erythromycin from Abbott Laboratories; puromycin dihydrochloride from Nutritional Biochemicals Corp.; neomycin sulphate,fusidic acid and thiostrepton from Squibb; sodium fusidate from Leo Pharmaceutical Products; pactamycin and lincomycin from Upjohn. Kasugamycin was kindly donated by Professor A.W. Linnane. Cyanoethyl phosphoryl-PANS-phe was kindly synthesised by Dr. R.H. Symons.

11. Enzyme substrates

(a) Yeast RNA of high molecular weight was prepared according to the method of Crestfield et al. (1955) and used in the ribonuclease assays.

(b) Soluble starch for α-amylase assays was supplied by British Drug Houses Ltd. (A.R. grade).

(c) Light, white soluble casein (British Drug Houses Ltd.) was used as substrate in protease assays.

(d) Remazobrilliant Blue/Hide substrate, which was used in an ultrasensitive assay for protease, was prepared according to the method of Rinderknecht et al., (1968.)

12. Double Distilled Water

Double distilled water was used throughout except that liquid growth medium was prepared with mono-distilled water. The second distillation was from an all-glass apparatus.

13. Glassware and General Equipment

To minimise ribonuclease contamination during cell extraction procedures and in the cell-free protein synthesising studies, all glassware and equipment, where necessary, were either sterilised before use or subjected to a washing procedure with 0.2 N NaOH and then several rinses with distilled water.

B. METHODS

1. Preparation of spore suspensions

B. amyloliquefaciens cultures, after growth overnight in liquid growth medium, were centrifuged down and resuspended to the same cell density in sterile growth medium which had been previously diluted 25-fold with sterile

water. The cultures were shaken in this diluted medium for 24 hr.; spores and intact cells were centrifuged down and resuspended in sterile water. To destroy the remaining vegetative cells, the suspension was heated at 80° for 30 min. and after cooling to room temperature, the spores were centrifuged down at 10,000 g for 30 min. under sterile conditions. The spores were washed with sterile water by resuspension and centrifugation and finally resuspended in a suitable volume of sterile water. The suspension was distributed into small sterile bottles and stored at 4° until required.

2. Culture of B. amyloliquefaciens

(a) Incubation conditions for growth of the organism

The organism was grown under sterile conditions in liquid growth medium; 125 ml. of 4% (w/v) autoclaved maltose was added to 375 ml. of autoclaved medium to give 500 ml. of growth medium in a 2 litre flask. The growth medium was inoculated from a platinum-loop from the spore suspension. Cultures were grown at 30° and aerated by shaking in a Gyrotory incubator (Model G25, New Brunswick Scientific Co., Inc.) at 250 cycles per min.

(b) Incubation conditions for washed-cell suspension experiments

In general, B. amyloliquefaciens cells were harvested after 25 hr. of growth (A_{600nm} = 3.6) by centrifuga-

tion at 3,700 g for 5 min. at 30°. The cells were washed twice briefly in the appropriate suspending medium by resuspension and centrifugation and finally resuspended in the appropriate medium to the original cell density. The suspending medium was previously equilibrated at 30° and the entire procedure was carried out as quickly as possible at room temperature. A sample of cell suspension (20 to 40 ml.) was shaken in a 250 ml. conical flask at 30° on a Gyrotory water bath shaker (Model G75, New Brunswick Scientific Co., Inc.) at 250 cycles per min. Samples (1.0 ml.) were withdrawn at appropriate times, centrifuged and the supernatants assayed for extracellular enzyme activity.

3. Cell densities

Cell densities were determined by measurement of the absorbance of the cell suspension at 600 nm in a 1 cm. cuvette using an Hitachi Model 101 Spectrophotometer.

4. Preparation of cell-free extracts from B. amyloliquefaciens

The procedure for the preparation of cell-free extracts from B. amyloliquefaciens was based on that developed by Nirenmberg (1963) for E. coli. All operations unless otherwise stated were carried out at 0-4°.

(a) French pressure cell extract

B. amyloliquefaciens cells were grown at 30°

for 18 - 20 hr. until A_{600nm} reached the range 0.3 - 0.45 (early logarithmic phase of growth). Cell growth was rapidly stopped by addition of cells to an equal volume of frozen, crushed 0.05 M tris-HCl buffer (pH 7.6) containing 10 mM magnesium acetate, 85 mM KCl and 6 mM 2-mercaptoethanol (hereafter referred to as TMK buffer). The chilled cells were harvested by centrifugation (5,000 g for 5 min.), washed three times with ice cold TMK buffer and resuspended in a small volume of TMK buffer prior to passage through a pre-chilled French pressure cell (Aminco, 18,000 psi). The resulting extract was centrifuged at 30,000 g for 30 min. to remove the cell debris. The upper four fifths vol. of this supernatant fraction (S30) was treated, if necessary, with 2 µg/ml. of ribonuclease-free deoxyribonuclease and retained as the source of soluble enzymes and ribosomal material.

The S30 fraction was further fractionated into soluble (S-100) and ribosomal fractions. The ribosomes were collected by one 2 hr. centrifugation at 105,000 g (Beckman 'Spinco', model L), washed once with TMK buffer and recentrifuged. The ribosomal pellet was resuspended in a small volume of TMK buffer and stored under liquid nitrogen. The S100 fraction, represented by the upper two-thirds of the supernatant remaining after centrifugation of the S30 fraction at 105,000 g for 2 hr., was dialysed for 3 hr. against 500 vol. of TMK buffer and then stored under liquid

nitrogen.

(b) Lysis under anaerobic conditions with lysozyme

Cells were harvested and washed as described for the French pressure cell extract. After the final washing, the chilled cells were resuspended in a small volume of TMK buffer and lysozyme (500 - 750 µg/ml.) was added. The cell suspension was flushed with nitrogen and allowed to stand in a tightly stoppered flask (occasionally shaken) at room temperature for 30 - 40 min., after which time lysis was complete. The clear supernatant fraction (S30) obtained after centrifugation of the lysate (30,000 g for 30 min.) could be further fractionated into S-100 and ribosomal fractions as described for the French pressure cell extract.

5. Amino acid Incorporating System for B. amyloliquefaciens cell extracts

The standard reaction mixture for endogenous mRNA-directed protein synthesis was similar to that described by Coleman (1969a) and the components are shown in the table below:

Component	Amount
Tris-HCl buffer (pH 7.6)	12.5 µmoles
Magnesium acetate	2.0 µmoles
KCl	21.25 µmoles
2-mercaptoethanol	1.5 µmoles
ATP	0.5 µmole
GTP	0.015 µmole
Potassium phosphoenolpyruvate (PEP)	1.0 µmole
Pyruvate kinase (PK)	4 µg
^{14}C-phenylalanine (spec.act. 455mCi/m-mole)	0.2 µCi
19 other ^{12}C-amino acids	0.05 µmole (each
Formyl-tetrahydrofolic acid	30 µg
B. amyloliquefaciens soluble (S100) extract (14 mg protein/ml)	0.07 ml
B. amyloliquefaciens ribosomal extract (12.5 mg RNA/ml)	0.08 ml

The reaction mixture is contained in a total volume of 0.25 ml. The above soluble and ribosomal extracts may be replaced with a suitable volume of B. amyloliquefaciens S30 fraction.

Incubations were carried out at 30° for 30 min., unless otherwise stated, after which time no further incorporation occurred. The reactions were stopped by the addition of 10% (w/v) trichloroacetic acid containing 1.0% (w/v) Casamino acids and left at 0° for 30 min. The precipitates were centrifuged and suspended in 1.0 M NaOH (1.5 ml.) for 30 min. at room temperature. After the addition of 6 ml. of 1% Casamino acids in 10% trichloroacetic acid, the tubes were heated at 95° for 30 min., cooled and the protein precipitates were collected on 2.5 cm. Oxoid membrane filters. Each tube was washed repeatedly with 10% trichloroacetic acid containing 1.0% Casamino acids (5 ml.) and the washings transferred to the filter. The filters were then washed with 10 ml. of 1% (v/v) acetic acid, dried and counted by liquid scintillation in a Packard Tri-Carb spectrometer (90% efficiency).

6. Analysis of ribosomal preparations by sucrose density gradient centrifugation

Cell extracts, containing approximately 2 mg RNA/ml, were analysed on linear sucrose density gradients (15 - 35% w/v) in TMK buffer. The volume of the gradients was 11.6 ml. The ribosomal sample (0.5 ml.) was layered on top of the gradient and centrifuged in the SW 41 rotor of a Beckman 'Spinco' L2-65 preparative ultracentrifuge at

38,000 rev./min. for 75 min. at 4°. The gradients were fractionated and the effluent was continuously monitored at 260 nm by an Optica spectrophotometer with flow cell and recorder attachments.

7. Measurement of total protein and RNA synthesis

(a) To measure total cellular protein synthesis, 2.0 ml. of a washed cell suspension were shaken at 30°, either with 0.5 µCi of uniformly labelled ^{14}C-leucine (spec. act. 316 mCi/m-mole) or with ^{14}C-phenylalanine (spec. act. 455 mCi/m-mole). At appropriate times 0.1 ml. samples were withdrawn and pipetted into 3.0 ml. of cold 1% (w/v) Casamino acids in 10% (w/v) trichloroacetic acid and left at 0° for 30 min. The precipitates were centrifuged and suspended in 1 M NaOH (1.5 ml.) for 20 min. at room temperature. After adding 6.0 ml. of 1% Casamino acids in 10% trichloroacetic acid, the tubes were heated at 95° for 30 min., cooled and the contents of each filtered through a 2.5 cm. Oxoid membrane filter. Each tube was washed repeatedly with 1% Casamino acids in 10% trichloroacetic acid (5.0 ml.) and the washings transferred to the filter. The filters were then washed with 10 ml. of 1% (v/v) acetic acid, dried and counted by liquid scintillation.

(b) 2-^{14}C-uracil incorporation into total cellular RNA was measured by shaking 3.0 ml. of a washed-cell suspension at 30° with 0.75 µCi of ^{14}C-uracil (spec. act.

52 mCi/m-mole) in suspending medium supplemented with 4.0 µg of unlabelled uracil/ml. Samples (0.1 ml.) were withdrawn at various times and pipetted into tubes at 0° containing 3.0 ml. of 5% (w/v) trichloroacetic acid, with an excess of unlabelled uracil (1.0 mg/ml.). The resulting preparations were kept at 0° for about 10 min. and then filtered through an Oxoid membrane filter. The filters were washed repeatedly with a total volume of 15 ml. of cold 5% trichloroacetic-acid-uracil mixture and finally with 5.0 ml. of 1% (v/v) acetic acid. After drying, the filters were counted by liquid scintillation.

8. Enzyme assay methods

(a) α-Amylase estimations

This enzyme was assayed in two ways:

(i) Procedure of May and Elliott (1968b).

A stock solution contained 2.3 ml. of 3% (w/v) starch in water, 250 ml. of 0.1 M potassium phosphate buffer (pH 6.2) with 0.025 M NaCl, 200 ml. of water and 1 ml. of 0.1 M $CaCl_2$. The assay mixture contained 4.5 ml. of the stock solution and 0.5 ml. of suitably diluted enzyme sample. The reaction was stopped with 0.9 ml. of 1 M HCl after 30 min. incubation at 37°. To each tube, 0.1 ml. of I_2 reagent (0.3% I_2, 3% (w/v) KI) and 4 ml. of water were added and the absorbance measured at 620 nm.

Control incubations without enzyme were carried out.

A unit of activity is defined as the amount of enzyme which under standard test conditions (30 min. incubation) gives a loss in absorbance at 620 nm of 1.76. The assay gave a linear relationship between $\Delta A^{1\ cm}_{620\ nm}$ and enzyme concentration up to 0.4.

(ii) Modified procedure of May and Elliott (1968b).

The stock solution now contained 10 ml. of 3% (w/v) starch in water and 300 ml. of buffer (0.1 M potassium phosphate buffer (pH 6.2) with 0.025 M NaCl and 0.1 M $CaCl_2$). The assay mixture was incubated and the reaction was stopped as described above. Aliquots (0.5 ml.) were added to 4.5 ml. of water in separate tubes and 0.1 ml. of iodine reagent (as above) was added and the absorbance was measured at 620 nm. Control incubations without enzyme were carried out.

A unit of α-amylase activity is defined as that amount of enzyme which under standard test conditions gives a decrease in $A_{620\ nm}$ of 0.7 and corresponds to the unit of May and Elliott (1968b).

(b) Protease estimations

This enzyme was assayed in three ways:

(i) Protease estimation using casein as substrate (May and Elliott, 1968a)

A stock solution of casein (British Drug Houses Ltd.) was prepared by boiling for 5 min. a solution containing 1.0 g of casein and 100 ml of 0.1 M Sorenson's buffer, pH 7.6 (12.2 g Na_2HPO_4 and 1.82 g KH_2PO_4 per 1 l. of water). The assay mixture contained 1.0 ml. of enzyme suitably diluted with Sorenson's buffer. After the appropriate time of incubation at 35°, the reaction was stopped with 3.0 ml. of a solution containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid. The precipitated protein was chilled at 4° for 60 min., centrifuged, and the absorbance of the supernatant measured at 280 nm. Blanks were run for appropriate samples by adding the trichloroacetic acid reagent before the enzyme. A unit of protease activity is defined as that amount of enzyme which produces an increase in $A_{280\ nm}$ of 0.05 in 40 min. at 35°. The relationship between $\Delta\ ^{1\ cm.}_{A_{280\ nm}}$ and enzyme concentration was linear up to a value of 1.0.

(ii) Protease estimation using casein as substrate (modified procedure of May and Elliott, 1968a)

The stock solution of casein in this case consisted of 4.0 g casein and 100 ml of 0.1 M Sorenson's buffer, pH 7.6. The assay procedure is the same as that above.

The unit of protease activity is defined as that

amount of enzyme which gives an increase in A_{280nm} of 0.1 after 40 min. incubation at 35°, and this corresponds to the original unit of May and Elliott (1968a).

(iii) Protease estimation using Remazobrilliant Blue/ Hide as substrate

Protease activity was estimated using a modification of the method of Rinderknecht et al. (1968). This procedure which depends upon the hydrolysis of an exceedingly sensitive insoluble substrate (hide powder covalently labelled with Remazobrilliant Blue), is approximately 115 times more sensitive than the casein digestion method of May and Elliott (1968a). The assay mixture contained 10 mg of the Remazobrilliant Blue/Hide powder and 1.5 ml. of enzyme suitably diluted in 50 mM tris-HCl buffer (pH 7.8). Assay tubes were incubated at 37° for 40 min. and the reaction was stopped by plunging the tubes into ice water. The tubes were centrifuged and the amount of dye released into the supernatant was measured at 595 nm. A blank containing no enzyme was incubated under identical conditions.

A unit of protease activity is defined as that amount of enzyme which produces an increase in $A_{595\ nm}$ of 5.7 in 40 min. at 37° and corresponds to the casein protease assay unit as described by May and Elliott (1968a). The assay gave a linear relationship between $\Delta\ ^{1\ cm}_{A_{595\ nm}}$ and enzyme concentration up to 1.1.

(c) Ribonuclease estimation

Ribonuclease activity was estimated using the method described by Coleman and Elliott (1965).

A sample (0.5 ml.) of suitably diluted enzyme solution together with 1.0 ml. of 0.25 mM tris-HCl buffer (pH 8.2) containing 0.25 mM EDTA, was equilibrated at 25° after which 1.0 ml. of aqueous 0.8% (w/v) yeast RNA solution was added. After 30 min. incubation the reaction was stopped by the addition of 0.5 ml. of 0.75% uranyl acetate in 25% (w/v) perchloric acid. The mixture was cooled on ice for 15 min. and the precipitate removed by centrifugation at 4°. A portion (0.5 ml.) of the supernatant was diluted in 3.5 ml. of water and the $A^{1\ cm.}_{260nm.}$ measured.

A unit of ribonuclease activity is defined as the amount of enzyme that under standard test conditions produces an increase in $A^{1\ cm.}_{260nm}$ of 1.0. The assay gave a linear relationship between $\Delta\ A^{1\ cm.}_{260nm.}$ and enzyme concentration up to 0.5.

9. Electron microscopy

This was kindly carried out by Miss P.Y. Dyer using a Siemens Elmiskop I microscope operated at 80 Kv with a 50 μ objective aperture.

(a) Preparation of negatively stained specimens

A drop of suspension was placed on a carbon-coated grid for about 20 seconds. Excess fluid was then removed with

a piece of filter paper and several drops of stain (2% aqueous uranyl acetate) were immediately placed on the grid. The fluid from the grid was removed after about 10 seconds with filter paper and the dried grid examined immediately.

(b) Preparation of sectioned specimens

Cells were pelleted by centrifugation and treated for 1 hour in glutaraldehyde (sodium cacodylate buffer). After washing for 30 min. in this buffer, the pellet was fixed in 1% osmium tetroxide for 1 hour and taken to 1% uranyl acetate for 30 min. The fixed material was progressively dehydrated in acetone and finally embedded in 'Araldite'. Thin sections were cut on a LKB microtome and post stained for 3 min. in lead citrate.

10. pH measurements

pH measurements were made with a Radiometer Model 25 pH meter fitted with a scale expander and micro-electrode.

11. Spectrophotometric measurements

Spectrophotometric measurements were made either on a Hitachi Perkin Elmer 139 UV-VIS spectrophotometer or on a Shimadzu Model QV 50 spectrophotometer with a Gilford Model 2443 Rapid Sampler attachment.

12. Sepraphore polyacetate strip electrophoresis

Sepraphore cellulose polyacetate strips (2.5 cm. x 17 cm.) from Gelman Instrument Co., Michigan, were soaked in 0.1 M tris-HCl buffer, pH 8.5, for 15 min. The strips were lightly blotted and after 20 µl. of the appropriate supernatants were applied, they were subjected to electrophoresis in 0.1 M tris-HCl buffer, pH 8.5, for 75 min. at 350 V.

Protease activity on the strips was detected by incubating them in contact with an agar (1.5%)/casein (2%) plate (pH 7.6) for 3 hr. at 37°. The strips were then removed and the plate was stained for 30 sec. with 0.5% Amido Black 10B in 7% acetic acid and washed with 7% acetic acid to remove excess dye. This procedure stained the plate blue, except for the protease digested areas of casein which remained relatively clear.

13. Polyacrylamide gel electrophoresis

Electrophoresis of protease, in glass tubes of 5 mm internal diameter, was carried out at pH 8.5 using the following solutions: 1 part solution A (4.8 g tris, 0.46 ml Temed, water to 100 ml and HCl to pH 8.5), 2 parts solution B (28 g acrylamide, 1.47 ml. ethylene diacrylate and water to 100 ml.), 1 part solution C (4 mg. riboflavin, water to 100 ml.) and 4 parts water. The electrolyte buffer was 0.025 M tris-HCl, pH 8.5, and no stacking gels were used.

Electrophoresis, at a constant current of 5 mA per tube, was performed at 4° for 2.5 hr. with the electrodes reversed.

To detect protease activity, the gels were frozen in solid CO_2, sliced longitudinally and the flat surface was incubated in contact with an agar/casein plate for 3 hr. at 37°. To detect proteins, the gels were fixed in 10% trichloroacetic acid for 30 min. and stained overnight with Coomassie brilliant blue (0.05% in 10% trichloroacetic acid). Destaining was carried out in 50% ethanol.

Standard analytical gels (7% polyacrylamide, Tris/glycine buffer system, pH 8.3) were prepared according to the method of Davis (1964) in glass tubes of 5 mm internal diameter. Electrophoresis, at a constant current of 3 mA per tube, was performed at room temperature for 2 hr. Staining and destaining techniques were the same as those specified for gels run at pH 8.5.

14. Protein determination

Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

15. RNA determination

The RNA contents of cell fractions were determined by a method based on the orcinol reaction (Schneider, 1957).

CHAPTER III

IN VITRO STUDIES ON PROTEIN SYNTHESIS BY CELL EXTRACTS FROM B. AMYLOLIQUEFACIENS

IN VITRO STUDIES ON PROTEIN SYNTHESIS BY CELL EXTRACTS FROM B. AMYLOLIQUEFACIENS

A. INTRODUCTION

The overall plan for elucidating the site and mechanism of extracellular enzyme secretion by B. amyloliquefaciens included the aim of achieving cell-free synthesis of these enzymes. As a first approach to this problem, it seemed desirable to examine the ability of B. amyloliquefaciens cell-free extracts to incorporate radioactive amino acids into protein before attempting the more ambitious synthesis of specific enzymes. Although the E. coli system, first described in 1961 by Niremberg and Matthaei, has been well characterised, much less is known of extracts of Bacillus species. Coleman (1967) had previously described the isolation of an extract from B. amyloliquefaciens which was active in incorporating amino acids into protein by a ribonuclease sensitive, energy dependent process. However, this study gave no indication whether the conditions used were those necessary for optimal cell-free protein synthesis or not. It was therefore deemed necessary to determine the optimal conditions in a systematic way, since without this information, any attempt at extracellular enzyme synthesis would be much

too hit or miss in nature.

In addition, it seemed logical to examine the cytoplasmic membrane as the possible site for extracellular enzyme synthesis. Circumstantial evidence at the time led to the proposal that extracellular enzymes are synthesised on membrane-bound ribosomes so that the polypeptide chains are extruded into or through the membrane to take up their tertiary structure outside the permeability barrier (May and Elliott, 1968a). This model was attractive since it explained the immunity of the cell to protease and ribonuclease and the apparent absence of extracellular enzymes within the cells. The presence of the inhibitor for ribonuclease within the cytoplasm (Smeaton and Elliott, 1967a), as previously mentioned in Chapter I, further suggested that, at least in the case of ribonuclease, the enzyme could have at no time existed in the cytoplasm since the enzyme-inhibitor complex is very stable and can only be separated by drastic conditions (Hartley, 1970). However, despite this, there was no direct evidence for the presence of ribosomes on the membranes or a role for the latter in extracellular enzyme synthesis. It was therefore decided to examine both the cytoplasmic fraction and the membrane fraction for protein synthesising ability.

This chapter describes the characteristics of the cell-free protein synthesising system and the isolation of

purified cytoplasmic membrane fragments from B. amyloliquefaciens together with their ability for protein synthesis.

B. POLYSOME EXTRACTION AND ANALYSIS BY SUCROSE DENSITY GRADIENTS

Since protein synthesis is associated with polyribosomes (Dresden and Hoagland, 1965), a study was first made of the cell treatments which resulted in extracts rich in polysomes. It is known from studies with other organisms that important factors are the use of rapidly cooled exponential cells, gentle cell breakage and avoidance of shear forces (Oppenheim et al., 1968; Scheinbuks et al., 1969). A preliminary check on these factors was made with B. amyloliquefaciens. This was necessary, since for extracellular enzyme synthesis studies, different aged cells have been used and the phenomenon of 'cold shock' in which cells are rendered permeable by sudden cooling (Smeaton and Elliott, 1967b) raised some problems so far as rapid cooling was concerned.

(i) Necessity for rapid cooling of cells

Exponential phase cells of B. amyloliquefaciens were harvested by pouring over frozen, crushed TMK buffer (cells cooled to 0° within 15 seconds) and an S30 extract was obtained using lysozyme under anaerobic conditions for cell lysis (see Chapter II). The sucrose density gradient

profile obtained (Fig. III.1a) shows the presence of polyribosomes. A similar profile is obtained when cells were cooled by swirling the flask in a dry ice/acetone mixture or when cells were first quickly centrifuged and frozen in liquid nitrogen prior to lysis with lysozyme. This latter technique was originally used by Coleman (1967). When cultures are cooled more slowly (for example, culture flask swirled slowly in an ice bath for 10 min. or cells washed twice with washed cell suspension media (30°) before pouring over frozen, crushed TMK buffer), S30 extracts prepared from them do not contain polysomes; a concomitant increase in the number of ribosomal subunits is seen (Fig. III.1c).

(ii) Method of cell breakage

Two methods for disrupting exponential B. amyloliquefaciens cells were compared; French pressure cell extraction and lysozyme lysis. The experimental procedures are described in Chapter II. Fig. III.1d shows that an extract resulting after passage of cells through a French press at 18,000 psi compares favourably with that from the lysozyme lysis technique seen previously (Fig. III.1a).

(iii) Effect of growth phase of cells on ribosomal profile of S30 extracts

A stationary phase culture (27 hr.) of B. amyloliquefaciens was harvested by pouring over frozen, crushed TMK

FIG. III.1

SUCROSE DENSITY GRADIENT PROFILES OF S30 LYSATES FROM *B. AMYLOLIQUEFACIENS* OBTAINED UNDER DIFFERENT CONDITIONS.

The experimental procedures are described in Chapter II.

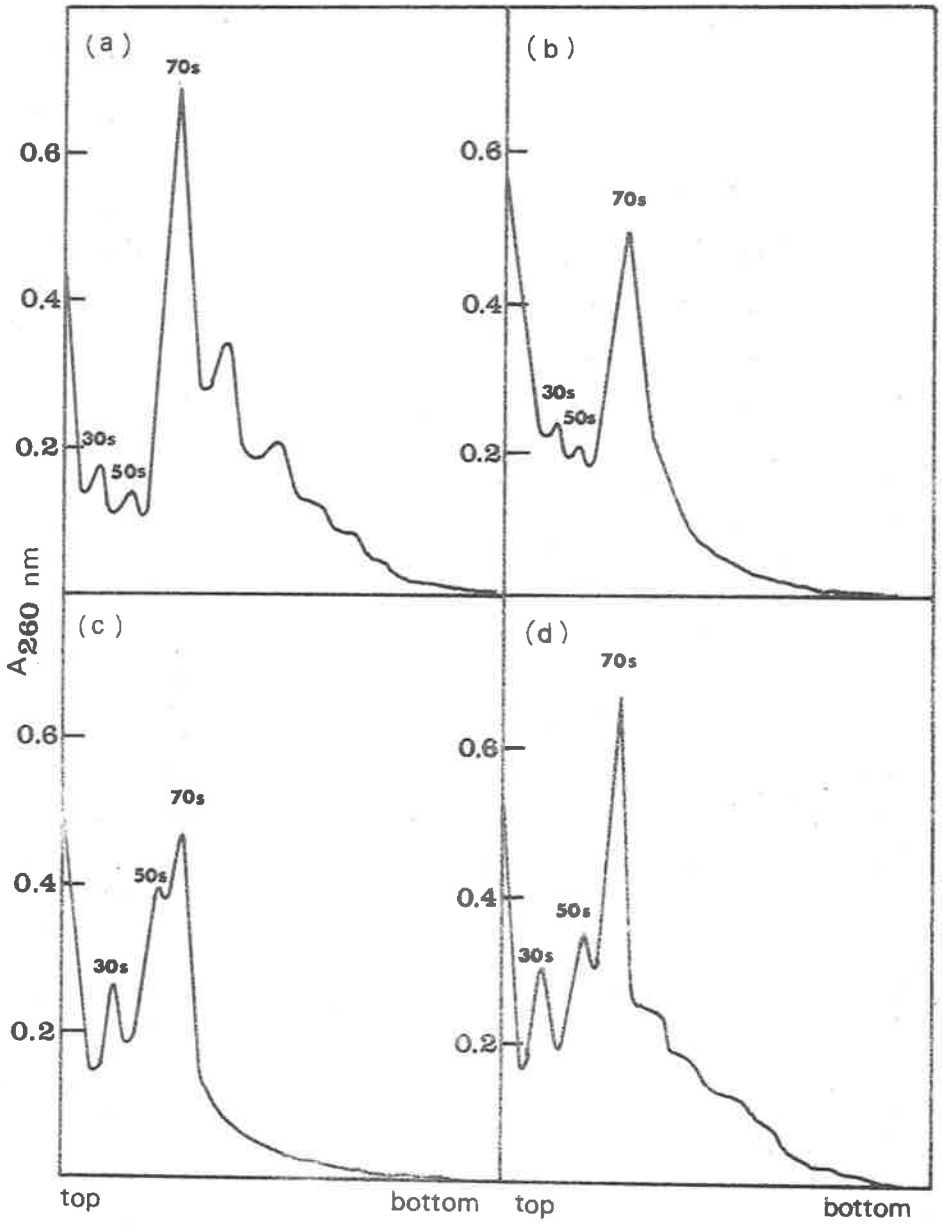
Lysates were obtained from:

(a) an exponentially growing culture which was harvested by pouring over frozen, crushed TMK buffer; the extract was prepared by lysozyme treatment under anaerobic conditions;

(b) a stationary phase culture, harvested and extracted as in (a);

(c) an exponentially growing culture which was cooled slowly; cells were washed twice with washed cell suspension media (30°) prior to pouring over frozen, crushed TMK buffer; the extract was prepared by lysozyme treatment under anaerobic conditions;

(d) an exponentially growing culture which was harvested by pouring over frozen, crushed TMK buffer; the extract was prepared by passage through the French pressure cell at 18,000 psi.


(a)
(b)
(c)
(d)
70s
30s
50s
0.6
0.4
0.2
A260 nm
top
bottom

buffer and an S30 extract was obtained using lysozyme under anaerobic conditions for cell lysis. It can be seen (Fig. III.1b) that polyribosomes are absent from extracts of stationary phase cells.

(iv) Studies on amino acid incorporation by the different preparations

The ability of the above preparations to incorporate ^{14}C-phenylalanine into protein was examined using the system described in Chapter II. The results are shown in Table III.1, where it can be seen that the ^{14}C-phenylalanine incorporating capacity correlates well with the presence of polyribosomes. Hence for future work standard B. amyloliquefaciens cell-free extracts were prepared from exponentially growing cells, poured onto frozen, crushed TMK buffer to instantaneously halt cell metabolism and lysed by either the French press or the lysozyme lysis technique.

C. CHARACTERISTICS OF THE CELL-FREE PROTEIN SYNTHESISING SYSTEM FROM B. AMYLOLIQUEFACIENS

This section deals with the properties of a cell-free amino acid incorporating system from B. amyloliquefaciens. During the course of these studies, Coleman (1969a,b) studied the effects of both K^+ and Mg^{2+} concentration on the amino acid incorporating ability by cell-free preparations of

TABLE III.1. INCORPORATION OF ^{14}C-PHENYLALANINE BY VARIOUS CELL-FREE EXTRACTS OF B. AMYLOLIQUEFACIENS.

Method of preparation			counts/min
Cell growth phase	Cooling procedure	Disruption method	per mg.RNA
exponential	rapid; frozen crushed TMK buffer	lysozyme, anaerobic conditions	4,020
stationary	rapid; frozen crushed TMK buffer	lysozyme, anaerobic conditions	720
exponential	rapid; dry ice/ acetone	lysozyme, anaerobic conditions	3,900
exponential	rapid; cells frozen in liquid nitrogen	lysozyme, anaerobic conditions	3,600
exponential	slow cooling (10 min.)	lysozyme, anaerobic conditions	600
exponential	rapid; frozen crushed TMK buffer	French press	4,110

Details of the complete incubation system are given in Chapter II.

All extracts were S30 preparations adjusted to the same RNA concentration.

Reaction mixtures were incubated at 30° for 30 min.

The sucrose density gradient profiles are shown in Fig. III.1.

gently disrupted cells of this organism. The optimum K^+ and Mg^{2+} levels for these in vitro protein synthesising studies were found to be 70 - 100 mM and 5 - 10 mM, respectively. Since similar concentrations have also been reported to be optimal in E. coli (Takeda, 1969), K^+ (85 mM) and Mg^{2+} (8 mM) were the concentrations chosen in the following studies.

(i) General properties of the incorporating system

Ribosomes and a soluble fraction were prepared from cells harvested in the early logarithmic phase of cell growth as described in Chapter II and used in the cell-free system. The requirements of the cell-free amino acid incorporating system are summarised in Table III.2. The incorporating system is seen to be dependent on the presence of ribosomes, soluble fraction and an ATP regenerating system. When GTP, N-formyl tetrahydrofolate or amino acids are omitted, the system is affected only slightly. Ribonuclease completely inhibits incorporation while deoxyribonuclease has no effect. The system is inhibited approximately 70% by chloramphenicol (300 µg/ml) and 95% by puromycin (100 µg/ml). In addition, it is of interest to note that in repeated experiments, the addition of polyuridylic acid (20 - 60 µg) increases the incorporation of phenylalanine in this system by only 5 - 10%.

The time course of ^{14}C-phenylalanine incorporation by

TABLE III.2. PROPERTIES OF THE ENDOGENOUS mRNA-DIRECTED CELL-FREE PROTEIN SYNTHESIS SYSTEM FROM B. AMYLOLIQUEFACIENS

Conditions	^{14}C-phenylalanine incorporation	
	counts/min.	% of complete system
Complete	5,840	[100]
- GTP	4,890	84
- ATP and ATP regeneration	750	13
- Amino acids	4,620	79
- Ribosomes	60	1
- Soluble fraction (S100)	1,110	19
- N. formyl-tetrahydrofolate	5,380	92
+ Ribonuclease (20 μg)	80	1
+ Deoxyribonuclease (20 μg)	5,980	102
+ Chloramphenicol (75 μg)	1,780	31
+ Puromycin (25 μg)	270	5
+ Polyuridylic acid (60 μg)	6,130	105

Details of the complete incubation system are given in Chapter II. The French pressure cell was used for cell disruption. Reaction mixtures were incubated at 30° for 30 min.

cell extracts obtained both by disruption through the French press and by lysozyme treatment are shown in Fig. III.2. It can be seen that extracts isolated by both methods behave similarly and resemble the incorporation pattern reported for other mesophilic and thermophilic strains of Bacillus (Algranati and Lengyel, 1966; Imsande, 1966; Takeda and Lipmann, 1966; Stenesh and Schechter, 1969). In the complete system, approximately 83% of the final level of incorporation is achieved during the first 15 min. with no further incorporation after 30 min. of incubation.

(ii) Effect of ribosome and soluble protein concentration on ^{14}C-phenylalanine incorporation

When the protein concentration of the soluble fraction was held constant in the standard incubation system, the incorporation of ^{14}C-phenylalanine into polypeptides was found to be proportional to the amount of ribosomes added, up to the final tested concentration (Fig. III.3). In order to express results directly as counts/min./mg RNA, the concentration of ribosomes used in all subsequent studies was 1 mg RNA per incubation system. Fig. III.4 shows the effect of protein concentration of the soluble fraction on incorporation; maximal incorporation of ^{14}C-phenylalanine was obtained with 420 µg protein, the amount falling with further addition.

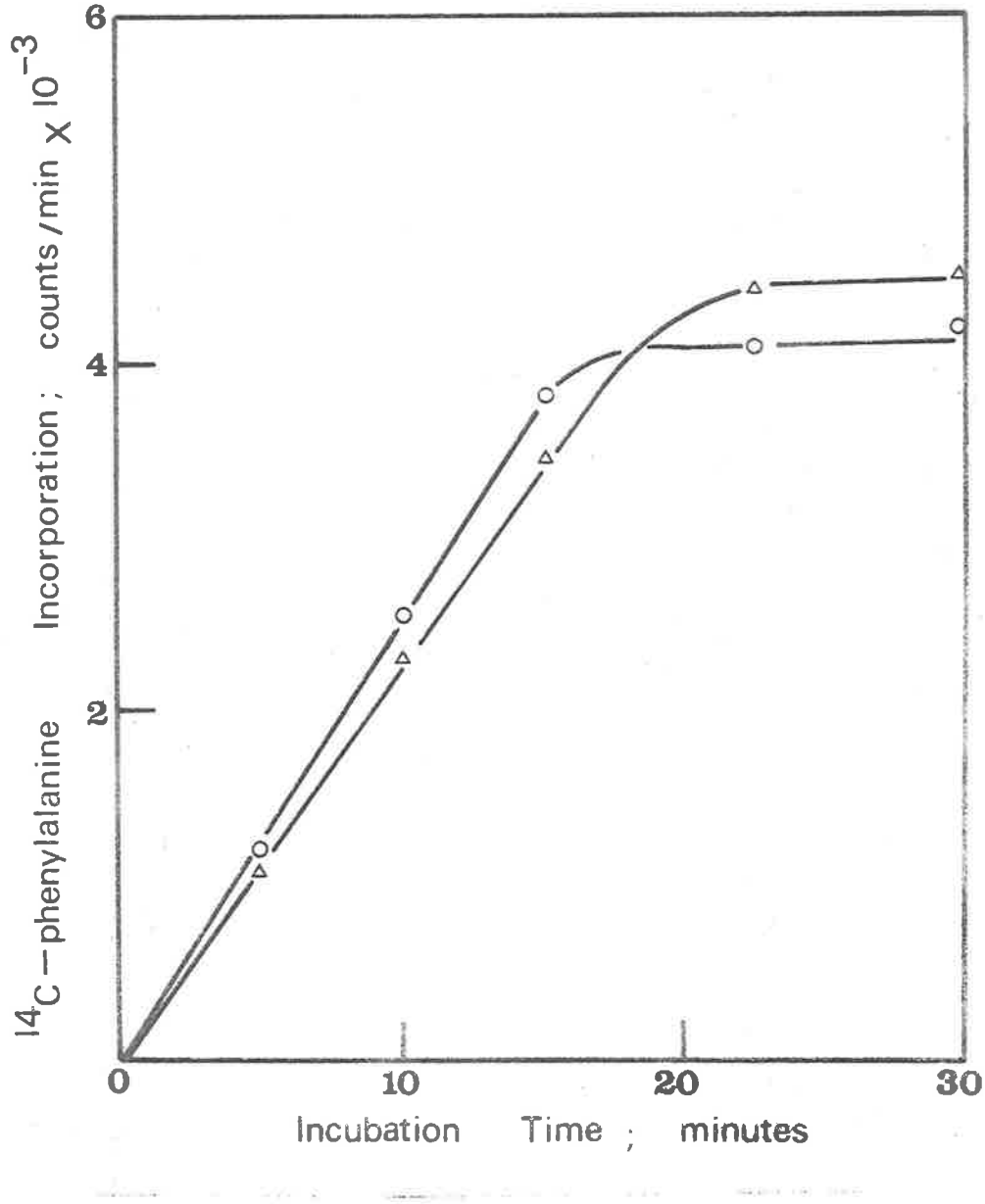


FIG. III.2 COMPARISON OF ^{14}C-PHENYLALANINE INCORPORATION BY CELL EXTRACTS ISOLATED UNDER DIFFERENT CONDITIONS.

—Δ—Δ— , extract prepared by disruption through the French pressure cell

—O—O— , extract prepared by lysozyme treatment under anaerobic conditions.

Measurements of ^{14}C-phenylalanine incorporation were carried out a described in Chapter II.

The reaction mixtures, containing ribosome (1 mg RNA) and soluble (560 µg protein) fractions, were incubated at 30° for 30 min.

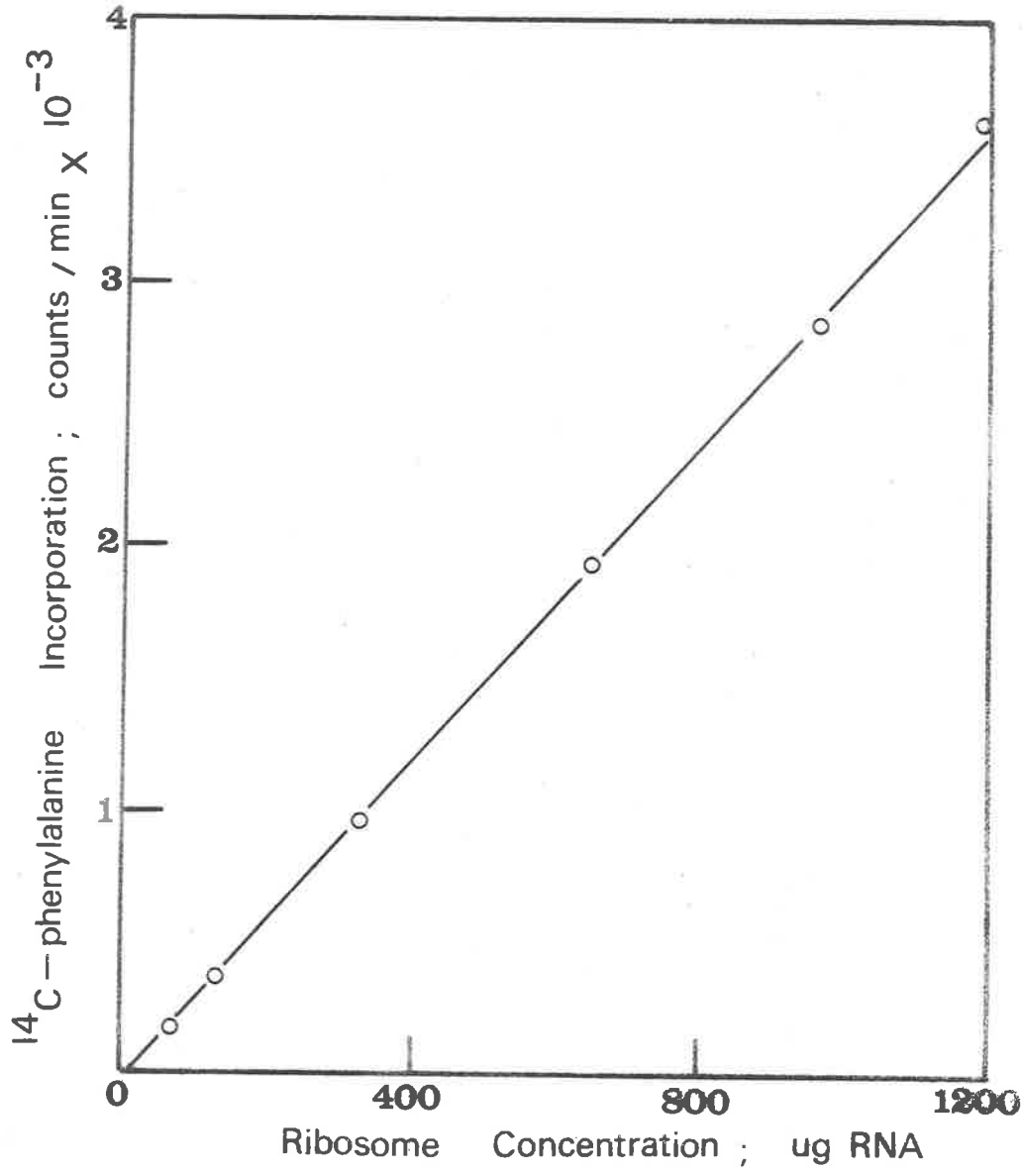


FIG. III.3. EFFECT OF RIBOSOME CONCENTRATION ON ^{14}C-PHENYLALANINE INCORPORATION.

Measurements of ^{14}C-phenylalanine incorporation were carried ou as described in Chapter II. The reaction mixtures, containing soluble fraction (560 µg protein), were incubated at 30° for 30 min. Logarithmic phase cells were disrupted in the French pressure cell.

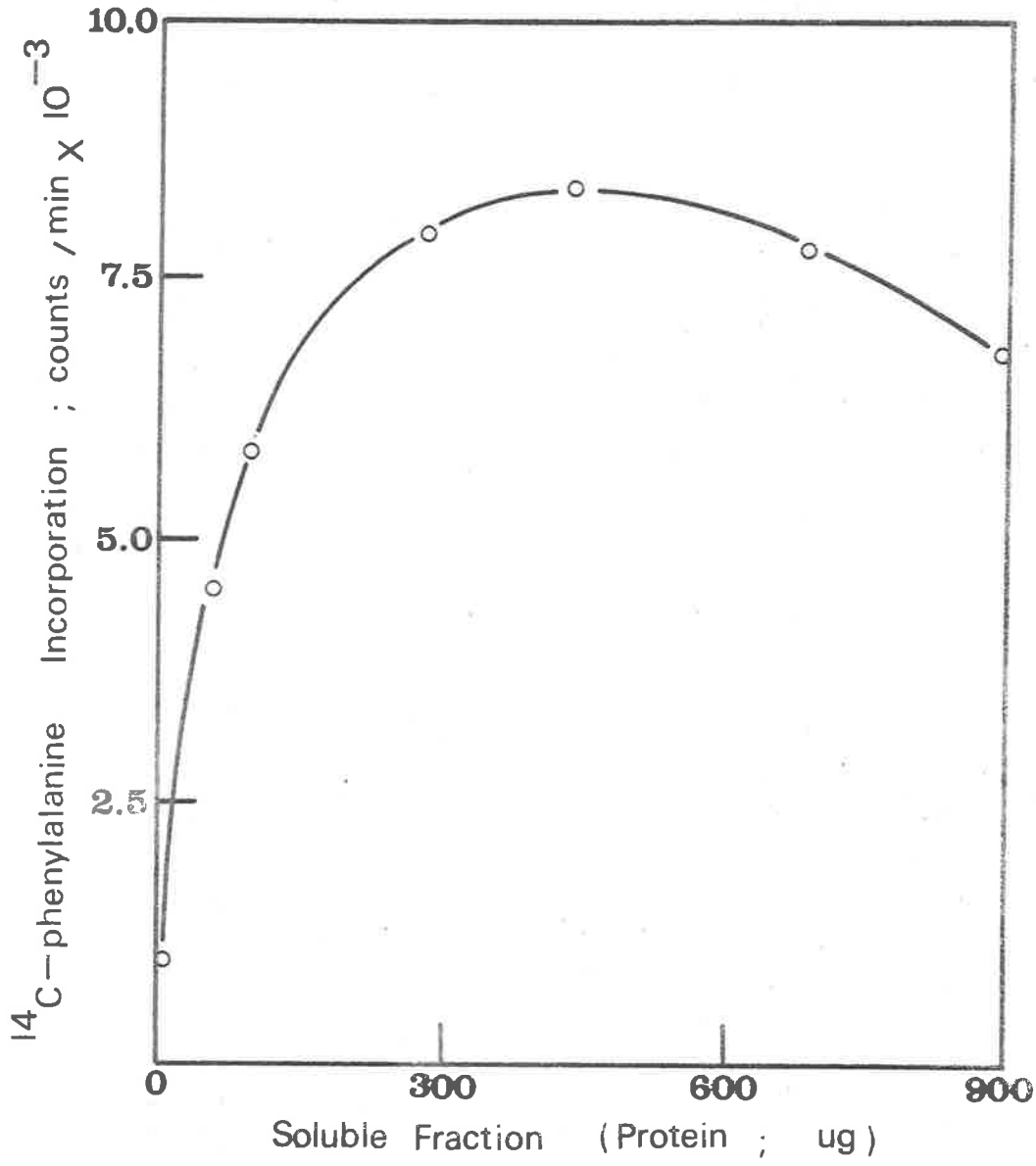


FIG. III.4. EFFECT OF PROTEIN CONCENTRATION OF THE SOLUBLE FRACTION ON ^{14}C-PHENYLALANINE INCORPORATION.

Measurements of ^{14}C-phenylalanine incorporation were carried out as described in Chapter II.

The reaction mixtures, containing ribosome fraction (1 mg RNA), were incubated at 30° for 30 min.

(iii) Cell-free studies on ^{14}C-phenylalanine incorporation with extracts from different aged cells

Studies already described in this chapter showed that logarithmic phase cells are necessary to obtain S30 extracts containing polyribosomes (Fig. III.1) and to incorporate ^{14}C-phenylalanine (Table III.1). As an additional check on this, the effect of different cell ages on the incorporating ability of derived cell-free extracts was examined more closely. Fractions were isolated from cells harvested after growth for 19 hr. (early logarithmic), 24 hr. (late logarithmic) and 29 hr. (stationary) respectively and their ability to incorporate ^{14}C-phenylalanine into protein was examined. Fig. III.5 shows that cell extracts from the late logarithmic and stationary phase cells possessed only 13% and 6% respectively of the endogenous activity of logarithmic phase cells.

In order to determine which component had become inactive or deficient in the older cell extracts, ribosome and soluble fractions were exchanged between the different aged cultures. It is apparent from the data (Table III.3) that the defect lies in both fractions; for each of the different aged soluble fractions, the incorporating ability by supplemented ribosomes falls with increasing ribosomal age, whilst ribosomes from each of the three stages of growth show a decreased incorporating ability with increasing age of the supplemented soluble fraction.

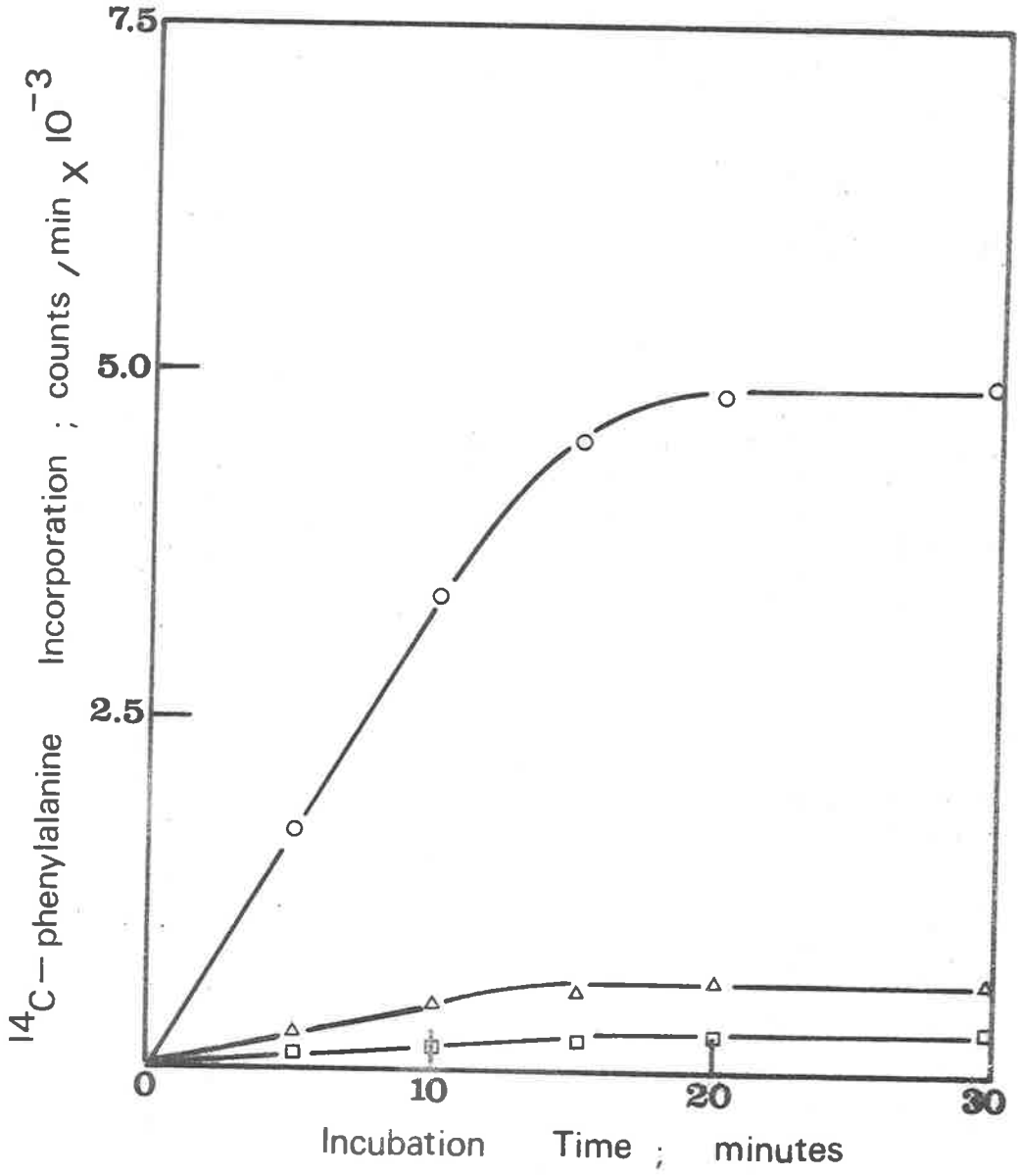


FIG. III.5. COMPARISON OF ^{14}C-PHENYLALANINE INCORPORATION BY EXTRACTS FROM DIFFERENT AGED CELLS.

—O—O— , early logarithmic
—Δ—Δ— , late logarithmic
—□—□— , stationary phase

Prior to incubation, the different cell extracts (prepared using the French pressure cell) were adjusted to identical ribosome ar soluble protein concentrations by the addition of TMK buffer. Measurements of ^{14}C-phenylalanine incorporation were carried out as described in Chapter II.
The reaction mixtures, containing ribosome (1 mg RNA) and soluble (420 µg protein) fractions, were incubated at 30°.

TABLE III.3. ^{14}C-PHENYLALANINE INCORPORATION BY HOMOLOGOUS AND MIXED FRACTIONS FROM DIFFERENT AGED CULTURES OF B. AMYLOLIQUEFACIENS

Ribosomes	Soluble fraction	^{14}C-phenylalanine incorporation	
		counts/min.	%
early logarithmic	early logarithmic	4,900	[100]
	late logarithmic	1,020	21
	stationary	590	11
late logarithmic	early logarithmic	2,240	46
	late logarithmic	650	13
	stationary	440	9
stationary	early logarithmic	1,050	21
	late logarithmic	350	7
	stationary	295	6

Prior to incubation, the different cell extracts were adjusted to identical ribosome and soluble protein concentrations by the addition of TMK buffer.

Measurements of ^{14}C-phenylalanine incorporation were carried out as described in Chapter II.

The reaction mixtures, containing ribosome (1 mg RNA) and soluble (420 µg protein) fractions, were incubated at 30° for 30 min.

It was important to establish whether the above effect was an intrinsic property of B. amyloliquefaciens cells or the result of the preparative procedure per se. Since this organism produces large amounts of protease and ribonuclease during the later stages of growth, the possibility existed that one or both of these exoenzymes in the older cell extracts could be responsible for their reduced activity. To test this, an equal volume of stationary phase supernatant from 28 hr. cells was added to exponentially growing cells (19 hr.) and shaken at 30° for 5 min. prior to harvesting in the normal manner by pouring over frozen, crushed TMK buffer. Ribosomes and a soluble fraction were then prepared in the standard way and their ^{14}C-phenylalanine incorporating ability was compared with that from control extracts prepared from exponential phase cells. Fig. III.6 shows that the incorporation was lowered 87% by the presence of the stationary phase supernatant during the extraction procedure. In a subsequent experiment carried out in the same way, it was found that the deleterious effect of culture supernatant was destroyed by heating for 15 min. at 100° (Fig. III.6).

The possibility therefore existed that the heat labile extracellular protease or ribonuclease plays a major role in determining the activity of cellular extracts. To test whether extracellular ribonuclease was involved, the specific inhibitor to this enzyme was isolated according

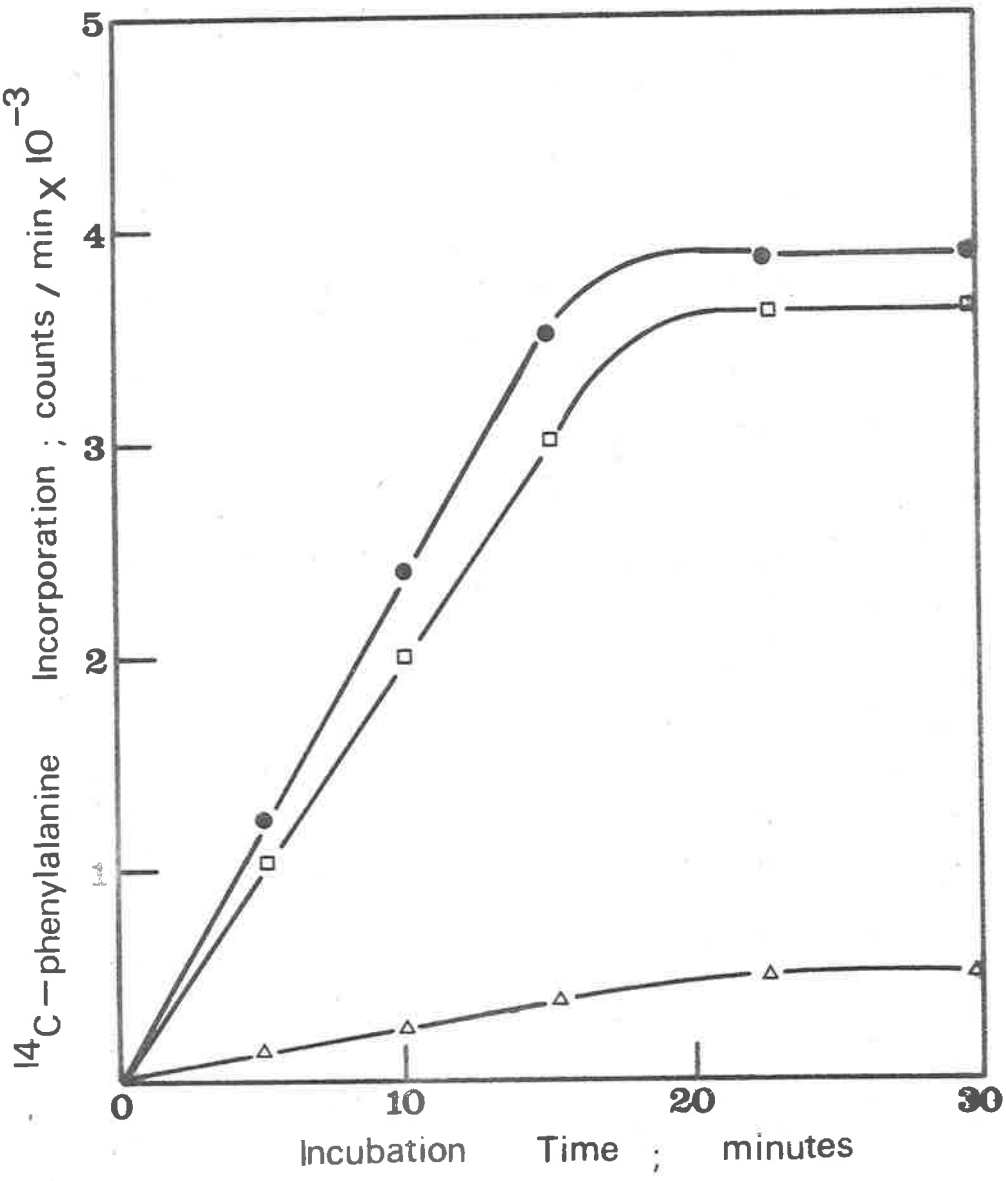


FIG. III.6. INFLUENCE ON ^{14}C-PHENYLALANINE INCORPORATION BY THE ADDITION OF STATIONARY PHASE SUPERNATANT TO EXPONENTIAL PHASE CELLS PRIOR TO CELL HARVESTING.

—●—●— , exponential phase cells (control)

—△—△— , exponential phase cells + stationary phase supernatant

—□—□— , exponential phase cells + stationary phase supernatant heated at 100° for 15 min.

Measurements of ^{14}C-phenylalanine incorporation were carried out as described in Chapter II.

The reaction mixtures, containing ribosome (1 mg RNA) and soluble (420 µg protein) fractions, were incubated at 30°.

to the method of Smeaton and Elliott (1967b) and an excess was added to a cell culture in the late logarithmic phase prior to harvesting. The finding that no significant difference occurred in the *in vitro* protein synthesising abilities of the resulting cell extracts (compared with a control culture) suggests that ribonuclease does not play a major role.

On the other hand, limited evidence does suggest a role for protease; studies with a *B. amyloliquefaciens* mutant, low producing in extracellular protease, yielded cell extracts from late logarithmic cells with much greater phenylalanine incorporating activity than control extracts prepared from cells harvested in the same physiological state. The use of a *B. subtilis* mutant, deficient in protease, to yield more active cell extracts has been previously reported (Takeda and Lipmann, 1966).

D. INVESTIGATION OF THE PROTEIN SYNTHETIC ACTIVITY OF CYTOPLASMIC MEMBRANE FRAGMENTS

This section deals with a study of the protein synthetic activity of membrane fragments from *B. amyloliquefaciens* using the cell-free system described previously.

In studies with bacterial cell lysates, the attachment to the cell membrane of ribosomal material which

cannot be removed after repeated washing, appears to be a function of a variety of factors (see Chapter I). One such important factor is the ionic environment. Coleman (1969c) studied the distribution of ribosomes between the soluble and membrane fractions of preparations of exponential phase cells of B. amyloliquefaciens, lysed with lysozyme, and showed this distribution was strongly influenced by the K^+ concentration in the medium in which cell lysis occurred. In a tris buffer (pH 7.6) containing 10 mM Mg^{2+}, 37% of the ribosomes were found to be membrane-bound in the absence of K^+, but this amount decreased on increasing the K^+ concentration to 100 mM, when less than 10% of the ribosomes remained attached. This author suggested that in the absence of a precise knowledge of the intracellular environment, the best procedure is to isolate membranes under conditions known to preserve maximal cell-free protein synthesis.

In the following studies, membrane fragments of B. amyloliquefaciens were prepared in the presence of TMK buffer containing 85 mM K^+, the optimal concentration for protein synthesis as determined by Coleman (1969a). The purity of membranes isolated by different procedures was followed by electron microscopy using negative staining (see Chapter II).

(i) Isolation of membrane fragments

To isolate membrane fragments, several authors

(for example, Schlessinger, 1963; Coleman, 1969c,d; 1970) have used lysozyme lysis of cells in the absence of sucrose. In this laboratory, however, this procedure was found to be unsatisfactory for membrane preparations for B. amyloliquefaciens; such preparations contained few membrane ghosts but consisted mainly of cell wall debris and unlysed cells.

It was clear from preliminary work that the most satisfactory approach for preparing 'clean' membrane fragments was to use the technique of gentle lysing protoplasts (Salton, 1967). To do so had an element of illogicality since it was known that protoplasts fail to produce extracellular enzymes rapidly but do synthesise intracellular proteins (May and Elliott, 1968b). However, it was considered possible that this failure might not be due to a failure to produce the polypeptide chain of extracellular enzymes but to some secondary effect needed to 'activate' the enzyme. Despite the uncertainty of this, it was considered that basic information on the membrane of B. amyloliquefaciens and its properties so far as protein synthesis was concerned were important and since the protoplast route offered the best approach, it was adopted.

Protoplasts were first prepared as follows: cells from 150 mL of a B. amyloliquefaciens culture (near end of exponential growth) were washed twice in TMK buffer, and resuspended in 60 mL of this buffer made 0.6 M with respect

to sucrose. Lysozyme (18 mg) was added and the suspension incubated at 30° as a thin layer in a 2 litre flask with gentle shaking. Complete conversion of cells to protoplasts occurred in 50 - 60 min. (For complete formation of protoplasts, the amount of lysozyme added was critical otherwise gross cell wall contamination was observed in the resulting membrane preparation; excessive amounts were as deleterious as too little.) The protoplasts were centrifuged at 20° for 10 min. at 6,000 g and the pellet gently suspended in 20 ml. of TMK buffer (without sucrose). This suspension was pipetted into 100 ml. of ice-cold buffer and left for 30 min. The resulting lysate was centrifuged at 20,000 g for 15 min. and the pellet examined for membrane fragments by electron microscopy. The preparation contained some membrane pieces and whole protoplasts but consisted mainly of collapsed protoplast ghosts.

It was felt that such ghosts were not the ideal preparation since it was likely that on collapse of the protoplast, cytoplasmic ribosomes would be trapped inside and complicate the examination for membrane-bound ribosomes. Therefore, membrane fragments were prepared from the ghosts by passage of the lysate through a French pressure cell operated at a very low pressure (1300 psi). The resulting membrane fragments were collected by centrifugation for 10 min. at 20,000 g, washed once with TMK buffer and resuspended in 4 ml. of this buffer. A final centrifugation

at 500 g for 1 min. sedimented any large aggregates which were then discarded.

Electron microscopy of this preparation revealed large membrane fragments with the only visible contamination being free ribosomes (Fig. III.7). At least five separate washes were required to completely remove material absorbing at 260 nm and electron microscopy confirmed that at this stage the preparation was almost entirely free of unattached ribosomes. This washing procedure was tedious, and 'clean' membrane fragments were more readily obtained by resuspending once washed membranes in 2 ml. of TMK buffer and centrifuging the suspension at 25,000 g for 30 min. through 25 ml. of TMK buffer containing 1.0 M sucrose. The supernatant, containing ribosomes, was removed and the membrane pellet was suspended in TMK buffer.

Electron microscopy confirmed the almost complete absence of free background ribosomes (Fig. III.8) and this procedure was therefore routinely adopted for subsequent membrane preparations. (Separate experiments showed that fragmentation of the ghosts by sonication rather than passage through the French pressure cell was unsatisfactory since it was not easily controlled to give fragments of a consistent size. Also, it was not feasible to prepare large membrane fragments by passage of protoplasts, rather than ghosts, through the French pressure cell even at low pressures, since the resulting fragments were extremely small and vesicular

FIG. III.7.

NEGATIVELY STAINED PREPARATION OF ONCE WASHED CYTOPLASMIC MEMBRANE FRAGMENTS.

Many free ribosomes (r) can be seen. The membranes are covered with small particles, some of which can be seen to be coming away from the membrane surface (p).

The preparation was stained with uranyl acetate as described in Chapter II.

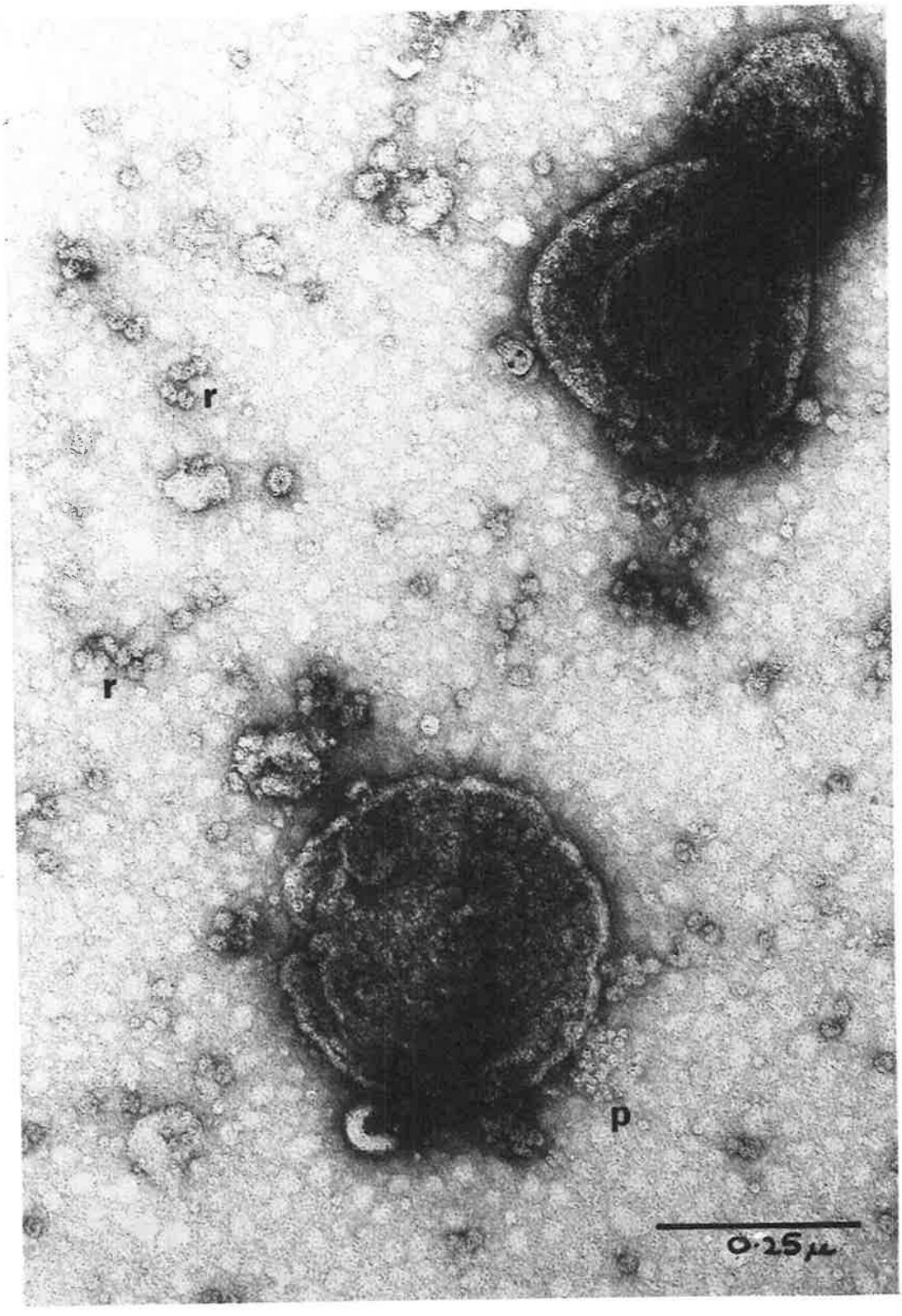
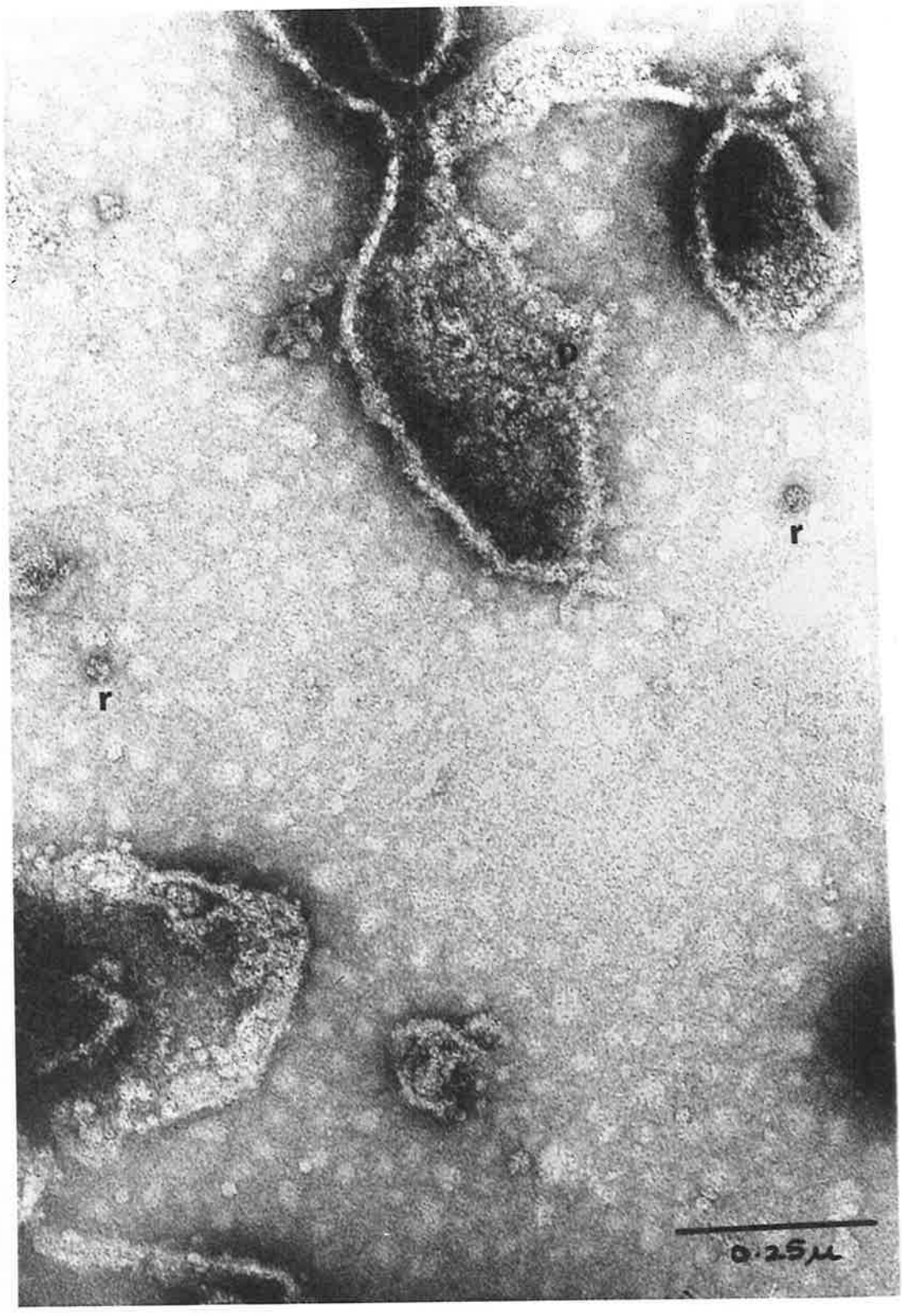

r
r
p
0·25 μ

FIG. III.8.

NEGATIVELY STAINED PREPARATION OF CYTOPLASMIC MEMBRANE FRAGMENTS SPUN THROUGH 1.0 M SUCROSE.

The membranes are covered with small particles, some of which are free (p). An occasional free ribosome (r) can be seen.

The preparation was stained with uranyl acetate as described in Chapter II.


r
r
0.25μ

in nature).

From the electron micrographs (Fig. III.7 and 8), small particles (diameter 110 - 140 Å), were seen to cover the membrane surface; (by comparison, the average diameter of ribosomes are 270 Å). These particles most probably represent adenosine triphosphatase as discussed later. However, despite the absence of visible membrane-bound ribosomes in the electron micrographs, the membrane fragments are capable of incorporating amino acids into protein (see below). Hence, preliminary experiments were carried out to confirm the presence of ribosomes associated with the membrane.

(ii) Association of RNA with membrane fragments

Two separate observations suggested that ribosomes were associated with the membrane fragments. The first observation involved the addition to the membrane sample of a 'protoplast-bursting factor'. This factor, a peptide-lipid, is isolated from the culture supernatant of B. amyloliquefaciens and is capable of rapidly lysing protoplasts and membranes (May and Elliott, 1970). The structure of this factor appears to be identical to that for 'Surfactin', isolated from B. subtilis supernatant by Arima et al., 1968 (Gould et al., 1971). When this factor was added to a purified membrane preparation showing no background of free ribosomes in the electron micrographs,

subsequent examination by electron microscopy revealed that the membrane was disrupted to give small vesicular pieces together with a significant number of unattached ribosomes. A similar release of ribosomes was observed after thawing out a membrane preparation which had been frozen overnight, although in this case, no apparent disruption of the membrane fragments had occurred.

In addition, determinations on these membrane preparations by the orcinol method (Schneider, 1957), indicated small amounts of RNA to be present. A more direct approach to this problem was then used in which RNA was extracted from membrane fragments from cells previously labelled with $^{32}PO_4^{3-}$ and analysed by sucrose density gradient analysis.

The procedure was as follows: 4 ml. of a logarithmic phase B. amyloliquefaciens culture was added to 200 ml. of growth medium containing 3.4 mM $(NH_4)_2HPO_4$. After the addition of $^{32}PO_4^{3-}$ (2.5 μCi/ml.), the cells were vigorously shaken at 30° for 8 hr. (A preliminary experiment had established that under these conditions, cells actively incorporated $^{32}PO_4^{3-}$ into trichloroacetic acid precipitable material.) The cells were spun down, washed twice with TMK buffer and membrane fragments were prepared as described above. Electron microscopy confirmed the presence of membrane fragments devoid of free ribosomes.

The membranes were then subjected to a RNA

extraction process based on the method of Loening (1967). Membranes in 0.8 ml TMK buffer, together with about 1 mg crude B. amyloliquefaciens RNA as a marker, were added to 25 ml. glycine-NaOH buffer, pH 9.5 containing 0.5% sodium dodecyl sulphate. (The crude RNA was prepared in a similar fashion to that described here.) The mixture was phenol extracted for 10 min. with 25 ml. of phenol containing 8% m-cresol, 1% tri-isopropyl naphthalene sulphonate and 0.1% 8-hydroxyquinoline. After phase separation by centrifugation, the phenol extraction was repeated twice using decreasing volumes (15 ml. and 10 ml.) of the phenol mixture. After three ether extractions of the aqueous phase, the RNA was precipitated by the addition of 2% sodium acetate and 2 vol. of ethanol at -20°. The RNA was then dissolved in a small amount of TMK buffer to give a final concentration of about 1 mg/ml.

A sample (0.3 ml.) was analysed on a linear sucrose density gradient (5 - 20% w/v) in TMK buffer by centrifugation in the SW41 rotor at 41,000 rev. per min. for 11 hr. at 4°. The gradient, collected from the top of the tube, was continuously monitored at 260 nm and collected as 15 drop fractions into 3.0 ml. of cold 5% (w/v) trichloroacetic acid containing 0.5% w/v $(NH_4)_2HPO_4$. After standing at 0° for 30 min., the precipitates were collected by Oxoid membrane filtration and washed repeatedly with a total volume of 15 ml. cold 5% trichloroacetic acid - 0.5% $(NH_4)_2HPO_4$

mixture and finally with 5 ml. of 1% (v/v) acetic acid. After drying, the filters were counted by liquid scintillation. The results are shown in Fig. III.9 where peaks of radioactivity are seen to correspond with the A_{260nm} profile of the B. amyloliquefaciens 4S - 5S, 16S and 23S RNA species. The positions of these species had been previously determined by suitable control studies in conjunction with G. Both. (The profiles also suggest that some RNA breakdown has occurred during the extraction procedure; the 23S rRNA peak is diminished in size compared with the 16S rRNA peak and a corresponding shoulder is noticeable alongside the 16S rRNA species.)

Although this initial finding suggests that both 30S and 50S ribosomal subunits are associated with the membrane fragments, the results should be treated with caution since these subunits may well be cytoplasmic ones which have adsorbed to the membrane during the isolation procedure. This possibility could be readily tested by the addition of radioactively labelled subunits to an unlabelled protoplast preparation, from which membranes are then prepared. A direct examination for the presence of ribosomal subunits (rather than rRNA) in these membrane fragments would also seem to be a better approach in future studies. This study was carried out only recently and there has been insufficient time to do this, though it is planned.

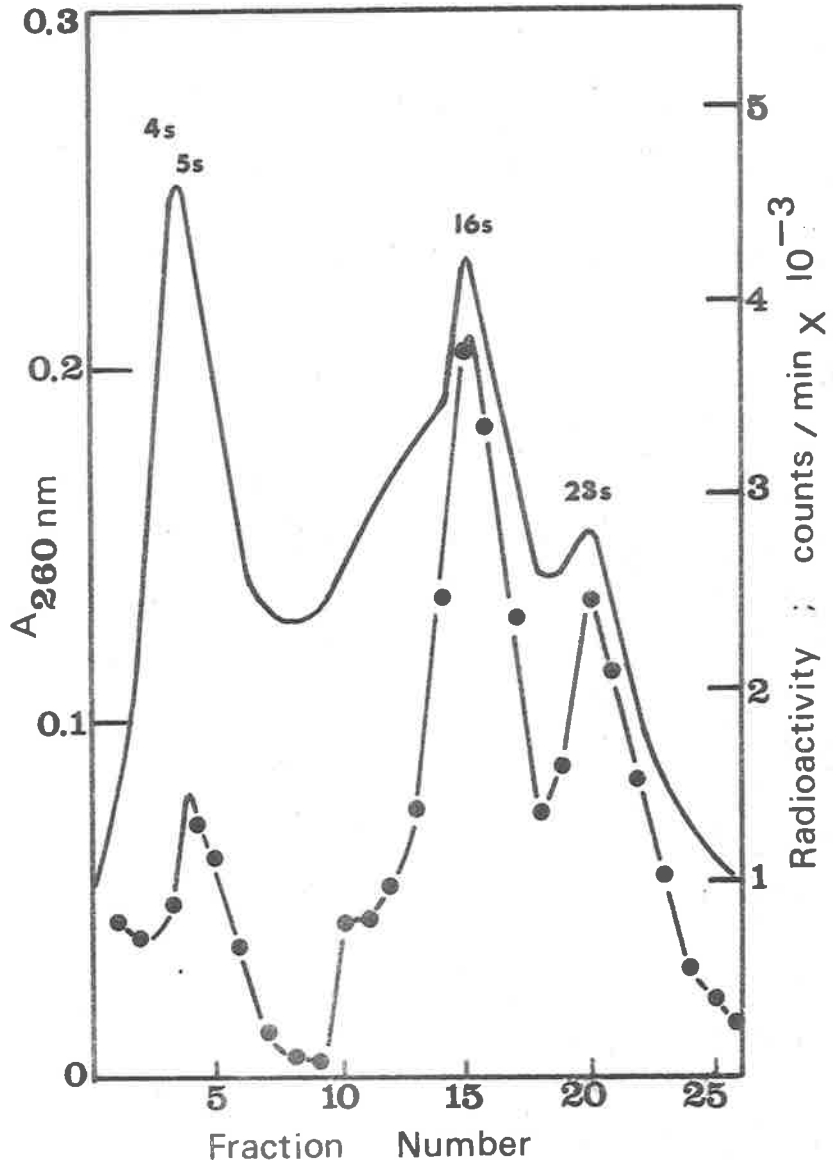


FIG. III.9. SUCROSE DENSITY GRADIENT ANALYSIS OF RNA EXTRACTED FROM B. AMYLOLIQUEFACIENS MEMBRANE FRAGMENTS.

Experimental details for the preparation of the cell extract are described in the text. A sample (0.3 ml) was loaded onto a 5-20% sucrose gradient in TMK buffer, centrifuged in the SW41 rotor at 41,000 rpm for 11 hr. at 4° and fifteen drop fractions were collected (see Chapter II for details).

(——) , absorbance 260 nm

—●—●— , radioactivity (counts/min. x 10^{-3}).

(iii) Study of protein synthesis by membrane fragments

As a prelude to an examination of their extracellular enzyme-forming abilities, a comparison was made between the abilities of membrane fragments and cytoplasmic ribosomes of B. amyloliquefaciens to incorporate amino acids into protein. For this comparison, the lysate obtained after osmotic lysis and French pressure cell treatment as described above, was centrifuged at 20,000 g for 10 min. The pellet of membrane fragments, was purified as described above while the supernatant fraction was centrifuged at 105,000 g for 2 hr. to give a ribosomal pellet which was resuspended in TMK buffer and recentrifuged. In all in vitro studies on protein synthesis, both the ribosomal fraction and the membrane fragments were reinforced with a soluble extract, isolated from exponential B. amyloliquefaciens cells using the standard extraction procedure (see Chapter II). The final concentration of Mg^{2+} (8 mM) present in the incubation mixtures permits natural initiation of protein synthesis to occur (Lengyel and Soll, 1969).

Because of the problem of intact cell (or protoplast) contamination in cell-free studies, control experiments were carried out to ensure that no contribution to amino acid incorporation into protein arose from these sources. The inhibitory effect of sodium azide on intact B. amyloliquefaciens cells was studied in the cell-free incubation mixture and a concentration of 20 mM was found necessary to give

complete inhibition of ^{14}C-amino acid incorporation. Hence, in addition to the cell-free incubation mixture, both membrane fragments and the free cytoplasmic ribosomal preparation were made 20 mM with respect to sodium azide during the latter stages of their preparation.

As a further control, viable cell counts were carried out on the various cell fractions prepared in the presence and absence of 20 mM sodium azide. In all samples tested (soluble fraction, membranes and free ribosomes), the viable cell count was insignificant. (For example, the membrane fragment preparation prepared in the absence of sodium azide gave the highest cell count of 40 colonies per ml.)• These studies were also confirmed by electron microscopy.

The membrane fragments and free cytoplasmic ribosomes, adjusted to the same RNA concentration and supplemented with soluble extract, were studied for their abilities to incorporate ^{14}C-labelled amino acids into protein. The results of two separate experiments (Table III.4) show that (per unit weight of RNA) membrane fragments were two or three times more active than free cytoplasmic ribosomes. Both systems were inhibited about 70% by chloramphenicol (300 µg/ml.) The reason for the increase in protein synthesising activity shown by the cell extracts in experiment 2 (compared with experiment 1) is possibly due to the age effect mentioned previously, since the cells were 2 hr. younger.

The time course of amino acid incorporation, as

TABLE III.4. COMPARISON OF FREE AND MEMBRANE-BOUND RIBOSOMES ISOLATED FROM EXPONENTIAL PHASE CELLS OF *B. AMYLOLIQUEFACIENS* TO INCORPORATE AMINO ACIDS INTO PROTEIN

Ribosome preparation	^{14}C-amino acid incorporation (counts/min/mg. RNA)
Experiment 1.	
Free	6,910
Membrane-bound	19,710
Experiment 2.	
Free	15,680
Membrane-bound	28,400

Experimental details for the preparation of the cell extracts are described in the text. ^{14}C-labelled amino acid incorporation experiments were carried out as described in Chapter II, except that 0.4 μCi of a reconstituted protein hydrolysate mixture (supplemented with ^{12}C-amino acids in which it was deficient) replaced 0.2 μCi of ^{14}C-phenylalanine and the reaction mixtures were made 20 mM with respect to sodium azide. The reaction mixtures, containing ribosome (20 μg RNA) and soluble (420 μg protein) fractions were incubated at 30° for 60 min.

shown for the first of these experiments (Fig. III.10), indicate that both free and membrane-bound ribosomes incorporate steadily over 45 min., reaching a plateau level at 60 min. This more prolonged protein synthetic activity (compared with previous studies), may be a consequence of the different extraction procedure used to obtain the two ribosomal classes. Coleman (1969d) in a similar study with B. amyloliquefaciens ribosomal and membrane fractions (but obtained differently), also indicated a prolonged amino acid incorporation, whilst other reports in different bacteria have shown linear incorporation in both ribosomal fractions for up to 90 min. (Schlessinger, 1963; Moore and Umbreit, 1965; Hirashima et al., 1967).

Since both the membrane and the ribosomal fractions incorporated amino acids into protein, the fractions were examined for their ability to synthesise extracellular enzymes. Both protease and α-amylase formation were studied in the cell-free system described above, the scale of which was increased two-fold and the radioactive amino acid mixture was replaced with a mixture of non-radioactive amino acids (0.2 mM each amino acid). Protease activity was determined by the ultrasensitive assay using the Remazo-brilliant Blue/Hide substrate. Initial studies, however, failed to detect any synthesis of protease or α-amylase during a 45 min. incubation of these fractions. In separate experiments, total cell lysates prepared by both the French

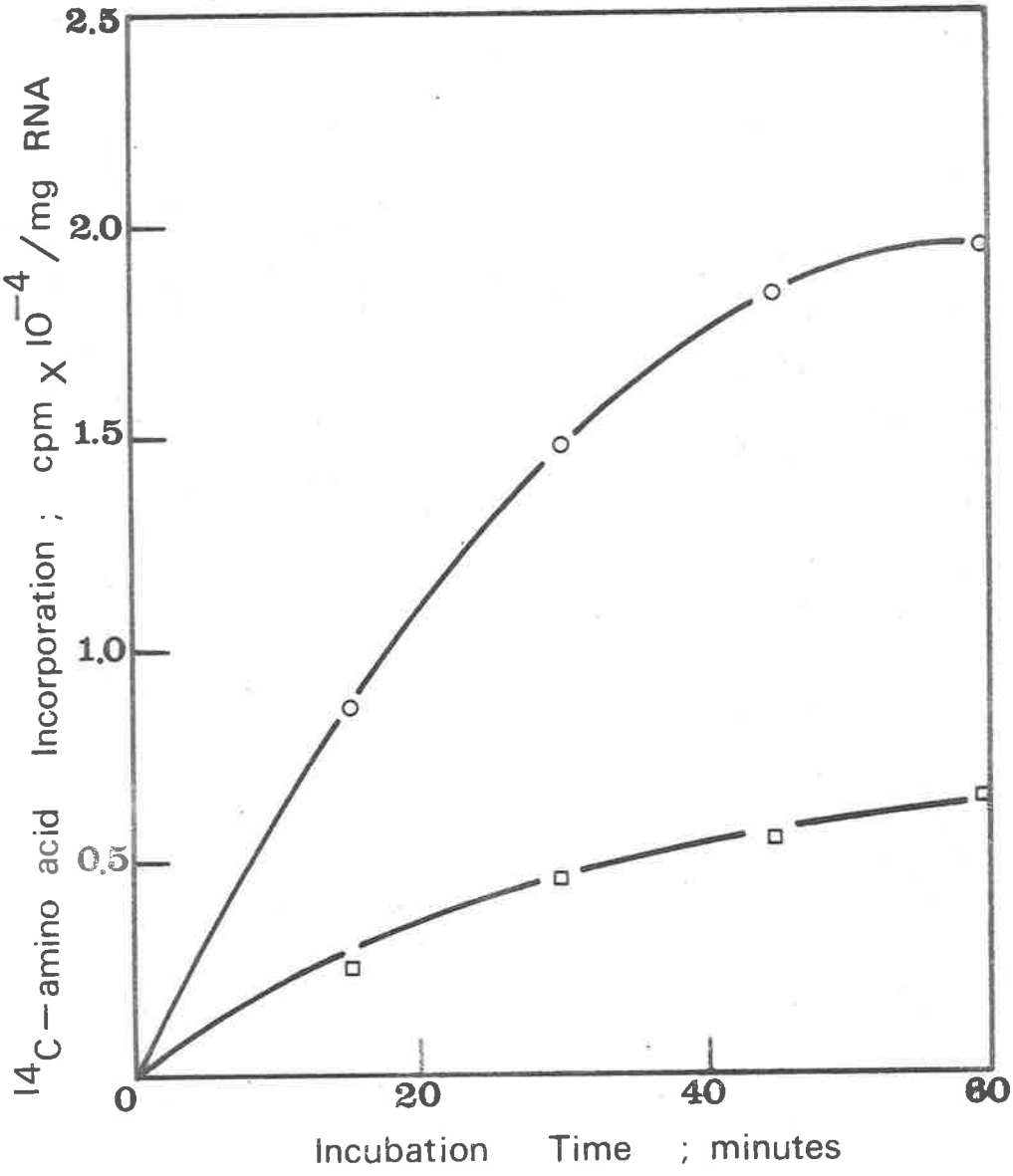


FIG. III.10. COMPARISON OF FREE AND MEMBRANE-BOUND RIBOSOMES ISOLATED FROM EXPONENTIAL PHASE CELLS OF B. AMYLOLIQUEFACIENS TO INCORPORATE AMINO ACIDS INTO PROTEIN.

The experimental details for the preparation of the cell extracts are described in the text.
Measurements of ^{14}C-labelled amino acid incorporation were carried out as stated in Table III.4. The reaction mixtures, containing ribosome (20 µg RNA) and soluble (420 µg protein) fractions were incubated at 30°.

—○—○— , membrane-bound ribosomes
—□—□— , free ribosomes.

pressure cell and lysozyme techniques (see Chapter II), were also examined in the cell-free system for their abilities to synthesise extracellular enzymes, but again no synthesis of the enzymes was detected.

E. DISCUSSION

This chapter describes *in vitro* studies on protein synthesis by cell extracts from *B. amyloliquefaciens.* In order to obtain active cell extracts containing polyribosomes from this organism, it is necessary to use exponential phase cells and to rapidly cool them. These results were as expected from previous studies with other bacterial systems.

The requirements of the endogenous mRNA-directed cell-free system from *B. amyloliquefaciens* were similar to those from other *Bacillus* species (Hirashima *et al.*, 1967; Gonzalez *et al.*, 1968) with respect to dependence on ribosomes, soluble fraction and energy generating system. The partial inhibition (approximately 70%) shown by chloramphenicol was reproducible over several experiments and has also been reported for the endogenous mRNA-directed cell-free system from *B. subtilis* (Hirashima *et al.*, 1967). The relative inability of *B. amyloliquefaciens* cell extracts to translate added polyuridylic acid has since been more fully examined and the results are presented in a later

chapter.

Findings that cell extracts prepared from the late logarithmic and stationary phases show a low *in vitro* amino acid incorporating activity agree with those of other workers who used different bacterial stains (Hirashima *et al.*, 1967; Gonzalez *et al.*, 1968; Changchien and Aronson, 1970; Scheps *et al.*, 1971; Himes *et al.*, 1972). The exact reason for this loss of activity in the older *B. amyloliquefaciens* cell extracts is not known and is complicated by the finding that both soluble and ribosomal fractions are implicated. Possible reasons, suggested as well by other workers, include reduced concentrations of amino-acyl-activating enzymes, N-formyl-methionine tRNA or some other tRNA, a lack of available initiation or dissociation factors or a decrease in mRNA content. The finding in the present work of a decrease in polysomes with a concomitant increase in ribosomal subunit number in ageing *B. amyloliquefaciens* cells, suggests a blockage in the initiation of protein synthesis. In this context, Scheps *et al.* (1971) showed that cell extracts from stationary phase *E. coli* cells were reduced in their polyribosomal content; these extracts were subsequently shown to be deficient in the three initiation factors of protein synthesis (Scheps and Revel, 1972) thus explaining the reduced protein synthesis seen by these preparations.

This chapter also describes for the first time the isolation of clean cytoplasmic membrane fragments by the

osmotic lysis of B. amyloliquefaciens protoplasts. Membrane preparations routinely contained large fragments with virtually no contaminating cell wall material or free ribosomes. Interestingly enough, even though these membrane fragments were apparently capable of protein synthesis as discussed below, no polysomes could be seen associated with them. Such a finding is in contrast with other reports involving different bacteria where membrane associated ribosomes were visible in electron micrographs (Hendler et al., 1964; Pfister and Lundgren, 1964; Abrams et al., 1964; Schlessinger et al., 1965; Brown and Abrams, 1970).

The small particles which were seen to cover the surface of the membrane fragments seem most likely to be adenosine triphosphatase, since morphologically identical particles were present on Micrococcus lysodeikticus membrane fragments and were identified as this enzyme (Munoz et al., 1968). On the other hand, there is the interesting possibility (yet to be tested) that these particles may in fact be 30S subunits which have their associated 50S subunits submerged in the membrane.

The in vitro amino acid incorporation experiments have shown that the membrane fragments are two to three times more active, per unit weight of RNA, as free ribosomes isolated from the same culture. A similar result was observed by other workers using different bacteria (Schlessinger, 1963; Moore and Umbreit, 1965; Krembel, 1971;

Scheinbuks et al., 1972). On the other hand, Coleman (1969d, 1970) reported no difference between the incorporating abilities of these fractions. It should be pointed out that with any study involving protein synthesis by isolated bacterial membranes, there is always the possibility that the observed amino acid incorporation could be due either to a few free or adsorbed cytoplasmic ribosomes. In the present work, while it is not possible to eliminate the first possibility, the second possibility seems less likely since no ribosomes are seen attached to the membrane fragments, though clearly, such negative evidence cannot be regarded as conclusive.

Although these membrane fragments appear to incorporate amino acids into protein, preliminary studies have shown that they are apparently incapable of synthesising active extracellular enzymes. This finding does not exclude the possibility that inactive extracellular polypeptide chains are being synthesised or that the amounts of active enzyme produced are too small to be detected by the assay procedure used. The recent isolation of antibodies to protease and α-amylase in this laboratory will permit a direct examination of these possibilities.

It should be pointed out that Coleman (1970) has claimed that membranes prepared from this organism in the absence of sucrose are capable of synthesising α-amylase. However, the small amount of enzyme synthesised could be due

to contamination by intact cells since his *in vitro* protein synthesising incubation medium did not contain sodium azide and moreover, in the present work, preparation of such membranes using Coleman's method, always resulted in considerable contamination by intact cells. In addition, Coleman failed to establish that the enzyme was synthesised *de novo*.

In summary, the studies presented in this chapter have established the requirements necessary to achieve endogenous mRNA-directed protein synthesis with *B. amyloliquefaciens* cell extracts. The fact that cytoplasmic membrane fragments from this organism are capable of *in vitro* protein synthesis gives encouragement for further studies aimed at achieving cell-free synthesis of extracellular enzymes. Further *in vitro* protein synthesis studies will be presented in a later chapter.

CHAPTER IV

INHIBITION OF EXTRACELLULAR ENZYME FORMATION IN B. AMYLOLIQUEFACIENS BY LYSOZYME

INHIBITION OF EXTRACELLULAR ENZYME FORMATION IN B. AMYLOLIQUEFACIENS BY LYSOZYME

A. INTRODUCTION

Earlier work (May and Elliott, 1968b) demonstrated that extracellular enzyme formation by protoplasts of B. amyloliquefaciens was zero or very small even though incorporation of ^{14}C-valine was almost the same as in untreated cells and that of ^{14}C-uracil, greater. In this earlier study, only protoplasts were examined and no attempt was made to see at what stage of the lysozyme treatment the apparent selective inhibition of extracellular enzyme synthesis occurred. Subsequent to these studies, reports from different workers indicated that protoplasts from other bacterial strains (that is, B. licheniformis and M. sodonensis) were active in the secretion of extracellular penicillinase and alkaline phosphatase (Sargent et al., 1969; Glew and Heath, 1971).

In view of the above findings and the possibility mentioned previously, that the failure by B. amyloliquefaciens protoplasts may not be due to a failure to produce the polypeptide chain of the extracellular enzymes, but rather to some secondary effect needed to 'activate' the enzyme, it was deemed necessary to examine more closely the effect of cell wall removal by lysozyme in this organism. This chapter describes such a study.

B. EFFECT OF LYSOZYME ON ^{14}C-PHENYLALANINE INCORPORATION INTO CELLS AND EXTRACELLULAR ENZYME FORMATION

It was previously established (Chapter III) that protoplasts may be prepared from B. amyloliquefaciens washed cells in a TMK-sucrose buffer. The complete conversion to protoplasts occurred over a 50 - 60 min. incubation period with gentle shaking at 30° in the presence of a critical concentration of lysozyme. This conversion process has now been studied using a mutant of B. amyloliquefaciens which was normal in extracellular enzyme production, but did not produce the protoplast-bursting factor (May and Elliott, 1970) mentioned previously. The use of this mutant avoided the danger of lysis by lytic factor synthesised during the incubation with lysozyme. The medium used in these studies consisted of TMK buffer containing 0.6 M sucrose, 4.25 mM sodium citrate, trace metal solution (0.25 ml/l.), 1% maltose and 3 mM $(NH_4)_2HPO_4$. This is referred to below as TMKE-sucrose buffer.

Total cellular protein synthesis by cells suspended in this medium was measured in the presence and absence of lysozyme as follows: 50 ml. of exponential cells were washed twice with TMKE buffer and resuspended in 20 ml. of TMKE-sucrose buffer. Cell samples, with and without lysozyme (300 µg/ml.), were incubated in the presence of ^{14}C-phenylalanine (spec. act. 455 mCi/m-mole) and ^{12}C-phenylalanine (100 µg/ml.)

at 30° as thin layers in 100 ml. flasks with gentle shaking over a 45 min. period. It can be seen (Fig. IV.1) that the cells, in the presence of lysozyme, incorporated linearly but at approximately 75% of the control rate. The change in rate appeared to occur 5 min. after the lysozyme addition. Phase contrast microscopy showed that no protoplasts were detectable before at least 30 min. after lysozyme addition and that a total period of 50 - 60 min. was required for complete conversion of the cells to protoplasts. This experiment is essentially in agreement with the previous work of May and Elliott (1968b), except that they did not observe the small inhibition.

An experiment was then carried out to determine over a 45 min. time period the ability of cells to produce extracellular protease in the presence and absence of lysozyme. In marked contrast to the above, cessation of enzyme appearance in the supernatant occurred about 5 to 8 min. after the lysozyme addition (Fig. IV.2). To test whether the enzyme which did appear in the presence of lysozyme during this 5 to 8 min. period represented de novo synthesis, the effect of chloramphenicol (25 μg/ml.) was examined. (This concentration of chloramphenicol immediately and almost completely inhibited general protein synthesis as measured by ^{14}C-phenylalanine incorporation.) The results show (Fig. IV.2) that some enzyme synthesis occurs in the presence of lysozyme during the first 5 min. The remaining enzyme appearing in the first 8 min. probably represents preformed enzyme, probably en route to secretion (Gould, unpublished results). A similar

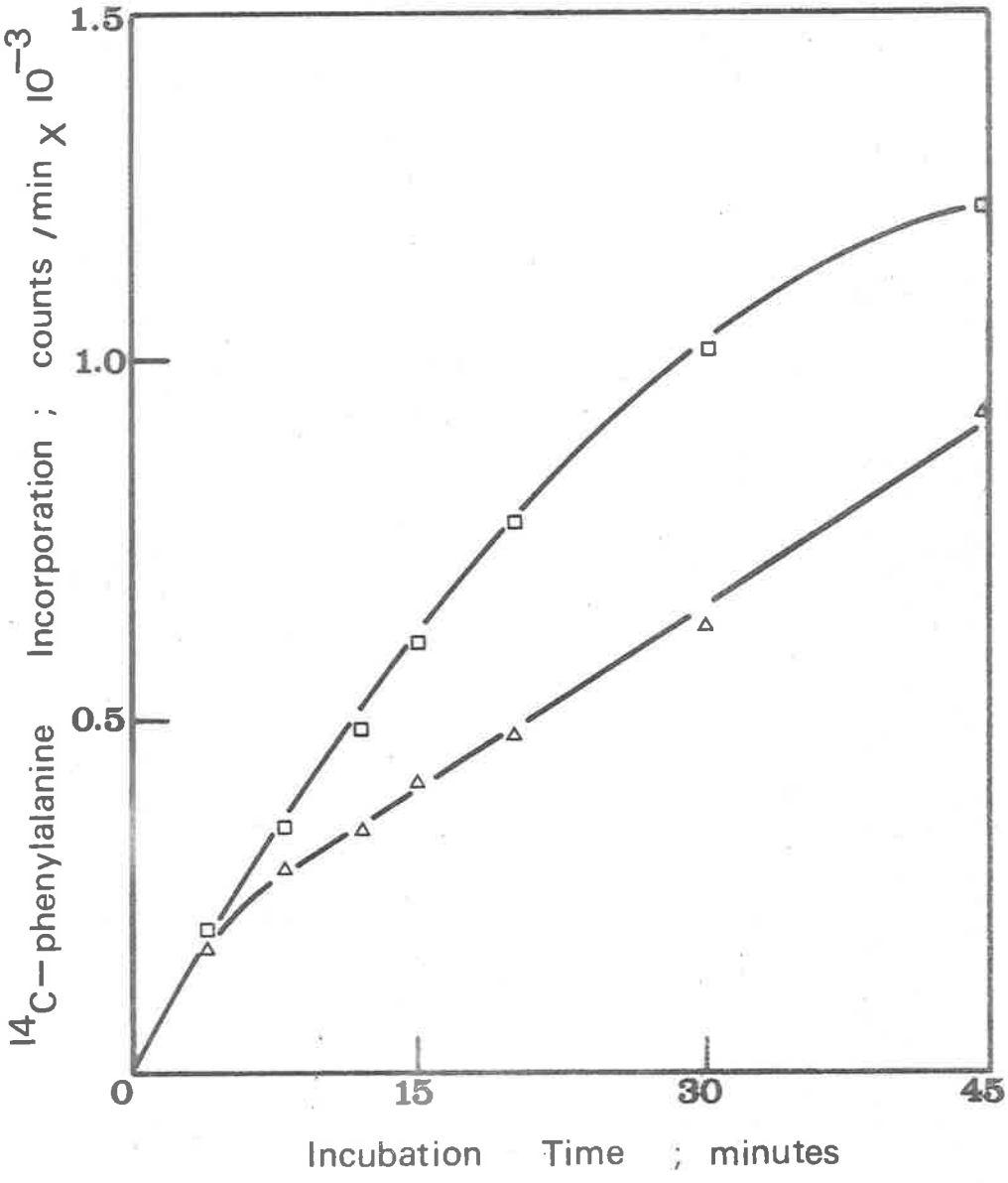


FIG. IV.1. EFFECT OF LYSOZYME ON ^{14}C-PHENYLALANINE INCORPORATION BY WASHED CELLS IN TMKE-SUCROSE BUFFER.

—□—□— , no addition
—△—△— , lysozyme (300 µg/ml).

Lysozyme and ^{14}C-phenylalanine were added at zero time.

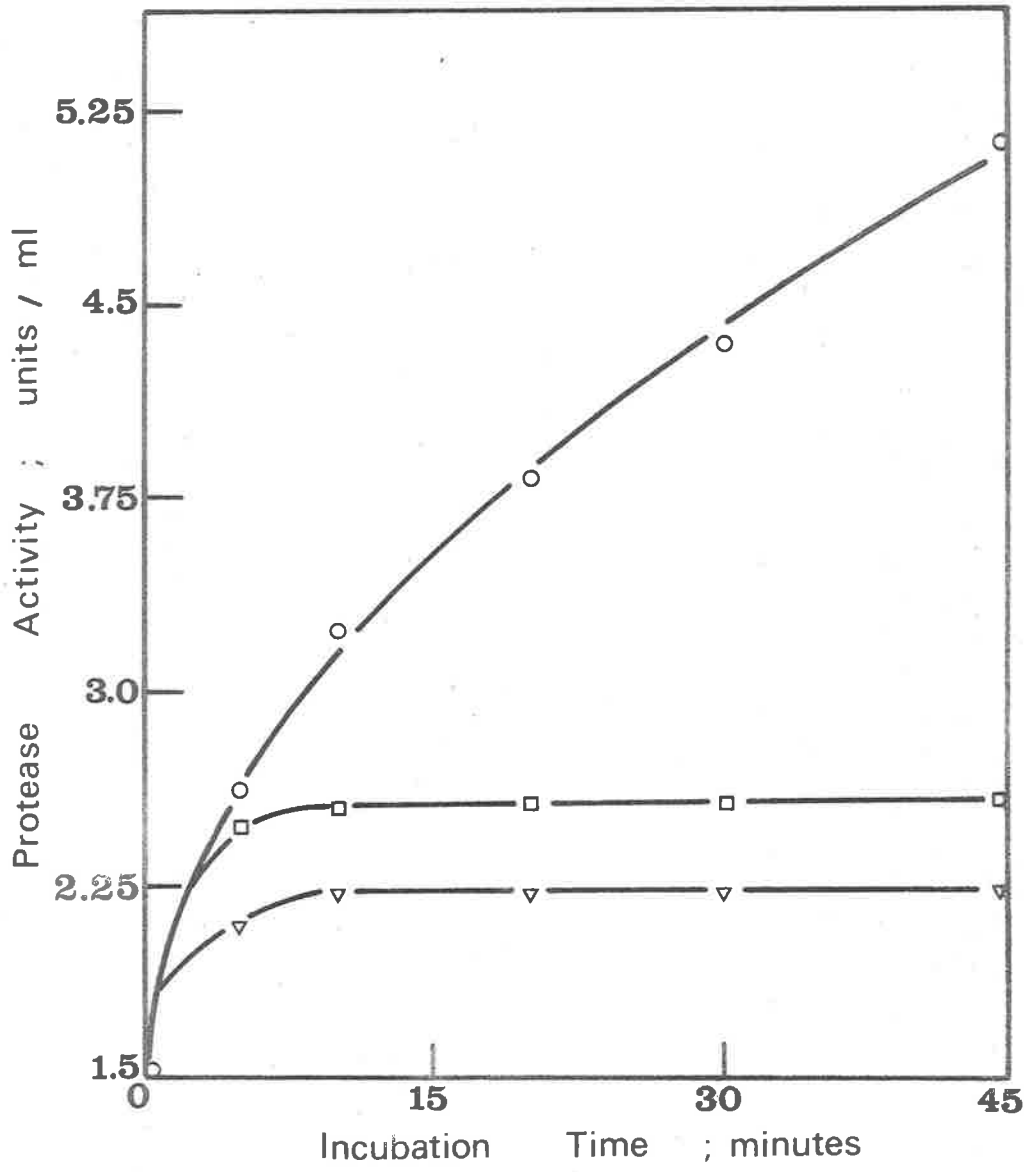


FIG. IV.2. EFFECT OF LYSOZYME AND CHLORAMPHENICOL ON PROTEASE FORMATION BY WASHED CELLS IN TMKE-SUCROSE BUFFER.

—○—○— , no addition
—□—□— , lysozyme (300 µg/ml)
—▽—▽— , lysozyme (300 µg/ml) and chloramphenicol (25 µg/ml).

Lysozyme and chloramphenicol were added at zero time. Protease activity was measured using Remazo brilliant blue/ Hide as substrate.

inhibition of α-amylase production by lysozyme (Fig. IV.3) suggests that this effect is a general one on extracellular enzyme production in this organism. Control studies have ruled out the possibility of a direct inhibitory effect of lysozyme on the enzymes themselves during the assays.

One possible explanation of these findings was that an inhibitory substance (for example, ribonuclease or protease) can enter the cell after slight cell wall digestion and damage the extracellular enzyme synthesising machinery. To test whether entry of ribonuclease was responsible for the effect, an experiment was carried out as described above but with an excess of RNase inhibitor present in the TMKE-sucrose buffer before the lysozyme addition. The fact that cessation of protease production still occurred after 5 min., as previously found, indicated that ribonuclease is not the cause of the phenomenon. At the time of these studies, no antibody to protease was available, so that a direct study of possible protease involvement has yet to be carried out.

C. ELECTRON MICROSCOPIC STUDY OF THE EFFECT OF LYSOZYME ON CELLS

The effect of lysozyme on B. amyloliquefaciens cells has also been studied by electron microscopy. It was found that small vesicles appear 4 min. after the addition of

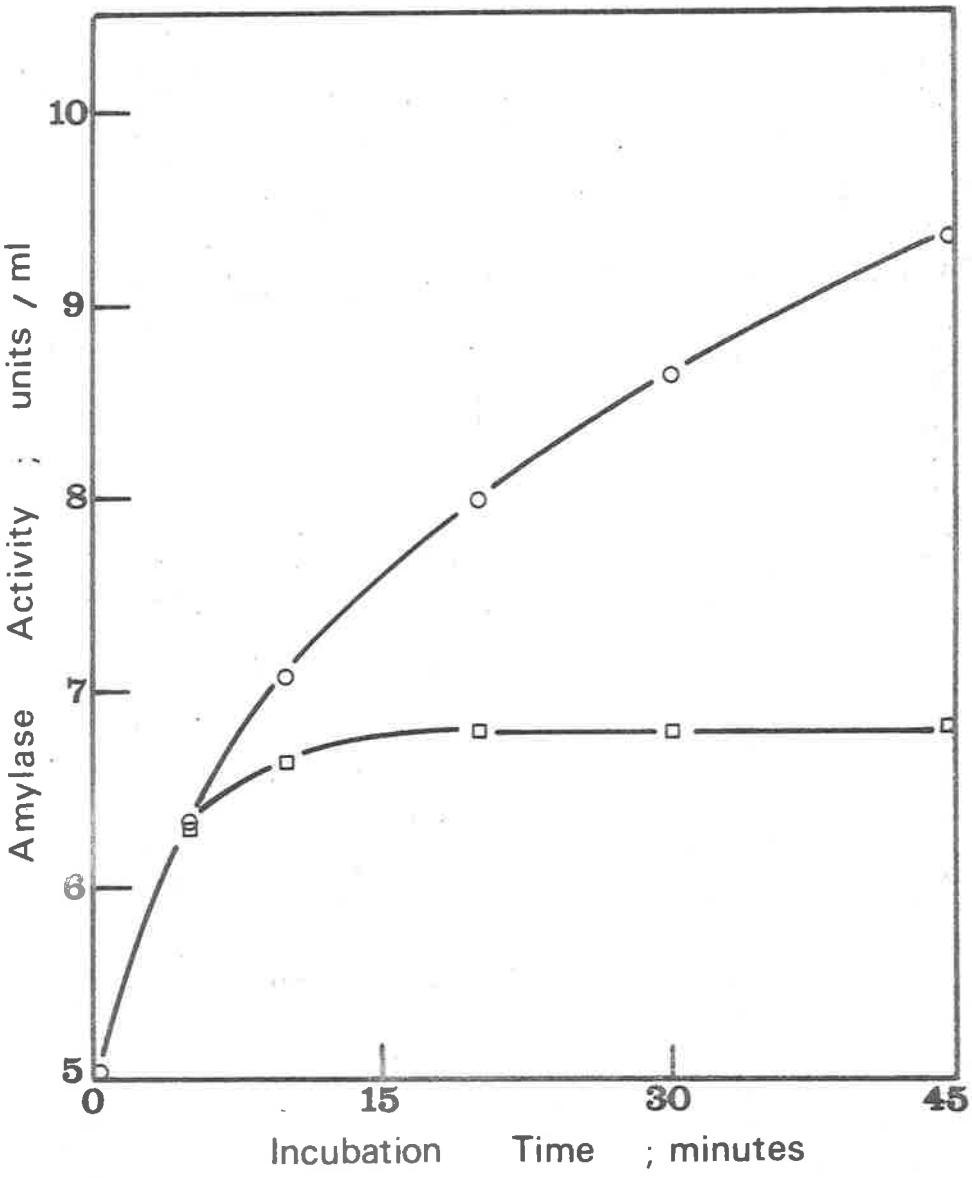


FIG. IV.3. EFFECT OF LYSOZYME ON α-AMYLASE FORMATION BY WASHED CELLS IN TMKE-SUCROSE BUFFER.

—○—○— , no addition
—□—□— , lysozyme (300 µg/ml), added at zero time.

lysozyme (Fig. IV.4-6). These are closely associated with the cells and are not found in the general background. These vesicles appear to be similar to the mesosomal vesicles found in this organism (B. May, Ph.D. Thesis). This effect of lysozyme was also studied by sectioning cells; again the small vesicles were seen in close association with lysozyme-treated bacteria. Whereas the cell wall of control cells appears sharp and distinct (Fig. IV.7), the cell wall of a 5 min. lysozyme-treated sample (Fig. IV.8) appears as a more diffuse structure; liberated vesicles can be seen lying nearby. Fig. IV.8 suggests on first examination that the membrane has apparently drawn away from the cell wall, but on closer examination (Fig. IV.9), it appears possible that a band of dense material lying near the cytoplasmic membrane has been removed from the cell wall by the lysozyme treatment, leaving the outer side of the membrane with some cell wall material still attached.

D. DISCUSSION

The results presented in this chapter show that total cell wall removal is not required for the loss of exoenzyme-forming ability as previously indicated by May and Elliott (1968b). Within an 8 min. period after lysozyme addition, enzyme synthesis was seen to be inhibited; no protoplasts were detectable during this time period, and indeed, a

FIG. IV.4.

NEGATIVELY STAINED PREPARATION OF B. AMYLOLIQUE-FACIENS CELLS.

This preparation served as a control for the subsequent preparations (Figs. IV.5 and 6).

The cells, after two washes in TMKE buffer, were suspended in TMKE-sucrose buffer and were negatively stained with uranyl acetate as described in Chapter II.

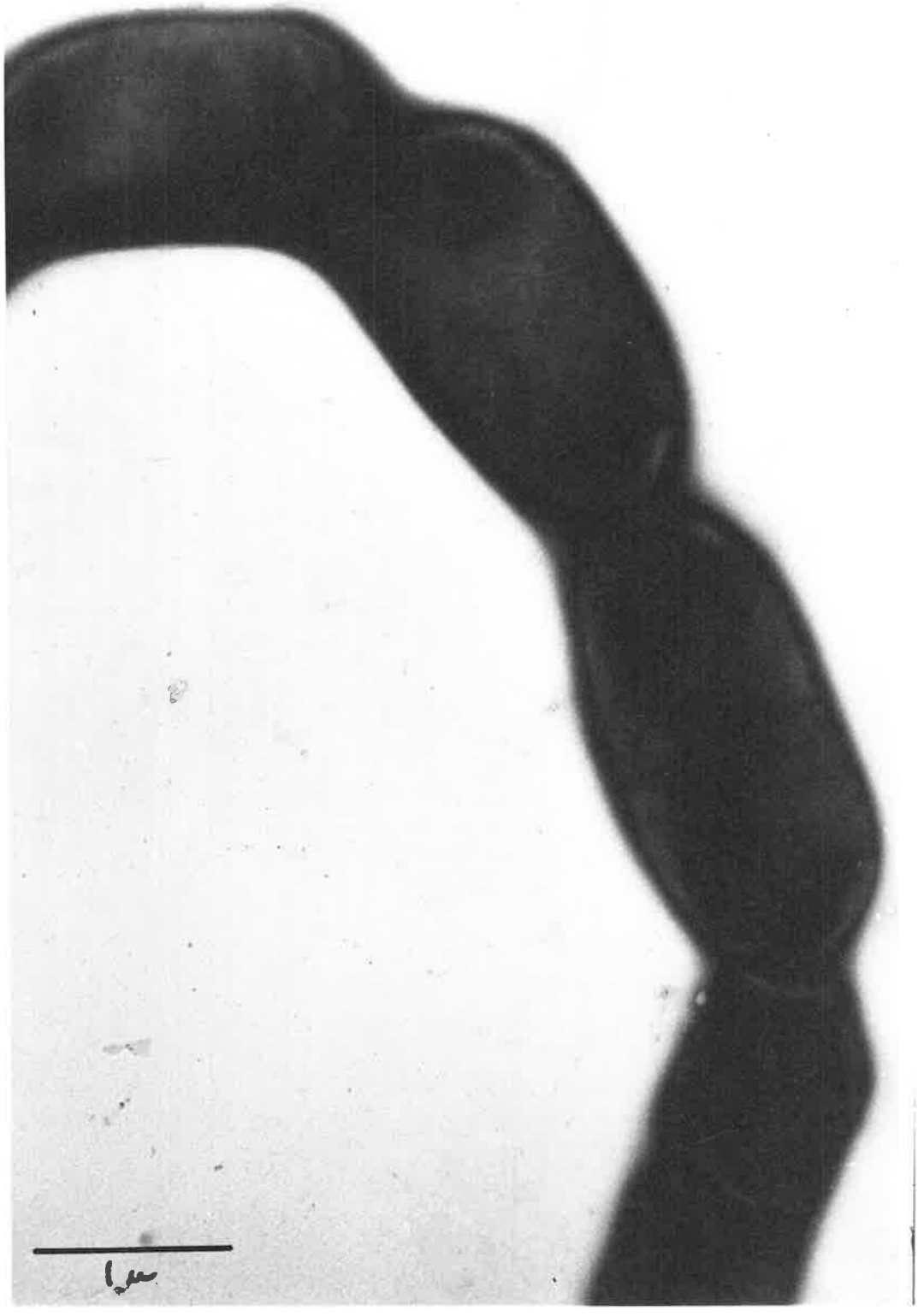

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FIG. IV.5.
NEGATIVELY STAINED PREPARATION OF B. AMYLOLIQUE-FACIENS CELLS AFTER A FOUR MINUTE TREATMENT WITH LYSOZYME.

The cells, after two washes in TMKE buffer, were suspended in TMKE-sucrose buffer and treated with lysozyme (300 µg/ml) for four min. The cells were negatively stained with uranyl acetate as described in Chapter II. Small vesicles (v) are seen in close association with liberated cell wall material (cw).


CW
V
V

FIG. IV.6.

NEGATIVELY STAINED PREPARATION OF B. AMYLOLIQUEFACIENS CELLS AFTER AN EIGHT MINUTE TREATMENT WITH LYSOZYME.

The cells, after two washes in TMKE buffer, were suspended in TMKE-sucrose buffer and treated with lysozyme (300 µg/ml) for eight min. The cells were negatively stained with uranyl acetate as described in Chapter II. Large numbers of small vesicles (v) are seen in close association with liberated cell wall material (cw).

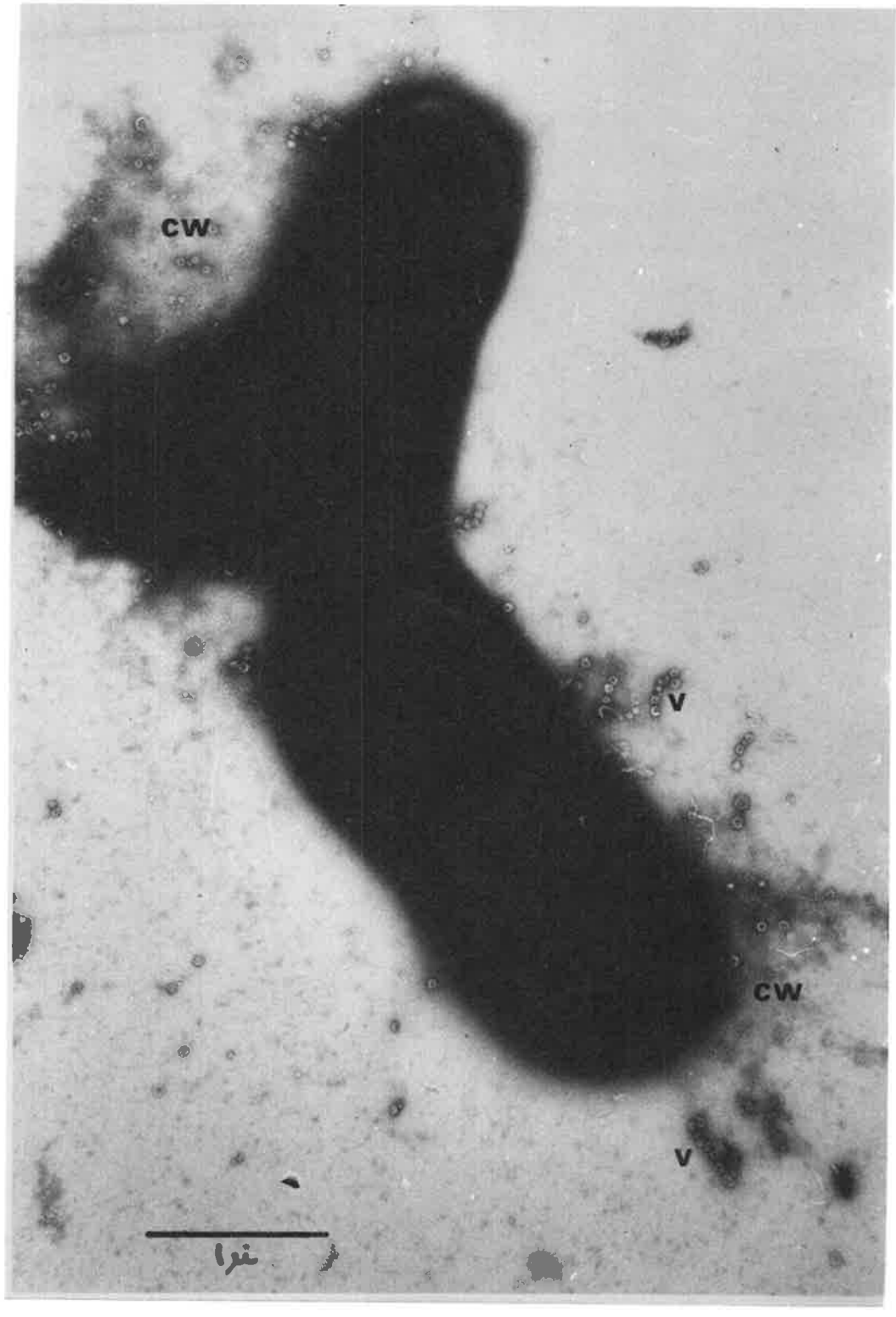

CW
V
CW
V
نفرا

FIG. IV.7.
ELECTRON MICROGRAPH OF A SECTIONED SPECIMEN SHOWING B. AMYLOLIQUEFACIENS CELLS WITH DISTINCT AND WELL-CHARACTERISED CELL WALLS.

This specimen served as a control for the following specimen (Fig. IV.8). The cells, after two washes in TMKE buffer, were suspended in TMKE-sucrose buffer. The specimen was then fixed and sectioned as described in Chapter II.

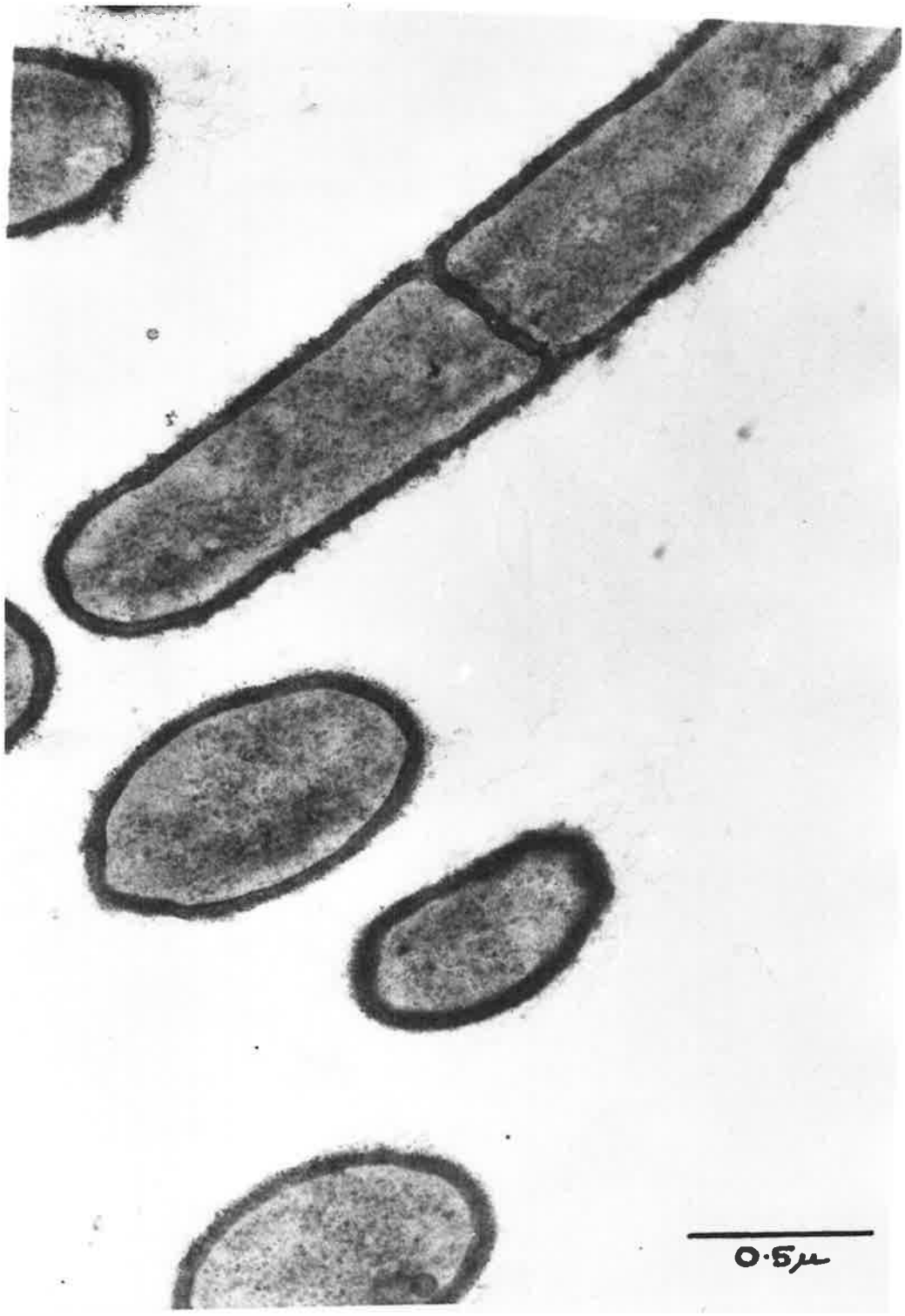

0·5μ

FIG. IV.8.

ELECTRON MICROGRAPH OF A SECTIONED SPECIMEN SHOWING B. AMYLOLIQUEFACIENS CELLS AFTER A FIVE-MINUTE TREATMENT WITH LYSOZYME.

The cells after two washes in TMKE buffer, were suspended in TMKE-sucrose buffer and treated with lysozyme (300 µg/ml) for five min. The specimen was fixed and sectioned as described in Chapter II.

The lysozyme treated cells show a more diffuse cell wall structure.

Liberated vesicles (v) can be seen near to the cells; cell wall digested areas (cw) are also visible.

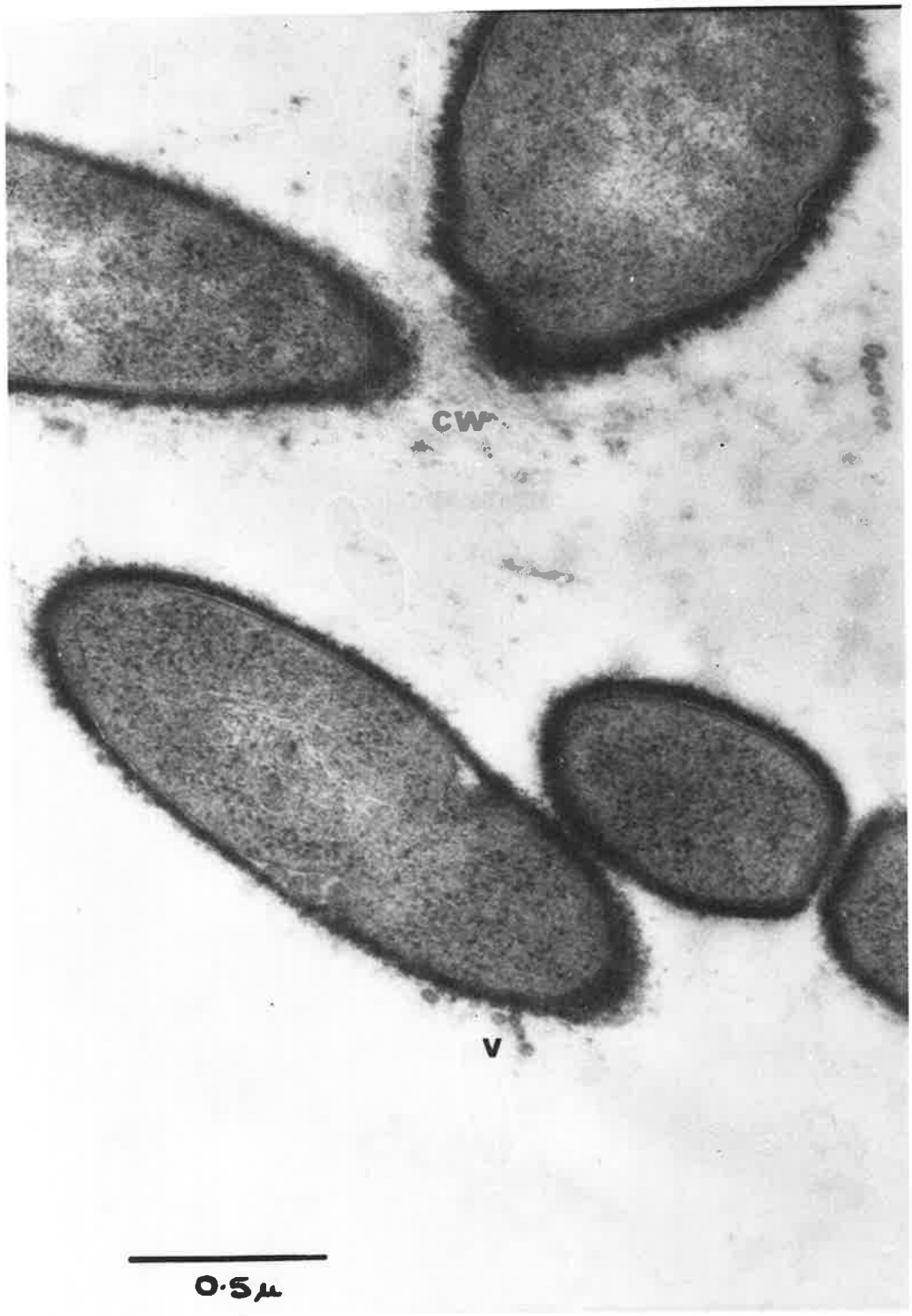

CW
V
0·5μ

FIG. IV.9.

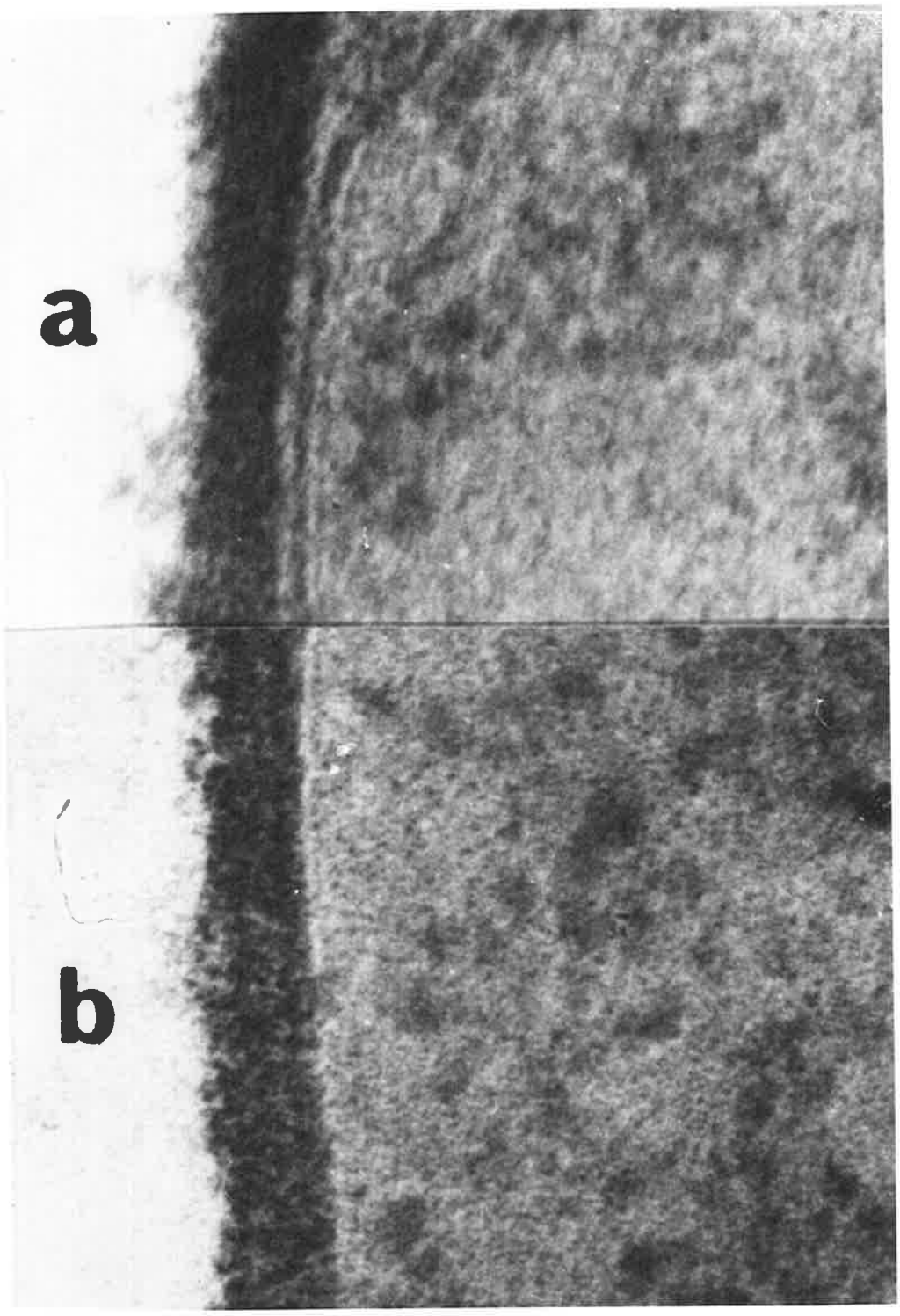
COMPOSITE ELECTRON MICROGRAPH OF SECTIONED SPECIMENS OF *B. AMYLOLIQUEFACIENS*:

(a) FIVE MINUTE LYSOZYME-TREATED CELLS, AND

(b) CONTROL CELLS.

A band of dense material lying near the cytoplasmic membrane appears to have been removed from the cell wall by the lysozyme treatment, leaving the outer side of the membrane with some cell wall material still attached.

Magnification: 408,000 x.


a
b

further 20 min. incubation was required before any of these structures were seen.

It is not possible to decide how lysozyme treatment inhibits extracellular enzyme formation. There are many possibilities: (a) The most trivial is that lysozyme binds to the cell wall and physically prevents enzyme secretion. This cannot be excluded but is rendered unlikely in that addition of lysozyme increases the rate of release of preformed enzyme believed to be en route to secretion (Gould, unpublished results).

(b) Lysozyme may give exoprotease or exoribonuclease access to sensitive structures involved in extracellular enzyme production. Since the presence of an excess of RNase inhibitor had no effect on this phenomenon, ribonuclease participation can most probably be ruled out; protease participation, on the other hand, has yet to be tested, but it may, for example, degrade the emerging polypeptide chains in the absence of cell wall. Indeed, the studies of Bettinger and Lampen (1971) may be directly relevant here, since they have shown, using protoplasts of B. licheniformis, stripped of their membrane-bound penicillinase, that proteases can act at the outer surface of the bacterial membrane and degrade the partially folded emerging polypeptide chains. With the availability of the protease antibody, the above possibility can now be studied.

(c) Lysozyme may release vesicles involved in extracellular

enzyme secretion. The electron micrographs showed that, at approximately the same time that extracellular enzyme synthesis ceased upon lysozyme addition, vesicles were liberated; this suggests that they may be related to extracellular enzyme production. These vesicles may possibly be mesosomal in nature. Lampen (1965) originally suggested a role for the mesosome in extracellular enzyme production but previous studies on the mesosomes of this organism (B. May, Ph.D. Thesis) have suggested that they are unlikely candidates for a role in extracellular enzyme secretion since neither ribosomal RNA nor active enzymes could be detected within or in association with these structures. However, the recent suggestion of ribosomes in tubules and vesicles of B. subtilis (Matheson and Kwong, 1972), if substantiated by more direct evidence, suggests that perhaps a reappraisal may be necessary in this organism to determine the role, if any, played by the mesosome in extracellular enzyme production.

(d) Lysozyme may remove a layer of cell wall mucopeptide which is involved in some unknown way in extracellular enzyme production. The electron micrographs of sections of B. amyloliquefaciens tentatively suggest the possibility of a layer being removed. Evidence does exist for a thin strand of peptidoglycan in the cell wall of E. coli and other Gram negative bacteria (Osborn, 1969) while the observed thickness of the wall and the derived peptidoglycan in most Gram positive bacteria implies the presence of several layers

of peptidoglycan (Archibald et al., 1973). Nermut (1967) in an electron microscopic study of B. megaterium, concluded that the peptidoglycan component of this organism was present as an inner layer, about 100 Å in thickness, covered by a teichoic acid surface layer of the same thickness. It has also been suggested that a lysozyme-sensitive structural component may join the membrane and cell wall in B. licheniformis (Ghosh et al., 1968).

A direct involvement of cell wall in enzyme secretion is also a possibility. An involvement of cell wall in extracellular protease formation has been reported previously by several authors. It has been shown that streptococcal proteinase is formed as an extracellular zymogen in which a mercaptan is bound by disulphide bonding to a half cystinyl residue (Ferdinand et al., 1965). Activation of the enzyme involves reduction of this disulphide and the cysteine groups of the cell wall were shown to be involved in the process (Liu and Elliott, 1965). This system is different, however, from that described in the present work, since zymogens do not appear to be involved and all three B. amyloliquefaciens extracellular enzymes are devoid of cyst(e)ine.

Hammel and Zimmerman (1966) also described a dependence on cell wall for proteinase formation by S. faecali though it was not established whether this was a differential effect with general protein synthesis unimpaired. More

recently, Gratzner (1972) has described cell wall alterations associated with the hyperproduction of extracellular enzymes in Neurosporra crassa. A more speculative explanation for cell wall involvement is that by limited cell wall digestion, ribosomal complexes embedded in the membrane may now become disorientated and it is this event which leads to the observed cessation of extracellular enzyme synthesis. This is obviously very vague as an hypothesis and is mentioned to illustrate the possible complexity of the system.

(e) Finally, it cannot be eliminated that limited cell wall removal still results in extracellular enzyme synthesis, but now the enzymes are synthesised in an inactive form. The lack of enzyme 'activation' could be due, for example, to a failure in the removal of N-formyl-methionine from the N-terminus of the polypeptide chain or to a failure in a deglycosylation step. Whilst there is no evidence for the attachment of sugars to the exoenzymes of B. amyloliquefaciens it is interesting to note that M. sodonensis elaborates soluble extracellular polysaccharide which is associated with the extracellular alkaline phosphatase through a number of steps in its purification (Glew and Heath, 1971). In addition, the extracellular nuclease of this same organism contains 21% carbohydrate chemically and immunologically identical to the cell wall carbohydrate (Berry et al., 1970). More recently, Yamane et al. (1973) have isolated and purified a glycoprotein serologically related to α-amylase

from the culture fluids of a B. subtilis mutant strain; the cross-reacting material was entirely free from α-amylase activity.

The possibility that extracellular enzyme production is still occurring in B. amyloliquefaciens, but in an inactive form, is under investigation at the present time.

CHAPTER V

EVIDENCE FOR AN ACCUMULATION OF MESSENGER RNA SPECIFIC FOR EXTRACELLULAR PROTEASE IN B. AMYLOLIQUEFACIENS AND ITS RELEVANCE TO THE MECHANISM OF ENZYME SECRETION

EVIDENCE FOR AN ACCUMULATION OF MESSENGER RNA SPECIFIC FOR EXTRACELLULAR PROTEASE IN B. AMYLOLIQUEFACIENS AND ITS RELEVANCE TO THE MECHANISM OF ENZYME SECRETION

A. INTRODUCTION

The studies reported previously involving *in vitro* protein synthesis with cell extracts from *B. amyloliquefaciens* were undertaken with the aim of elucidating the mechanism of extracellular enzyme synthesis and secretion. In conjunction with these studies, and with a similar ultimate aim, extracellular enzyme production by washed *B. amyloliquefaciens* cells has been investigated.

Washed cell suspensions of *B. amyloliquefaciens* produce large amounts of protease, α-amylase and ribonuclease into the external medium and it has been shown previously that production of each of these enzymes is subject to separate controls. Thus, ribonuclease, alone of the three, is subject to repression by inorganic phosphate (May *et al.*, 1968) while protease synthesis is almost completely repressed by amino acids (May and Elliott, 1968a). No specific control for α-amylase has been recognised.

Previous studies (May and Elliott, 1968a) established a biphasic time course for extracellular protease formation in a medium containing high levels of amino acids. There

is an initial rapid production for 30 min. followed by a plateau level for 50 min. after which synthesis resumes linearly. At the time it was believed that mRNA synthesis was needed during the initial phase since actinomycin D (10 µg/ml.) inhibited. However, it was pointed out (J. Mandelstam, personal communication) that the above concentration of actinomycin D would most likely have been toxic to the cells and is therefore not evidence of a transcriptional requirement.

It was therefore decided to reinvestigate the effects of inhibitors of RNA synthesis on protease production by washed cell suspensions of B. amyloliquefaciens. Such studies are described in this chapter.

B. PROTEASE PRODUCTION BY WASHED CELL SUSPENSIONS IN THE PRESENCE AND ABSENCE OF INHIBITORS OF RNA SYNTHESIS AND PROTEIN SYNTHESIS

(i) Nature of the proteases produced

Studies in this laboratory have shown that two extracellular proteases are produced by B. amyloliquefaciens and these may be separated by electrophoresis on either Sepraphore polyacetate strips or on polyacrylamide gels. The experimental details for both techniques are given in Chapter II.

Previous studies (W. Carey, Honours Thesis) have shown that crystallisation of protease from a 25 hr. culture medium of B. amyloliquefaciens by the method of Hagihara et al. (1958), described for B. subtilis, results in a preparation which yields two bands on Sepraphore strip electrophoresis; a major and minor band of activity were detected when the strips were incubated in contact with an agar/casein plate (see Chapter II for details). (These bands are identical to those shown in Fig. V.3.) The bands are a result of two distinct enzyme species; this was shown by the fact that on elution from the strips, followed by a second electrophoresis, the bands retained their original characteristics. The major and minor bands represent neutral and alkaline proteases with pH optima of 7.6 and 10.8, respectively.

When extracellular culture medium is electrophoresed on Sepraphore strips, the same two bands result. Similarly, when cells are harvested after 25 hr. of growth, washed and resuspended in a fresh suspending medium (see Chapter II), two proteases are produced which show the same electrophoretic pattern as those above. Quantitative protease estimations in this chapter were carried out using the casein digestion method, unless otherwise stated; the alkaline protease component contributed less than 5% of the total activity under the assay conditions and hence the results refer only to the neutral protease. This was established by experiments in which the two components were eluted from Sepraphore

strips and their activities independently determined (May, unpublished results). A method for isolating the neutral protease is described in this chapter, which was devised and carried out by Mrs. J. Hanlon, but no method for isolating the alkaline protease pure, in any significant amounts, has yet been obtained.

(ii) Time-course of extracellular protease production in different media

The production of protease is seen to occur almost linearly when washed cells are resuspended in a medium which has a low level of Casamino acids (0.025% w/v) while in contrast, addition of a high level of Casamino acids (0.5% w/v), results in a biphasic time-course of extracellular protease production (Fig. V.1). In the latter situation a rapid initial production occurs for 30 min. (phase 1) followed at 80 min., by a linear phase (phase 2). Between the phases there is a period of 50 min. during which the protease production is very low. Separate experiments (May and Elliott, 1968a) have shown this to be due to multivalent amino-acid repression with combinations of either proline and isoleucine or of glutamic and aspartic acids being the main effectors. Unless otherwise stated, the following studies were performed using a medium containing a high level (0.5% w/v) of Casamino acids.

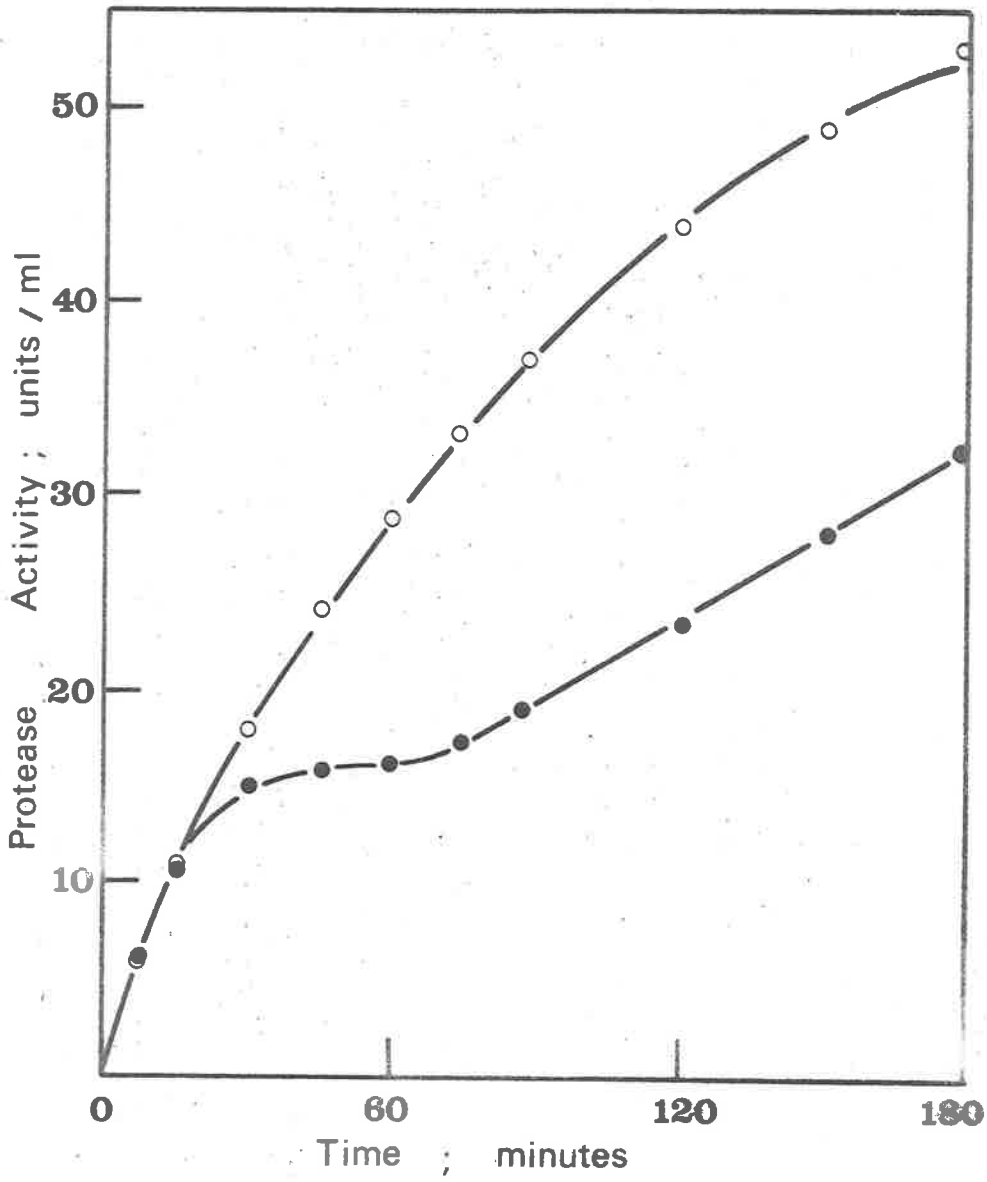


FIG. V.1. PROTEASE FORMATION BY WASHED CELLS IN THE PRESENCE OF LOW AND HIGH CASAMINO ACIDS.

—○—○— , 0.025% w/v Casamino acids
—●—●— , 0.5% w/v Casamino acids.

(iii) Effect of inhibitors of RNA synthesis on protease production by washed-cell suspensions

Fig. V.2 shows that addition of either rifampicin (0.5 µg/ml.) or actinomycin D (2 µg/ml) at zero time has little effect on protease production initially, and indeed, phase 1 production measured over 90 min. is invariably increased by these drugs. However, phase 2 protease production in the presence of these drugs does not occur at all. This agrees with the observation that the addition of rifampicin (or actinomycin D) at 90 min. results in the inhibition of phase 2 synthesis almost immediately (Fig. V.2).

Sepraphore strip electrophoresis of supernatants showed that during phase 1 both proteases are produced in the presence of rifampicin (0.5 µg/ml.). It can be seen (Fig. V.3) that the intensities of both protease bands increase over an 80 min. time period.

A trivial explanation of these results would be that the harvested cells are relatively insensitive to the drugs at first, but acquire sensitivity during incubation in the suspending medium. This, however, proved not to be the case since the incorporation of both 2-^{14}C-uracil and ^{14}C-leucine into trichloroacetic acid-precipitable material was stopped almost completely and immediately when either rifampicin or actinomycin D was added at zero time (Fig. V.4 and Fig. V.5).

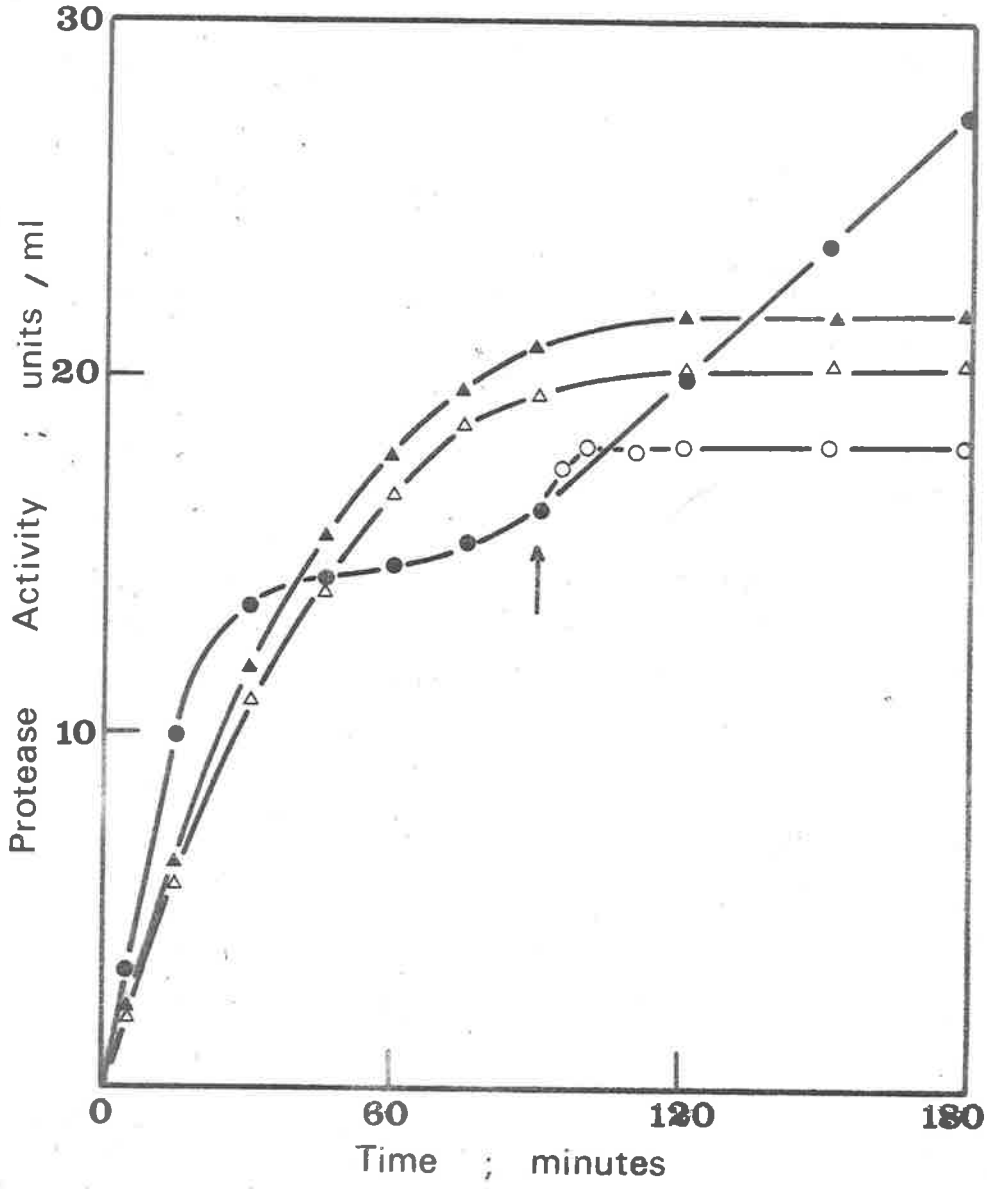


FIG. V.2. EFFECT OF ACTINOMYCIN D AND RIFAMPICIN ON PROTEASE FORMATION BY WASHED CELLS.

—●—●— , no addition of drugs

—△—△— , rifampicin (0.5 µg/ml) added at zero time

—▲—▲— , actinomycin D (2 µg/ml) added at zero time

—○—○— , rifampicin (0.5 µg/ml) added at 90 min. (arrow).

FIG. V.3.

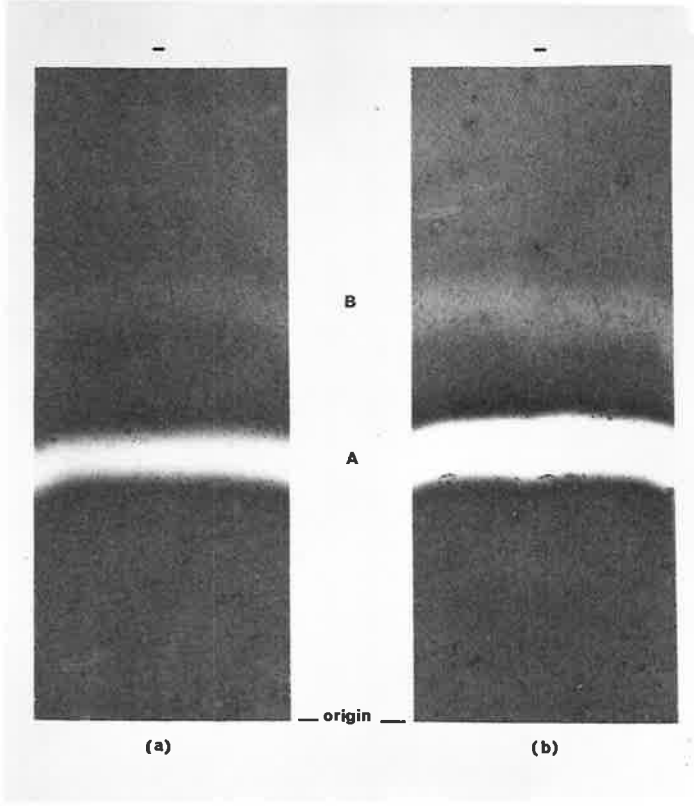
TWO PROTEOLYTIC BANDS OBTAINED AFTER SEPRAPHORE STRIP ELECTROPHORESIS.

Washed cells were incubated with rifampicin (0.5 µg/ml) and cell samples removed at 10 min. and 90 min. were centrifuged. 20 µl of each supernatant was electrophoresed and protease activity was detected.

(a) Two proteolytic bands after 10 min. treatment with rifampicin.

(b) Two proteolytic bands after 90 min. treatment with rifampicin.

A. Neutral protease; B. Alkaline protease.


B
A
origin
(a)
(b)

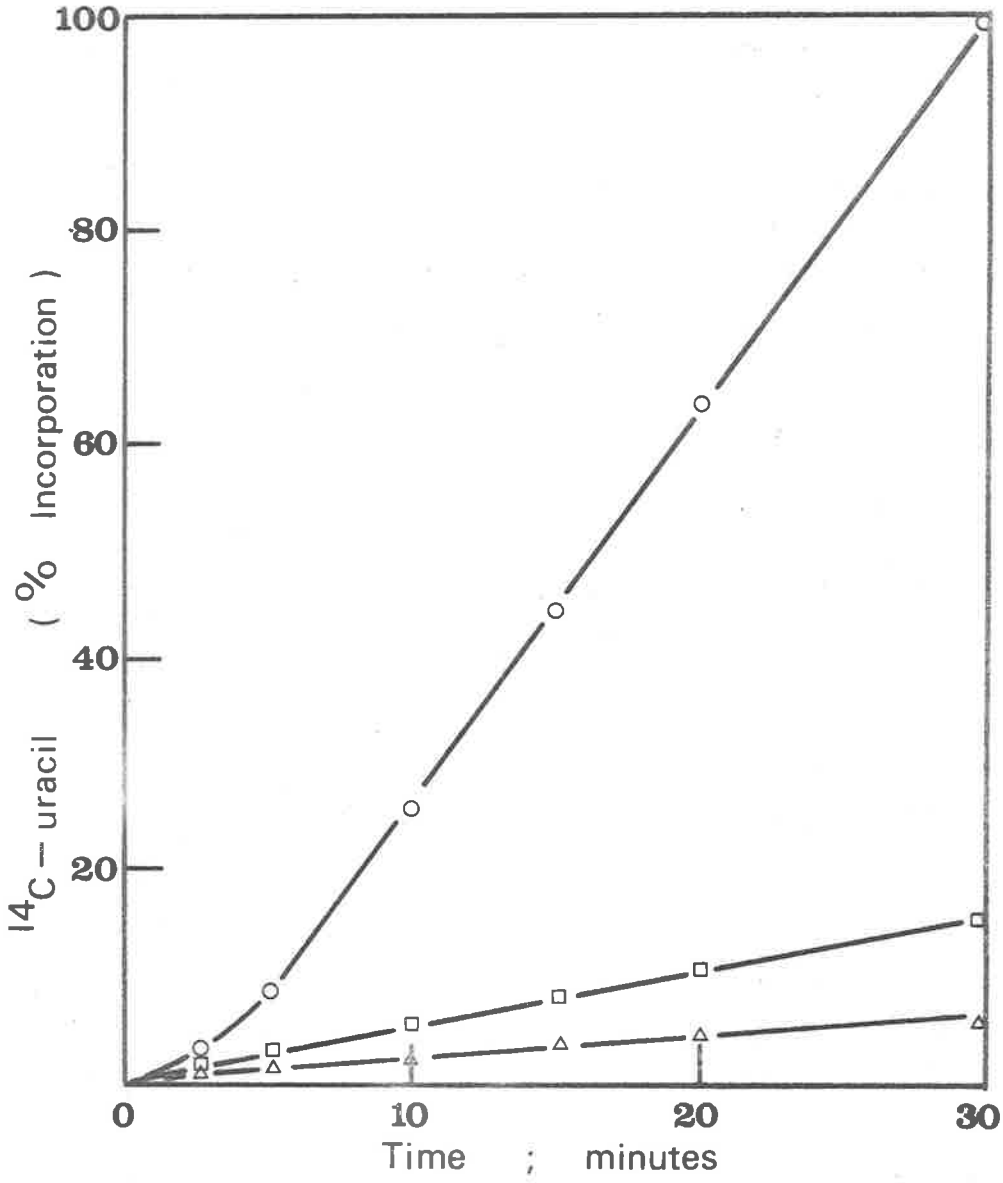


FIG. V.4. EFFECT OF ACTINOMYCIN D AND RIFAMPICIN ON ^{14}C-URACIL INCORPORATION BY WASHED CELLS.

Drugs and ^{14}C-uracil were both added at zero time.

—○—○— , no addition of drugs
—□—□— , actinomycin D (2 µg/ml)
—△—△— , rifampicin (0.5 µg/ml)

The 100% value for ^{14}C-uracil incorporation was 21,800 counts/min.

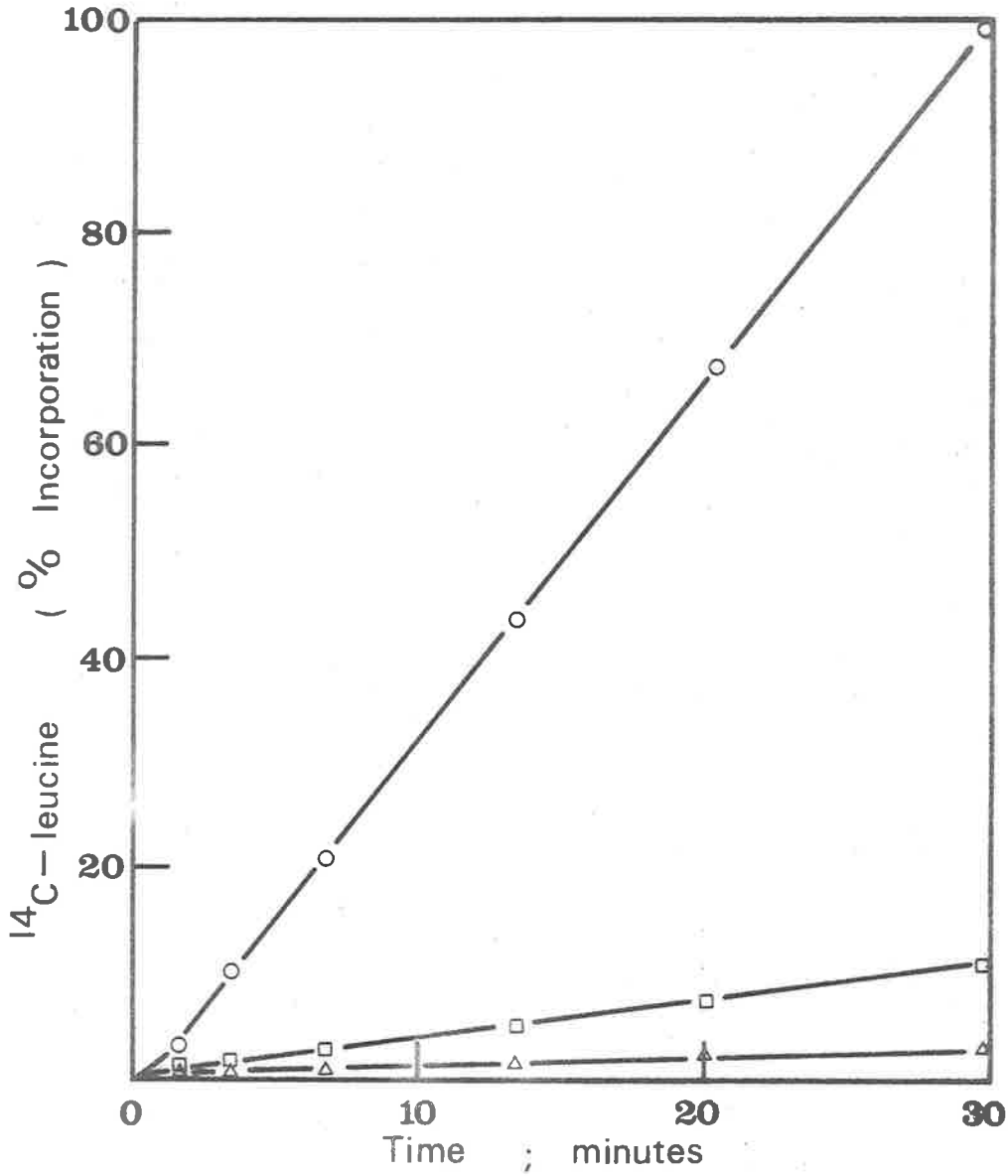


FIG. V.5. EFFECT OF ACTINOMYCIN D AND RIFAMPICIN ON ^{14}C-LEUCINE INCORPORATION BY WASHED CELLS.

Drugs and ^{14}C-leucine were both added at zero time.

—○—○— , no addition of drugs
—□—□— , actinomycin D (2 µg/ml)
—△—△— , rifampicin (0.5 µg/ml)

The 100% value for ^{14}C-leucine incorporation was 8,020 counts/min.

(iv) Effect of inhibitors of protein synthesis on protease production by washed-cell suspensions

The simplest explanation of the above findings is that phase 1 extracellular protease production represents secretion of preformed enzyme, while phase 2 represents de novo protease synthesis and secretion. However, studies with several specific inhibitors of protein synthesis suggest that phase 1 extracellular protease production is dependent on protein synthesis. Chloramphenicol (10 µg/ml.) immediately inhibits phase 1 protease production (Fig. V.6); furthermore, the actinomycin D insensitive appearance of extracellular protease is almost completely sensitive to chloramphenicol (Fig. V.6), thereby indicating a similar involvement of protein synthesis. In confirmation of this, fusidic acid (150 µg/ml.) and pactamycin (0.05 µg/ml.) completely inhibit phase 1 protease production (Fig. V.7).

There existed the possibility that these drugs might have been exerting a general toxic effect on the cells thereby preventing active secretion of the preformed enzyme rather than specifically inhibiting protein synthesis. Several lines of evidence suggest that they are not acting in such a non-specific manner. First, the chloramphenicol concentration which completely inhibits phase 1 protease formation does not affect incorporation of 2-^{14}C-uracil into RNA measured over a 90 min. incubation period. The second line of evidence concerns the production of protoplast-bursting

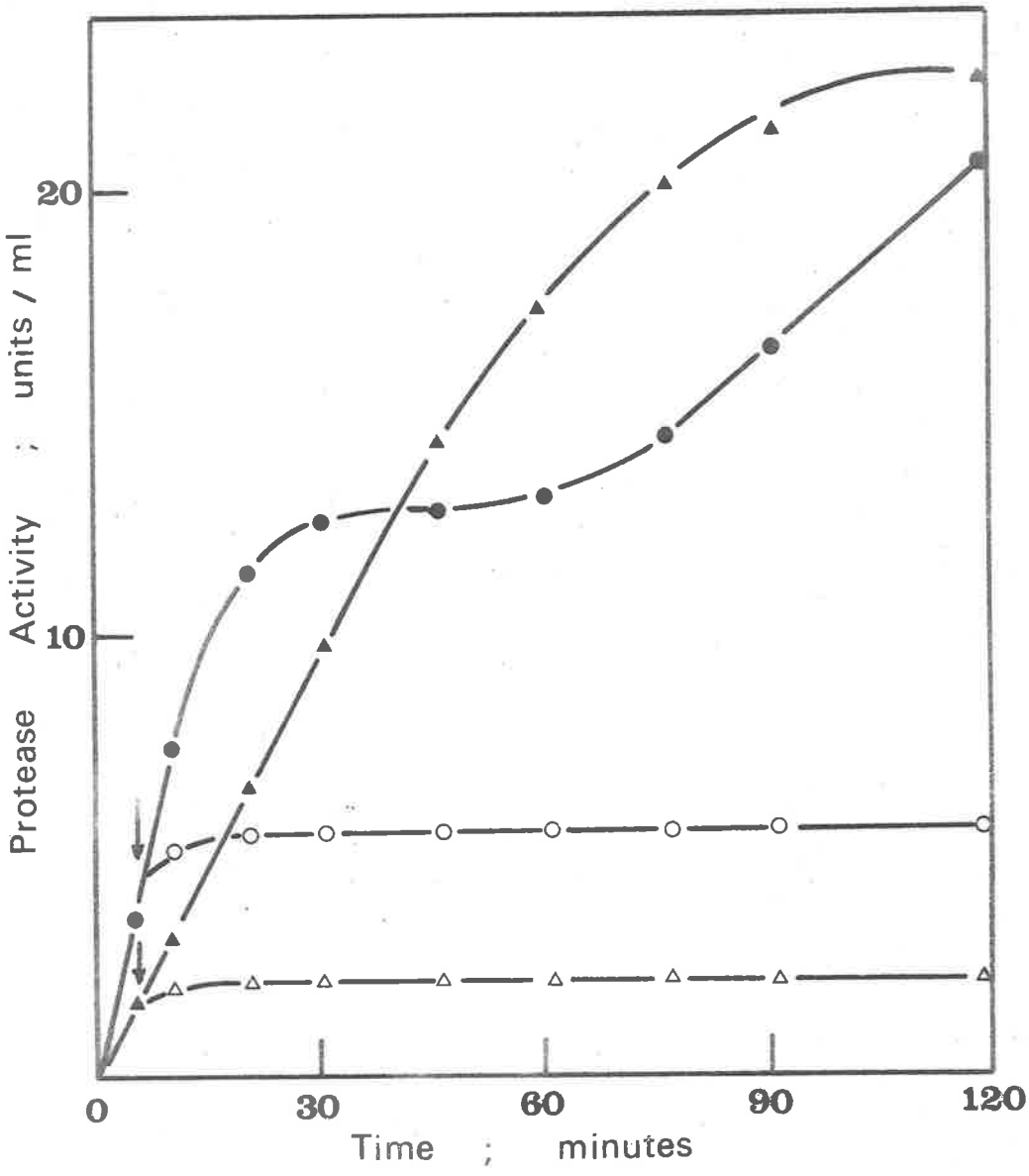


FIG. V.6. EFFECT OF CHLORAMPHENICOL ON PROTEASE PRODUCTION BY WASHED CELLS IN THE PRESENCE AND ABSENCE OF ACTINOMYCIN D.

Chloramphenicol was added (arrows) to cells after 5 min. preincubation. Actinomycin D was added at zero time.

—●—●— , no addition of drugs
—○—○— , chloramphenicol (10 µg/ml)
—▲—▲— , actinomycin D (2 µg/ml)
—△—△— , actinomycin D (2 µg/ml) + chloramphenicol (10 µg/ml.)

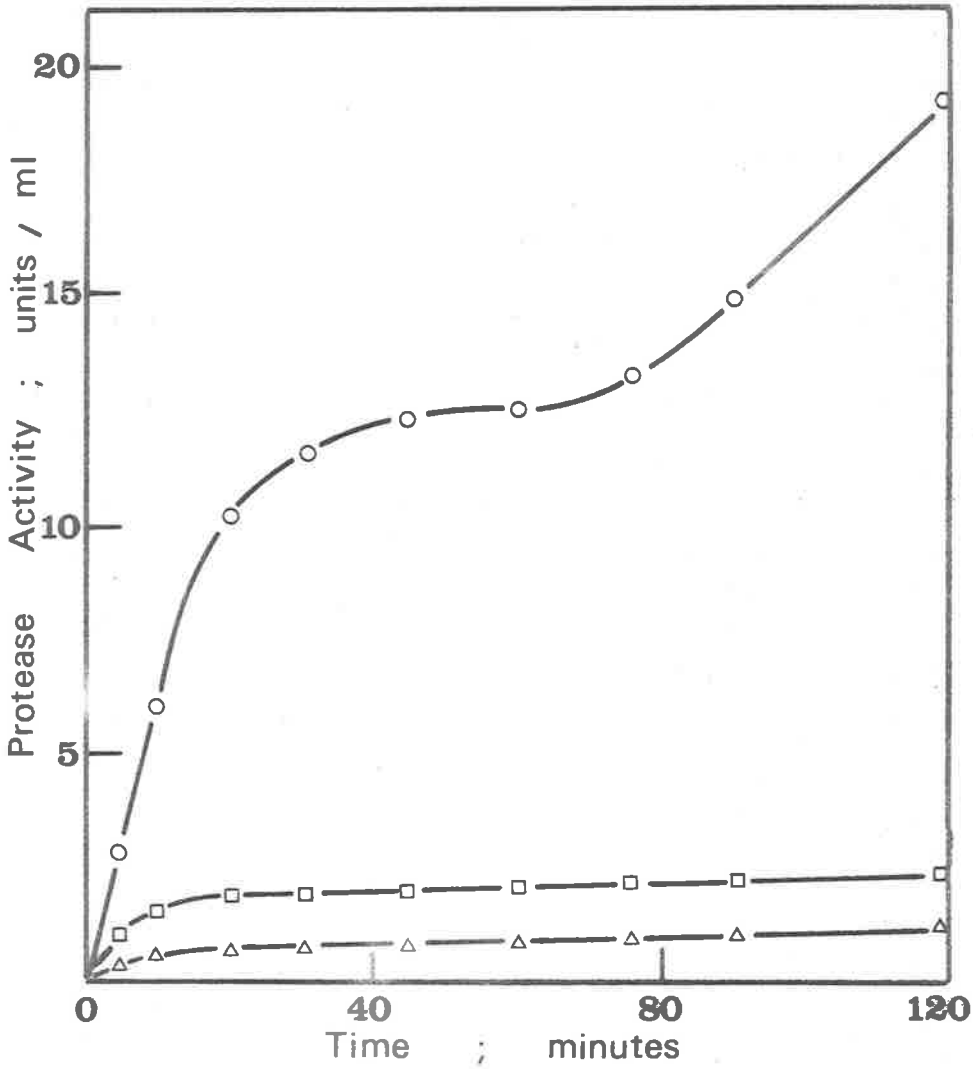


FIG. V.7. EFFECT OF FUSIDIC ACID AND PACTAMYCIN ON PROTEASE FORMATION BY WASHED CELLS.

—○—○— , no addition of drugs
—□—□— , pactamycin (0.05 µg/ml)
—△—△— , fusidic acid (150 µg/ml)

factor by B. amyloliquefaciens (May and Elliott, 1970). The production by a washed-cell suspension of this peptide-antibiotic is completely insensitive to chloramphenicol (10 µg/ml.) for greater than 60 min. Separate studies have shown that production of this antibiotic involves de novo synthesis of the molecule (B. May, Ph.D. Thesis). Both results imply that chloramphenicol is not having a general toxic effect on the cells.

Similarly, the effects of fusidic acid and pactamycin on protease formation are not due to non-specific toxicity. Fusidic acid (150 µg/ml.) and pactamycin (0.05 µg/ml.) selectively inhibit extracellular protease production without significantly impairing general protein synthesis as measured by ^{14}C-phenylalanine incorporation (Fig. V.7 and Fig. V.8). These results are incompatible with the antibiotics exerting general toxic effects. A possible explanation for this selective inhibition of extracellular protease formation lies in the proposed peripheral location of the sites for extracellular enzyme synthesis as discussed more fully later.

While these results suggest that de novo protein synthesis is required for phase 1 protease production, they do not conclusively establish de novo synthesis of the enzyme molecule itself. It may be, for example, that protein synthesis is merely needed for some other protein which is either required for the secretion of preformed enzyme or for some modification of the protease molecule. It became

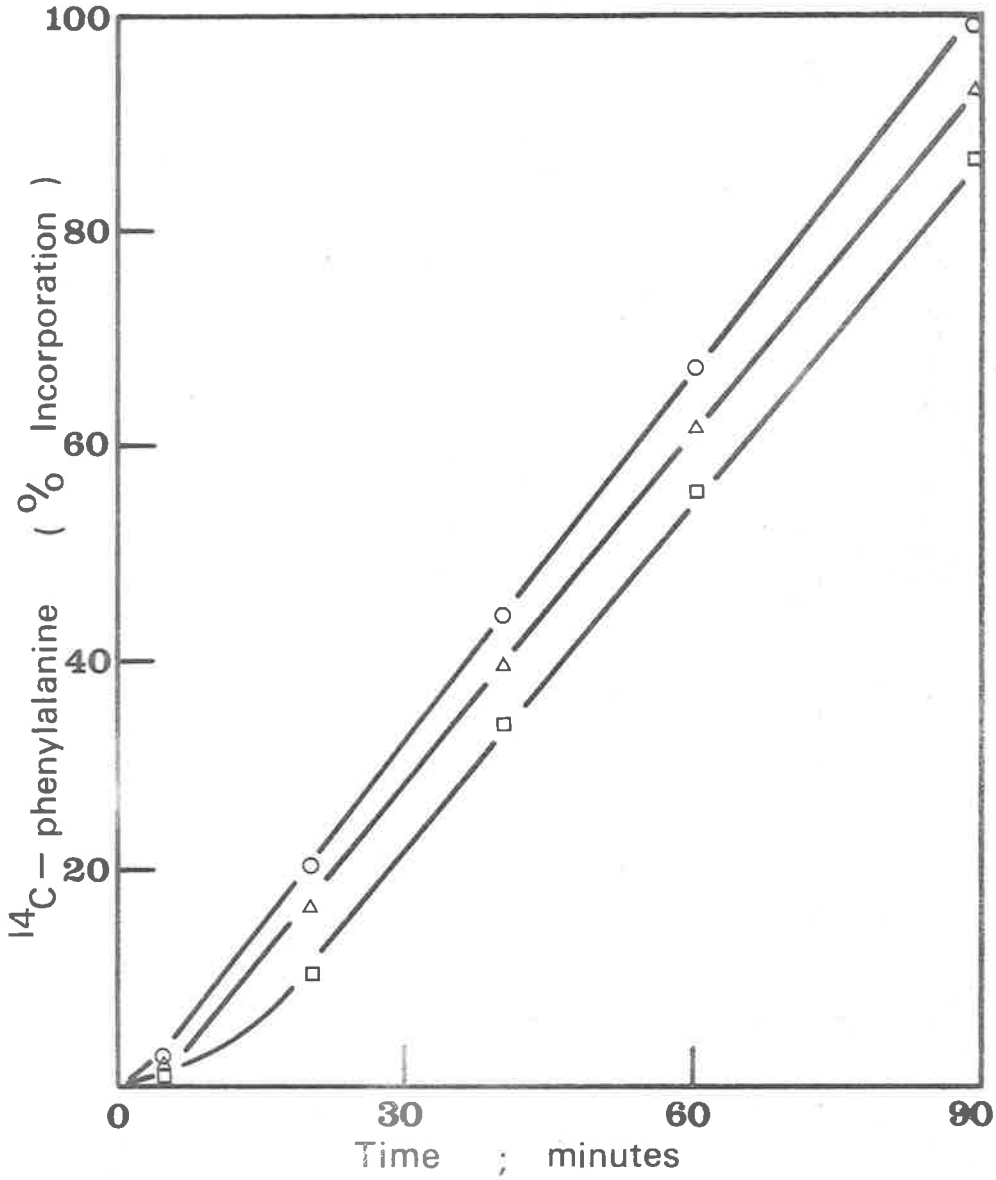


FIG. V.8. EFFECT OF FUSIDIC ACID AND PACTAMYCIN ON ^{14}C-PHENYLALANINE INCORPORATION BY WASHED CELLS.

Drugs and ^{14}C-phenylalanine were both added at zero time.

—○—○— , no addition of drugs
—△—△— , pactamycin (0.05 µg/ml)
—□—□— , fusidic acid (150 µg/ml)

The 100% value for ^{14}C-phenylalanine incorporation was 2,5[illegible] counts/min.

essential therefore to establish de novo synthesis of the enzyme molecule itself by appropriate labelling studies and these are reported below.

C. DE NOVO SYNTHESIS OF EXTRACELLULAR PROTEASE OCCURRING IN THE PRESENCE OF RIFAMPICIN OR ACTINOMYCIN D

To investigate whether the protein synthesis necessary for rifampicin-insensitive protease production involved de novo synthesis of the enzyme molecule itself, a ^{14}C-leucine labelling study of the protease was carried out. The medium chosen for this study contained, instead of 0.5% Casamino acids, a synthetic amino-acid mixture, the composition of which was identical to that of Casamino acids as found by amino-acid analysis, except that the leucine concentration was lowered to 24 µg/ml. Separate experiments showed that in this medium, protease production was identical to that in a medium containing 0.5% Casamino acids and that protease synthesis was again insensitive to rifampicin (0.5 µg/ml.). It was also shown that at this concentration of rifampicin, general protein synthesis (as measured by ^{14}C-leucine incorporation) was completely and almost immediately inhibited.

The experimental procedure for labelling was as follows. Washed cells were suspended in the above medium and incubated with shaking in the presence of rifampicin for

20 min. before 60 μCi of ^{14}C-leucine (spec. act. 316 mCi/ m-mole) was added. The 20 min. pre-incubation period was used to permit decay of mRNA species with a 'normal' short life-time. A separate experiment showed that the addition of extra leucine added as the source of ^{14}C had no effect on further protease production. After a further 60 min. incubation the cells were removed. To determine the amount of protease formed during this time-period a duplicate flask containing unlabelled leucine was incubated and the protease activity in the supernatant determined at 20 and 80 min. Unlabelled leucine (4 mg.) was added to the radioactive sample which was then dialysed for 2 hr. against 0.01 M Tris buffer (pH 7.6) containing 10 mM calcium acetate. As a source of carrier protease, the supernatant from a 30 hr. culture of B. amyloliquefaciens was dialysed similarly and added to the radioactive sample. The total enzyme activity in the mixture was determined.

This combined preparation was then fractionated on an SE-Sephadex column (Fig. V.9). A peak of labelled protease activity was separated from α-amylase and ribonuclease (also present in the carrier sample) and this protease was shown by polyacrylamide gel electrophoresis to correspond to the neutral species. The alkaline protease activity was found not to be associated with this peak, presumably due to its separation from the major protease or to its

FIG. V.9.

FRACTIONATION OF PROTEASE BY SE-SEPHADEX COLUMN CHROMATOGRAPHY.

Radioactive sample from labelling experiment (see text for details), containing 8.1 units of potentially labelled protease, was added to carrier protease (12,900 units) and loaded onto an SE-Sephadex column (2.8 cm x 48 cm). The column, equilibrated with 0.02 M KH_2PO_4/NaOH buffer (pH 6.7) containing 0.5mM calcium acetate, was eluted with 300 ml of this buffer and then with the same buffer containing 0.2 M NaCl (see arrows). The column was run at 4° at a flow rate of 14 ml/hr and 9 ml fractions were collected.

Radioactivity in the fractions were determined by taking 0.05 ml samples and adding them to 2.5 ml of Bray's scintillation fluid. The protease activity in the fractions was detected by a semi-quantitative assay which involved the clearing of a milk solution; a sample of the fraction was added to 5.0 ml of 1% aqueous solution of commercial powdered skim milk, incubated at 37° and the A_{600nm} was then read.

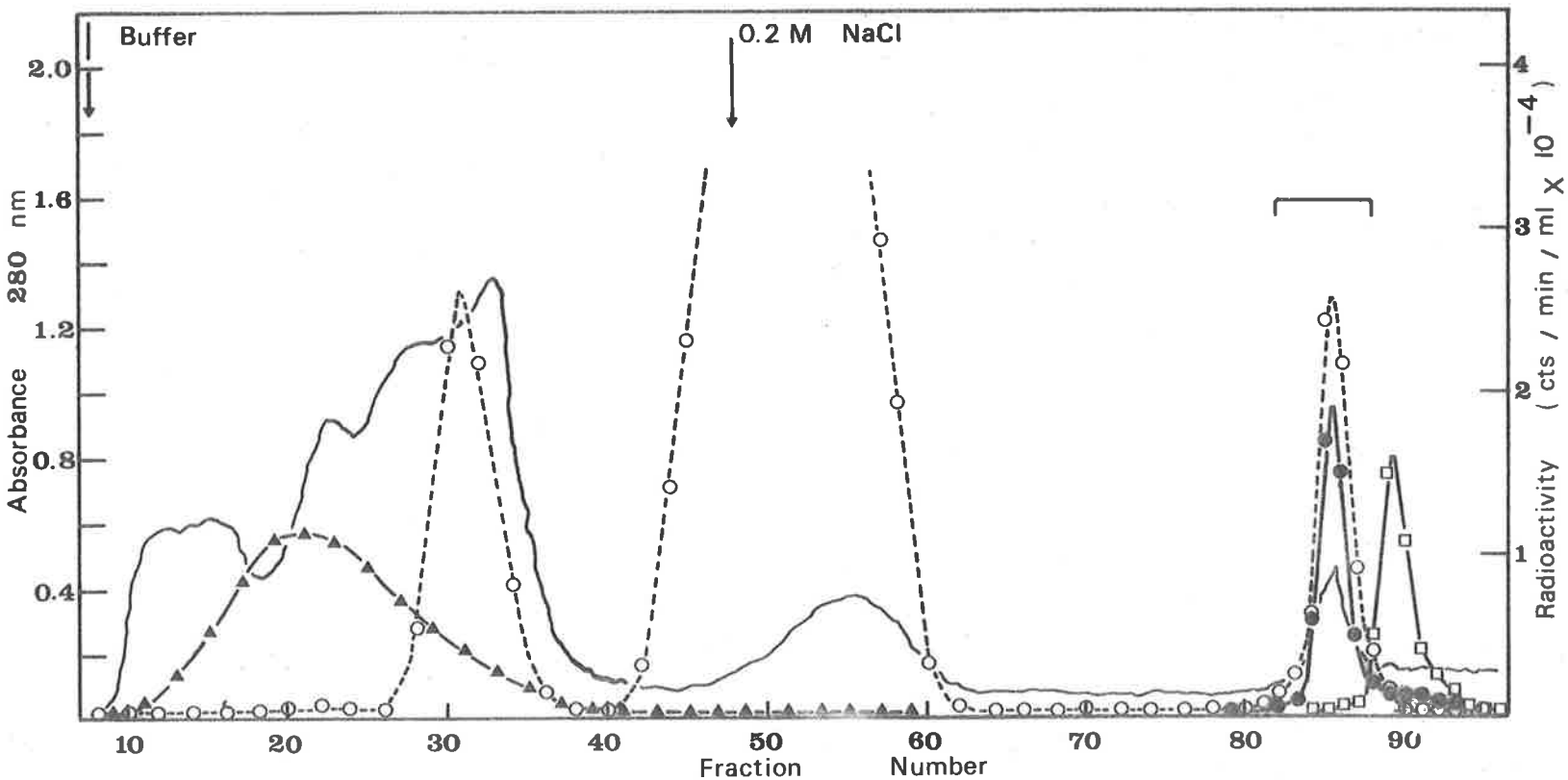
---○---○-- , Radioactivity.

—●—●— , protease activity. Fractions 82 to 88 contained a total of 11,330 units as measured by the casein digestion method.

—▲—▲— , α-amylase activity. Fractions 10 to 40 contained a total of 40,000 units of activity.

—□—□— , ribonuclease activity. Fractions 87 to 95 contained a total of 550 units of activity.

(——) , absorbance at 280 nm monitored by an LKB Uvicord II instrument.


Buffer
0.2 M NaCl
Absorbance 280 nm
2.0
1.6
1.2
0.8
0.4
Radioactivity (cts / min / ml X 10^{-4})
4
3
2
1
10
20
30
40
50
60
70
80
90
Fraction Number

inactivation on the column. The radioactivity found in fractions 41 to 61 was shown to be due to free ^{14}C-leucine whilst the labelled material in fractions 26 to 38 has not yet been identified. It was found that the peak of radioactivity in the protease region ran slightly behind the protease activity. This effect was confirmed and amplified by re-running the appropriate pooled fractions (82 to 88) on a Sephadex G75 column (Fig. V.10).

Fractionation on this column resulted in two labelled peaks; the first of these (fractions 22 to 31) showed protease activity again running slightly ahead of the radioactivity peak. Standard polyacrylamide gel electrophoresis at pH 8.3 (see Chapter II) did not reveal any protein bands from this peak. However, at pH 8.5 with the electrodes reversed, two bands were obtained thus confirming the presence of two protein species under this area; one corresponding to the neutral protease and the other to an unknown species. The additional labelled peak (fractions 37 to 45), showing no protease activity, gave one protein band on polyacrylamide gel electrophoresis at pH 8.5. The trace of ribonuclease present in the pooled protease fractions from the SE-Sephadex column was now completely separated from protease and detected in fractions 31 to 34.

To separate the two protein species in the main protease peak, the appropriate pooled fractions (22 to 31) from the Sephadex G75 column were fractionated by affinity

FIG. V.10.

FRACTIONATION OF PROTEASE BY SEPHADEX G75 COLUMN CHROMATOGRAPHY.

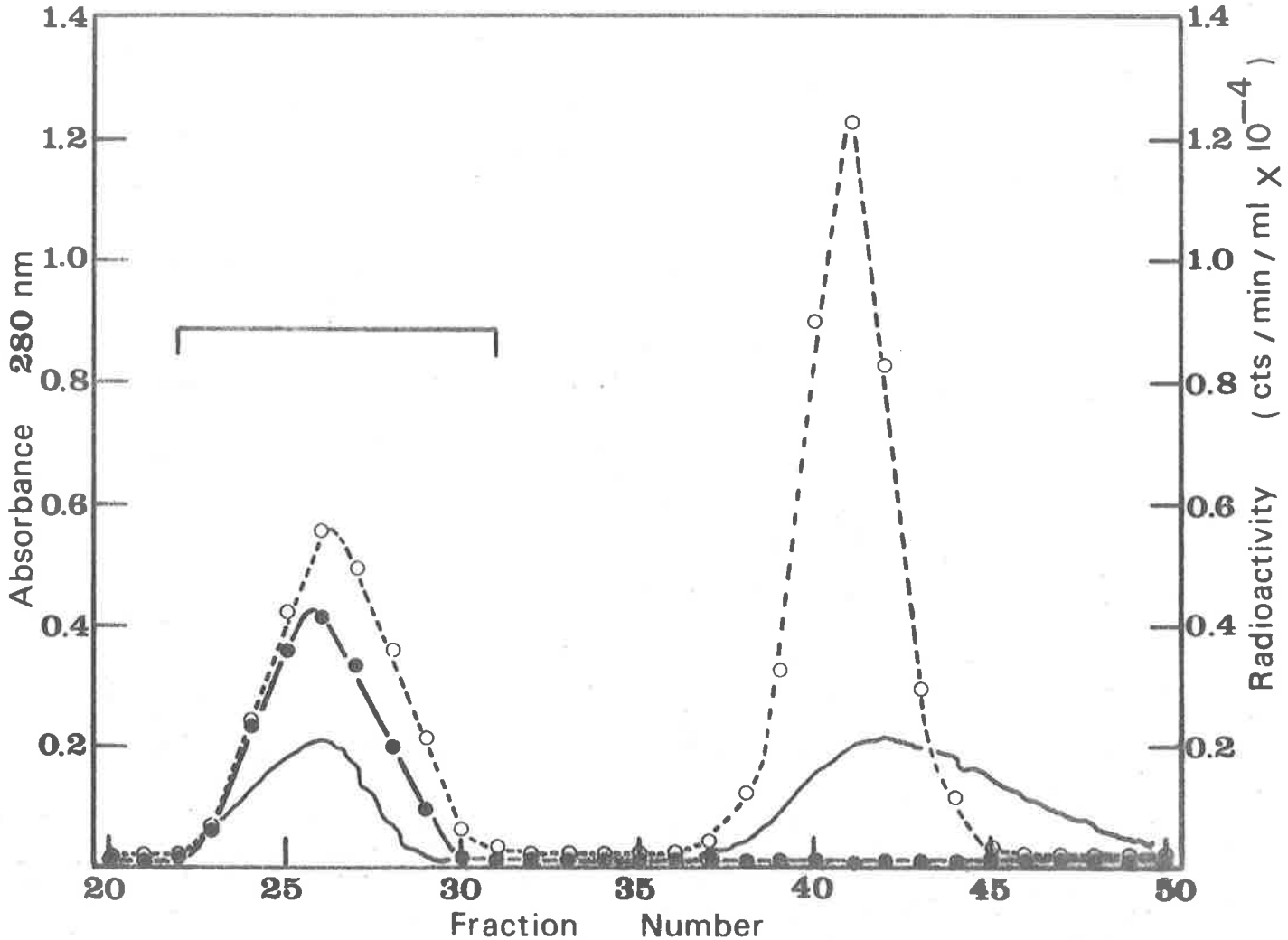
Fractions 82 to 88 from the SE-Sephadex column were pooled, dialysed for 2 hr. against water containing 10 mM calcium acetate and freeze-dried. The freeze-dried sample in KH_2PO_4/NaOH buffer (pH 6.7) containing 0.5 mM calcium acetate, was loaded onto a Sephadex G75 column (2 cm x 98 cm) and eluted with the same buffer. The column was run at 4° with a flow rate of 14 ml/hr. and 4 ml fractions were collected.

---O----O--- , Radioactivity

—●—●— , protease activity (measured as stated in Fig. V.9)

Fractions 22 to 31 contained a total of 3,540 units of activity .

(———) , absorbance at 280 nm.


1.4
1.2
1.0
0.8
0.6
0.4
0.2
Absorbance 280 nm
Radioactivity (cts / min / ml X 10^{-4})
20
25
30
35
40
45
50
Fraction Number

chromatography on a Sepharose/casein column prepared by the method of Cuatrecasas et al. (1968). The profile obtained is shown in Fig. V.11. Two peaks of radioactivity were obtained. The first of these contained the unknown protein (as confirmed by polyacrylamide gel electrophoresis) which was devoid of protease activity while the second peak of radioactivity coincided with a peak of protease activity. The latter peak gave only one protein band on polyacrylamide gel electrophoresis with the electrodes reversed at pH 8.5 (Fig. V.12). (No band was detected under standard gel conditions at pH 8.3). Corresponding areas of radioactivity and protease activity were associated with this protein band, as determined by slicing gels run under the same conditions (Fig. V.13).

The main peak of protease from the Sepharose/casein column contained 3,450 units of protease and 57,460 counts/min. of radioactivity. It was possible to calculate from this the proportion of the protease which had been synthesised de novo from ^{14}C-leucine. First it was established by acid hydrolysis of a sample of the protease, followed by amino-acid separation on the column of a Beckman amino-acid analyser connected to a fraction collector, that the ^{14}C in the protease was almost exclusively in leucine and no other amino acid. The protease produced during the rifampicin experiment between 20 and 80 min. was 8.1 units; the specific activity of crystalline protease is 5,000 units/mg. of protein (Carey,

FIG. V.11.

AFFINITY CHROMATOGRAPHY OF PROTEASE ON SEPHAROSE/CASEIN.

Fractions 22 to 31 from the Sephadex G75 column were pooled and loaded onto a Sepharose/casein column (1.3 cm x 34 cm). The column was eluted with 70 ml of KH_2PO_4/NaOH buffer (pH 6.7) containing 0.5 mM calcium acetate, followed by 80 ml of this buffer containing 0.4 M NaCl and then finally 320 ml of buffer containing 0.8 M NaCl (see arrows). The column was run at 4° with a flow rate of 25 ml/hr. and 5 ml fractions were collected.

--O---O-- , Radioactivity

—●— ●— , protease activity (measured as stated in Fig. V.9.)

Fractions 40 to 54 contained a total of 3,450 units of activity.

(——) , absorbance at 280 nm.

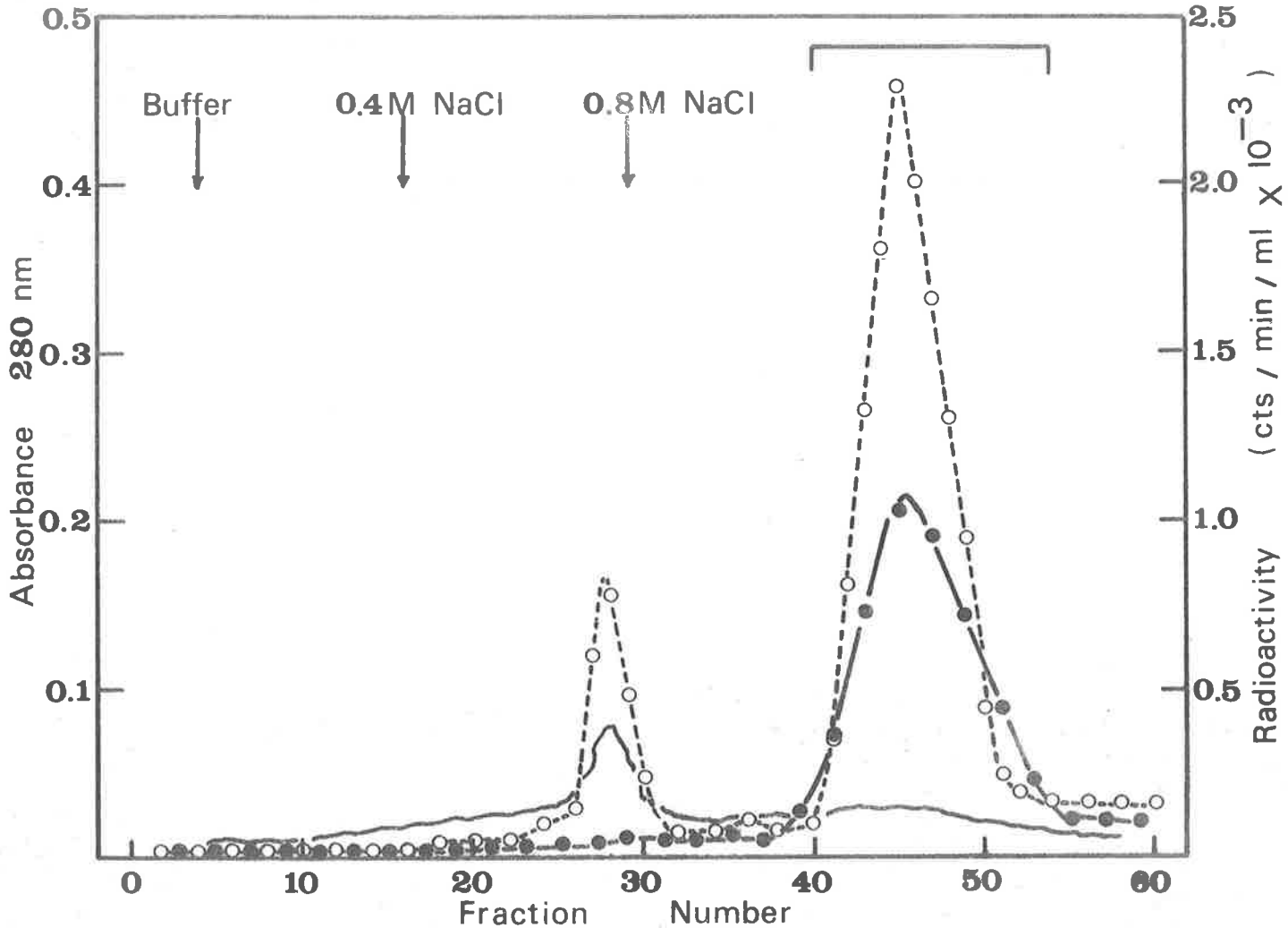

Buffer
0.4M NaCl
0.8M NaCl
Absorbance 280 nm
Radioactivity (cts / min / ml X 10^{-3})
Fraction Number
0.5
0.4
0.3
0.2
0.1
2.5
2.0
1.5
1.0
0.5
0
10
20
30
40
50
60

FIG. V.12.

PROTEIN BAND OBTAINED UPON POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEASE.

Fractions 40 to 54 from the Sepharose/casein column were concentrated by freeze-drying, dialysed and 20 µl. samples were electrophoresed at pH 8.5 with the electrodes reversed. Protein was detected by staining the gel with Coomassie brilliant blue. Identical gels were analysed for protease activity and radioactivity (see Fig. V.13). The anode is at the top of the photograph.

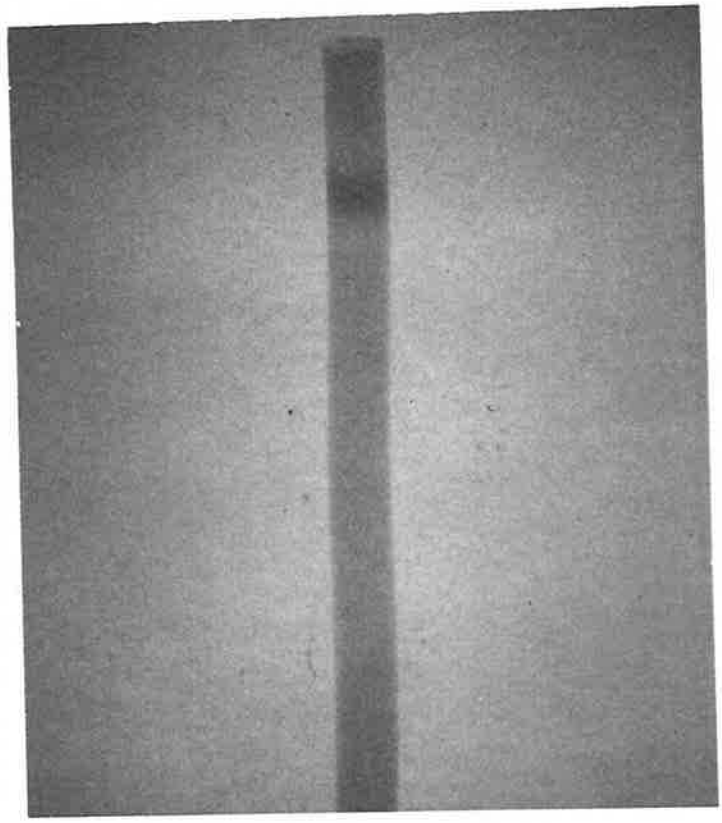


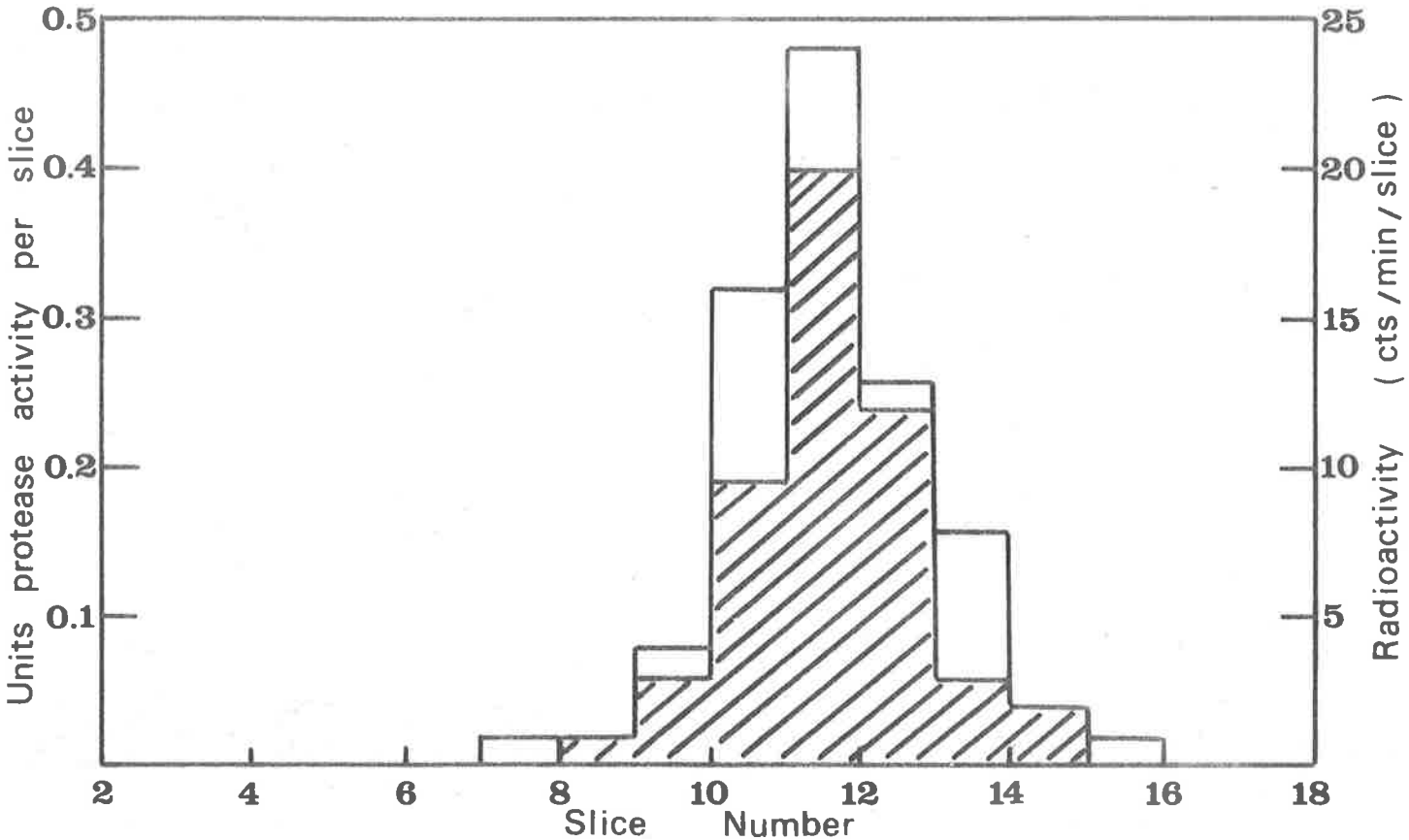
FIG. V.13.

PROFILES OF PROTEASE ACTIVITY AND RADIOACTIVITY OF POLYACRYLAMIDE GELS RUN AS DESCRIBED IN FIG. V.12.

Frozen gels were sliced into 1 mm sections using a Mickle gel slicer; protease activity in these slices was eluted with 0.05 M tris-HCl buffer (pH 7.8) for 6 hr. at 4° and determined by the ultrasensitive assay using the Remazo brilliant Blue/Hide substrate (see Chapter II). To determine ^{14}C radioactivity, gel slices were first digested with 1 M NH_4OH for 3 hr. at 60° in scintillation vials. After the addition of a glass-fibre filter paper, each vial was dried overnight at 110°. Scintillation fluid was added and vials were counted for 10 min.

Unshaded area, radioactivity (counts/min. above background).

Shaded area, protease activity.


0.5
0.4
0.3
0.2
0.1
Units protease activity per slice
25
20
15
10
5
Radioactivity (cts / min / slice)
2
4
6
8
10
12
14
16
18
Slice Number

unpublished results). The molecular weight of the enzyme determined by Sephadex chromatography fell in the range 25,000 to 30,000 and amino-acid analysis yielded a minimum molecular weight of 27,900 with 15 leucine residues per unit weight (Carey, unpublished results). The specific activity of the leucine was 129 mCi/m-mole and the counting efficiency determined directly was 84%. From this it can be calculated that the total expected radioactivity in the fully labelled enzyme allowing for a recovery of 26.7% is 58, 460 counts/min. This compared favourably with the observed result of 57,460 counts/min. (98.3% of theory).

In a repeat experiment carried out in an identical way, the enzyme recovery was 13.3%; the observed counts per minute were 35,460 as against an expected 32,010 counts/min. (110.8% of theory) for fully labelled enzyme.

In this calculation the assumptions are made that the specific activity of the ^{14}C-leucine remained unchanged during the experiment (the results show that no distribution of label to other amino acids occurred) and also that there was no difference in properties between protease synthesised in the presence of rifampicin and carrier protease such that a preferential loss of the latter occurred.

Confirmatory evidence that the protease produced in the absence of mRNA production was de novo synthesised was given by a similar labelling experiment but the protease was separated by Sepraphore polyacetate strip electrophoresis.

Washed cells were suspended in the previous medium except that leucine was restored to its normal concentration while the valine concentration was lowered to 40 µg/ml. (Separate experiments again showed that in this medium, protease production was normal and was insensitive to actinomycin D (2 µg/ml.) In addition, at this concentration of actinomycin D, general protein synthesis, as measured by ^{14}C-valine incorporation, was completely and almost immediately inhibited.)

The cell suspension was incubated with shaking in the presence of actinomycin D (2 µg/ml.) and 25 µCi ^{14}C-valine (spec. act. 260 mCi/m-mole) for 60 min. To determine the amount of protease formed during this time-period, a duplicate flask containing unlabelled valine was incubated at the same time and the protease activity in the supernatant after the 60 min. incubation was determined. Unlabelled valine (5 mg) was added to the radioactive sample which was dialysed as above. After dialysis, 20 µl samples of the crude enzyme were electrophoresed on Sepraphore strips (see Chapter II).

The major protease band was cut out from identical strips and used for radioactivity and protease determinations. The resulting profile (Fig. V.14) shows a peak of protease activity (0.13 units) with a corresponding area of radioactivity (3,725 counts/min.). The observed counts/min. amounted to 93.4% of that calculated for fully labelled

FIG. V.14.

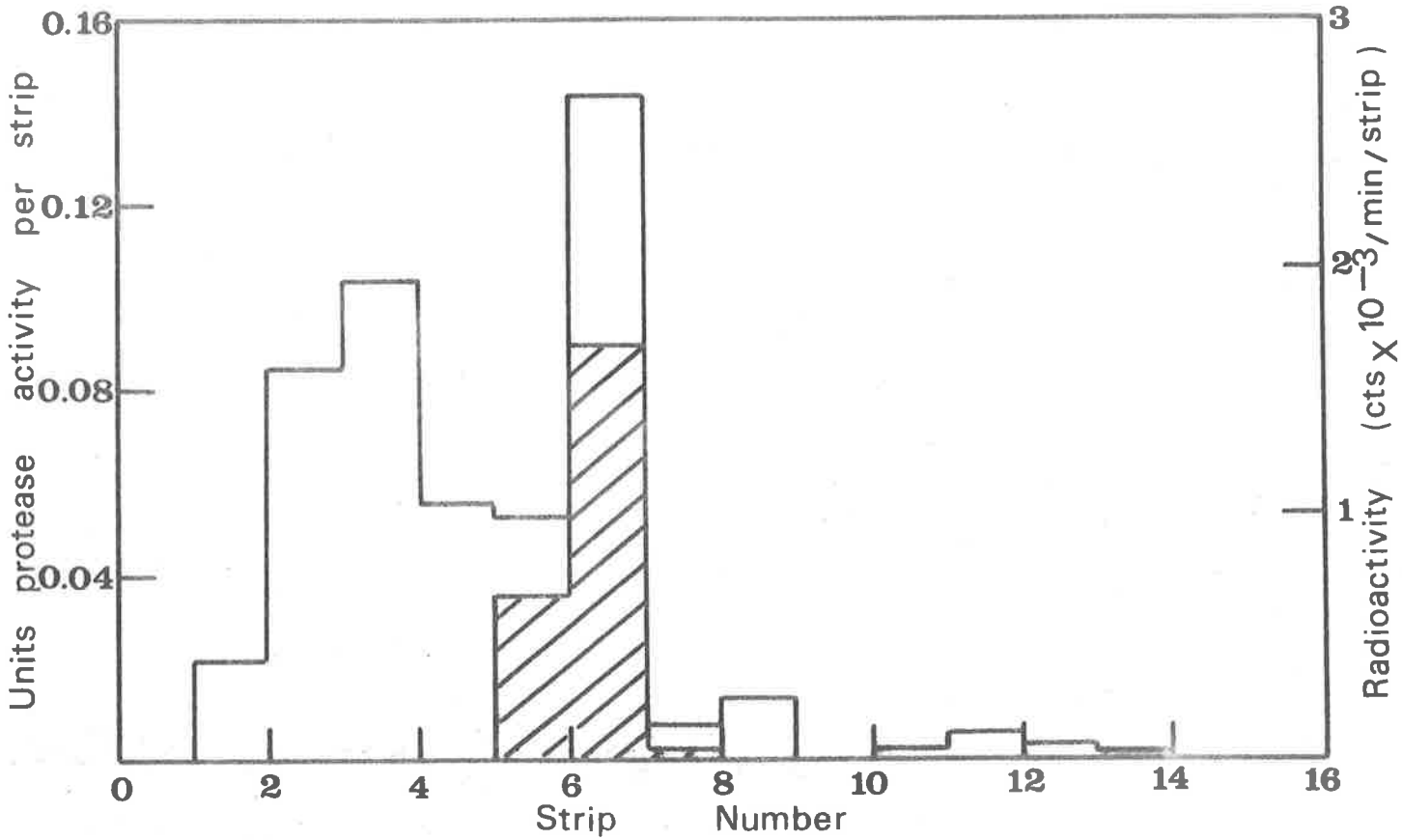
PROFILES OF PROTEASE AND RADIOACTIVITY OF SEPRAPHORE POLYACETATE STRIPS

The radioactive sample from the valine labelling experiment (see text for details) was dialysed and 20 µl. samples were electrophoresed on Sepraphore strips (see Chapter II).

The strips were cut into small pieces (0.3 cm); protease activity in these pieces was eluted with 0.05 M tris-HCl buffer (pH 7.8) for 1.5 hr at 4° and determined by the ultrasensitive assay using the Remazo brilliant Blue/Hide substrate (see Chapter II).

To determine ^{14}C-radioactivity, strips were added to scintillation vials and dried for 3.5 hr. at 55°. Scintillation fluid was added and vials were counted.

Unshaded area, radioactivity (counts/min. above background).
Shaded area, protease activity.


0.16
0.12
0.08
0.04
Units protease activity per strip
3
2
1
Radioactivity (cts X 10⁻³/min/strip)
0
2
4
6
8
10
12
14
16
Strip Number

enzyme. (Protease contains 25 valine residues per unit weight (Carey, unpublished results); the spec. act. of the valine was 48 mCi/m-mole and 8.2 units of protease were produced during the 60 min. experiment.)

A further experiment along similar lines in which ^{14}C-leucine was used, established that the radioactivity associated with the protease was not non-specifically adsorbed. No radioactivity was observed in a control experiment where the isotope was added at the end of the incubation while the experimental sample showed the expected incorporation.

Hence, it is concluded from these studies that prolonged production of protease by cells of B. amyloliquefaciens harvested at 25 hr., occurs in the almost complete absence of mRNA synthesis and that this involves de novo synthesis of the enzyme molecule. The labelling studies in which ^{14}C-label was incorporated into protease exclude the possibility that, for example, protein synthesis is needed merely to secrete preformed enzyme. The fact that the ^{14}C-labelling of the enzyme occurred after the cells had already been pre-incubated with rifampicin for 20 min. to allow 'normal' mRNA to decay, clearly demonstrated that an unusual situation exists in which preformed mRNA can support synthesis of the enzyme for as long as 80 min.

D. STUDIES RELATED TO THE STABILITY AND THE ACCUMULATION OF THE MESSENGER RNA POOL SPECIFIC FOR EXTRACELLULAR PROTEASE

As discussed earlier, the synthesis of protease, while biphasic in the presence of high levels of amino acids, proceeds almost linearly in the presence of low levels of amino acids. As the level of amino acids in the medium increases, the deviation from linearity becomes progressively more pronounced (May and Elliott, 1968a). The most likely explanation of this was that high levels of amino acids repress transcription of the protease gene for 80 min. and that only after this time is the amino-acid level reduced by metabolism to a point where transcription starts. To directly test this idea, 0.5% Casamino acids were added to cell incubations after 90 min. However, no repression of protease formation was observed and it must be further postulated that cells after phase 1 are resistant to amino-acid repression. This is not unreasonable since such cells may be able to maintain lowered intra-cellular levels of amino acids by virtue of their increased utilisation or metabolism. In apparent confirmation of this, when cells after 90 min. incubation are rapidly resuspended without washing in fresh medium, a linear production of protease is observed rather than a biphasic time-course.

In keeping with amino acids exerting their effect at the transcriptional level, it has been found that the

apparent pool of mRNA is maintained for a longer period in the presence of a low level of amino acids. When rifampicin is added to cells which have been incubated in the high amino-acids medium for 75 min. no further protease production occurs (Fig. V.15). However, a significant amount of protease formation is observed after rifampicin is added at 75 min. to cells which were initially resuspended in a low amino-acids medium (Fig. V.15). This strongly suggests that the amino-acid level determines the level of protease mRNA in the cells under these conditions; that is, the pool of mRNA disappears faster in a high amino-acids medium than in a low one. This is not withstanding the fact that protease production during the first 75 min. incubation is somewhat greater in low amino acids than in the higher amino-acids medium.

By contrast, when rifampicin (or actinomycin D) is added to cells at zero time in high or low amino-acids medium, the production of protease is the same in the two cases; therefore, protease synthesis, dependent on pre-existing mRNA, is totally independent of the level of amino-acids in the medium under these conditions (Fig. V.15). This is entirely compatible with the amino acids acting at the level of transcription, as predicted earlier. (It is to be noted that amino-acid repression occurs only with protease and not with the other two extracellular enzymes.)

Separate experiments suggest that it takes more

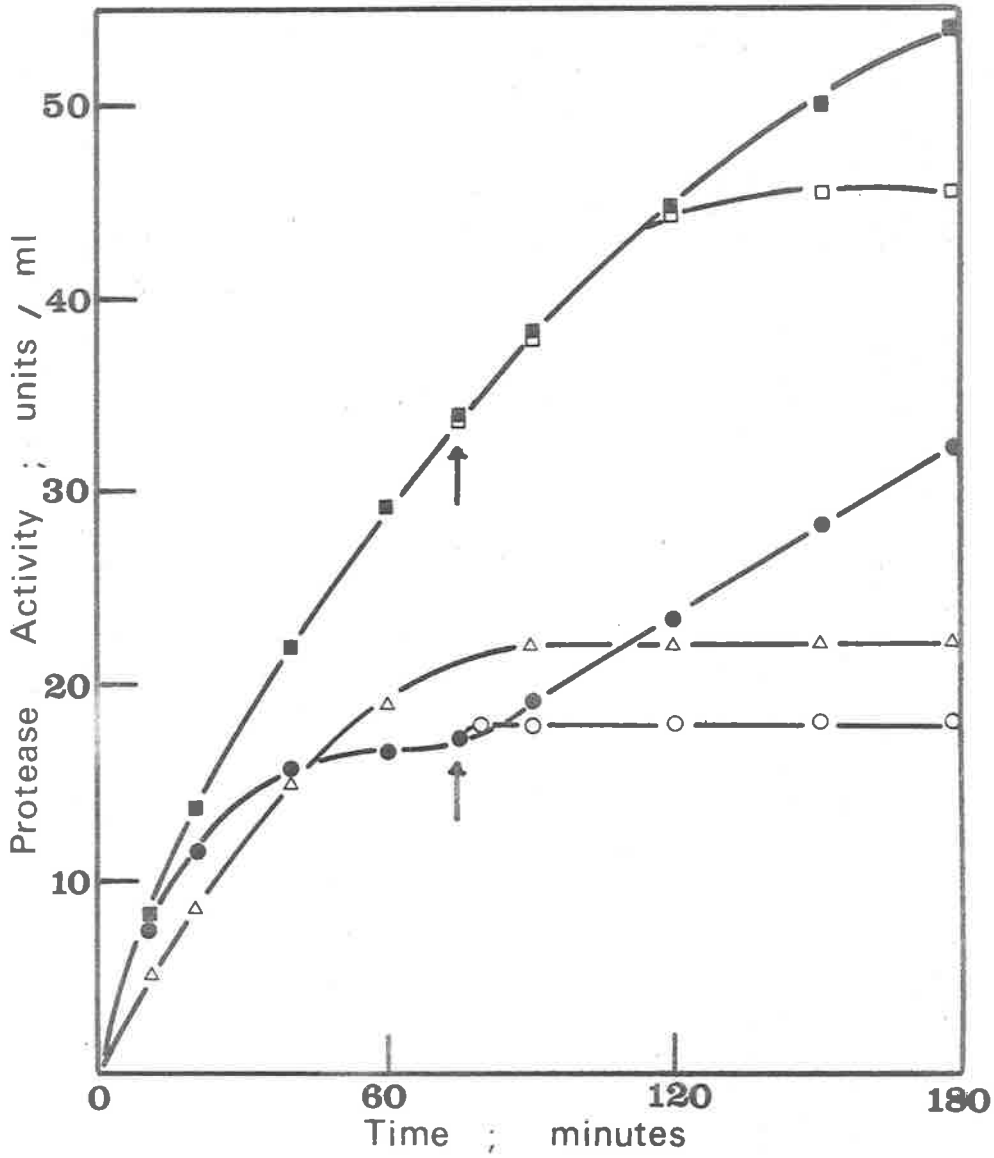


FIG. V.15. EFFECT OF RIFAMPICIN ADDITION AT 0 AND 75 MIN. ON PROTEASE PRODUCTION BY WASHED CELLS IN THE PRESENCE OF LOW AND HIGH AMINO ACID LEVELS.

—■—■— , 0.025% Casamino acids - no addition of drug

—□—□— , 0.025% Casamino acids - rifampicin (0.5 µg/ml) added at 75 min.

—●—●— , 0.5% Casamino acids - no addition of drug

—○—○— , 0.5% Casamino acids - rifampicin (0.5 µg/ml) added at 75 min.

—△—△— , 0.5% Casamino acids - rifampicin (0.5 µg/ml) added at 0 min. An identical curve was obtained in 0.025% Casamino acids medium.

than three hours to completely deplete the pool of protease mRNA when cells are suspended in the low amino-acids medium. Washed cells were incubated for different time periods in the low amino-acids medium and after centrifugation, were resuspended in high amino-acids medium containing rifampicin (0.5 µg/ml.). The resulting rifampicin-insensitive protease production was used as an indication of the level of messenger RNA pre-existing inside the cells. The result (Table V.1) shows that the rifampicin-insensitive protease production was reduced by only 26% during 75 min. incubation in low amino acids as compared with a loss of 81% after a 180 min. incubation period.

Some preliminary experiments have also been carried out to determine the rate and the stage at which the protease mRNA accumulates in the cell culture. A comparison was made of the rifampicin-insensitive protease production by different aged cell cultures. Cells were washed twice and resuspended in the high amino-acids medium containing rifampicin (0.5 µg/ml.) The resulting cell densities were adjusted to that of 25 hr. cells ($A_{600\ nm}$ 3.6). The resulting rifampicin-insensitive protease production was used as an indication of the level of messenger RNA pre-existing inside the different aged cells at the time of harvesting. (Control studies were carried out to ensure that the above rifampicin concentration was not toxic to those cultures harvested in the early stages of cell growth.) The results (Table V.2) suggest that the

TABLE V.1. RIFAMPICIN-INSENSITIVE PROTEASE PRODUCTION FOLLOWING INCUBATION FOR DIFFERENT TIME PERIODS IN LOW AMINO-ACIDS MEDIUM

Incubation time period in low amino-acids medium	Protease (units/ml.)	Percentage
0 min.	19.2	[100]
75 min.	14.2	74
180 min.	3.7	19

The harvested and washed cells (Chapter II) were incubated for the stated time periods in low amino-acids medium and after centrifugation, were resuspended in a high amino-acids medium containing rifampicin (0.5 µg/ml.); protease production over a period of 100 min. was measured by the casein-digestion method.

TABLE V.2. RIFAMPICIN-INSENSITIVE PROTEASE PRODUCTION BY DIFFERENT AGED CELL CULTURES

Cell culture age (hr.) at time of harvest	Protease (units/ml.)	Percentage
20	7.0	36
22	11.2	58
25	19.2	[100]
28	19.2	100

The different aged cells were harvested, washed twice (Chapter II) and resuspended in the high amino-acids medium containing rifampicin (0.5 µg/ml.); protease production over a period of 100 min. was measured by the casein-digestion method.

accumulation of the protease mRNA pool is a gradual one since significant levels are present several hours earlier when cell growth is proceeding most rapidly. It appears that once the cells have reached late logarithmic phase (25 hr.) that the maximum mRNA pool size has been attained.

E. DISCUSSION

The results conclusively show that phase 1 of protease production, in the presence of a high level of amino acids, is insensitive to rifampicin or actinomycin D and involves *de novo* synthesis of the enzyme. In marked contrast, phase 2 synthesis is almost immediately inhibited by the addition of rifampicin. Several possibilities exist to explain these findings.

It is conceivable that the protease synthesised during phase 1 has a long-lived mRNA while that synthesised during phase 2 is a different protein with a normal short-lived mRNA. This possibility seems unlikely since the enzymes produced in the two phases co-electrophorese on Sepraphore strips; however, rigorous exclusion of this possibility would require more complete characterisation of the proteins. A second and most unlikely possibility is that the same protease is synthesised during the two phases, but that its mRNA is more stable in phase 1 than phase 2; no precedent for such a change is known in bacteria.

The most likely interpretation of these results is that late logarithmic phase cells of B. amyloliquefaciens contain a pool of preformed mRNA capable of sustaining synthesis of extracellular protease for as long as 80 min. It is proposed that in the presence of a high level of amino acids, protease mRNA synthesis is repressed and phase 1 production consists of translating the pool of mRNA until it is exhausted. During this time, cell metabolism reduces the internal amino acid level to the point where derepression occurs at about 80 min. and protease gene transcription and mRNA translation results in phase 2 synthesis. Experiments in which rifampicin was added at 90 min. clearly show exhaustion of the mRNA pool and hence as expected, phase 2 synthesis is totally dependent on continued mRNA production.

Moreover, it seems that protease-specific mRNA is not intrinsically long-lived in the sense that it is able to support prolonged protein synthesis by being translated many more times than normal. This conclusion can be drawn from the experiments on phase 2 of protease synthesis; here the rate of enzyme production is almost as great as in phase 1, but addition of inhibitors of mRNA synthesis results in the rapid cessation of protease synthesis as is expected with bacterial mRNA molecules of short lifetime.

If by contrast, cells are suspended in low levels of amino acids, it would be predicted that continued transcription of the protease gene would occur and hence the accumulated

pool of mRNA known to be present in harvested cells would not be exhausted in 75 min. Such proved to be the case; when rifampicin was added at 75 min. to cells incubated in low amino acids, a significant pool of mRNA was found still to be present, even though the total production of protease during this time period is, in fact, greater than in high levels of amino acids. That the effect of amino acids is at the transcriptional level is confirmed by the finding that in the presence of rifampicin, protease production is identical in low and high amino acids media. The small stimulation of phase 1 protease production by rifampicin which is consistently observed after about 50 min. is not understood but could have a trivial explanation such as making available more intermediates for protease synthesis by eliminating competition from other mRNA species.

Several cases have been reported of the accumulation of specific mRNA in bacteria; an example is the case of ornithine transcarbamylase, in which the E. coli cells during arginine starvation, accumulate sufficient mRNA to support subsequent enzyme synthesis for 20 min. (McLellan and Vogel, 1970). Similarly, accumulation of histidine and tryptophan operon mRNA has been reported after starvation of cells for histidine (Venetianer, 1969) and tryptophan (Stubbs and Hall, 1968), respectively.

The accumulation of protease mRNA cannot be accounted for by an inability of the cells to synthesise protein due to

amino-acid depletion. Although the cell cultures at 25 hr. are nearing the end of their growth phase, cell multiplication is still occurring and moreover, extracellular enzyme synthesis is at its maximum rate and continues for at least a further five hr. at a rapid rate. The preliminary experiments reported here suggest that the protease mRNA pool is present several hours earlier when cell growth is proceeding rapidly and appears to be due to a gradual accumulation process. It is not known in this organism, whether the onset of protease production is in any way associated with the onset of sporulation as is postulated for B. subtilis (Mandelstam and Waites, 1968). (The level of protease production by B. amyloliquefaciens used here is of the order of 50 to 100 times greater than that in B. subtilis 168, commonly used for sporulation studies.)

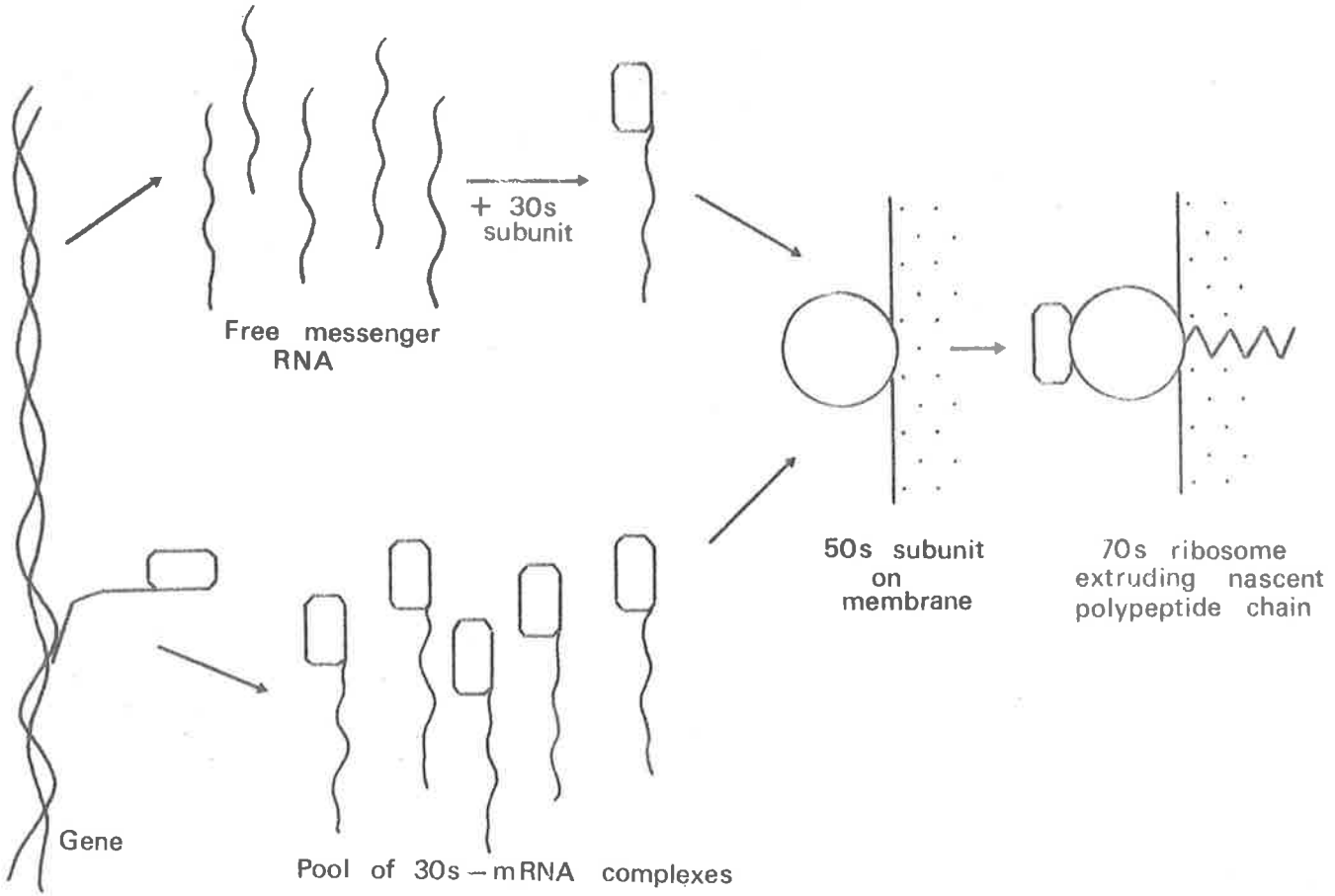
On the basis of these findings, it is proposed that the accumulation of the pool of protease mRNA is a repercussion of the mechanism of synthesis of extracellular enzymes. It was previously postulated (May and Elliott, 1968a) that extracellular enzyme synthesis by this organism occurs on ribosomes bound at special translational-extrusion sites located at the cell membrane. Synthesis and secretion would be a single inseparable event with the polypeptide chain being extruded directly through the membrane as it is synthesised to assume its three-dimensional form only outside the permeability

barrier. This hypothesis was based on indirect evidence (see Chapter I) and was analogous to that proposed for the endoplasmic reticulum in animal cells (Redman and Sabatini, 1966).

The observations of a highly selective inhibition of protease production by pactamycin and fusidic acid might be explained by the fact that the ribosomes synthesising this extracellular enzyme are peripherally localised and hence more sensitive to inhibition by low concentrations of drug than are cytoplasmic ribosomes. (Further studies related to this aspect will be reported in Chapter VII.) Such a peripheral location would mean that special problems exist. At present it is generally assumed that mRNA is translated while attached to the gene. Assuming that there is more than one protease translational-extrusion site per cell, as seems reasonable, translation of nascent mRNA seems unlikely in the present situation unless there exist many extrachromosomal plasmids with protease genes in contact with the extrusion sites. Attempts to find evidence for such plasmids in this organism have so far proved negative. Hence, if the translation-extrusion site hypothesis is correct, a unique situation must exist whereby mRNA would have to migrate from the chromosome to the membrane to be translated. If, in fact, such a migration occurs, a pool of mRNA could result from individual molecules having to 'queue-up' for a limited number of translational sites as shown in Fig. V.16; a

FIG. V.16.

PROPOSED ALTERNATIVE MODELS FOR THE ACCUMULATION OF A POOL OF PROTEASE MESSENGER RNA AND ITS SUBSEQUENT TRANSLATION AT THE MEMBRANE.


+ 30s subunit
Free messenger RNA
50s subunit on membrane
70s ribosome extruding nascent polypeptide chain
Gene
Pool of 30s – mRNA complexes

positive imbalance of transcription over translation would then explain this mRNA accumulation.

Several possibilities exist with regard to the form of the protease mRNA pool. The first possibility is that that there is an accumulation of free polysomes. However, the results with pactamycin would tend to rule this out, assuming that the pactamycin concentration used is specific for inhibiting the initiation of protein synthesis as is claimed by Cohen et al. (1969). However, the recent finding that the drug inhibits elongation as well as initiation in E. coli systems (Stewart and Goldberg, 1973; Tai et al. 1973), suggest that experiments with pactamycin in bacteria should be treated with caution. The most likely alternatives are that the messenger is either free, or bound to a 30S subunit (see Fig. V.16). In either situation, translation would only begin after the 70S ribosome is completely assembled at the membrane. In this scheme, the presence of the nascent polypeptide chains is not obligatory for attachment of the ribosomes to the membrane. In agreement with this, Andrews and Tata (1971) have shown that nascent polypeptide chains are not required for the binding of ribosomes to the endoplasmic reticulum. Similarly, Baglioni et al. (1971) and Rolleston (1972) have presented evidence suggesting that the large ribosomal subunit binds directly to the endoplasmic reticular membrane followed by the attachment of the smaller subunit-mRNA complex.

Two possibilities existed to account for the above situation; first the mRNA might somehow be stabilised in the cytoplasm for transport from gene to membrane, but that on translation, it acquired the normal lability of bacterial mRNA. The second possibility was that the observed presence of mRNA represents the product of a dynamic equilibrium betweeen synthesis and degradation. A study reported in full elsewhere (it is not given fully here since the candidate was not a major participant in this aspect) has now shown the second alternative to be correct (Glenn et al., 1973).

It was first established that the protease mRNA pool was degraded in the absence of translation by the following approach. Cells in the presence of a high level of amino acids were incubated with inhibitors of translation for 15 min. After collection on a Millipore filter, the cells were washed and resuspended into identical fresh medium containing rifampicin. The rifampicin-insensitive protease production was then taken as a measure of the mRNA pool size. It was found that the pool of mRNA was degraded even in the presence of inhibitors of translation. In addition, it was shown that preincubation of cells in the presence of a low level of amino acids with actinomycin D, followed by drug removal, greatly reduced the subsequent protease production in the presence of actinomycin D. This showed that the pool of protease mRNA is rapidly turning over

and depends for its maintenance on continued transcription. Thus the pool represents the product of a dynamic equilibrium between synthesis and degradation.

It was previously postulated that a limit in the number of membrane-localised ribosome translational sites could account for the pool accumulation but the question then arises as to why the cell should so overproduce protease-specific mRNA. These recent experiments now suggest, though somewhat speculatively, that the excessive production of mRNA may be a mechanism to ensure that sufficient mRNA reaches the membrane to satisfy the needs of extracellular enzyme production, despite its rapid degradation in the cytoplasm *en route*. As such, it could be regarded as a primitive prokaryote messenger transport system; while wasteful of mRNA, it could make biological sense to accept this waste rather than to develop specific mechanisms for transporting a few specific messengers.

Studies in this laboratory have now also shown that corresponding pools of mRNA exist in *B. amyloliquefaciens* for the other two extracellular enzymes, α-amylase and ribonuclease (Gould *et al.*, 1973). The proposed model, therefore, that the pool of mRNA represents mRNA migrating from the gene to the membrane translation-extrusion site can now be extended to all three extracellular enzymes of *B. amyloliquefaciens*.

It is important to realise that in the above model

the existence of a pool of mRNA need not be a prerequisite for all extracellular enzyme secretion. Whether such a pool accumulates or not may depend on secondary factors such as the relative rates of transcription and translation in the particular system. However, in this respect, recent findings in this laboratory (Semets, unpublished data) suggest that B. subtilis 168 also possesses a pool of protease-specific mRNA so that mRNA accumulation for extracellular enzymes may be a more universal phenomenon.

CHAPTER VI

IN VITRO TRANSLATION OF NATURAL AND SYNTHETIC MESSENGER RNA BY *B. AMYLOLIQUEFACIENS* AND *E. COLI* CELL EXTRACTS

IN VITRO TRANSLATION OF NATURAL AND SYNTHETIC MESSENGER RNA BY B. AMYLOLIQUEFACIENS AND E. COLI CELL EXTRACTS

A. INTRODUCTION

The studies reported in the previous chapter established that logarithmic phase cells of B. amyloliquefaciens contain a large pool of preformed mRNA capable of sustaining extracellular protease synthesis for over 60 min. This finding led to the definite possibility that the exo-protease mRNA (and possibly other messenger RNA species) could be isolated and purified from the above organism, translated in a suitable cell-free system and the polypeptide product characterised. Two new approaches were therefore undertaken in this laboratory. Firstly, different cell extraction methods were investigated to maximise conditions for messenger RNA isolation; this work has been carried out by G. Both. Secondly, it became necessary to establish a cell-free system, preferably devoid of endogenous mRNA-directed activity, which would be capable of translating purified B. amyloliquefaciens mRNA samples.

Previously, the in vitro protein synthesis studies (Chapter III) indicated an inability of B. amyloliquefaciens cell extracts to translate the added synthetic messenger, polyuridylic acid. Because of the potential importance of

this observation to our aims, it was decided to carry out more detailed studies of this effect and compare both endogenous and exogenous mRNA-directed protein synthesis by B. amyloliquefaciens extracts with E. coli extracts. This chapter describes such studies and at the same time presents preliminary findings with a cell-free system capable of translating both phage MS2 RNA and B. amyloliquefaciens mRNA as sources of natural messenger.

B. ENDOGENOUS mRNA AND POLY-U-DIRECTED PROTEIN SYNTHESIS

In vitro systems for the incorporation of amino acids have been previously reported for B. subtilis (Takeda and Lipmann, 1966; Hirashima et al., 1967; Migita and Doi, 1970). In particular, Takeda and Lipmann (1966) reported that the 30S ribosomal subunit was unstable and could be compensated for by the use of E. coli (30S) - B. subtilis (50S) hybrid ribosomes. In order to evaluate the cell-free system from B. amyloliquefaciens, a comparison has been made with the well-studied system of E. coli.

(i) Cell extracts used without pre-incubation

E. coli MRE 600, a strain deficient in RNase I, was grown with vigorous aeration at 37° in a medium containing 16 g Bacto-tryptone (Difco), 10 g yeast extract (Difco) and 5 g NaCl in 1 litre of water. The doubling time was 25 to 30

min. Exponential phase cells ($A_{600\ nm}$ 0.8) were harvested by pouring over frozen, crushed TMK buffer and ribosomal and soluble fractions were prepared as for the B. amyloliquefaciens system.

The ability of these fractions to promote the incorporation of ^{14}C-phenylalanine into protein, with and without poly-U, was compared with similar fractions prepared from exponential phase cells of B. amyloliquefaciens. The latter system was seen to be approximately 50% as active as the E. coli system when endogenous mRNA activity alone was measured (Table VI.1). A much greater difference was seen when poly-U-directed phenylalanine polymerisation was measured; the E. coli system responded with an over 20-fold increase in rate as compared with the extremely small increase shown by that from B. amyloliquefaciens.

In further experiments, the two types of ribosome and soluble fractions were exchanged and the abilities of such combinations to incorporate ^{14}C-phenylalanine into protein, with and without poly-U (60 µg), were measured (Table VI.2). The data shows that both in the presence and absence of poly-U, E. coli ribosomes, when supplemented with B. amyloliquefaciens soluble proteins, incorporate phenylalanine at approximately 15% of the rate of an all E. coli system. On the other hand, in the absence of poly-U, B. amyloliquefaciens ribosomes, supplemented with the E. coli soluble fraction, incorporate as efficiently as the homologous

TABLE VI.1. COMPARISON OF ^{14}C-PHENYLALANINE INCORPORATION IN *B. AMYLOLIQUEFACIENS* AND *E. COLI* CELL-FREE SYSTEMS.

System	^{14}C-phenylalanine incorporation (counts/min/mg RNA)	
	B. amyloliquefaciens	*E. coli*
No polyuridylic acid	6,280	13,450
+ polyuridylic acid (60 µg)	6,450	289,820

Exponential phase *E. coli* MRE 600 cells ($A_{600\ nm}$ 0.8) were harvested by pouring over frozen, crushed TMK buffer; ribosome and soluble fractions were prepared as for the *B. amyloliquefaciens* system (Chapter II).

Measurements of ^{14}C-phenylalanine incorporation by the different systems were carried out as described for *B. amyloliquefaciens* (Chapter II).

Reaction mixtures, containing ribosome (1 mg RNA) and soluble (420 µg) protein fractions were incubated at 30° for 30 min.

TABLE VI.2. EFFECT OF ADDITION OF POLYURIDYLIC ACID ON ^{14}C-PHENYLALANINE INCORPORATION BY HOMOLOGOUS AND MIXED FRACTIONS FROM *B. AMYLOLIQUEFACIENS* AND *E. COLI*

Ribosomes	Soluble enzymes	poly-U (60µg)	^{14}C-phenylalanine incorporation (counts/min)	Incorporation due to poly-U addition (counts/min)
B. amylo.	*B. amylo.*	-	6,390	290
		+	6,680	
E. coli	*E. coli*	-	14,580	215,730
		+	230,310	
E. coli	*B. amylo.*	-	2,350	31,920
		+	34,270	
B. amylo.	*E. coli*	-	14,960	25,860
		+	40,820	

Experimental details are as described in Table VI.1 and as stated in the text.

E. coli system; in the presence of poly-U, however, this incorporation reaches only a maximum 18% of that of the homologous E. coli system.

(ii) Activity of preincubated cell extracts

The above experiments were supplemented with studies in which the extracts were preincubated to deplete their endogenous mRNA; the ability of each cell-free system to translate poly-U was then studied.

Cell-free extracts (soluble and ribosome) from both B. amyloliquefaciens and E. coli exponential phase cells were prepared as described previously. Reaction mixtures without the ^{14}C-phenylalanine (Chapter II) were preincubated at 30° for 30 min. in order to allow time for the breakdown of endogenous mRNA. After preincubation, poly-U (60 µg) and ^{14}C-phenylalanine (0.2 µCi) were added to the reaction mixtures and incubations were carried out over a 45 min. period. Control experiments without poly-U were carried out at the same time.

As anticipated from the previous studies, the results (Fig. VI.1) show that B. amyloliquefaciens preparations were unable to translate poly-U while E. coli cell extracts did so efficiently. Since synthetic homopolynucleotide messengers may require an artificially high concentration of Mg^{2+} to promote formation of the messenger-ribosome aminoacyl tRNA complex (Revel and Gros, 1966), the incorporating ability of the Bacillus system was re-examined at a Mg^{2+} concentration

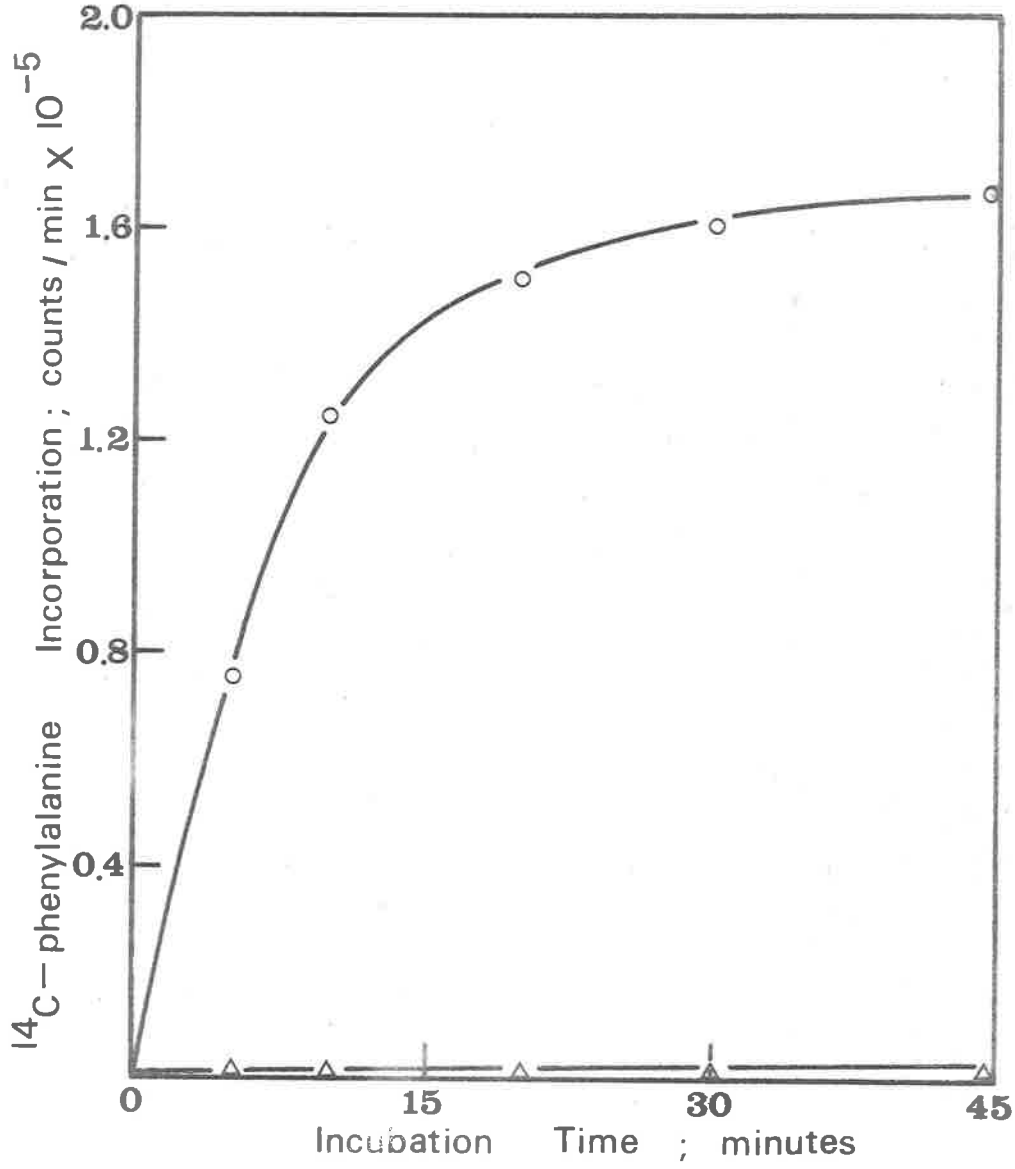


FIG. VI.1. COMPARISON OF POLY-U DIRECTED ^{14}C-PHENYLALANINE INCORPORATION IN PREINCUBATED B. AMYLOLIQUEFACIENS AND E. COLI CELL-FREE SYSTEMS.

Ribosome and soluble fractions were prepared from B. amyloliquefaciens and E. coli exponential cells as described in the text. Measurements of ^{14}C-phenylalanine incorporation by the two systems were carried out as described for B. amyloliquefaciens (Chapter II Reaction mixtures containing ribosome (1 mg RNA) and soluble (420 µg protein) fractions were first preincubated in the absence of ^{14}C-phenylalanine for 30 min. at 30° in order to allow endogenous mRNA breakdown. Poly-U (60 µg) and ^{14}C-phenylalanine (0.2 µCi) we added and the reaction mixtures were incubated at 30°.

—O—O— , E. coli.

—Δ—Δ— , B. amyloliquefaciens.

of 20 mM (compared to the 8 mM used above). However, the *Bacillus* extracts again failed to translate poly-U, since the same incorporation was seen at this higher Mg^{2+} concentration as that seen previously (Fig. VI.1).

C. PHAGE MS2 RNA-DIRECTED PROTEIN SYNTHESIS

As a pre-requisite for the translation of the natural *B. amyloliquefaciens* mRNA, once isolated, it seemed essential to establish a cell-free system capable of translating other natural messengers; for example, the RNA of bacteriophages R17, MS2, f2 or QB. In this context, Doi (1971) reported little or no incorporation when MS2 phage RNA was used as a source of natural messenger in a *B. subtilis* system; in fact, MS2 RNA inhibited the endogenous mRNA-directed protein synthesis of *B. subtilis* vegetative cells. In view of these findings and the inability of *B. amyloliquefaciens* cell extracts to engage in poly-U-directed protein synthesis, it seemed desirable to examine natural messenger RNA translation in the *E. coli* system as a basis for comparison. At this stage, an informative study was published (Modollel, 1971) of an *E. coli* cell-free system capable of translating MS2 phage RNA; a similar system was adopted in this laboratory and is described below.

E. coli MRE 600 was grown and harvested as before except that the cells were poured over a frozen, crushed 0.01 M tris-HCl buffer (pH 7.8) containing 10 mM magnesium acetate,

60 mM NH_4Cl and 6 mM 2-mercaptoethanol (hereafter referred to as TMN buffer). All subsequent operations were carried out at 0 - 4°. The chilled cells were harvested by centrifugation (5,000 g for 5 min.), washed twice with TMN buffer and resuspended in a small volume of the above buffer prior to passage through a pre-chilled French pressure cell (18,000 psi). The extract was centrifuged for 30 min. at 30,000 g and the upper 4/5 vol. of the supernatant was dialysed against 500 vol. of standard TMN buffer for 4 hr. and clarified by centrifugation at 30,000 g for 20 min. This dialysed S30 extract was stored under liquid nitrogen in 0.5 ml lots. A sample of undialysed S30 extract was centrifuged at 105,000 g for 2.5 hr. and the upper 2/3 vol. of the supernatant, after dialysis and clarification as stated above, was stored as 0.25 ml. lots under liquid nitrogen. This fraction constituted the dialysed S100 supernatant.

Table VI.3 shows the composition of the reaction mixture for incorporating ^{14}C-labelled amino acids with E. coli cell extracts and phage MS-2 RNA as a source of natural messenger. A concentration of 7.5 mM Mg^{2+} was chosen for the incorporation studies since this is the concentration required for natural translation of MS2 RNA (Modollel, 1971). The MS2 RNA was prepared according to the method of Sugiyama (1971).

An initial experiment was carried out to determine the most suitable preincubation time for the E. coli S30 extract to maximise the depletion of its endogenous mRNA-

TABLE VI.3. COMPONENTS OF THE E. COLI AMINO ACID INCORPORATING SYSTEM DIRECTED BY MS2 PHAGE RNA MESSENGER

Component	Concentration
Tris-HCl buffer (pH 7.8)	50 mM
NH_4Cl	60 mM
Magnesium acetate	7.5 mM
2-mercaptoethanol	8 mM
ATP	1 mM
GTP	0.02 mM
Potassium phosphoenolpyruvate (PEP)	5 mM
Pyruvate kinase (PK)	30 µg/ml.
^{14}C-leucine (spec. act. 312 mCi/m-mole)	0.2 µCi
^{14}C-valine (spec. act. 250 mCi/m-mole)	0.2 µCi
18 other ^{12}C-amino acids	0.05 mM
Phage RNA messenger	80 µg
Incubated S30 fraction (iS-30)	0.2 vol.
Unincubated S100 supernatant	0.1 vol.

The S30 fraction contained 15 mg RNA/ml.
The reaction mixture volume was 0.25 ml.
Incubations were carried out at 34° for 20 min. unless otherwise stated.
Reactions were stopped by the addition of 4 ml. of 10% trichloroacetic acid containing 1% casamino acids and chilled on ice for 30 min. The tubes were heated at 90° for 20 min., cooled, filtered and counted by liquid scintillation as described previously (see Chapter II).

directed activity and yet still retain a high activity in subsequent exogenous mRNA-directed protein synthesis. S30 extracts were incubated, just prior to their addition to the reaction mixtures, for various times at 34° after being mixed with 0.02 vol. of 2.0 M tris-HCl buffer (pH 7.8) and 0.1 vol. of the energy generating system (potassium phosphoenol-pyruvate, pyruvate kinase, ATP and GTP at 10 times the concentration in the reaction mixture). After this pre-incubation step, these iS-30 extracts were chilled, mixed with 0.5 vol. of S100 supernatant and then incubated in the reaction mixtures (see Table VI.3) in the presence and absence of the MS2 phage RNA for a 20 min. period.

The resulting ^{14}C-amino acid incorporations (Fig. VI.2) show that a preincubation time of 60 min. seems to be the best; the net incorporation achieved from such a preincubation shows a maximal 25-fold increase against a small residual endogenous mRNA activity. Hence, for all future studies a 60 min. preincubation was chosen.

To determine the maximum amount of MS2 phage RNA to add to this cell-free system, the ability of different phage RNA concentrations to incorporate ^{14}C-amino acids into protein was studied. Fig. VI.3 shows that a linear relation-ship between incorporation and the amount of phage RNA added existed up to 50 µg, with a maximal incorporation being reached with 80 µg RNA. Using this amount of phage RNA as the messenger source, the time course of ^{14}C-amino acid

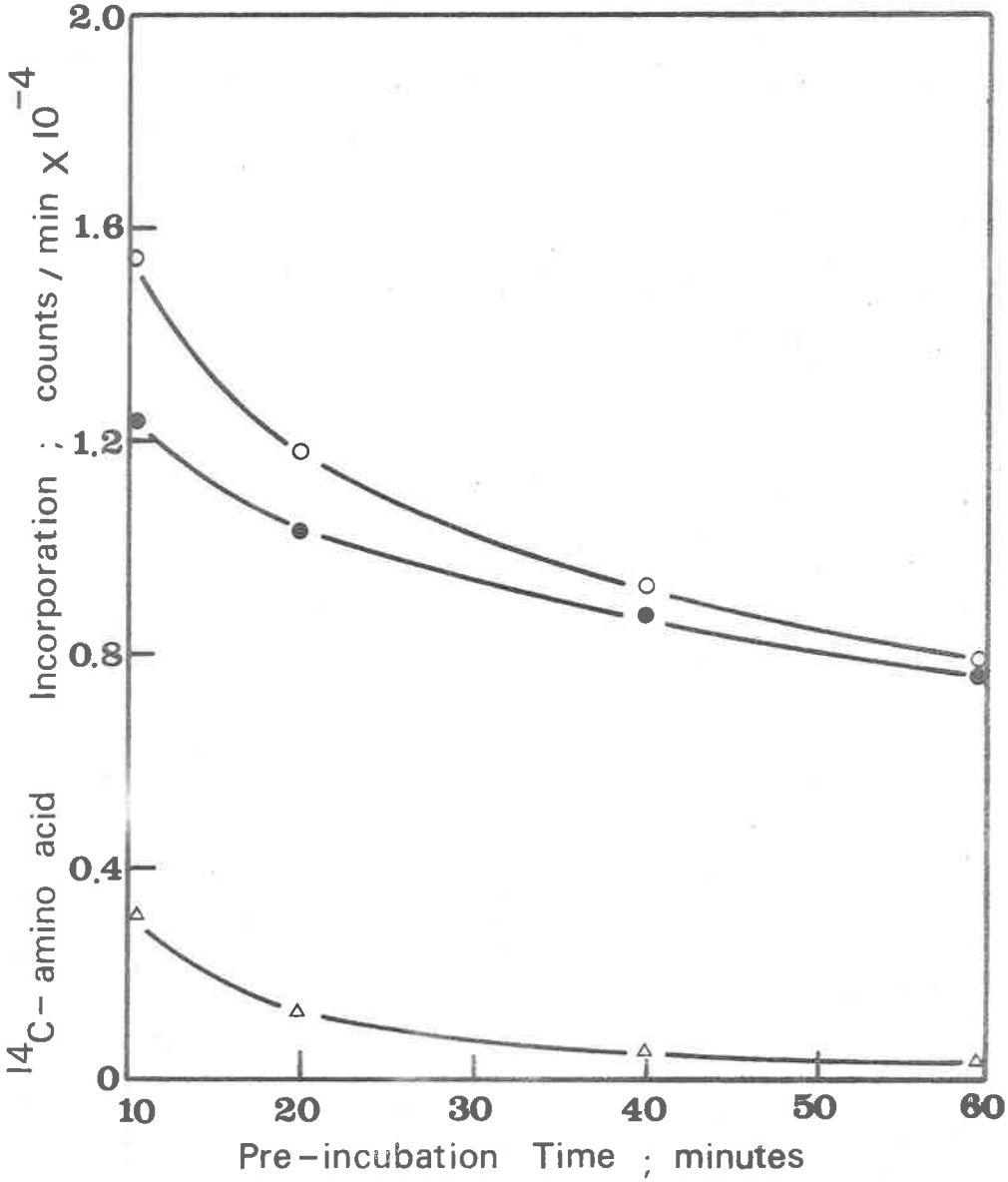


FIG. VI.2. PHAGE MS2 RNA-DIRECTED PROTEIN SYNTHESIS IN E. COLI CELL EXTRACTS: EFFECT OF PREINCUBATION TIME ON SUBSEQUENT ^{14}C-AMINO ACID INCORPORATION.

E. coli S30 extracts were incubated for various times at 34° afte being mixed with 0.02 vol. of 2.0 M tris-HCl buffer (pH 7.8) and 0.1 vol. of the energy generating system (see text for details). After this preincubation step, the iS30 extracts were chilled, mixed with 0.5 vol. of S100 supernatant and incubated at 34° for 20 min. in the reaction mixtures (see Table VI.3) in the presence and absence of MS2 phage RNA (80 µg).

—○—○— , MS2 RNA present (endogenous+exogenous incorporati
—△—△— , MS2 RNA absent (endogenous incorporation)
—●—●— , net exogenous incorporation

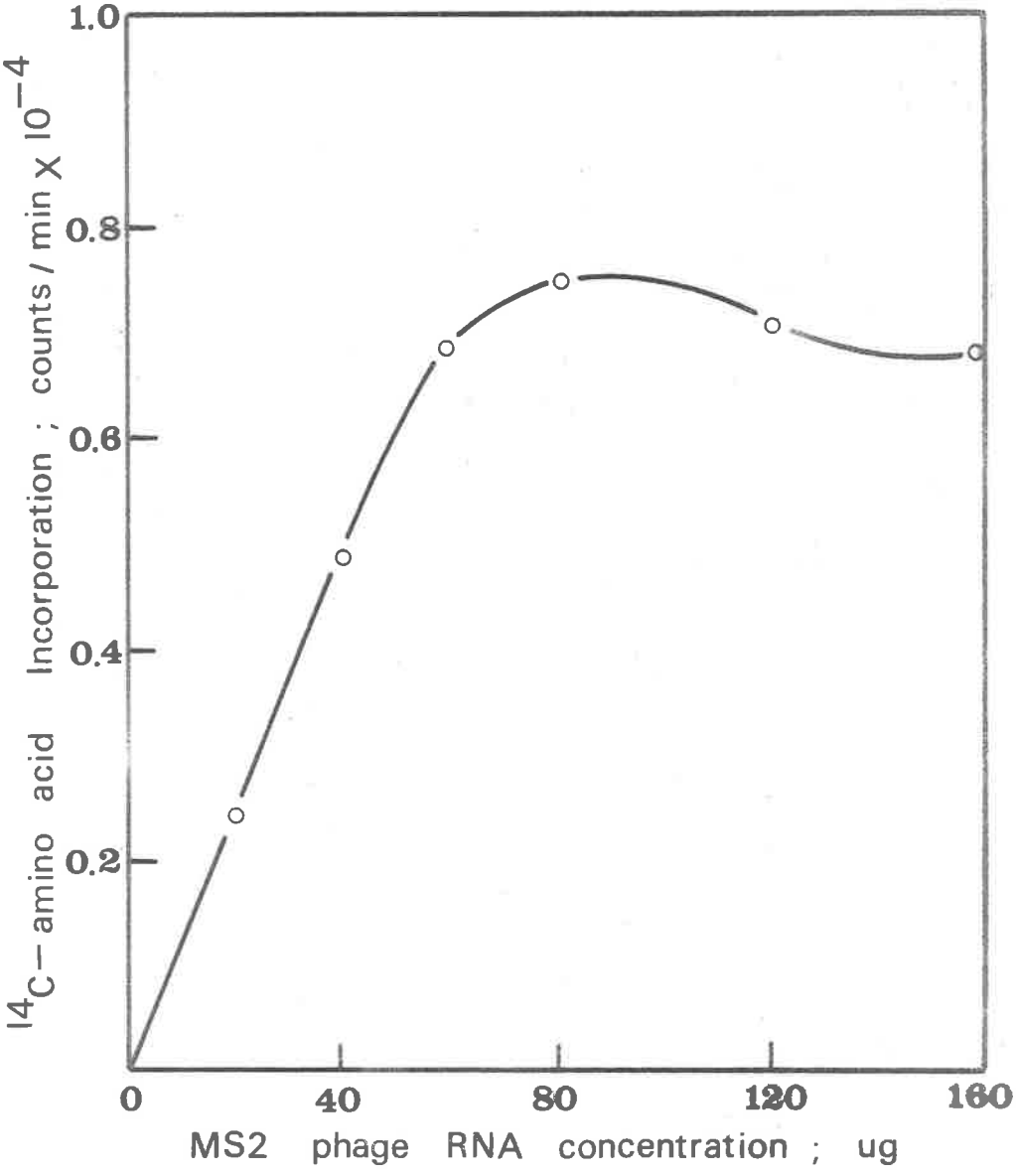


FIG. VI.3. EFFECT OF MS2 PHAGE RNA CONCENTRATION ON ^{14}C-AMINO ACID INCORPORATION BY E. COLI CELL EXTRACTS.

E. coli S30 extracts were preincubated for 60 min. at 34° as described in the text. After preincubation, the iS30 extracts were chilled, mixed with 0.5 vol. of S100 supernatant and incubated at 34° for 20 min. in the reaction mixtures (see Table VI.3). Residual endogenous mRNA activity was subtracted from each sample.

incorporation by the E. coli cell extracts was studied (Fig. VI.4). It can be seen that, under the incubation conditions tested (34°), approximately 95% of the final level of incorporation is achieved during the first 10 min. with no further incorporation after 20 min. of incubation.

D. B. AMYLOLIQUEFACIENS mRNA-DIRECTED PROTEIN SYNTHESIS BY E. COLI EXTRACTS

The ability of the E. coli cell-free system to translate the natural MS2 coliphage RNA provided encouragement for the translation in the same system of purified mRNA samples from B. amyloliquefaciens. Preliminary in vitro studies involving ^{14}C-amino acid incorporation into protein have now been carried out as described below.

The isolation and purification of B. amyloliquefaciens RNA was carried out by G. Both. RNA from this organism was extracted from exponential phase cells using a phenol extraction technique similar to that described in Chapter III. A sample (10 ml.) containing approximately 30 mg RNA was then layered onto a 5 - 20% (w/v) sucrose density gradient in a tris-HCl buffer (pH 7.4) containing 50 mM KCl and 1.5 mM EDTA. Centrifugation was performed at 7° in a Beckman Zonal rotor (650 ml. capacity) for 16 hr. at 45000 rev./min. The gradient was continuously monitored at 260 nm and collected as 6 ml. fractions. Under these conditions the 23S RNA sediments nearly to the bottom of the gradient

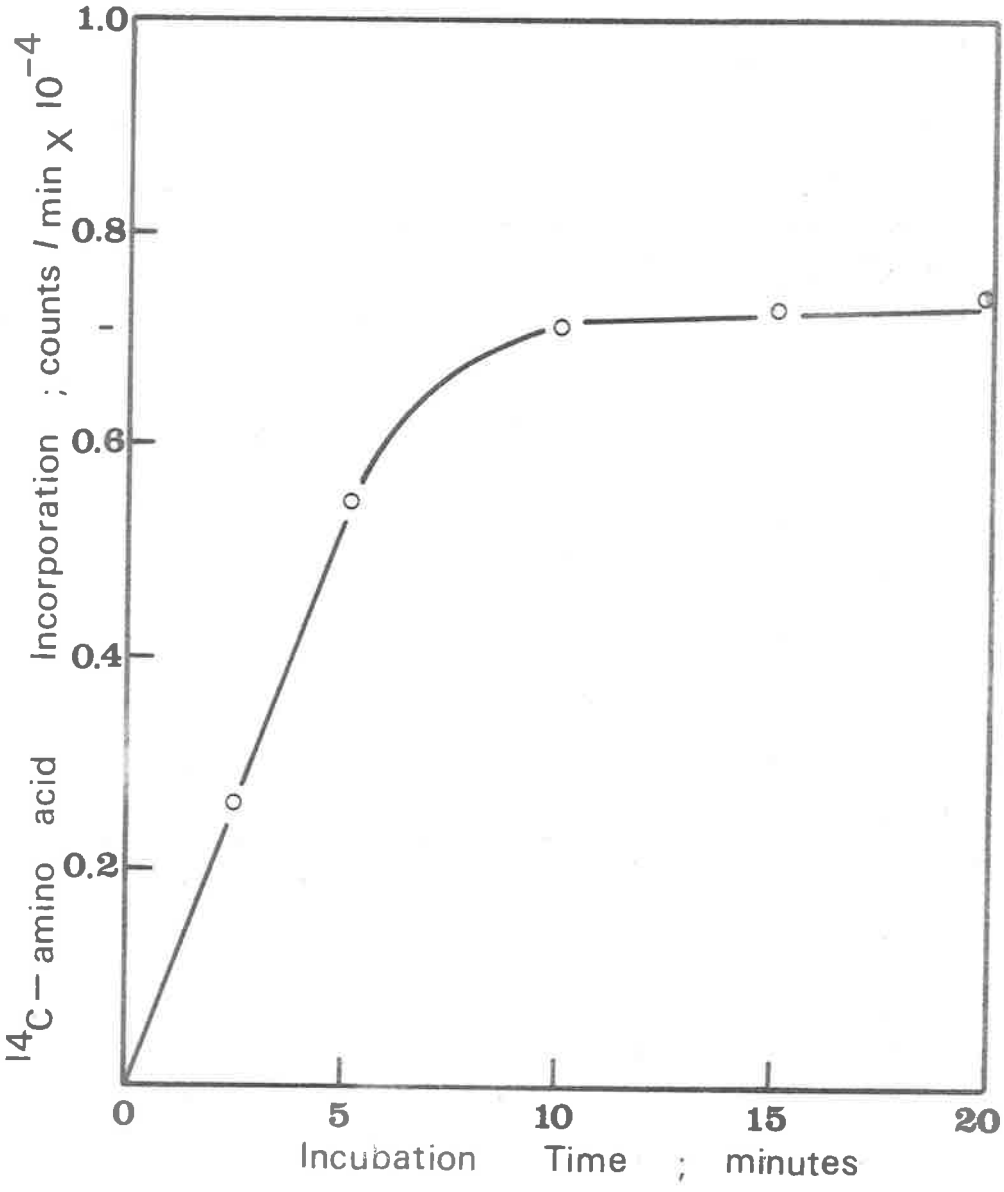


FIG. VI.4. TIME COURSE OF MS2 PHAGE RNA-DIRECTED PROTEIN SYNTHESIS IN E. COLI CELL EXTRACTS.

E. coli S30 extracts were preincubated for 60 min. at 34° as described in the text. After preincubation, the iS30 extracts were chilled, mixed with 0.5 vol. of S100 supernatant and incubated at 34° in the reaction mixtures (see Table VI.3) in the presence of 80 µg MS2 RNA. Residual endogenous mRNA activity was subtracted from each sample.

(Fig. VI.5a).

The RNA fractions most likely to possess messenger characteristics were thought to sediment somewhere between the 4S and 16S RNA species; appropriate fractions (Fig. VI.5a, shaded area) were therefore pooled. RNA was precipitated by addition of 2% sodium acetate and 2 vol. of ethanol at -20°. After dissolving in a small volume of distilled water, the RNA was reanalysed on a linear 5 - 20% sucrose density gradient by centrifugation in the SW41 rotor at 41000 rev./min. for 16 hr. at 4°. The gradient was fractionated and appropriate pairs of fractions were separately pooled. After precipitation with ethanol, each RNA sample was tested for messenger activity in the preincubated E. coli cell-free system. Control samples of B. amyloliquefaciens 4S and 16S RNA were included at the same time.

Fig. VI.5b shows the Absorbance (260 nm) trace of the final gradient of the RNA used for translation experiments and the resulting incorporation shown by each of the combined fractions. It can be seen that ^{14}C-amino acid incorporation promoting ability is spread well over the gradient and shows a peak at approximately 7S - 8S. The control samples of 4S and 16S B. amyloliquefaciens RNA, in comparison, gave only poor incorporation as expected (3% and 8%, respectively, of the highest fraction). The exact nature of the ^{14}C-amino acid incorporation shown by the combined fractions is not known at the present time.

FIG. VI.5.

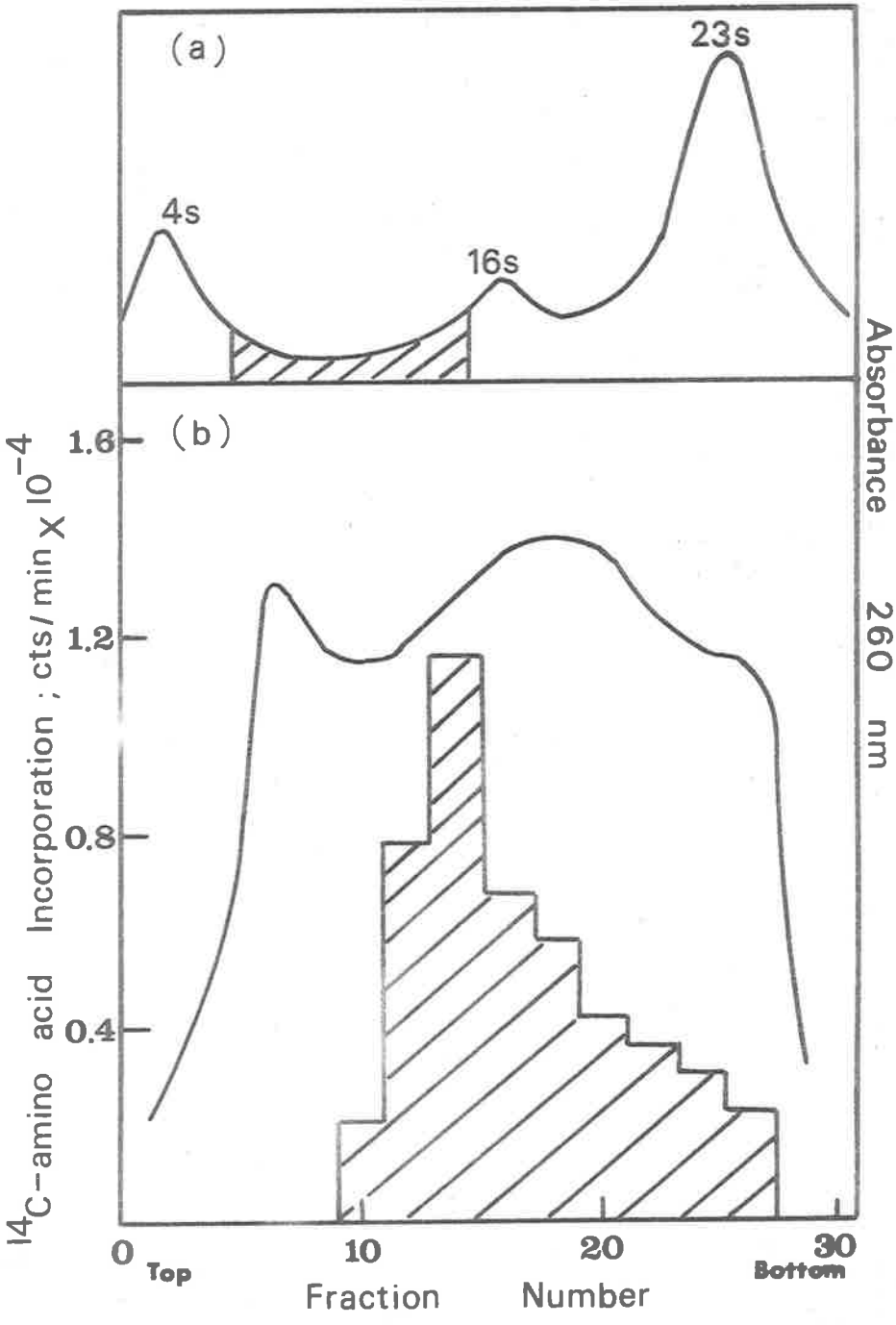
(a) ABSORBANCE TRACE OF RNA EXTRACTED FROM *B. AMYLOLIQUEFACIENS* EXPONENTIAL PHASE CELLS.

Sedimentation was through a 5-20% sucrose gradient in a tris-HCl buffer (pH 7.4) containing 50 mM KCl and 1.5 mM EDTA at 45,000 rev./min. for 16 hr. (7°) in a Beckman zonal rotor. The shaded area was pooled and the RNA was precipitated prior to further analysis.

(b) ABSORBANCE TRACE OF THE FINAL GRADIENT OF THE RNA USED FOR TRANSLATION AND THE RESULTING ^{14}C-AMINO ACID INCORPORATION PROFILE.

Sedimentation of the pooled RNA from the zonal rotor was through a 5 -20% sucrose gradient at 41,000 rev./min. for 16 hr. (4°) in a SW41 rotor. Pairs of fractions were separately pooled, the RNA was precipitated and tested for messenger activity in the preincubated *E. coli* cell-free system (Table VI.3).

Shaded area; radioactive incorporation (counts/min.).


(a)
23s
4s
16s
(b)
1.6
1.2
0.8
0.4
^{14}C-amino acid Incorporation ; cts/min X 10^{-4}
Absorbance 260 nm
0
Top
10
20
30
Bottom
Fraction Number

E. DISCUSSION

Several significant findings emerged from the comparison of the B. amyloliquefaciens and E. coli cell-free systems. In the first instance, using ^{14}C-phenylalanine incorporation as a measure of protein synthesising activity directed by endogenous mRNA, extracts from exponential phase B. amyloliquefaciens cells had only approximately 50% of the activity of an E. coli system. Takeda and Lipmann (1966) reported similar findings in a comparison of B. subtilis and E. coli cell-free systems. Exchange experiments between soluble and ribosome fractions from the two systems suggested that either the soluble fraction from B. amyloliquefaciens is deficient in some component(s) necessary for endogenous mRNA-directed protein synthesis or else it contains some component(s) detrimental to the translating abilities of both ribosomal species.

The two homologous systems were also found to differ enormously in their abilities to translate polyuridylic acid. Whereas E. coli ribosomes showed a large increase in phenylalanine incorporation, the B. amyloliquefaciens ribosomes showed little response to the added messenger. Similar results were obtained when the cell extracts were first preincubated to deplete their endogenous mRNA content. These findings can be compared with those from Takeda and Lipmann (1966) who showed that a B. subtilis system responded with only about a four-fold increase in rate when poly-U-directed phenylalanine

polymerisation was measured as compared with a nearly forty-fold increase with E. coli. More recently, Doi (1971) has shown that poly-U-directed polyphenylalanine synthesis by vegetative cell extracts from B. subtilis is relatively low (10%) compared with that obtained with E. coli extracts.

It is not possible at the present time to account for the poor response shown by B. amyloliquefaciens ribosomes to poly-U addition. However, several possibilities do exist. B. amyloliquefaciens ribosomes may require a different Mg^{2+} concentration for optimal translation of the synthetic message than do E. coli ribosomes. Such a situation has been recently shown to exist with Clostridium pasteurianum ribosomes (Himes et al., 1972); here the higher Mg^{2+} concentration required was involved with initiation of the poly-U directed translation process. This does not seem to be the case with B. amyloliquefaciens; ribosomes from this organism, in a preincubated cell-free system, failed to translate the poly-U at 20 mM Mg^{2+} concentration. Moreover, the finding that B. amyloliquefaciens ribosomes were able to translate the synthetic message to a limited extent when E. coli soluble enzymes were substituted suggested that some factor other than Mg^{2+} concentration is involved here.

Another possibility is that E. coli ribosomes may be saturated at a much lower level of poly-U than the B. amyloliquefaciens ribosomes, so that the 60 µg level chosen for these studies may be too low for the Bacillus

system but may be a near-saturating amount for the E. coli system. Indeed, a requirement for a high level of poly-U has been reported for other Bacillus systems (Algranati and Lengyel, 1966; Kobayashi and Halvorson, 1966; Stenesh and Schechter, 1969); often a high nuclease content in these organisms has been quoted as the reason for the poor response to poly-U. In this context, Himes et al. (1972) recently showed that the C. pasteurianum ribosomes mentioned above require a much higher saturation level of this synthetic messenger than do E. coli ribosomes.

The establishment in this laboratory of the E. coli cell-free system capable of translating the natural MS2 phage RNA enabled preliminary translation studies to be carried out with purified RNA from B. amyloliquefaciens. The significant ^{14}C-amino acid incorporation into protein achieved with this RNA suggests that it is 'messenger-like' in nature. Unequivocal evidence, however, that this is the case can only be obtained by showing that this RNA directs the synthesis of one or more specific polypeptide products. By the use of an immunological precipitation technique with purified antibodies to the B. amyloliquefaciens extracellular enzymes, it is hoped in the near future to characterise the particular mRNA species which promote in vitro extracellular enzyme synthesis. In this context, it will be of extreme importance to establish whether a species specificity exists with respect to translation of the natural B. amyloliquefaciens mRNA using the E. coli

in vitro protein synthesising system. Species specificity with respect to translation of natural mRNA has been reported before (Lodish, 1970; Szer and Brenowitz, 1970).

CHAPTER VII

SELECTIVE INHIBITION OF EXTRACELLULAR ENZYME SYNTHESIS IN *B. AMYLOLIQUEFACIENS* BY PROTEIN SYNTHESIS INHIBITORS

SELECTIVE INHIBITION OF EXTRACELLULAR ENZYME SYNTHESIS IN B. AMYLOLIQUEFACIENS BY PROTEIN SYNTHESIS INHIBITORS

A. INTRODUCTION

Preliminary studies reported in Chapter V with two inhibitors of protein synthesis, pactamycin and fusidic acid, indicated that these drugs selectively inhibited extracellular protease synthesis in B. amyloliquefaciens while having little effect on general protein synthesis as measured by ^{14}C-phenylalanine incorporation into trichloroacetic acid precipitable material. Concurrent with these studies, Glew and Heath (1971) established that synthesis of Micrococcus sodonensis extracellular alkaline phosphatase was preferentially inhibited by antibiotics specific for protein synthesis. With the three drugs under study (chloramphenicol, puromycin and sparsomycin) these workers established that 6 to 17 times as much inhibitor was required to achieve 50% inhibition of ^{3}H-leucine incorporation into total cellular protein as was necessary for comparable inhibition of alkaline phosphatase secretion.

Both of these sets of findings suggested that ribosomes engaged in extracellular enzyme synthesis are more sensitive to inhibition by lower concentrations of these drugs than cytoplasmic ribosomes owing to a peripheral location.

Such a peripheral location is a necessary requirement for the proposed model of extracellular enzyme synthesis and secretion mentioned previously (Chapter V). In view of the potential significance of these observations, it was decided to examine the effect of a range of inhibitors of protein synthesis on both B. amyloliquefaciens α-amylase and protease formation and compare the effect of the same inhibitors on total cellular protein synthesis. This chapter describes such studies.

B. THE EFFECT OF 30S and 50S RIBOSOMAL SUBUNIT INHIBITORS ON PROTEASE, α-AMYLASE AND TOTAL PROTEIN SYNTHESIS

It was previously established (Chapter V), that extracellular protease synthesis by washed cell suspensions of B. amyloliquefaciens shows a non-linear time course in the presence of 0.5% Casamino acids; there is an initial rapid phase lasting approximately 30 min. followed by a period during which enzyme formation does not increase appreciably. However, after 90 min., the time course of protease synthesis is rapid and linear and lasts for at least another 80 min. The synthesis of α-amylase by washed cell suspensions of B. amyloliquefaciens shows essentially the same characteristics (Gould et al., 1973). In contrast, total protein synthesis as measured by ^{14}C-phenylalanine incorporation into trichloroacetic acid precipitable material, shows an almost linear time course during the entire 170 min.

incubation period (May, unpublished results).

The inhibition by translational inhibitors of extracellular α-amylase and protease synthesis during the 90 - 170 min. incubation period has been compared with the effect of the same inhibitors on total cellular protein synthesis. The latter can be taken as a measurement of intracellular protein synthesis since total extracellular protein synthesis constitutes only about 5% of total cellular protein synthesis (see below).

Control experiments have shown that none of the drugs tested affect α-amylase or protease activity; both enzymes were assayed by the modified procedures of May and Elliott (1968a,b) (see Chapter II). Ribonuclease was not examined in these studies since any trace of cell lysis produced by the drugs would release the internal ribonuclease inhibitor (Smeaton and Elliott, 1967a) which would render interpretation of results impossible.

(i) Proportion of total cellular protein synthesis represented by total extracellular protein synthesis

The following experiment was carried out to determine what proportion total extracellular protein synthesis is of total protein synthesis of the cell. A 6.0 ml. sample of washed B. amyloliquefaciens cell suspension (Chapter II) was shaken at 30° in the presence of 1.5 μCi of ^{14}C-phenylalanine (spec. act. 455 mCi/m-mole). A sample (0.5 ml.) was withdrawn

after 120 min. incubation, centrifuged and the supernatant kept. The cell pellet was washed once by recentrifugation with 0.5 ml. of 0.9% (w/v) NaCl solution and the washings were added to the supernatant. The cell pellet was processed for incorporation of ^{14}C-phenylalanine into trichloroacetic acid precipitable material and counted by liquid scintillation as described in Chapter II. The resulting incorporation gave a measurement of total intracellular protein synthesis. Treatment of the supernatant and washings in a similar fashion gave a measurement of total extracellular protein synthesis.

Similar experiments were carried out using different radioisotopes (^{14}C-leucine and a ^{14}C-reconstituted protein hydrolysate mixture). The results (Table VII.1) show that, based on the average of the three determinations, total extracellular protein synthesis constitutes about 5% of total cellular protein synthesis in B. amyloliquefaciens.

(ii) Inhibitor studies

Washed cell suspension experiments were carried out in a medium containing 0.5% Casamino acids, as previously described (Chapter II), except that cells were first shaken for 90 min. in this medium. (The 90 min. preincubation gave linear production of extracellular enzymes during the experimental period.) After this time, 20 ml. samples of cell suspensions were withdrawn for incubation with

TABLE VII.1. COMPARISON OF TOTAL CELLULAR PROTEIN SYNTHESIS WITH EXTRACELLULAR PROTEIN SYNTHESIS IN B. AMYLOLIQUEFACIENS

^{14}C-Label	^{14}C-amino acid incorporation (counts/min.)			
	(a) Extracellular	(b) Intracellular	Total (c) cellular protein synthesis	% (a)/(c)
Phenylalanine	620	15,550	16,170	3.8
Leucine	820	10,150	10,970	7.5
Reconstituted protein hydrolysate	430	11,430	11,860	3.6
			Average ..	5.0

Experimental details for the washed cell experiment are described in the text.

A sample of the cell suspension (0.5 ml.) was withdrawn at 120 min., centrifuged and the supernatant kept. The cell pellet was washed once by recentrifugation with 0.5 ml. of 0.9% NaCl solution and the washings were added to the supernatant. 3.0 ml. of cold 1% (w/v) Casamino acids in 10% (w/v) trichloroacetic acid was added separately to the cell pellet and to the supernatant and washings; each was processed and counted by liquid scintillation as described in Chapter II.

The resultant incorporation from the supernatant and washings gave a measurement of total extracellular protein synthesis (a); that from the cell pellet gave a measurement of total intracellular protein synthesis (b).

an appropriate concentration of drug. During a further incubation of 80 min., samples (1.0 ml.) were withdrawn at appropriate times, centrifuged and the supernatants assayed for enzyme activity. From the preincubated washed cell suspension, samples (2.0 ml.) were also taken for incubation with 0.5 µCi of ^{14}C-phenylanine (spec. act. 455 mCi/m-mole) and an appropriate drug concentration. At appropriate times, samples (0.1 ml.) were withdrawn and incorporation of the label into protein was measured (Chapter II).

Initially, a study with 10 different translational inhibitors was carried out. In the first instance, the percentage (as compared with the control) of extracellular enzymes produced and of ^{14}C-phenylalanine incorporated into cellular protein at 170 min. in the presence of each drug concentration was determined (Table VII.2). The drugs examined fall into two quite distinct groups; those in group I (kanamycin, neomycin and streptomycin), inhibited both total protein synthesis and extracellular enzyme synthesis to approximately the same extent at any given concentration. The drugs in this group all act on the 30S ribosomal subunit (Pestka, 1971). However, drugs in group II (puromycin, chloramphenicol, erythromycin, lincomycin, tetracycline, sodium fusidate and thiostrepton) showed a differential effect in that they inhibited extracellular enzyme formation to a much greater extent than total protein synthesis. These latter drugs all act on the 50S ribosomal subunit (Bodley and Lin, 1970; Pestka, 1971).

TABLE VII.2. THE EFFECT OF INHIBITORS OF PROTEIN SYNTHESIS ON ^{14}C-PHENYLALANINE INCORPORATION AND EXTRACELLULAR ENZYME PRODUCTION

Group	Inhibitor	Concentration (µg/ml.)	Percentage of control			Ribosome subunit bound by inhibitor
			^{14}C-phenylalanine incorporation	protease production	α-amylase production	
Group I	kanamycin	1	87	83	83	30S
		2	75	66	67	
		4	46	44	42	
		8	24	30	33	
	neomycin	1	73	67	60	30S
		2	37	45	42	
		4	18	28	20	
		8	9	17	8	
	streptomycin	1	88	86	92	30S
		2	81	78	79	
		5	57	57	55	
		10	36	39	41	
Group II	puromycin	2	82	68	67	50S
		4	74	43	50	
		6	67	33	33	
		10	48	17	22	
	chloramphenicol	1	73	33	40	50S
		2	43	20	27	
		4	26	9	4	
	erythromycin	0.05	88	58	62	50S
		0.10	63	38	42	
		0.25	29	18	17	

TABLE VII.2 Continued ..

Group	Inhibitor	Concentration (µg/ml)	Percentage of control			Ribosome subunit bound by inhibitor
			^{14}C-phenylalanine incorporation	protease production	α-amylase production	
Group II (contd.)	lincomycin	0.5	89	68	77	50S
		1.0	74	44	48	
		2.5	38	14	26	
		5.0	12	6	14	
	tetracycline	0.25	84	61	69	30S and 50S
		0.5	74	47	54	
		1.0	60	28	39	
	sodium fusidate	10	88	67	72	50S
		15	64	33	33	
		20	45	15	16	
		25	37	9	13	
	thiostrepton	1	92	70	80	50S
		2	77	27	40	
		3	40	16	25	

The percentages in the table are those of either ^{14}C-phenylalanine incorporation or extracellular enzyme synthesis occurring at 170 min. (compared with the control). Bodley and Lin (1970) and Pestka (1971) are the sources of reference for the ribosomal subunit type(s) bound by the particular inhibitors.

From Table VII.2, the concentrations of inhibitor required for 50% inhibition of ^{14}C-phenylalanine incorporation, protease and α-amylase formation were determined. The results are shown in Table VII.3. It can be seen that equal concentrations of Group I inhibitors are required for the 50% inhibition of both protease and α-amylase production and total protein synthesis. However, in the case of Group II inhibitors, approximately 2 to 3 times as much inhibitor is required to achieve 50% inhibition of ^{14}C-phenylalanine incorporation as is required for a comparable inhibition of exoenzyme production.

The results presented so far involve a direct study of the inhibition produced by the various drugs over an 80 min. time period and as such give no indication as to the nature of the inhibition with respect to time. It was decided therefore to study more closely the time courses of inhibition of the different inhibitors.

It can be seen in the case of kanamycin (Fig. VII.1), that the time courses of both extracellular enzymes and total protein synthesis are similar and show comparable degrees of inhibition with time at any given drug concentration. A similar result was obtained with the other Group I inhibitors, neomycin and streptomycin (Fig. VII.2 and 3).

On the other hand, the situation appears to be more complex in the case of Group II inhibitors, as shown by the profiles obtained with chloramphenicol (Fig. VII.4). If

TABLE VII.3. COMPARISON OF INHIBITOR CONCENTRATIONS REQUIRED FOR 50% INHIBITION OF ^{14}C-PHENYLALANINE INCORPORATION AND EXOENZYME PRODUCTION

Group	Inhibitor	Concentrations required for 50% inhibition (µg/ml.)			Ratio of concentrations	
		^{14}C-phenylalanine incorporation (a)	protease production (b)	α-amylase production (c)	(a)/(b)	(a)/(c)
Group I	kanamycin	3.6	3.3	3.2	1.1	1.1
	neomycin	1.5	1.65	1.5	0.9	1.0
	streptomycin	6.6	6.8	6.4	1.0	1.0
Group II	puromycin	9.6	3.2	3.5	3.0	2.7
	chloramphenicol	1.7	0.55	0.6	3.1	2.8
	erythromycin	0.15	0.06	0.07	2.5	2.1
	lincomycin	1.9	0.7	0.9	2.7	2.1
	tetracycline	1.6	0.44	0.54	3.5	3.0
	sodium fusidate	19.0	12.0	12.5	1.6	1.5
	thiostrepton	2.5	1.5	1.6	1.7	1.6

FIG. VII.1.

EFFECT OF KANAMYCIN ON (a) ^{14}C-PHENYLALANINE INCORPORATION INTO TOTAL CELLULAR PROTEINS (b) PROTEASE FORMATION AND (c) α-AMYLASE FORMATION BY WASHED CELL SUSPENSIONS.

Kanamycin and ^{14}C-phenylalanine were added at 90 min.

—●—●— , no addition of drug
—○—○— , kanamycin (1 µg/ml)
—□—□— , kanamycin (2 µg/ml)
—△—△— , kanamycin (4 µg/ml)
—▲—▲— , kanamycin (8 µg/ml)

The 100% value for ^{14}C-phenylalanine incorporation was 4,060 cpm.

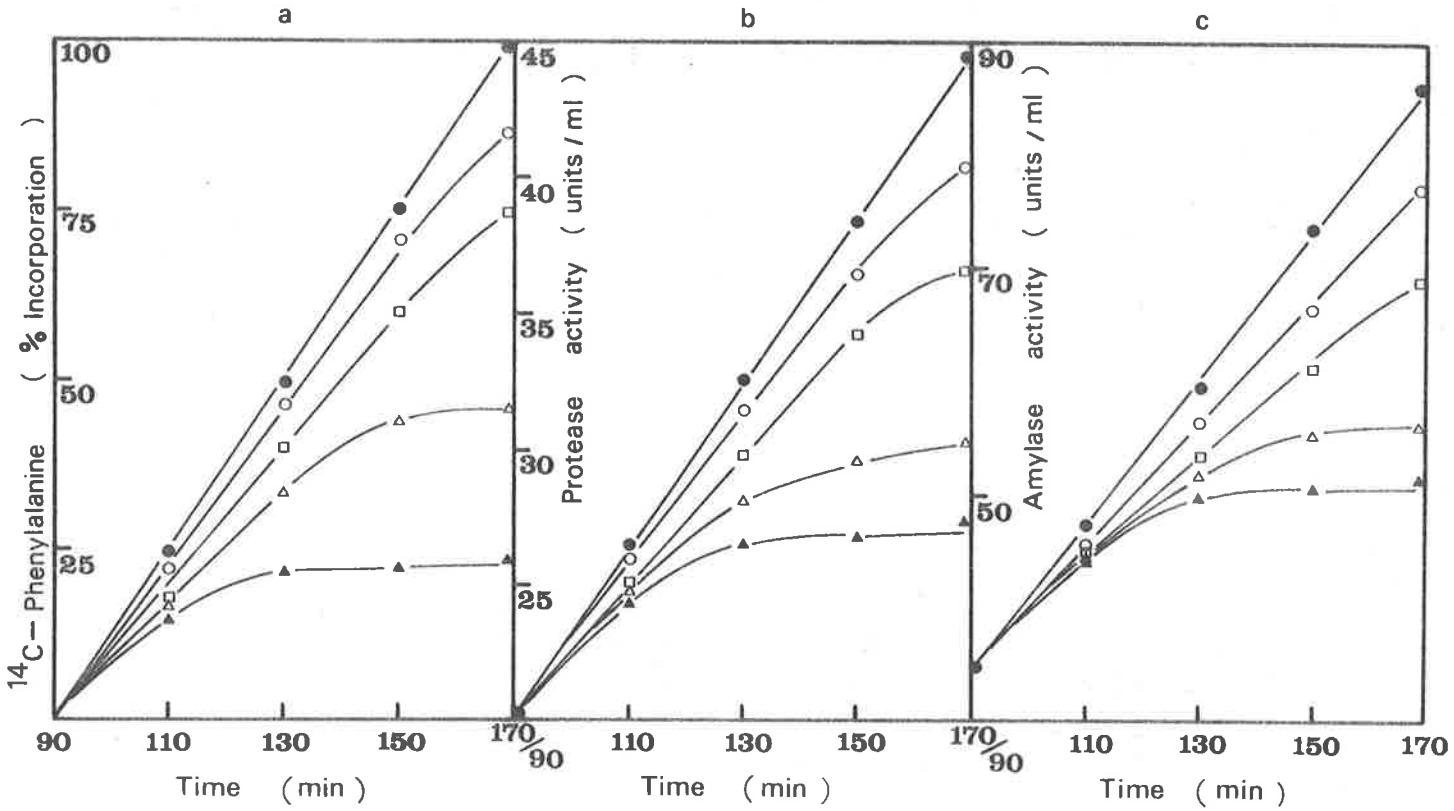

a
b
c
100
75
50
25
45
40
35
30
25
90
70
50
^{14}C— Phenylalanine (% Incorporation)
Protease activity (units / ml)
Amylase activity (units / ml)
90
110
130
150
170
Time (min)

FIG. VII.2.

EFFECT OF NEOMYCIN ON (a) ^{14}C-PHENYLALANINE INCORPORATION INTO TOTAL CELLULAR PROTEINS (b) PROTEASE FORMATION AND (c) α-AMYLASE FORMATION BY WASHED CELL SUSPENSIONS.

Neomycin and ^{14}C-phenylalanine were added at 90 min.

—●—●— , no addition of drug
—○—○— , neomycin (1 µg/ml)
—□—□— , neomycin (2 µg/ml)
—△—△— , neomycin (4 µg/ml)
—▲—▲— , neomycin (8 µg/ml)

The 100% value for ^{14}C-phenylalanine incorporation was 3,860 cpm.

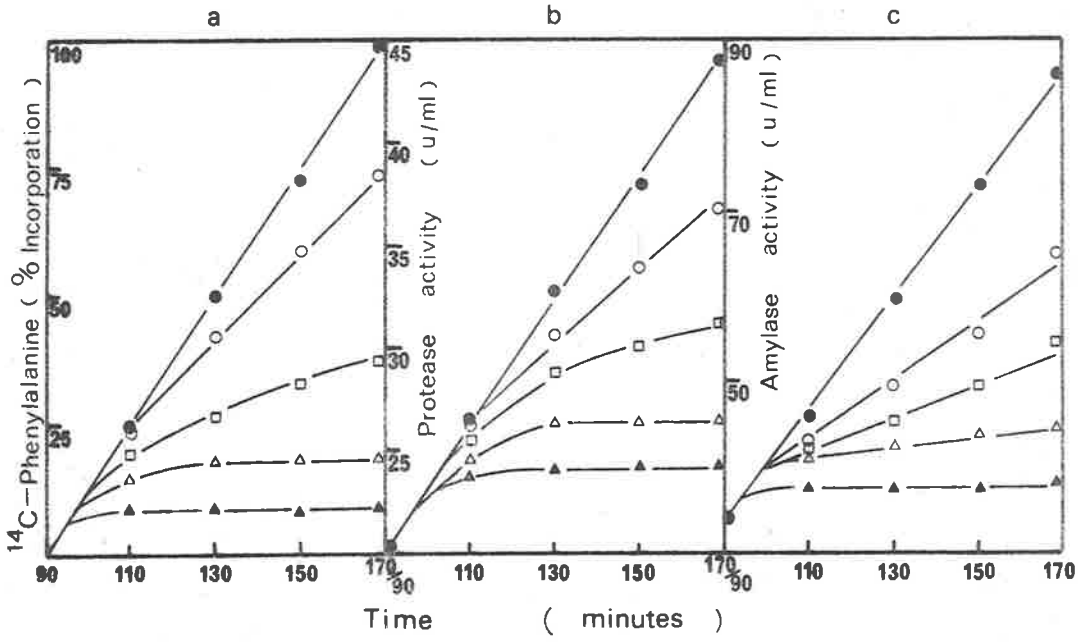
FIG. VII.3.

EFFECT OF STREPTOMYCIN ON (a) ^{14}C-PHENYLALANINE INCORPORATION INTO TOTAL CELLULAR PROTEINS (b) PROTEASE FORMATION AND (c) α-AMYLASE FORMATION BY WASHED CELL SUSPENSIONS.

Streptomycin and ^{14}C-phenylalanine were added at 90 min.

—●—●— , no addition of drug
—○—○— , streptomycin (1 µg/ml)
—□—□— , streptomycin (2 µg/ml)
—△—△— , streptomycin (5 µg/ml)
—▲—▲— , streptomycin (10 µg/ml)

The 100% value for ^{14}C-phenylalanine incorporation was 4,220 cpm.


a
b
c
^{14}C—Phenylalanine (% Incorporation)
Protease activity (u/ml)
Amylase activity (u/ml)
Time (minutes)

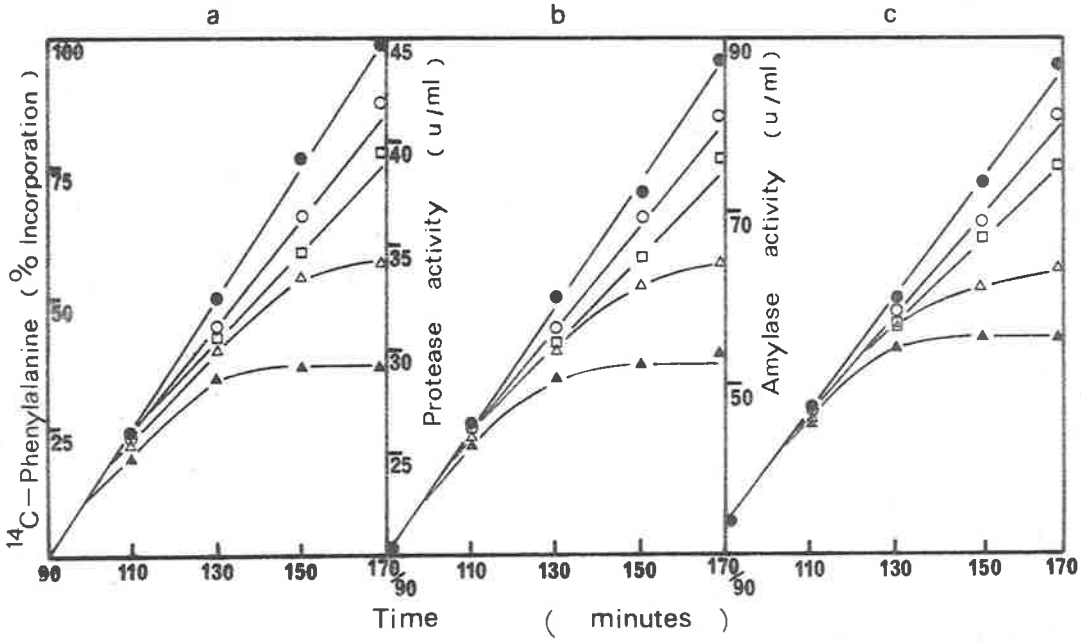

a
b
c
^{14}C—Phenylalanine (% Incorporation)
Protease activity (u/ml)
Amylase activity (u/ml)
Time (minutes)

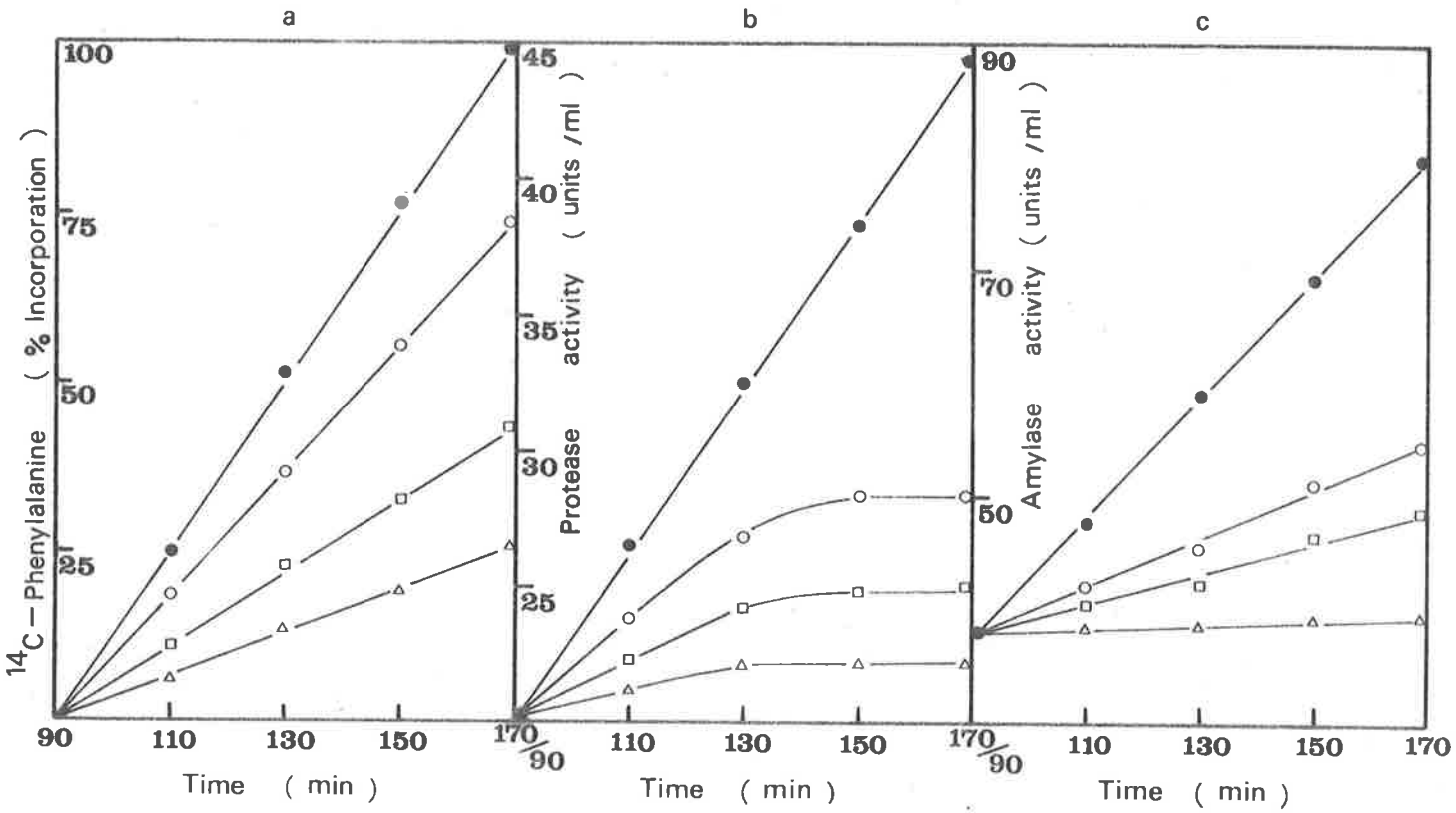
FIG. VII.4.

EFFECT OF CHLORAMPHENICOL ON (a) ^{14}C-PHENYLALANINE INCORPORATION INTO TOTAL CELLULAR PROTEINS (b) PROTEASE FORMATION AND (c) α-AMYLASE FORMATION BY WASHED CELL SUSPENSIONS.

Chloramphenicol and ^{14}C-phenylalanine were added at 90 min.

—●—●— , no addition of drug
—○—○— , chloramphenicol (1 µg/ml)
—□—□— , chloramphenicol (2 µg/ml)
—△—△— , chloramphenicol (4 µg/ml)

The 100% value for ^{14}C-phenylalanine incorporation was 4,060 cpm.


a
b
c
100
75
50
25
45
40
35
30
25
90
70
50
^{14}C – Phenylalanine (% Incorporation)
Protease activity (units /ml)
Amylase activity (units /ml)
90
110
130
150
170
Time (min)

one compares, first of all, the time courses of total cellular protein production with that of α-amylase formation, it can be seen that because of the linear production of all proteins, the degree of inhibition, and hence the differential effect, is the same at any given time for a given drug concentration. However, with protease formation, it can be seen that the degree of inhibition increases markedly with time. Although it may not be directly evident from the profiles, there is on calculation, a slight differential effect at early times; however, the major effect is dependent upon the progressive increase in inhibition.

Any attempted explanation of this effect was confounded by the profiles obtained with the other Group II inhibitors (Figs. VII.5-10); in the case of some of the inhibitors, the effect was not seen at all, and yet with others, it appeared with either one or both of the two extracellular enzymes. Overall, there seemed to be no general pattern and the effect is not readily explained. All that clearly emerges is that the 50S subunit - specific inhibitors will give the large differential effect with time.

To account for the differential effect observed with inhibitors which act on the 50S subunit, the possibility existed, although unlikely, that the 50S subunit for extracellular enzyme synthesis was directly exposed to the external environment. To test this hypothesis, the effect of a derivative of puromycin, 5'-O-cyanoethyl phosphoryl-(3'-N-

FIG. VII.5.

EFFECT OF PUROMYCIN ON (a) ^{14}C-PHENYLALANINE INCORPORATION INTO TOTAL CELLULAR PROTEINS (b) PROTEASE FORMATION AND (c) α-AMYLASE FORMATION BY WASHED CELL SUSPENSIONS.

Puromycin and ^{14}C-phenylalanine were added at 90 min.

—●—●— , no addition of drug
—○—○— , puromycin (2 µg/ml)
—□—□— , puromycin (4 µg/ml)
—△—△— , puromycin (6 µg/ml)
—▲—▲— , puromycin (10 µg/ml)

The 100% value for ^{14}C-phenylalanine incorporation was 4,200 cpm.

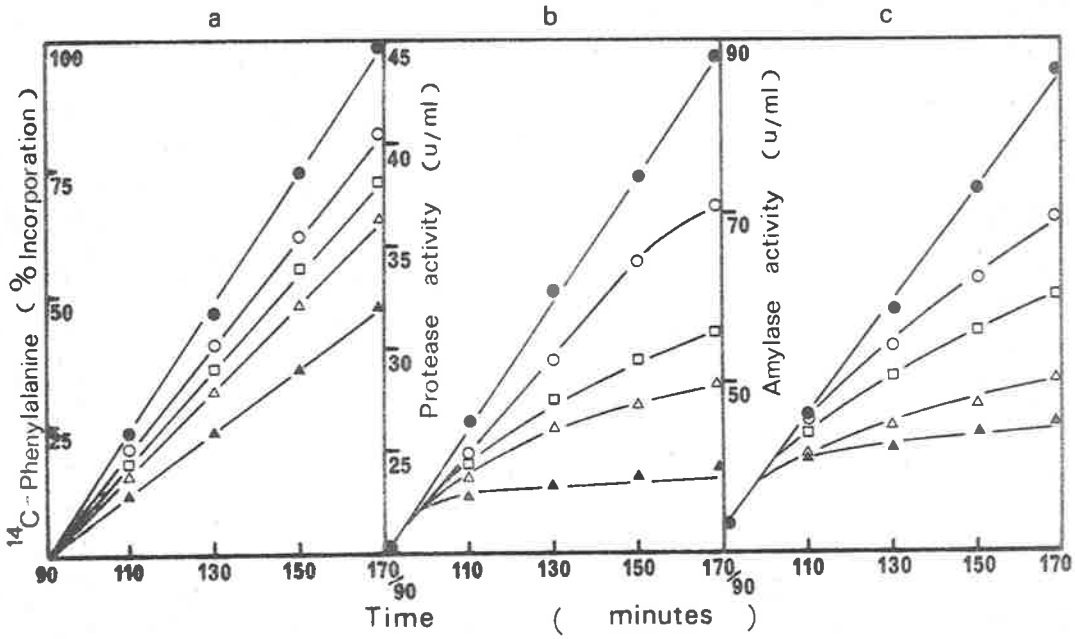
FIG. VII.6.

EFFECT OF ERYTHROMYCIN ON (a) ^{14}C-PHENYLALANINE INCORPORATION INTO TOTAL CELLULAR PROTEINS (b) PROTEASE FORMATION AND (c) α-AMYLASE FORMATION BY WASHED CELL SUSPENSIONS.

Erythromycin and ^{14}C-phenylalanine were added at 90 min.

—●—●— , no addition of drug
—○—○— , erythromycin (0.05 µg/ml)
—□—□— , erythromycin (0.1 µg/ml)
—△—△— , erythromycin (0.25 µg/ml)

The 100% value for ^{14}C-phenylalanine incorporation was 3,830 cpm.


a
b
c
^{14}C-Phenylalanine (% Incorporation)
Protease activity (u/ml)
Amylase activity (u/ml)
100
75
50
25
45
40
35
30
25
90
70
50
90
110
130
150
170
Time (minutes)

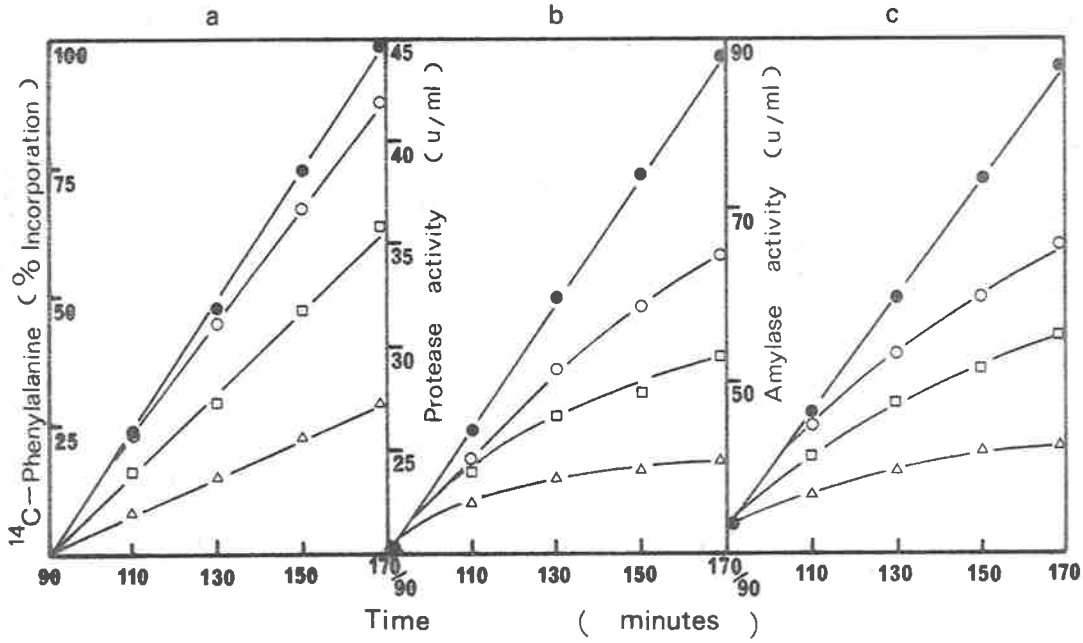

a
b
c
^{14}C-Phenylalanine (% Incorporation)
Protease activity (u/ml)
Amylase activity (u/ml)
100
75
50
25
45
40
35
30
25
90
70
50
90
110
130
150
170
Time (minutes)

FIG. VII.7.

EFFECT OF LINCOMYCIN ON (a) ^{14}C-PHENYLALANINE INCORPORATION INTO TOTAL CELLULAR PROTEINS (b) PROTEASE FORMATION AND (c) α-AMYLASE FORMATION BY WASHED CELL SUSPENSIONS.

Lincomycin and ^{14}C-phenylalanine were added at 90 min.

—●—●— , no addition of drug
—○—○— , lincomycin (0.5 µg/ml)
—□—□— , lincomycin (1.0 µg/ml)
—△—△— , lincomycin (2.5 µg/ml)
—▲—▲— , lincomycin (5.0 µg/ml)

The 100% value for ^{14}C-phenylalanine incorporation was 3,770 cpm.

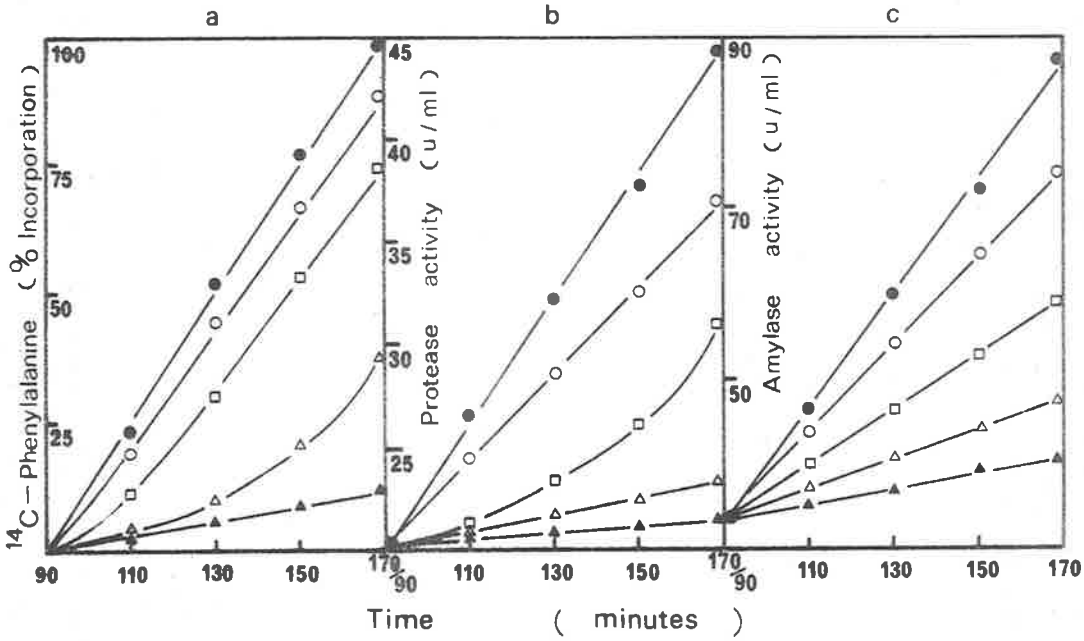
FIG. VII.8.

EFFECT OF TETRACYCLINE ON (a) ^{14}C-PHENYLALANINE INCORPORATION INTO TOTAL CELLULAR PROTEINS (b) PROTEASE FORMATION AND (c) α-AMYLASE FORMATION BY WASHED CELL SUSPENSIONS.

Tetracycline and ^{14}C-phenylalanine were added at 90 min.

—●—●— , no addition of drug
—○—○— , tetracycline (0.25 µg/ml)
—□—□— , tetracycline (0.5 µg/ml)
—△—△— , tetracycline (1.0 µg/ml)

The 100% value for ^{14}C-phenylalanine incorporation was 4,560 cpm.


a
b
c
^{14}C – Phenylalanine (% Incorporation)
Protease activity (u/ml)
Amylase activity (u/ml)
Time (minutes)

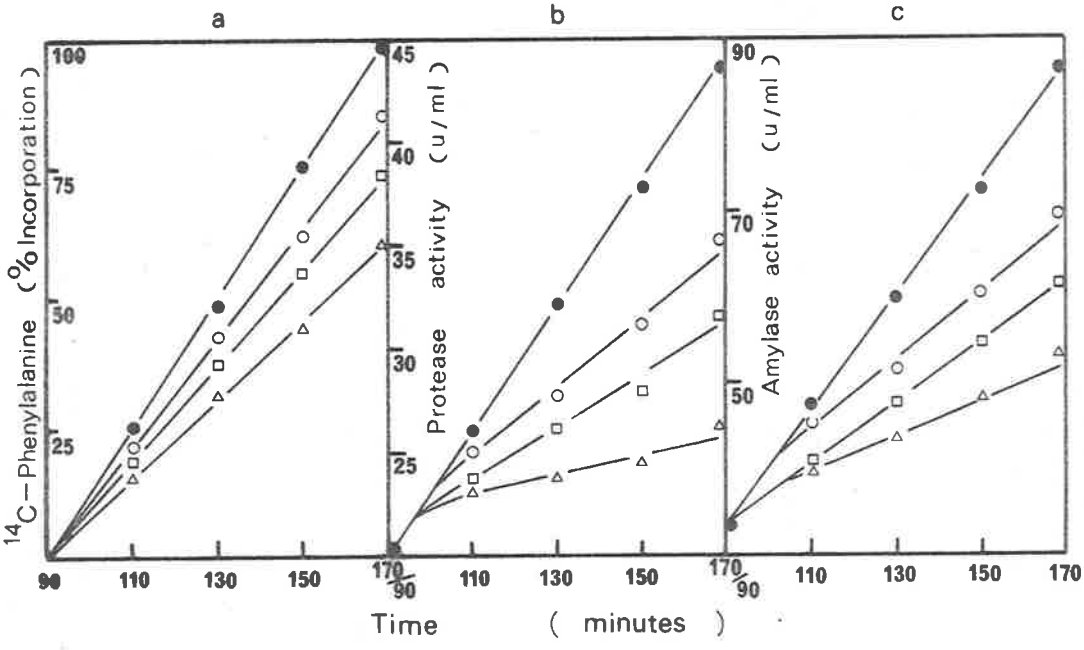

a
b
c
^{14}C – Phenylalanine (% Incorporation)
Protease activity (u/ml)
Amylase activity (u/ml)
Time (minutes)

FIG. VII.9.

EFFECT OF SODIUM FUSIDATE ON (a) ^{14}C-PHENYLALANINE INCORPORATION INTO TOTAL CELLULAR PROTEINS (b) PROTEASE FORMATION AND (c) α-AMYLASE FORMATION BY WASHED CELL SUSPENSIONS.

Sodium fusidate and ^{14}C-phenylalanine were added at 90 min.

—●—●— , no addition of drug
—○—○— , sodium fusidate (10 µg/ml)
—□—□— , sodium fusidate (15 µg/ml)
—△—△— , sodium fusidate (20 µg/ml)
—▲—▲— , sodium fusidate (25 µg/ml)

The 100% value for ^{14}C-phenylalanine incorporation was 2,460 cpm.

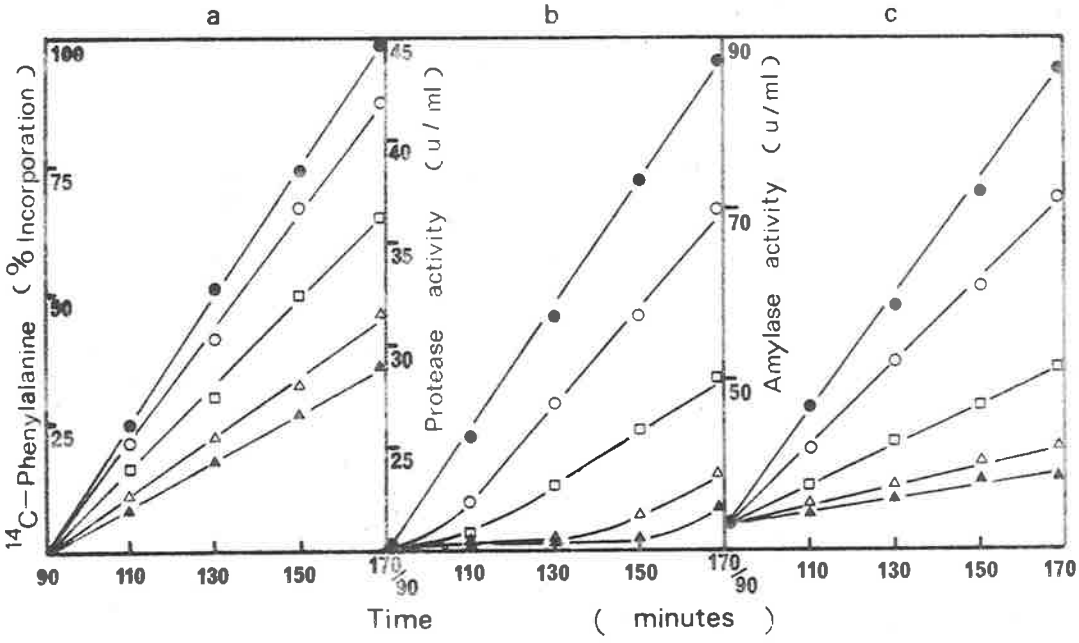
FIG. VII.10.

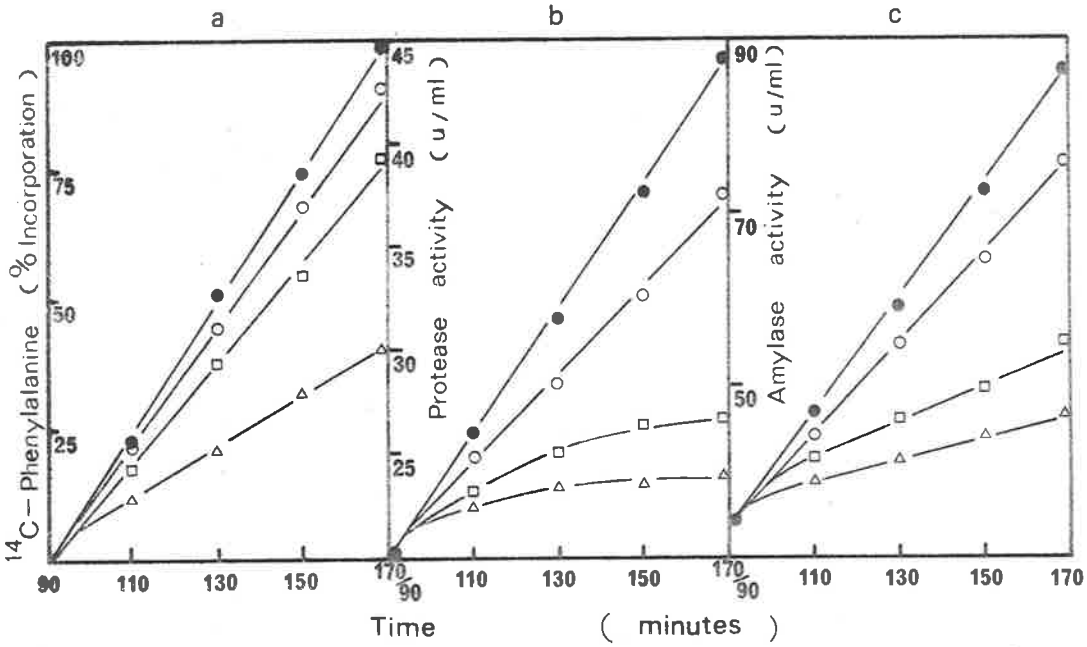
EFFECT OF THIOSTREPTON ON (a) ^{14}C-PHENYLALANINE INCORPORATION INTO TOTAL CELLULAR PROTEINS (b) PROTEASE FORMATION AND (c) α-AMYLASE FORMATION BY WASHED CELL SUSPENSIONS.

Thiostrepton and ^{14}C-phenylalanine were added at 90 min.

—●—●— , no addition of drug
—○—○— , thiostrepton (1 µg/ml)
—□—□— , thiostrepton (2 µg/ml)
—△—△— , thiostrepton (3 µg/ml)

The 100% value for ^{14}C-phenylalanine incorporation was 3,980 cpm.


a
b
c
^{14}C–Phenylalanine (% Incorporation)
Protease activity (u / ml)
Amylase activity (u / ml)
Time (minutes)


a
b
c
^{14}C–Phenylalanine (% Incorporation)
Protease activity (u / ml)
Amylase activity (u / ml)
Time (minutes)

L-phenylalanyl)-puromycin aminonucleoside [cyanoethyl phosphoryl-PANS-phe] on protease formation, was examined. It was reasoned that if the 50S subunit was externally located, the drug might inhibit extracellular enzyme formation even though its passage across the membrane would be prevented by the presence of the ionised phosphate group. However, at a concentration of 50 µg/ml., this drug had no effect on protease formation suggesting that penetration of the cell membrane by this drug is necessary for inhibition. (This assumes that the compound can penetrate the surface layers and reach the cell membrane.) A control experiment with the B. amyloliquefaciens cell-free protein synthesising system utilising endogenous mRNA (Chapter II), showed that the drug was 50% as active as puromycin.

Recent studies have also been carried out with kasugamycin and pactamycin, both of which bind to the 30S subunit (Pestka, 1971) and are known to be capable of acting at the level of initiation of protein synthesis (Tai et al., 1973; Stewart and Goldberg, 1973). The profiles obtained for kasugamycin and pactamycin are shown in Figs. VII.11 and 12, respectively. The results with pactamycin are difficult to interpret because recovery of extracellular enzyme synthesis and total protein synthesis occurs. This finding was not unexpected since Both et al. (1971) showed that total protein synthesis in B. amyloliquefaciens recovers from pactamycin treatment. However, it can be seen that with both drugs,

FIG. VII.11.

EFFECT OF KASUGAMYCIN ON (a) ^{14}C-PHENYLALANINE INCORPORATION INTO TOTAL CELLULAR PROTEINS (b) PROTEASE FORMATION AND (c) α-AMYLASE FORMATION BY WASHED CELL SUSPENSIONS.

Kasugamycin and ^{14}C-phenylalanine were added at 90 min.

—●—●— , no addition of drugs

—○—○— , kasugamycin (800 μg/ml)

—□—□— , kasugamycin (1600 μg/ml)

The 100% value for ^{14}C-phenylalanine incorporation was 1,800 cpm.

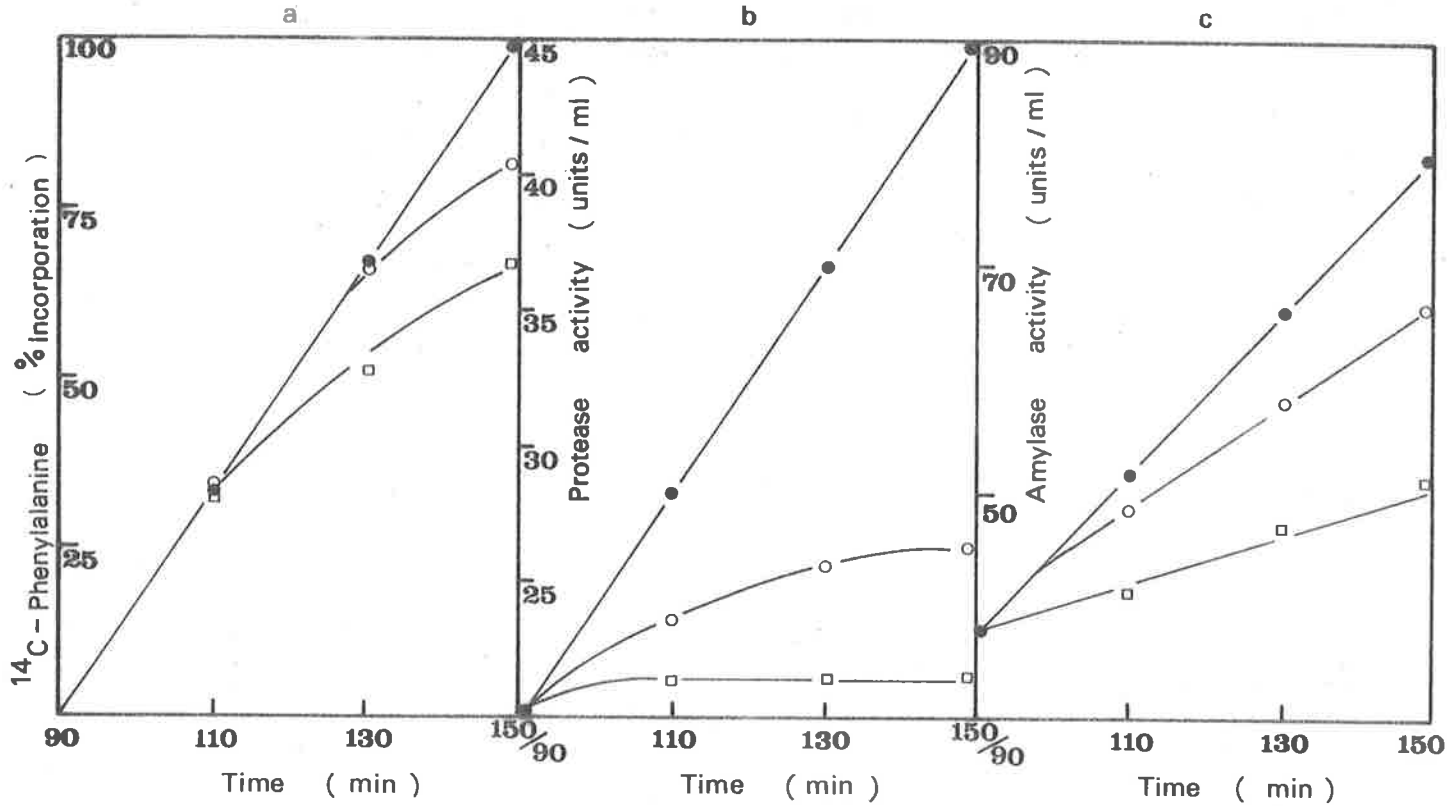

a
b
c
100
75
50
25
45
40
35
30
25
90
70
50
^{14}C – Phenylalanine (% Incorporation)
Protease activity (units / ml)
Amylase activity (units / ml)
90
110
130
150
Time (min)

FIG. VII.12.

EFFECT OF PACTAMYCIN ON (a) ^{14}C-PHENYLALANINE INCORPORATION INTO TOTAL CELLULAR PROTEINS (b) PROTEASE FORMATION AND (c) α-AMYLASE FORMATION BY WASHED CELL SUSPENSIONS.

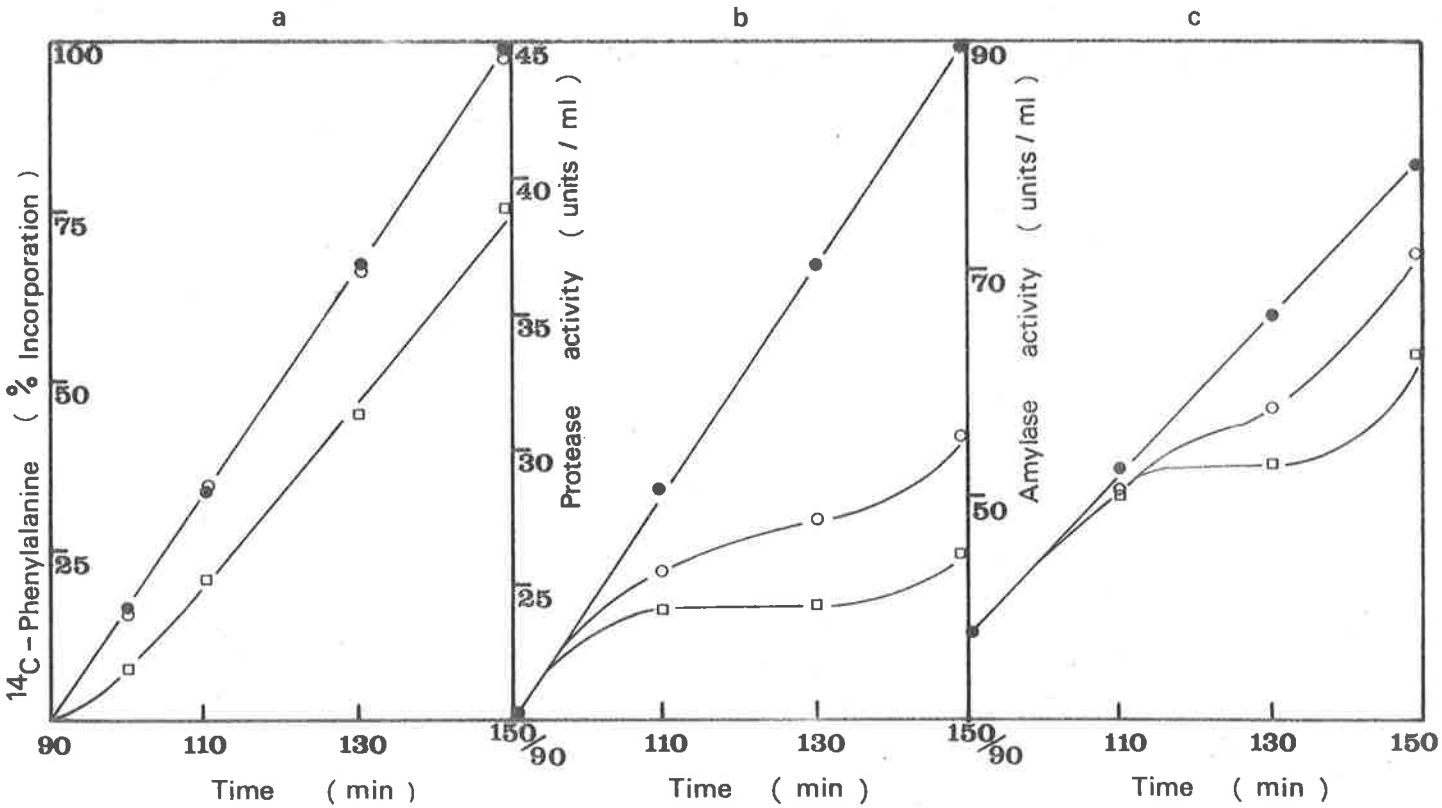
Pactamycin and ^{14}C-phenylalanine were added at 90 min.

—●—●— , no addition of drug

—○—○— , pactamycin (0.025 µg/ml)

—□—□— , pactamycin (0.05 µg/ml)

The 100% value for ^{14}C-phenylalanine incorporation was 3,100 cpm.


a
b
c
100
75
50
25
45
40
35
30
25
90
70
50
^{14}C - Phenylalanine (% Incorporation)
Protease activity (units / ml)
Amylase activity (units / ml)
90
110
130
150
Time (min)

extracellular enzyme formation is more sensitive than total protein synthesis. This effect is most pronounced with the lower concentration of each drug, where although total protein synthesis is almost unaffected, protease formation and α-amylase formation to a lesser extent, are both markedly inhibited.

C. DISCUSSION

The results show that inhibitors of protein synthesis which act on the 50S ribosomal subunit apparently inhibit extracellular enzyme synthesis to a greater degree than intracellular protein synthesis. In contrast, inhibitors which act on the 30S subunit do not show such a differential effect except in the case of kasugamycin and pactamycin as discussed below. This conclusion is complicated by the initial smallness of the effect in some cases and in view of the complex nature of the time course studies it is unfortunately impossible to offer any full explanation of the results observed with the different inhibitors. It should be noted that the findings of Glew and Heath (1971) are still in agreement with those presented here. Although these workers did not distinguish between different inhibitor types, the three inhibitors used were all inadvertently 50S subunit-specific.

If, for the time being, these results are accepted as a real differential effect on cellular and extracellular

protein synthesis, several possible explanations exist. Firstly, it is possible that the 50S ribosomal subunits synthesising extracellular enzymes are inherently different from those involved in cytoplasmic protein synthesis. While the possibility of distinct ribosomes is by no means discounted, it is nevertheless unlikely that the difference would be such that they would be simultaneously more sensitive to a variety of drugs inhibiting in different ways. However, in this context, it is conceivable that the attachment of a 50S subunit to the membrane may alter its sensitivity to a number of drugs acting at different ribosomal sites (although no basis other than vague concepts of conformational changes can be offered for such an effect).

An alternative and more likely postulate is that the 50S subunits engaged in extracellular enzyme synthesis are in a location more available to external drugs than are the attached 30S subunits or ribosomal subunits engaged in cytoplasmic protein synthesis, One might imagine that the 50S subunit for extracellular enzymes protrudes externally through the membrane or is located over a pore in the membrane, but these possibilities seem unlikely since cyanoethyl phosphoryl-PANS-phe did not affect protease formation by washed cell suspensions and presumably needs to penetrate the cell membrane to exert its effect. An alternative compromise situation may be that the 50S subunit for extracellular enzymes is built into the membrane and is therefore exposed to an effectively

higher concentration of drug than its associated 30S subunit which is assumed to be localised in an intracellular environment. However, all such hypotheses must remain speculative until substantiated by *in vitro* studies. It should be noted that the explanation of Glew and Heath (1971) that the peripheral ribosomes are 'titrated out' by the drugs seems untenable since this would offer no distinction between 50S and 30S inhibitors.

In the present work, kasugamycin and pactamycin are exceptions in that they selectively inhibit extracellular enzyme synthesis very markedly but do not combine so far is known with the 50S subunit. Although initiation of protein synthesis in *E. coli* can be inhibited by pactamycin, it is no more sensitive to the drug than elongation (Tai *et al.*, 1973). However, kasugamycin does selectively inhibit initiation (Tai *et al.*, 1973). The discrepancies observed with these drugs in the present studies could be related to their action on initiation of protein synthesis since the proposed model (Chapter V) requires a unique recognition process occurring between the 30S - mRNA complex for extracellular enzymes and the membrane-bound 50S subunit and possibly therefore a unique initiation process. It is interesting to note that kasugamycin has been recently shown to differentially inhibit the initiation of phage MS2 proteins (Kozak and Nathans, 1972).

Finally, the conclusions reported here depend upon

the comparison between inhibition of production of an active extracellular enzyme and of the incorporation of amino acids into total cellular proteins. (The latter was taken as a measurement of intracellular protein synthesis, as explained previously.) Over very short time periods it may be argued that completion of active enzyme might be intrinsically more sensitive to drugs, but over the long experimental time periods used in these studies there is no reason to believe that the two are not comparable indices of the effects of inhibitors on the two types of protein synthesis. Attempts to find a suitable system for directly measuring synthesis of an intracellular enzyme in B. amyloliquefaciens either failed or the measurements were too inaccurate for the purposes of these studies.

CHAPTER VIII

FINAL SUMMARY AND DISCUSSION

FINAL SUMMARY AND DISCUSSION

The exact mechanism by which microorganisms synthesise and secrete extracellular enzymes remains unclear at the present time. The studies presented in this thesis with B. amyloliquefaciens, while not solving the problem, nevertheless have revealed several significant findings and have allowed the hypothesis proposed by May and Elliott (1968a) to be expanded. Although some of the points mentioned below have been stated earlier in the thesis, a recapitulation will be given here to permit an overall discussion of the problem.

It has been established that release of extracellular enzymes occurs without impairment of cell viability and without cell lysis. There is no evidence in bacteria for the existence of secretory granules which exist in the pancreas. The first question that arises is whether the process of synthesis and secretion are two separate events. It has been previously established (Coleman and Elliott, 1965; May and Elliott, 1968a) that for all three extracellular enzymes of B. amyloliquefaciens, inhibition of protein synthesis inhibits secretion instantly, so far as can be measured. There are no detectable reserves of preformed enzymes within the cell (Coleman and Elliott, 1962; May and Elliott, 1968a) although current studies suggest that small amounts of preformed enzyme are associated with the

cell and are believed to be en route to secretion (Gould, unpublished results).

Labelling studies have shown that secreted α -amylase is synthesised de novo from amino acids during the period of secretion by washed cell suspensions and not from any significant pool of preformed protein built up during the growth period (Grant and Coleman, 1972). There is no evidence for any of the extracellular enzymes of B. amyloliquefaciens existing as zymogens. These facts suggest either there are no enzyme molecules within the cell destined for secretion, or else there is a pool of undetectable size. (The small amounts of enzyme detected in disrupted cell preparations probably represents enzyme adsorbed to the cell wall.)

In the case of the extracellular ribonuclease from B. amyloliquefaciens, Smeaton and Elliott (1967a) have shown the existence within the cell cytoplasm of a soluble protein, coded for by a gene distinct from that for the enzyme, which completely inhibits the enzyme virtually instantaneously. Formation of the enzyme-inhibitor complex is essentially irreversible (Hartley, 1970) since only drastic conditions in denaturing solvents will recover active enzyme when the inhibitor and the ribonuclease are mixed together. It seems therefore unlikely that the active enzyme could ever have existed in the cytoplasm. (This line of reasoning, however, can never be more than suggestive since it is conceivable

that the membrane is capable of dissociating the enzyme-inhibitor complex and secreting the enzyme.)

The previous proposal by May and Elliott (1968a) in which extracellular enzymes are synthesised by ribosomes bound at special translational-extrusion sites located at the cell membrane is supported and can be expanded by the experiments reported in this thesis. It was shown that protein synthesis inhibitors specific for the 50S ribosomal subunit apparently inhibit the synthesis of both protease and α-amylase to a greater degree than the synthesis of total cellular proteins. The fact that 30S subunit-specific inhibitors do not show such a differential effect except in the case of two drugs both of which are capable of acting at the initiation level (discussed below), suggests that extracellular enzymes are synthesised on peripherally located ribosomes with the 50S subunit built into the membrane. In this context, membrane fragments from B. amyloliquefaciens were isolated and their ability to synthesise extracellular enzymes was examined in in vitro incubation studies. Although no enzyme synthesis was detected, the fragments, nevertheless, are capable of incorporating labelled amino acids into protein and so provide encouragement for future in vitro studies.

The work has also shown that a pool of mRNA exists in harvested B. amyloliquefaciens cells which can support extracellular protease synthesis for 80 min. It

is proposed that this mRNA accumulation and the similar ones for α-amylase and ribonuclease (Gould et al., 1973) are a repercussion of the mechanism of extracellular enzyme synthesis. Since it is generally accepted that in bacteria, nascent mRNA is translated while attached to the gene, these observations together with the proposed peripheral location for enzyme synthesis, suggests a unique situation exists in which extracellular enzyme-specific mRNA would have to migrate from the chromosome to the membrane prior to translation.

If one postulates that the mRNA for extracellular enzymes is somehow stabilised until translated (to permit this migration), then a positive imbalance of transcription over translation would cause 'queueing up' of mRNA molecules for a limited number of translational sites and so cause a pool of mRNA to accumulate. Although this possibility seemed the most likely at the time, it now seems more likely from the recent findings of Glenn et al. (1973) that the pool of protease mRNA represents a balance between mRNA synthesis and degradation. The excessive mRNA production may be a mechanism to enable sufficient intact mRNA to reach the membrane translational sites despite its rapid breakdown. The nascent polypeptide chain is then thought to be extruded directly into or through the membrane as it is synthesised where it assumes its tertiary structure (discussed below). The possibility that the excessive transcription

and accumulated messenger pools is an aberration of the strain of organism used is rendered unlikely by the recent finding of an identical situation in the genetically unrelated organism, B. subtilis 168.

With regards to the nature of the mRNA pools for extracellular enzymes, a most recent study by the author seems to eliminate the possibility that free polysomes for protease bind to the membrane. Phase 1 production of protease by washed cell suspensions of B. amyloliquefaciens was found to be markedly inhibited by kasugamycin (800 µg/ml.); this drug has been shown to selectively inhibit initiation of protein synthesis in prokaryotic cells (Tai et al., 1973). The most likely possibilities, therefore, are that the messenger is either free or is attached to a 30S subunit and that the translation mechanism begins only after final assembly of the 70S ribosome at the membrane. It should be noted that in this scheme, no requirement exists for a participation by the nascent polypeptide chain for ribosomal attachment to the membrane.

The model of extracellular enzyme secretion in B. amyloliquefaciens proposed above, raises several problems such as (a) the nature of the translational extrusion sites (b) whether special ribosomes exist and (c) the basis of the specific interaction between the messenger and the ribosomes. These aspects are discussed below.

The precise location of the formation of extra-

cellular enzymes in their active forms in B. amyloliquefaciens is unknown, but given the concept of extrusion of the polypeptide chain, there are several possibilities. The enzyme might asssume its folded configuration in the membrane (either in an intermediate or in its final form) before release, or in the periplasmic space or in the external medium outside the cell wall (see Fig. VIII.1). Although in B. licheniformis the first possibility is favoured for penicillinase secretion (Lampen, 1972), there appears to be no corresponding accumulation of extracellular enzymes in the membrane of B. amyloliquefaciens. (The location of enzyme en route to secretion in the latter organism has not, as yet, been determined.)

There is an indirect (and perhaps slender) piece of evidence which might lead to the speculation that in B. amyloliquefaciens the nascent polypeptide traverses the cell wall. It is based on the production by the organism of the protoplast-bursting factor which instantly disorganises the protoplast membrane. It is suggested (Gould et al., 1971) that this peptide-lipid antibiotic leaves the cell in an extended inactive form and assumes an active cyclic configuration outside the wall by lactone ring formation. The intact cell is totally immune to this molecule despite the extreme sensitivity to it of the protoplast membrane of the same cell. This might suggest that the permeability of the cell wall is limited to

FIG. VIII.1.

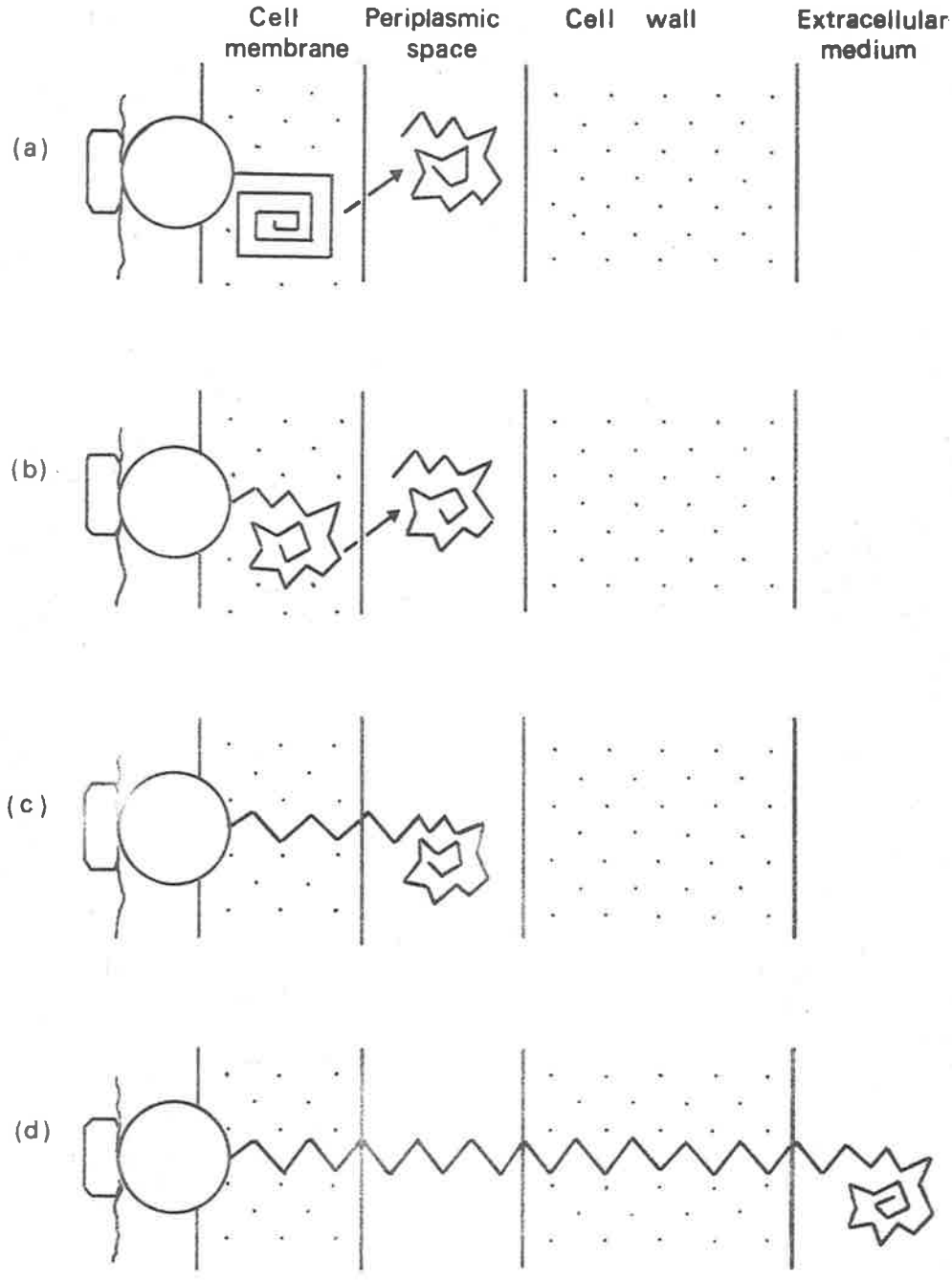
POSSIBLE SITES WHERE THE EXTRUDING POLYPEPTIDE CHAIN CAN ASSUME ITS THREE-DIMENSIONAL CONFIGURATION.

(a) In the cell membrane as an intermediate form which assumes its final configuration in the periplasmic space.

(b) In the cell membrane in its final configuration.

(c) In the periplasmic space.

(d) In the external medium.


Cell membrane
Periplasmic space
Cell wall
Extracellular medium
(a)
(b)
(c)
(d)

molecules smaller than that of the antibiotic (Mol. Wt. approximately 1,000). If this were the case, it might suggest that much larger enzyme molecules could not diffuse from the periplasmic space through the cell wall and this would favour the idea of extrusion of the nascent chain through the cell wall.

Such a scheme would necessitate a mechanism for the survival of the polypeptide chain emerging into a medium rich in proteases. This is a formidable difficulty but may be explained in terms of the observed protection of protease from digestion by loosely bound calcium ions (B. May, Ph.D. thesis) or in terms of the emerging chain being protected by an α-helical configuration. It might also offer an explanation for the striking fact that all three bacterial enzymes are devoid of disulphide bonds, unlike their mammalian cell counterparts. If the polypeptide chains need to assume tertiary configuration in an undefined external medium, any sulphydryl groups on the extruded chains might combine with other sulphydryl reactive molecules in the medium. In this event, cells which produce extracellular enzymes not dependent on disulphide bond formation for activity would presumably have an evolutionary selective advantage. Until much more is known however, such ideas must remain very speculative since the absence of disulphide bonds could be for other reasons not yet appreciated.

It is of interest to note the recent findings

of other authors in relation to the above possibilities. Bettinger and Lampen (1971) and Bissel et al. (1971), in studies investigating the secretion of extracellular penicillinase and proteinase, respectively, have presented evidence that the emerging proteins are not in their normal extracellular conformation. By studying the effects of proline analogues on the formation of alkaline phosphatase (an enzyme which is localised exterior to the cell cytoplasm in the periplasmic space) in E. coli, Morris and Schlesinger (1972) concluded that disruption of the secondary and tertiary structure of the protein does not affect its transport. A similar suggestion that tertiary structure of extracellular proteins is not a characteristic determining secretion has arisen from an analysis of the extracellular proteinase secreted by a B. subtilis mutant (Shoer and Rappaport, 1972).

On the question of the actual site of synthesis, it was shown in this thesis that the addition of lysozyme to washed B. amyloliquefaciens cell suspensions almost immediately inhibited extracellular enzyme formation but only slightly impaired total cellular protein synthesis. Although numerous explanations are possible to account for this observation (see Chapter IV for details), there is the possibility for an involvement, either direct or indirect, of the cell wall in extracellular enzyme synthesis. One likely possibility, under investigation at the present time, is that limited cell wall removal in B. amyloliquefaciens still results in extracellular

enzyme synthesis, but the enzymes are no longer synthesised in an active form; perhaps some cell wall 'activation' step is required during the secretion process. A possible involvement of mesosomes in extracellular enzyme production was suggested by the electron microscopic study of the lysozyme effect on B. amyloliquefaciens cells; small vesicles, mesosomal in nature, were seen to be released at the same time extracellula enzyme production ceased. Additional studies will be necessary to determine the exact role, if any, played by the mesosomes in extracellular enzyme production.

It is interesting to note proposals by other authors with regard to the site of synthesis. Lampen (1972) has suggested that the sites of synthesis and secretion for penicillinase (and by analogy to other exoenzymes) may be limited on the bacterial cell membrane. This suggestion arose as a result of the findings of Bettinger and Lampen (1971) that stripped B. licheniformis protoplasts incorporate nascent penicillinase in an amount approximating that previously removed, thereby indicating a fixed number of penicillinase binding sites. In apparent agreement with this postulate, limited sites of alkaline phosphatase activity in exponentially growing B. subtilis cells were located at the membrane by an electron microscopic histochemical technique (Ghosh et al., 1971).

In an interesting report, the ultrastructure of

Clostridium perfringens, *B. subtilis* and *B. licheniformis* cells were studied by electron microscopy in the process of toxin and enzyme secretion (Smirnova *et al.*, 1971). It is proposed that products to be secreted may either accumulate in special cavities known as 'lock-chambers' prior to their release or release occurs immediately through naturally occurring cell wall channels. Although both were visible in the electron micrographs presented, the evidence was not convincing.

Given that the 50S subunits for extracellular enzyme synthesis are fixed in the matrix of the membrane as postulated above, a number of possibilities arise as to how the preferential synthesis of secretory proteins by membrane-bound ribosomes is achieved. While the possibility cannot be ignored that unique 50S subunits exist for translating extracellular enzyme mRNA, it nevertheless seems unlikely that their difference from the 50S subunits involved in cytoplasmic protein synthesis would be such that they would be simultaneously more sensitive to a variety of drugs inhibiting in different ways as proved to be the case with the experiments reported in this thesis. Evidence for special membrane-bound ribosomes has been previously reported; Rosbash and Penman (1971), studying HeLa cells, have shown that two different classes of membrane-associated ribosomes exist which can be distinguished by their strength of attachment to the membranes. From the bacterial view-point, Vambutas and Salton (1970)

presented evidence suggesting that special membrane-bound ribosomes synthesise membrane proteins in M. lysodeikticus, which although similar to cytoplasmic ribosomes, have lost the ability to bind chloramphenicol.

It also seems reasonable to suggest that either the 30S subunits involved in extracellular enzyme synthesis or the messengers possess some property which enable them to discriminate between free and membrane-bound 50S subunits. Whilst a sequence of nucleotides in mRNA may provide for the localisation of the extracellular enzyme messenger to a specific site on the membrane, it seems more likely that specific initiation factors may exist to select the class of mRNA and that additional factors may then direct the binding of the initiation complex to the membrane-bound 50S subunit. The present studies indicate that this indeed may be the case since both kasugamycin and pactamycin, two inhibitors known to be capable of acting at the initiation level, selectively inhibit extracellular enzyme synthesis in B. amyloliquefaciens.

Uenoyama and Ono (1972b) have recently presented evidence indicating that mRNA from free and bound polysomes of rat liver contains the specificity for a distinct species of ribosomes. Messenger specificity to explain selectivity in secretion has also been suggested in the model proposed by Blobel and Sabatini (1971); here, all mRNAs to be translated on membrane-bound ribosomes are assumed to possess

a common feature, such as several codons near their 5' end, not present in other mRNAs. The resulting common sequence of amino acids near the N-terminal end of the nascent chains (or a modification of it) would then be recognised by a factor mediating the binding of the ribosome to the membrane. The recent experiments of Milstein et al. (1972) suggest that such a mechanism, in which polypeptide synthesis begins in the cytoplasm and the polypeptide itself provides the recognition signal for membrane binding, may indeed be operating with the secretion of light chain immunoglobulin, since the protein is synthesised as a precursor molecule with additional amino acids at the N-terminus. The possibility that such a mechanism occurs in B. amyloliquefaciens is not supported by the kasugamycin studies which imply that initiation of peptide synthesis on the accumulated mRNA has not occurred.

The recent discovery that many eukaryotic mRNA species possess large polyadenylic acid sequences raised the question of whether similar sequences occur in prokaryotic messengers and possibly a means of specificity for the membrane-translational process. However, since the mRNA of E. coli lacks poly-A sequences (Perry et al., 1972) and newly synthesised mRNA isolated from polyribosomes bound to the endoplasmic reticulum of mouse myeloma cells contains a poly-A sequence similar to that isolated from free polyribosomes (Baglioni et al., 1972), the above possibility seems most unlikely.

Despite all of the above problems, the extrusion model at present appears to fit the facts better than any other. It explains the immunity of the cell to protease and ribonuclease and also explains how the cell is able to secrete ribonuclease despite the presence in the cytoplasm of a vast excess of an almost irreversible inhibitor of the enzyme. Finally, passage of the protein through the membrane would be facilitated when it is in the form of an extended polypeptide chain.

A final note must be added concerning the current status of the *in vitro* protein synthesis studies. The finding that mRNA accumulation for the extracellular enzymes in *B. amyloliquefaciens* is a general phenomenon (Gould *et al.*, 1973), gives encouragment for the characterisation of all three extracellular enzyme mRNA species by *in vitro* studies. It is hoped in the near future to isolate and characterise these mRNAs by translating them in the established *E. coli* cell-free system or in an ascites cell system. It remains to be established whether or not a species specificity exists in these studies. In this context, it may be feasible in the future to utilise the Xenopus oocyte system developed by Gurdon *et al.* (1971) for the translation of the *B. amyloliquefaciens* mRNA species, once isolated. This assay system is an extremely sensitive one showing very little species specificity with respect to the type of mRNA which is

translated. Whether a bacterial mRNA can be translated or not in this system, however, remains to be verified.

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