



STUDIES ON EXTRACELLULAR PROTEASE FORMATION

BY BACILLUS AMYLOLIQUEFACIENS

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by

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SUMMARY

1. Extracellular protease synthesis by B. amylolique-faciens continues for 80 minutes in the presence of rifampicin or actinomycin D concentrations sufficient to prevent mRNA synthesis. Despite this the transcription-independent protease production is inhibited by antibiotics specific for protein synthesis and direct labelling studies have confirmed that de novo synthesis of protease occurs under these conditions.
2. The evidence indicates that there exists in harvested cells, a pool of protease-specific mRNA, capable of supporting protease synthesis, in the absence of RNA synthesis, for up to 80 minutes.
3. The protease mRNA is not intrinsically long-lived and has a half-life of the order of a few minutes.
4. The results imply that the mRNA pool is being constantly turned over by a degradation process unrelated to translation, and therefore the mRNA pool may be a result of a dynamic equilibrium between mRNA synthesis, degradation and translation.
5. The time course of protease production in a medium containing a high level of amino acids is biphasic due to

amino acid repression, while that in the presence of a low level of amino acids is essentially linear. The results presented here are compatible with amino acids acting at the level of transcription of the protease mRNA. The biphasic production of protease in a medium containing a high level of amino acids can be accounted for in the following way. The first phase of synthesis, which is insensitive to rifampicin, is due to amino acid repression of mRNA transcription and the translation to exhaustion of the accumulated pool of mRNA. Subsequent derepression of mRNA synthesis and translation of nascent mRNA accounts for the second phase of synthesis which is therefore sensitive to rifampicin and actinomycin D.

6. The protein synthesis inhibitors, pactamycin and fusidic acid, at certain concentrations, completely inhibit protease production without affecting general intracellular protein synthesis. This is interpreted as supporting the concept that protease synthesis occurs on ribosomes located at the periphery of the cell.

7. Pactamycin, at higher concentrations, inhibits general protein synthesis in B. amyloliquefaciens but the cells recover from this inhibition. The recovery is not due to the acquisition of resistance; the results are compatible with the metabolic removal of the antibiotic from the cells.

8. Preliminary attempts to isolate the protease mRNA species have been made. RNA has been recovered apparently



intact from B. amyloliquefaciens and from this, fractions containing some mRNA-like RNA species have been isolated. Preliminary attempts to identify the protease mRNA by translating it in vitro were unsuccessful.

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 3 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:

GERALD W. BOTH

### ABBREVIATIONS

The abbreviations used in this thesis are acceptable to the Journal of Molecular Biology, or are defined in the text.

PUBLICATIONS

- Both, G.W., McInnes, J.L., May, B.K. and Elliott, W.H.  
(1971). "Evidence for a unique accumulation of stable protease m-RNA from Bacillus amyloliquefaciens and implications on the mechanism of extracellular enzyme synthesis".  
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for extracellular protease in Bacillus amylolique-  
faciens: Its relevance to the mechanism of  
enzyme secretion".

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A prokaryote messenger transport mechanism from  
gene to membrane?"

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G.W. Both, A.R. Glenn, J. McInnes, A. Gould, B.K. May and  
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## INTRODUCTION

Work described in this thesis is concerned with the synthesis and secretion of extracellular enzymes by bacteria. The organism Bacillus amyloliquefaciens\* which has been used throughout this study, secretes large amounts of  $\alpha$ -amylase, ribonuclease and protease into the external medium. The work has been aimed at elucidating the mechanism of synthesis and secretion of these enzymes at the molecular level.

The production of extracellular proteins is a widespread phenomenon and many different types of cells secrete large amounts of protein into the external medium. These include plasma proteins, antibodies and hormones such as insulin. 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  $\alpha$ -amylase, protease and ribonuclease produced by the pancreatic exocrine cells, while in plants these enzymes are synthesised by the aleurone-cell layer of germinating barley seeds (Varner and Ram Chandra, 1964). Micro-

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\*The strain of organism, previously known as Bacillus subtilis, was designated Bacillus amyloliquefaciens by Welker and Campbell (1967) on the basis of DNA base composition and hybridization studies.

organisms and bacteria also produce a wide variety of extracellular enzymes; the subject of bacterial enzyme secretion has been reviewed by Pollock (1962) and Lampen (1965).

Apart from the physiological interest of extracellular enzymes, their secretion poses some interesting biochemical problems. These are concerned with the mechanism by which a cell can synthesise large amounts of potentially lethal enzymes such as proteases and ribonucleases and secrete them from the cell, without either the protein synthesising capacity of the cell being affected or the cell itself undergoing lysis and, indeed, without the loss of the normal membrane permeability barrier to small molecules.

Some cells, such as the pancreatic cell, overcome this latter 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, Siekevitz and Caro, 1962). However, there is still the primary problem of explaining how the enzymes are secreted through the endoplasmic reticular membrane before enclosure in secretory granules and this process will now be discussed in detail.



## A. MECHANISM OF SECRETION OF MAMMALIAN SECRETORY PROTEINS

### (1) Site of synthesis

It is now well established that proteins destined for export from secretory cells are synthesised on ribosomes bound to the rough endoplasmic reticulum. This was first established for chymotrypsinogen (Siekevitz and Palade, 1958, 1960), later for  $\alpha$ -amylase (Siekevitz and Palade, 1966; Redman, Siekevitz and Palade, 1966), both of which are secreted by guinea pig pancreas and also for serum albumin secretion by rat liver (Campbell, Greengard and Kernot, 1960; Redman, 1968; Tagaki, Tanaka and Ogata, 1969).

Free polysomes, it appears, synthesise those proteins not destined for secretion (Goldberg and Green, 1964; Redman, 1969; Ganoza and Williams, 1969; Hicks, Drysdale and Munro, 1969; Sherr and Uhr, 1970; Andrews and Tata, 1971; Uenoyama and Ono, 1972b). It should be noted, however, that non-secretory cells, for example, brain and muscle cells, also contain some membrane-bound ribosomes, but it has been suggested that the proteins of membranes are synthesised on membrane-bound ribosomes and this might explain their presence in cells which have no obvious secretory function (Dallner, Siekevitz and Palade, 1966).

The sequence of events occurring after the synthesis of secretory proteins on the endoplasmic reticulum is also well established; radioautography has shown that the proteins are released into the cisternal space of the endoplasmic reticulum from which they move to be incorporated into secretory granules in the Golgi apparatus. The secretory granules

then migrate to and fuse with the membrane, releasing their contents into the external medium (Caro and Palade, 1964). However, the process of protein ejection from the cell is not directly related to the work in this thesis and it will not be further discussed. Instead, attention will be focussed on the primary process of secretion which occurs during the synthesis of secretory proteins on the rough endoplasmic reticulum.

(2) Evidence for simultaneous synthesis and secretion of proteins made on the endoplasmic reticulum

Redman, Siekevitz and Palade (1966) claimed that proteins, synthesised by ribosomes attached externally to microsomes, are vectorially released into the interior of the microsomes. These workers prepared a pigeon pancreas microsomal fraction which could incorporate labelled amino acids uniformly into  $\alpha$ -amylase. After different periods of labelling, the microsomal system was fractionated. Initially, most of the total  $\alpha$ -amylase radioactivity was associated with membrane-bound ribosomes, identifying them 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 content of the microsomes. Hence, it was concluded that newly synthesised  $\alpha$ -amylase had been preferentially transferred from its site of synthesis at the ribosome into the microsomal cavity (the microsomal equivalent of the cisternal space). Similarly, Redman and Sabatini (1966) presented evidence that unfinished proteins are also vectorially discharged into the interior

of guinea pig liver microsomes when they are released from the ribosomes with puromycin. Streptovaricin 'A', which inhibits protein synthesis without causing the release of incompleated peptides did not show this effect.

Redman (1967), using a rat liver microsomal system, observed a similar transfer of labelled nascent proteins across the microsomal membrane following the addition of puromycin. This transfer occurred in the absence of adenosine triphosphate, at 0° and in the absence of changes in the membrane phospholipid metabolism, suggesting that the only requirement for the transport of nascent protein across the microsomal membrane was that it be released from the membrane-bound ribosome.

These results imply that in vivo, the nascent polypeptide chain is unidirectionally transferred through the membrane of the endoplasmic reticulum while it is being synthesised. This process then appears to be a fundamental difference between secretory and non-secretory cells since it has been reported that membrane ribosomes in the latter do not release their nascent proteins into the interior of the microsome. It has been suggested that there is a major difference in the attachment of ribosomes to the membranes of the endoplasmic reticulum of secretory and non-secretory tissues which results in a tissue-specific difference in the unidirectional release of nascent proteins (Andrews and Tata, 1971).

(3) Model for secretion of proteins through the endoplasmic reticulum

Sabatini, Tashiro and Palade (1966) examined the arrangement of ribosomes on the microsomal membrane and showed that EDTA-treatment of rat liver microsomes caused a stepwise release of attached ribosomal subunits, the smaller subunit being preferentially lost while the larger subunit remained attached to the membrane. Even at high EDTA concentrations, a considerable proportion of large subunits remained attached to the membrane and these were found to contain most of the newly-made proteins. It was concluded that the ribosomes were attached to the microsomal membrane via the large ribosomal subunit and electron microscopy supported this conclusion. The EDTA release of ribosomal subunits was primarily due to the chelation of  $Mg^{++}$ , needed for their attachment to the membrane. However, the fact that the large ribosomal subunit contained newly synthesised protein suggested that the latter may contribute to the membrane attachment of the subunit.

On the basis of their observations, Redman and Sabatini (1966) proposed the following model for protein synthesis and secretion. The large membrane-bound ribosomal subunit is assumed to contain a central channel which is continuous with the cisternal space via a discontinuity in the membrane. (The existence of this channel was suggested by electron micrographs of negatively stained large subunits while discontinuities in the membrane were seen under the subunits in sectioned specimens. The peptides being synthesised are assumed to grow within a central channel in

an environment continuous with the cisternal space such that the unidirectional nature of peptide chain release is explained simply by structural restrictions. Based on the puromycin experiments described above, these authors suggested that release of the polypeptide chain directly into the cisternal space might occur when protein synthesis is terminated.

Compatible with this model is the data of Blobel and Sabatini (1970) who showed that part of the nascent polypeptide chain could be protected from proteolytic digestion by the large ribosomal subunit, suggesting that the protein chain was associated with it. However, this protection was not specific to membrane-bound ribosomes since free ribosomes offered similar protection (Sabatini and Blobel, 1970). Nevertheless, the association of the nascent polypeptide chain with the large ribosomal subunit appears to be an intimate part of the secretion process since proteolysis at 0° of microsomes labelled in vivo or in vitro resulted in the cleavage of the nascent chain into a fraction associated with the large ribosomal subunit and one associated with the deoxycholate-soluble fraction of the microsomes. This is compatible with the passage of the nascent polypeptide chain through the ribosome into the microsomal membrane or the cisternal space, as suggested by the model.

However, recent experiments by Sauer and Burrow (1972) suggest that for rat liver, the polypeptide chain could be discharged into the membrane preceding its release into the

cisternal space. This interaction may have physiological significance in that glycosyl transferases, known to be associated with liver microsomal membranes would be able to add carbohydrate moieties as the polypeptide chain becomes associated with the membrane. This idea is supported by the recent findings of Redman and Cherian (1972) who reported that glycoproteins were indeed glycosylated during their association with the rough endoplasmic reticular membrane, while non-glycoproteins, for example, albumin, passed directly into the microsomal lumen after their release from the ribosomes. However, that the albumin was transiently associated with the membrane, but remained unmodified, cannot be eliminated.

In spite of all previous evidence, it seems that the concept of vectorial transfer of nascent protein chains across the microsomal membrane is in doubt. This conclusion is derived again from the work of Sauer and Burrow (1972), who showed that exogenous labelled intact proteins could bind to microsomal membranes *in vitro*, thereby contaminating the deoxycholate-soluble fraction. Therefore, the appearance of labelled protein in this fraction may be artefactual and not due to specific vectorial transfer of nascent polypeptide chains into, or through, the microsomal membranes.

In agreement, Burke and Redman (1973) have recently showed that  $C^{14}$ -polyphenylalanine released from the ribosomes by puromycin can absorb non-specifically to membranes in vitro. They suggest that the appearance of acid-insoluble radioactivity in the detergent-soluble fraction of microsomes alone is not

sufficient evidence for vectorial transport, as previously thought. Therefore, the concept of vectorial transport of nascent proteins across the microsomal membrane can only be substantiated by future experiments which eliminate artefactual binding of proteins to the membrane.

(4) Binding of ribosomes to the endoplasmic reticular membrane

Studies on the means by which the ribosomes bind to the endoplasmic reticular membrane are currently in a confused state. Generally, two approaches to the problem have been used to study the membrane-ribosome interaction. The first involves the detachment of ribosomes from microsomal membranes under defined conditions; the second, attempts to reconstitute the rough endoplasmic reticulum in vitro. It now seems that the latter approach is doubtful, as will be described below.

There are conflicting reports regarding the possible role of the polypeptide chain in the binding of the large (60S) ribosomal subunit to the endoplasmic reticular membrane. It was suggested (Sabatini, Tashiro and Palade, 1966) that the polypeptide chain was responsible for the binding of 60S subunits, resistant to release by EDTA, to the membrane since newly synthesised protein was associated with them. Consistent with this was the observation that rat liver ribosomes could be released from their membrane attachment by trypsin or puromycin in vitro (Chefurka and Hayashi, 1966). Similarly, the distribution of labelled nascent protein, after proteolysis, between the 60S subunit and the membrane also

suggests the involvement of the polypeptide chain in binding the ribosome to the membrane.

In conflict with these observations, however, are other reports (Blobel and Potter, 1967; Rosbash and Penman, 1971; Andrews and Tata, 1971) which claim that puromycin treatment does not reduce the number of membrane-bound ribosomes. However, perhaps the most convincing evidence that the polypeptide chain is not involved in the binding comes from the in vivo studies of Baglioni, Bleiberg and Zauderer (1971) who found that the 60S subunit could bind to the membrane in the absence of protein synthesis. This suggested the existence of an alternative binding mechanism which may be ionic in nature as postulated for the binding of polysomes to the endoplasmic reticular membrane in vitro (Shires, Narurkar and Pitot, 1971b). However, the validity of this approach is doubtful as discussed later.

Recent work (Adelman, Sabatini and Blobel, 1973) has clarified somewhat, the method by which ribosomes bind to the membrane in vitro. It appears that there are at least two types of interaction between the ribosomes and the endoplasmic reticular membrane. The first of these can be disrupted by high concentrations (750 mM of KCl, suggesting that it is of an electrostatic type. The second is believed to result from an interaction between the subunit-attached polypeptide chain and the membrane, perhaps due to the partially assumed tertiary structure of the protein chain, or its interaction with modifying enzymes in the membrane. It is shown that relatively low concentrations of KCl (100-



200 mM), in the absence of puromycin, release a mixture of inactive membrane-bound ribosomes and those active ones having a short nascent polypeptide chain. As the KCl concentration is increased (up to 750 mM) a gradient of release of ribosomes with longer polypeptide chains occurs. Some ribosomes can only be released by combined puromycin/KCl treatment and it therefore seems that the polypeptide chain is involved in their binding. In agreement, Tanaka and Ogata (1972) also claim that two fractions of membrane-bound ribosomes exist in rat liver; one of these is releasable by ribonuclease. Only those ribosomes not released by ribonuclease are involved in secretory protein synthesis, again suggesting the existence of two types of membrane-ribosome interactions.

As mentioned earlier, a second approach to the study of the ribosome-membrane interaction involves the binding of ribosomes, subunits, or polysomes to stripped rough endoplasmic reticular membrane in vitro (Suss, Blobel and Pitot, 1966; Campbell, 1970; Ragland, Shires and Pitot, 1971; Shires, Narurkar and Pitot, 1971a,b; Rolleston, 1972). However, the following work raises serious doubts about the validity of such attempts to reconstitute the rough endoplasmic reticulum in vitro.

Hochberg, Stratman, Zahlten, Morris and Lardy (1972) examined the binding of rat liver and hepatoma polysomes to stripped rough endoplasmic reticulum in vitro. They found that thiol reagents, known to dissociate ribosomes, did not significantly reduce binding of P<sup>32</sup>-labelled polysomes to stripped rough endoplasmic reticulum. Similarly, denaturing the protein of P<sup>32</sup>-labelled polysomes or stripped rough

endoplasmic reticulum of liver of hepatoma with heat, trichloroacetic acid or perchloric acid did not alter the binding in vitro.

In addition, Burke and Redman (1973) have examined the distribution of puromycyl peptides recovered from a variety of recombination systems in order to determine whether these can duplicate in vitro the specific ribosome-membrane interaction of hepatic rough endoplasmic reticulum found in vivo. The systems tested included the binding of polysomes to endoplasmic reticular membranes preconditioned with citrate and pyrophosphate (Ragland, Shires and Pitot, 1971), with EDTA and ribonuclease (Shires, Narurkar and Pitot, 1971b), or with puromycin in the presence of 0.75 M KCl (Adelman, Blobel and Sabatini, 1970). The binding of ribosomal subunits to puromycin/salt-conditioned membranes in the presence of polyuridylic acid was also demonstrated. It was found that the distribution of puromycyl peptides following detergent fractionation indicated that vectorial transport did not occur in any of these in vitro recombination systems and therefore their use as models of the in vivo situation must be reconsidered. Alternatively, these results raise the question as to whether vectorial transport occurs at all in vivo.

(5) Models for cellular discrimination between secretory and non-secretory protein synthesis

It has already been mentioned that secretory cells synthesise proteins for both intracellular and extracellular

functions. The question therefore arises; how does the cell determine whether a given protein will be synthesised at the membrane and secreted, or synthesised on free polysomes and used intracellularly?

There are two basic mechanisms by which this might occur. Blobel and Sabatini (1972) have suggested that polysome formation in the cytoplasm might precede the membrane attachment of the polysome and the subsequent secretion of the nascent protein. They suggest that the information as to whether a polysome becomes membrane-bound may lie in the mRNA sequence itself. All mRNA's for secretory proteins might have a common feature such as several codons near their 5' end, not present in mRNA's of non-secretory proteins. The resulting common sequence of amino acids near the N-terminal of the nascent chains might then be recognised by a factor mediating the binding of the polysomes to the membrane after protein synthesis has been initiated in the cytoplasm. The binding factor could be a soluble protein which recognises both a site on the large ribosomal subunit and a site on the membrane. Presumably, if this scheme were to operate, any secretory proteins completed before the polysome attached to the membrane would somehow be degraded in the cytoplasm. There is no direct support for this model.

The second mechanism involves the binding of the ribosomal subunits to the endoplasmic reticular membrane with the subsequent initiation of secretory protein synthesis and direct secretion of the protein. Evidence for this is provided by Baglioni, Bleiberg and Zauderer (1971) who

showed that in mouse myeloma cells, the 60S subunit alone could attach to the membrane in vivo. For initiation of protein synthesis to occur, the 40S-mRNA complex normally formed as part of the process of initiation, would have to recognise a 60S subunit located on the membrane. Compatible with this model, Borgese, Blobel and Sabatini (1973) present evidence that in rat liver, only the 40S subunit of membrane-bound ribosomes can exchange with the free subunit pool after puromycin release of the nascent polypeptide chain in vitro. It appears that the 60S subunit remains membrane-bound; however, this may result from the in vitro system lacking the factors necessary to exchange the large ribosomal subunit.

Assuming that the initiation of protein synthesis does occur on the endoplasmic reticular membrane, how then might a 40S-mRNA complex recognise a 60S membrane-bound subunit and ultimately determine whether or not a protein will be synthesised on membrane-bound ribosomes and secreted?

There are several ways in which a specific mechanism for this process could be provided, depending on whether the specificity resides in the ribosomes, the mRNA, initiation factors, or in all or some of these components. It seems that there may indeed exist a special broad class of mRNA molecules for secretory proteins since it has been found that in rat liver, mRNA contained in free polysomes is labelled with  $^{14}\text{C}$  orotic acid more rapidly than that present in membrane-bound polysomes (Murty and Sidransky,

1972), implying that a basic difference in turnover rate exists between the two classes. This difference is not due, however, to the variations in the poly-adenylic acid sequences known to be attached to the 3'-OH end of most eukaryotic mRNA species (Lee, Mendecki and Brawerman, 1971). Baglioni, Pemberton and Delovitch (1972) have shown that in mouse myeloma cells the poly A sequences from free and membrane-bound polysomes are very similar, implying that these sequences are not involved in directing mRNA into one or other type of polysome.

Whether ribosomes themselves have any intrinsic specificity in the secretion process is unclear. It has been reported that active free and membrane-bound polysomes differ in several ways, for example, in their  $Mg^{2+}$  plus  $Ca^{2+}$  and their  $Mg^{2+}$  dependence (Vernie, Bont and Emelot, 1972), their sensitivity to growth hormone (McDonald and Korner, 1971) and antibiotics (Glazer and Sartorelli, 1972). In addition, Shafritz and Isselbacher (1972) claim that in contrast to free ribosomes of rat liver, membrane-bound ribosomes show a lesser requirement for KCl, a reduced ratio of endogenous to poly U-dependent protein synthesis and a preference for membrane-ribosomal wash for stimulation of endogenous messenger translation in vitro. However, whether these differences are due to specificities inherent in the ribosomes or whether they are due to the membrane-ribosome interaction is not clear. It is interesting to note, in this context, that on the large subunit of free ribosomes from rat liver, there appears to be a protein present only

in small amounts in bound large subunits (Borgese, Blobel and Sabatini, 1973). Perhaps the loss of this protein coupled with the binding of the 60S subunit to the membrane is sufficient to alter the characteristics of the subunit such that it is now recognised as a specific component in secretory protein synthesis. In any event, it seems that there exists at least a structural similarity between free and membrane-bound ribosomes since they protect a similar length of nascent polypeptide chain from proteolytic digestion (Sabatini and Blobel, 1970). Therefore, ribosomal modification might be necessary to impart a specific feature in ribosome.

A convenient way to render specific the process of secretory protein synthesis might arise if the cell contained initiation factors specific for secretory protein synthesis. Work by Uenoyama and Ono (1972) suggests that this may be so, but in addition it seems that a measure of specificity resides in other factors of protein synthesis also. From rat liver cells, these workers isolated free and membrane-bound polysomes and prepared from each type, single ribosomes, polysomal mRNA and 0.8 M KCl ribosome-wash (which contained crude initiation factors). Incorporation of  $H^3$ -phenylalanine into protein was maximal in each case only in the homologous system, that is, mRNA, ribosomes and 0.8 M KCl ribosome-wash prepared only from free polysomes or membrane-bound polysomes. Heterologous systems gave reduced  $H^3$  phenylalanine incorporation, indicating the existence of a distinct specificity between the three fractions involved in free or membrane-bound protein synthesis. A scheme might be envisaged whereby initiation

factors discriminate between secretory and non-secretory protein synthesis by allowing initiation of protein synthesis to occur with selected mRNA molecules bound only to the appropriate ribosomes situated at the correct membrane site.

It is quite evident from the preceding discussion that most of the mechanism of secretory protein synthesis in eukaryotes remains speculative. Nonetheless, some of the ideas presented in this section have been adapted to proposed mechanisms of bacterial secretory protein synthesis as will now be discussed.

#### B. The mechanism of protein secretion by bacteria

The mechanism of protein secretion by bacteria is not as well understood as that for mammalian systems. Many proteins secreted by bacteria are potentially lethal enzymes, for example, protease and ribonuclease, and these cells too must overcome the problem of secreting such molecules without damage to themselves. A simple solution to the problem can be found if it is postulated that the cytoplasmic membrane is the bacterial equivalent of the mammalian endoplasmic reticulum and that a basic mechanism of vectorial discharge of secretory proteins is similar in the two cell types. There is indeed evidence claimed to indicate that protein synthesis occurs at the cytoplasmic membrane, although this has not been shown to be that for extracellular enzymes.

(1) Evidence that the cytoplasmic membrane is a site of protein synthesis

The evidence that the cytoplasmic membrane is a site of protein synthesis comes primarily from the detection of membrane-bound polysomes in cell lysates which could incorporate labelled amino acids into protein (Schlessinger, 1963; Schlessinger, Marchesi and Kwan, 1965; Moore and Umbreit, 1965). Additionally, Moore and Umbreit found that 30S subunits were released from the membrane during fractionation, while the 50S subunit remained attached. Similarly, Cundliffe (1970) was able to isolate 50S membrane-bound ribosomal subunits, suggesting that polysome may be bound to the membrane via the large subunit as found for rat liver microsomes by Sabatini et al. (1966). In both S. faecalis and Azotobacter vinelandii, an extra protein has been found in association with membrane-bound ribosomes and it may be responsible for binding them to the membrane (Brown and Abrams, 1970; Scheinbuks, Kalschmidt and Marcus, 1972).

However, the apparent proportion of membrane-bound ribosomes appears to vary greatly. Fluctuations have been found to be caused by such factors as the method of cell lysis (Hendler and Tani, 1964), the use of lysozyme to disrupt cells (Patterson, Weinstein, Nixon and Gillespie, 1970), the  $Mg^{++}$  concentration (Schlessinger, Marchesi and Kwan, 1965), the age of the cell culture (Moore, Kocun and Umbreit, 1966) and the  $K^+$  concentration (Coleman, 1969).

Therefore, it is evident that many factors influence the number of membrane-bound ribosomes found in cell preparations



and indeed, whether those found in vitro actually exist in vivo is entirely uncertain.

(2) Possible models for protein secretion in bacteria

The two most studied bacterial enzyme secretion systems are those of penicillinase production in Bacillus licheniformis and the production of  $\alpha$ -amylase, ribonuclease and protease by B. amyloliquefaciens, with which this thesis is concerned. These will now be discussed in turn.

(a) Models for penicillinase secretion

Lampen (1965) proposed a mechanism of extracellular enzyme secretion in which enzyme synthesis is coupled with membrane synthesis, occurring possibly at the mesosome, which is a complex internal invagination of the cytoplasmic membrane. 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 invagination was dependent on the membrane growing out of the mesosome. Mesosomes were implicated in penicillinase secretion by Staphylococcus aureus when it was found that they were structurally altered upon release of the enzyme (Beaton, 1968).

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

(Sargent, Ghosh and Lampen, 1968). Further studies (Sargent, Ghosh and Lampen, 1969) showed that these structures were not essential for the actual synthesis of penicillinase since protoplasts, which lacked these structures, were able to synthesise and secrete the enzyme. However, the pattern of release was abnormal in that the enzyme did not first become cell-bound. Presumably, therefore, on their concepts, the enzyme is normally incorporated into the membrane of the mesosome-like structures as part of the secretion process.

Further work has shown that there is an extracellular form of penicillinase which is secreted directly, as well as two membrane-bound forms of the enzyme (Sargent and Lampen, 1970a). One of these, the vesicle penicillinase, is a precursor of the exoenzyme, but the other, the plasma membrane enzyme is a stable resident of the membrane. Variations in conformation and hydrophobicity are believed to account for the differences in membrane binding properties of the three forms of penicillinase.

Sargent and Lampen (1970b) have proposed the following model for penicillinase secretion: a hydrophobic form of the enzyme, capable of entering the membrane, is produced and inserted into the plasma membrane at a growing point where other membrane components are also inserted. The enzyme then either undergoes a conformational change to the hydrophilic exoenzyme form which is secreted, or it polymerises and assumes one of the two membrane-bound forms. Since penicillinase synthesis and secretion are fairly closely coupled, the majority of the enzyme must be secreted directly.

It is proposed that the conversion of the enzyme from the hydrophobic to the hydrophilic form is a crucial step in extracellular enzyme secretion in micro-organisms.

(b) Model for extracellular enzyme secretion  
in *B. amyloliquefaciens*

A question fundamental to the model of secretion is whether the enzymes are synthesised and then secreted as two separate events. It has been established that for all three extracellular enzymes of *B. amyloliquefaciens* inhibition of protein synthesis stops secretion rapidly. The amount of preformed enzyme detectable in lysates of cells is small and of the order that could be accounted for by adsorption to the cell surface. At no time is there a substantial accumulation of enzyme within the cell (Coleman and Elliott, 1962; Coleman and Elliott, 1965; May and Elliott, 1968a). Furthermore, labelling studies have shown that at least 76% of secreted  $\alpha$ -amylase is synthesised de novo from amino acids and not formed from any significant pool of preformed protein (Grant and Coleman, 1972). There is no evidence for the existence of radioactive zymogens within the cell.

These facts suggest either that there are no enzyme molecules within the cell destined for secretion, or else there is a pool of small size. It is impossible to decide between these two possibilities by measurement of levels, but accumulative evidence has been obtained which suggests that extracellular enzymes have never existed as such

inside the cell. In the case of extracellular ribonuclease production, Smeaton and Elliott (1967) have shown that there exists a protein in the cell cytoplasm which specifically inhibits the enzyme. 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 ribonuclease are mixed together. It is therefore unlikely, though not excluded, that the active enzyme could have existed in the cytoplasm. On this indirect evidence it was postulated that B. amyloliquefaciens extracellular enzymes at no time exist in a completed form inside the cell, but that the nascent polypeptide chain is extruded directly through the membrane as it is synthesised to take up its tertiary structure with enzyme activity only outside the cell (May and Elliott, 1968a).

Bettinger and Lampen (1971) have since proposed a similar hypothesis. It was shown that penicillinase synthesis by protoplasts partially stripped of their cell-bound enzyme is sensitive to trypsin and chymotrypsin; implying that the emerging polypeptide chain is degraded before it can assume an active configuration. It was suggested that penicillinase too is formed by membrane-bound polysomes from where the growing chain is extruded into the membrane in an extended form. Both models are formally analogous to that proposed by Redman (1967) for secretion of enzymes through the endoplasmic reticulum. The basic postulate for extracellular enzyme secretion in B. amyloliquefaciens will be discussed further and extended by the work in this thesis.

## C. Properties of bacterial extracellular enzymes

### (a) General properties

Although enzymes have been found in the culture media of many bacterial species, particularly those in old cultures, the presence of such an activity is not sufficient evidence that the enzyme is a truly extracellular one. An extracellular enzyme is regarded as one which is physiologically liberated from the cell and not released by death or cell lysis. There are now many examples where the extracellularity of enzymes has been established. For example, various species of Bacillus, Clostridium and Streptococcus have been shown to secrete polysaccharidases, proteases, nucleases, penicillinases and toxins.

Most of the enzymes secreted by bacterial cells are concerned with the degradation of large molecular weight substances (for example, proteins, polysaccharides and nucleic acids which cannot penetrate the cell. Presumably, the function of extracellular enzymes (for example, protease,  $\alpha$ -amylase and ribonuclease) is to degrade such external molecules to products that are small enough to enter the cell where they can be utilised. The exception to this is the bacterial penicillinase which may fulfil a protective role by degrading external penicillin which can readily penetrate the cell. Apparently, bacteria have developed extracellular enzymes as an alternative to phagocytosis and intracellular digestion of large molecules which would be made impossible by the rigidity of the bacterial cell wall.

Gram-positive organisms produce extracellular

enzymes almost exclusively and it may be significant that Gram-negative bacteria have a generally more complex cell wall structure which may be impermeable to the release of enzymes from the cell. However, Gram-negative organisms do produce a number of 'surface' enzymes which are specifically localised in the periplasmic space between the cytoplasmic membrane and the cell wall. These enzymes are all degradative and include, in the case of E. coli, phosphatases, phosphodiesterases and penicillinase.

Bacterial extracellular enzymes are generally proteins of molecular weight not exceeding about 80,000 and many are well characterised. For example, the B. subtilis alkaline proteases (for example, BPN' and subtilopeptidase A) and the neutral protease are single chain polypeptides of molecular weights 30,000, 27,000 and 45,000 respectively.

A curious and striking difference is observed between bacterial and mammalian extracellular enzymes in that in the former the cyst(e)ine content never exceeds one half-cysteine residue per molecule, 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 only slightly greater than the B. amyloliquefaciens enzyme which is completely devoid of cyst(e)ine (Hartley and Barker, 1972). There are exceptions, however, Hofsten and Tjeder (1965) reported two disulphide bridges in an extracellular protease from a strain of Arthrobacter while an extracellular streptococcal protease was shown to contain

a thiol group (Ferdinand, Stein and Moore, 1965). The streptococcal protease is unique in that it is released as an inactive extracellular zymogen which is activated by reduction, due primarily to cyst(e)ine residues in the cell wall (Liu and Elliott, 1965).

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's external barriers. These authors reasoned that the differences between the disulphide bond content of mammalian and bacterial extracellular enzymes probably reflects a fundamental difference in the membrane structure and secretory mechanisms of animal and bacterial cells. Bacterial enzymes, although lacking disulphide bridges, do not 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 for activity on the proteins (Pollock, 1962). If however, the secretion of extracellular enzymes occurs by the mechanism proposed by May and Elliott (1968a), the polypeptide chain would have to assume its tertiary configuration in an undefined external medium where any -SH groups on the extruded chain might combine with other -SH reactive molecules in the medium. The absence of S-S bonds in extracellular enzymes would presumably have an evolutionary selective advantage in this regard (Elliott and May, 1969).

Many proteases and  $\alpha$ -amylases in particular have

$\text{Ca}^{++}$  associated with their molecules; the B. subtilis  $\alpha$ -amylase requires  $\text{Ca}^{++}$  for stability, immunity to protease digestion and catalytic activity (Stein and Fischer, 1958) as does the proteinase of Sarcina strain (coccus P) (Bissell, Tosi and Gorini, 1971). Hsiu, Fischer and Stein (1964) proposed that  $\text{Ca}^{++}$ , bound inside the  $\alpha$ -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  $\text{Ca}^{++}$  is not a universal phenomenon of extracellular enzymes. There is, for example, no evidence that the Bacillus cereus strain 569 penicillinase requires this cation (Pollock, 1962).

(b) Properties of Bacillus amyloliquefaciens  
extracellular enzymes

It seems necessary to briefly summarise the general properties of the extracellular enzymes of B. amyloliquefaciens, to complete this introduction.

It has been shown previously that production of each of these enzymes is subject to separate controls (though whether they have some primary control mechanism, such as activation of a sporulation genome, is unknown). None of the enzymes is substrate induced; ribonuclease synthesis is partially repressed by inorganic phosphate (May, Walsh, Elliott and Smeaton, 1968) while the synthesis of protease and  $\alpha$ -amylase is almost completely repressed by amino acids (May and Elliott, 1968a; Gould, May and Elliott, 1973). Protease production is



most effectively repressed by combinations of proline and isoleucine, or of glutamic and aspartic acids (May and Elliott, 1968a). There is no carbohydrate repression of  $\alpha$ -amylase production (Coleman and Elliott, 1962).

The time-courses of enzyme appearance in washed-cell experiments vary from one to another; ribonuclease production occurs linearly (Coleman and Elliott, 1965) while protease production in the same medium is biphasic (May and Elliott, 1968a). Depending on the age of the cells,  $\alpha$ -amylase production can be either biphasic or produced with a pronounced lag phase (Gould, May and Elliott, 1973; Coleman and Elliott, 1962).

At least two species of protease are produced by B. amyloliquefaciens and these are referred to as the neutral (pH optimum 7.6) and the alkaline (pH optimum 10.5 - 11.0) proteases. The molecular weight of both species is in the range of 25-30,000; the neutral protease contains no more than one cyst(e)ine residue and possibly zero (Carey, 1966). The neutral protease at least requires  $\text{Ca}^{2+}$  for activity (May, unpublished observations).

$\alpha$ -Amylase has a molecular weight of 48,000 (Grant, 1967) and appears to be composed of two subunits of molecular weight 24,000 (Connellan and Shaw, 1970). The enzyme also requires  $\text{Ca}^{2+}$  for its activity (May, unpublished observations), and it contains no half-cystine residues (Grant, 1967).

The amino acid sequence of ribonuclease has been completely determined (Hartley and Barker, 1972). The molecule is a single polypeptide chain of molecular weight 12,382,

containing 110 amino acid residues. There are no half-cystine residues and  $\text{Ca}^{2+}$  is not required for activity.

With this background to the concepts of extra-cellular enzyme synthesis and secretion, the work comprising this research project can now be presented.

D. The aims of the work in this thesis

The work in this thesis was directed at the basic aim of determining in molecular terms how and where extra-cellular enzymes are synthesised and how they are secreted in Bacillus amyloliquefaciens. The project was based on extending or refuting the model for secretion proposed on indirect evidence by May and Elliott (1968a).

Unexpected results, reported in Chapter 3 were obtained and this major discovery provided a new approach to the problem and the incentive to establish a cell-free system with which it is hoped to obtain the final solution.

CHAPTER 2

MATERIALS AND METHODS

## A. Methods

### (1) Growth of the organism

The organism was previously described as an unclassified strain of Bacillus subtilis; however, it has now been identified as Bacillus amyloliquefaciens (Welker and Campbell, 1967). It was grown for about 25 hours as follows. Culture medium was inoculated with a platinum loop from a suspension of spores and shaken at 30°C until the  $A_{600\text{nm}}$  of twenty-fold diluted culture was 0.18. This was measured by diluting a sample of the culture with saline at 30°C.

The culture medium contained 34 mM- $(\text{NH}_4)_2\text{HPO}_4$ , 1 mM- $\text{MgSO}_4$ , 5 mM-KCl, 4.25 mM-sodium citrate, 0.125 mM- $\text{CaCl}_2$ , 0.0125 mM  $\text{ZnSO}_4$ , 0.5 mM  $\text{FeCl}_3$ , 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 pH was adjusted to 7.3 with  $\text{H}_3\text{PO}_4$ . The trace metal solution contained 0.5 mg  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.5 mg ammonium molybdate, 5.0 mg  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  and 0.01 mg  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  dissolved in 1 l. of water.

### (2) Washed-cell experiments

Cells, harvested when the  $A_{600\text{nm}}$  of twenty-fold diluted culture reached 0.18, were washed twice by resuspension and centrifugation at 30°C with the appropriate suspending medium and finally resuspended to the original cell density, except where stated in the text. The suspending medium was the same as the growth medium, but  $\text{FeCl}_3$  and yeast extract were omitted, thereby limiting cell growth. In

later experiments, a modified suspending medium was used and this will be described when appropriate.

A sample of the cell suspension (20 to 40 ml) was shaken in a 250 ml conical flask at 30°C and samples (1.0 ml) were withdrawn at appropriate times, centrifuged and the supernatants assayed for enzyme activity. In appropriate experiments, casamino acids were replaced with L-amino acids which were 'Mann assayed' products of Mann Research Laboratories, New York. The Casamino acid concentrations used in the suspending medium were either 0.5% (w/v) or 0.025% (w/v) and these will be referred to as high and low amino acid medium, respectively.

### (3) Measurement of total protein and RNA synthesis

To measure total cellular protein synthesis, 2.0 ml of a washed-cell suspension were shaken either with 0.5  $\mu$ Ci of uniformly labelled L-[ $^{14}$ C]leucine (spec. act. 316 mCi/mmole), with L-[ $^{14}$ C]phenylalanine (spec. act. 455 mCi/mmole), or with 1.0  $\mu$ C of L-[ $^{14}$ C]valine (spec. act. 260 mC/mmole). At appropriate times, samples (0.1 ml) were withdrawn and treated as described previously (May and Elliott, 1968a) except that the Oxoid filters were counted by liquid scintillation in a Packard Tri-Carb spectrometer (90% efficiency) using scintillation fluid containing 3 g of 2,5 diphenyloxazole (PPO) and 0.3 g of 1,4-bis-[2-(4 methyl-phenyloxazolyl)]benzene (POPOP) per litre of toluene.

2-[ $^{14}$ C]Uracil incorporation into total cellular RNA was measured by shaking 3.0 ml of a washed-cell suspension

with 0.75  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]uracil (spec. act. 52 mCi/mmmole) in suspending medium supplemented with 4.0  $\mu\text{g}$  of unlabelled uracil/ml. Samples (0.1 ml) were withdrawn at various times and processed as described previously (May and Elliott, 1968a) except that liquid scintillation counting was again used.

In experiments where  $^{32}\text{Pi}$  (of varying spec. act.) was incubated with a washed-cell suspension, incorporation was measured as follows. Samples of cell suspension were precipitated at  $0^\circ\text{C}$  for 20 min in 3 ml of 5% TCA containing 0.5% (w/v  $(\text{NH}_4)_2\text{HPO}_4$ ), filtered onto Oxoid filters and washed with 5 ml of TCA- $(\text{NH}_4)_2\text{HPO}_4$  mixture and finally with 5 ml of 1% (v/v) acetic acid. Liquid scintillation counting was again used.

#### (4) Enzyme assay methods

Quantitative protease estimations were carried out using the casein digestion method at pH 7.6 described previously (May and Elliott, 1968a).

The protease activity in the fractions obtained from the column was detected by a semi-quantitative assay which involved the clearing of milk solution; a sample of the fraction was added to 5.0 ml of 1% aqueous solution of commercial powdered skim milk, incubated at  $37^\circ\text{C}$  and the  $A_{600\text{nm}}$  was then read.

$\alpha$ -Amylase and ribonuclease activities were estimated using the methods described earlier by May and Elliott (1968b) and Coleman and Elliott (1965), respectively.

(5) 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  $\mu$ l. 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 350V.

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°C. 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.

(6) Polyacrylamide gel electrophoresis

(a) Electrophoresis of protein samples

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°C

for 2.5 hr with the electrodes reversed.

To detect protease activity, the gels were frozen in solid CO<sub>2</sub>, sliced longitudinally and the flat surface was incubated in contact with an agar/casein plate for 3 hr at 37°C.

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.

(b) Electrophoresis of RNA samples

Total RNA from B. amyloliquefaciens and ribosomal RNA from E. coli were electrophoresed on polyacrylamide gels according to the method of Loening (1967). Conditions for electrophoresis and staining of RNA are specified in the legends to the appropriate figure.

(7) Preparation of equipment for the isolation of RNA

Wherever possible, equipment used for isolation of RNA was sterilised either by heating at 180°C for 3 hr or autoclaving. Articles not suited to this treatment such as



plastic centrifuge tubes, were soaked in 1 N NaOH, rinsed four times with distilled water and dried.

Buffers, made up using sterile or alkali-washed equipment, were treated with diethylpyrocarbonate and left overnight before use.

(8) Sucrose density gradients

Ribonuclease-free sucrose (Schwarz/Mann, Orangeburg, N.Y.) was used exclusively in buffer solutions.

The sucrose density gradients used in this work were linear gradients of 5 to 20% (w/v) sucrose in 10 mM-Tris HCl buffer, pH 7.4 containing 10 mM-EDTA. The volume of the gradients was 11.6 ml.

No more than 600  $\mu$ g of crude RNA was loaded onto the gradients in this work. All RNA samples analysed were centrifuged on gradients in the Spinco SW41 rotor at 41,000 rev./min. at 4°C in the Beckman L<sub>2</sub>65B ultracentrifuge. The length of the run varied and is specified in the legend to the appropriate figure.

Gradients were fractionated as follows. The bottom of the gradient tube was punctured with a needle and sucrose (40%, w/v) was pumped in, thereby displacing the gradient through the top of the tube. An LKB Uvicord II flow cell was used to monitor the A<sub>260nm</sub> profile of the gradient and this was recorded by an Hitachi QPD53 recorder. Fractions (15 drops) were collected from the end of a piece of tubing supported in a retort clamp into alkali-washed tubes, if RNA fractions were being prepared, or directly, at

0°C, into 3 ml of 5% trichloroacetic acid containing 0.5% (w/v)  $(\text{NH}_4)_2\text{HPO}_4$ . These latter samples were processed as described in Methods (3).

(9) Zonal centrifugation

Sucrose solutions were made up in 0.001 M-Tris HCl buffer, pH 7.4 containing 0.05 M KCl and 0.0015 M EDTA. Ribonuclease-free sucrose was used exclusively. The gradient, of volume 560 ml varied from 5 to 20% (w/v) sucrose and was prepared using an MSE gradient former. It was linear except for the first 10% of the volume which was 5% sucrose and the last 10% of the volume which was 20% sucrose. The underlay solution, 110 ml of 25% (w/v) sucrose, was used to expel some of the 5% sucrose from the top of the gradient before the RNA sample (containing up to 40 mg of RNA in 10 ml of distilled water) was loaded. Finally, overlay solution, 50 ml of 2.5% (w/v) sucrose, was loaded. RNA samples were centrifuged in the Beckman 650 ml titanium rotor at 7°C for 17 hr at 45,000 rev./min. in the Beckman L<sub>2</sub>65B ultracentrifuge.

The zonal gradient was fractionated by displacing it from the rotor with 40% sucrose, pumped in at a constant rate. The  $A_{260\text{nm}}$  profile of the gradient was monitored by an Isco recorder connected to an Isco fraction collector and 6 ml fractions were collected.

(10) Preparation of *E. coli* ribosomal RNA

*E. coli* ribosomes were a gift from Dr. P. Greenwell. The ribosomes were suspended in TE buffer (50 mM-Tris.HCl, pH 7.5; 10 mM-EDTA) containing SDS (1%, w/v) and Macaloid (0.1%, w/v). The mixture was incubated at 37°C for 10 min before 20 ml of phenol containing 8-hydroxy-quinoline (0.1%, w/v) was added. The solution was gently shaken and the layers separated by centrifugation. The aqueous solution was twice more extracted with 20 ml and 10 ml of the phenol mixture, respectively. Following three extractions with equal volumes of ether, N<sub>2</sub> was bubbled through the aqueous layer. *E. coli* ribosomal RNA (r-RNA) was precipitated, by the addition of sodium acetate (2%, w/v) and two volumes of ethanol, overnight at -15°C. The RNA precipitate was centrifuged down, dissolved in water and stored in liquid N<sub>2</sub>.

(11) Estimation of DNA and RNA

Crude RNA samples, prepared as described in the text, were assayed for DNA content by the method of Burton (1956) and for RNA as follows. Crude RNA (2-3 A<sub>260nm</sub> units) was precipitated with an equal volume of 1 N HClO<sub>4</sub> at 0°C for 15 min. and centrifuged down. The supernatant was decanted, 0.5 ml of 0.3 N KOH was added to the pellet and the RNA was hydrolysed at 37°C for 60 min. After this time, 0.05 ml of 3 N HClO<sub>4</sub> and 0.05 ml of 6 N HClO<sub>4</sub> were added and the solution cooled to 0°C for 15 min before centrifugation. A sample (0.5 ml) of the supernatant was assayed by adding 3.0 ml of orcinol reagent and heating the solution at 100°C

for 25 min. The orcinol reagent, freshly made, contained 1.0 ml of 1% (w/v) orcinol, 40 ml of conc. HCl and 1.0 ml of 10%  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ . The  $A_{660\text{nm}}$  of the solutions was read and the RNA concentration was calculated from a standard RNA curve.

#### B. Materials

All radioactive compounds, except  $^{32}\text{Pi}$ , were obtained from Schwarz/Mann, Orangeburg, New York.  $^{32}\text{Pi}$  radioisotope was supplied as carrier-free orthophosphate by the Australian Atomic Energy Commission.

Rifampicin was obtained from Scharz/Mann; actinomycin D was a gift from Merck, Sharp and Dohme. Chloramphenicol was obtained from Parke-Davis, fusidic acid from Squibb, and pactamycin from Upjohn. Pactamycin was also a gift from Dr. H.F. Lodish.

Tri-iso-propylnaphthalene sulphonic acid was obtained from Eastman Kodak and 8-hydroxyquinoline and sodium dodecyl sulphate from Sigma. Macaloid was obtained from Baroid Division, National Lead Co., Houston, Texas.

CHAPTER 3

EVIDENCE FOR AN ACCUMULATION OF mRNA SPECIFIC FOR EXTRACELLULAR  
PROTEASE IN BACILLUS AMYLOLIQUEFACIENS

## A. INTRODUCTION

It was earlier shown (May and Elliott, 1968a) that extracellular protease formation by washed-cell suspensions of B. amyloliquefaciens follows a biphasic time course due to multivalent amino acid repression in a medium containing high levels of amino acids (0.5% Casamino acids). This result has been confirmed (Fig. 3.1). There is an initial rapid production for 30-40 min. (phase 1) followed by a period during which extracellular protease does not increase. At approximately 90 min., linear extracellular protease production recommences and goes on indefinitely (phase 2). However, protease formation in a medium containing low levels of amino acids (0.025% Casamino acids) is not biphasic (Fig. 3.1).

It was previously shown (May and Elliott, 1968a) that protease synthesis by washed cells in a high amino acids medium is sensitive to actinomycin D and chloramphenicol, implying that de novo synthesis of the enzyme was occurring. However, it was suggested that high concentrations of actinomycin D may exert toxic effects on the cells (Professor J. Mandelstam, person communication). Therefore, it was decided to re-examine the effect of this drug on protease formation by washed cells. In addition, the effect of rifampicin, another RNA synthesis inhibitor, was also examined.

## B. The nature of the proteases produced by Bacillus amyloliquefaciens

The proteases produced by Bacillus amyloliquefaciens have not been investigated as thoroughly as those of other organisms.

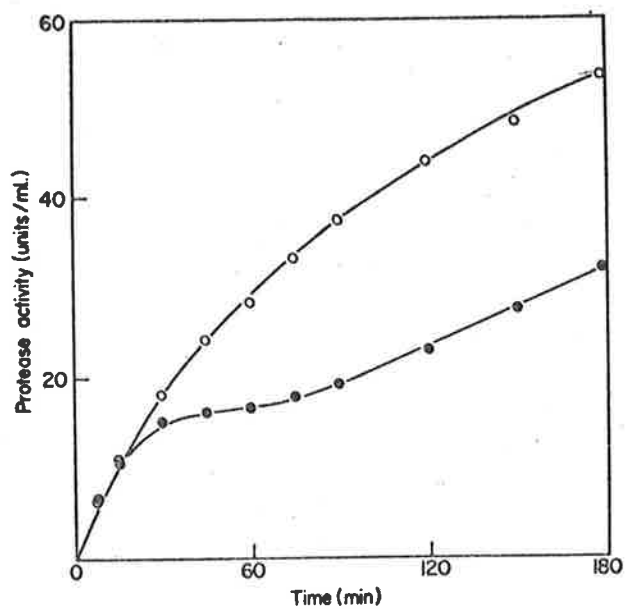


FIG. 3.1. Protease formation by washed cells in the presence of 0.025% w/v (—○—○—) and 0.5% w/v (—●—●—) Casamino acids.

However, work described below shows that at least two extracellular proteases are formed and these may be separated by electrophoresis on either polyacrylamide gels at pH 8.5 with electrodes reversed (due to the positive charge on the molecule) or on Sepharose polyacetate strips. When extracellular culture medium is electrophoresed on Sepharose strips (see Methods (5)), a major and minor band of activity are detected when the strips are incubated in contact with an agar/casein plate at pH 7.6. (These bands are identical to those shown in plate 3.1.) Crystallisation of protease from 25-hour culture medium of B. amyloliquefaciens by the method of Hagihara, Matsubara, Nakai and Okunuki (1958), described for Bacillus subtilis, resulted in a preparation which yields the same two bands on electrophoresis (Carey, 1966). These bands are a result of two distinct enzyme species; this was shown during the present work 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 (Carey, 1966). A method for isolating the neutral protease is described in this thesis but no method for isolating the alkaline protease pure, in any significant amounts, has yet been obtained.

When cells are harvested as described in Methods (1), washed and resuspended in suspending medium (see Methods (2)), (with or without actinomycin D), two proteases are produced which show the same electrophoretic pattern as those in the culture medium (Plate 3.1).



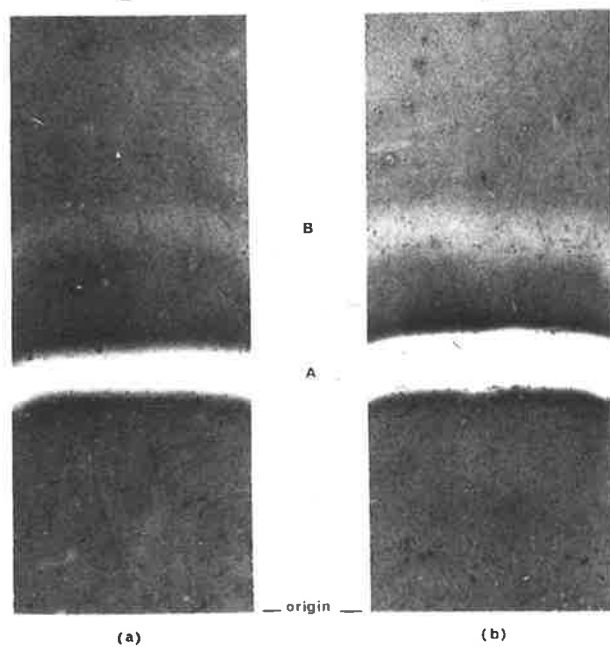


Plate 3.1 Two proteolytic bands obtained after Sephadex strip electrophoresis.

Washed cells were incubated with rifampicin (0.5  $\mu\text{g}/\text{ml}.$ ) and cell samples removed at 10 min and 90 min were centrifuged. 20  $\mu\text{l}.$  of each supernatant was electrophoresed and protease activity was detected.

- (a) 2 proteolytic bands after 10 min treatment with rifampicin.
  - (b) 2 proteolytic bands after 90 min treatment with rifampicin.
- A Neutral protease; B alkaline protease.

In the protease casein digestion assay used in this work, the alkaline protease component probably contributes less than 15% of the total activity and hence the results refer essentially to the neutral protease. This was established as follows. Supernatant from a three hour washed-cell suspension experiment was concentrated ten-fold by freeze-drying. Samples (5  $\mu$ l) were loaded onto six Sephadex strips and electrophoresed at 350 volts for 105 min. One strip was incubated in contact with an agar-casein plate and the position of the separated proteases was determined. The remaining five strips were cut into fractions containing the separated proteases and the appropriate strips were pooled. The enzymes were eluted with 1.5 ml of Sørensen's buffer (Rappaport, Riggsby and Holden, 1965) for 90 min. at 4°C. To measure the total enzyme activity 5 x 5  $\mu$ l of supernatant were spotted on a single strip, dried, eluted and assayed. The assay mixture contained 1.2 ml of eluent 0.3 ml of Sørensen's buffer and 0.5 ml of casein (2%), Assay tubes were incubated at 35°C for 75 min. The results are shown in Table 3.1. There may have been some slight contamination of the alkaline protease band by the neutral protease species if the bands did not run completely straight on the Sephadex strips. Therefore, the alkaline protease probably contributes less than 15% to the total enzyme activity. Nevertheless, qualitative evidence (shown below) suggests that in fact both types of enzyme vary in amount in a parallel manner.

TABLE 3.1.

RECOVERY OF PROTEASE ACTIVITY AFTER ELECTROPHORESIS  
ON SEPRAPHORE STRIPS

The experimental details are given in the text.

---

Enzyme	Protease activity (units/ml)	% Total activity
Total (not electrophoresed)	6.0	[100]
Neutral protease	5.10	85
Alkaline protease	0.91	15.2

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C. Insensitivity of *B. amyloliquefaciens* extracellular protease formation to rifampicin and actinomycin D

The concentration of each drug which almost completely inhibits total cellular RNA and total protein synthesis, as measured by 2-[<sup>14</sup>C]uracil and L-[<sup>14</sup>C]leucine incorporation respectively, was determined (Fig. 3.2 and Fig. 3.3). It was found that 2 µg/ml of actinomycin D and 0.5 µg/ml of rifampicin each inhibited cellular RNA synthesis and total protein synthesis very rapidly at these concentrations.

Surprisingly, however, these concentrations of rifampicin and actinomycin D added at zero time to a washed-cell suspension had little effect on phase 1 protease accumulation (defined above) in the external medium, and indeed, the total amount of extracellular enzyme at 90 min. is increased by the drugs (Fig. 3.4). In marked contrast to this, when rifampicin is added to a washed-cell suspension after 90 min. incubation (that is, during phase 2 synthesis), protease production is sensitive to the drugs as expected, inhibition being complete within a few minutes (Fig. 3.4). A similar result was obtained with actinomycin D (2 µg/ml).

A trivial explanation, that the cells only become permeable to these drugs after 90 min., is ruled out by the fact that total cellular protein and RNA synthesis are almost immediately inhibited (Figs. 3.2 and 3.3).

A further possible explanation of the results was that phase 1 synthesis represents secretion of preformed enzyme while phase 2 synthesis represents de novo enzyme synthesis. However, phase 1 and actinomycin D-insensitive

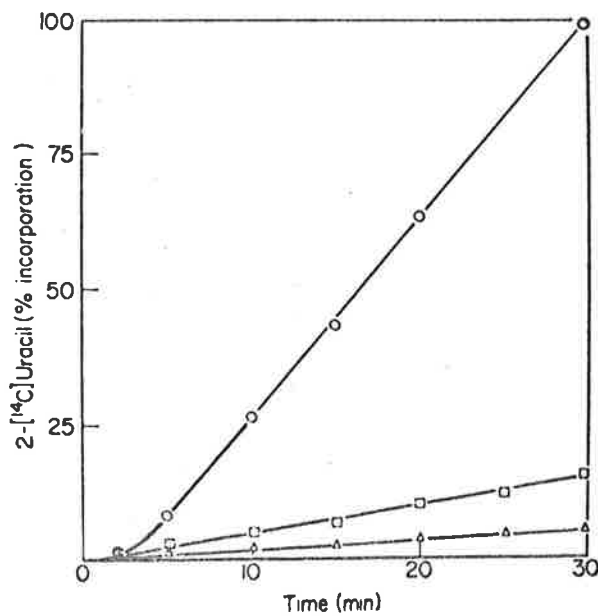


FIG. 3.2. Effect of actinomycin D and rifampicin on  $[^{14}\text{C}]$ uracil incorporation by washed cells. Drugs and  $[^{14}\text{C}]$ uracil were both added at zero time. —○—○—, No addition of drugs; —□—□—, actinomycin D (2  $\mu\text{g}/\text{ml}$ ); —△—△—, rifampicin (0.5  $\mu\text{g}/\text{ml}$ ). The 100% value for  $[^{14}\text{C}]$ uracil incorporation was 21,800 cts/min.

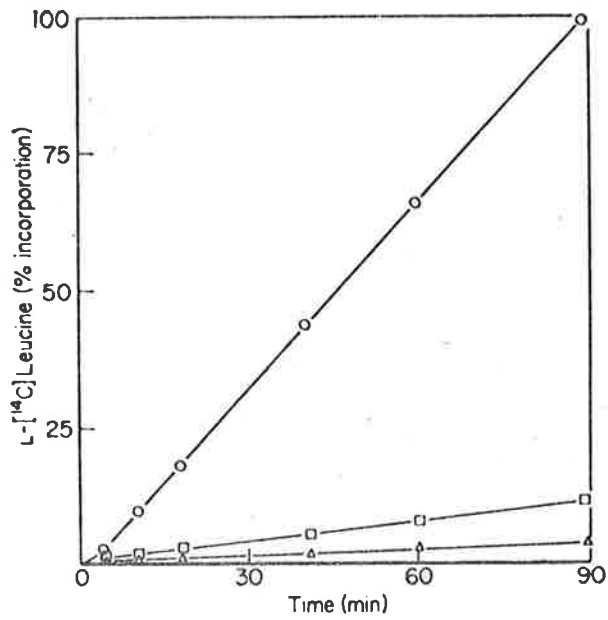


FIG. 3.3. Effect of actinomycin D and rifampicin on  $[^{14}\text{C}]$ leucine incorporation by washed cells. Drugs and  $[^{14}\text{C}]$ leucine were both added at zero time. —○—○— No addition of drugs; —□—□—, actinomycin D ( $2\ \mu\text{g}/\text{ml}$ ); —△—△—, rifampicin ( $0.5\ \mu\text{g}/\text{ml}$ ). The 100% value for  $[^{14}\text{C}]$ leucine incorporation was 8020 cts/min.

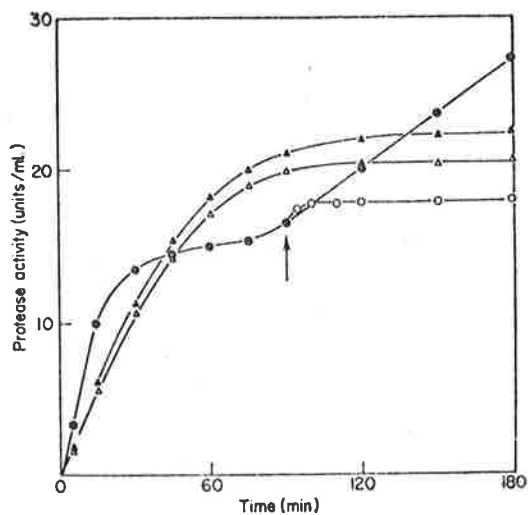


FIG. 3.4. Effect of actinomycin D and rifampicin on protease formation by washed cells. —●—●—, No addition of drugs; —Δ—Δ—, rifampicin (0.5 µg/ml) added at zero time; —▲—▲—, actinomycin D (2 µg/ml) added at zero time; —○—○—, rifampicin (0.5 µg/ml) added at 90 min (arrow).

protease synthesis are both sensitive to chloramphenicol (Fig. 3.5), suggesting that protein synthesis is required for protease formation. Similarly, when fusidic acid, pactamycin or puromycin were used, phase 1 protease synthesis was again totally inhibited (Fig. 3.6).

However, there still existed the possibility that these inhibitors of protein synthesis might have been exerting a general toxic effect on the cells thereby preventing active secretion of preformed enzyme rather than specifically inhibiting protein synthesis. Several indirect 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-[<sup>14</sup>C]uracil into RNA measured over a 90 min. incubation period. The second line of evidence concerns the production of protoplast-bursting 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  $\mu$ g/ml.) for greater than 60 min. Separate studies have shown that production of protoplast-bursting factor involves de novo synthesis of the molecule (May and Elliott, 1970). Both results imply that chloramphenicol is not having a general toxic effect on the cells and that therefore de novo protein synthesis is required for phase 1 protease production and the actinomycin D-insensitive protease formation.

Similarly, the effects of fusidic acid and pactamycin on protease formation are not due to non-specific



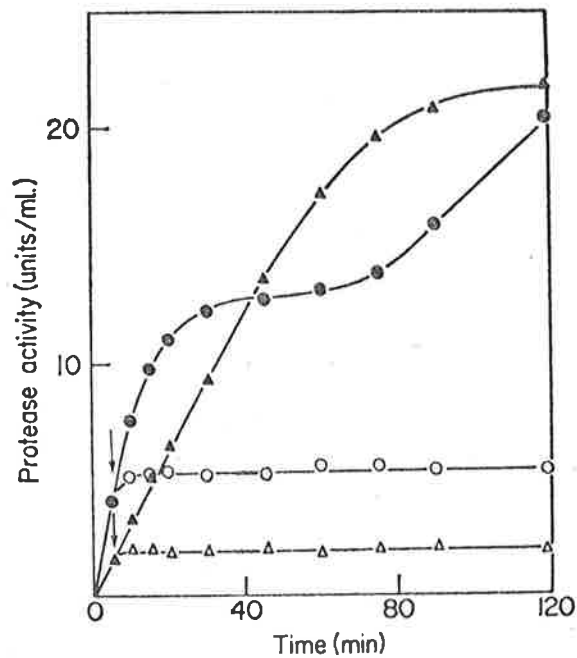


FIG. 3.5. Effect of chloramphenicol on protease production by washed cells in the presence and absence of actinomycin D. Chloramphenicol was added (arrows) to cells after 5 min preincubation. Actinomycin D was added at zero time. —●—●—, No addition of drugs; —○—○—, chloramphenicol (10 µg/ml); —▲—▲—, actinomycin D (2 µg/ml); —△—△—, actinomycin D (2 µg/ml) + chloramphenicol (10 µg/ml).

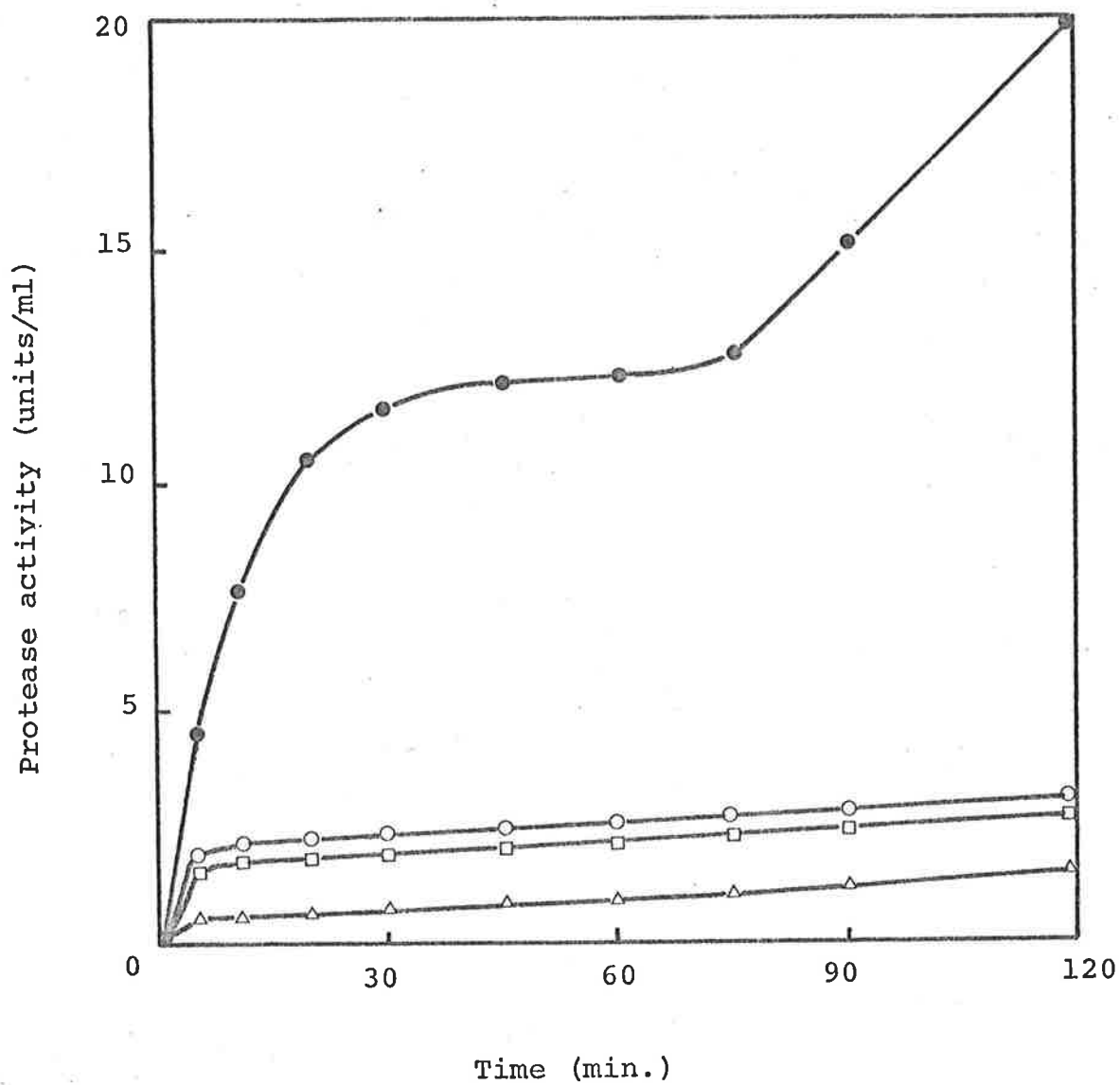


Fig. 3.6. Effect of fusidic acid, pactamycin and puromycin on protease formation by washed cells.  
 —●—●—, No addition of drugs; —○—○—, puromycin (20  $\mu\text{g}/\text{ml}$ ); —□—□—, pactamycin (0.05  $\mu\text{g}/\text{ml}$ ); —△—△—, fusidic acid (150  $\mu\text{g}/\text{ml}$ ).

toxicity. Fusidic acid and pactamycin selectively inhibit extracellular protease production without significantly impairing general protein synthesis as measured by L-[<sup>14</sup>C]-phenylalanine incorporation (Figs. 3.6 and 3.7). Higher concentrations of pactamycin do inhibit general protein synthesis; however, the cells rapidly recover from this inhibition, as discussed in the addendum. 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 production as discussed more fully later.

D. Incorporation of L-[<sup>14</sup>C]leucine into protease in the presence of rifampicin

The question next examined was whether the protein synthesis necessary for rifampicin-insensitive protease production involved de novo synthesis of the enzyme molecule itself, or alternatively the synthesis of some other protein, perhaps one required for the secretion of preformed enzyme. A [<sup>14</sup>C]leucine labelling study of the protease was carried out in the presence of rifampicin. 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%

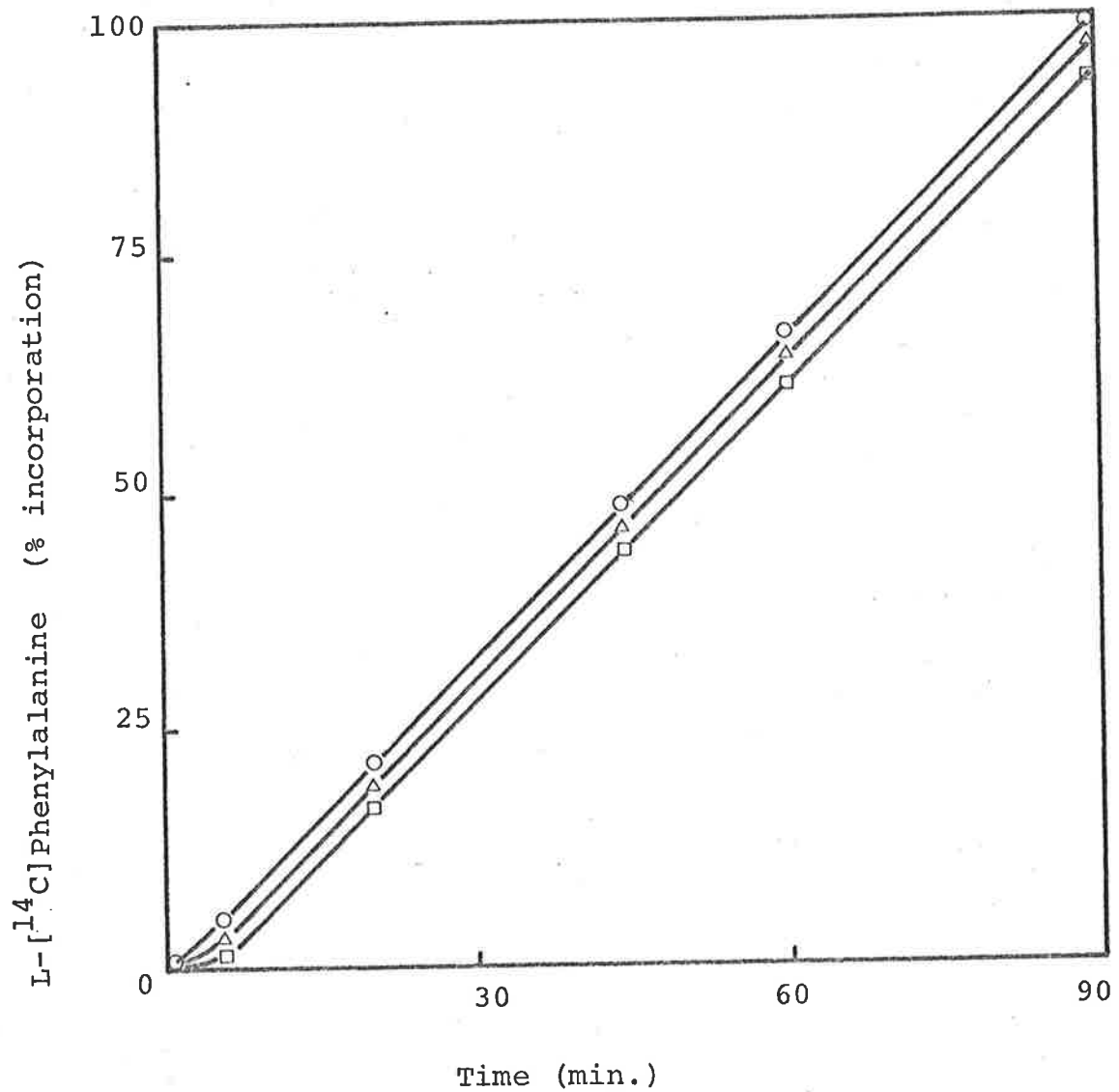


Fig. 3.7. Effect of fusidic acid and pactamycin on <sup>14</sup>C phenylalanine incorporation by washed cells. Drugs and [<sup>14</sup>C]phenylalanine were both added at zero time.

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

The 100% value for [<sup>14</sup>C]phenylalanine incorporation was 2550 cts./min.

Casamino acids and that protease synthesis was again insensitive to rifampicin (0.5  $\mu\text{g/ml.}$ ). It was also shown that at this concentration of rifampicin, general protein synthesis (as measured by [ $^{14}\text{C}$ ]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  $\mu\text{Ci}$  of L-[ $^{14}\text{C}$ ]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 lifetime. A separate experiment showed that the addition of extra leucine added as the source of  $^{14}\text{C}$  had no effect on further protease production. After a further 60 min. incubation the cells were removed and the supernatant retained. To determine the amount of protease formed during this time period a duplicate flask was incubated, identical except that unlabelled leucine was added at 20 min. and the protease activity in the supernatant determined at 20 and 80 minutes. Unlabelled leucine (4 mg) was added to the supernatant from the incubation with [ $^{14}\text{C}$ ]leucine and it was then dialysed for two hours 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-hour 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. 3.8). (The actual chromatographic procedures were developed and carried out by Ms. J. Hanlon.) A peak of labelled protease activity was separated from  $\alpha$ -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 its inactivation on the column. The radioactivity found in fractions 41 to 61 was shown to be due to free [ $^{14}\text{C}$ ]leucine whilst the labelled material in fractions 26 to 38 has not yet been identified. It was found that the peak of radioactivity found 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. 3.9). 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 (as described in Methods (6a)) 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 corresponded 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

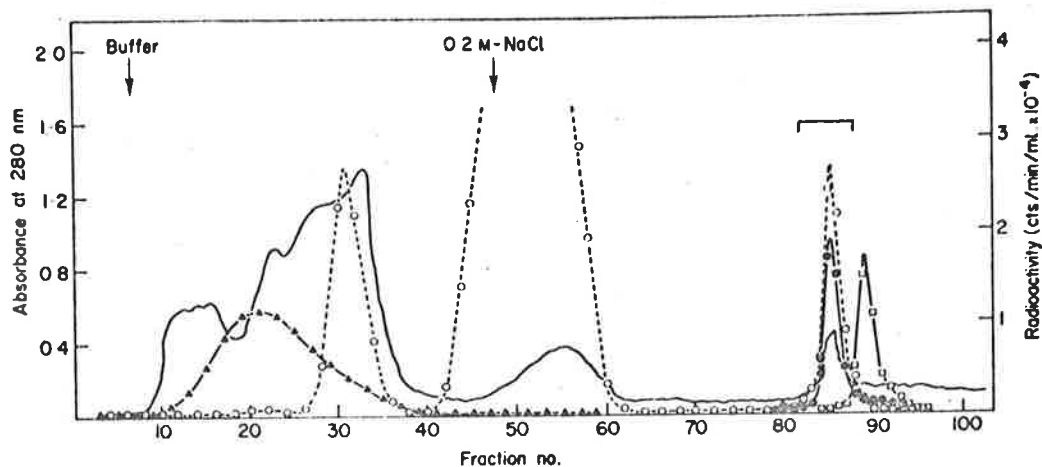


FIG. 3.8. Fractionation of protease by SE-Sephadex column chromatography. Radioactive sample from labelling experiment (see text for details), containing 8.1 units of potentially labelled protease, was added to carrier protease (12,900 units) and loaded on to an SE-Sephadex column (2.8 cm x 48 cm). The column, equilibrated with 0.02 M  $\text{KH}_2\text{PO}_4/\text{NaOH}$  buffer (pH 6.7) containing 0.5 mM-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°C 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. Protease activity in the fractions was detected by a milk assay described under Materials and Methods.

---○---○---, Radioactivity;—●—●—, protease activity. Fractions 82 to 88 contained a total of 11,330 units as measured by the casein digestion method;—▲—▲—,  $\alpha$ -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.

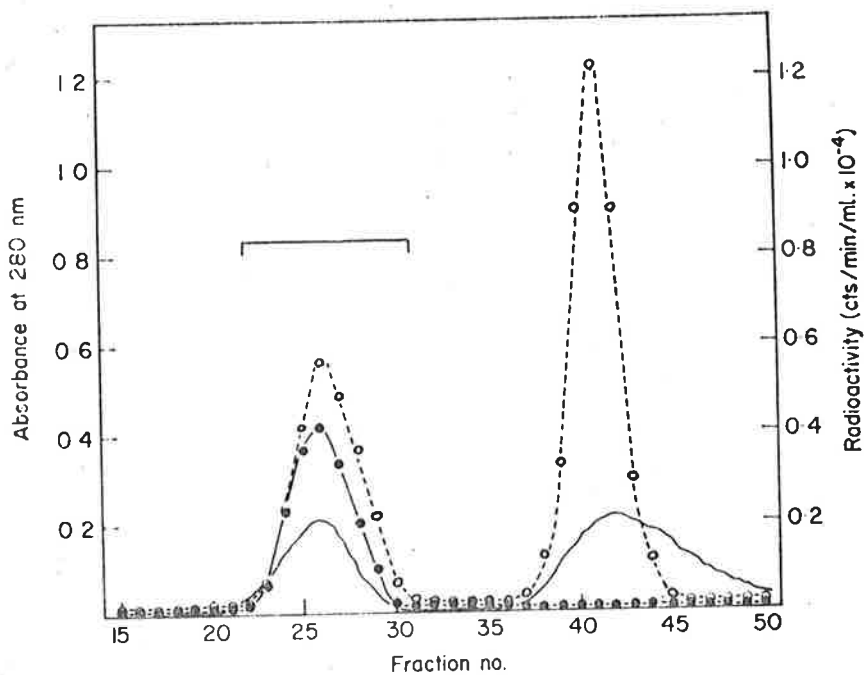


FIG. 3.9. Fractionation of protease by Sephadex G75 column chromatography. Fractions 82 to 88 from the SE-Sephadex column were pooled, dialysed for 2 hr against water containing 10 mM-calcium acetate and freeze dried. The freeze dried sample in  $\text{KH}_2\text{PO}_4/\text{NaOH}$  buffer (pH 6.7) containing 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°C with a flow rate of 14 ml/hr and 4 ml fractions were collected.

--○--○-- Radioactivity; —●—●—, protease activity. fractions 22 to 31 contained a total of 3540 units of activity; ( ——— ) absorbance at 280 nm.



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 chromatography on a Sepharose/casein column prepared by the method of Cuatrecasas, Wilchek and Anfinsen (1968). The profile obtained is shown in Fig. 3.10. 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 at pH 8.5 with electrodes reversed (Plate 3.2). (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. 3.11).

The main peak of protease from the Sepharose/casein column contained 3450 units of protease and 57,460 cts/min. of radioactivity. It was possible to calculate from this the proportion of the protease which had been synthesised de novo from [ $^{14}\text{C}$ ]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

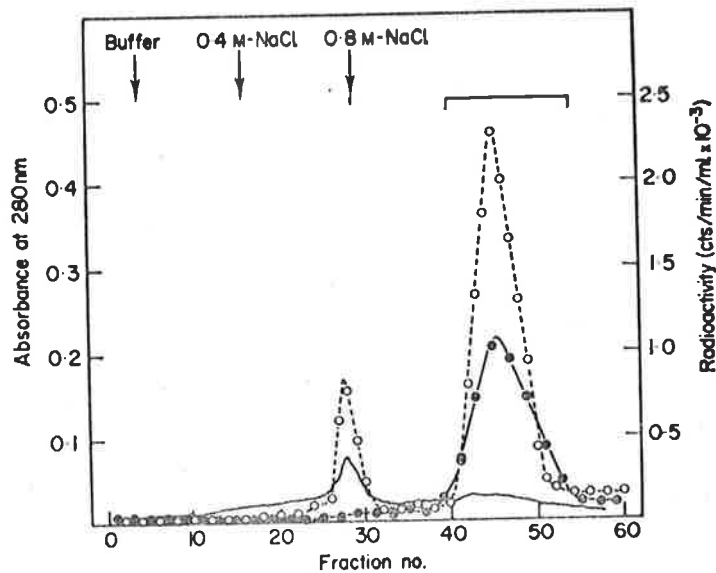


FIG. 3.10. Affinity chromatography of protease on Sepharose/casein. Fractions 22 to 31 from the Sephadex G75 column were pooled and loaded onto a Sepharose/casein column (1.3 cm x 34 cm). The column was eluted with 70 ml of  $\text{KH}_2\text{PO}_4/\text{NaOH}$  buffer (pH 6.7) containing 0.5 mM-calcium 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°C with a flow rate of 25 ml/hr and 5 ml fractions were collected. --○--○--, Radioactivity; —●—●—, protease activity. Fractions 40 to 54 contained a total of 3450 units of activity; ( — ) absorbance at 280 nm.

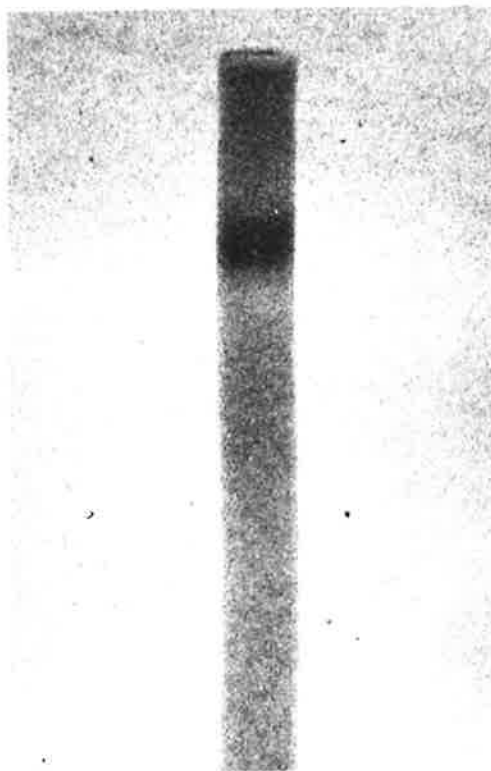
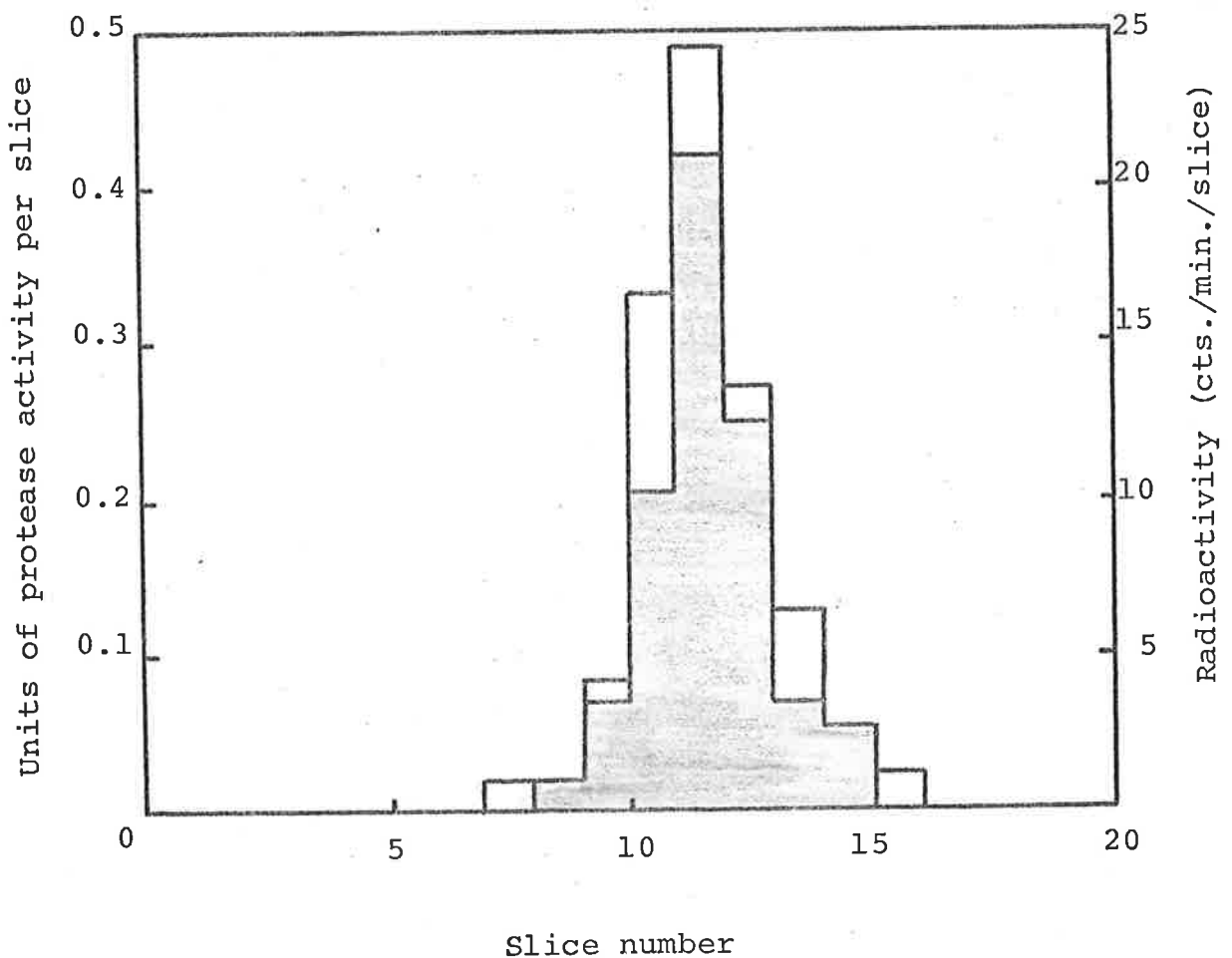


Plate 3.2 Protein band obtained upon polyacrylamide gel electrophoresis of protease. Fractions 40 to 54 from the Sepharose/casein column were concentrated by freeze drying, dialysed and 20  $\mu$ l. samples were electrophoresed at pH 8.5 with the electrodes reversed. Protein was detected by staining the gel with Coomassie brilliant blue. Identical gels were analysed for protease activity and radioactivity (see Fig.3.11). The anode is at the top of the photograph.



**Fig. 3.11.** Profiles of protease activity and radioactivity of polyacrylamide gels run as described in Plate 3.2.

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 buffer (pH 7.8) for 6 hr at 4°C and determined by an ultrasensitive assay using a Remazobright Blue/Hide substrate (Rinderknecht, Geokas, Silverman and Haverback, 1968). To determine <sup>14</sup>C radioactivity, gel slices were first digested with 1 M-NH<sub>4</sub>OH for 3 hr at 60°C in scintillation vials. After the addition of a glass-fibre filter paper, each vial was dried overnight at 110°C. Scintillation fluid was added and vials were counted for 10 min. Unshaded area, radioactivity (cts./min. above background); shaded area, protease activity.

connected to a fraction collector, that all of the  $^{14}\text{C}$  in the protease was in leucine and no other amino acid (Fig. 3.12). The protease produced during the rifampicin experiment between 20 and 80 minutes was 8.1 units; the specific activity of crystalline protease is 5000 units/mg of protein (Carey, 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 cts/min. This compared favourably with the observed result of 57,460 cts/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 cts/min. (110.8% of theory) for fully labelled enzyme.

In this calculation the assumptions are made that the specific activity of the [ $^{14}\text{C}$ ]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

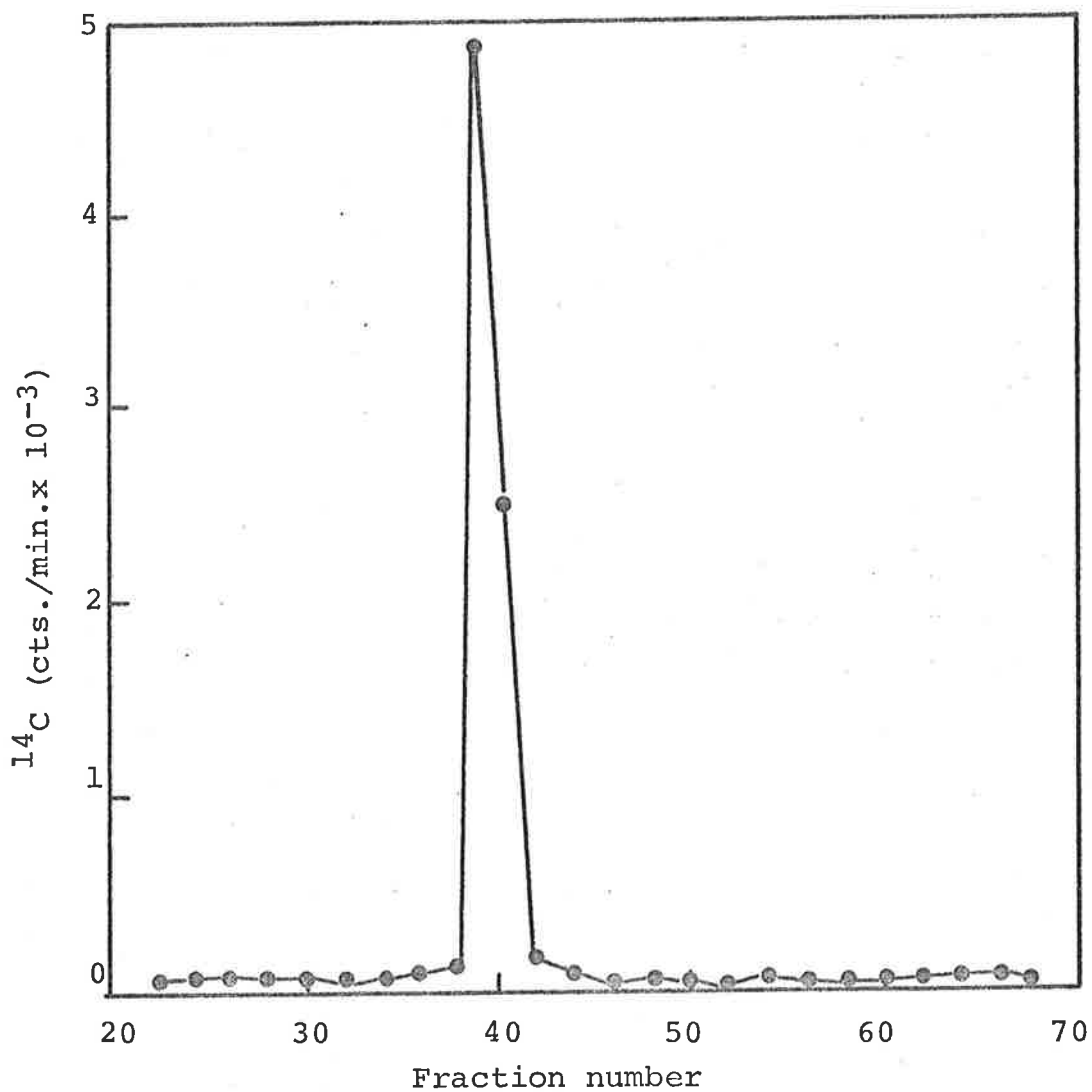


Fig. 3.12. Amino acid analysis of acid hydrolysed  $[^{14}\text{C}]$ -labelled protease.

The  $[^{14}\text{C}]$ -labelled protease, purified as described in the text, was hydrolysed in 6N HCl at  $110^\circ\text{C}$  for 16 hours and loaded on the column of a Beckman amino acid analyser. The peak containing the radioactivity was shown to correspond to leucine by using standard amino acid markers.

production was de novo synthesised was given by a labelling experiment carried out in a different way by J.L. McInnes. Cells were incubated with actinomycin D (2 µg/ml) and [<sup>14</sup>C]valine for 60 minutes. After dialysis, the crude enzyme was electrophoresed on Sepharose strips (see Methods (5)). The major protease band was cut out and used for radioactivity and protease determination. The observed counts per minute amounted to 93.4% of that calculated for fully labelled enzyme.

A further experiment along similar lines in which [<sup>14</sup>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 the production of extracellular protease which occurs in the presence of rifampicin represents de novo synthesis of the enzyme molecule and this occurs even after a 20 minute preincubation with rifampicin to permit normal mRNA to decay. Therefore, it appears that an unusual situation exists whereby harvested cells contain a pool of preformed mRNA capable of supporting protease synthesis for 80 minutes.

E. Studies on the accumulation and stability of the protease-specific mRNA pool

(1) Amino acid repression of transcription of protease mRNA

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 more pronounced (May and Elliott, 1968a). A likely explanation of these results is that amino acids repress transcription of protease mRNA. If this were the case, it might then be expected that phase 1 protease production in high amino acids medium would be due entirely to the translation of the mRNA pool. Its complete utilisation might then account for the plateau phase during protease production. The following experiment was done to test this idea. Cells harvested from 20 ml of culture medium were washed twice and suspended in high amino acids medium and rifampicin was added at varying times after resuspension. The rifampicin-insensitive protease production was measured and taken as an indication of the amount of protease-specific mRNA remaining in the cells. The results show (Fig. 3.14a) that immediately after the cells were resuspended in the high amino acids medium, the mRNA pool size decreased rapidly and was exhausted after about 40 minutes, when protease production enters the plateau phase. A similar result was obtained using actinomycin D.

It appears therefore, that high levels of amino acids repress the transcription of protease mRNA and phase 1 protease synthesis is independent of mRNA production. It might be expected then, that when the amino acid level has been sufficiently reduced by metabolism and protein synthesis,



escape from repression, that is, phase 2 protease synthesis would occur. This phase would therefore be sensitive to rifampicin and actinomycin D as observed (Fig. 3.4).

To test directly for amino acid repression at the transcriptional level, 0.5% Casamino acids were added to cell incubations after 90 minutes. 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 intracellular levels of amino acids by virtue of their increased utilisation or metabolism. In apparent confirmation of this, when cells after 90 minutes incubation are rapidly resuspended without washing in fresh medium, a linear production of protease is observed, rather than a biphasic one.

It might be further predicted that if amino acids are acting at the level of transcription, the protease mRNA pool might be maintained for longer in a medium containing a low level of amino acids. When rifampicin is added to cells which have been incubated in the high amino acids medium for 75 minutes no further protease production occurs (Fig. 3.13). In contrast, a significant amount of protease formation is observed after rifampicin is added to cells which were initially resuspended in a low amino acids medium (Fig. 3.13). 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 protease mRNA disappears faster in a high amino acids medium than in a low one. This is notwith-

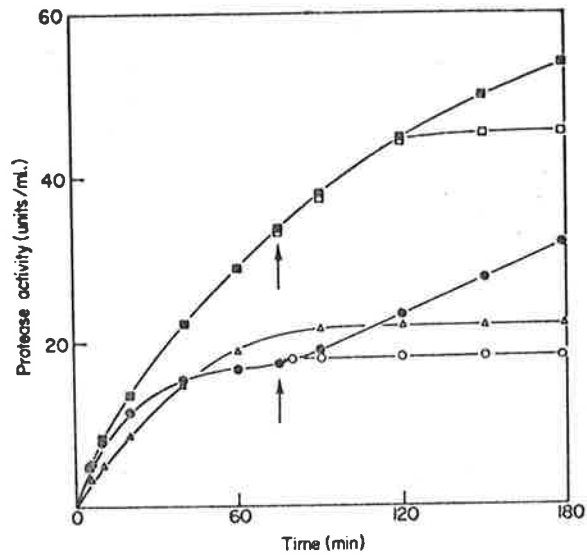


FIG. 3.13. Effect of rifampicin addition at 0 and 75 min on protease production by washed cells in the presence of low and high amino acid levels.

—■—■—, 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.

standing the fact that protease production during the first 75 minutes incubation is somewhat greater in low amino acids than in the higher amino acids medium. Separate experiments suggest that it takes approximately three hours to completely deplete the pool in the low amino acids medium.

In addition, 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 (Fig. 3.13); therefore protease synthesis, dependent on pre-existing mRNA, is totally independent of the level of amino acids in the medium under these conditions. This is entirely compatible with the amino acids acting at the level of transcription, as suggested earlier.

(2) Increased protease production by washed-cells in high amino acids medium in the presence of rifampicin

It has been shown (Fig. 3.4) that protease production by washed-cells continues for up to 80 minutes in the presence of rifampicin (or actinomycin D) when the drug is added at zero time and this is due to the presence in the cells of a pool of protease-specific mRNA. In high amino acids medium, this mRNA pool is exhausted after some 40 minutes due to amino acid repression of protease mRNA synthesis (Fig. 3.14a). It would be expected, therefore, that the addition of rifampicin to washed-cells at various times after resuspension in high amino acids medium would result in a progressive reduction in the amount of rifampicin-insensitive protease synthesis due

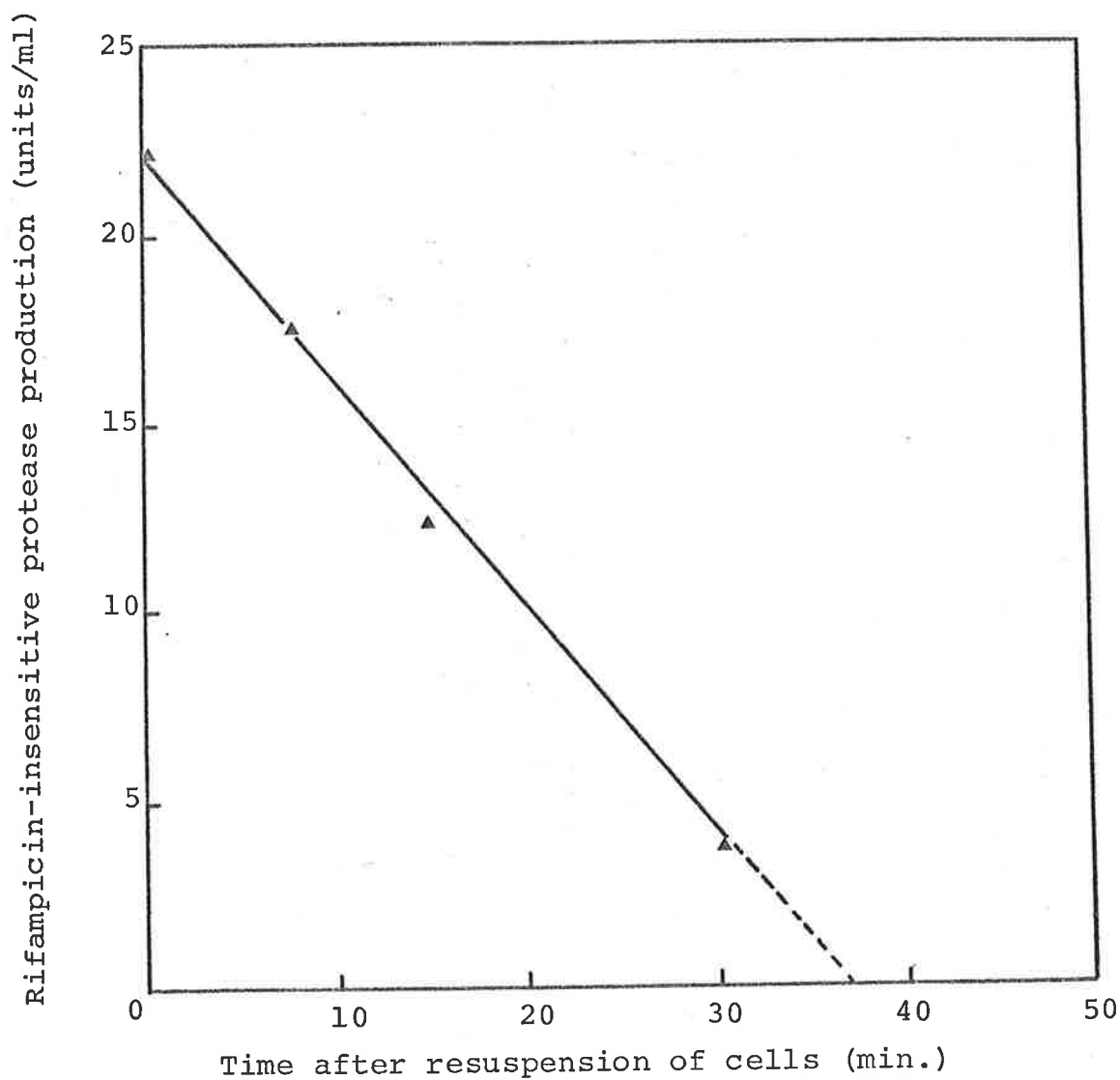


Fig. 3.14a. Rifampicin-insensitive protease production by cells incubated for various times in high amino acids medium.

Rifampicin ( $0.5 \mu\text{g/ml}$ ) was added at 0, 7.5, 15 and 30 min., to cells incubated in high amino acids medium. Protease was assayed in the supernatant after incubation for 120 min. as described in Methods (4).

to the decreasing level of protease mRNA. This is observed, as shown by the decrease in the amount of rifampicin-insensitive protease production plotted as a function of the time of incubation of cells in high amino acids medium (Fig. 3.14a). However, superimposed on this is a puzzling effect. It can be seen (Fig. 3.14b) that the total amount of protease production finally achieved is increased by the addition of rifampicin, the effect being maximal when the drug is added at 7.5 minutes after resuspension in high amino acids medium. In fact, there is 20% more enzyme produced when rifampicin is added at this time compared with that synthesised after its addition at zero time. This effect is fully reproducible and observed also with actinomycin D. From Fig. 3.14b it is evident that the amount of protease produced between 7.5 and 60 minutes in the presence of rifampicin is 19 units per ml., while during the same time period in the absence of the drug, only 10.6 units per ml. are produced, that is, addition of rifampicin to washed cells at 7.5 minutes has resulted in a 79% increase in the total amount of protease synthesised by the cells.

One obvious possible explanation is that amino acid repression of transcription of the protease mRNA is not exerted until 7.5 minutes, that is, sufficient mRNA is produced during the first 7.5 minutes to account for the increased protease production. However, this explanation is not supported by the data in Fig. 3.14a where it can be seen that the level of rifampicin-insensitive protease production starts to decline linearly from zero time, implying that amino

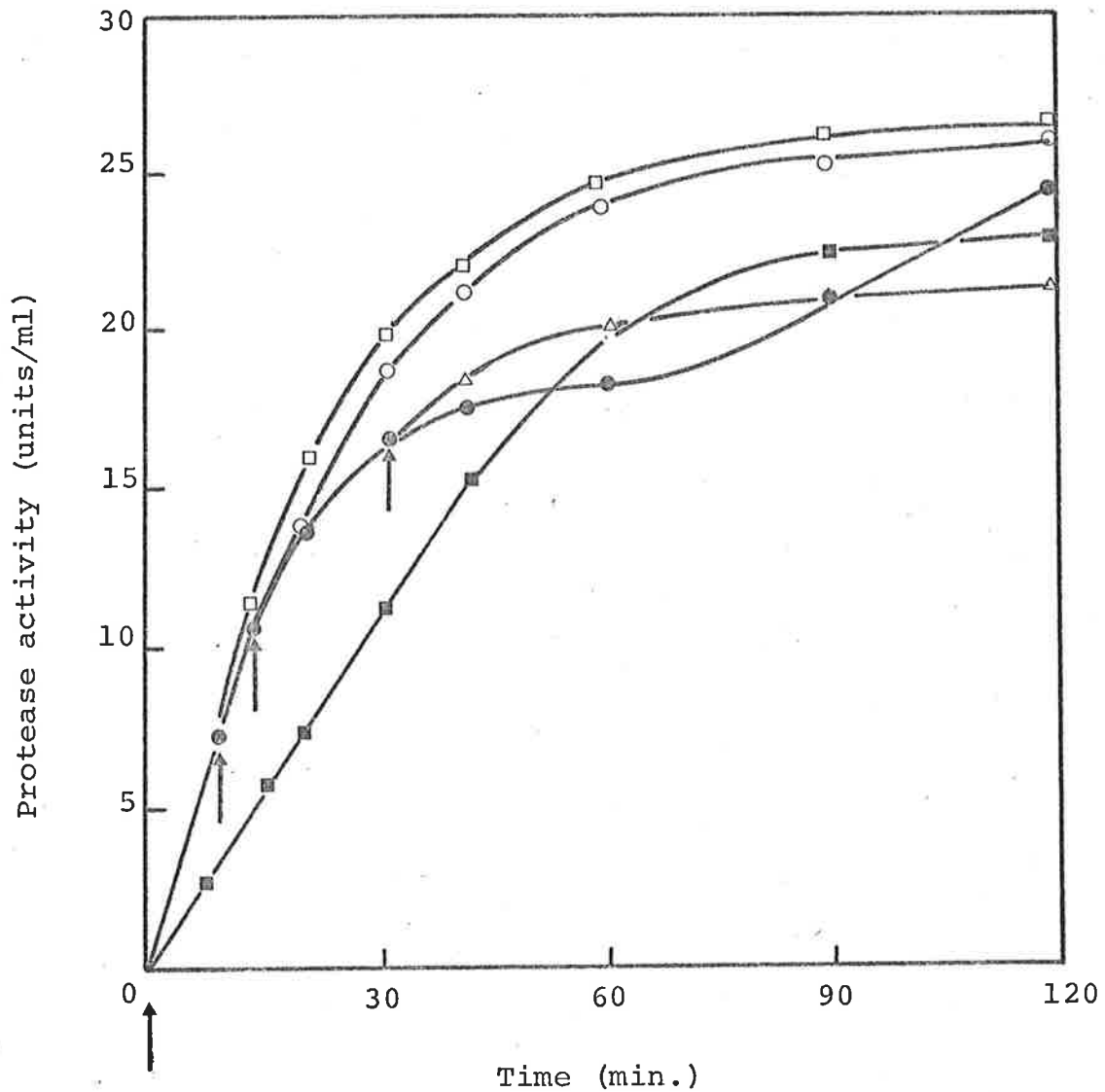


Fig. 3.14b. Protease production in high amino acids medium by cells incubated for varying times with rifampicin.

Rifampicin (0.5  $\mu\text{g/ml}$ ) was added at times indicated (arrows) to cells resuspended in high amino acids medium. Protease was assayed in the supernatant as described in Methods (4).

—●—●—, No addition of rifampicin; —■—■—, rifampicin added at zero time; —□—□—, rifampicin added at 7.5 min.; —○—○—, rifampicin added at 15 min.; —△—△— rifampicin added at 30 min.

acid repression is rapidly established. An alternative explanation is that not all of the protease mRNA is translated in control cells, that is, mRNA degradation quite unrelated to its translation, is occurring. This is different from the generally accepted concept that in bacteria, the translation and degradation of mRNA molecules are coupled (Morikawa and Imamoto, 1969; Morse, Mosteller, Baker and Yanofsky, 1969; Kuwano, Kwan, Apirion and Schlessinger, 1969). In this event, it is conceivable that rifampicin somehow increases the degree of translation of the protease mRNA pool, though how this might occur is not even speculated upon. The obvious possibility, that inhibition of general protein synthesis by rifampicin makes available more intermediates for protease synthesis is not tenable because in this event, rifampicin added at time zero should result in more protease production than when it is added at 7.5 minutes, and this is not observed.

Despite this, a series of experiments was performed in an attempt to find out if the protease mRNA pool decays in the absence of translation. The method used was to incubate cells for varying periods of time with chloramphenicol. The chloramphenicol was washed away by centrifugation and resuspension and the amount of rifampicin-insensitive protease production potential retained by the cells was determined. The results implied that the presence of chloramphenicol made little difference to the apparent rapid decay of the protease mRNA pool determined in this way. However, control experiments showed that cells took almost 40 minutes to completely recover

the normal rate of protease production (in the absence of rifampicin) after washing away chloramphenicol by this method. Under these circumstances the interpretation of the results was equivocal since reduced protease production might be due to cell damage rather than loss of mRNA. For this reason these experiments are not reported in detail. As will be seen in the discussion, subsequent work in this laboratory by another technique has shown conclusively that the above implied finding of translation-independent protease mRNA degradation was in fact correct.

#### F. Discussion

The results in this chapter conclusively show that prolonged production of protease by B. amyloliquefaciens harvested after about 25 hours growth, occurs in the absence of mRNA synthesis and that this involves de novo synthesis of the enzyme molecule. The labelling studies in which L-[<sup>14</sup>C]leucine was incorporated into protease exclude the possibility that protein synthesis is needed merely to secrete preformed enzyme. The fact that the <sup>14</sup>C labelling of the enzyme occurred after the cells had already been pre-incubated with rifampicin for 20 minutes rather clearly demonstrates that an unusual situation exists in which preformed mRNA can support synthesis of the enzyme for as long as 80 minutes.

It would seem that the 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 is 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 the addition of inhibitors of mRNA synthesis results in the rapid cessation of protease synthesis as is normally expected with mRNA molecules of short life-time.

The most likely explanation of the biphasic curve in the presence of high levels of amino acids is that phase 1 protease synthesis represents translation to exhaustion of the mRNA pool, while phase 2 synthesis is a result of the translation of newly synthesised protease mRNA. However, unlikely though it seems, the two enzymes produced in phases 1 and 2 could conceivably be different; although the enzymes co-electrophorese, rigorous exclusion of this possibility would require more complete characterisation of the proteins. Similarly, it is also conceivable that the same mRNA molecules are more stable in phase 1 than in phase 2, though no precedent for such a change is known and it again seems unlikely.

Given the concept of a short-lived protease mRNA, it follows that protease synthesis could only occur for 80 minutes in the presence of rifampicin if there was a huge excess of mRNA molecules present in the cells initially. For example, if one messenger molecule remains in the cell after 80 minutes of rifampicin treatment and if the mRNA half-life is of the order of five minutes (deduced from Fig. 3.4), then approximately 32,000 mRNA molecules must have been present initially to support protease synthesis through sixteen half-life decay periods. This is a unique situation for prokaryotes.

Several other cases, of lesser magnitude, of the accumulation of specific mRNA molecules in bacteria have been reported. The most striking example is perhaps the case of ornithine transcarbamylase, in which the E. coli cells during arginine starvation, accumulate sufficient mRNA to support subsequent enzyme synthesis for 20 minutes (McLellan and Vogel, 1970). Similarly, accumulation of histidine and tryptophan operon mRNA has been reported after starvation of cells for histidine (Venetanier, 1969) and tryptophan (Stubbs and Hall, 1968), respectively. In addition, Sells and Sayler (1971a,b) have shown that four ribosomal protein mRNA's can accumulate in E. coli treated with puromycin or chloramphenicol.

The accumulation of protease mRNA in this system cannot be accounted for by an inability of the cells to synthesise protein due to amino-acid depletion. Although the cell cultures at 25 hours 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 hours at a rapid rate. Preliminary experiments furthermore suggest that the protease mRNA pool is present several hours earlier when cell growth is proceeding rapidly (J. McInnes, unpublished result).

There is no direct evidence as to the form in which the mRNA pool exists. The first possibility is that there is an accumulation of cytoplasmic polysomes, but the results with pactamycin were interpreted as ruling this out, based on the assumption that the drug is specific for inhibiting the initiation of protein synthesis (Cohen, Herner and Goldberg,

1969). However, recent work suggests this may not be so; in  $f_2$  mRNA-directed polypeptide synthesis, no evidence was obtained that initiation of protein synthesis was more sensitive to pactamycin than elongation (Stewart and Goldberg, 1973). Very recent work (McInnes, May and Elliott, submitted for publication) with kasugamycin, believed to specifically inhibit initiation of protein synthesis (Tai, Wallace and Davis, 1973) has agreed with the conclusion derived from the experiment with pactamycin. Therefore, it seems that the protease mRNA does not exist as a pool of polysomes. The other alternatives are that it exists free in the cytoplasm or bound only to a 30S ribosomal subunit (Cundliffe, 1970).

Work by Dr. A.R. Glenn in this laboratory has shown that the protease mRNA pool is quickly lost from cells in high amino acids medium in the presence of chloramphenicol, pactamycin or fusidic acid, indicating that the protease mRNA pool is turning over rapidly. This agrees with the short half-life of the mRNA deduced from Fig. 3.4. Furthermore, this mRNA turnover occurs quite independently of the translation process as suspected earlier. Therefore, it appears that the protease mRNA pool is a dynamic equilibrium between transcription, translation and translation-independent degradation. Recently, Fry, Israeli-Reches and Artman (1972) also reported the loss, in the presence of chloramphenicol, of newly made mRNA in E. coli which did not require polysome formation for its degradation.

In addition, it has also been shown by Dr. Glenn that the mRNA pool can be lost from cells in the presence of

both actinomycin D and chloramphenicol, or from cells resuspended in high amino acids medium with chloramphenicol. This result shows, as suspected, that amino acids act by repressing the synthesis of protease mRNA. This is in keeping with the loss of the mRNA pool in the presence of rifampicin being independent of the amino acid level (Fig. 3.13).

These facts support the explanation of the biphasic nature of protease production already given above, namely that in the presence of high levels of amino acids protease mRNA synthesis is repressed and phase 1 protease production therefore consists of translating the pool of mRNA until it is exhausted. During this time, cell metabolism and protein synthesis may reduce the internal amino acid level to the point where de-repression occurs at about 80 minutes and protease gene transcription and nascent mRNA translation results in phase 2 protease synthesis. This would therefore be rifampicin sensitive, as observed. The maintenance of the protease mRNA pool in low amino acids medium is therefore explained by incomplete amino acid repression which allows synthesis of protease mRNA to proceed.

The effect of increased protease production by cells resuspended with rifampicin after incubation for varying times in high amino acids medium appears significant, but is not at all understood.

The existence of a dynamic pool of protease mRNA is unprecedented in prokaryotes. The question arises as to what possible biological significance could exist for such an

overproduction of mRNA. It is proposed that the accumulation of the mRNA pool may be a repercussion of the mechanism of extracellular enzyme synthesis as discussed below. It has been postulated (May and Elliott, 1968a) that extracellular enzyme synthesis by this organism occurs on ribosomes bound at special translation-extrusion sites located at the cell membrane. In this model synthesis and secretion occur as inseparable events with the polypeptide chain being extruded directly through the membrane as it is synthesised to take up its three-dimensional form only outside the permeability barrier. The model was proposed on indirect evidence as discussed in Chapter 1.

Further indirect evidence for this hypothesis might be obtained from the observations in this work of a highly selective inhibition of protease production by pactamycin and fusidic acid. In addition, recent work (McInnes, May and Elliott, submitted for publication) has shown that protease production is more sensitive to a variety of antibiotics which bind specifically to the 50S ribosomal subunit than is general protein synthesis. This might be explained by the fact that the 50S ribosomal subunits synthesising this extracellular enzyme are peripherally located in a position rendering them more sensitive to inhibition by low concentrations of the drug (or more accessible to it) than are cytoplasmic ribosomes. A similar differential inhibition by antibiotics of the synthesis of extracellular alkaline phosphatase by Micrococcus sodonensis was observed by Glew and Heath (1971).

Such a peripheral location of the ribosomes would

mean that special problems exist. At present it is generally assumed that translation of mRNA in prokaryotes begins while it is still being transcribed from the gene (Morse, Baker and Yanofsky, 1968). However, translation, coupled to transcription cannot be occurring in this system because protease synthesis occurs de novo for up to 80 minutes in the presence of actinomycin D, and this drug immediately inhibits the elongation of mRNA chains. Hence, completed protease mRNA molecules must exist within the cell. Furthermore, if the translation-extrusion hypothesis is correct, a unique situation must exist whereby mRNA has to migrate from the gene-transcription site to the membrane-translation site (Fig. 3.15). If, in fact, such a migration of messenger from the gene to the membrane sites occurs, the pool of mRNA could be the result of individual mRNA molecules 'queuing up' for translation at a limiting number of membrane sites. Such a migration and 'queuing up' of mRNA molecules implies that there must be a specific recognition process between the protease mRNA (perhaps with a 30S subunit attached) and a membrane-bound 50S subunit to prevent initiation of protease synthesis occurring in the cytoplasm. Such a scheme is analogous to the model for the specific initiation of protein synthesis on the endoplasmic reticular membrane in myeloma cells proposed by Baglioni, Bleiberg and Zauderer (1971). It is interesting to note that no differential inhibition of protease production was observed with antibiotics which bind specifically to the 30S ribosomal subunit (McInnes, May and Elliott, submitted for publication), except for kasugamycin and pactamycin, both

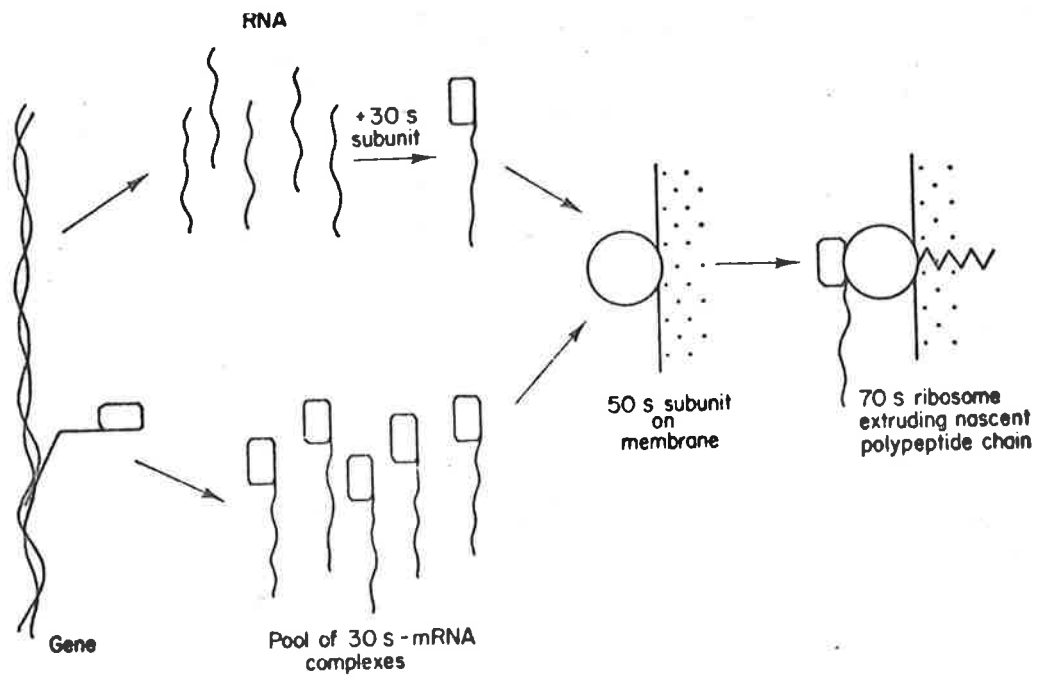


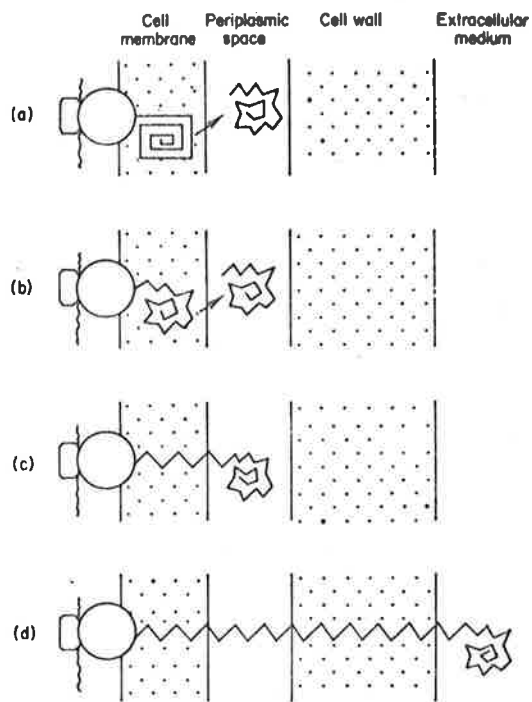
FIG. 3.15. Proposed alternative models for the accumulation of a pool of protease messenger RNA and its subsequent translation at the membrane.

of which are capable of affecting the initiation of protein synthesis (Tai, Wallace and Davis, 1973; Cohen, Herner and Goldberg, 1969). Perhaps there is some special feature(s) involved in the initiation of protease synthesis.

The precise location of the formation of the protease in its active form is unknown, but given the concept of extrusion of the polypeptide chain, there are several possibilities.

The enzyme might assume 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. 3.16). Although in B. licheniformis the first possibility is favoured (Sargent and Lampen, 1970b), there is no corresponding accumulation of extracellular enzymes in the membrane of B. amyloliquefaciens. Since protoplasts of B. amyloliquefaciens are not lysed by purified protease it is not possible to eliminate any of the above models. There is an indirect piece of evidence which might suggest that in B. amyloliquefaciens the nascent polypeptide chain traverses the cell wall. It is based on the production by the organism of the peptide/lipid antibiotic mentioned previously, which instantly disorganises the protoplast membrane. It is suggested (Gould, May and Elliott, 1971) that this molecule leaves the cell in an extended inactive form and assumes an active cyclic configuration outside the cell 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.





**FIG. 3.16** 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.

This might suggest that the permeability of the cell wall is limited to the molecules smaller than that of the antibiotic (mol. wt. approx. 1000). If this were the case it would imply 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 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 (May, unpublished data) or in terms of the emerging chain being protected by an  $\alpha$ -helical configuration (Hartley and Crick, personal communication).

It is important to realise that in this proposed model, the existence of a pool of mRNA is not a pre-requisite for all extracellular enzyme secretion. However, recent work has shown that similar pools exist for extracellular  $\alpha$ -amylase and probably ribonuclease in B. amyloliquefaciens (Gould, May and Elliott, 1973) and for extracellular protease in B. subtilis (Semets and Glenn, in preparation). Whether such a pool of mRNA exists depends on the relative rates of transcription and translation in a particular system.

The situation could be quite different in Staphylococcus aureus where the genes for extracellular penicillinase are carried on plasmids (Novick, 1963; Harmon and Baldwin, 1964; Hashimoto, Kono and Mitsuhashi, 1964) which may be in direct contact with the secreting sites.

CHAPTER 3ADDENDUMRECOVERY OF B. AMYLOLIQUEFACIENS PROTEIN SYNTHESIS  
FROM INHIBITION BY PACTAMYCINA. Introduction

Pactamycin is an antibiotic which is believed to inhibit initiation and elongation of protein synthesis (Cohen, Herner and Goldberg, 1969; Stewart and Goldberg, 1973). During the course of studies concerning the effect of pactamycin on protease synthesis, it was found that general protein synthesis rapidly recovers from inhibition by pactamycin and so far as is known, this has not been previously observed. At the time, it seemed useful and relevant to the main work to understand what was happening and a short investigation of the phenomenon was made, leading to the discovery of a pactamycin-removal system. This work is now presented briefly as an addendum, since it is not part of the main extracellular enzyme work.

B. Characterisation of the recovery phenomenon

Washed cells, resuspended in medium containing 0.5% Casamino acids, used throughout this work, incorporate L-[<sup>14</sup>C]valine linearly after 20 minutes when the label is added at zero time (Fig. 3A.1). Separate studies showed that pre-incubation of cells before the addition of

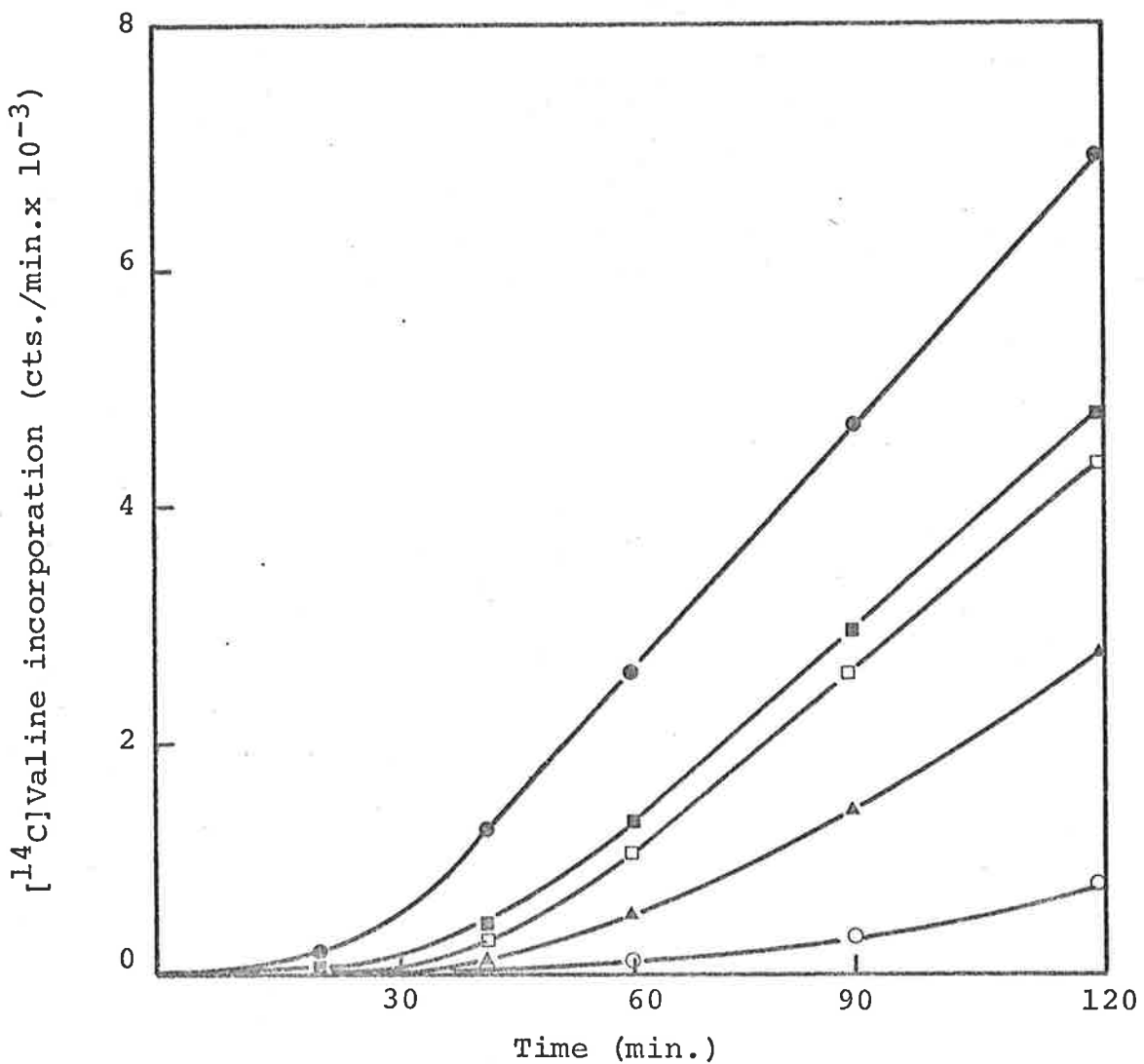


Fig. 3A.1. Effect of pactamycin on general protein synthesis by washed cells.

Pactamycin and L- $^{14}\text{C}$ valine were both added at zero time.

No addition (—●—●—); pactamycin (0.1 µg/ml) (—■—■—); pactamycin (0.5 µg/ml) (—□—□—); pactamycin (1.0 µg/ml) (—▲—▲—); pactamycin (5.0 µg/ml) (—○—○—).

[<sup>14</sup>C]valine decreased the time taken for the establishment of its linear incorporation; if the cells were pre-incubated for 40 min. before the addition of the label, its incorporation was linear almost immediately. This effect is not understood, but it does not produce artefactual results, as discussed later.

When pactamycin is added at varying concentrations to washed cells, there is pronounced inhibition of general protein synthesis, but the cells soon recover (Fig. 3A.1). The time taken for the recovery from inhibition increases with increasing concentrations of pactamycin; with 0.1 µg/ml of the antibiotic, recovery begins at about 40 minutes, but with 1.0 µg/ml, recovery only begins after about 60 minutes.

The effect of pactamycin on [<sup>14</sup>C]valine incorporation is not due to a transient general toxic effect on the cells. This is shown by the fact that pactamycin (0.5 µg/ml) does not inhibit 2-[<sup>14</sup>C]uracil incorporation into RNA over 40 minutes (Fig. 3A.2), while during this time general protein synthesis is almost totally inhibited. No stimulation of RNA synthesis was observed in the presence of pactamycin as has been previously reported for Bacillus subtilis (Kersten, Kersten, Emmerich and Chandra, 1967; Bhuyan, 1967).

The possibility existed that recovery from pactamycin inhibition is artefactual, in the sense that faulty protein synthesis in the presence of the antibiotic may allow the synthesis of nonsense protein to occur. This does not appear to be the case; the synthesis of extracellular α-amylase and protease recovers to the same rate as control cells after

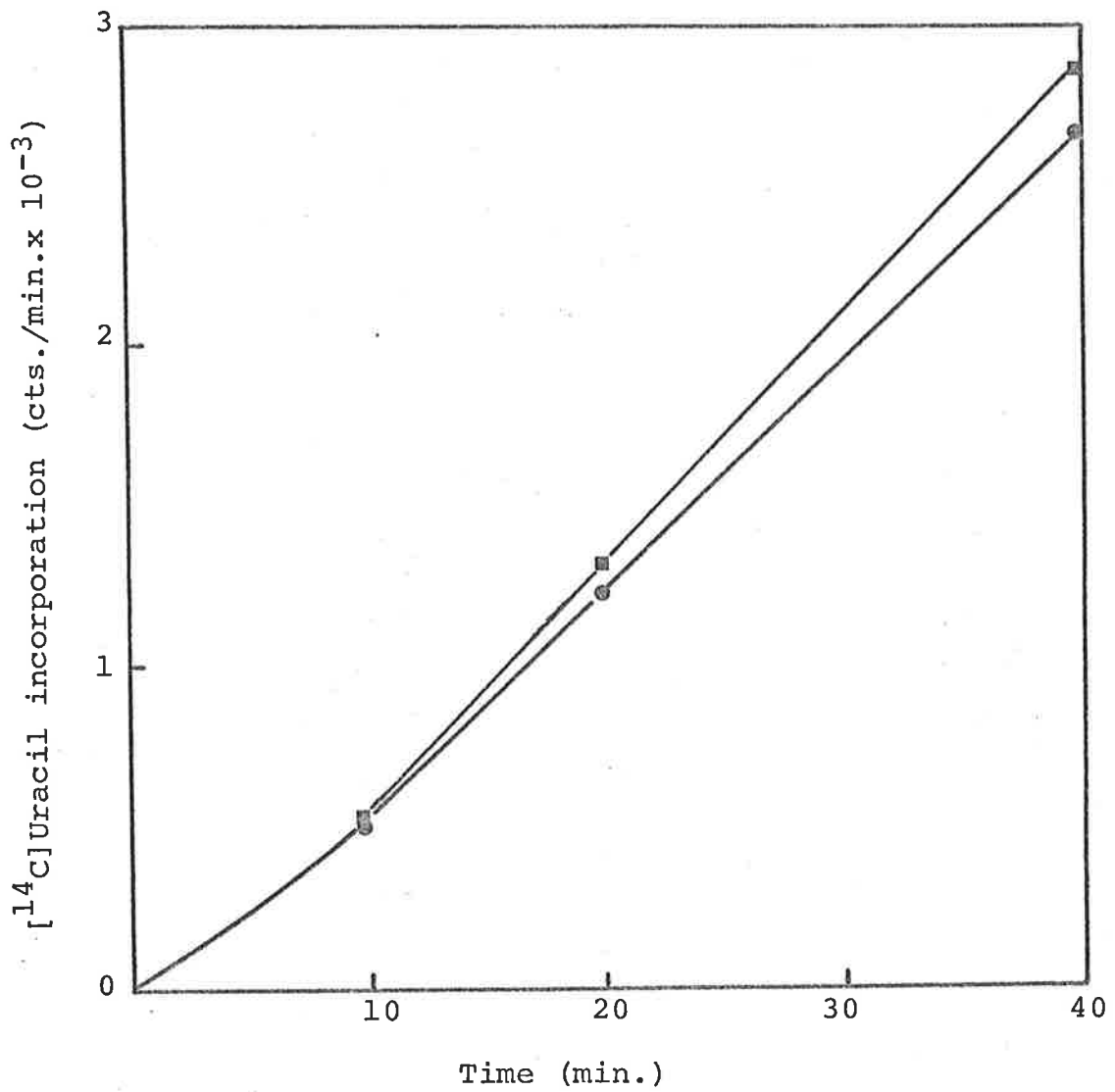


Fig. 3A.2. Effect of pactamycin on total RNA synthesis by washed cells.

Pactamycin and 2- $[^{14}\text{C}]\text{uracil}$  were both added at zero time. No addition (—●—●—); pactamycin (0.5 µg/ml) (—■—■—).

recovery from pactamycin inhibition. Protease synthesis, after pactamycin treatment, recovers to the rate of phase 2 synthesis in control cells, presumably because the mRNA pool is lost during incubation with the antibiotic. However, this recovery is only observed after 120 minutes and the reason for the delay compared with general protein synthesis, is not understood. It may be related to the level of amino acids in the medium and the derepression of protease mRNA transcription, or the apparent greater sensitivity of protease production to pactamycin as discussed earlier.

Recovery from pactamycin inhibition does not depend on the acquisition of resistance to the drug. When cells were pre-incubated for 70 minutes with pactamycin (0.5  $\mu\text{g/ml}$ ) (during which time the cells completely escape from inhibition) and then transferred to new medium containing pactamycin (0.5  $\mu\text{g/ml}$ ) (Fig. 3A.3; closed triangles), the rate of incorporation of [ $^{14}\text{C}$ ]valine was identical to that of control cells pre-incubated for 70 minutes in the absence of pactamycin (Fig. 3A.3; closed squares). As expected, both cultures were initially inhibited as compared with control cells without pactamycin. This result clearly indicates that cells, once incubated with pactamycin, are equally susceptible to a second incubation with the antibiotic.

It was thought that the most likely explanation for the recovery of protein synthesis from pactamycin inhibition was that the antibiotic was progressively removed from the medium. To test this idea, cells pre-incubated without pactamycin for 70 minutes were resuspended in super-

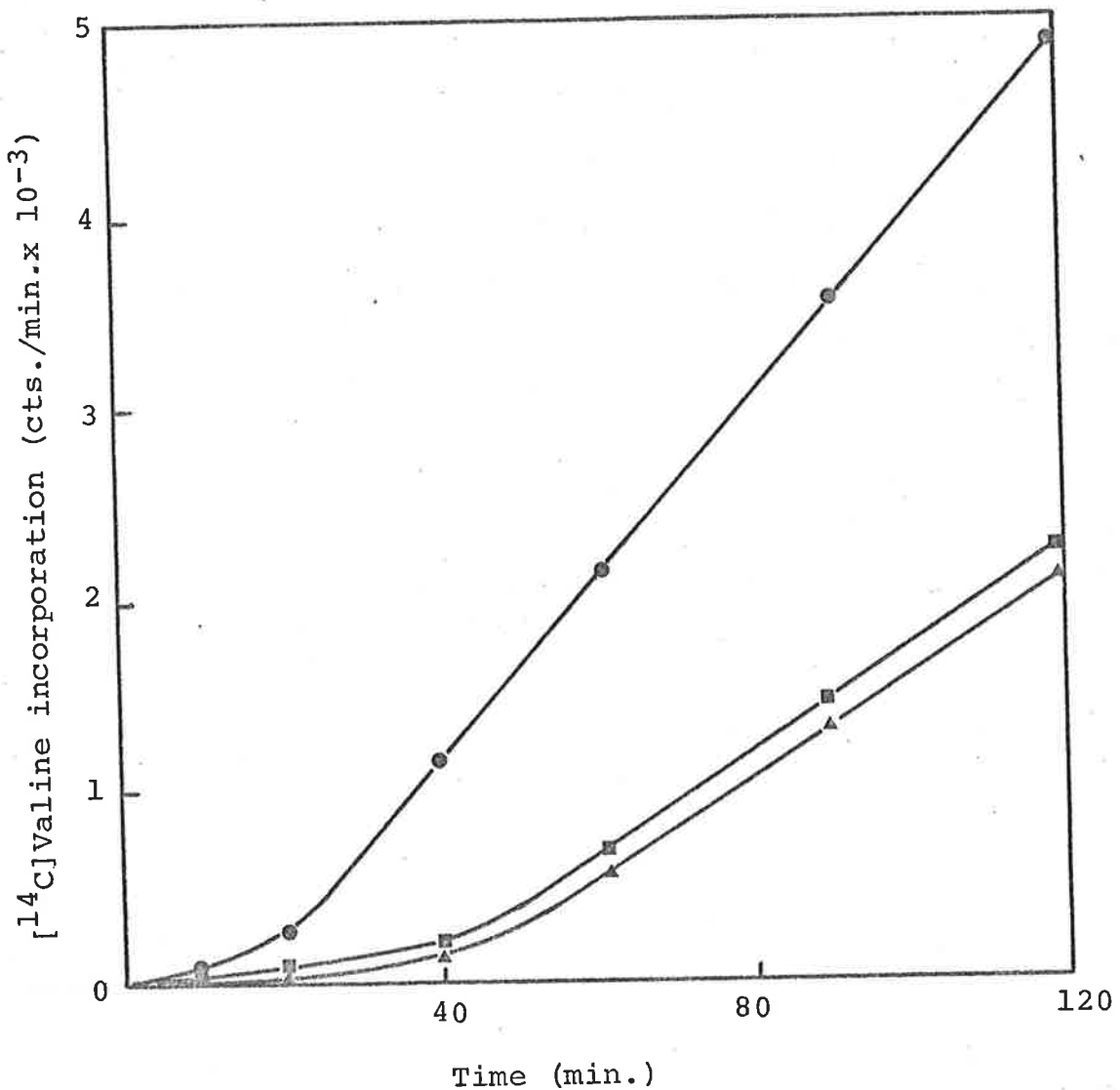


Fig. 3A.3. Effect on protein synthesis of treating washed cells twice with pactamycin.

Washed-cell cultures were pre-incubated in suspending medium without pactamycin (culture 1) and with pactamycin (0.5  $\mu\text{g/ml}$ ) (culture 2). After 70 min., cells were centrifuged and resuspended in new suspending medium. Pactamycin (0.5  $\mu\text{g/ml}$ ), where required and L-[<sup>14</sup>C]valine (0.5  $\mu\text{C/ml}$ ) were added together immediately after resuspension. Cells from culture 1, (—●—●—); cells from culture 1 plus pactamycin (0.5  $\mu\text{g/ml}$ ) (—■—■—); cells from culture 2 plus pactamycin (—▲—▲—).



natant from cells which had recovered from pactamycin inhibition (Fig. 3A.4; closed triangles). Control cells were resuspended in identical medium which had not at any stage contained pactamycin (Fig. 3A.4; closed squares). It is evident that there was no inhibition of [ $^{14}$ C]valine incorporation in the cells suspended in medium which originally contained pactamycin. This shows that the antibiotic was totally removed from the medium by the cells during the initial 70 minute pre-incubation. (The absence of a significant lag phase for the top two curves (Fig. 3A.4) as compared with the control (Fig. 3A.4; closed circles) will be discussed later.)

Finally, the mechanism which removes pactamycin from the medium appears to be intracellular. Pactamycin (0.5  $\mu$ g/ml) added to the supernatant of cells pre-incubated with the antibiotic at a concentration of 0.5  $\mu$ g/ml for 70 minutes, was not degraded during a further 70 minute incubation of this supernatant. Therefore, it seems that no extracellular product is produced by the cells which destroys the pactamycin in the external medium.

### C. Discussion

As mentioned earlier, the lag in the incorporation of L-[ $^{14}$ C]valine into the cells is not understood. It may have a trivial explanation such as the presence initially of a large intracellular pool of unlabelled valine, or the competitive inhibition of L-valine uptake by L-isoleucine in the medium, described for E. coli K12 (Britten and McClure,

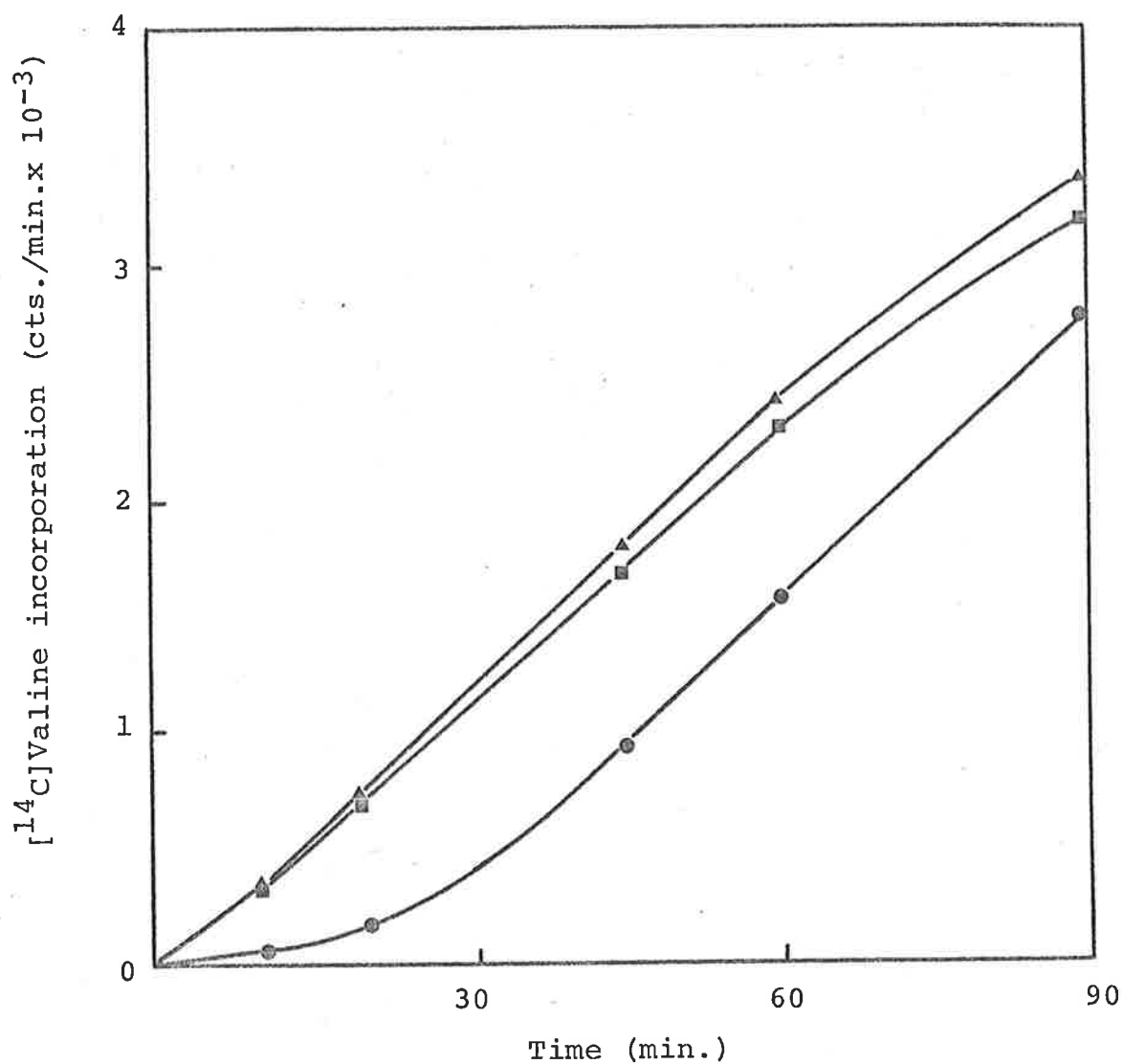


Fig. 3A.4. Effect on protein synthesis of resuspending new cells in medium in which cells have recovered from pactamycin inhibition.

Washed-cell cultures were pre-incubated in suspending medium without pactamycin (culture 1) and with pactamycin (0.5  $\mu\text{g}/\text{ml}$ ) (culture 2). At 70 min., culture 1 cells were centrifuged and resuspended with L- $[^{14}\text{C}]$ valine (0.5  $\mu\text{C}/\text{ml}$ ) in the following media: fresh suspending medium (—●—●—); supernatant from culture 1 (—■—■—); supernatant from culture 2 (—▲—▲—).

1962). If the latter explanation were correct, it might explain the absence of a lag in [ $^{14}\text{C}$ ]valine incorporation when fresh cells are resuspended in supernatant from cells pre-incubated in suspending medium for 70 minutes (Fig. 3A.4), since the level of isoleucine would have been reduced by cell metabolism.

In any event, the lag does not produce artefactual results since similar recovery of general protein synthesis from pactamycin (0.5  $\mu\text{g}/\text{ml}$ ) treatment has been observed using L- [ $^{14}\text{C}$ ]phenylalanine and L- [ $^{14}\text{C}$ ]leucine where incorporation is linear. However, the time taken for recovery from inhibition, measured with these radioisotopes, is reduced by an amount equal to the lag time of [ $^{14}\text{C}$ ]valine incorporation.

The results indicate that there exists in washed cells of B. amyloliquefaciens a factor capable of destroying pactamycin activity, though how this might be achieved remains speculative.

In some cases, resistance to an antibiotic can be acquired by a cell due to a decreased uptake of the compound. Inoue et al. (1970) and Sompolinsky et al. (1970) have characterised an acquired inducible resistance to tetracyclines in Staphylococcus aureus and in this system, a decrease in the ability of the cells to actively accumulate the drug accounts for their resistance. This resistance declined when cells were transferred to a tetracycline-free medium. However, in the present situation, permeability effects seem unlikely to be involved because all the pactamycin is removed from the medium. It seems more probable that there exists an enzyme

system in the cells which inactivates the pactamycin.

Two analogous enzyme systems have been reported. First, several bacillus species are capable of inactivating nisin (Jarvis, 1967) and this is believed to be due to a dihydropeptide reductase (Jarvis and Farr, 1971). Secondly, an acetyl-transferase, inducible by sub-inhibitory concentrations of chloramphenicol in Staphylococcus epidermidis has been shown to acylate and inactivate the antibiotic (Shaw, Bentley and Sands, 1970). Similar acylations of chloramphenicol have been reported in Streptomyces coelicolor (Argoudelis and Coates, 1971) and E. coli carrying antibiotic resistance factors (Suzuki and Okamoto, 1967).

It appears likely then that there could be an intracellular enzyme system in B. amyloliquefaciens which inactivates the pactamycin. In addition, it seems that the mechanism responsible for inactivating the pactamycin may be operating constantly since the time taken for protein synthesis to escape is the same during the first and second treatments with the antibiotic. Whether other antibiotics can be inactivated by B. amyloliquefaciens has not been determined.

The nature of the pactamycin inactivating system has not been further examined.

CHAPTER 4

DEVELOPMENT OF TECHNIQUES FOR THE ATTEMPTED ISOLATION OF  
EXTRACELLULAR PROTEASE mRNA FROM B. AMYLOLIQUEFACIENS

## A. Introduction

It has been established that there exists in washed-cells of B. amyloliquefaciens a pool of protease-specific mRNA whose synthesis is repressed by a high level of amino acids and which is the result of a dynamic equilibrium between synthesis and degradation. The existence of what appears to be a large amount of this mRNA is in complete contrast to the generally accepted concept that mRNA is limiting in protein synthesis in bacteria. This situation therefore provides an opportunity for the isolation of a prokaryotic mRNA species.

The isolation of this mRNA would be particularly useful for the establishment of an in vitro system in which the protease synthesis and secretion process could be studied. The isolation has not yet been achieved, but this chapter describes initial exploratory work which will provide a basis for further work now in progress in this laboratory.

Preliminary attempts at RNA isolation from B. amyloliquefaciens were somewhat discouraging in that when cells were broken by French pressure cell treatment and the RNA isolated by conventional phenol extraction procedures, it was obvious from electrophoresis on polyacrylamide gels and sucrose gradient analyses that extensive breakdown of ribosomal RNA was occurring. Initially, therefore, the work was concerned only with the limited objective of determining conditions of extraction in which degradation of r-RNA was minimised, the r-RNA merely being a convenient

indicator.

B. Attempted isolation of RNA in the presence of ethanol

It was reasoned that degradation might be minimised if, prior to cell breakage, cells were resuspended in 66% ethanol and treated in the French pressure cell at  $-15^{\circ}\text{C}$ . The idea was that RNA would be precipitated while ribonucleases might be inactivated or left in the supernatant after centrifuging. However, this approach proved abortive, the main obvious reason being that cell breakage under these conditions was poor, even after two passages of the cell suspensions through the French pressure cell. A modified approach, described below, was therefore used.

A sample (200 ml) of washed-cell suspension was centrifuged and the cells resuspended in 50 ml of TES buffer (10 mM-Tris HCl, pH 7.5; 10 mM-EDTA; 0.5% (w/v) SDS) at  $4^{\circ}\text{C}$ . This suspension was passed through the French pressure cell and the lysate collected directly into two volumes of ethanol containing SDS (0.35%, w/v), at  $-15^{\circ}\text{C}$ . (The presence of the ethanol made it possible to use low temperatures in the hope of minimising RNA degradation.) Sodium acetate (2%, w/v) was dissolved in the mixture and after one hour the lysate was centrifuged at  $25,000 \times g$  for 15 min. at  $-15^{\circ}\text{C}$  and the pellet resuspended in 50 ml of TE buffer (10 mM-Tris HCl, pH 7.5; 10 mM-EDTA) to extract the RNA. The suspension was centrifuged at  $10,000 \times g$  for 15 min. at  $4^{\circ}\text{C}$  to remove cell debris. The supernatant was extracted with phenol as follows. An equal volume of water-saturated redistilled phenol was

shaken with the supernatant for 5 min. and the layers separated by centrifugation at 7,000 x g for 5 min. at 20°C. The aqueous layer was retained, extracted twice more with water-saturated phenol, then shaken three times with an equal volume of ether and the layers separated each time by brief centrifugation. Ether was removed by suction and finally by bubbling N<sub>2</sub> through the aqueous solution. The RNA was precipitated overnight at -15°C after the addition of sodium acetate (2%, w/v) and two volumes of ethanol. This precipitate was centrifuged down at 30,000 x g for 15 min. at -10°C and redissolved in distilled water. The absorbance spectrum and the A<sub>260nm</sub> reading of the solution indicated that about 15 mg of crude total RNA had been isolated from the cells. The RNA and DNA contents of this preparation, determined as described in Methods (11), were 86% and 14% respectively.

This crude RNA preparation was analysed on a sucrose gradient and compared with marker E. coli ribosomal RNA (r-RNA), prepared as described in Methods (10) (Fig. 4.1.a.b). Most of the A<sub>260nm</sub>-absorbing material from B. amyloliquefaciens sedimented as a broad peak near the top of the gradient (Fig. 4.1.a), indicating that extensive degradation of the RNA had occurred.

It was decided therefore, to use added E. coli r-RNA as a marker for RNA degradation during the extraction of RNA from B. amyloliquefaciens. Cells from culture medium were washed twice and resuspended in TES buffer, passed through the French pressure cell and collected into two



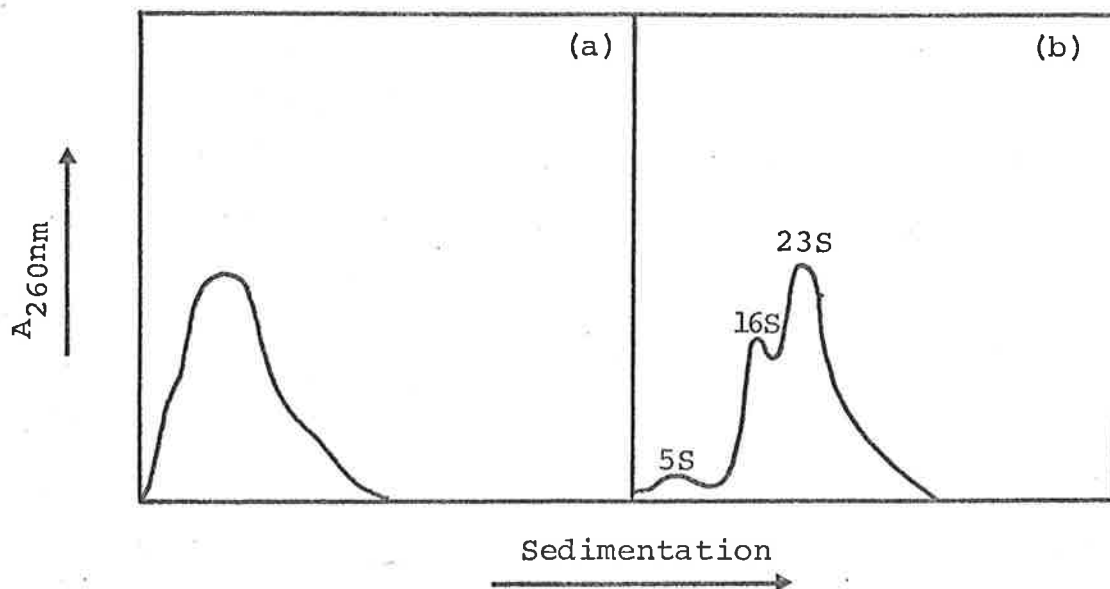


Fig. 4.1. Sucrose density gradient analysis of RNA from B. amyloliquefaciens and r-RNA from E. coli.

RNA from B. amyloliquefaciens was prepared as described in the text. E. coli r-RNA was prepared as described in Methods (10). Approximately 200  $\mu\text{g}$  of each sample was applied to a sucrose gradient and centrifuged for 4 hr as described in Methods (8).

All  $S^{\circ}_{20,w}$  values cited are approximate and used for identification purposes only.

- (a) RNA extracted from B. amyloliquefaciens.
- (b) r-RNA extracted from E. coli ribosomes.

volumes of ethanol containing SDS (0.35%, w/v) at  $-15^{\circ}\text{C}$ , as described above. E. coli r-RNA was added to the mixture and allowed to precipitate for 60 min at  $-15^{\circ}\text{C}$ . After centrifugation the pellet was resuspended in TE buffer and the cell debris was removed by centrifugation as described above. The supernatant was extracted as previously described with phenol mixture of the following composition: phenol (90%, v/v), water (10%, v/v) and 8-hydroxy-quinoline (0.1%, w/v). The latter reagent was included because it was used by other workers (Kirby, 1965; Loening, 1967). Following extraction of the aqueous phase with ether and bubbling  $\text{N}_2$  through it, as previously described, the RNA was precipitated by the addition of sodium acetate (2%, w/v) and two volumes of ethanol. The RNA (which was a mixture of added E. coli r-RNA and RNA from lysed cells of B. amyloliquefaciens) was analysed on polyacrylamide gels. In comparison with E. coli r-RNA run separately (Fig. 4.2.a), it is apparent that the 23S RNA species is absent from the extracted mixture of E. coli r-RNA and B. amyloliquefaciens RNA (Fig. 4.2.b), indicating that extensive degradation has occurred.

A similar attempt to extract intact RNA from B. amyloliquefaciens was made with the ribonuclease inhibitor Macaloid (0.1%, w/v), included in the mixture of ethanol and SDS (0.35%, w/v) before the cell lysate was added. However, RNA obtained by this procedure was still extensively degraded (Fig. 4.3.a,b,c). Therefore, these conditions are not suitable for the isolation of intact RNA.

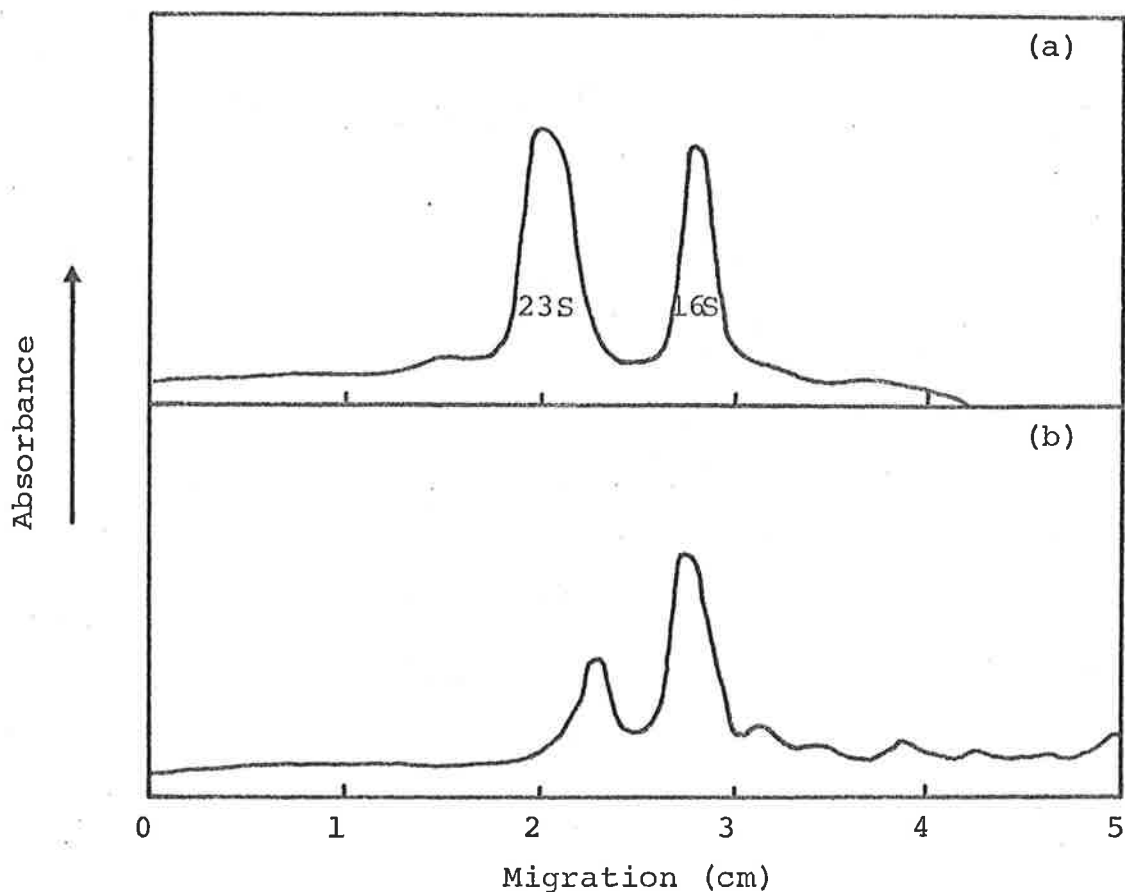


Fig. 4.2. Electrophoretic analysis, on polyacrylamide gels, of E. coli r-RNA and a mixture of E. coli r-RNA extracted together with RNA from cell lysate of B. amyloliquefaciens.

E. coli r-RNA was prepared as described in Methods (10). A sample of E. coli r-RNA was mixed with a cell lysate from B. amyloliquefaciens and extracted with phenol as described in the text. Approximately 50  $\mu\text{g}$  of each RNA sample, applied to a 3% gel, was electrophoresed at 5 mA/gel for 2  $\frac{1}{4}$  hours at room temperature as described in Methods (6b). Gels were stained for 60 min. in a solution of 0.2 M methylene blue in 0.2 M acetic acid, containing 0.2 M sodium acetate and destained overnight in dilute acetic acid. The absorbance profile of destained gels was obtained by scanning them with a densitometer (Photovolt, N.Y., N.Y.).

(a) E. coli r-RNA alone.

(b) Extracted mixture of E. coli r-RNA and B. amyloliquefaciens RNA.

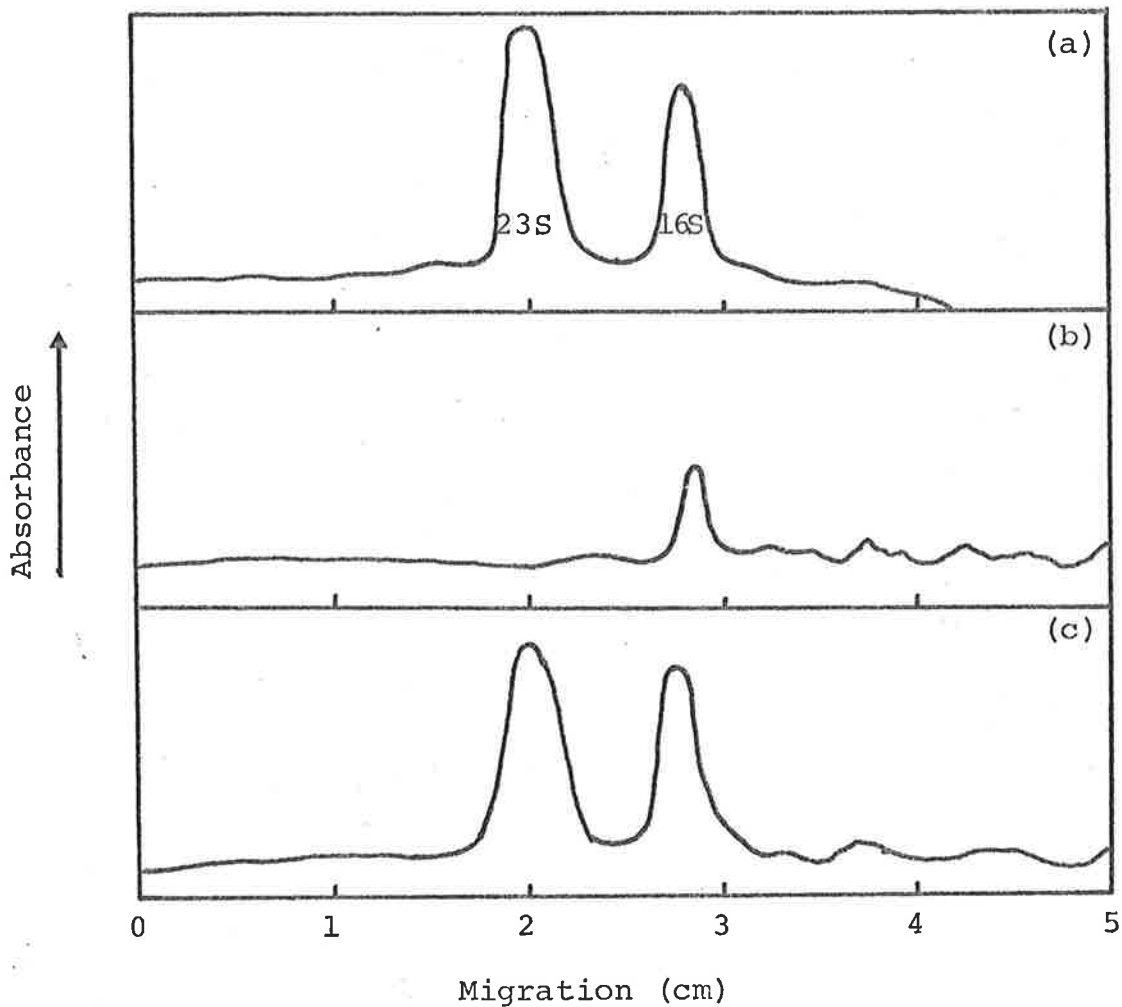


Fig. 4.3. Electrophoretic analysis on polyacrylamide gels, of E. coli r-RNA and B. amyloliquefaciens RNA.

E. coli r-RNA was prepared as described in Methods (10). B. amyloliquefaciens RNA was prepared as described in the text. Approximately 50  $\mu\text{g}$  of RNA, applied to a 3% gel, was electrophoresed at 5 mA/gel for 2.1/4 hours at room temperature as described in Methods (6b). Gels were stained and scanned for absorbance as described in the legend to Fig. 4.2.

(a) E. coli r-RNA alone (50  $\mu\text{g}$ ).

(b) B. amyloliquefaciens RNA alone (50  $\mu\text{g}$ ).

(c) E. coli r-RNA (25  $\mu\text{g}$ ) mixed with B. amyloliquefaciens RNA (25  $\mu\text{g}$ ).

### C. The use of alkaline conditions in RNA isolation

An attempt was made to extract the RNA at alkaline pH; it was hoped to inactivate an acidic ribonuclease (Smeaton and Elliott, 1967) by this method. The method and solutions used were identical to the extraction procedure described above, except that the Tris.HCl buffer, pH 7.5, was replaced by either 100 mM- $\text{Na}_2\text{CO}_3 \cdot \text{NaHCO}_3$ , pH 10.0, or 50 mM-diethanolamine.HCl buffer, pH 9.0. The resultant two RNA preparations were analysed on polyacrylamide gels (Figs. 4.4.a,b,c).

It appears that a small amount of 23S r-RNA was present in each preparation, but the ratio of 23S/16S RNA was far less than that obtained for E. coli r-RNA species (Fig. 4.4,c). Degradation products were clearly present in the RNA samples prepared at pH 9.0 and 10.0. However, that some 23S r-RNA was present indicated that the increased pH resulted in less RNA degradation than that when RNA was prepared at pH 7.5.

### D. RNA isolation from lysozyme-treated cells

#### (1) Establishment of conditions for cell breakage

To further improve the extraction of RNA, it was decided to shorten the time period between cell lysis and phenol extraction. Osmotic lysis following lysozyme treatment of cells was investigated.

Cells from 90 ml of culture medium were washed twice in TM buffer (50 mM-Tris.HCl, pH 7.6; 10 mM-magnesium acetate) and resuspended in 40 ml of TM buffer containing

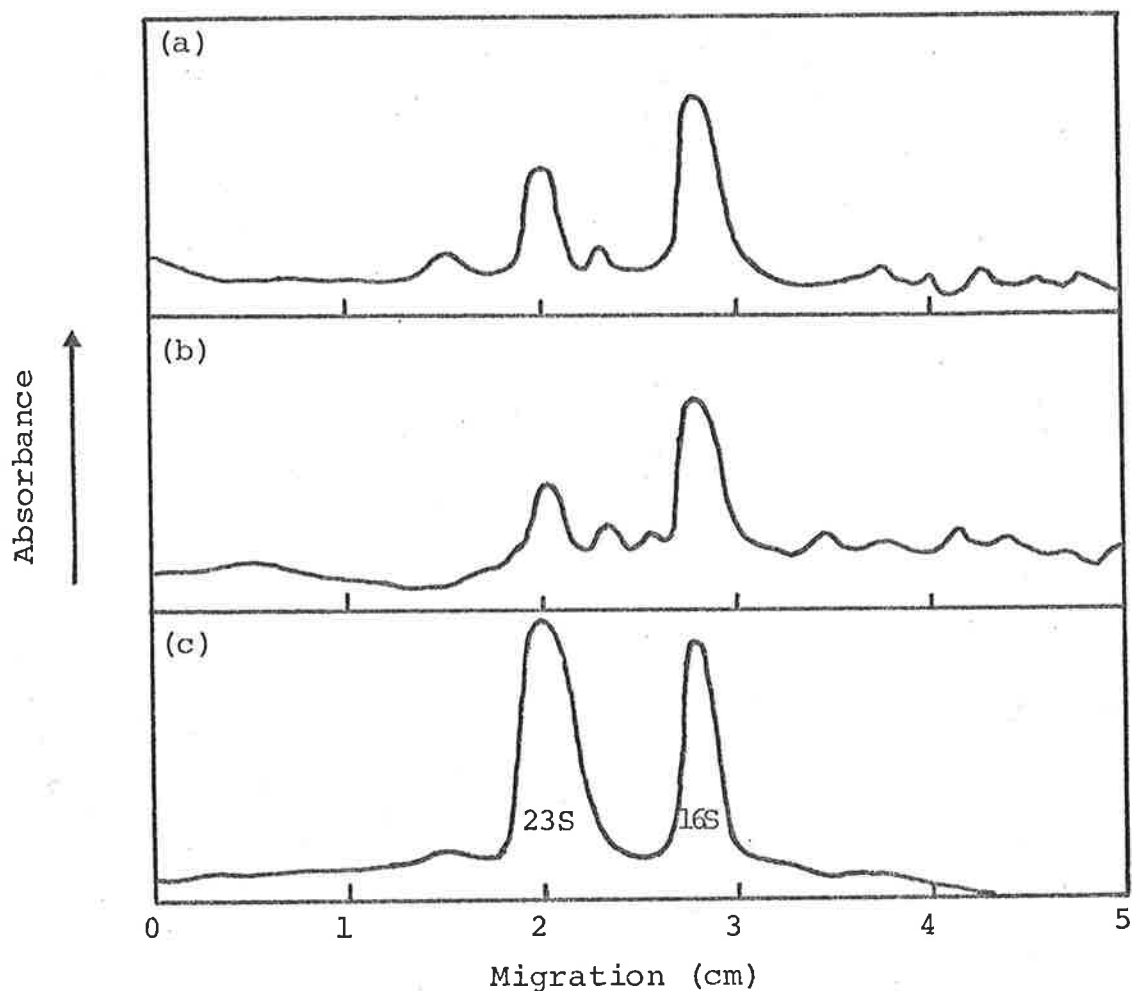


Fig. 4.4. Electrophoretic analysis on polyacrylamide gels, of *E. coli* r-RNA and RNA from *B. amyloliquefaciens* prepared at alkaline pH.

*E. coli* r-RNA was prepared as described in Methods (10). *B. amyloliquefaciens* RNA was prepared as described in the text. Approximately 50  $\mu$ g of each RNA sample, applied to a 3% gel, was electrophoresed at 5 mA/gel for 2 1/4 hours at room temperature as described in Methods (6b). Gels were stained and scanned for absorbance as described in the legend to Fig. 4.2.

- (a) *B. amyloliquefaciens* RNA prepared at pH 10.0.
- (b) *B. amyloliquefaciens* RNA prepared at pH 9.0.
- (c) *E. coli* r-RNA alone.

sucrose (25%, w/v). Lysozyme (6 mg), was added (this was the optimum amount, J. McInnes, unpublished result), and the progressive susceptibility of the cells to osmotic lysis was measured by the reduction in  $A_{600\text{nm}}$  after thoroughly mixing cell suspension (1.0 ml) with 1.0 ml of GS buffer (50 mM-glycine.NaOH buffer, pH 9.5: 0.5% sodium dodecyl sulphate (SDS)). After a 10 min. incubation with lysozyme, there was a 50% reduction in the  $A_{600\text{nm}}$  of cells mixed with GS buffer, compared with the  $A_{600\text{nm}}$  of cells diluted at zero time or cells diluted after incubation without lysozyme.

## (2) Extraction of RNA

The extraction of RNA from cells pre-incubated with lysozyme was attempted as follows. Cells from 90 ml of culture medium were harvested, washed twice in TM buffer, resuspended in 40 ml of TM buffer containing sucrose (25%, w/v) and lysozyme (6 mg) and incubated with shaking for 10 min at 30°C. The cell suspension was then rapidly cooled to 4°C by swirling in an ice-water bath and the pH was adjusted to approximately 9.5 by the addition of glycine (169 mg) and 0.31 ml of 10 N NaOH. The suspension was then added to 60 ml of GS buffer, shaken vigorously for about five seconds and added quickly to 100 ml of phenol mixture. The phenol mixture now had the following composition: phenol (82%, w/v), water (10%, v/v), m-cresol (8%, v/v), 8-hydroxy-quinoline (0.1%, w/v) and SDS (0.5%, w/v). m-Cresol and SDS were included to improve deproteinisation of the RNA (Kirby, 1965; Loening, 1967). The mixture was shaken at

room temperature for 10 min and the layers separated by centrifugation. Two further extractions using 75 ml and 50 ml of the phenol mixture respectively, were carried out. Ether extraction and N<sub>2</sub> treatment of the aqueous layer preceded RNA precipitation, as described earlier. The crude RNA was centrifuged at 30,000 x g for 15 min at -10°C and dissolved in water; the solution was centrifuged again at 30,000 x g for 30 min at 4°C to remove undissolved material. About 3 mg of A<sub>260<sub>nm</sub></sub>-absorbing material was recovered and this contained 70% RNA and 30% DNA as determined by the assays described in Methods (11).

The RNA was analysed on a sucrose density gradient (Fig. 4.5); this showed 16S and 23S r-RNA species, similar to those obtained from E. coli ribosomes (Fig. 4.1.b), to be present. In addition, a large peak of 4S RNA was present near the top of the gradient. Most of the DNA had presumably sedimented during centrifugation.

Polyacrylamide gel analysis of this sample (Plate 4.1) resolved the RNA into species similar to those observed in total RNA extracted from E. coli (Loening, 1967). The DNA in the sample presumably migrates at, or near, the origin. The identity of the faint bands migrating between the 4S and 16S RNA species is unknown but they are reproducible. It appears then, that this extraction procedure provides a suitable method of isolating relatively intact rRNA and tRNA from B. amyloliquefaciens.



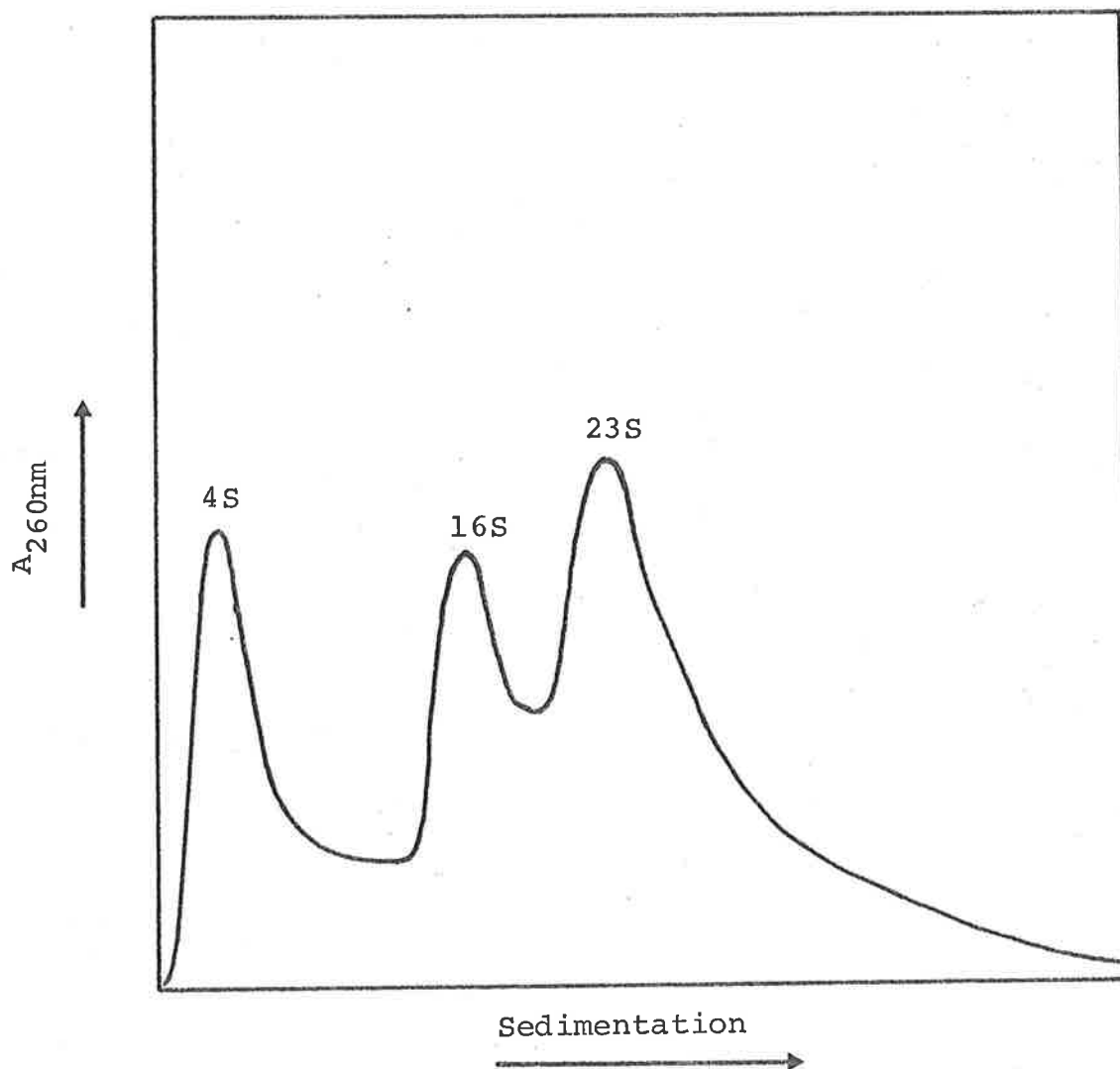
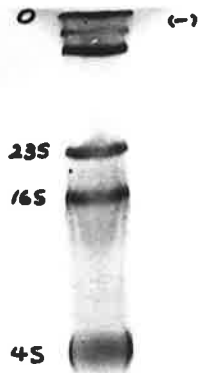


Fig. 4.5. Sucrose density gradient analysis of RNA from B. amyloliquefaciens.

RNA was prepared from lysozyme-treated cells at pH 9.5, as described in the text. Approximately 150  $\mu$ g of RNA was applied to the gradient and centrifuged for 5½ hours as described in Methods (8).



(\*)

Plate 4.1 Polyacrylamide gel electrophoresis of total cellular RNA from B. amyloliquefaciens. RNA (50  $\mu\text{g.}$ ) was electrophoresed at 4mA/gel for 1.5 hours on 2.4% gels. These were stained for 2 minutes in 0.05% toluidine blue and destained for 30 minutes in distilled water.

E. Recovery of bacteriophage MS2 RNA in the RNA of  
B. amyloliquefaciens

In order to test whether the RNA extraction procedure described above was suitable for the isolation of mRNA species, it was decided to determine whether a known mRNA molecule could be recovered intact from a cell lysate. Bacteriophage MS2 RNA was used as a ready source of an intact mRNA molecule and the following experiment was performed.

Cells from 45 ml of culture medium were washed twice, resuspended in Tris-low amino acids medium (described later) and incubated at 30°C for 10 minutes. Sucrose (11.25 gm) and lysozyme (3 mg) were then added. After 20.5 minutes, the cell suspension was cooled, the pH was adjusted to 9.5, as described earlier and the cells mixed with 50 ml of GS buffer, to which had been added  $^{32}\text{P}$ -MS2 RNA, prepared by the method of Sugiyama (1971). The lysate was shaken for about five seconds and added to the phenol mixture. The solutions used and the extraction procedure were as described above for extraction of RNA at pH 9.5. The mixture of  $^{32}\text{P}$ -MS2 RNA and B. amyloliquefaciens RNA recovered by this procedure was analysed on a sucrose density gradient (Fig. 4.6). This showed that an almost symmetrical peak of  $^{32}\text{P}$ -MS2 RNA was obtained and this migrated in a position on the gradient consistent with its molecular weight of  $1.1 \times 10^6$  daltons (Strauss and Sinsheimer, 1963), indicating that MS2 RNA, exposed to the same conditions as B. amyloliquefaciens mRNA species, had been recovered apparently intact. The slight

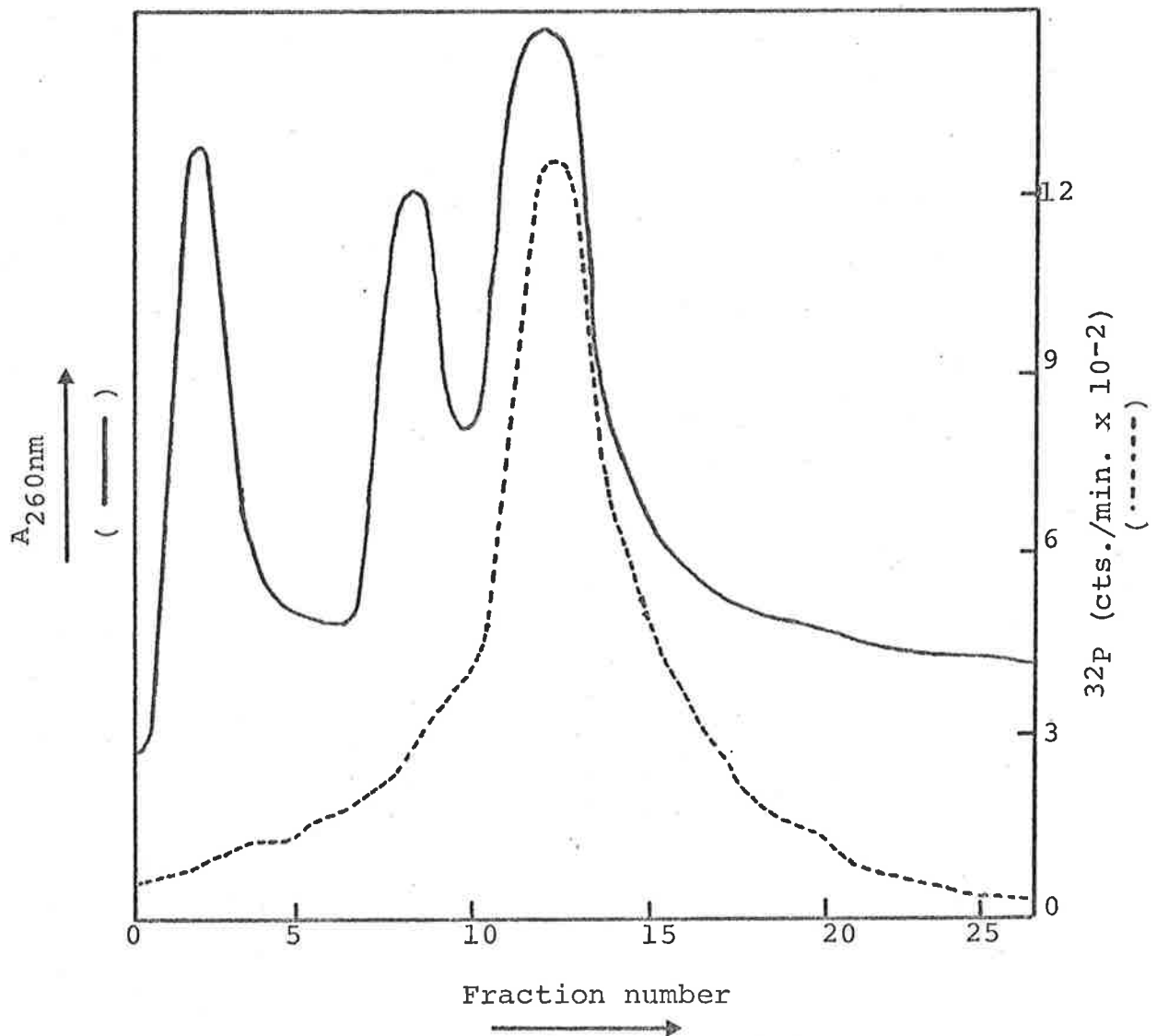


Fig. 4.6. Sucrose density gradient analysis of a mixture of <sup>32</sup>P-MS2 RNA and unlabelled *B. amyloliquefaciens* RNA extracted together from a cell lysate.

The mixture of <sup>32</sup>P-MS2 RNA and *B. amyloliquefaciens* RNA was extracted from a cell lysate as described in the text. Approximately 500 μg of the RNA mixture was applied to a gradient, centrifuged for 5½ hours and fractionated as described in Methods (8).

shoulder on the left side of the radioactivity peak does not appear to be the result either of exposing the MS2 RNA to the B. amyloliquefaciens cell lysate, or the extraction conditions since it was also observed (Fig. 4.7) when unlabelled MS2 RNA was run separately on a sucrose density gradient.

The above results show that r-RNA can be isolated from B. amyloliquefaciens with little or no detectable degradation by this procedure. In addition, in this way, a known mRNA molecule can be recovered apparently intact from a lysate of cells of B. amyloliquefaciens. It therefore seemed a reasonable basis for attempting to isolate the protease mRNA.

#### F. Attempts to label the protease mRNA pool

At the time when these experiments were done, it was believed that the mRNA pool was stable until translated (Kuwano, Schlessinger and Apirion, 1970). Therefore, the initial approach and rationale was as follows. Since the mRNA pool appeared to accumulate over several hours during cell growth (J. McInnes, unpublished result), cells were incubated with  $^{32}\text{P}$ i for two generations (about 5 hr). It was predicted from Fig. 3.4, that if the protease mRNA remained stable until translated, about 75% of the pool would remain in the cells after rifampicin treatment for 15 minutes, while after 90 minutes of incubation with the drug, the pool would be gone. It was reasoned, therefore, that there might be a significant difference between the  $^{32}\text{P}$ -radioactivity

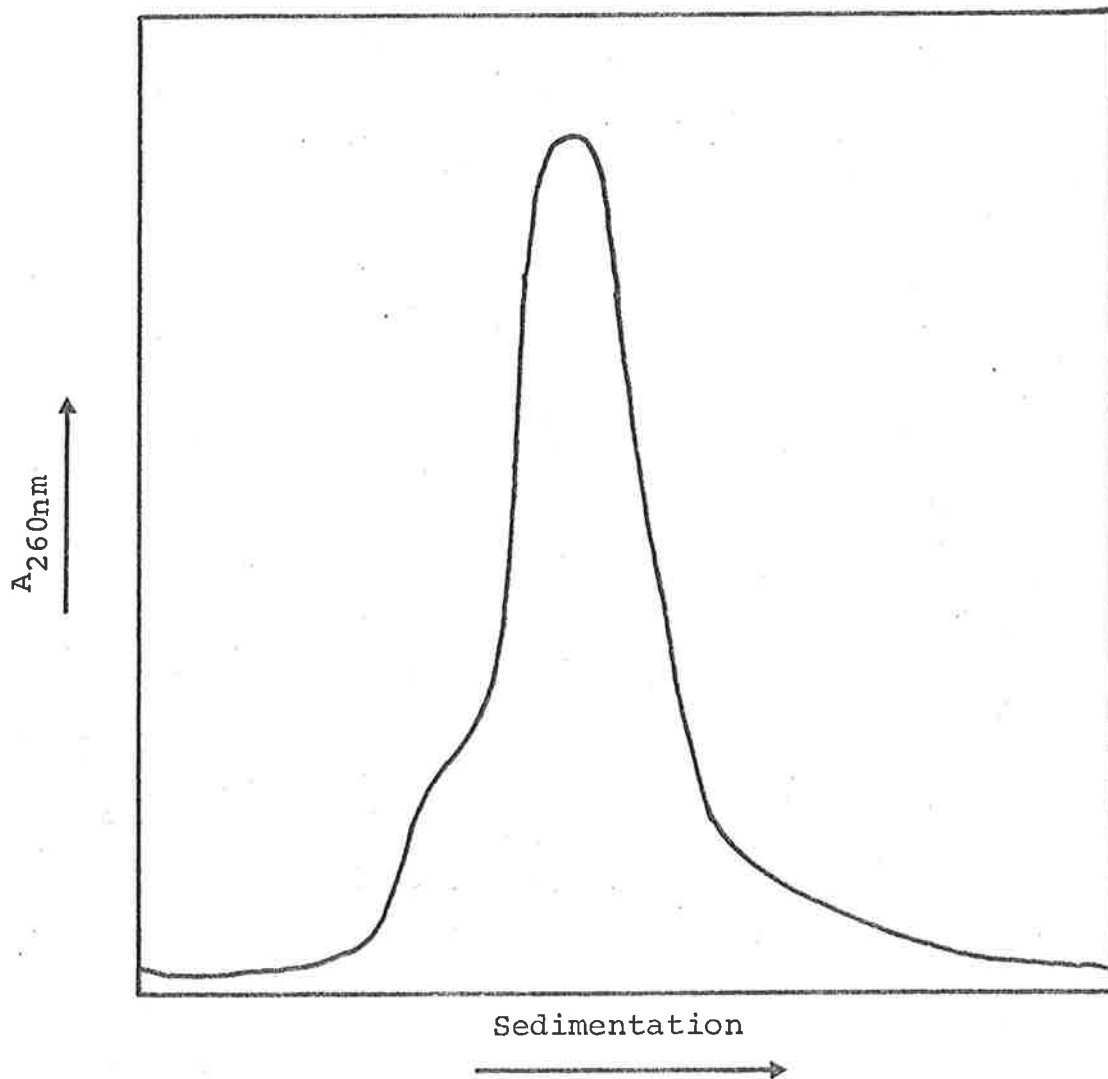


Fig. 4.7. Sucrose density gradient analysis of unlabelled MS2 RNA.

Unlabelled MS2 RNA was prepared by the method of Sugiyama (1971), 400  $\mu\text{g}$  was applied to the gradient, and centrifuged for 5½ hours as described in Methods (8).

profiles of total RNA isolated from cells treated with rifampicin for these different time periods.

Cells were incubated in culture medium with  $^{32}\text{P}_i$  for two generations and then treated in suspending medium with rifampicin (0.5  $\mu\text{g}/\text{ml}$ ) for either 15 or 85 minutes. At these times,  $^{32}\text{P}$ -RNA was prepared from the cells at pH 9.5 by the method described earlier, and each sample was analysed on a sucrose density gradient. There was no significant difference between the radioactivity profiles of the two RNA samples.

The discovery by Dr. Glenn of the dynamic state of the mRNA pool (Chapter 3) subsequently gave a possible explanation for this failure, since most of the mRNA pool would have decayed during 15 min treatment with rifampicin. An alternative approach using shorter labelling times was therefore adopted.

For shorter term labelling of mRNA, it was preferable to resuspend cells in a low amino acids suspending medium, for under these conditions, protease mRNA transcription is believed to proceed maximally (Fig. 3.13). Preliminary studies showed that if the cells were treated with lysozyme in this medium following labelling and the addition of sucrose, the final RNA precipitation, after phenol extraction, was accompanied by large amounts of an insoluble complex, probably due to the phosphate in the suspending medium. This problem had not been previously encountered because in earlier studies, cells were harvested from the culture medium and resuspended in TM buffer containing sucrose

(that is, in the absence of phosphate) for treatment with lysozyme. In the present experiments, it was undesirable to do this because mRNA breakdown might occur during the centrifugation and resuspension. Accordingly, it was necessary to label the cells in a medium in which they could be directly processed for RNA extraction. The new medium devised differed from the original low amino acids suspending medium in the following ways; 50 mM-Tris was substituted for  $(\text{NH}_4)_2\text{HPO}_4$  and 9 mM-magnesium acetate and 5 mM- $\text{NH}_4\text{Cl}$  were added. The pH was adjusted to 7.3 with HCl. This will be referred to as Tris-low amino acids medium.

A control experiment involving rifampicin-insensitive protease production by cells incubated for 25 minutes in Tris-low amino acids medium, showed that the protease mRNA pool had been effectively maintained, despite the lack of phosphate in this new medium. In addition, a separate experiment showed that cells incubated in this medium with lysozyme in the presence of sucrose osmotically lysed when added to GS buffer, as described earlier.

Checks were also made that the period of lysozyme treatment in the presence of sucrose did not affect the ability of the cells to synthesise RNA as follows. Cells from culture medium were washed twice and resuspended in Tris-low amino acids medium with either  $^{32}\text{Pi}$  (0.1  $\mu\text{C}/\text{ml}$ ) or 2- $^{14}\text{C}$ uracil (0.25  $\mu\text{C}/\text{ml}$  plus 4  $\mu\text{g}/\text{ml}$  of unlabelled uracil) in the presence or absence of sucrose (25%) and lysozyme (0.065 mg/ml). The incorporation of radioactivity was followed as described in Methods (3). The results (Fig. 4.8a,b) show



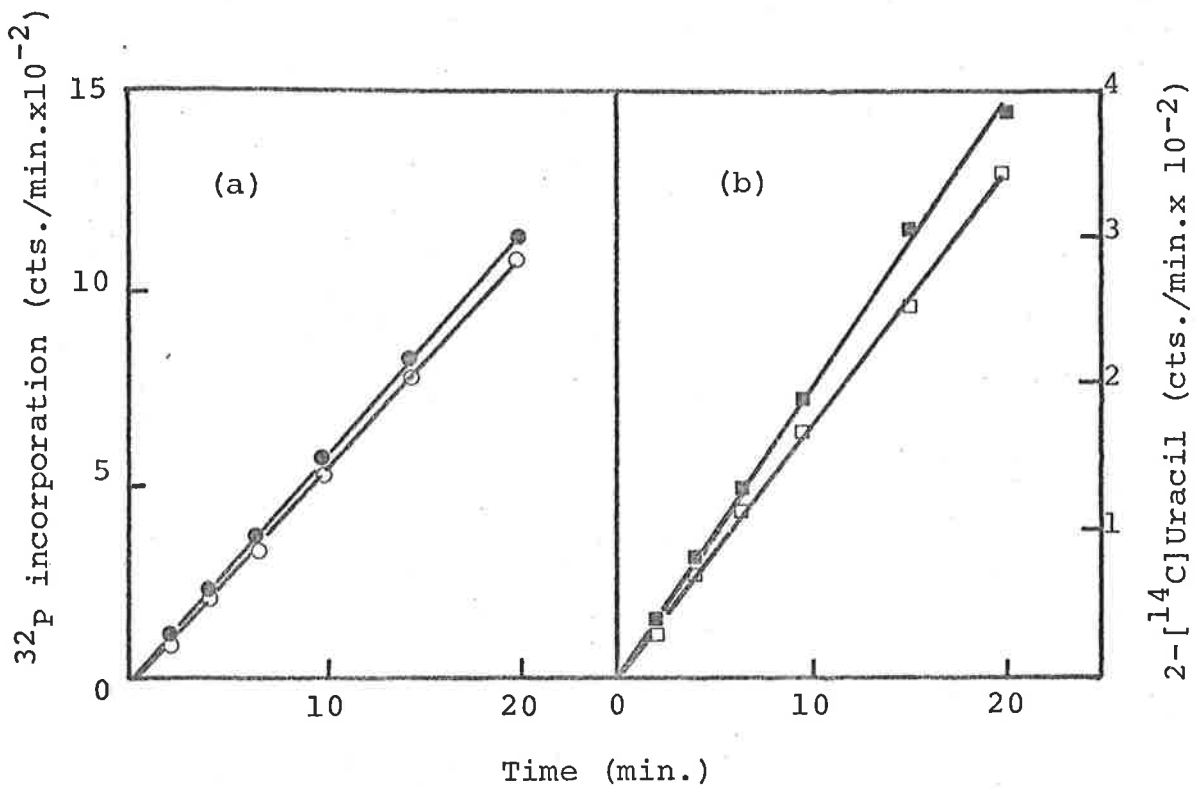


Fig. 4.8. Incorporation of radioactivity into cells in the presence and absence of sucrose and lysozyme.

The experimental details are given in the text. Radioactivity, lysozyme and sucrose were added at zero time. (a) Incorporation of  $^{32}\text{P}$  into cells in the absence (—●—●—) and presence (—○—○—) of sucrose and lysozyme. (b) Incorporation of 2- $^{14}\text{C}$ uracil into cells in the absence (—■—■—) and presence (—□—□—) of sucrose and lysozyme.

that the incorporation of these labelled compounds was unaffected by the presence of lysozyme and sucrose in this medium, measured over a 20 minute time period. From this, it seemed reasonable to conclude that mRNA synthesis was unlikely to be impaired and that labelling of mRNA species and lysozyme treatment of the cells could proceed simultaneously.

As a further improvement to the overall procedure, tri-isopropyl-naphthalene sulphonic acid (1%, w/v) was also added to the phenol mixture described for the extraction of RNA at pH 9.5. This compound was also reported to promote more efficient deproteinisation of RNA (Kirby, 1965; Loening, 1967).

#### G. Isolation of labelled RNA

In these experiments, cells were washed twice and resuspended in Tris-low amino acids medium with  $^{32}\text{P}_i$  (2  $\mu\text{C.ml}$ ) for either 10 or 20 minutes. Lysozyme and sucrose were added together at either zero time (for a 10 min. labelling period) or at 10 minutes after resuspension (for a 20 minute labelling period). In this way, the cells could be rapidly lysed and the RNA isolated by extraction with phenol at the end of the labelling time as described previously. RNA, labelled in this way, was isolated and analysed on sucrose gradients (Fig. 4.9,a). It is evident that most of the label was incorporated into species migrating between the 4S and 16S RNA species, the region in which small, monocistronic mRNA molecules are expected to migrate. In particular, calculated

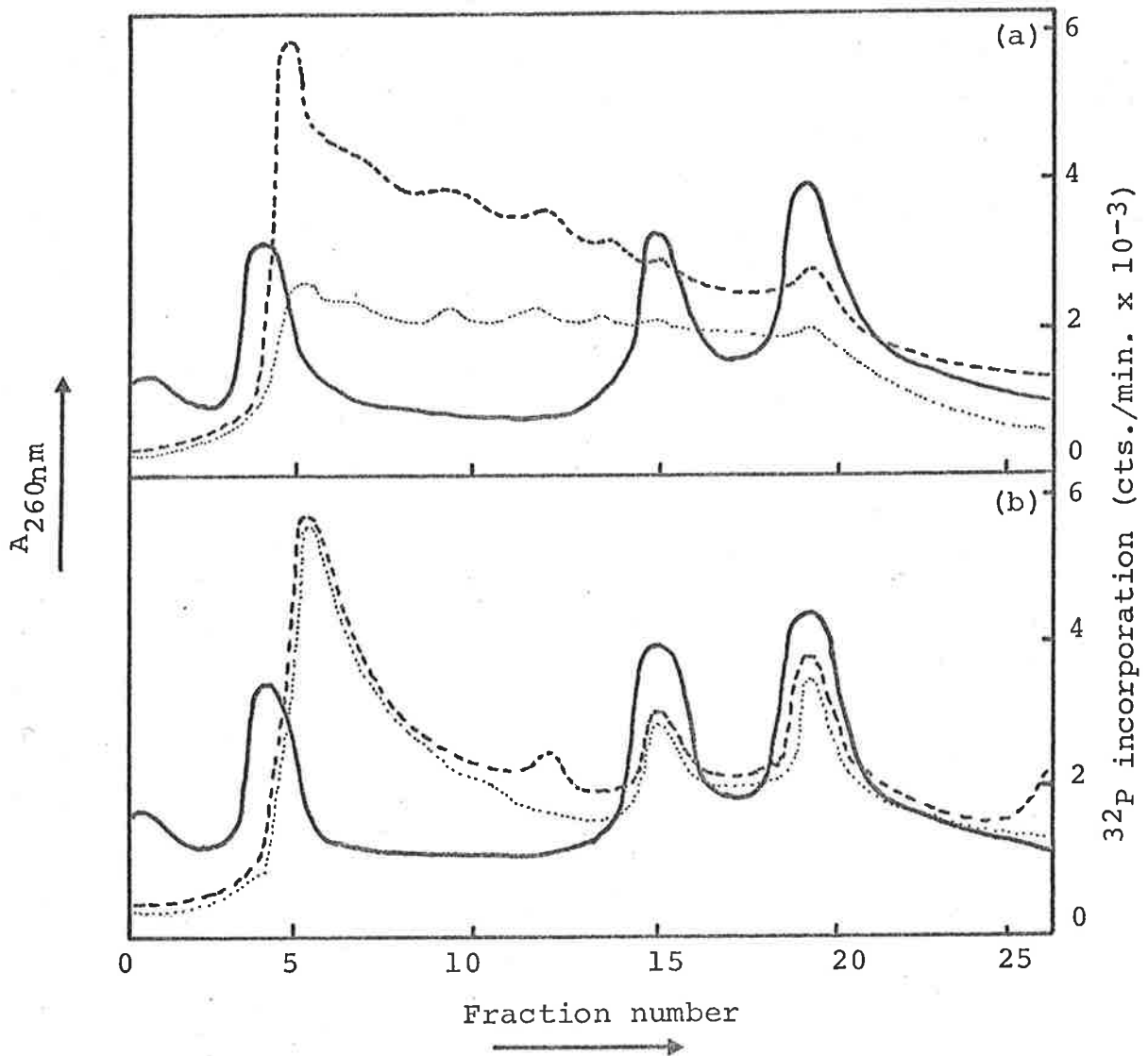


Fig. 4.9. Effect of rifampicin on the radioactivity  $^{32}\text{P}$  associated with RNA isolated from cells labelled with  $^{32}\text{P}$ .

$^{32}\text{P}$ -RNA samples were prepared as described in the text. Approximately 250  $\mu\text{g}$  of each RNA sample was applied to a sucrose density gradient and centrifuged for 9 hr. as described in Methods (8).

(a) RNA from a cell suspension labelled with  $^{32}\text{P}$  for 10 min. (.....) and 20 min. (-----).

(b) RNA from a cell suspension labelled with  $^{32}\text{P}$  for 20 min. then incubated with rifampicin for 10 min. (-----) or 20 min. (.....).

from a molecular weight of 27,500 for extracellular protease (Carey, 1966), a monocistronic protease mRNA might be expected to migrate between fractions 11-13 (Fig. 4.9,a), that is, 11S, and this region of the gradient contains rapidly labelled RNA species.

#### H. Incubation of labelled cells with rifampicin

It was reasoned that if mRNA species had been labelled by the above procedure, the radioactivity incorporated should rapidly disappear during incubation of the cells with rifampicin. To test this, it was first necessary to show that the presence of lysozyme and sucrose in Tris-low amino acids medium did not affect the decay of mRNA species in the presence of rifampicin. Therefore, samples of washed cells were suspended in Tris-low amino acids medium containing 4  $\mu\text{g/ml}$  of unlabelled uracil with 0.25  $\mu\text{C/ml}$  of 2- $^{14}\text{C}$ uracil and incubated for 10 minutes. At this time, sucrose (25%) and lysozyme (0.065 mg/ml) were added to one sample, followed at 10.5 min. by the addition of rifampicin (0.5  $\mu\text{g/ml}$ ) and unlabelled uracil (400  $\mu\text{g/ml}$ ) to both. The loss of incorporated radioactivity was followed (Fig. 4.10) and found to be the same in both cases.

To determine whether radioactivity incorporated in Fig. 4.9.a was in labile RNA species, washed-cells were labelled for 20 minutes in Tris-low amino acids medium, as described above, before rifampicin (0.5  $\mu\text{g/ml}$ ) was added and the culture divided. Sucrose and lysozyme were added immediately to one half and the RNA extracted 10 minutes

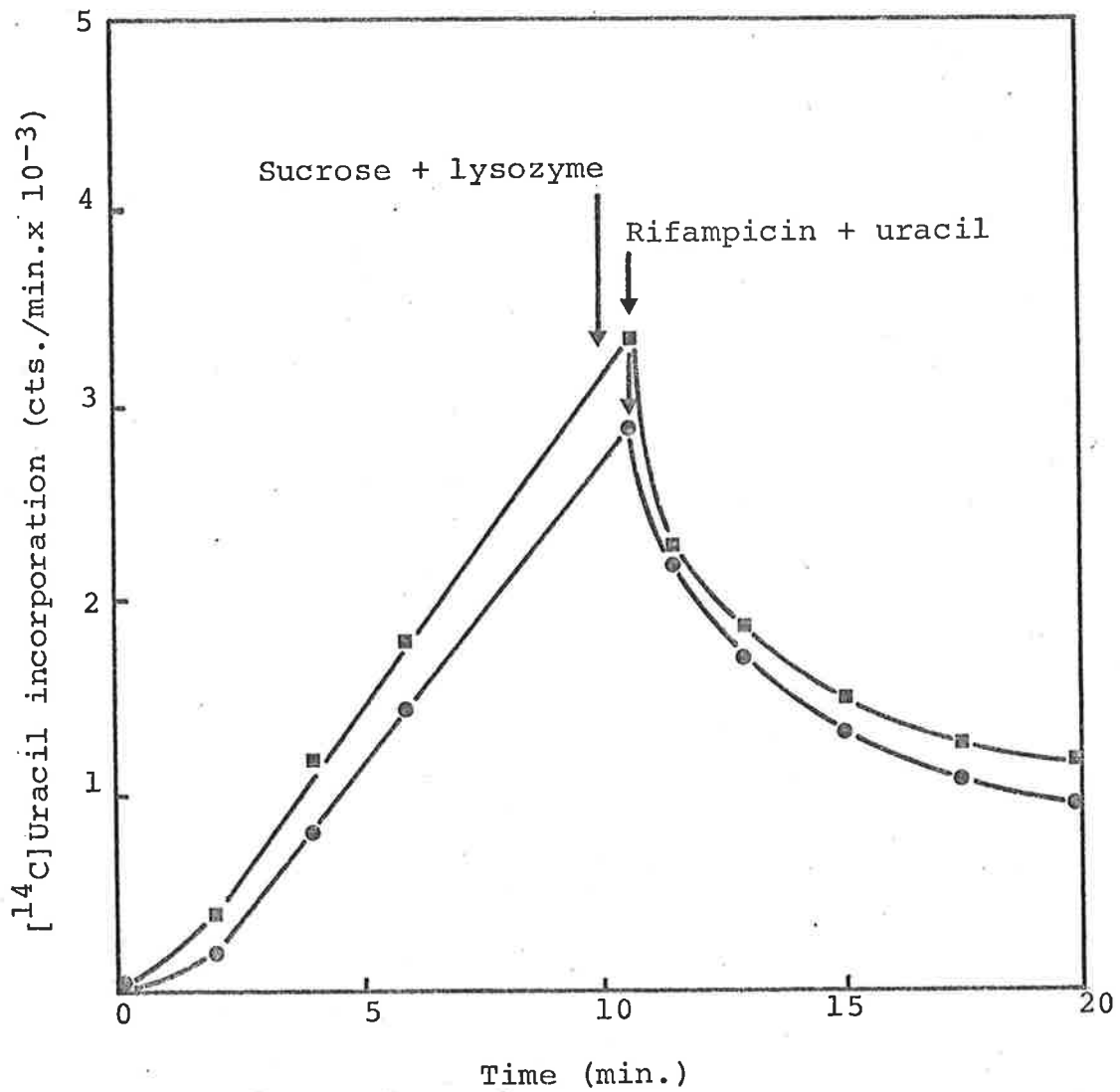


Fig. 4.10. Decay of labelled RNA in cells resuspended with rifampicin in the presence and absence of sucrose and lysozyme.

The experimental details are given in the text. Decay of mRNA in the absence (—●—●—) and in the presence (—■—■—) of sucrose and lysozyme.

later. To the second half, sucrose and lysozyme were added 10 minutes after rifampicin, resulting in a 20 minute incubation in the presence of the drug before the RNA was extracted. The results (Fig. 4.9,b) indicate that much of the radioactivity migrating between the 4S and 16S RNA species after a 20 minute labelling period (Fig. 4.9,a) is unstable in the presence of rifampicin and this is indicative of mRNA species. A very similar result was obtained when cells labelled with 2-[<sup>14</sup>C]uracil were treated with rifampicin for 10 minutes. Of particular interest (Fig.4.9a,b) is the small peak of radioactivity in fractions 11-13 which remains after 10 minutes of rifampicin treatment, but disappears after 20 minutes of incubation with the drug. These decay properties might approximate those expected from the protease mRNA pool.

The most striking feature of Fig. 4.9 a and b is the large unidentified peak of radioactivity near the 4S RNA peak. It is probably not tRNA because the radioactivity profile has never been seen to coincide with the 4S peak in the  $A_{260nm}$  profile except when cells were labelled for two generations. Moreover, there is a band which runs very close to the 4S species on polyacrylamide gels (not visible in Plate 4.1) and it may be this species which is labelled. Possibly, the peak of radioactivity represents degradation products of RNA or perhaps it is a precursor to tRNA. It is appreciated that it may be an undesirable consequence of using a 10 or 20 minute labelling period, rather than much shorter times. The reason these labelling

times were adopted (for this preliminary work at any rate) was to ensure significant labelling of the protease mRNA pool since its half life is not precisely known.

I. Translation of possible mRNA species in an *E. coli* cell-free system measured by amino acid incorporation into proteins

The fractions showing messenger-like characteristics (Fig. 4.9,a,b) were prepared and tested for template activity in a cell-free system in the following way. A sample of  $^{32}\text{P}$ -labelled RNA was prepared from 90 ml of washed-cell suspension after a 20 minute labelling period, as described above. The RNA was distributed between six identical sucrose gradients and these were centrifuged for 16 hours, the longer time being to improve the resolution in the 4S - 16S region. Identical fractions from these gradients were pooled and a sample (50  $\mu\text{l}$ ) was taken from each to determine the  $^{32}\text{P}$ -radioactivity profile (Fig. 4.11). Based on this, the pooled gradient fractions indicated (Fig. 4.11) were combined, the RNA isolated by precipitation and re-centrifuged on identical sucrose gradients for 16 hours. The radioactivity profile of each gradient was determined (Fig. 4.12a-f) and the fractions from these gradients indicated on the figure, were pooled. The  $A_{260\text{nm}}$  was read and the RNA, isolated by precipitation, assayed in an *E. coli* cell-free system (Modolell, 1971) for mRNA activity in directing amino acid incorporation into protein. (The  $^{32}\text{P}$  radioactivity in the RNA fractions was removed from the cell-free incubation

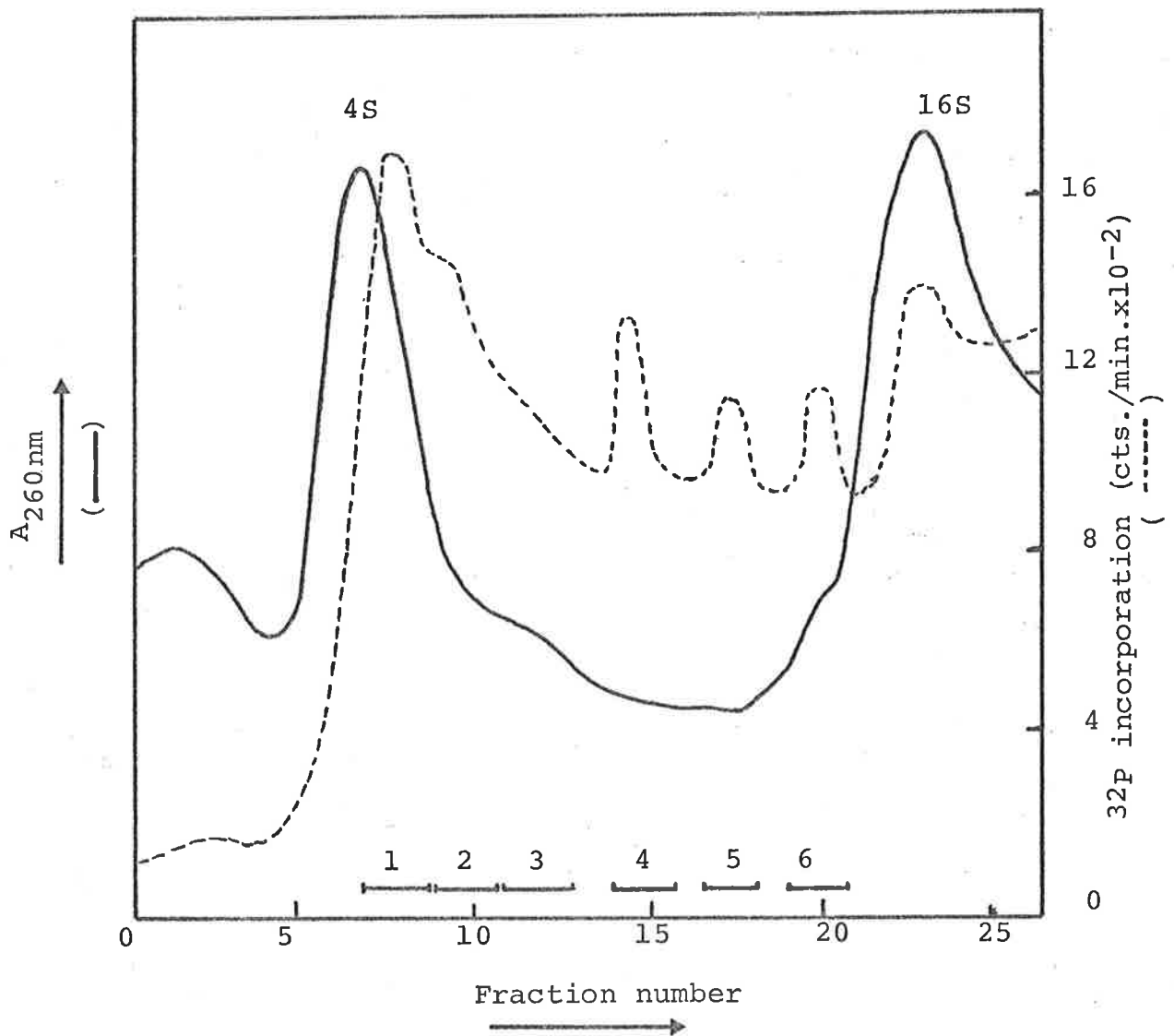


Fig. 4.11. Preparation of messenger-like RNA fractions from *B. amyloliquefaciens* for assay in an *E. coli* cell-free system.

The experimental details are given in the text. Approximately 500  $\mu$ g of RNA was applied to each gradient and these were centrifuged for 16 hr. as described in Methods (8).



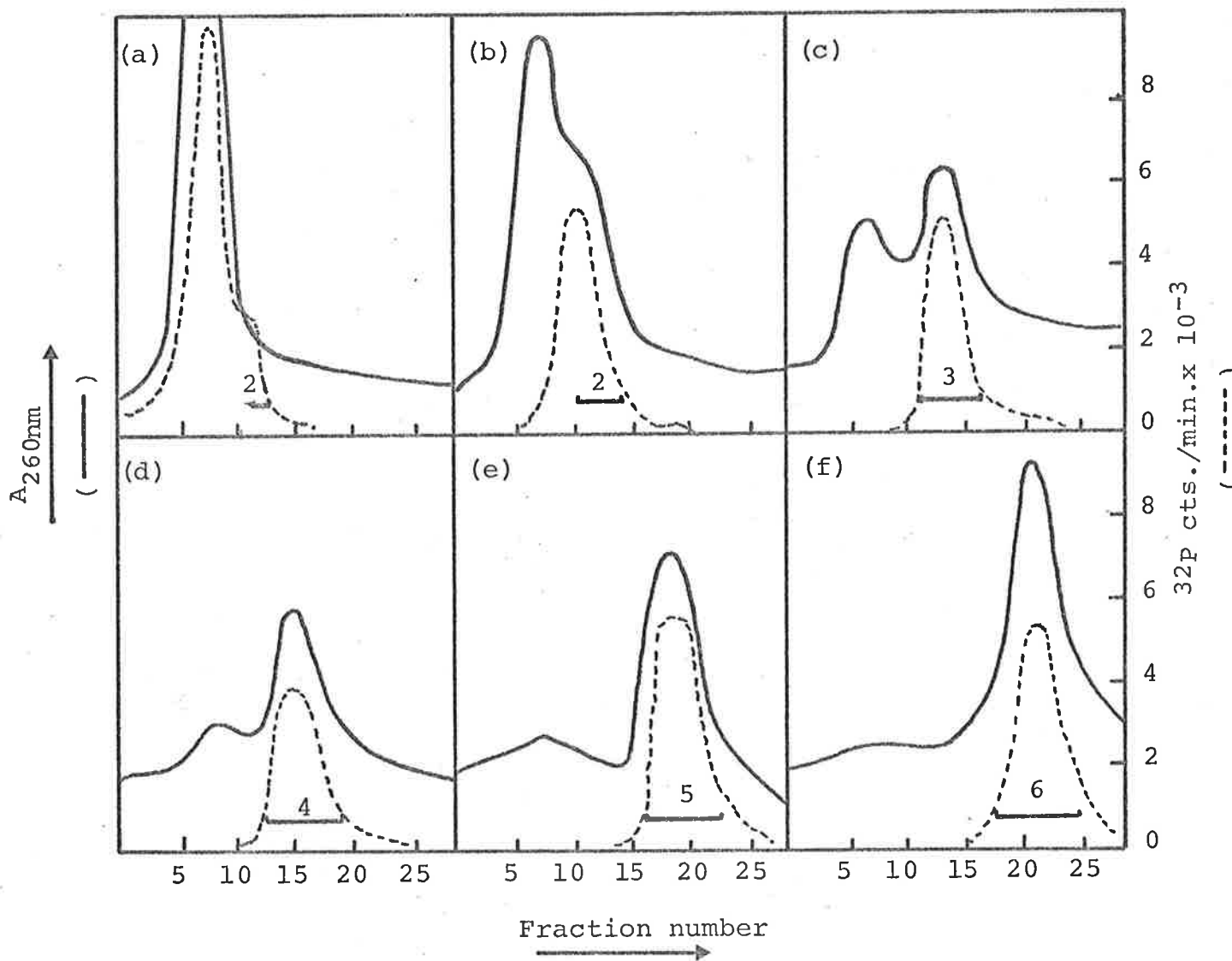


Fig. 4.12. Further fractionation of messenger-like RNA fractions from *B. amyloliquefaciens* for assay in an *E. coli* cell-free system.

RNA isolated from the fractions pooled in Fig. 4.11 were centrifuged on identical sucrose gradients for 16 hr. as described in Methods (8). The amount of RNA isolated from each fraction is given in Table 4.1.

mixtures by hydrolysis with alkali when the samples were processed as described in Methods (3).) Fraction 1 was not tested since it was not separated from the tRNA peak; fraction 6 was lost due to a faulty centrifuge tube. The results given in Table 4.1, are promising since the highest mRNA activity is found in the region of the gradient where most of the radioactivity decays in the presence of rifampicin (Fig. 4.9,b). However, the amount of RNA isolated in these individual fractions (Table 4.1) was insufficient for routine analysis in a cell-free system.

After this preliminary work, a decision had to be made as to which line of research to follow. The considerations were that the peaks observed between fractions 11-21 (Fig. 4.11) showed the characteristics of mRNA species and were the only obvious such peaks present in the relatively large amounts anticipated for extracellular mRNA. At the same time, it was appreciated that, while corresponding roughly to the expected positions of monocistronic extracellular enzyme mRNA, if the mRNA for these enzymes is polycistronic, they would be elsewhere. The argument that most bacterial mRNA molecules are polycistronic did not carry much weight since little concerning extracellular enzyme mRNA species, and in particular protease mRNA, seems to be typical. The alternative approach of taking fractions throughout the gradient and attempting to translate them has logical advantages (and is now being pursued), but has the disadvantage that mRNA activity may be inhibited by the excess of other RNA.

TABLE 4.1.

[<sup>14</sup>C]AMINO ACID INCORPORATION INTO PROTEINS DIRECTED  
BY ISOLATED RNA FRACTIONS

RNA samples were prepared and assayed for mRNA activity as described in the text.

Fraction	1	2	3	4	5	6	Blank
RNA ( µg)	-	30	25	20	35	50	Nil
Incorporation of [ <sup>14</sup> C]amino acids (cts./min)	-	610	2900	1975	3100	-	267
Cts./min. incorporated per 80 µg of RNA	-	1495	9238	7474	6855	-	Nil

Despite, therefore, the fact that it could prove to have nothing to do with extracellular enzymes, it nevertheless seemed best, in the time available, and considering other approaches going on in this laboratory, to attempt to isolate, translate and identify the peaks observed. In view, however, of the small amounts of these RNA fractions available, it was necessary to scale up the RNA preparation in order to increase the amount of these RNA species available for analysis in a cell-free system.

J. Large-scale preparation of total RNA from

B. amyloliquefaciens

The major difficulty in devising a large-scale preparative technique, was that of working with cumbersome volumes of the extracting solutions. The following modified procedure was adopted for the isolation of up to 25 mg of total RNA.

Cells, harvested from culture medium (375 ml) were washed twice and resuspended in Tris-low amino acids medium (with or without  $^{32}\text{P}_i$  (2  $\mu\text{C}/\text{ml}$ ) and incubated with shaking in a 2 l. conical flask for 10 minutes at 30°C. At this time, sucrose (93.75 gm) and lysozyme (25 mg) were added and the incubation continued for a further 10.5 minutes. The cell suspension was then poured into a beaker, cooled in an ice-water bath and stirred for two minutes, during which time glycine (1.412 gm) and 10 N NaOH (2.58 ml) were

added. Following this, 375 ml of GS buffer was added and five seconds later the whole suspension was poured into a separate vessel containing 750 ml of phenol mixture, the composition of which was defined earlier. The suspension was stirred vigorously at room temperature for 10 minutes, then extra sucrose (20 gm) was added so that the aqueous layer was heavier than the phenol layer. The layers were separated by centrifugation for 5 min. at 15°C at 20,000 x g and about 90% of the phenol layer was removed by suction. Two further extractions of the aqueous layer were performed with 560 ml and 400 ml, respectively, of the phenol mixture. The remaining aqueous layer was extracted three times with equal volumes of ether which was separated by brief centrifugation and removed by suction. Finally, ether was removed from the aqueous layer by bubbling N<sub>2</sub> through the solution. The final volume of the aqueous layer varied between 250 and 300 ml. Following the addition of sodium acetate (2%, w/v), the crude RNA was precipitated overnight at -15°C with two volumes of ethanol. The crude RNA was centrifuged at 30,000 x g for 15 min. at -5°C and dissolved in distilled water (10 ml). The solution was quite viscous; undissolved material was removed by centrifugation at 30,000 x g for 60 min. at 4°C. The supernatant which was carefully removed with a pasteur pipette, contained the crude RNA in yields of up to 25 mg and this was stored in liquid N<sub>2</sub> until required.

K. Fractionation of crude total RNA from *B. amyloliquefaciens* using zonal centrifugation

Isolation of mRNA species from total RNA of *B. amyloliquefaciens* on a large scale was achieved using the zonal rotor. Large-scale preparations were first analysed by centrifuging a small sample for 16 hours on a sucrose gradient in the Spinco SW41 rotor, as described in Methods (8). The procedures employed for zonal analysis (described in Methods (9)) were essentially those of Williamson *et al.* (1971). However, a 17 hour centrifugation time was used to obtain adequate separation of RNA species on the zonal gradient. Up to 40 mg of crude RNA were treated in a single run. The  $A_{254\text{nm}}$  profile obtained from a typical centrifugation is shown in Fig. 4.13. It can be seen that there is good separation between the 4S and 16S RNA species, while the 23S RNA has almost pelleted. The fractions between 4S and 16S (indicated in Fig. 4.13) were pooled and the RNA isolated by precipitation with sodium acetate and ethanol. This RNA was re-centrifuged on sucrose gradients in the Spinco SW41 rotor for 16 hours and the  $A_{260\text{nm}}$  profile is shown in Fig. 4.14. Fractions from various sections of the gradient were pooled, the  $A_{260\text{nm}}$  of these measured and the RNA isolated by precipitation. By this method, up to about 2 mg of RNA was obtained in the total fraction taken between the 4S and 16S RNA species on the zonal gradient (Fig. 4.13). Of this, about 600  $\mu\text{g}$  migrated on the second sucrose gradients, after centrifugation for 16 hours, in the SW41 rotor, in fractions 17-20. It was

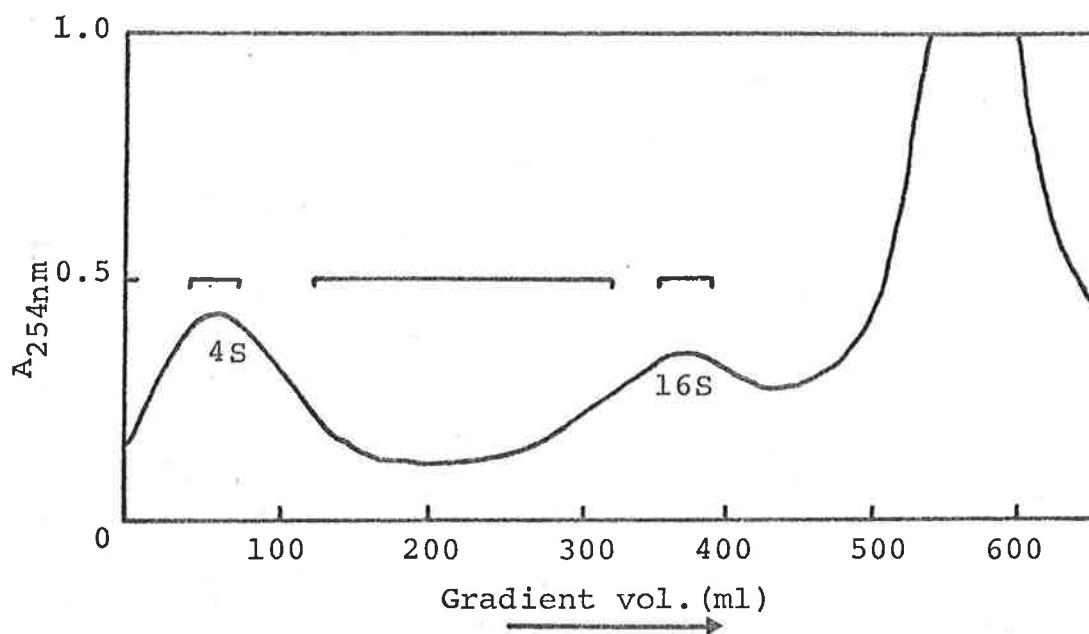


Fig. 4.13. Fractionation of RNA from B. amyloliquefaciens using zonal centrifugation.

Approximately 20 mg of RNA applied to the gradient, was centrifuged and fractionated as described in Methods (9). The fractions indicated on the figure were pooled and the RNA isolated by precipitation.

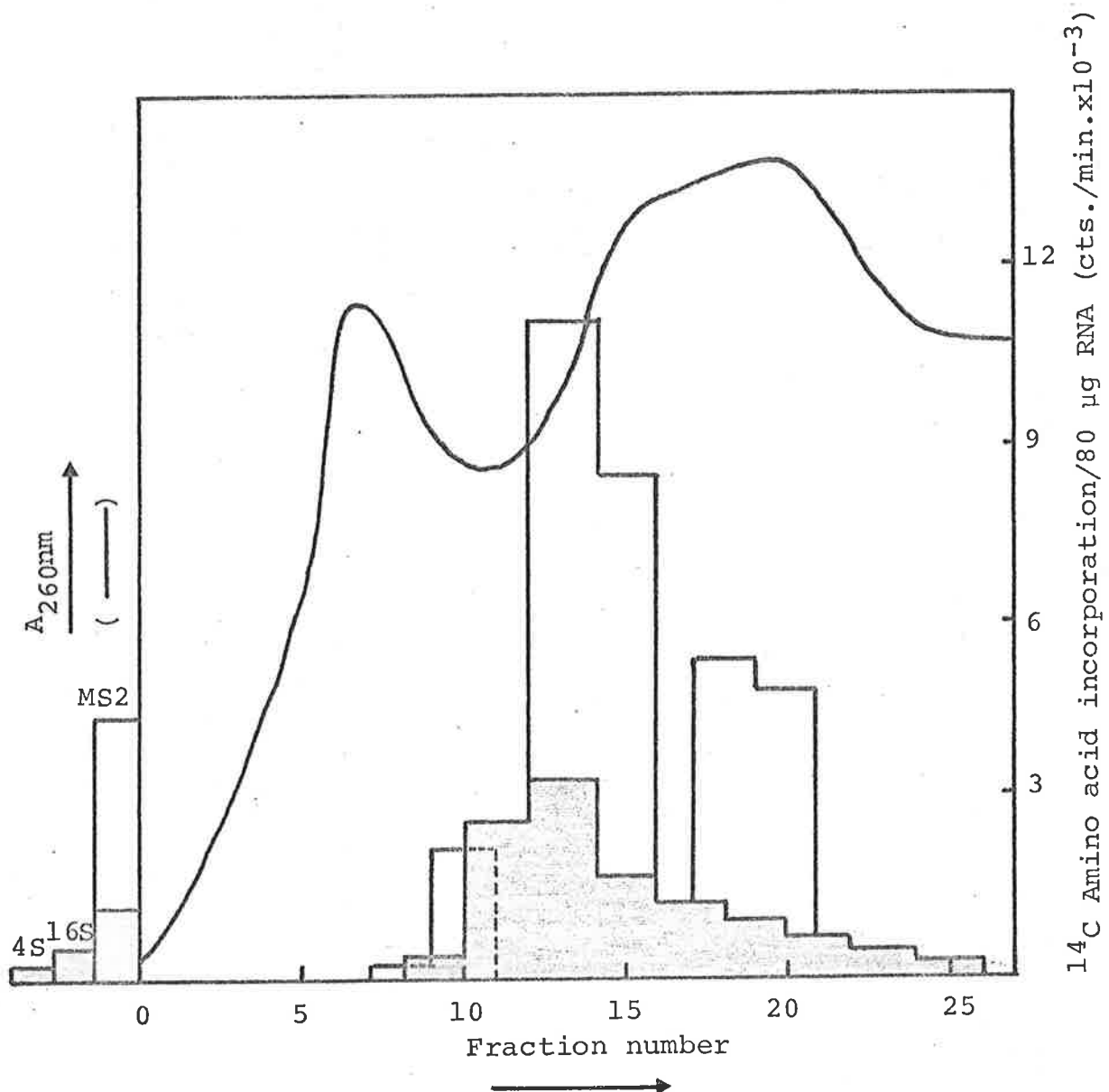


Fig. 4.14. Sucrose density gradient sedimentation of RNA isolated from the zonal gradient and its mRNA activity as assayed in an *E. coli* cell-free system.

Samples (500 µg) of RNA, isolated by precipitation from the solution between the volumes of 120 and 350 ml on the zonal gradient (Fig. 4.13), were centrifuged in the Spinco SW41 rotor for 16 hr on identical sucrose gradients and fractionated as described in Methods (8). Corresponding gradient fractions from different tubes were pooled. Adjacent fractions across the gradient (shown on the figure) were pooled, the RNA isolated by precipitation and assayed in an *E. coli* cell-free system for mRNA activity as described in the text.

Experiment 1. Puromycin was added to the cell-free incubation mixture as described in the text. Incorporation of [<sup>14</sup>C]amino acids into released protein chains was determined (shaded area).

Experiment 2. Incorporation of [<sup>14</sup>C]amino acids into total nascent protein was determined (unshaded area) as described in the text. Results are expressed as counts per minute incorporated per 80 µg of RNA.



expected that a monocistronic protease mRNA would migrate in this area, as discussed earlier. Samples of this RNA were assayed for messenger like activity in a cell-free system.

L. Messenger activity of RNA fractions from large-scale preparations measured by amino acid incorporation into proteins

A satisfactory cell-free protein synthesising system from B. amyloliquefaciens had not yet been established (J. McInnes, unpublished results). Therefore, translation of possible mRNA species was attempted using an E. coli cell-free system (Modollel, 1971). Two experimental approaches were used; both gave similar results. In the first experiment, puromycin (0.1 mM) was added to the cell-free incubation mixtures after 18 minutes. The purpose of this was to maximise the release of nascent polypeptide chains from the ribosomes into the supernatant. Two minutes later, the ribosomes were centrifuged at 105,000 x g for 60 minutes at 4°C and the radioactivity incorporated into released protein chains in the supernatant was determined (Fig. 4.14, shaded area). In the second experiment, using a separate preparation of RNA fractions, radioactivity incorporated by the cell-free system into total TCA-precipitable protein material (that is, without puromycin release) was measured (Fig. 4.14, unshaded area). All results are expressed as counts per minute incorporated per 80 µg of RNA, which was

the amount of 16S, 4S and MS2 RNA used in the controls.

It is evident that the relative patterns of amino acid incorporation directed by the RNA fractions is similar in both experiments. The results presented in Table 4.1 also agree with these. Most of the putative mRNA fractions direct the incorporation of [ $^{14}$ C]amino acids into protein as well as, or better than, comparable amounts of MS2 RNA, with which the system was calibrated. Moreover, incorporation directed by the 16S and 4S RNA fractions (isolated by precipitation from the regions indicated on the zonal gradient (Fig. 4.13) is negligible.

M. Preliminary attempts to obtain exogenous mRNA-directed protease synthesis

There was available in this laboratory, an antibody which cross-reacts with the extracellular neutral protease from B. amyloliquefaciens, prepared using commercial 'Nargase' as antigen. To measure protease synthesis, immunological precipitation was used. Cell-free extracts of E. coli were incubated with radioactive amino acids in the presence of the RNA fraction under test. The protease antibody was then added to either the total E. coli cell-free incubation mixture, or to the supernatant from an incubation mixture treated with puromycin to release nascent polypeptide chains. Only RNA taken from fractions 17-20 (Fig. 4.14) directed the synthesis of a radioactive product which was precipitated by the antibody, as compared with a control containing no added RNA. However, a comparable amount of

radioactivity was precipitated in a control cell-free incubation mixture to which MS2 RNA had been added. The results were therefore inconclusive.

#### N. Discussion

The work described in this chapter is of a preliminary nature; the results seem reasonably encouraging and provide a basis for future work.

It appears that the extraction procedure developed has prevented major degradation of r-RNA in the cell lysate and thus, although it is obviously not certain, the isolation of undegraded mRNA from lysozyme-treated cells, by rapid phenol extraction, may reasonably be expected to have been achieved. The percentage of total cell lysis during the extraction is unknown and this parameter may influence the yield of RNA obtained and perhaps, the relative proportion of low molecular weight RNA and the larger r-RNA species, since low molecular weight (4S) RNA can be selectively extracted from cells without cell lysis (Brubaker and McCorquodale, 1963). Nevertheless, the  $A_{260\text{nm}}$  and  $^{32}\text{P}$ -radioactivity profiles obtained from sucrose density gradient analysis of B. amyloliquefaciens RNA and the corresponding bands observed on polyacrylamide gels are fully reproducible and the relative proportion of the major RNA species changes little.

The technique for the extraction of RNA appears suitable for the objective of isolating the protease mRNA since MS2 RNA was recovered apparently intact from a

B. amyloliquefaciens cell lysate by this method. However, it has since been appreciated that this experiment should be repeated and the whole RNA mixture subjected to formamide treatment during its analysis. This would ensure, by destroying the tertiary structure of the RNA, that no single breaks in the MS2 RNA, hidden because the tertiary structure was maintained by complementary base pairing, had occurred during the RNA extraction. This experiment is being done, but the present candidate had insufficient time available to do it. At least the present work eliminates extensive degradation of the MS2 RNA.

It is encouraging also, in this respect, that the relative incorporation of [ $^{14}$ C]amino acids into protein, directed by the putative mRNA fractions isolated from sucrose gradients, was similar for each analysis in the E. coli cell-free system (Table 4.1; Fig. 4.14). If random degradation of RNA was occurring during its isolation, this pattern would probably not be reproducible.

It seems that the labelling experiments were successful in labelling messenger-like RNA species, as judged by the rifampicin sensitivity of radioactivity migrating in the region of the sucrose gradient where small monocistronic mRNA molecules would be found. It cannot be determined whether or not the protease mRNA is monocistronic, but assuming that it is and that it has no extra nucleotide sequences which perform some special function, such as directing it to the membrane for translation, it would be expected to migrate approximately in fractions 11-13 (Fig.

4.9,a), that is, about 11S. A peak of radioactivity with decay characteristics consistent with it being the protease messenger was observed in this region (Fig. 4.9,b), but although this result is compatible with the protease mRNA being monocistronic, the possibility that it is polycistronic has not been discounted and work considering this is in progress.

Unfortunately, a cell-free system from B. amylo-liquefaciens is not yet fully established. The E. coli system was used as a matter of convenience, although it is realised that other systems such as the ascites cell-free system could have been used. The fact is also appreciated, that the E. coli cell-free system and others, may intrinsically be incapable of translating extracellular enzyme mRNA from B. amyloliquefaciens because of the specific recognition process implied for the initiation of extracellular enzyme synthesis in the model discussed in Chapter 3. Nevertheless, it seems that reproducible mRNA activity has been demonstrated in an RNA fraction which, based on its decay properties in the presence of rifampicin, is a candidate to be the protease mRNA.

Further attempts are being made in this laboratory to translate the observed mRNA species into recognisable products in an ascites cell-free system while work on establishing the B. amyloliquefaciens cell-free system continues. An effort is also being made to selectively isolate, by low temperature phenol extraction, low molecular weight and unbound RNA, that is, possibly the protease mRNA,

from intact cells. The isolation and attempted translation of RNA fractions from right across the sucrose gradient is also proceeding.

CHAPTER 5

FINAL SUMMARY AND DISCUSSION

It was the aim of this thesis to extend or refute the model for extracellular enzyme synthesis and secretion proposed by May and Elliott (1968a). The results obtained are fully compatible with the model and knowledge about how the synthesis of extracellular enzymes (in particular, protease) might begin has been expanded. The major conclusions from the work in this thesis will now be briefly reviewed to permit an overall discussion of the results.

It is apparent that the extracellular neutral protease of B. amyloliquefaciens is synthesised de novo by the cells. Furthermore, the synthesis of the enzyme is repressed by amino acids (May and Elliott, 1968a) and this has now been shown to be due to the repression of protease mRNA transcription by high levels of amino acids. This is teleologically reasonable because protease is presumably produced extracellularly to provide free amino acids for the cell.

Surprisingly, there appears to exist in B. amyloliquefaciens a pool of protease-specific mRNA capable of supporting protease production in the absence of RNA synthesis for up to 80 minutes. It appears that this mRNA is not intrinsically long-lived, but has a half-life of the order of 5-10 minutes, as deduced from the rifampicin-sensitivity of protease production during phase 2 synthesis. Consistent with this result is the implication from this work and the results of Dr. Glenn which showed that the protease mRNA pool is rapidly turning over due to a translation-independent degradation process. Therefore, the apparent contradiction



between the short lifetime of the protease mRNA and the ability of the cells to support protease synthesis in the absence of RNA synthesis for up to 80 minutes can best be explained if the mRNA is present initially in such excess that even after several half-life decay periods, sufficient remains in the cells to saturate the translational sites.

It is generally accepted that the synthesis of a specific protein by bacterial cells in the absence of RNA synthesis is a measure of the mRNA level in the cell. However, the excess of protease mRNA in the cell and its degradation by a process unrelated to translation makes it unlikely that this assumption is tenable in this case. It follows that protease production cannot be assumed to be a measure of the amount of protease-specific mRNA in the cells; no hybridization assay is available as an alternative. Despite this, gross changes in rifampicin-insensitive protease production have been taken to indicate gross changes in the level of mRNA within the cell.

The protease mRNA pool appears to be the result of a dynamic equilibrium between mRNA transcription, translation and translation-independent degradation. This situation is unprecedented so far as is known in prokaryotes; as to the biological significance of such a relationship, it is proposed that it is related to the mechanism of extracellular enzyme production. If, as postulated, mRNA migrates from the gene to the membrane-translational sites, then excess mRNA production may occur to ensure that sufficient messenger reaches the membrane to satisfy the needs of extracellular

enzyme production, despite its rapid breakdown in the cytoplasm en route. As such, it could be regarded as a primitive mRNA transport mechanism. 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. Verification of this hypothesis will depend on studies with other systems and other organisms; the existence of mRNA pools for extracellular  $\alpha$ -amylase and ribonuclease from B. amyloliquefaciens (Gould, May and Elliott, 1973) and for extracellular protease from B. subtilis (Semets and Glenn, in preparation) are consistent with this hypothesis.

The apparent existence of the protease mRNA pool and metabolic conditions under which it can be maintained despite its rapid turnover, that is, in low amino acids medium, have made possible an approach to the isolation of a prokaryotic mRNA species. RNA has been isolated from B. amyloliquefaciens and from this, fractions believed to contain some mRNA species have been prepared. Proof that it is mRNA for extracellular enzymes can only come from its translation into a recognisable product in a cell-free system. This has not yet been achieved; obviously there is great potential for research in this area. If the mRNA could be isolated and translated in a cell-free system from B. amyloliquefaciens (or in an ascites cell-free system) not only could the protease synthesis and secretion process be studied in vitro, but the mRNA molecule itself could be characterised. The accumulation of protease mRNA is a unique

situation and it provides much incentive for further research.  
This is in progress.

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