STUDIES ON EXTRACELLULAR ENZYME SYNTHESIS BY *BACILLUS AMYLOLIQUEFACIENS*

A THESIS

SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN THE

UNIVERSITY OF ADELAIDE

BY

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April, 1978.

TO MY FAMILY,

PAST AND PRESENT

AND FUTURE.

TABLE OF CONTENTS

SUMMARY

1. Harvested cells of Bacillus amyloliquefaciens have the capacity to synthesize extracellular enzymes for prolonged periods in the presence of transcriptional inhibitors. Protease gene transcription is repressed by high levels of amino acids. Incubation in high amino acids medium (repressive) for 75 minutes exhausts the capacity of cells of B.amyloliquefaciens to produce extracellular protease in the presence of rifampicin or actinomycin D. When such exhausted cells are then transferred to a low amino acids medium (non-repressive) this capacity is rapidly regained.

2. The amount of protease produced in the presence of rifampicin increases linearly for 75 minutes from a zero value to an amount equivalent to that produced by control cells without rifampicin during the same 75 minute period.

3. The acquired capacity to produce protease in the presence of rifampicin is still chloramphenicol-sensitive.

4. At all stages the cells are fully sensitive to rifampicin as judged by complete inhibition of ^{3}H-uracil incorporation.

5. In the presence of actinomycin D the same phenomenon is observed.

6. When the capacity for rifampicin-insensitive protease production is followed over a longer time period it increased to a maximum in 75 minutes, then rapidly decreased and thereafter oscillated.

7. The oscillating capacity for rifampicin-insensitive protease production can be explained by bursts of protease gene activity alternating with transcriptionally inactive periods. It is proposed that during gene transcription two forms of protease mRNA are made, one immediately translated, the other forming a reserve pool of non-translatable mRNA which, on cessation of transcription, converts to the translatable form and supports protease production during the transcriptionally inactive period. Thus it appears as though transcription of the protease gene is switched on when the reserve mRNA pool is low, and off when it is high.

8. When total cellular RNA is prepared from cells at various stages of the oscillations and analysed by sucrose density gradient centrifugation, a species of RNA is observed to also apparently oscillate in concert with the capacity for rifampicin-insensitive protease production.

9. This RNA species, when added to a wheat-germ cell-free system, stimulates incorporation of labelled amino acids.

10. There is evidence that protease is synthesized during this cell-free translation.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma. The studies were carried out in the Department of Biochemistry, University of Adelaide, under the supervision of Prof. W.H. Elliott and Dr. B.K. May. The work was done entirely by myself, except that of Fig.(5,3), which was carried out in collaboration with Dr. B.K. May. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made in the text.

signed

REINA O'CONNOR

ABBREVIATIONS

The abbreviations used in this thesis are acceptable to the JOURNAL OF MOLECULAR BIOLOGY or are defined in the text.

FIGURES AND FIGURE LEGENDS

The figures and figure legends for the work described in each chapter will be found together at the end of each chapter.

ACKNOWLEDGMENTS

I wish to warmly thank Professor W.H. Elliott for guidance, support and encouragement. I thank also Dr. B.K. May for guidance and rewarding discussions.

I am also grateful to the other members of our group, Julie Bielicki, Gerry Both, Andrew Glenn, Allan Gould, Don Love, Jim McInnes, Ted McMurchie, Julian Mercer, James Paton, Phil Pearce, Lance Sanders, Vilnis Sêmets and Mal Whiting, and also, to many other members of this Department for their advice, co-operation and interest during the course of this work.

My thanks to Ann Heylen of our group for competent technical assistance and also to Jane Somerville for her excellent typing of this thesis.

I also thank my family and friends, and particularly my husband, Ralph, for encouragement and support.

The financial support of a Commonwealth Post-graduate Research Award is also acknowledged.

CHAPTER 1

GENERAL INTRODUCTION

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1,1 INTRODUCTION

The work to be described in this thesis concerns the synthesis of extracellular enzymes, in particular, the synthesis of extracellular protease by Bacillus amyloliquefaciens. This (gram-positive) organism secretes into the external medium large quantities of extracellular enzymes, namely, protease, α-amylase and ribonuclease. It can actively produce these enzymes under conditions where the cells are not growing and these cells can be easily washed free of accumulated extracellular enzymes and resuspended in test solutions thus making it very amenable to experimental study.

Two excellent reviews of the extracellular enzyme field have been published in the last two years. They are:

1) 'Production of extracellular proteins by Bacteria', by A.R. Glenn (1976); and
2) 'Extracellular enzyme synthesis in the genus Bacillus', by F.G. Priest (1977).

These two reviews adequately cover this large field and thus the material they review will not be covered again here except to emphasize points of interest and relevance to this thesis.

1,2 EXTRACELLULAR ENZYMES

Extracellular enzymes are largely scavenger enzymes, their function being to degrade large molecules in the bacterial environment so that they may be assimilated and used by the organism concerned. Claims have also been made for a function of exoenzymes in sporulation since maximal exoenzyme synthesis occurs just before sporulation (Priest, 1977). However, both these phenomenon may be independently related to a decrease of nutrients in the external medium, and thus, an amylase deficient mutant of B.subtilis can grow and sporulate normally (Schaeffer, 1969). Many of these enzymes are potentially lethal to the cells that produce them, e.g., ribonuclease, protease, and do not occur in detectable amounts in active form inside the cells. Many extracellular enzymes do not have intracellular substrates (e.g., penicillinase) and, in the case of the extracellular ribonuclease of B.amyloliquefaciens, an intracellular inhibitor exists for this enzyme to which it binds irreversibly (Smeaton and Elliott, 1967). The sizes of bacterial extracellular enzymes vary widely, but they are mostly in the range of 20,000 to 40,000 (Glenn, 1976). Much of the interest in this field is thus centered around the molecular biology of enzyme secretion, the main question being of how these relatively large and, in many cases, potentially lethal enzymes can be synthesized and secreted with immunity by the bacterial cells.

This problem is not confined to bacterial cells. Eukaryote cells also secrete extracellular enzymes and

other proteins, e.g., the liver, pancreas and various hormone producing organs. In the liver and pancreas, for example, the extracellular enzymes are secreted across the endoplasmic reticular membrane from where they are transported to the Golgi complex to be processed and packaged in secretory vesicles which fuse with the plasma membrane and release their contents by reverse pinocytosis (Palade et al., 1962; Andrews and Tata, 1971; Castle et al., 1972). The initial problem of how these enzymes are synthesized and secreted across the endoplasmic reticular membrane may be similar to that in bacteria. As major advances have recently been made in the study of secretion in mammalian systems these will now be discussed.

1,3 EXTRACELLULAR ENZYME SECRETION IN EUKARYOTES

A considerable body of evidence suggests that in mammalian cells exportable proteins are synthesized on membrane-bound polysomes, whereas non-exportable proteins are synthesized on free-polysomes (Borgese et al., 1974; Ganoza and Williams, 1969; Redman, 1969; and Takagi and Tanaka, 1970). It was originally proposed by Redman and Sabatini (1966) that enzymes secreted by the pancreas are synthesized on ribosomes bound to the rough endoplasmic reticular membrane and are secreted, in nascent form, as they are translated. This basic idea has been considerably elaborated to form the 'signal hypothesis' which is discussed below.

In recent years evidence has accumulated that secreted

proteins are produced as precursors which are converted to mature protein in the Golgi complex. With the isolation of mRNAs for secretory proteins and translation of these in heterologous cell-free systems it has also been shown that secretory proteins (some in precursor form) also have a characteristic peptide at their N-terminus (e.g., prolactin and insulin. For references and discussion see Campbell and Blobel, 1976). These findings support the signal hypothesis originally proposed by Blobel and Sabatini (1971), and which has been considerably extended and supported by results of their own work (Blobel and Dobberstein, 1975; Dévillers-Thiery et al., 1975; Campbell and Blobel, 1976). The 'signal' hypothesis suggests that secretory proteins have a common N-terminal sequence of amino acids (encoded by the mRNA for these enzymes) which causes the ribosome synthesizing this protein to bind to the membrane of the endoplasmic reticulum. They further proposed that the signal peptide, on associating with the membrane, causes the association of several ribosomal receptor proteins of the endoplasmic reticular membrane thus forming a tunnel through which the polypeptide is extruded into the intracisternal space. The signal peptide is removed, before the chain is completed, by a membrane-bound protease. The model thus provides for the selective synthesis and secretion of exportable proteins. A key feature of this mechanism is that the transport through the membrane depends upon concomittant protein synthesis by membrane-bound ribosomes, since the polypeptide chain moves through the tunnel in an extended form as it is being lengthened.

Dévillers-Thiery et al. (1975) determined the sequence of up to 24 amino terminal residues of several putative precursors for pancreatic secretory proteins which had been synthesized in vitro in cell-free systems. These studies revealed an extensive homology of the 16 amino terminal residues and also that they comprised an unusually large percentage of hydrophobic residues. A similar result was obtained for IgG light chain precursor 'signal' sequence (Schechter et al., 1975). The hydrophobic residues may facilitate interaction with the membrane and this further supports the signal hypothesis.

J. Rothman and Lodish (1977) also provide evidence in support of the 'signal' hypothesis. They investigated the synthesis of G protein (glycoprotein) of vesicular stomatitis virus, which is synthesized on ribosomes bound to the endoplasmic reticulum of infected cells and inserts into the membrane before being further processed and transported to the plasma membrane. They showed that G protein could be synthesized on free ribosomes, but if membranes were added after 40 residues had extended outside the ribosome the proper insertion into the endoplasmic reticular membrane failed to occur. Thus the N-terminal residues appeared to act as a signal directing both the insertion of the protein into the membrane and the binding of the ribosomes to the endoplasmic reticulum. The nascent chain apparently spans the lipid bilayer so that the protein is extruded across the membrane as it is being elongated and this causes the protein to cross the membrane.

However, as recent evidence suggests, there may be other mechanisms by which proteins cross membrane barriers.

Recent studies have suggested that secreted proteins, many of them potentially lethal, e.g., proteases, lipases, may not be lost from the organism after secretion to the intestine, but may be reabsorbed for reuse by the body much as secreted bile salts are recycled by absorption from the gut. This evidence has been provided by Rothman and colleagues (Götze and Rothman, 1971; Isenman and Rothman, 1977), and is also recently summarized by Diamond (1978). They showed that radioactive chymotrypsinogen is absorbed from the gut and reappears in the pancreas unaltered, and that approximately 80-90% of the pancreatic enzymes may be recirculated.

Another result of interest is that of Highfield and Ellis (1978) who showed that the small subunit of chloroplast ribulose biphosphate carboxylase was synthesized *in vitro* as a higher molecular weight precursor. This precursor was then taken up into intact isolated chloroplasts and cleaved to its final size in the absence of protein synthesis. They deduce that the signal hypothesis does not apply but that a carrier protein in the chloroplast envelope is involved. Their preliminary observations suggest that the extra precursor sequence is acidic rather than hydrophobic and they suggest that the conformational change caused by cleavage of this acidic peptide by a membrane protease leads to transport across the membrane.

These findings do not necessarily detract from the 'signal' hypothesis, but they do illustrate that selective transport across biological membranes is a complex process and that, in the light of recent evidence, many more factors have to be taken into consideration before a suitable theory (or theories) for this is found.

1,4 EXTRACELLULAR ENZYME SECRETION IN BACTERIA

In the bacterial extracellular enzyme field as well, recent developments have resulted from cell-free synthesis of extracellular enzymes. Several of these appear to be synthesized as precursor molecules.

Yamamoto and Lampen (1976) found that the membrane-bound penicillinase of *Bacillus licheniformis*, which appears to be a precursor of the extracellular enzyme, was a phospholipoprotein that carries an N-terminal chain of 24 amino acids and a phosphatidylserine. This form is produced during cell-free synthesis in a homologous cell-free system (Dancer and Lampen, 1975). It is perhaps significant that the phospholipopeptide, and indeed the hydrophobic 'signals' on mammalian proteins, is long enough to extend across the lipid bilayer of the membrane. The authors point out that the proposed mRNA fragment coding for the precursor segment would have a high purine content (80%) and thus would have a structure very similar to that of poly-A, which occurs at the 3'-end of mRNAs from eukaryotes and has been reported to have an affinity for the membrane (Milcarek and Penman, 1974; Lande *et al.*, 1975), and

thus it is possible that the mRNA for membrane penicillinase (5'-end) and the nascent peptide contains structures that would favour the translation of these mRNAs at the membrane rather than on free ribosomes in the cytoplasm.

Alkaline phosphatase of E. coli, a secreted periplasmic protein, appears to be synthesized as a larger molecular weight precursor and there is also evidence that it is synthesized on membrane-bound ribosomes and extruded through the membrane as it is synthesized. Cancedda and Schlessinger (1974) demonstrated that in E. coli nascent alkaline phosphatase is mainly found on membrane-bound ribosomes. Inouye and Beckwith (1977) synthesized E. coli alkaline phosphatase in a DNA-directed (Zubay, 1973) cell-free system. A higher molecular weight product was found and this could be processed to approximately the mature size by an E. coli outer membrane fraction in vitro. The precursor was highly hydrophobic and this property appeared to result from the extra segment. They suggested that the 'signal' hypothesis of Blobel may apply in prokaryotes as well. Smith et al. (1977) provided evidence that secreted proteins of E. coli may traverse the membrane as growing chains, i.e., they may be extruded directly as they are synthesized. Spheroplasts of E. coli were labelled with a reagent that reacts with amino groups but does not cross the membrane. Polysomes isolated from the membrane-polysome fraction contained radioactivity, and furthermore, when allowed to complete the translation in an in vitro system, Smith et al. (1977) found a portion of the protein

made was alkaline phosphatase.

Whether or not proteins were synthesized on membrane-bound ribosomes in bacteria, and indeed whether they existed at all was previously much disputed (Glenn, 1976). Recently Randall and Hardy (1977) used a membrane-bound fraction of polysomes from E. coli [isolated without the use of lysozyme which has been reported to cause ribosome-membrane binding (Patterson et al., 1970)] and looked at their translation products in vitro. They found two types of protein were made, one which associated with the membrane, and a secreted periplasmic protein. Soluble ribosomes, however, produced a different class of proteins. They concluded that membrane-bound polysomes preferentially synthesize proteins which are exported from the cytoplasm.

May and Elliott (1968) and Both et al. (1972) proposed that extracellular enzymes in Bacillus amyloliquefaciens are synthesized on membrane-bound polysomes and are directly extruded in nascent form as they are being synthesized. Sanders and May (1975) showed that extracellular enzymes being secreted by protoplasts of B.amyloliquefaciens were susceptible to trypsin suggesting that the emerging protein is in a form different from the final form. McMurchie (1977), however, found no evidence for membrane-bound ribosomes in B.amyloliquefaciens when examining membrane preparations by electron microscopy.

In summary, it seems that there is still a long way to go before we can understand precisely how proteins are

secreted and how many different mechanisms exist. The work to be described in this thesis continues this investigation, the aim being to isolate the mRNA for extracellular protease of B.amyloliquefaciens to make possible future in vitro studies on extracellular enzyme synthesis.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2,1 MATERIALS

Bacterial Strain

The organism was previously described as an unclassified strain of Bacillus subtilis; however, it has since been classified as a distinct species, Bacillus amyloliquefaciens on the basis of DNA base composition and DNA hybridization studies by Welker and Campbell (1967).

Liquid Growth Medium

The culture medium contained 34 mM-$(NH_4)_2HPO_4$, 1 mM-$MgSO_4$, 5 mM-KCl, 4.25 mM-sodium citrate, 0.125 mM-$CaCl_2$, 0.0125 mM-$ZnSO_4$, 0.5 M-$FeCl_3$, 0.5% (w/v)-Bacto casamino acids, 0.05% (w/v) Bacto yeast extract (Difco), trace metal solution (0.25 ml/litre, and 1% (w/v)-maltose. The medium was adjusted to pH 7.3 with H_3PO_4 and sterilized by autoclaving. Maltose was autoclaved separately and was added just before use. The trace metal solution contained 0.5 mg $CoCl_2.6H_2O$, 0.5 mg ammonium molybdate, 5.0 mg $MnCl_2.4H_2O$, and 0.01 mg $CuSO_4.5H_2O$ dissolved in 1 litre of water.

Cell Suspending Medium - the high or low casamino acids medium.

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

either 'high casamino acids' (0.5% casamino acids) or 'low casamino acids' (0.025%).

Radioisotopes

^{3}H-uracil (spec. act. 17 Ci/mmole), ^{14}C-uracil (spec. act. 55 mCi/mmole) and L-[^{14}C]-phenylalamine (spec. act. 460 mCi/mmole) were obtained from Schwartz-Mann, Orangeburg, N.Y. L-leucine (4,5 - ^{3}H) (spec. act. 50 Ci/mmole) was obtained from The Radiochemical Centre, Amersham, England.

Antimicrobial Agents

Actinomycin D was a generous gift from Merck, Sharp and Dohme Research Lab., Rahway, N.J. Rifampicin was from Sigma Chemical Co. Chloramphenicol (chloromycetin) was from Parke-Davis & Co., Sydney.

Enzyme Substrates

Light, white soluble casein (British Drug Houses Ltd., Poole, England) was used as substrate for protease assays. 'Phadebas' amylase test tablets were purchased from Pharmacia (South Seas) Pty. Ltd., Lane Cove, New South Wales.

Scintillation Fluid

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

Melbourne.

NCS-tissue solubilizer from the Amersham/Searle Corp.

Reagents for RNA Extraction and Analysis

Phenol (Anax Pty. Ltd., Adelaide) and m-cresol (British Drug Houses, Poole, England) were redistilled before use.

Formamide (British Drug Houses, Poole, England) was deionized by stirring for at least two hours with Zeo-karb 225 resin (3% suspension) to lower the conductivity of the formamide solution. The resin (Permutit Co. of Australia, Brookvale, New South Wales) was removed by filtration. The resin had been prepared by washing with IN-NaOH, water, IN-HCl and then finally water again, using a large number of washes at each step. The resin was stored moist but dried thoroughly (freeze-drier) before use.

Ribonuclease-free sucrose was purchased from Schwarz/Mann, Orangeburg, N.Y.

Acrylamide (E. Merck, Darmstadt) and bis-acrylamide (N',N'-methylenebisacrylamide) (Sigma Chemical Co.) were recrystallized by the method of Loening (1967).

Other Reagents

SDS (sodium dodecyl sulphate) from Sigma Chemical Co.

TEMED(N,N,N',N'-tetramethylethylenediamine) from Eastman Kodak Co., Rochester, N.Y.

Ammonium persulphate from British Drug Houses (Australia) Pty. Ltd., Victoria.

Tris buffers: crystalline tris was obtained from Sigma Chemical Co. as 'Trizma' base, reagent grade. Solutions were adjusted to the required pH value by the addition of HCl.

TINSA (tri-iso-propylnaphthalenesulphonic acid) was from Eastman Organic Chemicals.

Ten Buffer

20 mM-Tris pH 8.5, 100 mM-NaCl and 1 mM-EDTA.

Reagents for Cell-free Synthesis

Adenosine triphosphate (ATP; disodium salt), guanosine triphosphate (GTP; disodium salt), creatine phosphokinase (rabbit muscle), creatine phosphate (disodium salt), DTE (dithioerythritol), HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethansulfonic acid) and 2-mercaptoethanol were from Sigma Chemical Co. L-^{12}C-amino acids were from Mann Research Labs., Inc., N.Y.

Spermine (tetrahydrochlorine) was obtained from Calbiochem.

Double-distilled Water

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

Treatment of Equipment and Solutions to Minimize Ribonuclease Contamination

All glassware and equipment, where necessary, was washed in IN-KOH, rinsed well with distilled water and, if possible, sterilized by dry heat. Disposable plastic, or washable rubber gloves were worn when handling ribonuclease-free equipment. Solutions (and glass-distilled water) were autoclaved wherever possible.

2,2 METHODS

Preparation of Spore Suspensions

B.amyloliquefaciens cultures, after growth overnight in liquid growth medium, were centrifuged down and resuspended to the same cell density in sterile growth medium which had been previously diluted 25-fold with sterile water. The cultures were shaken in this diluted medium for 24 hours; spores and intact cells were centrifuged down and resuspended in sterile water. To destroy the remaining vegetative cells, the suspension was heated at 80° for 30 minutes and after cooling to room temperature, the spores were centrifuged down at 10,000 g for 30 minutes under sterile conditions. The spores were washed with sterile water by resuspension and centrifugation and finally resuspended in a suitable volume of sterile water. The suspension was distributed into small sterile bottles and stored at 4° until required.

Growth of the Organism

Culture medium was inoculated with a platinum loop from a suspension of spores of Bacillus amyloliquefaciens and incubated at 30°C in a gyratory shaker to a cell density giving an optical density at 600 nm (OD_{600}) of 3.6 (late log phase, approximately 25 hours after inoculation).

Washed-cell Experiments

Late log phase cells (OD_{600} = 3.6) were harvested,

washed twice by resuspension and centrifugation at 30°C with the appropriate suspending medium. A sample of cell suspensions (20-40 ml) was shaken in a 250 ml conical flask at 30°C in a gyratory shaker. Samples (1.0-2.0 ml) were withdrawn at appropriate times, centrifuged and the supernatants assayed for extracellular enzyme activity.

Enzyme Assays

(a) Protease

Protease was estimated using a casein substrate. A stock casein solution was prepared by heating 1 g of casein with 100 ml of Sørensen's buffer, pH 7.6 (12.2 g Na_2HPO_4, 1.82 g KH_2PO_4 per 1.0 litre of water). Assay tubes contained 1.0 ml of stock casein and 1.0 ml of suitably diluted enzyme. Tubes were incubated for the required time at 35° and the reaction was stopped by the addition of 3 ml of 5% (w/v) TCA. Tubes were cooled on ice for 30 minutes, centrifuged for 15 minutes, and the absorbance of the supernatant at 280 nm was determined. Control tubes were prepared by adding the TCA solution to the stock casein prior to the addition of enzyme and were otherwise treated in the same manner as the assay tubes. The relationship between enzyme concentration and absorbance at 280 nm was linear to 0.7. A unit of enzyme is defined as that amount of enzyme which will produce a change in absorbance at 280 nm of 0.05 in 40 minutes at 35°.

(b) Amylase

α-Amylase was assayed by using Phadebas (Pharmacia) Amylase test tablets. The stock buffer solution contained 2.5 litres of 0.1 M potassium phosphate, 0.025 M NaCl, pH 6.2; 2.0 litres of water; 10 ml of 0.1 M $CaCl_2$; 11 ml of 5 mM $ZnSO_4$.

An even suspension was prepared by mixing one "Phadebas" tablet with 5 ml of stock buffer, and 1 ml aliquots of this suspension were pipetted into assay tubes. Suitably diluted enzyme (0.5 ml) was added and the tubes incubated at 37°C for 30 minutes. Development of soluble blue product was terminated by the addition of 0.2 ml of 0.5 N NaOH. The tubes were centrifuged for 10 minutes at full speed on a M.S.E. bench centrifuge and the absorbance at 620 nm of the supernatant determined. Control tubes were incubated with stock buffer instead of enzyme.

The relationship between enzyme concentration and absorbance at 620 nm was linear to 6.0. A unit of α-amylase activity was defined as that amount of enzyme which produces an increase in absorbance at 620 nm of 4.0 in 30 minutes at 37°. This unit corresponds to the unit of α-amylase activity previously defined using a starch-iodine method (May and Elliott, 1968b).

Measurement of Total RNA Synthesis

^{3}H-Uracil incorporation into total cellular RNA was measured by shaking 3.0-6.0 ml of a washed-cell suspension with 0.8 µCi of ^{3}H-uracil/ml in suspending medium supplemented with 10 µg of unlabelled uracil/ml. Samples (0.1 ml) were withdrawn at various times and added to 3 ml cold 5% (w/v) trichloroacetic acid containing 0.1% (w/v) unlabelled uracil. After standing on ice for 30 minutes insoluble precipitate was collected by filtration on to GF/C filters and washed repeatedly with a total volume of 15 ml of cold trichloroacetic acid-uracil mixture and finally 9 ml of 1% (v/v) acetic acid. Filters were dried and counted by liquid scintillation (Packard Tri-Carb Spectrophotometer).

Measurement of Total Protein Synthesis

To measure total protein synthesis by cells, 2.0 ml of a washed cell suspension was shaken with 0.5 µCi of L-^{14}C-phenylalanine (specific activity 460 mCi/mmole).

At appropriate times, 0.1 ml samples were pipetted into 3 ml of ice-cold 1% (w/v) Bacto casamino acids in 10% (w/v) trichloroacetic acid and left at 0° for 30 minutes. The tubes were heated at 95°C for 30 minutes, then cooled on ice for 30 minutes.

The precipitates were collected on to GF/A filters and washed 5 times with 5 ml of 1% (w/v) casamino acids in 10% (w/v) trichloroacetic acid, and then 3 times with 5 ml

of 1% (w/v) acetic acid. The filters were dried and counted by liquid scintillation.

Preparation of RNA from Bacillus amyloliquefaciens

This is discussed in Chapter 4, but a summary of the final procedure used is as follows.

Cell pellets were resuspended in a small quantity of Tris buffer (25 mM-Tris, pH 8.5, 5 mM-magnesium acetate) and poured into a pre-chilled (-25°C) X-press frozen cell disintegrator such that they froze instantly. [If it were necessary to prepare many samples of cells, these were firstly snap-frozen in a tube of the same diameter as the X-press cell-chamber, stored in liquid N_2 until needed, and then transferred in frozen state to the X-press.] The frozen lysate (3 ml) was thawed in an SDS solution [0.5% (w/v)-SDS, 1% (w/v)-TINSA, 100 mM-NaCl and 10 mM-EDTA]. To this aqueous mixture was added an equal volume of phenol: chloroform, 1:1, where the phenol comprised 78% (w/v)-redistilled phenol and 8% (v/v)-redistilled m-cresol in 50 mM-Tris, pH 9.0. This mixture was shaken vigorously for 15 minutes then the aqueous and phenol layers separated by centrifugating at 7,000 x g for 7 minutes at room temperature. The aqueous layer was carefully removed and re-extracted a further two times with phenol, as above. In most instances, the phenol and interface region resulting from the first extraction was re-extracted with an equal volume of SDS solution and the resulting aqueous phase re-extracted with phenol a further two times along

with the aqueous phase mentioned above. RNA was precipitated from the final aqueous phase by the addition of 2.5 volumes of ethanol-10 mM-sodium acetate, pH 6.0 and leaving the solution overnight at -20°C. The precipitate was removed by centrifugation at 30,000 x g for 30 minutes at 2°C, dried and resuspended in the appropriate buffer or 0.1 mM EDTA.

RNA concentrations were determined spectrophotometrically, assuming that one A_{260} unit equals 40 µg RNA/ml.

Gradient Analysis

Linear density gradients of either 5-20% (w/v) sucrose or 10-40% (w/v) sucrose in TEN buffer were prepared using a gradient former. The sucrose solutions were prepared using stocks of 50% (w/v) ribonuclease-free sucrose, 5 times concentrated TEN buffer and sterile glass-distilled water. The gradients were centrifuged on either an MSE Super Speed 65 (6 x 14 ml swing-out rotor) or a Beckman L265B (SW41 rotor) for the times and speeds indicated in the figure legends. A_{254} profiles were obtained using an Isco density gradient flow cell, and fractions were collected either manually or by using a Gilson microfractionator (Gilson Medical Electronics, Inc.). RNA samples were in TEN buffer. To check the intactness of the RNA by the gradient method, the samples were dissolved in TEN buffer containing 0.2% (w/v) SDS and heated at 60°C for 2 minutes before loading on to gradients.

Gel Electrophoresis of RNA

(a) Aqueous polyacrylamide tube gels

Aqueous (non-denaturing) gel electrophoresis in 40 mM-Tris-acetate buffer pH 7.2 containing 20 mM-sodium acetate and 2 mM-EDTA was by the method of Loening (1967). The 3% acrylamide-0.15% methylene-bisacrylamide gels were cast 9 cm deep and 0.7 cm diameter in perspex tubes. Tertiary butanol was layered over the gels before polymerization to obtain a flat loading surface, and washed off with eletrode buffer after polymerization. RNA samples in TEN buffer were made 10% (w/v) with sucrose. Gels were pre-electrophoresed for 1 hour at 5 mA/gel, samples (10-100 µg RNA in 5-50 µl) were loaded and gels subjected to 2 to 5 hour electrophoresis at 5 mA/gel at 4°C. RNA bands were located by staining for 30 seconds in 0.2% Toluidine blue, followed by destaining in distilled water. Profiles were obtained by scanning the gels at 600 nm in a Gilford linear gel transport.

(b) Formamide-polyacrylamide gel electrophoresis

Electrophoresis of RNA in 98% formamide-0.02 M-NaCl was by the system of Staynov *et al*. (1972). Gels of 3.8% acrylamide-0.6% methylenebisacrylamide were cast 9 cm deep and 0.7 cm in diameter in perspex tubes. Gels were carefully overlayed with t-butanol

to form a level surface. Gels were pre-electrophoresed for 60 minutes at 1.0 mA/gel with electrode solutions of formamide-aqueous 1 M-NaCl (1:0.02, by volume). RNA samples dried *in vacuo* were dissolved in loading solution (prepared by dissolving 0.5 g sucrose in 4 ml electrode buffer), heated at 60°C for 2 minutes, cooled quickly, loaded on the gels and electrophoresed at 1.0 mA/gel for 12-15 hours at room temperature. RNA bands were visualized as above.

SDS-Polyacrylamide Gel Electrophoresis

The 10% acrylamide gels were prepared by the method of Weber and Osborn (1969), the gels being cast into silicone-treated glass tubes, 10 cm deep and 0.5 cm in diameter. The samples (antibody precipitates, 100 µg protein) were prepared by adding 60 µl of loading buffer containing 2% (w/v)-SDS, 25% (v/v)-glycerol, 2% (w/v) Dithiothreitol, and 0.005% (w/v)-bromophenol blue and boiling this for 3 minutes immediately. Electrophoresis was carried out at 8 mA/gel for the times indicated in the figure legends. Staining for protein was by the method of Fairbanks *et al.* (1971).

The Wheat-Germ Amino Acid Incorporating System

Wheat germ extracts were prepared by a method based on that of Roberts and Paterson (1973). Wheat germ was obtained commercially, freshly milled non-toasted, from

Adelaide Milling, South Australia. 6 g Wheat germ was ground in a chilled mortar with 6 g of sterilized sand and 28 ml of 20 mM-HEPES buffer, pH 7.6, containing 100 mM-potassium chloride, 1 mM-magnesium acetate, 2 mM-calcium chloride and 1 mM-dithioerythritol. The wheat germ was ground for 1 minute. The homogenate was centrifuged at 30,000 x g for 10 minutes at 2°C. The supernatant was carefully removed, avoiding both the surface layer of fat and the pellet. The S-30 fraction was made up to 3.5 mM-magnesium acetate and preincubated with 1 mM ATP (neutralized), 20 µM-GTP, 2 mM-dithierythretol, 8 mM-creatine phosphate and 40 µg of creatine phosphokinase per ml of homogenate for 15 minutes at 30°C. 10 ml of the preincubated S-30 fraction was passed through a column (2.5 x 28 cm) of Sephadex G-25 (course), equilibrated with 20 mM HEPES (pH 7.6), 120 mM-potassium chloride, 5 mM-magnesium acetate, and 6 mM-2-mercaptoethanol at a flow rate of 3 ml/minute. The peak of the turbid fraction was pooled. 0.25 ml Lots were dispensed into vials containing liquid nitrogen and then stored at -80°C.

Construction and Incubation of In Vitro Reaction Mixtures

The assays contained in a final volume of 50 µl, 20 µl-preincubated wheat germ S-30 fraction, 20 mM-HEPES (pH 7.6), 2 mM-dithierythrytol, 1 mM-ATP, 20 µM-GTP, 8 mM-creatine phosphate, 40 µg/ml of creatine phosphokinase, 25 µM unlabelled amino acids, 60 mM-KCl, 2.6 mM-magnesium acetate, 40 µM-spermine, 3.6 µM-unlabelled leucine, 2 µCi ^{3}H-leucine. These conditions gave maximum incorporation

of ^{3}H-leucine into TCA-precipitable material in the presence of CMV-RNA (1 µg). In each experiment, CMV-RNA directed incorporation was measured to check the activity of the particular wheat germ S-30 sample. Reactions were incubated at 25°C for the times indicated in the figure legends.

Measurement of In Vitro Amino Acid Incorporation

After the required incubation time, reaction tubes were placed on ice. 10-20 µl Aliquots were pipetted into 150 µl of cold 10% (w/v)-TCA containing 0.5% (w/v) unlabelled leucine, allowed to stand for 15 minutes, centrifuged and the supernatant discarded. 500 µl Of 5% (w/v)-TCA containing 0.25% unlabelled leucine was added, the samples heated at 90°C for 15 minutes, and the pellet washed a further two times with the 5%-TCA solution. Finally, 200 µl of NCS solubilizer was added and the sample counted by liquid scintillation.

Preparation of TCA-Precipitates for Electrophoresis

The samples were prepared as above, except that after washing in 5% (w/v)-TCA containing 0.1% (w/v)-unlabelled leucine, the pellets were further washed two times in 2 ml of acetone:ether (3:1), the pellet dried thoroughly in vacuo and then resuspended in SDS-dissociation buffer as described below for immunoprecipitates.

Immunoprecipitation

The antisera were prepared by a standard method (as described by Sanders, 1974) and characterized by Ouchterlony immunodiffusion and titration experiments.

(a) Single antibody procedure

420 µg Rabbit antiprotease gamma globulins were added to the samples on ice and incubated for 20 minutes. 80 Units of protease were then added and the incubation continued at 4°C overnight.

(b) Washing of immunoprecipitates

The precipitate was removed by centrifugation at 3,000 x g for 10 minutes at 4°C. The pellet was washed 3-4 times with ice-cold 0.9% (w/v) NaCl in KPO_4 buffer, pH 7.0. 60 µl Of SDS buffer [2% (w/v)-SDS, 2% (w/v)-dithiothreitol, 25% (v/v)-glycerol and 0.005% (w/v)-bromophenol blue in 10 mM-$NaPO_4$ pH 7.0] and immediately boiled for 3-4 minutes. The sample was then electrophoresed on SDS-acrylamide gels as described above. A small sample of dissolved immunoprecipitate was dissolved in NCS-tissue solubilizer to estimate the radioactivity it contained.

CHAPTER 3

RESULTS

RESULTS

OSCILLATION OF APPARENT POOL OF mRNA FOR EXTRACELLULAR PROTEASE

3,1. INTRODUCTION

Production of extracellular enzymes by Bacillus amyloliquefaciens has been studied using washed-cell suspensions of this organism. Typically, cells were harvested in late log phase of growth (approximately 25 hours after inoculation) at an OD_{600} value of 3.6, washed twice in medium lacking the growth factors Fe^{++} and yeast extract and finally resuspended in the same medium. The basis of the work to be described in this thesis is that of Both et al. (1972). This will now be described by way of introduction.

When cells are resuspended in medium containing low concentrations of casamino acids (0.025%), protease production is virtually linear. However, in high concentrations of casamino acids (0.5%) protease production is repressed but the resultant curve is unusual in that enzyme production occurs in two distinct phases, 1 and 2 (Fig. 3, 1). The first phase lasts approximately 60 minutes and the second starts at about 70 minutes and proceeds linearly thereafter. Response to rifampicin is unusual in that phase 1 is not inhibited while phase 2 is inhibited within a few minutes: i.e., if rifampicin is added at zero time protease production continues for approximately 60 minutes,

however, if the drug is added at 75 minutes then production of enzyme stops almost immediately. Actinomycin D, another inhibitor of transcription, but with a completely different mode of action from rifampicin, also gives the same results. This precludes the possibility of the results being due to a rifampicin-insensitive RNA polymerase. At the levels of the antibiotics used, RNA synthesis was immediately inhibited more than 95% and general cellular protein synthesis ceased within a few minutes. Two factors ruled out the possibility of the continued production of protease (in the presence of rifampicin) being due to release of preformed enzyme. Firstly, chloramphenicol prevented the rifampicin-insensitive protease production, indicating the requirement for protein synthesis. Secondly, all the protease formed during this time could be totally labelled with ^{14}C-leucine indicating that this production was due to *de novo* synthesis of protease molecules.

To account for protease synthesis showing different responses to inhibition of transcription in phase 1 and 2 alternative explanations have been suggested (Glenn *et al.*, 1973). The stability of the mRNA may be modulated in some way such that it is long-lived in phase 1 conditions and short-lived in phase 2. Alternatively, protease production may be limited by availability of specific translational sites, the mRNA being in excess; in this event phase 1 synthesis of protease would be supported for a prolonged period by the excess mRNA pool. Given a sufficient excess over translational requirements, protease synthesis could

be supported over many half-life periods of the mRNA. No direct evidence exists for either hypothesis, nor are they mutually exclusive.

This chapter is concerned with further investigations into the existence, nature and control of this proposed mRNA pool. The aim was to provide a means of identifying the protease mRNA for subsequent isolation.

3,2. INITIAL APPROACH

The ultimate aim of this work was to identify and isolate the mRNA coding for extracellular protease. It was thought possible to isolate this RNA by direct extraction procedures because of the possible existence of a pool of mRNA for protease. Thus, on analysis by polyacrylamide gel electrophoresis, the mRNA may appear as a distinct peak in the optical density profile of total RNA extracted from Bacillus amyloliquefaciens.

One means of identifying this mRNA peak would be to compare the optical density profile of total RNA extracted from cells making protease mRNA with that extracted from cells lacking protease mRNA. This procedure would be more sensitive if the RNA in both situations were radioactively labelled. A suitable situation for achieving this appeared to exist in Bacillus amyloliquefaciens. High concentrations of amino acids repress protease production (May and Elliott, 1968a). Little new protease mRNA appears to be synthesized when cells are resuspended in high amino acids medium,

whereas protease mRNA is apparently still synthesized when cells are suspended in low amino acids. [The evidence for this is that after 75 minutes incubation in high amino acids-medium cells are incapable of further protease production in the presence of rifampicin but are capable of prolonged synthesis of the enzyme when the same experiment is conducted in low amino acids (Fig. 3,1., taken from Both et al., 1972). This implies that mRNA continues to be made, thus preserving the apparent mRNA pool in low amino acids, but that none (or very little) is made in high amino acids thus causing decay of the pool.] The approach was therefore to label the RNA made in these two situations, isolate and investigate the RNA using polyacrylamide gel electrophoresis and a double-labelling technique. That is, RNA in the low amino acid conditions would be labelled with ^{14}C-uracil and that in phase 1 in the high amino acids conditions with ^{3}H-uracil. Equal amounts of the two RNAs would then be co-electrophoresed and, using predetermined conditions of counting, the gel analysed for both ^{14}C and ^{3}H derived radioactivity simultaneously. A peak with a higher than normal ^{14}C/^{3}H ratio would indicate the position of an RNA species unique to the low amino acid conditions, such as would be expected of protease mRNA.

3,3. EFFECT OF AMINO ACID CONCENTRATION ON URACIL INCORPORATION BY WASHED CELLS OF B.AMYLOLIQUEFACIENS

The first experiment was to see whether the amino

acid level in the media affected ^{14}C- or ^{3}H-uracil incorporation into RNA. Ten minutes after resuspension of harvested cells into either high or low amino acids medium, radioactively labelled uracil was added and the cells incubated for a further 50 minutes. The 10 minute delay before adding the labelled uracil was to avoid labelling any protease mRNA made before the amino acids had exerted repression. Uracil incorporation was measured by pipetting 0.1 ml aliquots into TCA-uracil as described in Methods, Chapter 2. Uracil incorporation by cells was greater in high amino acids medium than in low (Fig. 3,2). This was true whether ^{3}H- or ^{14}C-uracil was used as the radioactive label. The labelled RNA was extracted from the above mentioned cells, by a method to be discussed in Chapter 4, and analysed by polyacrylamide gel electrophoresis. It appeared from this analysis that the increased incorporation of radioactively labelled uracil in the high amino acids situation could be accounted for by increased production of ribosomal RNA (Fig. 3,3). This effect is most likely due to the fact that the high amino acids medium is an enriched medium for cells harvested after 25 hours in culture medium. Such a "step-up" condition, as the situation with enriched medium is called, has been shown to stimulate ribosomal RNA synthesis in bacteria (Neidhardt, 1964).

May and Elliott (1968a) showed that only certain amino acids were responsible for the repression of protease, namely, isoleucine, proline and glutamine, whereas arginine did not repress at all. It was of interest to know

whether protease repression and the stimulation of ribosomal RNA synthesis were the same or separate mechanisms. Cells were resuspended in the low amino acids (0.025%) medium supplemented with a high concentration (0.5%) of the amino acid being investigated. Arginine which did not repress protease did not stimulate ribosomal RNA production either (Fig. 3,4 and 5). Glutamine, or a mixture of isoleucine and proline did repress protease, but did not stimulate uracil incorporation (Fig. 3,4 and 5). Therefore, step-up conditions were not necessary for repression of protease synthesis, but a high rate of uracil incorporation never occurred without also repressing protease production.

The results obtained necessitated a change of plan for identifying the protease mRNA. The relatively small amount of radioactively labelled uracil incorporated by cells synthesizing protease mRNA in low amino acids and the small amount of RNA which can be loaded on to gels during a double-labelling experiment made analysis difficult.

3,4. SECOND APPROACH: THE USE OF STEP-UP CONDITIONS TO BUILD UP A POOL OF PROTEASE mRNA

It was thought that the step-up phenomenon could be used to advantage in building up the apparent pool of protease mRNA within the cells. Incubation in high amino acids medium stimulates ribosomal RNA production and hence

cells would be expected to have a large population of ribosomes at the end of phase 1. At this stage cells would also be depleted of the protease mRNA pool which was present on harvesting the cells. If the cells were then transferred to the non-repressing low amino acids medium, production of protease would resume again. It was also considered possible that protease would be made at a faster rate than in harvested cells transferred directly to low amino acids medium because of the increase in ribosome population which had occurred. It was also speculated that during this subsequent incubation in low amino acids a pool of protease mRNA would build up and thus provide an ideal means for radioactively labelling the mRNA pool.

To test this hypothesis, cells were incubated in high amino acids medium for 75 minutes; at this stage they are incapable of rifampicin-insensitive protease production. They were then washed free of this medium by centrifugation and finally resuspended in the low amino acids medium. Protease production was measured by the casein assay (Methods, Chapter 2). The rate of protease production in low amino acids was only slightly greater in cells which had first been preincubated in high amino acids medium (Fig. 3,6). Uracil incorporation (Fig. 3,7) is identical to that in cells not first preincubated in high amino acids.

The size of the presumptive mRNA pool, as measured by the amount of protease produced in the presence of rifampicin, was investigated. Firstly, the concentration of rifampicin which almost completely inhibits total cell-

ular RNA and total protein synthesis, as measured by ^{3}H-uracil and ^{14}C-phe incorporation respectively, was determined (Fig. 3,8). It was found that a concentration of 0.075 µg/ml of rifampicin inhibits cellular RNA synthesis and total protein synthesis very rapidly. This amount is different from that used by Both et al. (1972) because the new source of the antibiotic was found to be more effective.

The apparent protease mRNA pool size* was measured by the addition of 0.075 µg/ml of rifampicin to cells as discussed above. When the drug was added at zero time, protease synthesis stopped immediately. If, however, the addition of rifampicin was delayed until 75 minutes after transfer to low amino acids, then protease production continued unchecked during the time it was measured (Fig. 3, 9). This situation appeared to be the reverse of that seen in high amino acids medium (Fig. 3,1). The experiment was repeated with rifampicin being added at various times between 0 and 75 minutes. The mRNA pool size appeared to increase steadily during this time (Fig. 3,10), in fact, the amount of protease produced in the presence of rifampicin increases linearly with time of incubation before addition of the drug (Fig. 3,10 insert). The pool

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*Footnote: The terms "apparent mRNA pool" or "mRNA pool size" will be used for brevity since repetitive use of terms such as "rifampicin/actinomycin insensitive capacity for protease synthesis" becomes cumbersome. Nevertheless, it is appreciated that measurement of protease produced may not necessarily be a measure of mRNA amount and the abbreviated term should be taken strictly to mean the longer term above. A discussion of interpretation of the work comes later.

size was measured by calculating the amount of protease produced from the time the drug is added to the time protease production appears to plateau. It was not entirely clear from this experiment whether the pool actually built up gradually from zero time or whether there was a lag of 20 minutes before the pool could be measured. To check this, the mRNA pool size was measured at five minute intervals from zero time to 20 minutes of incubation. It was found that the pool did indeed build up gradually during this time (Fig. 3,11). The experiment was then repeated adding rifampicin to cells at suitable intervals from zero time to 75 minutes incubation. The results from this experiment (Fig. 3,12) confirmed the previous observations that the mRNA pool, as measured by the amount of rifampicin-insensitive protease produced, increased during the incubation in low amino acids medium and that this increase was linear with time.

3,5. INITIAL RATES OF RIFAMPICIN-INSENSITIVE PROTEASE PRODUCTION

In the above experiments when the apparent mRNA pool was measured, a sample of cells was taken, rifampicin added, and a progress curve of protease production with time was made so as to ensure that the final amount reached was a true plateau level. However, as well as yielding information on the total amount of protease produced in the presence of rifampicin, it also showed the rate at which the enzyme was produced. This can be seen from Fig. 3,10, but it is more clearly illustrated in Fig. 3,13

in which three progress curves from Fig. 3,10 have been redrawn to illustrate the observation that the final amount of protease produced is proportional to the initial rate of protease synthesis in the presence of rifampicin. This point is important in the interpretation of results in this chapter as discussed later.

3,6. ACCUMULATION OF APPARENT mRNA POOL IN HIGH AMINO ACIDS MEDIUM

It was of interest to know whether this dramatic increase in pool size occurred when cells were transferred to fresh high amino acids medium after the preliminary 75 minute incubation in high amino acids medium. This was not expected on the basis of the original reasoning. It was found that the mRNA pool size, i.e., the capacity for rifampicin-insensitive protease production, did increase but at approximately 20% the rate observed if the cells were transferred to the low amino acids medium (Fig. 3,14). In fact, the mRNA pool size increased at a slow rate if the cells were allowed to remain in the original high amino acids medium (Fig. 3,15). Fig. 3,16 shows a comparison of the rates of apparent mRNA pool increase in these three conditions.

3,7. EFFECT OF CHLORAMPHENICOL ON PROTEASE SYNTHESIS DEPENDENT ON THE 'ACQUIRED' POOL

Work now reverted back to the situation in which cells, depleted of pool in high amino acids were then

resuspended in low amino acids. Chloramphenicol (20 µg/ml) immediately inhibited the rifampicin-insensitive protease production observed after 75 minutes incubation in the low amino acids medium (Fig. 3,17). This is consistent with the earlier direct evidence (Both *et al.*, 1972) that protease secreted from cells in the presence of rifampicin is synthesized *de novo*.

3,8. EFFECT OF ACTINOMYCIN-D ON PROTEASE PRODUCTION

It was important to determine whether, by using actinomycin-D, another inhibitor of transcription, an increase in drug-resistant protease synthesis would also be observed. This antibiotic prevents transcription by binding to the guanine residues of the DNA molecule, whereas rifampicin binds to the RNA polymerase molecule inhibiting its action. The amount of *Act D, which just maximally inhibits total cellular RNA and protein synthesis, was determined in the same manner as for rifampicin. 2.25 µg/ml of actinomycin D was found to be suitable (i.e., it caused 96% inhibition of ^{3}H-uracil incorporation into cells). When added at zero time to cells transferred to low amino acids medium, protease production stopped immediately, while if added after 75 minutes incubation, protease production continued for at least 60 minutes (Fig. 3,17). Thus actinomycin D had the same effect as rifampicin. It was considered extremely unlikely that a situation could exist in which both the protease gene was resistant to *Act D and that a special rifampicin-resistant RNA polymerase existed for the protease gene.

*Act D = actinomycin D.

3,9. EFFECT OF RIFAMPICIN ON URACIL INCORPORATION BY TRANSFERRED CELLS

Another possible explanation for this phenomenon was that the cells were variably resistant, possibly due to varying permeability to rifampicin. To test this, rifampicin was added to the cells at various times and ^{3}H-uracil incorporation into RNA measured. Cells were found to be fully sensitive to rifampicin throughout the 75 minute incubation (Fig. 3,18). The unusual pattern of uracil incorporation by control cells is consistently observed when cells are transferred from high amino acids medium to low amino acids medium. This is not fully understood. A reasonable hypothesis would be that the initial rapid rate of incorporation represents ribosomal RNA synthesis stimulated by the high level of amino acids still in the cell from the previous incubation. When the internal pool of amino acids has equilibrated with the new medium, this rapid synthesis stops, some of the RNA degrades, and then RNA synthesis proceeds at the rate supported by the low concentration of amino acids. However, no attempt has been made to see whether this idea has any experimental basis.

3,10. APPARENT mRNA POOL SIZE AFTER LONGER INCUBATION

The amount of rifampicin-insensitive protease produced after 75 minutes incubation in low amino acids, as described above, was several times greater than that pro-

duced by cells directly after harvesting; e.g., 8 units/ml of protease compared with 60 units/ml (Fig. 3,15 and Fig. 3,12). However, it was of interest to determine whether the pool size could be increased further. Thus the amount of rifampicin-insensitive protease production was measured at the times, 75, 120, 180, 240 and 300 minutes. The results were very surprising (Fig. 3,19). It appeared that the pool size increased for 75 minutes, then decreased until 180 minutes and then increased once again. It was also observed that the rate of protease production in control cells appeared to change at intervals. To improve the resolution of this observation, the amount of rifampicin-insensitive protease produced between 0 and 300 minutes incubation was measured in shorter stages. The pool size was again clearly shown to be decreasing rapidly during the time period 75 to 150 minutes (Fig. 3,20).

3,10.a. Effect of medium on change in apparent mRNA pool size

It was possible that this decrease was due to changes in the medium caused by cellular metabolism; perhaps an increase or decrease of some regulatory factor had occurred. The switch in mRNA pool from build-up to breakdown occurred at around 75 minutes. To test the effect of the external medium on this, cells were incubated in the low amino acids medium for 60 minutes, then transferred to fresh low amino acids medium and further incubated for 60 minutes. After this time the pool size was measured and compared to that

in cells which had been maintained in the same medium. It appeared that fresh medium made no difference to the change in pool size; in both cases the pool size decreased to the same extent (Fig. 3,21). The change in rate of protease production (approximately 80 minutes after the initial transfer to low amino acids) was also maintained in the cells which were transferred to fresh medium at 60 minutes.

In the next experiment the change in pool size was determined at half-hourly intervals between zero and 240 minutes. However, the results were difficult to interpret because of the scattering of points. It appeared that the high background level of protease in these experiments was causing this. The transfer of cells by a brief centrifugation step to fresh low amino acids medium immediately before rifampicin addition overcame this problem. As seen from Fig. 3,21, this procedure appeared to reduce the mRNA pool only slightly. It was also found essential to maintain the cells in one batch (500 ml) and remove a sample (20 ml) of cells from this to measure the pool size at the appropriate times. This was to avoid the slight scattering in pool size which occurred if cells were incubated in smaller batches.

Thus, using this method, the apparent mRNA pool size (the amount of rifampicin-insensitive protease production) was measured at half-hourly intervals from zero time to 300 minutes of incubation. In Fig. 3,22 are shown the individual curves of production of protease by cell samples taken at half-hourly intervals and incubated in fresh

medium with rifampicin. The plateau value of enzyme produced in each case was taken as a measure of the apparent protease mRNA pool size. These plateau values are plotted in Fig. 3,23 as also is the production of protease over the whole experiment in the absence of rifampicin. It was found that the pool size increased to a maximum value after nearly 90 minutes incubation, then followed an abrupt drop and thereafter the size of the pool continued to oscillate. It perhaps should be exphasized that this pattern has been found to be reproducible.

3,11. RATE OF CONTROL PROTEASE PRODUCTION

The rate of production of extracellular protease by control cells in the absence of rifampicin appeared to be essentially linear, but with wobbling (Fig. 3,23a) in a manner which at first inspection was attributed to experimental error. However, it appeared from examination of many experiments (those described here as well as similar and duplicate experiments) that the line showed two slopes, a rapid rate over the period when the mRNA pool was increasing and a slower rate while it was decreasing. The point of inflection of these two rates corresponded to the time at which the change from increase in pool size to decrease, or vice-versa, appeared to have occurred. From Fig. 3,21 it was seen that even on transferring cells to fresh medium this pattern was maintained.

3,12. DISCUSSION

The results in this chapter rather clearly confirm that the synthesis of extracellular protease by Bacillus amyloliquefaciens is a system whose response to inhibitors of transcription is very unusual when compared with other prokaryote systems. Previously, Both et al. (1972) had observed that washed cells of B.amyloliquefaciens are capable of synthesizing extracellular protease de novo in the presence of transcriptional inhibitors. It was also shown (May and Elliott, 1968a) that high concentrations of amino acids repress protease synthesis while low concentrations do not. The data here show that preliminary incubation of harvested cells in a high amino acids medium exhausts their capacity for rifampicin-insensitive protease production and that when transferred to a low amino acids medium the cells rapidly regain this capacity. The maximum capacity achieved is several times greater than that seen in the same cells directly after harvesting from culture medium. The observed increase in capacity for rifampicin-insensitive protease production is not due to increasing cellular resistance to rifampicin nor to the development of a specific resistant polymerase since actinomycin D gives identical results.

There appears to be no known precedent in bacteria for the progressive insensitivity of protease synthesis to transcriptional inhibitors. Two explanations are possible. As proposed by Both et al. (1972), a pool of mRNA could be

built up during transcription and this pool then supports translation in the presence of the transcriptional inhibitors rifampicin, or actinomycin D. If this mRNA were short-lived, large amounts would be required to support the prolonged protease synthesis, but if it were stabilized the required reserve would be smaller. An alternative to a pool of mRNA is modulation of its stability. Thus at the beginning of incubation in low amino acids the mRNA for protease would be unstable. Then, during the incubation it would become progressively more stable. In this situation, however, as the new mRNA synthesized became more stable, the amount of mRNA in the cell would increase if transcription remained unchanged. Thus, the rate of protease synthesis would continually increase. This does not happen. To avoid returning to the concept of mRNA forming a reserve pool, it is necessary to add to this hypothesis a mechanism for maintaining the absolute amount of mRNA constant. This hypothesis also requires that in high amino acids the mRNA becomes unstable and then regains its stability in phase 2. However, it is known (Both et al., 1972) that for any given 'pool' the synthesis in the presence of rifampicin is the same in both high and low amino acids. Thus the effect of the amino acids must be considered secondary. This hypothesis also requires that the mRNA stability oscillates.

Considering all of this, the idea of a pool of excess mRNA appears at present to be a more likely one. However, it is to be noted that as yet no definitive evidence is

available for discriminating between the two hypotheses. Indeed, they need not be entirely mutually exclusive.

The observation was made in this work that over a period of time the capacity for rifampicin-insensitive protease production oscillates. This again appears to be an unprecedented situation and in the face of its surprising nature the reproducibility of the phenomenon is stressed. In such a situation a speculative hypothesis is perhaps more useful than none at all and the following model is suggested on the basis of this philosophy.

The original model of Both et al. was that protease mRNA was transcribed at the chromosomal gene from where it was released into the cytoplasm with no or only incomplete translation. This is in complete contrast to what is considered normal for a prokaryotic system, such as the lac operon, where translation and transcription occur at the genome virtually simultaneously. The biological rationale for the proposition above was that protease may be synthesized at the membrane and directly extruded to the external medium. This provided an answer to the major question of how potentially lethal enzymes such as extracellular ribonuclease and protease could be synthesized with immunity by these cells. Protease synthesis at the cell membrane meant the mRNA may have to diffuse through the cytoplasm from genome to membrane in a non-translated or incompletely translated form. This concept provided a rationale for the existence of a pool of mRNA. If the translational sites (at the membrane or prior) for protease

were limited and transcription off the protease gene was nevertheless rapid, then a pool of mRNA might accumulate. The results presented in Fig. 3,10 would appear to support this view. Here it is shown that the size of the apparent mRNA pool increases with time after the transfer of cells from high to low amino acids medium. During this increase the rate of protease synthesis is essentially linear. (Or to be clear on this point, the rate of synthesis does not increase in concert with the apparent mRNA pool increase.) Now if this apparent pool increase is due to an increase in amount of available mRNA, then it seems reasonable to conclude that the translation of the mRNA is limiting. Thus the concept arrived at is that of a pool of excess mRNA in effect, queuing up for translational sites.

The next aspect to be discussed is the observed oscillating nature of the apparent mRNA pool. This phenomenon appears to be a very remarkable one, but a biological rationale is at least possible on the basis of the mRNA pool concept. If it can be assumed that the cell produces mRNA which migrates to the membrane for translation, then it also becomes reasonable that a control mechanism should exist to ensure that the amount of mRNA is allowed neither to exceed a maximum limit nor to fall below a minimum value required for continuous protease production. Thus it can be postulated that the cell switches on transcription when pool mRNA levels are low, but after a certain concentration of mRNA builds up within the cell, transcription is stopped and protease synthesis

'coasts' on the reserve of mRNA until this reaches a low level when the cycle is started again. It is not necessarily envisaged that transcription stops entirely, but rather may be considerably reduced. The observation, that the rate of decrease of the apparent pool of mRNA is linear (e.g., Fig. 3,20b) compared with the decay of the mRNA pool in the presence of rifampicin which is exponential, suggests this.

The size of the peaks of mRNA pool size during the oscillations appear to decline and build up more slowly with time (Fig. 3,23). This could be due to the fact that the transfer procedure the cells have undergone synchronizes them and this synchrony is gradually lost.

As there is little cell growth in the medium (approximately 6% during the 12 hour incubation) the oscillations could not directly correspond with phases of the cell growth cycle which would be perhaps the most obvious explanation of the observation.

So far the discussion on the oscillations have lead to the concept shown in diagram 1.

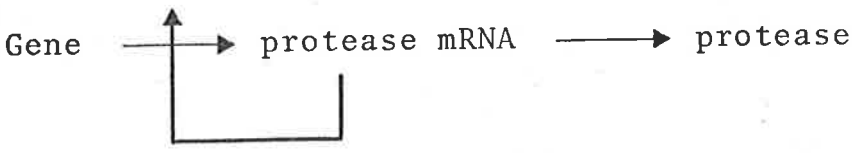


Diagram 1

Diagram 1 illustrates the feedback control system so far described.

3,12.a. THE INITIAL RATE PARADOX

So far this discussion has dealt with the simplest interpretation for the accumulation of the mRNA pool. Thus to summarize the argument so far - the apparent mRNA pool can vary enormously; for example, at 75 minutes in low amino acids medium it is approximately five times larger than at 15 minutes. It is also clear that the rate of protease synthesis does not increase five-fold over the same time period. Hence, the conclusion is formed that translation is limiting. This deduction seems to be a simple and self-evident one. However, careful analysis of the same results referred to above leads to a very paradoxical situation. From the progress curves of rifampicin-insensitive protease production (Fig. 3,13) it can be seen that the initial rates of protease production are proportional to the total size of the apparent mRNA pool (the total amount of rifampicin-insensitive protease produced). The conclusion from this, also apparently simple and self-evident, is that the mRNA itself is the limiting factor, not the translation of it. This appears to be a direct contradiction of the conclusion above, that mRNA is not limiting, but rather translation is.

It seems impossible to reconcile these contradictory conclusions without involving a second form of the protease mRNA, a reserve form, which constitutes the pool of mRNA. The reserve mRNA is in a non-translatable form that can be converted to the translatable form. If the reserve is stabilized, smaller quantities of RNA would be involved

than if it were not.

i.e., mRNA reserve (non-translatable) ⟶ mRNA ⟶ protease

It is necessary to add the following conditions to the hypothesis:

(1) that the conversion of reserve to mRNA is relatively slow so that mRNA availability still limits protease, and

(2) that on conversion the amount of translatable mRNA in the cell is proportional to the total amount of reserve.

This latter point is necessary to explain the observation, mentioned above (Fig. 3,13) that the initial rate of rifampicin-insensitive protease production is proportional to the total reserve.

In considering the relationship of transcription to the two forms of mRNA, three possibilities may be considered:

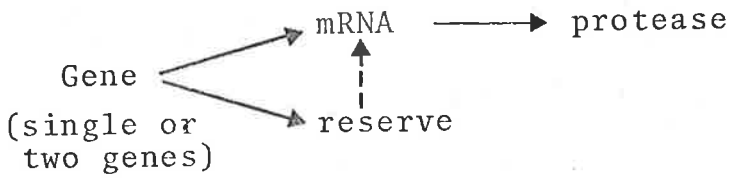
(1) mRNA may be produced directly, a proportion is converted to reserve, the rest immediately translated.

i.e., Gene ⟶ mRNA ⟶ protease
mRNA ⇅ reserve

(2) reserve mRNA may be the form transcribed such that,

Gene ⟶ reserve ⟶ mRNA ⟶ protease

(3) both forms may be transcribed,



Of these, scheme (2) seems unlikely since in this case the amount of translatable mRNA, and hence the rate of protease synthesis, would be seen to be proportional to the reserve in the absence of rifampicin as well as in its presence and this was shown not to be so. There seems to be little evidence to decide between (1) and (3) and either of these hypotheses would appear to fit the observed facts at present.

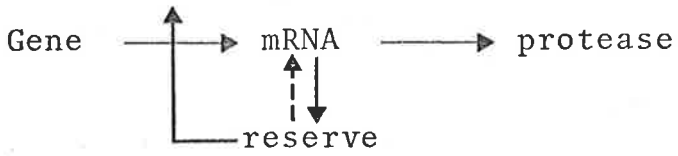
Thus far the theory is as follows: when transcription occurs mRNA is directly produced for immediate translation and at the same time reserve builds up without altering drastically the rate of portease synthesis. When the reserve has reached a maximum size transcription from the protease gene(s) is considerably reduced and the reserve becomes depleted by slow conversion to the translatable form. The level of mRNA available for translation, which results from these processes, may be less than when transcription is rapid and thus give rise to the different rates of protease synthesis observed in the two situations. In the case of transcription being stopped by rifampicin, the amount of translatable mRNA, and thus of protease synthesis, is directly proportional to the size of the reserve. This theory then also accommodates the observation that at maximum reserve the rate of rifampicin-insensitive protease

production can be greater than the rate in the absence of the drug.

3,12.b. POSSIBLE CONTROL MECHANISMS

The question now arises as to how the above schemes fit the apparent oscillatory control and the amino acid repression of protease synthesis.

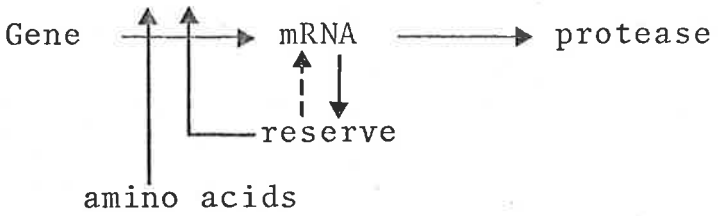
To explain the oscillations, a feedback control from the reserve messenger might exist. Supposing (without implication of favour) that scheme (1) operates.



If the reserve form controlled transcription, such that when the cells were replete with reserve transcription was dramatically reduced, and vice-versa when reserve was very low, then the oscillations would automatically result. But it should be pointed out that from the data presently available it must be concluded that only the maximum reserve is able to turn off transcription (and also, only very high levels of amino acids). If this were not so then the oscillations would not occur. (It is noted that in high amino acids (Fig. 3,14) the reserve continues to build up slowly such that even after 180 minutes the pool has not reached a maximum size and oscillations have not occurred.) However, it is quite possible that some other factor (e.g., an amino acid or protein) is primarily res-

ponsible for producing these oscillations, but data is not available to expand on this point.

It is still necessary to consider control by amino acids. High levels of amino acids repress protease production and low levels appear to stimulate. Incubation in high amino acids causes the reserve pool of mRNA to be depleted (Fig. 3,1). Amino acids appear to act at the level of transcription.



Thus it is proposed that dual control of protease gene transcription, by amino acids and reserve, exists. This hypothesis would accommodate phase 2 synthesis, a phenomenon previously difficult to explain. As shown in Fig. 3,1, when cells are suspended in high amino acids medium, phase 2 synthesis (dependent on mRNA reserve) terminates but almost immediately a new phase 2 of synthesis occurs, dependent on transcription. When this spontaneous derepression occurs the medium is still rich in amino acids. The dual control hypothesis would explain this phenomenon since the cells would be exhausted of mRNA at this time and hence repression would be partially lifted.

It was shown (Fig. 3,15) that a reserve pool again builds up in phase 2 of synthesis. This, of course, is accommodated in the hypothesis above. But the point of interest is that both the rate of pool build-up and that

of protease synthesis is slow and certainly slower than if the cells had been transferred to low amino acids medium (Fig. 3,16). High amino acids are not directly repressing the rate of protease translation since it is quite clear (Fig. 3,1) that the initial rate of protease synthesis in phase 1 is faster than that observed in phase 2. High amino acids are thus still repressing mRNA synthesis. The conclusion that this leads to is that mRNA appears to be the limiting factor and that the amount of reserve that builds up is dependent directly on transcription and does not build up as a result of limiting translation. A further observation which also supports this view is the following: it was noted that after transfer of cells from high to low amino acids medium the size of the reserve which built up varied from one experiment to another, and this appeared to be related to the amount of protease produced by control cells during the same time period. When the rate of portease synthesis by control cells in these various experiments was calculated, and compared to the rate of apparent pool increase over the same time period (0-75 minutes), the two were found to vary in concert (Table 3,1). This again suggests that transcription off the protease gene is the limiting factor in protease synthesis and reserve formation. If translation were the limiting factor, then perhaps it could be expected that if the rate of translation were reduced (relative to the rate of transcription) that the rate of reserve build-up would increase.

It is interesting to note that a similar conclusion was reached by Priest (1977) after considering the following published experiments. The level of extracellular α-amylase of B.subtilis Marburg was increased five-fold by introduction of the amyR marker from B.natto 1212 through transformation (Yamaguchi et al., 1974). AmyR appeared to be a promotor region controlling the rate of amylase transcription. Thus a greater yield of amylase was obtained as a result of an increased rate of transcription. This suggests that in B.subtilis Marburg the rate of α-amylase synthesis is not principally limited by the availability of translation sites but by the rate of transcription. A similar experiment (Uehara et al., 1974) involving B.subtilis 6160 and indicated that the rate of neutral protease synthesis by this organism was controlled by a specific genetic regulatory element.

Thus, although it was originally envisaged that the apparent mRNA pool built up because translational sites were limiting, it now seems that this may not be the case. Indeed, it is not necessary to propose that the extracellular protease is translated on membrane-bound ribosomes. Thus many questions now arise: what causes formation of the reserve mRNA, is it a result of post-transcriptional modification, or does the gene produce two forms of mRNA, perhaps of different lengths? What controls the rate of mRNA formation from reserve, and how is this conversion related to controls at the transcriptional level? While it is possible to continue speculating, it seems fruitless to do so until more information is available. It must be

strongly emphasized that the above hypotheses are speculative, there is no direct evidence for them, they are simply offered in the spirit that, notwithstanding the surprising nature of the observations, they nonetheless may be amenable to molecular explanations.

3,12.c. CRITICISMS OF THE mRNA POOL CONCEPT IN THE LITERATURE

While this work was in progress two papers by Brown and Coleman (1975a, 1975b) rejected the concept of a mRNA pool as put forward by Both et al. (1972), and a later paper (Coleman and Brown, 1975) continued this criticism. It should perhaps be pointed out that their statement that they were unable to confirm the results of Both et al. meant, in fact, that they disagreed with the latter group's conclusions since they neither repeated nor challenged the experimental work. In all experiments the authors appear to have used growing cells, but have not taken the increase in cell population into account when analysing their results.

There are two major areas of disagreement:

I. They claimed that although rifampicin totally inhibits cellular protein mRNA synthesis, exoenzyme mRNA synthesis is inhibited by only 50%. They suggest that the continued protease synthesis in the presence of rifampicin is simply due to this resistance. However, they do not comment on the observation of

Both et al. (1972) that actinomycin D gives identical results to rifampicin, and also, that in early phase 2 of protease synthesis cells have regained complete sensitivity to rifampicin. Priest (1977), in a review of the extracellular enzyme field, also criticises the lack of reference to this supportive evidence.

II. They concluded that exoenzyme synthesis does not occur during exponential phase of growth but is apparently switched on at the beginning of stationary phase. This observation has been the basis of both their own control models and their criticisms of those of Both et al. However, it is possible that the direct comparison of a semi-log plot of growth of the organism (dry weight) with a linear plot of enzyme production (not corrected for cell increase) has led to the erroneous assumption that exoenzyme synthesis is switched on in stationary phase (Coleman, 1967). The results from this laboratory show a parallel formation of protease production and cell growth when both are plotted on a logarithmic scale, that is, enzyme produced per dry weight of cells is linear with time.

To illustrate this point, Fig. 3,24 has been reproduced from Coleman (1967). If the rate of protease synthesis from 18-20 hours (log phase cells) is extrapolated the slope shows a rate of 35 units/hour. The dry weight at 19 hours is 0.24 mg/ml, so that

the rate/mg dry weight = 145 units/hour/mg dry weight. If the cell density and slope of the line at 20 hours only is used a figure of 142 units/hour/mg dry weight is arrived at. Now consider cells at 28 hours (stationary phase). The slope of the line at 28 hours = 250 units/hour. The cell density at 28 hours = 2.0 mg/ml. Therefore, the rate of protease synthesis per mg of dry weight = 125 units/hour. From this it would appear that log phase cells are at least as active, if not more active, in protease production than stationary phase cells.

Thus the statement that exoenzyme formation in B.amyloliquefaciens is "a process which occurs almost exclusively in the post-exponential phase of the growth cycle" seems unjustified from the author's own data.

In the two J. Mol. Biol. papers, Brown and Coleman dismissed both the idea of a stable mRNA or a pool of mRNA for extracellular enzymes. They showed that exoprotein mRNA had a normal short half-life of from 4.5 to 6 minutes, thus ruling out the possibility of a stable exoenzyme mRNA. Then, having made the assumption that mRNA for both the cellular and extracellular proteins were translated with equal efficiency during the growth cycle and that therefore the relative amounts of the two sets of mRNAs would be equal to the relative rates of protein synthesis of these fractions, they showed that the amount of exoprotein mRNA increased in stationary phase until it equalled 50%

of total mRNA. At the same time their hybridization studies showed that in stationary phase no one species of mRNA predominated and therefore the idea of a pool of mRNA need not be invoked. The authors apparently do not see these two results as being contradictory. The situation may be unfortunate as the authors themselves recognised that the interpretation of their hybridization data relied on there being no significant amount of exoprotein mRNA present in exponential phase cells, i.e., that such cells do not produce extracellular enzymes. As illustrated above, their assumption that exoprotein mRNA is not made in exponential phase is, so far as can be judged from their published data, erroneously based.

Against these criticisms other reports have appeared in the literature supporting the view that the existence of a pool of mRNA for extracellular enzymes may be a general phenomenon associated with extracellular enzyme synthesis. These are summarised below.

Gould et al. (1973) showed that the existence of an apparent pool of mRNA in Bacillus amyloliquefaciens was not peculiar to protease but extended to extracellular α-amylase and ribonuclease as well.

Sêmets et al. (1973) on investigating extracellular protease production by Bacillus subtilis 168 found that the pattern of response was qualitatively similar to that observed in B.amyloliquefaciens, a genetically distinct organism.

Boethling (1975) showed that de novo synthesis of extracellular protease by Pseudomonas maltophilia could be supported for at least 30 minutes in the presence of the transcriptional inhibitor, rifamycin-SV, although the cells were fully sensitive to this drug.

A similar phenomenon was reported by Stinson and Merrick (1974) to exist for poly-β-hydroxybutyrate depolymerase secretion by Pseudomonas lemoignei.

The results of studies by Priest (1975) showed the characteristics of α-amylase secretion by washed cells of Bacillus subtilis B20 to be essentially the same as that described by Gould et al. (1973) for B.amyloliquefaciens. α-Amylase production was biphasic [in much the same fashion as is protease production described by Both et al. (1972)], phase 1 being sensitive to rifampicin while phase 2 was not. Priest concluded that phase 1 synthesis corresponded to the translation of an existing mRNA pool and phase 2 to the resumption of coupled transcription and translation.

Kinoshita et al. (1968) showed that while RNA synthesis was completely stopped by uracil starvation of a uracil-negative mutant of B.subtilis KA63, α-amylase synthesis nevertheless continued for a considerable period.

3,12.d. CONCLUSIONS IN THIS CHAPTER

The work here clearly shows that the molecular biology of protease synthesis by Bacillus amyloliquefaciens cannot be explained by conventional prokaryote mechanisms.

The work is compatible with a reserve pool of mRNA whose synthesis is controlled in an unprecedented way such as to give oscillations of the pool. The work of Coleman's group does not constitute a valid objection to the concept.

It is also self-evident that the tentative conclusions the data point to are somewhat remarkable and thus can never be finally accepted on the basis of indirect evidence of the type obtained so far. The only way the hypotheses developed can be verified or rejected is to actually isolate the proposed pool of mRNA. The remainder of this thesis is concerned with this.

FIGURE 3,1. EFFECT OF RIFAMPICIN ADDITION AT ZERO AND 75 MINUTES ON PROTEASE PRODUCTION BY WASHED CELLS OF B.AMYLOLIQUEFACIENS IN THE PRESENCE OF LOW AND HIGH AMINO ACID LEVELS.

This fugure is reproduced from Both et al. (1972), p.212.

Cells were harvested at an OD_{600} of 3.6, washed twice and resuspended to the same cell density in either low (0.025%) or high (0.5%) casamino acids medium and incubated with shaking at 30°C. Rifampicin (0.5 µg/ml) was added at zero and 75 minutes to 20 ml samples of these cells. 1 ml Samples were taken, centrifuged and the supernatants assayed for protease activity as described in Chapter 2.

-■——■- , low casamino acids, no addition of drug.

-●——●- , high casamino acids, no addition of drug.

-△——△- , high casamino acids, rifampicin added at zero minutes. An identical curve was obtained in low casamino acids medium.

-○——○- , high casamino acids, rifampicin added at 75 minutes.

-□——□- , low casamino acids, rifampicin added at 75 minutes.

The arrows indicate time of rifampicin addition.

Fig 3, 1

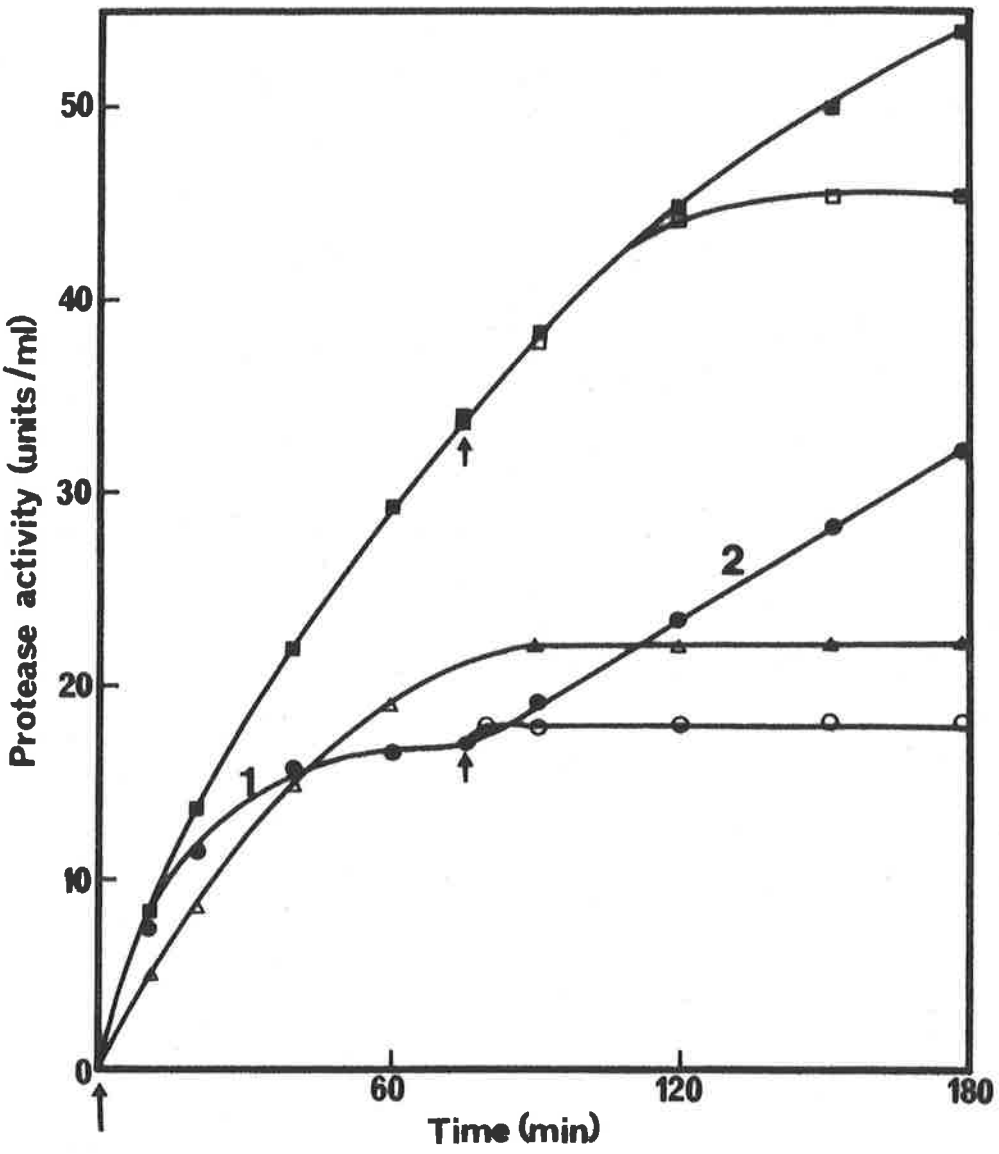

Protease activity (units/ml)
50
40
30
20
10
0
60
120
180
Time (min)
1
2

FIGURE 3,2. EFFECT OF AMINO ACID CONCENTRATION ON ^{3}H-URACIL INCORPORATION BY WASHED CELLS OF B.AMYLOLIQUEFACIENS.

Cells were harvested at an OD_{600} of 3.6, washed twice and resuspended to the same cell density in either high (0.5%) or low (0.025%) casamino acids medium and incubated with shaking at 30°C. At zero time, 1.6 µCi of ^{3}H-uracil (spec. act. 17C/mmole) and 20 µg of unlabelled uracil were added to a 2 ml cell sample. 0.1 ml Samples were taken and assayed for radioactivity as described in Chapter 2.

-●——●- , high amino acids.

··○·····○·· , low amino acids.

Fig 3,2

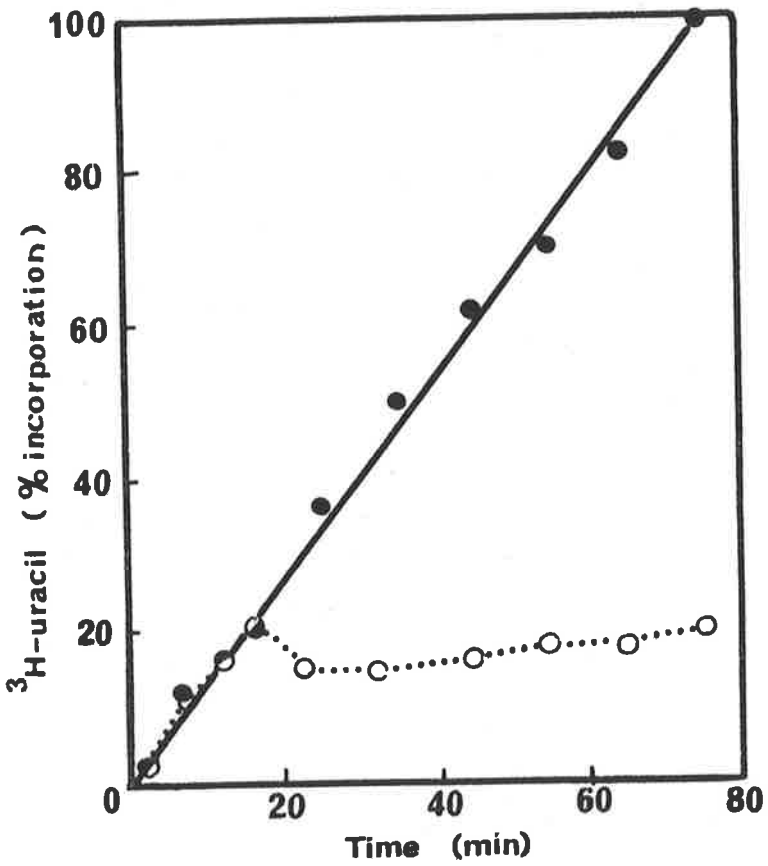


FIGURE 3,3. COMPARISON OF B.AMYLOLIQUEFACIENS TOTAL CELLULAR RNA LABELLED WITH RADIOACTIVE URACIL DURING INCUBATION IN LOW OR HIGH CASAMINO ACIDS MEDIUM.

Cells were treated as described in Fig. 3,2 except that radioactively labelled uracil (0.8 µc/ml of either ^{3}H-uracil, spec. act. 17C/mmole or ^{14}C-uracil, spec. act. 55 mC/mmole) and unlabelled uracil (10 µg/ml) was added 10 minutes after incubation started. RNA was extracted as described (Methods, Chapter 2) after a further 50 minutes incubation. 20 µg RNA was electrophoresed on 3% polyacrylamide gels for 3 hours at 5 mA/gel. 1 mm Gel slices were treated with NCS and assayed for radioactivity as described in Chapter 2.

a) High casamino acids medium

———— , OD profile of total cellular RNA.

--•----•-- , ^{3}H-uracil incorporated into RNA.

b) Low casamino acids medium

———— , OD profile of total cellular RNA.

--•----•-- , ^{14}C-uracil incorporated into RNA.

Similar results were obtained when the radioactive labels were reversed.

Fig 3,3

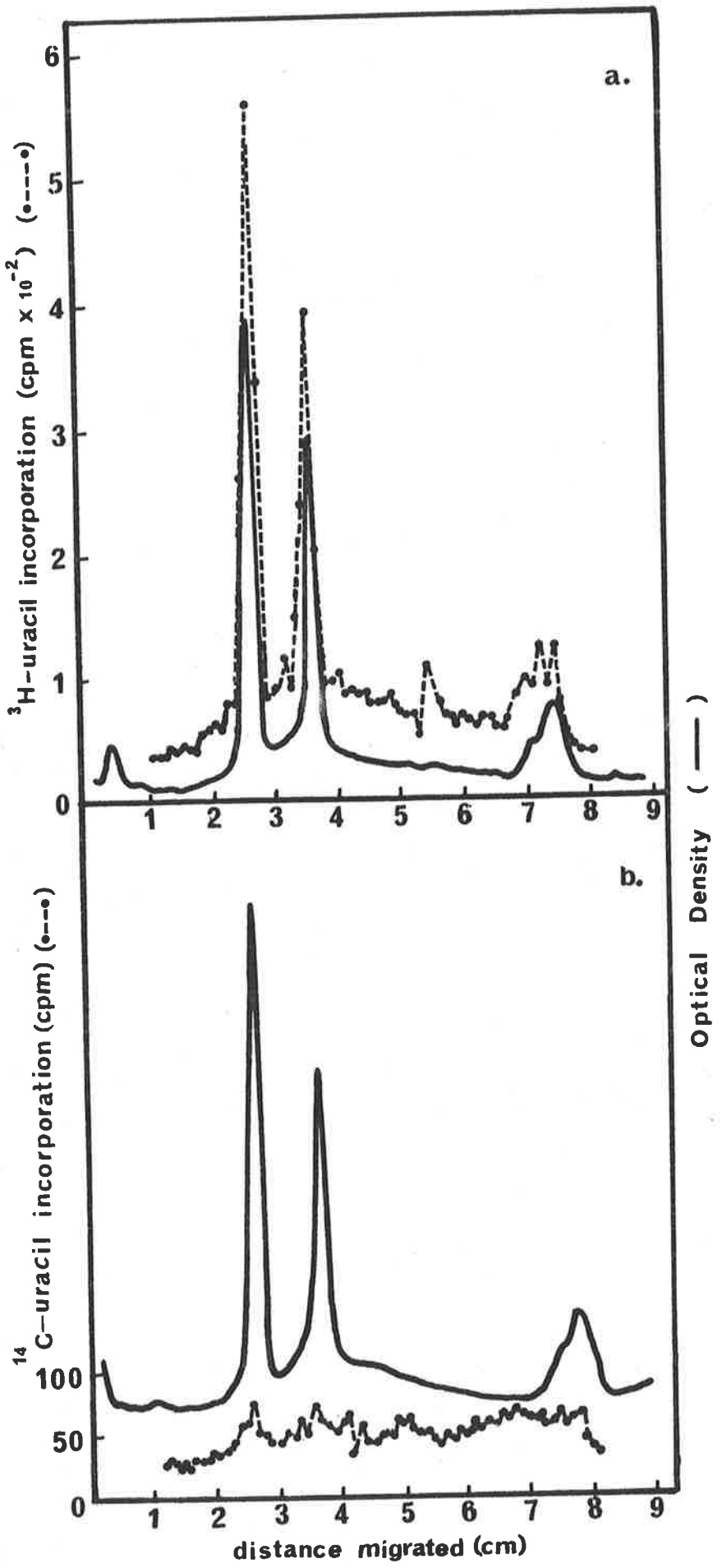

a.
b.
^{3}H-uracil incorporation (cpm x 10^{-2}) (•---•)
^{14}C-uracil incorporation (cpm) (•---•)
Optical Density (——)
distance migrated (cm)
0
1
2
3
4
5
6
7
8
9
50
100

FIGURE 3,4. EFFECT OF HIGH CONCENTRATIONS OF GLU OR ARG ON PROTEASE PRODUCTION BY WASHED CELLS OF B.AMYLOLIQUEFACIENS.

Cells were harvested at an OD_{600} of 3.6, washed twice and resuspended to the same cell density in low (0.025%), high (0.5%) or low casamino acids medium supplemented with either Glu (0.5%) or Arg (0.5%) and incubated with shaking at 30°C. 1 ml Samples were taken, centrifuged and the supernatants assayed for protease activity as described in Chapter 2.

-●--●- , high casamino acids.

-○—○- , low casamino acids.

..▲....▲.. , low casamino acids plus 0.5% (w/v) Arg.

..△.....△.. , low casamino acids plus 0.5% (w/v) Glu.

A mixture of Ileu and Pro (0.25% each) gave similar results to Glu.

Fig 3,4

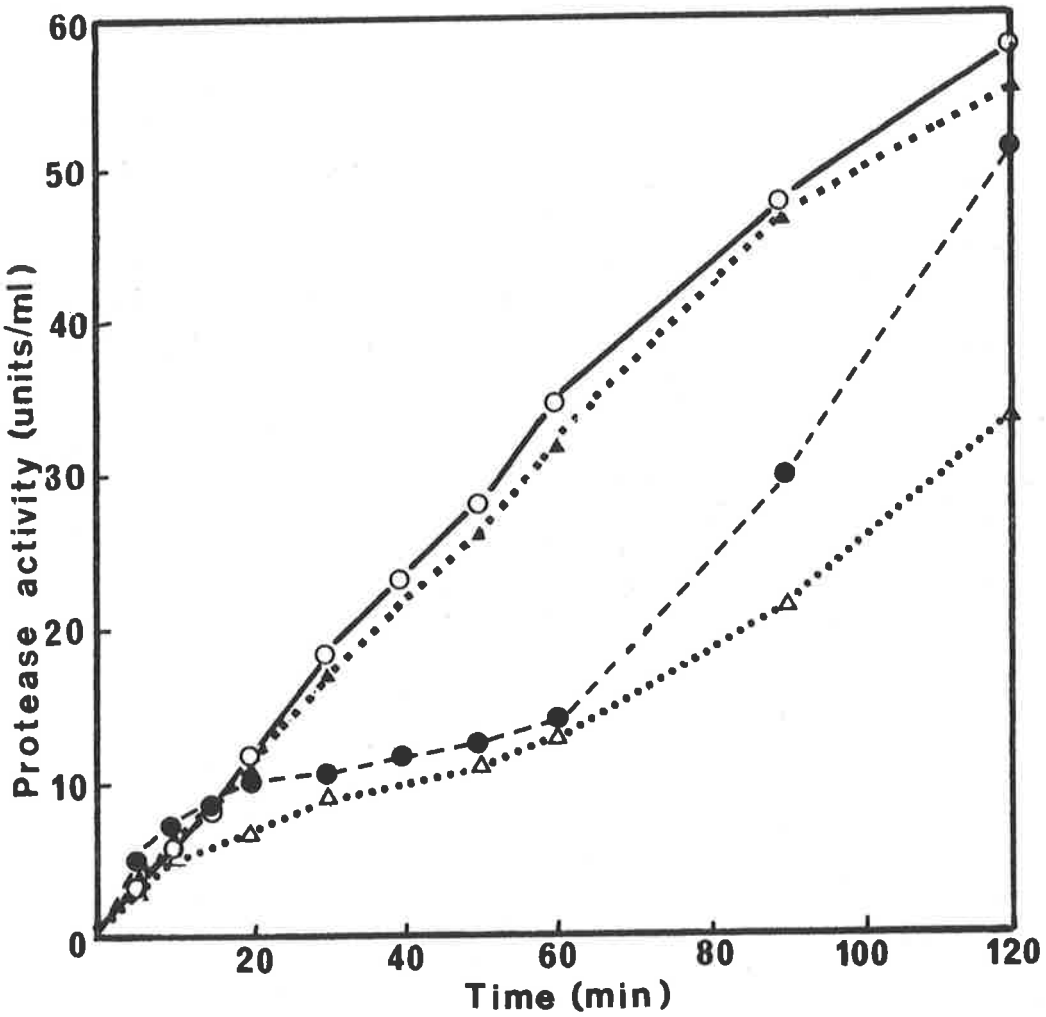

Protease activity (units/ml)
Time (min)
0
10
20
30
40
50
60
20
40
60
80
100
120

FIGURE 3,5. EFFECT OF HIGH CONCENTRATION OF GLU OR ARG ON ^{3}H-URACIL INCORPORATION BY WASHED CELLS OF B.AMYLOLIQUEFACIENS.

Cells were treated as described in Fig. 3,4. To a sample of cell (3 ml) 2.4 µc of ^{3}H-uracil (spec. act. 17 C/mmole) and 30 mg of unlabelled uracil was added at the beginning of incubation. 0.1 ml Samples were taken and assayed for radioactivity as described in Chapter 2.

-●—●- , high casamino acids.

-○--○- , low casamino acids.

··▲·····▲·· , low casamino acids plus 0.5% (w/v) Arg.

··△·····△·· , low casamino acids plus 0.5% (w/v) Glu.

100% incorporation was 27,000 c.p.m.

Fig3,5

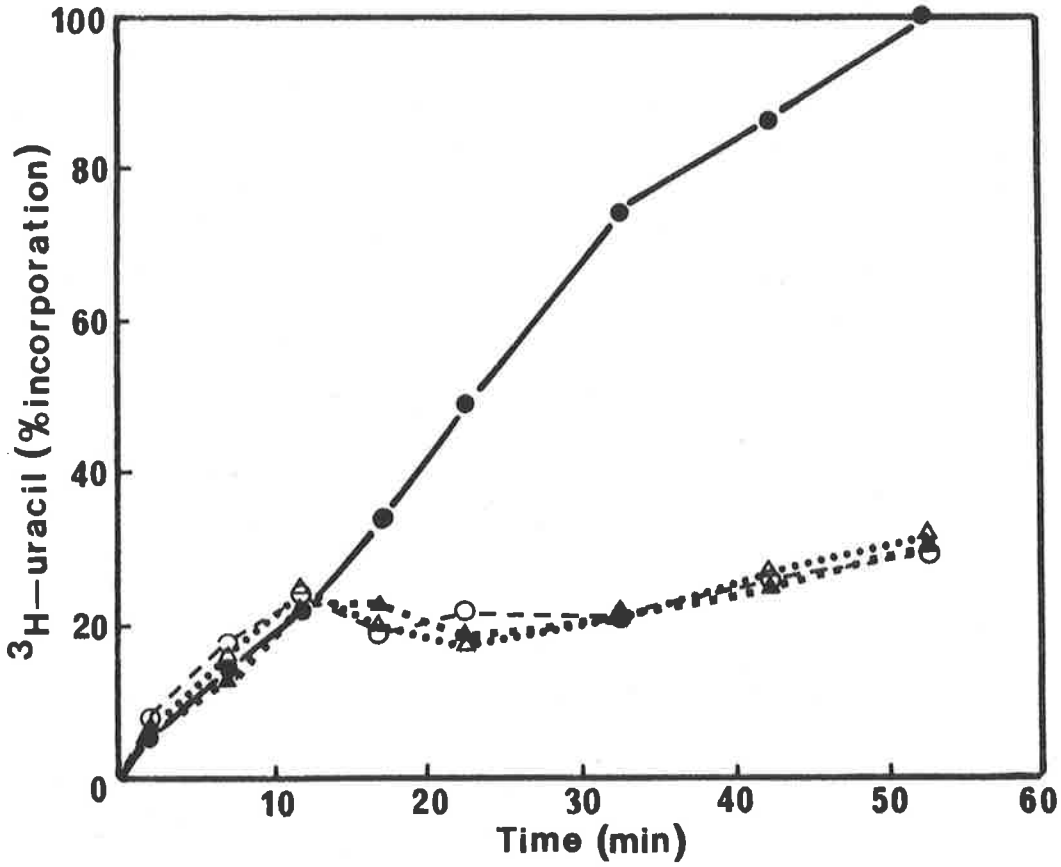

100
80
60
40
20
0
10
20
30
40
50
60
Time (min)
^{3}H—uracil (%incorporation)

FIGURE 3,6. COMPARISON OF RATES OF PROTEASE PRODUCTION BY WASHED CELLS OF *B.AMYLOLIQUEFACIENS* IN LOW OR HIGH CASAMINO ACIDS, AND AFTER TRANSFER FROM HIGH TO LOW CASAMINO ACIDS.

Cells were harvested at an OD_{600} of 3.6, washed twice and resuspended to the same cell density in either high (0.5%) or low (0.025%) casamino acids medium and incubated with shaking at 30°C. After 75 minutes incubation in high amino acids, a sample of cells (40 ml) was centrifuged, washed twice and resuspended in low casamino acids medium and incubation continued. 1.0 ml Samples were taken, centrifuged, and the supernatants assayed for protease activity as described in Chapter 2.

-●—●- , high casamino acids.

-○--○- , low casamino acids.

··▲·····▲·· , low casamino acids after transfer from high casamino acids.

Fig 3,6

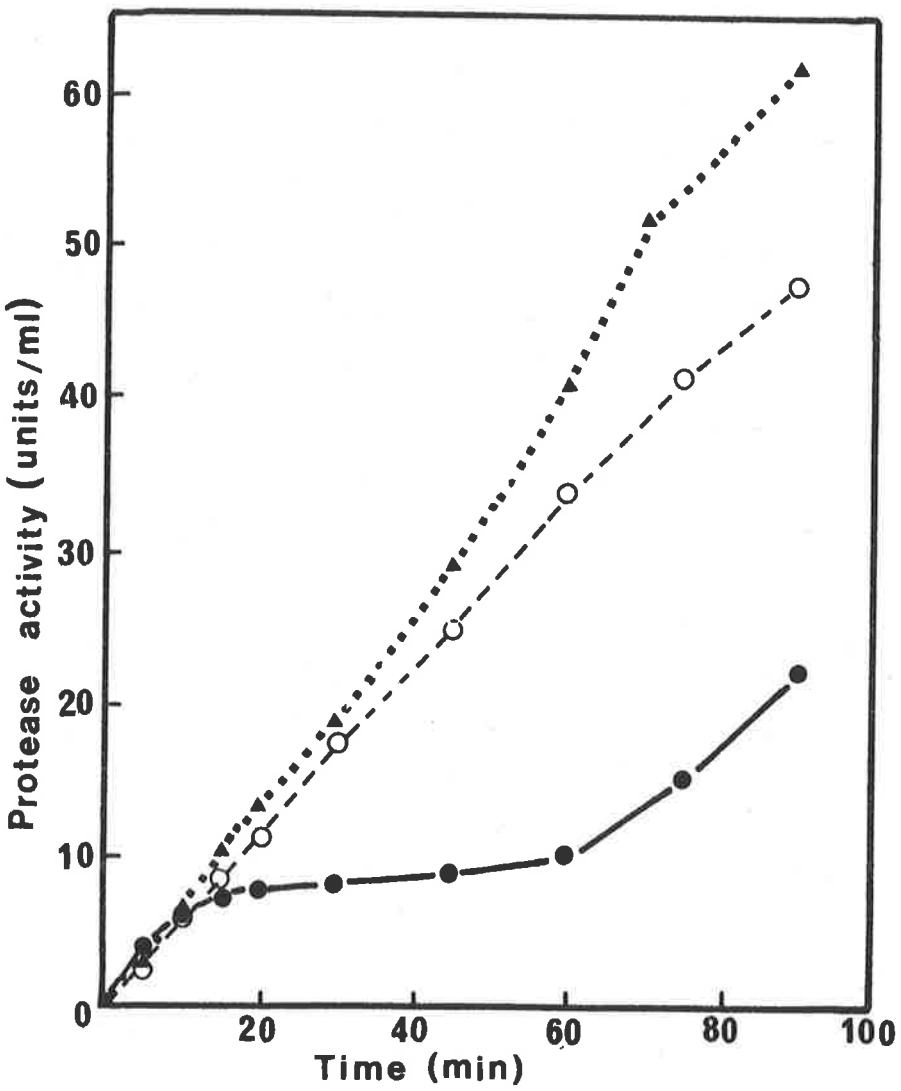

Protease activity (units/ml)
0
10
20
30
40
50
60
20
40
60
80
100
Time (min)

FIGURE 3,7. EFFECT ON ^{3}H-URACIL INCORPORATION OF TRANSFER OF B.AMYLOLIQUEFACIENS CELLS FROM HIGH TO LOW CASAMINO ACIDS MEDIUM.

Cells were prepared as described in Fig. 3,6. At the beginning of incubation in high or low casamino acids medium and again after transfer from high to low amino acids, 2.4 µCi of ^{3}H-uracil (spec. act. 17 C/mmole) and 30 µg of unlabelled uracil was added to a 3 ml sample of cells and the incubation continued as described. 0.1 ml Samples were taken and assayed for radioactivity as described in Chapter 2.

-●—●- , high casamino acids.

-○--○- , low casamino acids.

··▲····▲·· , low casamino acids after transfer from high amino acids.

Fig 3,7

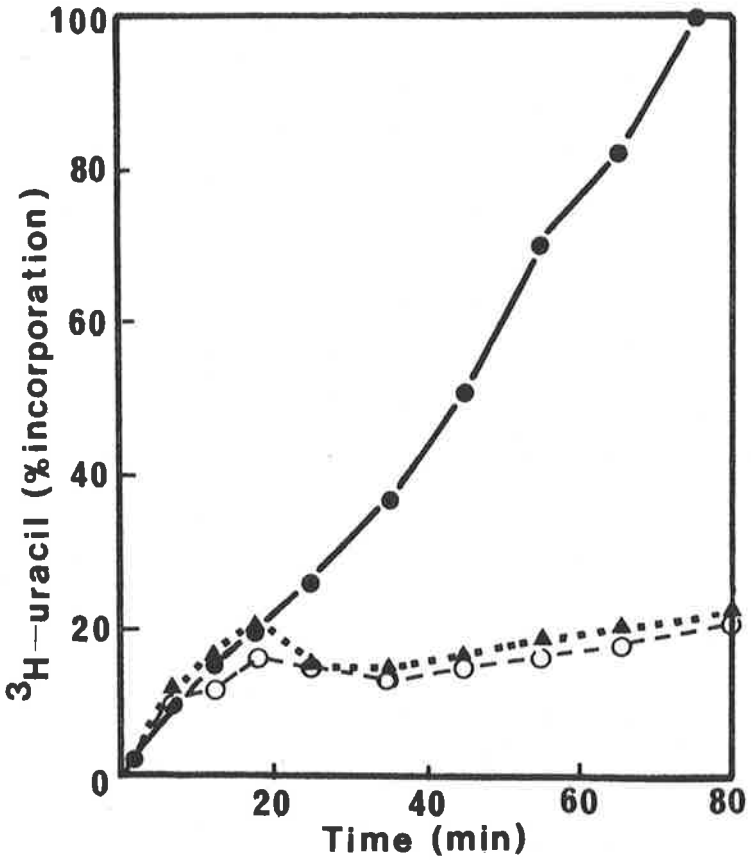


FIGURE 3,8. EFFECT OF RIFAMPICIN ON GENERAL PROTEIN AND RNA SYNTHESIS BY WASHED CELLS OF B.AMYLOLIQUEFACIENS.

Cells were harvested at an OD_{600} of 3.6, washed twice and resuspended to the same cell density in high (0.5%) casamino acids medium.

a) Cells were incubated with shaking at 30°C in the presence of 0.8 µCi/ml of ^{3}H-uracil (spec. act. 17 C/mmole) and 10 µg/ml unlabelled uracil. At zero time 0.075 µg/ml of rifampicin was added to a sample of cells (3 ml). 0.1 ml Samples were taken and assayed for radioactivity as described in Chapter 2.

-●—●- , no addition of drug.

-○--○- , rifampicin (0.075 µg/ml) added at zero time.

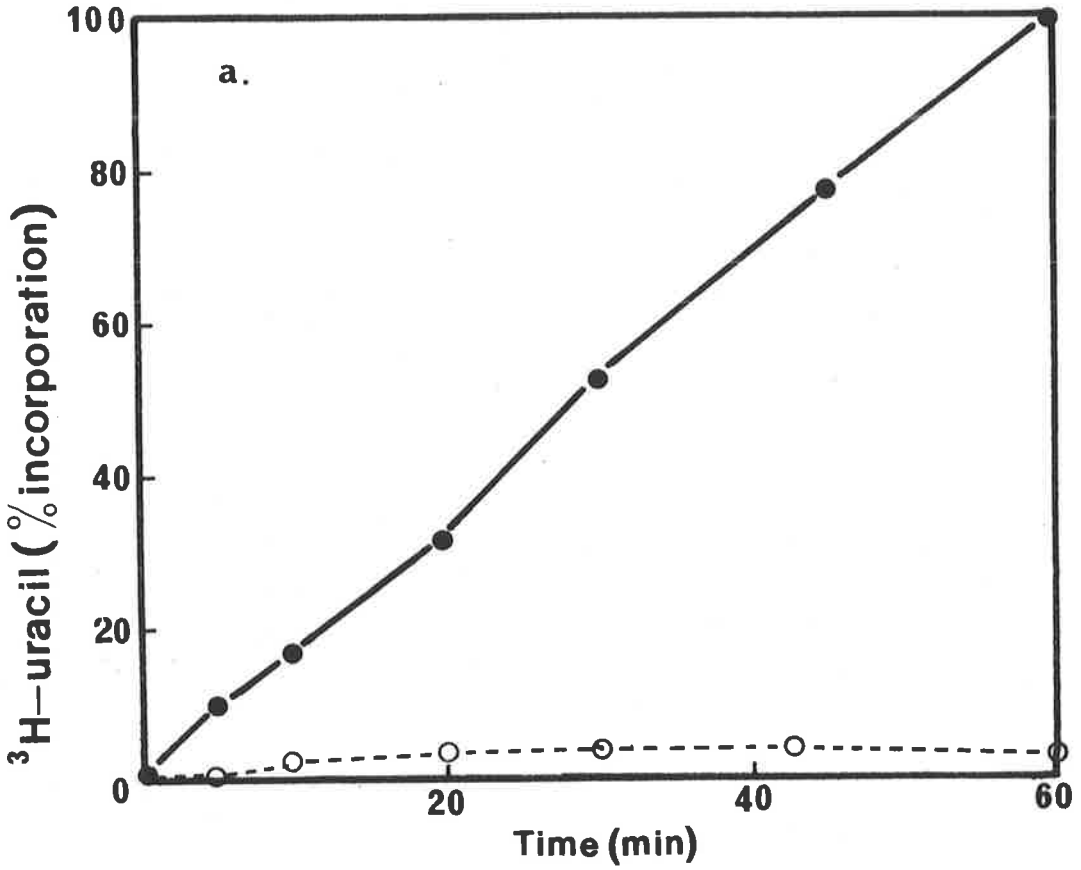
100% incorporation was 28,500 cp.m.

b) Cells were incubated in the presence of 0.5 µc/ml of ^{14}C-Phe (spec. act. 460 mc/mmole). Rifampicin (0.075 µg/ml) was added to a sample (3 ml) of these cells at zero time. 0.1 ml Samples were taken and assayed for radioactivity as described in Chapter 2.

-●—●- , no addition of drug.

-○--○- , rifampicin (0.075 µg/ml) added at zero time.

100% incorporation was 7,500 c.p.m.


a.
100
80
60
40
20
0
20
40
60
Time (min)
^{3}H—uracil (% incorporation)

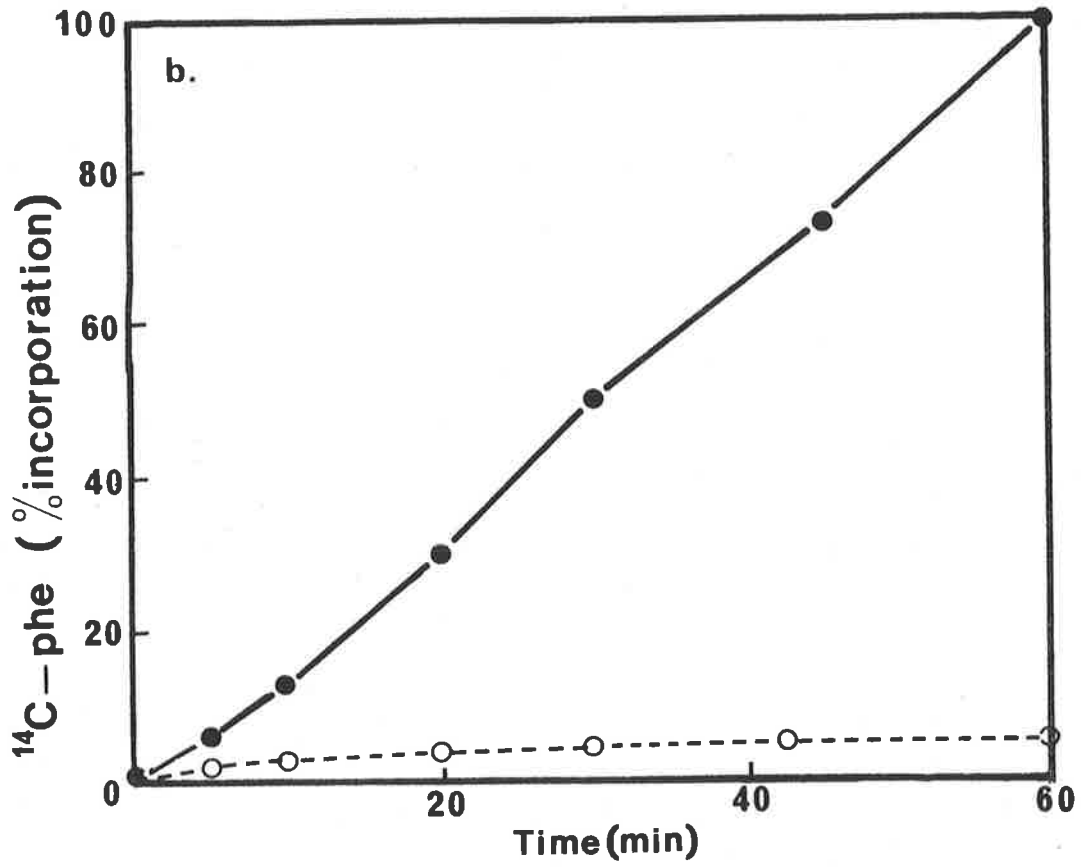

b.
100
80
60
40
20
0
20
40
60
Time (min)
^{14}C—phe (% incorporation)

FIGURE 3,9. EFFECT ON PROTEASE PRODUCTION OF RIFAMPICIN ADDITION TO CELLS AT ZERO AND 75 MINUTES AFTER TRANSFER FROM HIGH TO LOW AMINO ACIDS MEDIUM.

B.amyloliquefaciens cells were harvested at an OD_{600} of 3.6, washed twice and resuspended to the same cell density in high casamino acids medium and incubated at 30°C with shaking for 75 minutes. Cells were then centrifuged, washed twice and resuspended to the original cell density in low casamino acids and further incubated. Rifampicin (0.075 µg/ml) was added to a sample (40 ml) of cells at zero and 75 minutes after resuspension to low amino acids. 1 ml Samples were taken, centrifuged and the supernatants assayed for protease activity as described in Chapter 2.

All curves are of protease production in low amino acids medium.

-●——●- , no addition of drug.

··O····O·· , rifampicin added at zero time.

-▲----▲- , rifampicin added at 75 minutes.

Arrows indicate the time of rifampicin addition.

Fig 3,9

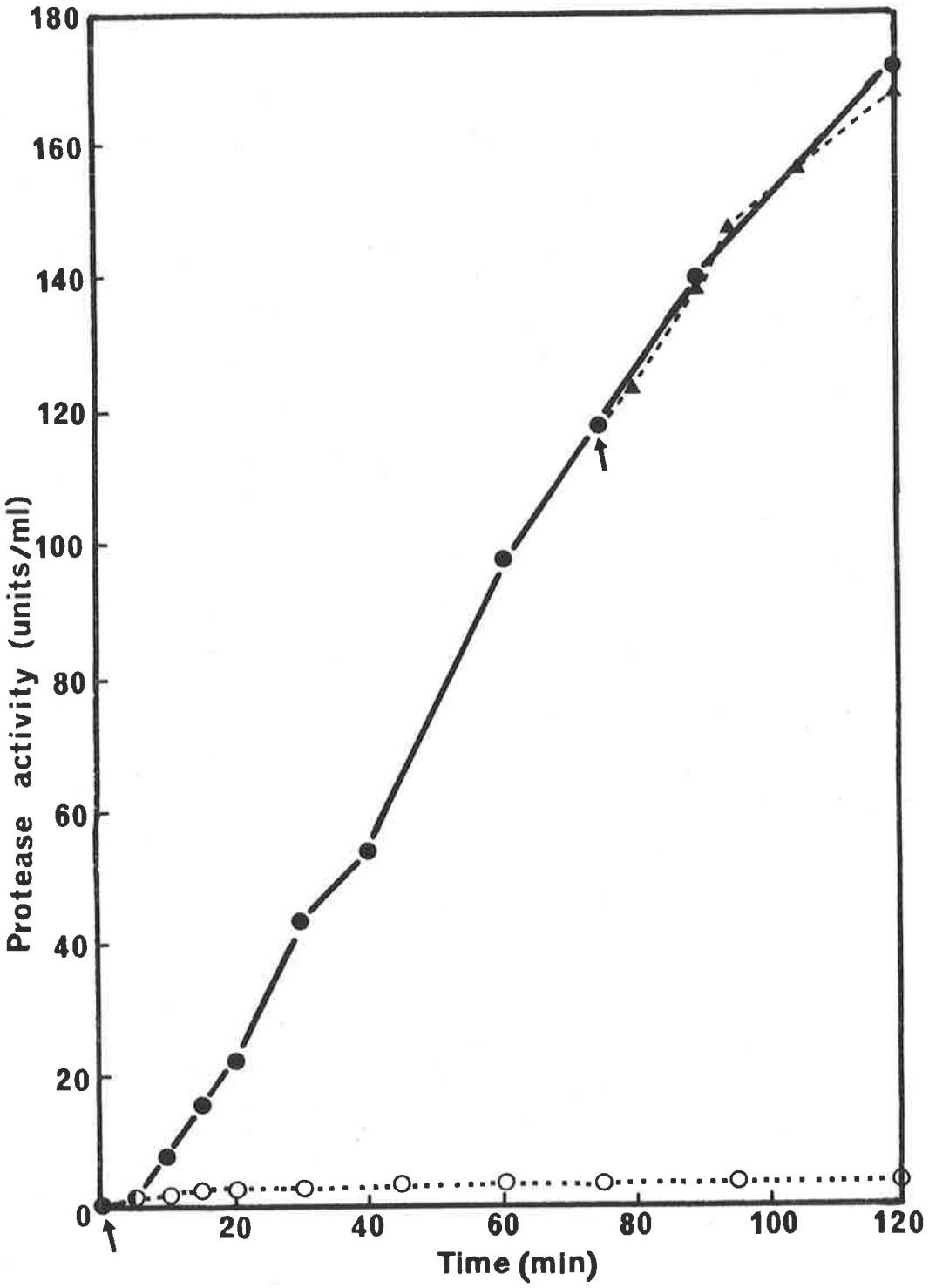

180
160
140
120
100
80
60
40
20
0
Protease activity (units/ml)
20
40
60
80
100
120
Time (min)

FIGURE 3,10. EFFECT OF RIFAMPICIN ADDITION AT VARIOUS TIMES ON PROTEASE PRODUCTION BY CELLS TRANSFERRED FROM HIGH TO LOW AMINO ACIDS MEDIUM.

B.amyloliquefaciens cells were prepared as described in Fig. 3,9 except that rifampicin (0.075 µg/ml) was now added at zero, 20, 45, 60 and 75 minutes and protease production assayed as described.

Main figure

All curves are of protease activity in low amino acids medium.

-●—●- , no addition of drug.

The remaining curves are of protease production after rifampicin was added at the following times:

-○—○- , zero minutes.

-▲---▲- , 20 minutes.

-△---△- , 45 minutes.

-■---■- , 60 minutes.

--□----□-- , 75 minutes.

The arrows indicate the time of rifampicin addition.

INSERT

Each point represents the final amount of protease produced (plateau level) after the addition of rifampicin at the times indicated.

Fig 3,10

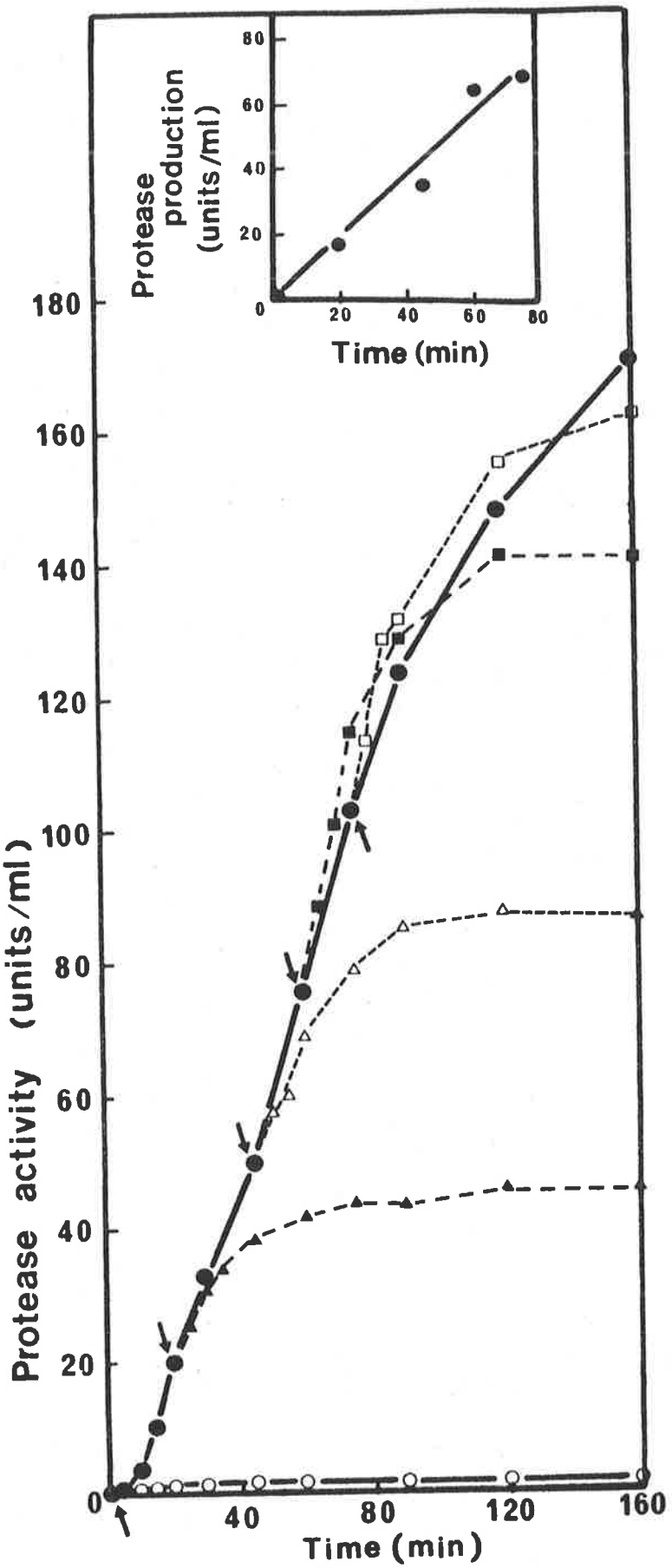

Protease production (units/ml)
80
60
40
20
0
20
40
60
80
Time (min)
Protease activity (units/ml)
180
160
140
120
100
80
60
40
20
0
40
80
120
160
Time (min)

FIGURE 3,11. EFFECT OF RIFAMPICIN ADDITION AT TIMES FROM ZERO TO 20 MINUTES ON PROTEASE PRODUCTION BY CELLS TRANSFERRED FROM HIGH TO LOW AMINO ACIDS MEDIUM.

B.amyloliquefaciens cells were prepared as described in Fig. 3,9 except that rifampicin (0.075 µg/ml) was added at zero, 5, 10, 15 and 20 minutes and protease production measured as described.

Main figure

All curves are of protease production in low amino acids medium.

-●——●- , no addition of drug.

The remaining curves are of protease production after rifampicin was added at the following times:

-○——○- , zero minutes.

-▲- -▲- , 5 minutes.

--△----△-- , 10 minutes.

-■——■- , 15 minutes.

-□- - -□- , 20 minutes.

Arrows indicate the times of rifampicin addition.

INSERT

Each point represents the total amount of protease produced (plateau level) after the addition of rifampicin at the times indicated.

Fig 3,11

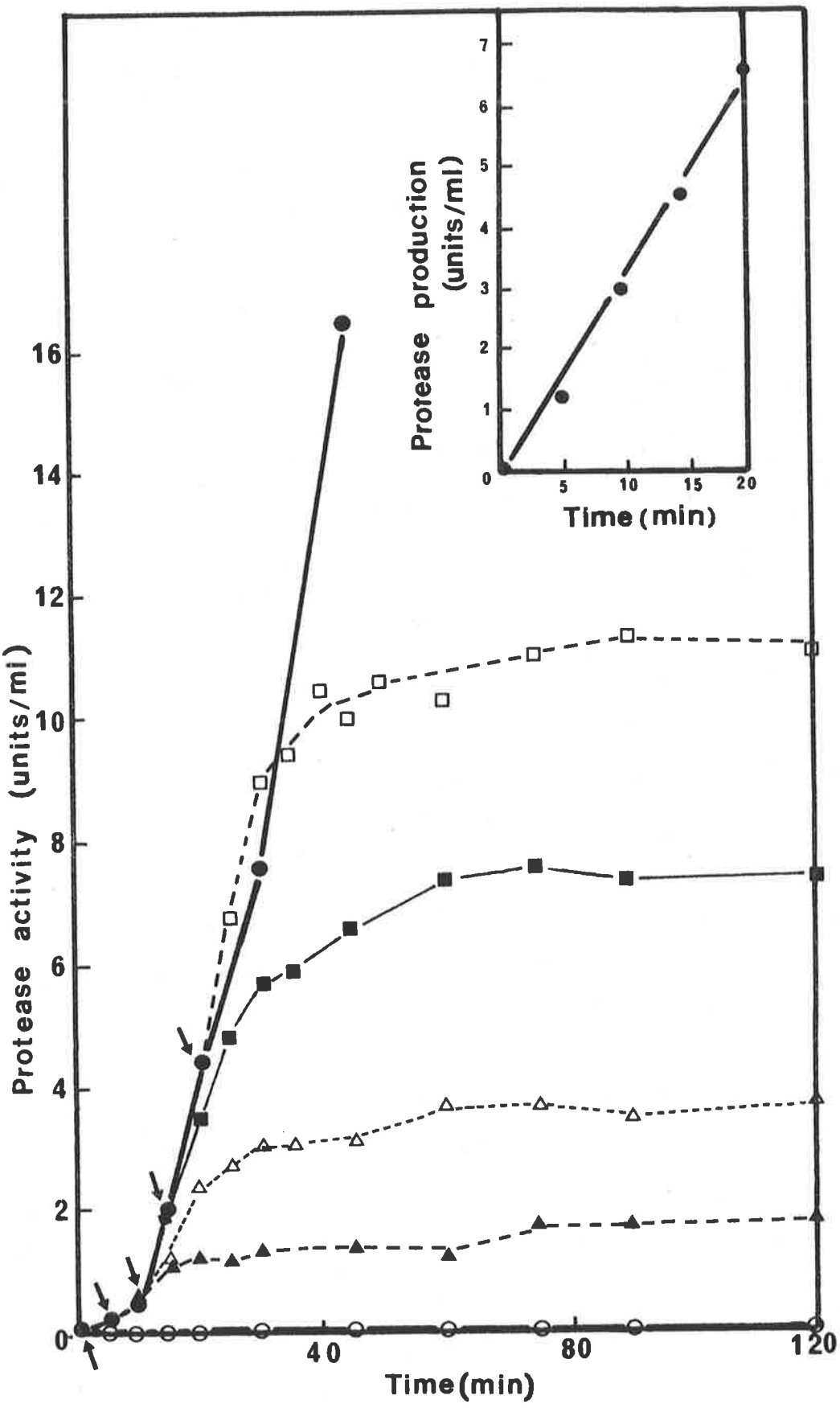

Protease activity (units/ml)
16
14
12
10
8
6
4
2
0
40
80
120
Time (min)
Protease production (units/ml)
7
6
5
4
3
2
1
0
5
10
15
20
Time (min)

FIGURE 3,12. EFFECT OF RIFAMPICIN ADDITION AT TIMES FROM ZERO TO 75 MINUTES ON PROTEASE PRODUCTION BY CELLS TRANSFERRED FROM HIGH TO LOW AMINO ACIDS MEDIUM.

This experiment is essentially the same as that described in Fig. 3,10 except that rifampicin (0.075 µg/ml) was added at zero, 5, 10, 20, 45, 60 and 75 minutes and the subsequent protease production measured as described.

Main figure

All curves are of protease production in low amino acids medium.

-●——●- , no addition of drug.

The remaining curves are of protease production after rifampicin was added at the following times:

-○——○- , zero minutes.

..▲....▲.. , 5 minutes.

--△--△-- , 10 minutes.

..■.....■.. , 20 minutes.

—□—□— , 45 minutes.

..▼.....▼.. , 60 minutes.

--▽----▽-- , 75 minutes.

INSERT

Each point represents the total amount of protease produced (plateau level) after the addition of rifampicin at the times indicated.

Fig 3,12

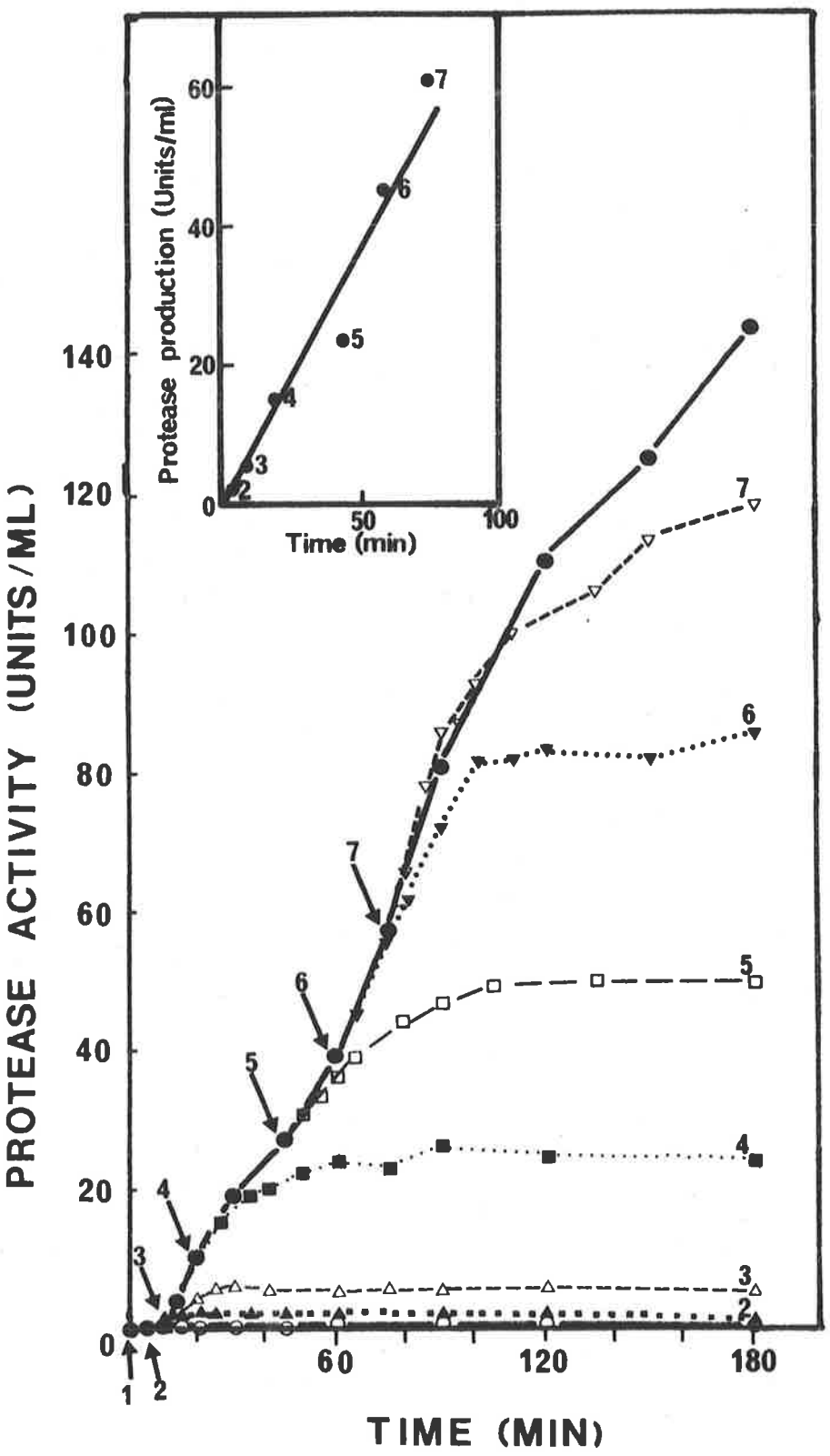

PROTEASE ACTIVITY (UNITS/ML)
TIME (MIN)
0
20
40
60
80
100
120
140
60
120
180
1
2
3
4
5
6
7
Protease production (Units/ml)
Time (min)
50
100

FIGURE 3,13. PROGRESS CURVES OF PROTEASE PRODUCTION IN THE PRESENCE OF RIFAMPICIN.

These three graphs were taken from Fig. 3,10. They show protease production in the presence of rifampicin which was added to cells at 20 (--▲-----▲--), 45 (-△---△-) and 60 minutes (-■——■-).

The curves were adjusted to zero by subtracting the amount of protease present in the supernatant at the time of rifampicin addition from the subsequent amount of protease produced.

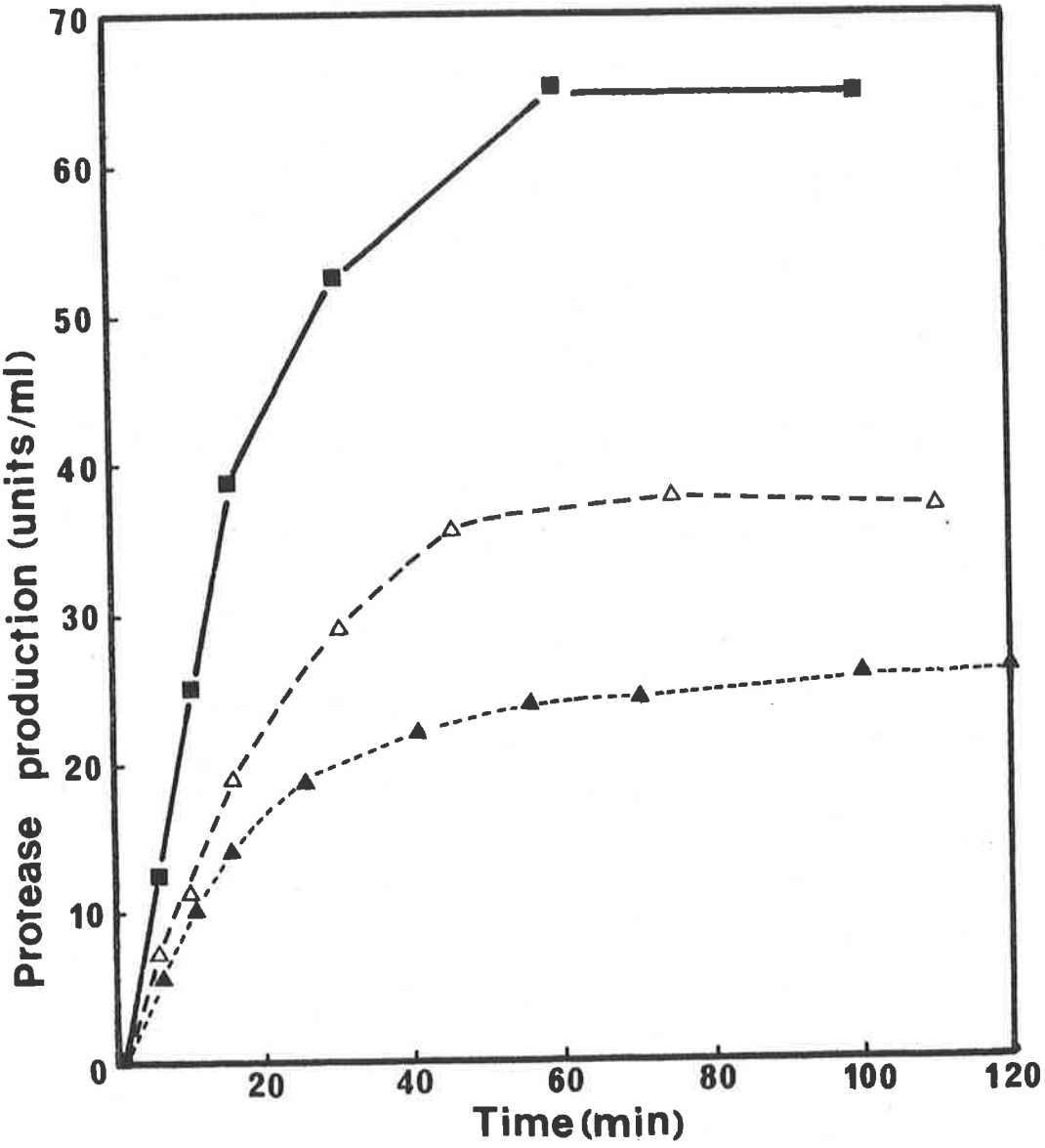

Protease production (units/ml)
70
60
50
40
30
20
10
0
20
40
60
80
100
120
Time (min)

FIGURE 3,14. EFFECT OF RIFAMPICIN ADDITION AT VARIOUS TIMES ON PROTEASE PRODUCTION BY CELLS TRANSFERRED FROM HIGH TO FRESH HIGH AMINO ACIDS MEDIUM.

B.amyloliquefaciens cells were harvested at an OD_{600} of 3.6, washed twice and resuspended to the same cell density in high casamino acids medium and incubated at 30°C with shaking for 75 minutes. Cells were then centrifuged, washed twice and resuspended to the original cell density in fresh high amino acids medium and further incubated. Rifampicin (0.075 µg/ml) was added to samples (40 ml) of cells at zero, 30, 90, 120, 150 and 180 minutes. 1 ml Samples were taken, centrifuged and the supernatants assayed for protease activity as described in Chapter 2.

Main figure

All curves are of protease production in the fresh high amino acids medium.

-●—●- , no addition of drug.

The remaining curves are of protease production after rifampicin was added at the following times:

-○—○- , zero minutes.

-▲--▲- , 30 minutes.

--△-----△-- , 90 minutes.

-■—■- , 120 minutes.

-□--□- , 150 minutes.

--▼----▼-- , 180 minutes.

INSERT

Each point represents the total amount of protease produced (plateau level) after rifampicin was added at the times indicated.

Fig 3,14

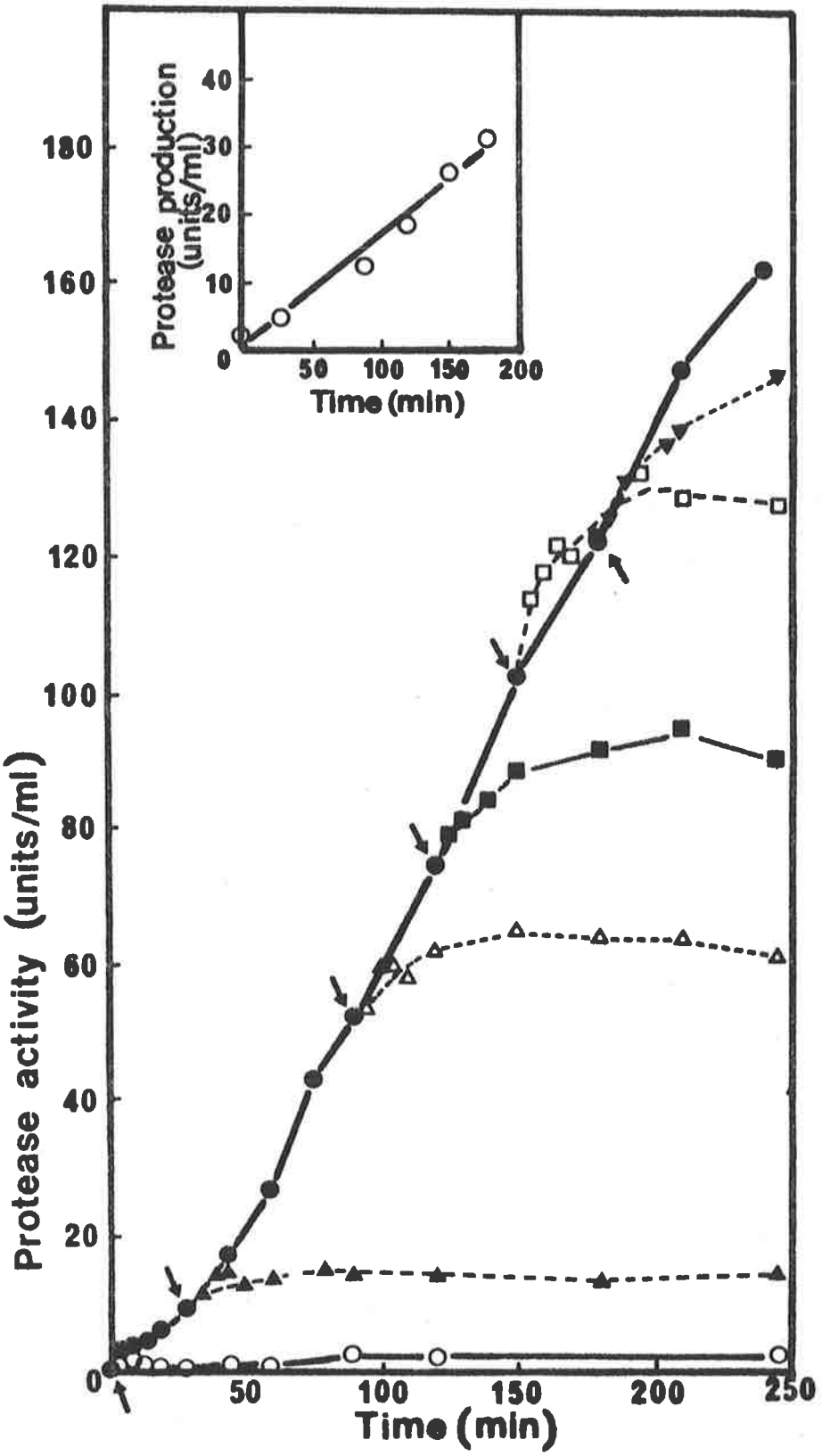

Protease activity (units/ml)
Time (min)
0
20
40
60
80
100
120
140
160
180
50
100
150
200
250
Protease production (units/ml)
Time (min)
0
10
20
30
40
50
100
150
200

FIGURE 3,15. EFFECT OF RIFAMPICIN ADDITION AT VARIOUS TIMES ON PROTEASE PRODUCTION DURING PHASE 2 OF PROTEASE SYNTHESIS IN HIGH AMINO ACIDS.

B.amyloliquefaciens cells were harvested at an OD_{600} of 3.6, washed twice and resuspended to the same cell density in high amino acids medium and incubated with shaking at 30°C. Rifampicin (0.075 µg/ml) was added to samples (40 ml) of cells at 60, 90, 120, 150 and 180 minutes. 1 ml Samples were taken, centrifuged and the supernatants assayed for protease activity as described in Chapter 2.

Main figure

All curves are of protease production in high amino acids medium.

-●——●- , no additon of drug.

The remaining curves are of portease production after rifampicin was added at the following times:

-○——○- , 60 minutes.

-▲- - -▲- , 90 minutes.

--△----△-- , 120 minutes.

-■- - -■- , 150 minutes.

--□----□-- , 180 minutes.

INSERT

All points represent the total amount of protease produced (plateau level) after addition of rifampicin at the times indicated.

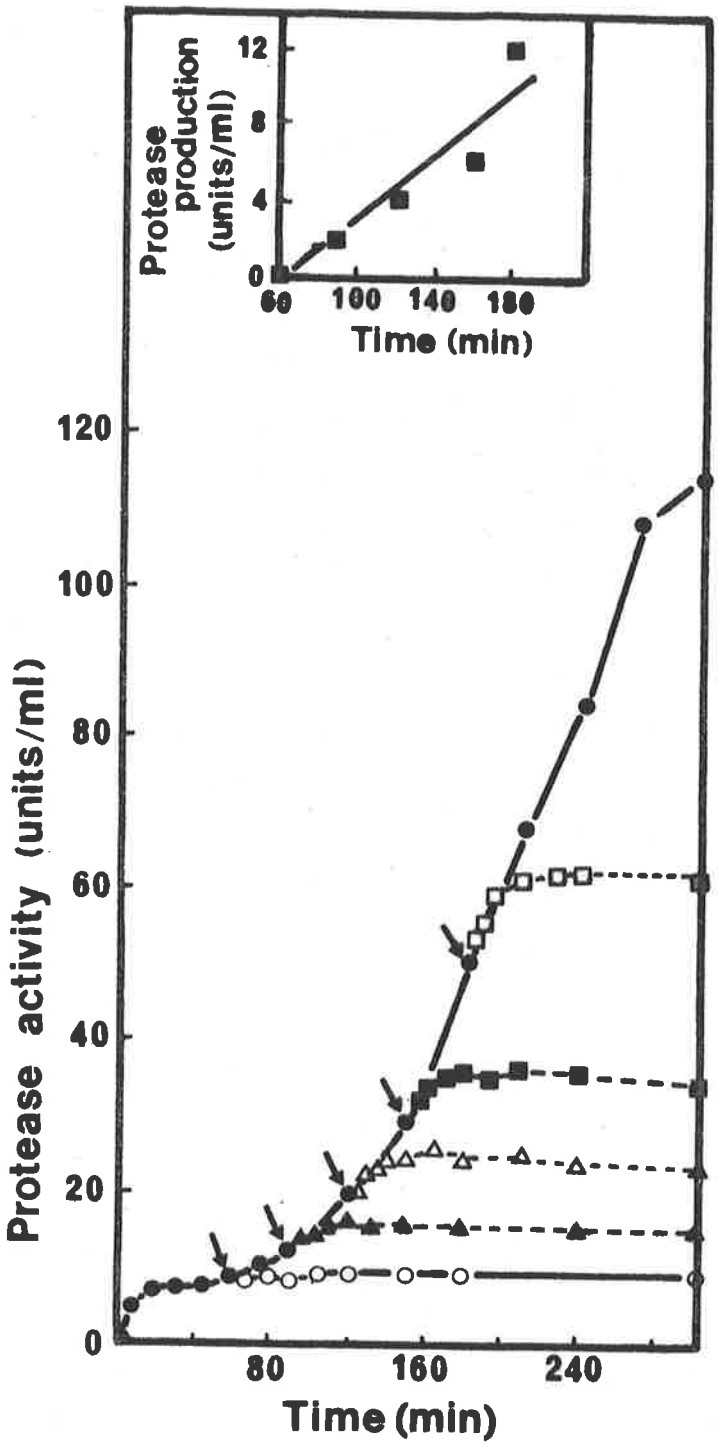

Protease production (units/ml)
12
8
4
0
60
100
140
180
Time (min)
120
100
80
60
40
20
0
Protease activity (units/ml)
80
160
240
Time (min)

FIGURE 3,16. COMPARISON OF RATES OF INCREASE OF RIFAMPICIN-INSENSITIVE PROTEASE PRODUCTION IN VARIOUS CONDITIONS.

The inserts from Figures (3,12), (3,14) and (3,15), which showed the increase in rifampicin-insensitive protease production with time, are presented here on the same graph for comparison.

-●—●- , from Fig. 3,12; increase in rifampicin-insensitive protease production in low casamino acids medium after transfer from high amino acids medium.

-○—○- , from Fig. 3,14; increase in rifampicin-insensitive protease production in fresh high casamino acids medium after transfer from high casamino acids medium.

-■—■- , from Fig. 3,15; increase in rifampicin-insensitive protease production during phase 2 of protease synthesis in high casamino acids medium. For purposes of comparison, 60 minutes, the beginning of phase 2 synthesis was equated with zero time of incubation.

Fig 3 ,16

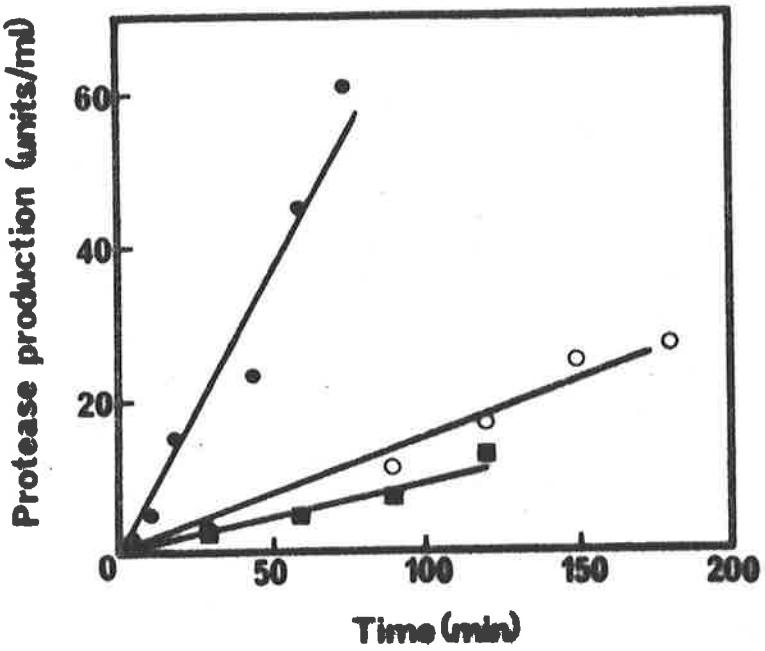

Protease production (units/ml)
60
40
20
0
50
100
150
200
Time (min)

FIGURE 3,17. EFFECT OF ACTINOMYCIN D AND CHLORAMPHENICOL ON PROTEASE PRODUCTION BY CELLS TRANSFERRED FROM HIGH TO LOW CASAMINO ACIDS MEDIUM.

B.amyloliquefaciens cells were harvested at an OD_{600} of 3.6, washed twice and resuspended to the same cell density in high casamino acids medium, and incubated at 30°C with shaking for 75 minutes. Cells were then centrifuged, washed twice and resuspended to the original cell density in low casamino acids medium and further incubated. Actinomycin D (2.25 µg/ml) or chloramphenicol (20 µg/ml) plus rifampicin (0.075 µg/ml) were added to samples (40 ml) of cells at zero and 75 minutes as indicated. 1 ml Samples were taken, centrifuged and the supernatants assayed for protease activity as described in Chapter 2.

All curves are of protease production in low casamino acids medium.

-●—●- , no addition of drug.

-○—○- , actinomycin D added at zero time. An identical curve was obtained when chloramphenicol plus rifampicin was added at zero time.

-▼--▼- , chloramphenicol plus rifampicin added at 75 minutes.

--□--□-- , rifampicin added at 75 minutes.

-■—■- , actinomycin D added at 75 minutes.

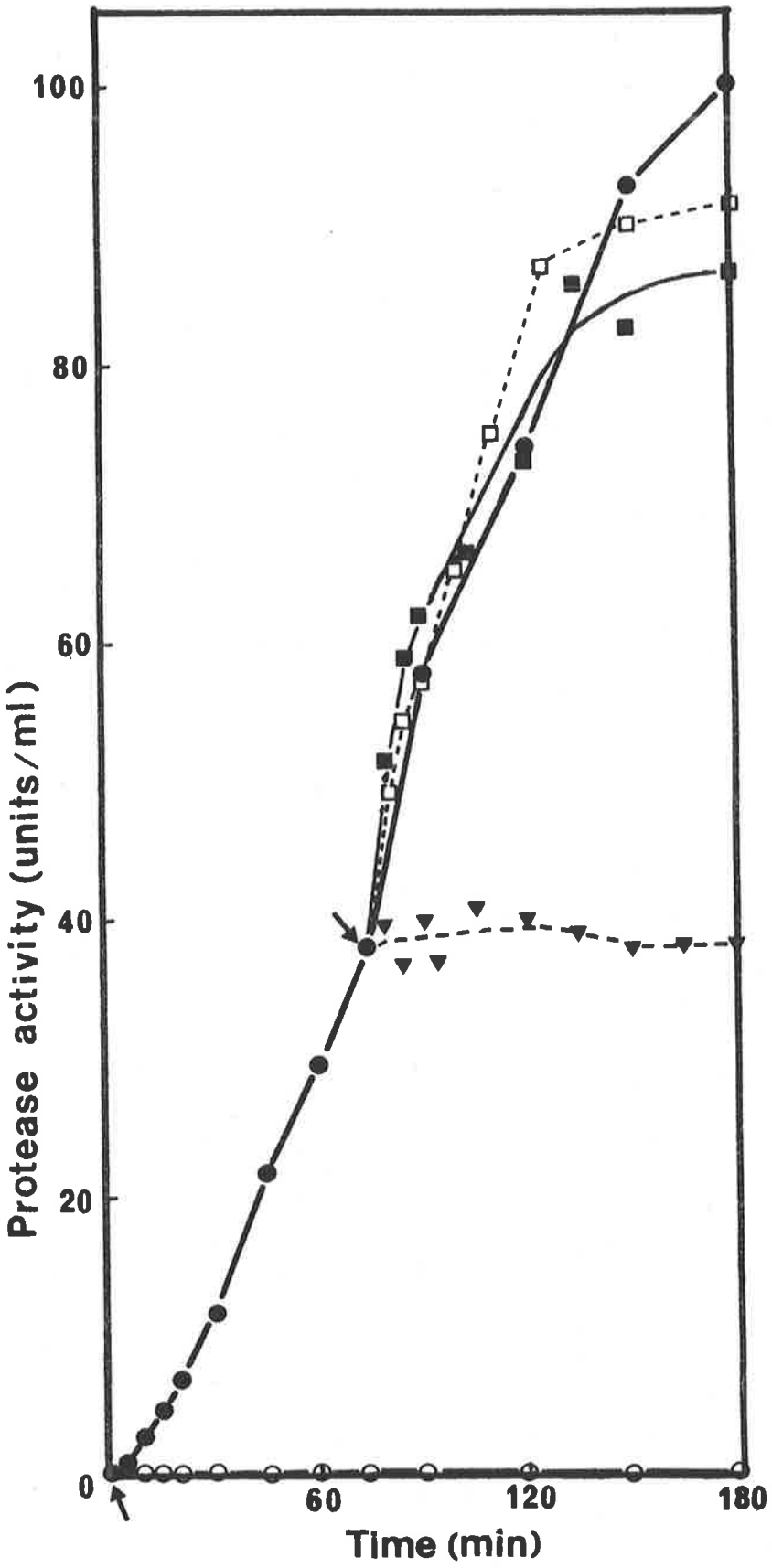

100
80
60
40
20
0
Protease activity (units/ml)
60
120
180
Time (min)

FIGURE 3,18. EFFECT OF RIFAMPICIN ADDITION AT ZERO AND 75 MINUTES ON ^{3}H-URACIL INCORPORATION BY CELLS TRANSFERRED FROM HIGH TO LOW CASAMINO ACIDS MEDIUM.

B.amyloliquefaciens cells were harvested at an OD_{600} of 3.6, washed twice and resuspended to the same cell density in high casamino acids medium and incubated with shaking at 30°C for 75 minutes. Cells were then centrifuged, washed twice and resuspended to the original cell density in low casamino acids medium. 0.8 µc/ml Of ^{3}H-uracil (spec. act. 17 C/mmole) plus 10 µg/ml unlabelled uracil was added at zero time. To make sure that rifampicin inhibition at 75 minutes was not due to reduced level of labelled uracil the addition of label and carrier was repeated after 20 minutes incubation. Rifampicin (0.075 µg/ml) was added to samples of cells at zero and 75 minutes. 0.1 ml Samples were taken and assayed for radioactivity as described in Chapter 2.

-●——●- , no addition of drug.

-□——□- , rifampicin added at zero minutes.

-△——△- , rifampicin added at 75 minutes.

100% incorporation was 9,300 c.p.m.

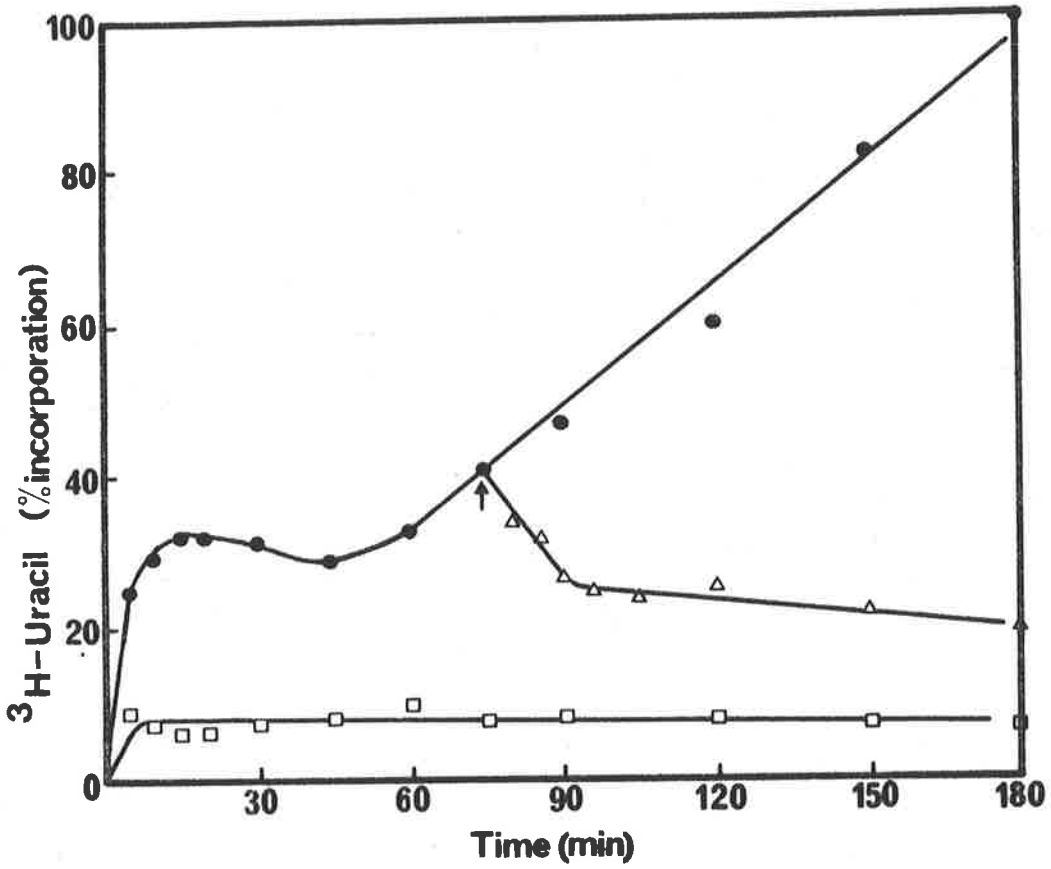

100
80
60
40
20
0
30
60
90
120
150
180
Time (min)
^{3}H-Uracil (%incorporation)

FIGURE 3,19. EFFECT OF RIFAMPICIN ADDITION AT VARIOUS TIMES ON PROTEASE PRODUCTION BY CELLS DURING A PROLONGED INCUBATION IN LOW AMINO ACIDS AFTER TRANSFER FROM HIGH AMINO ACIDS MEDIUM.

B.amyloliquefaciens cells were harvested at an OD_{600} of 3.6, washed twice and resuspended to the same cell density in high amino acids medium and incubated with shaking for 75 minutes at 30°C. Cells were then centrifuged, washed twice and resuspended to the original cell density in low casamino acids medium and incubation continued. Rifampicin (0.075 µg/ml) was added to samples (40 ml) of cells at 75, 120, 180, 240 and 300 minutes. 1 ml Samples were taken, centrifuged and the supernatants assayed for protease activity.

Main figure

All curves are of protease production in low amino acids medium.

-●—●- , no addition of drug.

The remaining curves are of protease production after rifampicin was added at the following times:

-○--○- , 75 minutes.

-▲---▲- , 120 minutes.

-△——△- , 180 minutes.

-■--■- , 240 minutes.

--□---□-- , 300 minutes.

Arrows indicate times of rifampicin addition.

INSERT

Each point represents the total amount of protease produced (plateau level) when rifampicin was added at the times indicated.

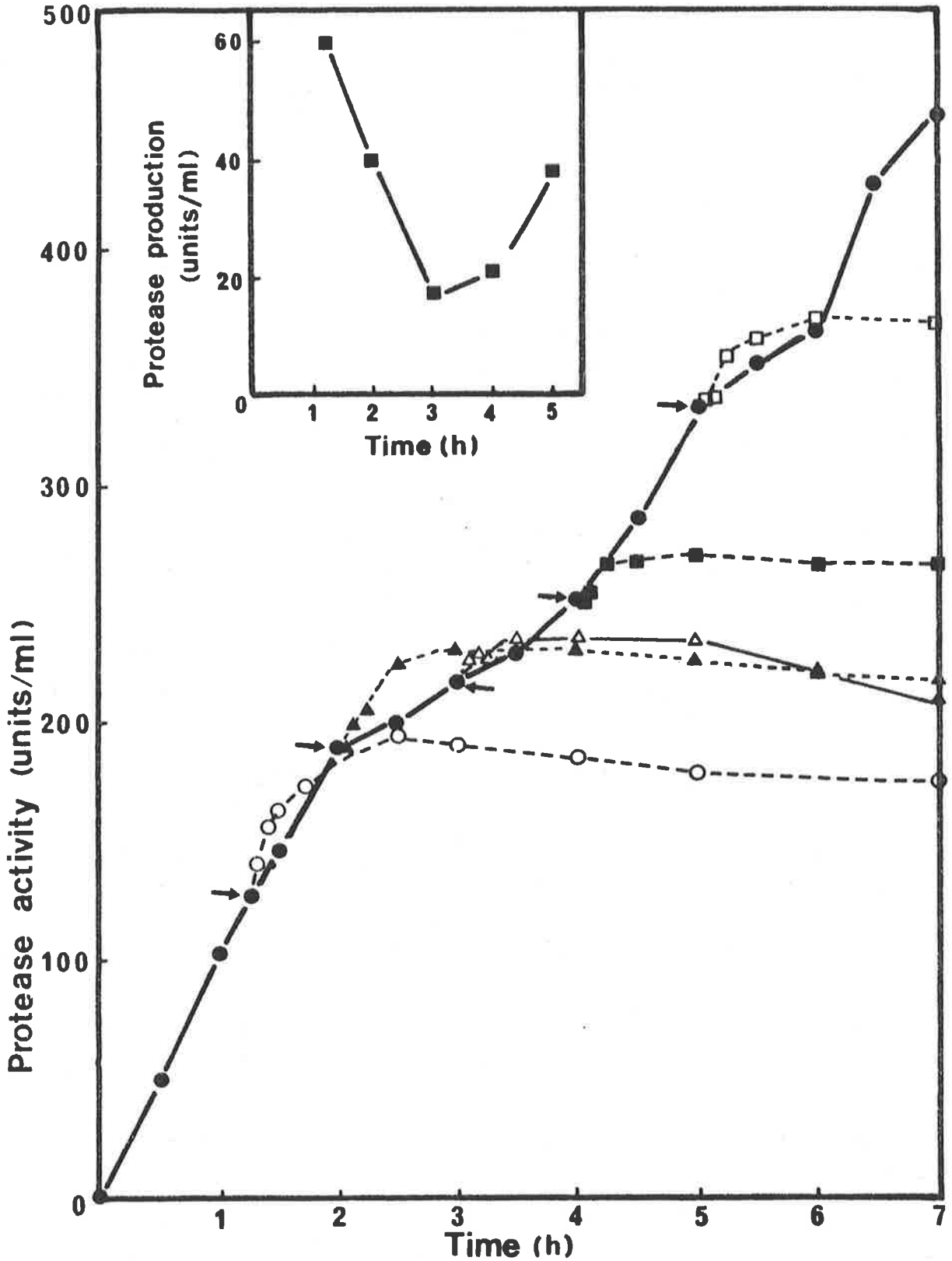

Protease activity (units/ml)
Time (h)
Protease production (units/ml)
Time (h)

FIGURE 3,20. DECREASE IN RIFAMPICIN-INSENSITIVE PROTEASE PRODUCTION DURING INCUBATION IN LOW CASAMINO ACIDS MEDIUM.

B.amyloliquefaciens cells were prepared as described in Fig. 3,19 except that rifampicin (0.075 µg/ml) was added at 75, 90, 120 and 150 minutes and protease production measured as described.

a) All curves are of portease production in low amino acids medium in the presence of rifampicin. For the purposes of illustration the time of rifampicin addition was taken as zero time and the values of protease activity adjusted accordingly. Rifampicin was added at the following times:

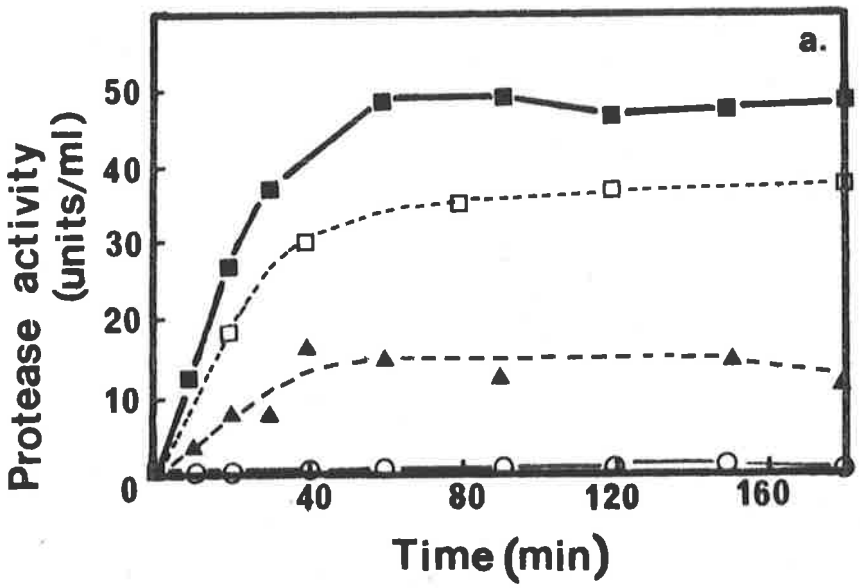
-■—■- , 75 minutes.

--□---□-- , 90 minutes.

-▲--▲- , 120 minutes.

-○—○- , 150 minutes.

b) Each point represents the total amount of protease produced (plateau level) when rifampicin was added at the times indicated.


a.
Protease activity (units/ml)
0
10
20
30
40
50
40
80
120
160
Time (min)

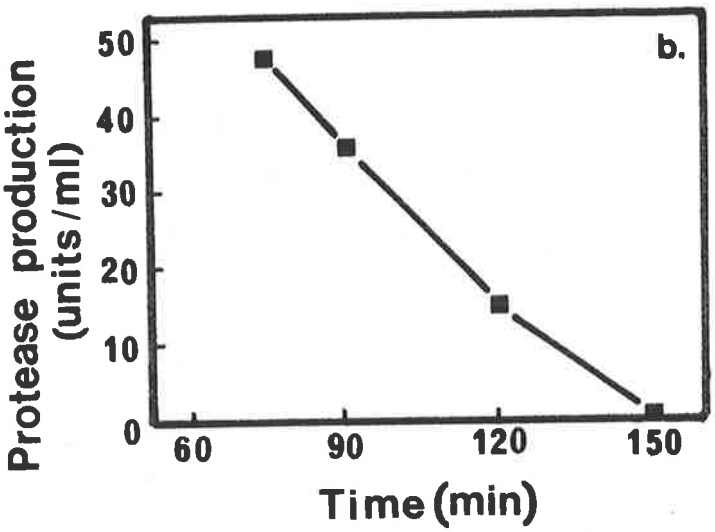

b.
Protease production (units/ml)
0
10
20
30
40
50
60
90
120
150
Time (min)

FIGURE 3,21. EFFECT OF CELL SUPERNATANT ON OBSERVED DECREASE IN RIFAMPICIN-INSENSITIVE PROTEASE PRODUCTION BY CELLS DURING INCUBATION IN LOW AMINO ACIDS MEDIUM.

B.amyloliquefaciens cells were harvested at an OD_{600} of 3.6, washed twice and resuspended to the same cell density in high casamino acids medium and incubated for 75 minutes at 30°C with shaking. Cells were then centrifuged, washed twice and resuspended to the original cell density in low casamino acids medium. Rifampicin (0.075 µg/ml) was added to samples of cells (40 ml) at 60 and 120 minutes. Samples of cells were also centrifuged at 60 minutes and resuspended in fresh low amino acids medium. Rifampicin (0.075 µg/ml) was added at zero and 60 minutes (equivalent of 120 minutes incubation) after this resuspension to samples of these cells. 1 ml Samples were taken, centrifuged and the supernatants assayed for protease activity as described in Chapter 2.

Main figure

Curves are of protease production by control cells (no rifampicin added) in low amino acids medium.

-■——■- , no resuspension.

-□--□- , cells fresh medium at 60 minutes.

INSERT

Curves are of protease production after rifampicin was added at the times indicated below:

-■——■- , no resuspension; rifampicin added at 60 minutes.

-▲——▲- , no resuspension; rifampicin added at 120 minutes.

-□--□- , cells resuspended at 60 minutes and rifampicin immediately added.

-△--△- , cells resuspended at 60 minutes and rifampicin added 60 minutes later (i.e., equivalent to 120 minutes).

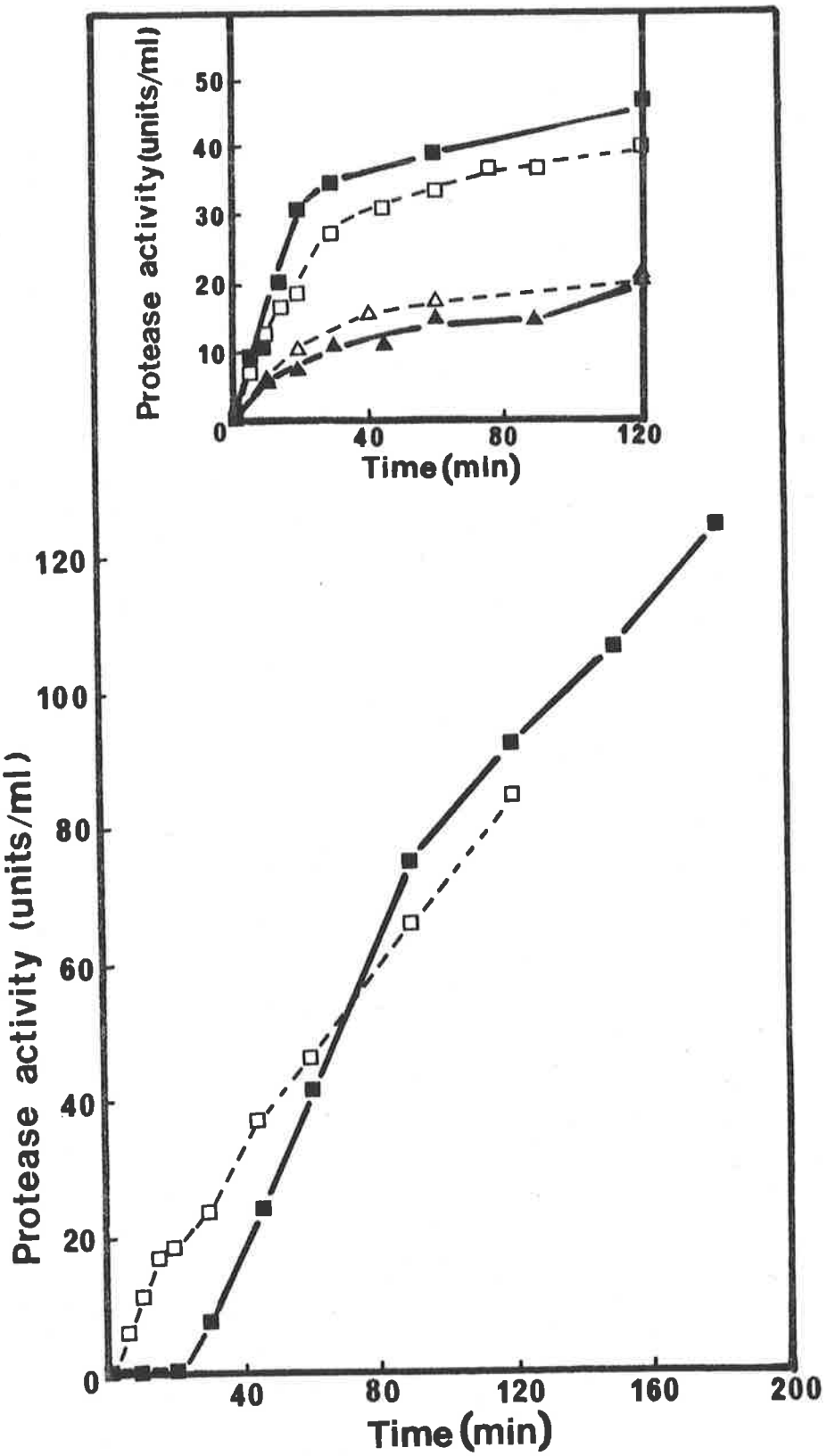

Protease activity(units/ml)
50
40
30
20
10
0
40
80
120
Time(min)
Protease activity (units/ml)
120
100
80
60
40
20
0
40
80
120
160
200
Time (min)

FIGURE 3,22. EFFECT OF RIFAMPICIN ADDITION AT 30 MINUTE INTERVALS ON PROTEASE PRODUCTION BY CELLS DURING A 12 HOUR INCUBATION IN LOW AMINO ACIDS MEDIUM.

B.amyloliquefaciens cells were prepared as described in Fig. 3,19 except that rifampicin (0.075 µg/ml) was added to samples (20 ml) of cells taken at 30 minute intervals during a 12 hour incubation in low amino acids medium. Protease activity was assayed as described.

All curves are of protease production when rifampicin was added at the times indicated below:

a) -●—●- , zero minutes
-○--○- , 30 minutes
-△---△- , 60 minutes

b) -●—●- , 90 minutes
-○--○- , 120 minutes
-△---△- , 150 minutes

c) -●—●- , 180 minutes
-○--○- , 210 minutes
-△---△- , 240 minutes

d) -●—●- , 270 minutes
-○--○- , 300 minutes
-△---△- , 330 minutes

c) -●—●- , 360 minutes
-○--○- , 390 minutes
-△---△- , 420 minutes

f) -●—●- , 450 minutes
-○--○- , 480 minutes
-△---△- , 510 minutes

g) -●—●- , 540 minutes
-○--○- , 570 minutes
-△---△- , 600 minutes
-▲—▲- , 630 minutes
-□--□- , 660 minutes

Protease production by control cells is shown in Fig. 3,23.

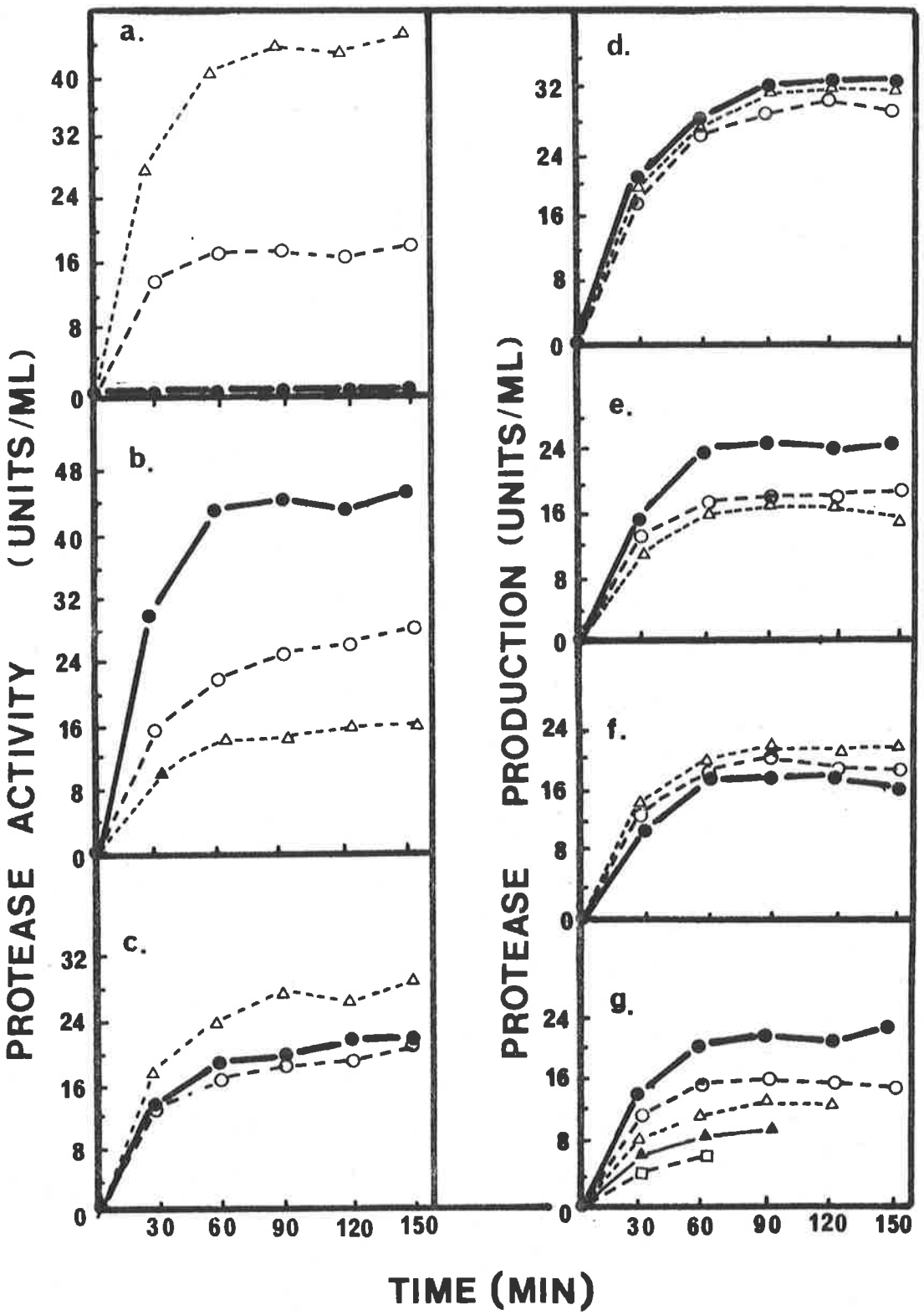
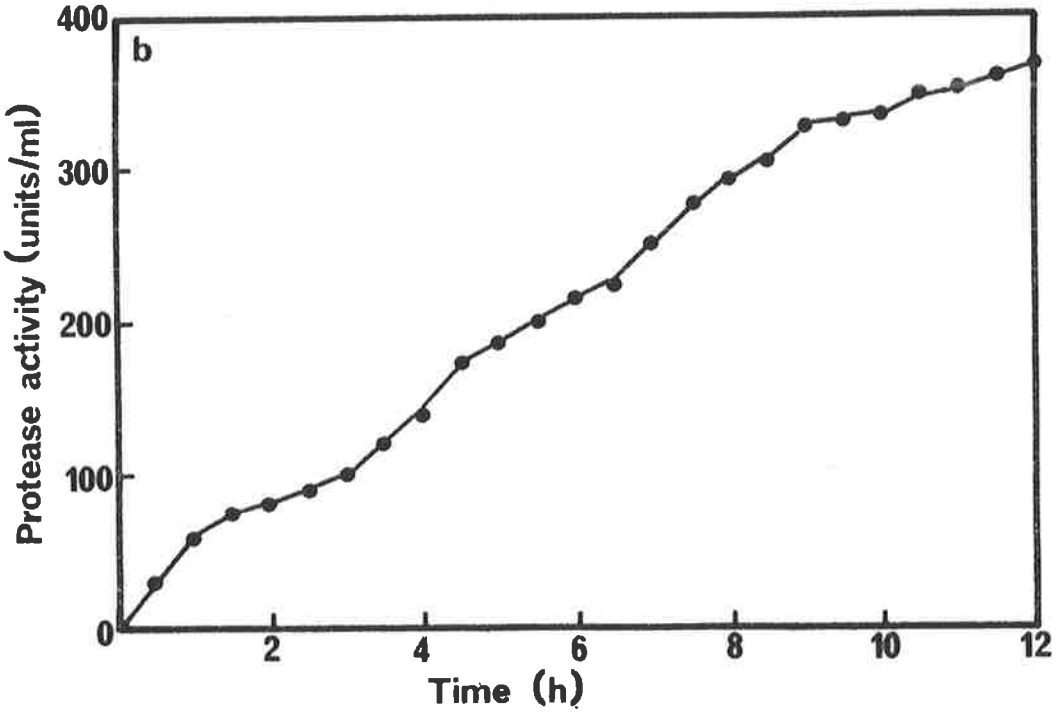

a.
b.
c.
d.
e.
f.
g.
PROTEASE ACTIVITY (UNITS/ML)
PROTEASE PRODUCTION (UNITS/ML)
TIME (MIN)
0
8
16
24
32
40
48
30
60
90
120
150

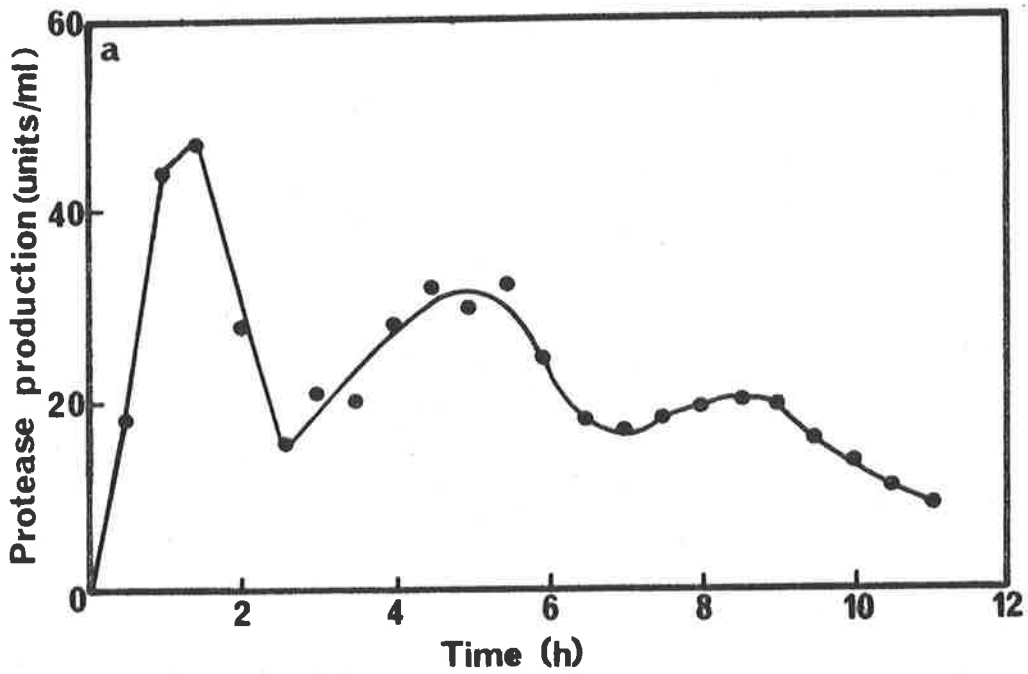
FIGURE 3,23. PROTEASE PRODUCTION AND CHANGE IN RIFAMPICIN-INSENSITIVE PROTEASE PRODUCTION BY CELLS OF B.AMYLOLIQUEFACIENS DURING A 12 HOUR INCUBATION IN LOW AMINO ACIDS MEDIUM.

The details of this experiment are described in Fig. 3,22.

a) Each point represents the total amount of protease produced (plateau level) when rifampicin was added at the times indicated. The individual curves of rifampicin-insensitive protease production are presented in Fig. 3,22.

b) This graph is of protease production by cells during the incubation in low amino acids medium. No rifampicin was added.


b
Protease activity (units/ml)
400
300
200
100
0
2
4
6
8
10
12
Time (h)


a
Protease production (units/ml)
60
40
20
0
2
4
6
8
10
12
Time (h)

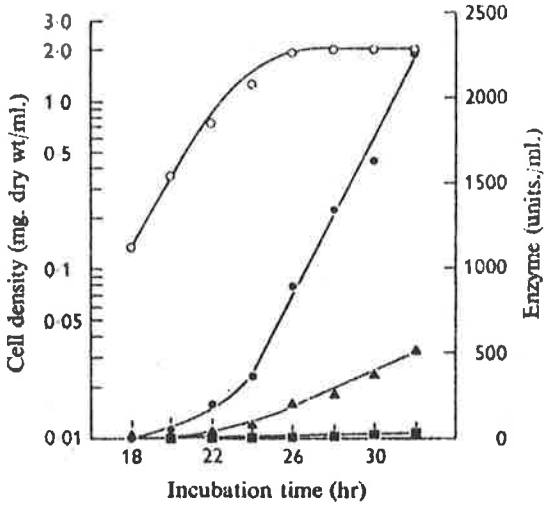


Fig. 4

Fig. 4. Time courses of growth (○) and of proteinase (●), α-amylase (▲) and ribonuclease (■) secretion by *Bacillus subtilis* growing in a complex medium with 1% maltose as the carbon source.

N.B.: This figure was reproduced from Coleman, G. (1967) page 425.

TABLE 3,1

RATES OF PROTEASE SYNTHESIS AND OF INCREASE OF CAPACITY FOR RIFAMPICIN-INSENSITIVE PROTEASE PRODUCTION DURING INCUBATION OF B.AMYLOLIQUEFACIENS CELLS IN LOW AMINO ACIDS.

Experiment	*Rate A	†Rate B
1,2	1.10	1.07
3	0.22	0.23
4	0.32	0.29
5	0.59	0.65
6	0.67	0.75

*Rate A: rate of protease synthesis (units/ml/min).

†Rate B: rate of increase in the capacity for rifampicin-insensitive protease production (units/ml/min), i.e., rate of apparent mRNA pool increase.

- - - - - - - - - - -

The experiments 1 to 6 were all as described in Fig. 3,10. That is, cells were incubated for 75 minutes in high amino acids medium and then transferred to low amino acids medium. Protease synthesis in low amino acids was measured (Rate A). To samples of these cells in low amino acids rifampicin (0.075 µg/ml) was added and the amount of protease produced in its presence was measured. The total amount of rifampicin-insensitive protease produced was plotted against the time of rifampicin addition (as in the insert to Fig. 3,10). The rate of increase of this production was calculated (Rate B).

CHAPTER 4

RESULTS

ISOLATION OF THE PUTATIVE PROTEASE mRNA

4,1 INTRODUCTION

This chapter deals firstly with work directed towards achieving a suitable procedure for the extraction of intact total cellular RNA from Bacillus amyloliquefaciens. This method was then used in the search for the protease mRNA. The oscillation of the apparent mRNA pool for protease described in the previous chapter was used as an aid to identifying the protease mRNA.

4,2 PROCEDURE FOR TOTAL CELLULAR RNA EXTRACTION

The isolation of total cellular RNA from Bacillus amyloliquefaciens had previously been attempted (Both, 1973). It was found that the RNA of this organism was very subject to degradation during normal phenol extraction procedures. When E. coli cells were disrupted and the RNA immediately isolated by conventional phenol extraction techniques, the resultant RNA appeared to be undegraded. In sharp contrast, when B.amyloliquefaciens cells were treated in an identical manner the results were very discouraging. Extensive degradation of ribosomal RNA was clearly evident in preparations. Attempts to avoid this degradation in cells disrupted in the French Pressure Cell were also discouraging. Use of alkaline conditions, inclusion of ribonuclease inhibitors, inclusion of alcohol coupled with lower than freezing temperatures, collection of the lysate directly into a phenol extraction mixture

all failed. Either the cells failed to disrupt (e.g., with ethanol present) or extensive RNA degradation occurred. It was clear from this that disruption of cells in the French Pressure Cell was unsatisfactory and an alternative was sought. The "X-Press" was chosen because in this (relatively little used apparatus) cells are forced through an orifice at temperatures of -25°C. Excellent disruption was obtained with B.amyloliquefaciens and the 'lysate' is recovered in a still frozen state. This apparatus made it possible to take cells directly from whatever experimental conditions were being used and immediately snap freeze them. The frozen lysate could then be allowed to thaw out in an extraction mixture containing suitable ribonuclease inhibitors. Thus the possibility of ribonuclease activity was minimized throughout the whole lysis procedure.

The extraction mixture decided upon for the initial trials was of the following composition: an SDS mixture containing 0.5% (w/v) SDS (sodium dodecyl sulphate), 100 mM-NaCl and 10 mM-EDTA; and a separate solution of 78% (v/v) redistilled phenol in 50 mM Tris, pH 9.0. SDS, an ionic detergent and protein denaturant, inhibits ribonuclease activity. Sodium chloride provides the high ionic strength necessary to maintain RNA conformation and stability. EDTA was included to inhibit any Mg-dependent ribonucleases.

The extraction procedure used is described below. Harvested cells were suspended in a Tris buffer (25 mM-

Tris, pH 8.5, 5 mM-Mg acetate), snap frozen in the 'X-Press' cell (precooled in ethanol-dry ice), lysed and the frozen lysate (3 ml) added to the above mentioned SDS solution (12 ml). An equal volume (15 ml) of the phenol solution was then added. This mixture was shaken vigorously for 15 minutes and the aqueous and phenol layers separated by centrifugation at 7,000 x g for seven minutes at room temperature. The aqueous layer was carefully aspirated and re-extracted in similar fashion a further two times. The final aqueous layer was then extracted twice with ether to remove residual phenol, the ether being then removed by bubbling nitrogen through the solution. Finally, the RNA was precipitated by addition of 2.5 volumes of ethanol containing 100 mM sodium acetate at pH 6.0 and allowing this solution to stand overnight at -20°C. The precipitate was collected by centrifugation at 30,000 x g for 30 minutes at 2°C. The RNA was analysed by polyacrylamide gel electrophoresis using 3% acrylamide gels (Fig. 4,1a). Using this technique, RNA was prepared which appeared reasonably free from degradation except for the possibility of slight breakdown of the 23S ribosomal RNA. To overcome this possibility two compounds, which had been reported by Kirby (1965) and Loening (1967) to promote more efficient deproteinization of RNA, were included in the extraction mixtures. 1% (w/v)-TINSA (tri-iso-propyl-naphthalene-sulphonic acid), a detergent, was added to the SDS-solution, and redistilled m-cresol (8%, v/v) was added to the phenol mixture. The RNA prepared using these solutions was analysed on 4% polyacrylamide gels containing 98% (v/v)-

formamide. The RNA appeared to be intact and not nicked or degraded (Fig. 4,1b)

A final alteration to the above method was replacement of a portion of the phenol in the extraction mixture with chloroform, such that phenol and chloroform were present in the ratio 1:1. Chloroform is routinely added during the extraction of poly-A-containing mRNAs to overcome the poor yield resulting from these hydrophobic RNA molecules tending to remain at the phenol-aqueous phase interface. It was included in this case to insure against the possible losses which would occur if the protease mRNA were in any way hydrophobic (in view of speculation that the mRNA may associate with membranes, a not impossible situation). As a further precaution, the first phenol and interface regions were also re-extracted with an equal volume of SDS-solution and the resultant aqueous phases from the two extractions pooled. These additional steps neither improved nor impaired the RNA profile as shown (Fig. 4,1b).

It is of interest to note that a method successfully employed for the extraction of mammalian poly-A containing mRNAs (Dr. D. Kemp, personal communication) was totally unsuccessful for the extraction of RNA from Bacillus amyloliquefaciens. In this procedure the lysate was extracted in 1 M-Tris, pH 9.0 containing 10% (w/v) Sarkosyl (a detergent) and phenol : chloroform in the ratio 1:1. This resulted in a preparation of completely degraded RNA and indicated that the inclusion of particularly EDTA in the

extraction mixture was essential. More recently, the use of guanidine thiocyanate, a denaturant which was used by Ullrich et al. (1977) so successfully on pancreatic RNA, was applied to B.amyloliquefaciens, the frozen lysate being thawed in the denaturant solution. Extensive degradation was evident in the preparation of RNA obtained and the method was not used again.

4,3 EXTRACELLULAR RIBONUCLEASE ACTIVITY

To extract RNA from cells containing the maximum apparent mRNA pool it was important to reduce the time between achieving this pool in the cells and lysing them. Routinely, cells were twice washed in fresh medium to remove the accumulated extracellular enzymes, including ribonuclease, from the external medium. Although an internal inhibitor for extracellular ribonuclease does exist (Smeaton et al., 1965; Smeaton and Elliott, 1967), removal of this enzyme was a further precaution against RNA degradation. Briefly centrifuging the cells and then resuspending them in a Tris-buffer (25 mM-Tris, pH 8.5 containing 5 mM-Mg acetate), a process taking four minutes was found to reduce the extracellular ribonuclease activity from 40 units per ml to a virtually zero value and resulted in a preparation of intact RNA.

4,4 USE OF SUCROSE DENSITY GRADIENTS FOR RNA ANALYSIS

At this stage it was decided to change from using

polyacrylamide gel electrophoresis as a means of RNA analysis to sucrose density gradient analysis. Larger quantities of RNA could be loaded on a gradient and there was not the interference of background 'noise' that had become a problem with the gel-system. The sucrose gradients were prepared and analysed as described in Chapter 2, Methods.

4,5 RNA PREPARED FROM CELLS AFTER INCUBATION FOR VARYING TIMES IN LOW AMINO ACIDS

Cells of Bacillus amyloliquefaciens harvested in late log phase of growth are capable of synthesizing protease de novo for at least 60 minutes in the presence of inhibitors of transcription. It was proposed that this capacity was due to the presence of a pool of mRNA for protease which sustained protease production in the absence of transcription (Both et al., 1972). In Chapter 3, it was shown that this capacity could be depleted by incubation in high amino acids medium for 75 minutes and then, by transferring these cells to low amino acids medium, this capacity was rapidly restored to a level greater than that originally present. Thus cells at zero time of incubation in low amino acids do not contain a pool of protease mRNA (assuming, of course, that the hypothesis is correct), and those after 75 minutes incubation contain a large pool. Therefore, as a preliminary experiment to identify this mRNA, total cellular RNA was prepared from cells in these two conditions, by the method discussed above, and analysed by sucrose density gradient centrifugation (Fig. 4,2).

A small peak of RNA in the 13S region was present on the optical density profile of total cellular RNA prepared from cells after 75 minutes incubation in low amino acids medium (Fig. 4,2a), that is, from cells presumed to contain a pool: this peak was not obvious in the profile of RNA prepared from cells presumed to have no pool (Fig. 4,2b). [Note that on the former gradient 50 µg RNA was loaded and 100 µg on the latter.] The molecular weight of protease had been estimated by amino acid analysis to be 27,950 (W. Carey, 1966). A protein of this size would require an mRNA molecule with an S-value of approximately 12. Unfortunately, some breakdown products of ribosomal RNA also appear near this 13S region.

4,5a Effect of Rifampicin on the RNA Profile

Next, RNA was again prepared from cells incubated for 75 minutes in low amino acids medium, but on this occasion ^{14}C-uracil was added to the cells at the time of transfer to the low amino acids medium. After 75 minutes of incubation rifampicin (0.075 µg/ml) was added to the cells. In the presence of rifampicin the capacity for protease production decays exponentially such that it is completely lost after 60 minutes (Chapter 3). It would be expected that after 10 minutes incubation in the presence of rifampicin almost all mRNA species of normal short half-life would have decayed (as is evidenced by cessation of ^{14}C-phe incorporation into cellular protein - see Chapter 3). However, if a pool of protease mRNA existed, some of this will still be present after 10 minutes incubation with

rifampicin and a smaller amount after 20 minutes incubation. Thus, after 0, 10 and 20 minutes incubation in the presence of rifampicin samples of cells were removed for RNA extraction. Equal amounts of the RNA preparations were then run on sucrose density gradients. Optical density measurements (Fig. 4,3a, solid line) showed that the 13S peak was present in RNA prepared from cells at the time of rifampicin addition, but was seen to decrease during the subsequent incubation (Fig. 4,3b and 3c). The 23S and 16S ribosomal RNA peaks did not change. The gradients (12 ml) were fractionated into 0.3 ml samples and the radioactivity in them measured by TCA-precipitation of the RNA and liquid scintillation counting of the precipitates. The profiles constructed from this analysis (Fig. 4,3a, dashed line) showed that a large amount of radioactivity was associated with the 4-16S RNA. After rifampicin treatment for 10 minutes the amount of radioactivity in this region drops markedly (Fig. 4,3b). After a further 10 minutes rifampicin treatment there is a further drop in radioactivity associated with RNA around the 13S region (arrow) and there is a slight increase in radioactivity associated with the lower molecular weight RNA species (5 to 8S) (Fig. 4,3c). The whole experiment was repeated and exactly the same pattern of results were obtained (Fig. 4, 4a, b and c). Also reproduced was the initial decrease and subsequent increase in radioactivity associated with the RNAs of S-values 5 to 8. The optical density profiles reflect this trend to a certain extent. The radioactivity profiles are difficult to interpret because of the rela-

tively large amount of radioactivity associated with RNA species of 4 to 16 S-value. There does, however, appear to be a discrete peak of radioactivity associated with the 13S region (Fig. 4,3 and 4,4) which decreases during the incubation in the presence of rifampicin.

In the experiment described above, some cells were also incubated for 100 and 150 minutes in the low amino acids medium. During this time the size of the apparent mRNA pool would be expected to decrease (Chapter 3). In the optical density profile obtained from cells incubated for 100 minutes (Fig. 4,5a) the 13S peak is still clearly visible. The amount of radioactivity associated with RNA from the 5 to 16S region is reduced (compared with the 75 minutes profile; Fig. 4,3a or 4,4a) though a discrete peak of radioactivity is still apparently associated with the peak of 13S RNA (arrow). From the optical density tracing of RNA from cells incubated for 150 minutes in low amino acids, it appears that the 13S RNA peak is reduced, but by no means absent (Fig. 4,5b). The radioactivity in the 4 to 13S region is also reduced. These latter profiles bear some resemblance to those of Fig. 4,4c, where cells incubated for 75 minutes were then treated with rifampicin for 20 minutes.

Next, RNA was prepared from cells which had been incubated for 150 and 300 minutes in low amino acids medium. It had been shown (Chapter 3) that after 300 minutes incubation cells had once again regained the capacity for rifampicin-insensitive protease production. On this

occasion the 13S RNA peak was clearly absent from the optical density profile of RNA from cells incubated for 150 minutes in low amino acids medium (Fig. 4,6a). It is, however, present in the profile of RNA from cells incubated for 300 minutes (Fig. 4,6b). From the radioactivity profiles of these two RNA preparations it appears that there is a small amount of radioactivity associated with RNA around the 13S region in the 150 minute RNA and that this is increased in the 300 minute profile (Fig. 4,6a and 4,6b).

4,6 RNA FROM CELLS AT VARIOUS STAGES DURING OSCILLATION OF THE 'POOL'

The aim of the next experiment was to obtain a more definitive answer to the question of whether the 13S peak of RNA oscillated in concert with the apparent pool of mRNA. If it did it would constitute evidence, albeit circumstantial, that the 13S peak was worth investigating further. To do this cells were incubated in high amino acids medium for 75 minutes, transferred to low amino acids medium and samples of cells taken at 30 minute intervals for RNA analysis. It was necessary also to determine the pattern of oscillations of the apparent mRNA pool, or more accurately, to pinpoint the inflexion points at which mRNA pool accumulation switched to pool decrease. The direct way to do this was to measure rifampicin-insensitive protease synthesis at each point. However, such a procedure would have rendered the experiment extremely cumbersome and a simpler method was used. It had been noted that

the change in rate of protease synthesis corresponded to the change from apparent mRNA pool increase to decrease or vice-versa. Reference has already been made to this in Chapter 3. As samples for RNA analysis were to be taken at no shorter than half-hourly intervals, the use of the change in rates of protease synthesis to determine whether the apparent mRNA pool was increasing or decreasing was deemed adequate. From the progress curve of protease synthesis, constructed from the results of the above experiment (Fig. 4,7), it could be deduced that the apparent pool of mRNA increased for 90 to 120 minutes, then decreased until approximately 240 minutes of incubation and then increased again. 100 µg Of RNA prepared from each cell sample was analysed by sucrose density gradient analysis (Fig. 4,8a to 4,81). From the optical density tracings it could be seen that the size of the 13S peak gradually increased during the first 90 minutes of incubation. It then decreased rapidly from 120 to 180 minutes and then, in the 300 minute sample, the last sample taken, the peak was present again. Thus the 13S RNA peak appeared to oscillate in the same fashion as did the capacity for rifampicin-insensitive protease production. That is, it appeared to be closely associated with the proposed pool of mRNA for protease. This result at any rate encouraged further work on the 13S RNA.

4,7 ISOLATION OF THE 13S RNA

The best proof that this 13S RNA species was in fact

the protease mRNA would be if this RNA directed the synthesis of protease in a cell-free system. To this end the 13S RNA was purified by further sucrose density gradient analysis. That is, the 13S peak was removed (hand fractionated) from the sucrose gradient (10-40%) of total cellular RNA. This RNA was then precipitated and re-analysed on another sucrose density gradient (5-20%) and again the 13S peak removed. The final 13S peak (representing 4.6-4.9% by weight of the total RNA extract) appeared as a single peak on the 5-20% sucrose density gradient (Fig. 4,9b), though on many occasions it appeared that there could be two species of very similar molecular weight as suggested here by the slight skewing of the peak (Fig. 4,9b).

4,8 PREPARATION OF LARGER QUANTITIES OF RNA

The next task was to isolate the 13S RNA species in sufficiently large quantities to be useful for cell-free analysis.

4,8a Poly-A Content

It had been reported that some (unclassified) bacterial RNAs contained poly-A tracts (Nakazato *et al.*, 1975; Ohta *et al.*, 1975 and also Edmonds and Kopp, 1970). If the poly-A tract on the mRNA is at least 15-20 residues long the mRNA will bind tightly to oligo-dT tracts attached to a cellulose matrix, thus providing an ideal method for preparation of large quantities of the mRNA concerned.

However, when both the isolated 13S RNA and the total cellular RNA prepared from Bacillus amyloliquefaciens were analysed for poly-A content on an oligo-dT-cellulose column, no species of RNA remained bound, thus indicating that none of the RNA prepared from this organism contained suitably long tracts of poly-A.

4,8b French Pressure Cell Lysis

It had been shown by Both (1973) that the French Pressure Cell was not suitable for preparation of intact RNA from B.amyloliquefaciens. However, because of its sheer convenience for the large scale lysis of cells, it was attempted again using the extraction solutions described here. Lysis was also performed with the ribonuclease inhibitor heparin (5 mg/ml) being present. Each attempt was unsuccessful.

It was decided not to persue the use of the French Pressure Cell any further for the present. Instead, large numbers of smaller RNA preparations were made using the 'X-Press' apparatus and cell suspensions of B.amyloliquefaciens which had been concentrated after incubation in low amino acids medium to build up the apparent mRNA pool.

4,8c Storage of Cells and of 13S RNA

It was found that storage in liquid nitrogen of snap-frozen cells containing an apparent mRNA pool with the view to preparing RNA from these cells at a later stage, was

satisfactory if the storage time did not exceed six weeks. After this time some degradation of ribosomal RNA was apparent.

A suitable method of storage of extracted RNA was found to be storage of small, freeze-dried aliquots at -20°C with dessicant.

4,9 DISCUSSION

The use of the RNA extraction technique described in this chapter resulted in total cellular RNA preparations which, on analysis by formamide gels, appeared undegraded (Fig. 4,1b). The 'X-Press' apparatus, which made it possible to keep cells frozen throughout the whole lysis procedure, was important in overcoming the problems of RNA degradation previously encountered (Both, 1973). When the French Pressure Cell was used again at a later stage for the large scale lysis of cells, the RNA thus prepared was degraded. Two other extraction techniques successfully used on other systems were not suitable for extraction of RNA from Bacillus amyloliquefaciens. Thus it seems that conventional RNA extraction techniques, proven on other systems, are not necessarily successful for preparation of B.amyloliquefaciens RNA.

As discussed earlier in this thesis, the capacity of harvested cells of B.amyloliquefaciens to synthesize protease de novo in the presence of inhibitors of transcription had been attributed to the presence in these cells of a pool of preformed protease mRNA which allowed continued protease production in the absence of transcription. Cells which were incubated for 75 minutes in high amino acids medium lost this capacity, but on transfer to low amino acids medium this capacity was rapidly regained. The first approach to identifying the protease mRNA was, therefore, to compare the RNA prepared from cells with this capacity to that from cells without, that is, from

cells incubated for 75 minutes and 0 minutes in low amino acids, respectively. This comparison indicated that a 13S species of RNA may be associated with the capacity for rifampicin-insensitive protease production. In the previous chapter it was shown that this capacity oscillated such that at 75, 100 and 300 minutes a pool of mRNA existed while at 150 minutes it was considerably reduced. When RNA was extracted from cells incubated for these times, a 13S species of RNA was found to be present when the proposed mRNA pool was present (Fig. 4,3; 4,4; 4,5 and 4,6). In a subsequent experiment RNA was prepared from cell samples taken at half-hourly intervals during a 300 minute incubation in low amino acids medium (Fig. 4,7 and 4,8). During this time the apparent mRNA pool increased, decreased and then increased again. The 13S RNA peak also appeared to oscillate in the same fashion, thus suggesting that this peak of RNA could be the mRNA pool being sought.

It was thought that the dramatic increase in size of apparent mRNA pool seen during the first 75 minute incubation in low amino acids (Fig. 3,10) would provide an ideal means of labelling this mRNA with radioactive uracil and subsequently identifying it. When RNA was prepared from such cells, a large amount of radioactivity was found to be associated with RNA sedimenting in the 4 to 16S region. Nevertheless, there did appear to be a discrete peak of radioactivity associated with the 13S RNA, but the large amount of radioactivity incorporated into species sedimenting in the immediate area of the 13S RNA reduced

its significance. This difficulty may be overcome by using shorter labelling periods, though it is to be expected that in the low amino acids medium mRNA synthesis for other enzymes, e.g., amino acid synthesizing enzymes, would also be rapid.

When rifampicin was added to the above mentioned cells at 75 minutes and incubation continued for a further 10 and 20 minutes before RNA extraction, the 13S RNA peak decreased (Fig. 4,3 and 4,4 parts b and c). The amount of radioactivity in this region also decreased, but the pattern of change in radioactivity in other regions was such that the results are at this stage difficult to interpret. [These changes may not be insignificant. It was also noted that the amount of RNA in the 5 to 8S region increases on incubation in the presence of rifampicin (Fig. 4,3 and 4,4). This region also increases when the apparent mRNA pool decreases during the oscillations (Fig. 4,8). The meaning of these observations remains unclear, but are noted.] The only clear observation is one arising from the comparison of the optical density and radioactivity profiles of RNA prepared from cells incubated for 150 and 300 minutes in low amino acids (Fig. 4,6a and 6b). Here it can be seen that at 150 minutes both the 13S RNA peak and the radioactivity associated with it are reduced in comparison to the 300 minute profiles, as would be expected.

From these results the tentative conclusion can be made that a 13S RNA species oscillates in concert with the capacity for rifampicin-insensitive protease production,

that is, with the proposed mRNA pool. Thus the 13S RNA species has properties consistent with it being the protease mRNA and is worthy of further investigation.

To further investigate the phenomenon of the oscillating mRNA pool described above, experiments using cDNA copied from the 13S RNA would be useful. Firstly, by hybridizing this cDNA to fractions taken from a sucrose density gradient of total RNA, it could be determined whether the 13S RNA is a ribosomal RNA breakdown product, and also, if there are any lower molecular weight RNAs derived from the 13S RNA. Secondly, the amount of 13S RNA present at various stages during the oscillations could be more accurately determined by hybridization experiments with cDNA prepared from 13S RNA. In the experiment envisaged the time taken for maximum hybridization of cDNA to a given quantity of total RNA containing the maximum mRNA pool would be taken as the 100% mRNA value. The time taken for maximum hybridization of cDNA to total RNA samples from various stages of pool oscillation would be related to this value. It is predicted that the times of maximum hybridization would reflect the oscillation pattern. Hybridization of cDNA to fractions of total RNA samples (from various times during the oscillations) taken from sucrose density gradients may also reveal a precursor relationship for the protease mRNA and the processing to smaller molecular weight RNAs during its breakdown. Of course, any conclusions from this work about the protease mRNA has to await conclusive proof that the 13S RNA in

question is in fact the protease mRNA.

It was noted during this work that total RNA extracted from cells after pool decrease, e.g., at 150 minutes (Fig. 4,6a), was relatively more highly labelled than that extracted after pool increase, e.g., at 75 minutes (Fig. 4,3a) and 300 minutes (Fig. 4,6b). This occurred even though the same quantities of ^{3}H-uracil were added on each occasion and the rate of incorporation of ^{3}H-uracil into whole cells appeared constant and linear throughout the whole time period. These results could indicate that the internal levels of nucleotides and amino acids may change during the incubation, perhaps due to ribosome metabolism resulting from the incubation in the low amino acids medium, and cause this effect. This phenomenon has not been studied further, but it may be worthy of future investigation because of the possibility of its involvement, directly or indirectly, in the regulation of protease synthesis.

However, any further realistic studies on the regulation of protease synthesis, and the concomitant regulation of the apparent mRNA pool, must await definite proof that the protease mRNA has been identified. The next chapter is concerned with this.

FIGURE 4,1. OPTICAL DENSITY PROFILES OF TOTAL CELLULAR RNA FROM B.AMYLOLIQUEFACIENS.

Total cellular RNA from B.amyloliquefaciens was prepared as described in the text.

a) 3% Polyacrylamide gel electrophoresis (aqueous)

The gel system is described in Chapter 2. 20 µg RNA in 10 µl loading solution was electrophoresed for 3 hours at 5 mA/gel at 4°C. Gels were stained and the optical density (600 nm) of the RNA bands measured as described in Chapter 2.

b) Formamide-polyacrylamide gel electrophoresis

The gel system is described in Chapter 2. 20 µg RNA in 10 µl loading solution was first heated at 60°C for 2 minutes, cooled and electrophoresed for 15 hours at 1 mA/gel. Gels were stained and the optical density (600 nm) of the RNA bands measured as described in Chapter 2.

FIG. 4,1

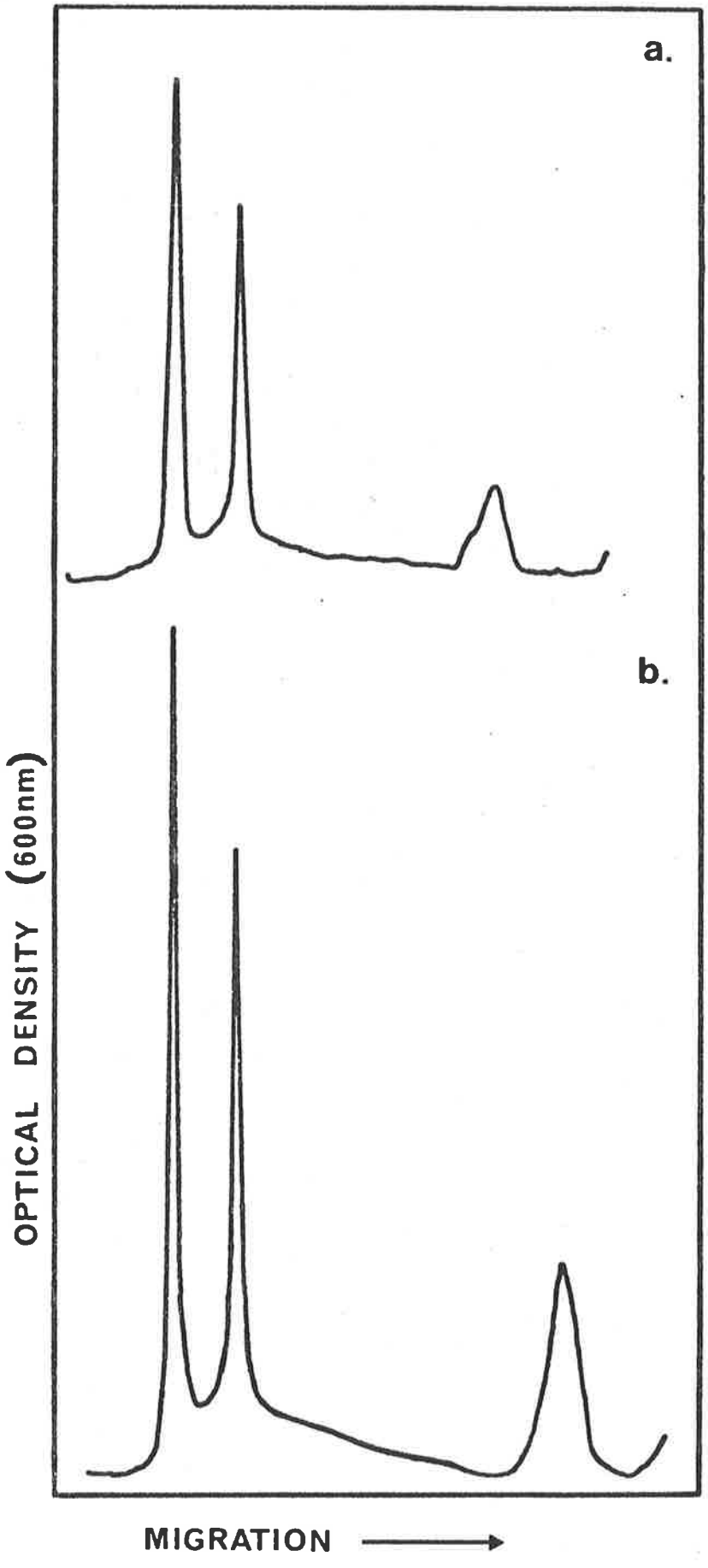


FIGURE 4,2. OPTICAL DENSITY PROFILES OF TOTAL CELLULAR RNA PREPARED FROM CELLS INCUBATED IN LOW AMINO ACIDS MEDIUM FOR ZERO AND 75 MINUTES.

B.amyloliquefaciens cells were incubated in high amino acids medium and transferred to low amino acids medium as described in Fig. 3,10. Cell samples were removed for RNA extraction after zero and 75 minutes incubation in low amino acids.

Total cellular RNA was prepared as described in the text.

The RNA sample, dissolved in TEN buffer was centrifuged on a 10-40% sucrose-density gradient for 15¼ hours at 34,000 r.p.m. and 3°C in a 6 x 14 ml swing-out rotor in an MSE Super Speed 65 ultracentrifuge. The OD_{254} of the gradient was recorded as described in Chapter 2.

a) 50 µg RNA prepared from cells incubated for 75 minutes in low amino acids as described.

b) 100 µg RNA prepared from cells at the start of incubation in low amino acids.

The arrow indicates the position of 13S RNA

FIG. 4,2

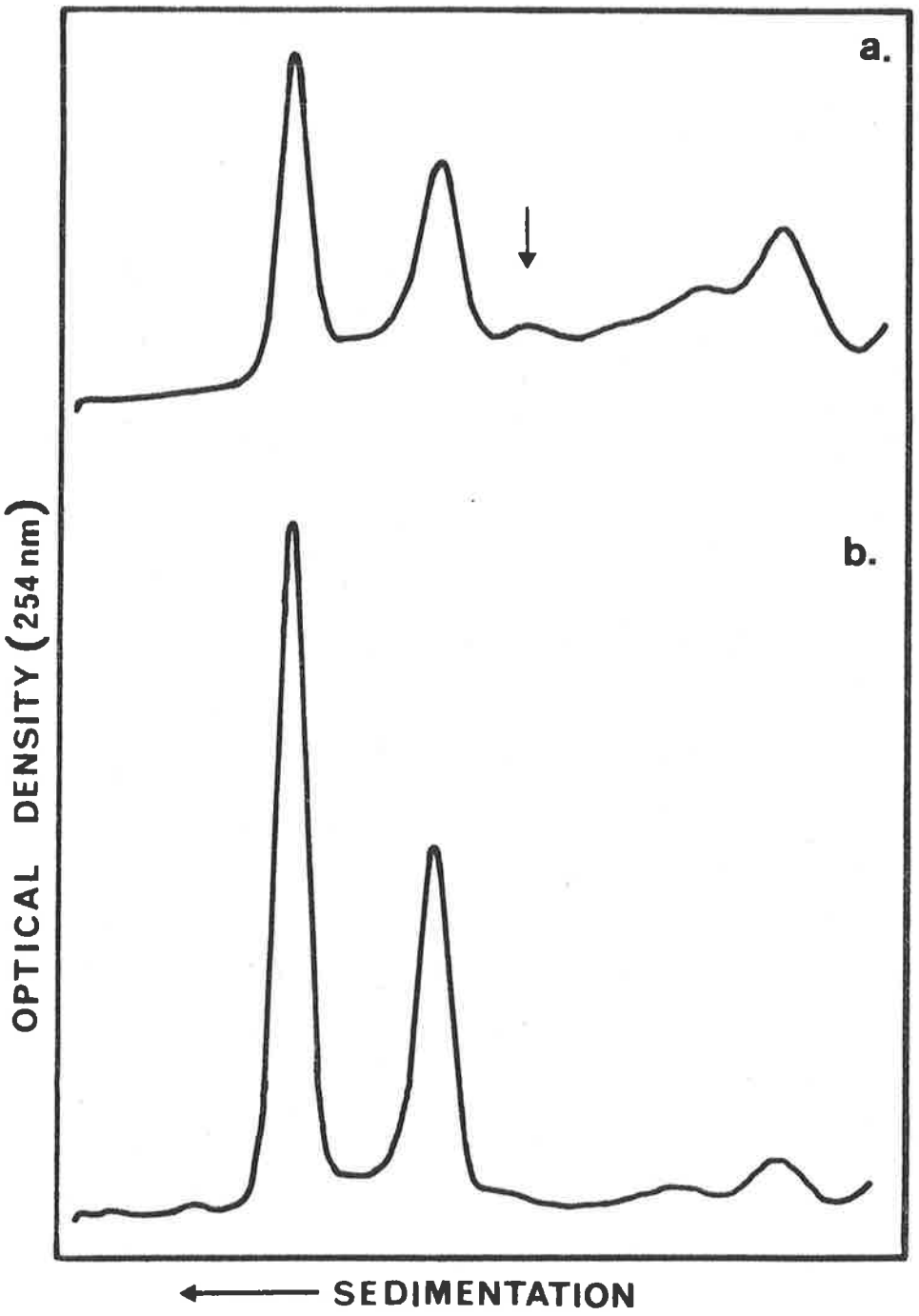


FIGURE 4,3. THE OPTICAL DENSITY AND RADIOACTIVITY PROFILES OF TOTAL CELLULAR RNA PREPARED FROM CELLS INCUBATED FOR 75 MINUTES IN LOW AMINO ACIDS AND TREATED WITH RIFAMPICIN.

B.amyloliquefaciens cells were prepared as described previously (Fig. 3,10). 12 µC Of ^{14}C-uracil (spec. act. 55 mC/mmole) plus 150 µg of unlabelled uracil was added to 15 ml of cells at the beginning of incubation in low amino acids. After 75 minutes incubation, 5 ml of cells were removed for RNA extraction (Fig. 4,3a). Rifampicin (0.075 µg/ml) was added to the remaining cells. After 10 minutes and 20 minutes incubation in the presence of rifampicin, 5 ml samples of cells were removed for RNA extraction (Fig. 4,3b and 3c, respectively).

Total cellular RNA was prepared as described in the text.

100 µg Of each RNA dissolved in TEN buffer (200 µl) was centrifuged on a 10-40% sucrose density gradient at 34,000 r.p.m. and 3°C for 15¼ hours in a 6 x 14 ml swing-out rotor in an MSE Super Speed 65 ultracentrifuge. The OD_{254} was recorded and 0.3 ml fractions were taken for radioactivity measurements as described in Chapter 2.

Fig. 4,3 comprises three separate diagrams presented on three pages.

They are OD_{254} and radioactivity profiles of RNA prepared from:

a) cells incubated for 75 minutes, i.e., zero minutes of rifampicin treatment;

b) 10 minutes of rifampicin treatment;

c) 20 minutes of rifampicin treatment.

The arrow indicates the position of 13S RNA.

FIG. 4,3a

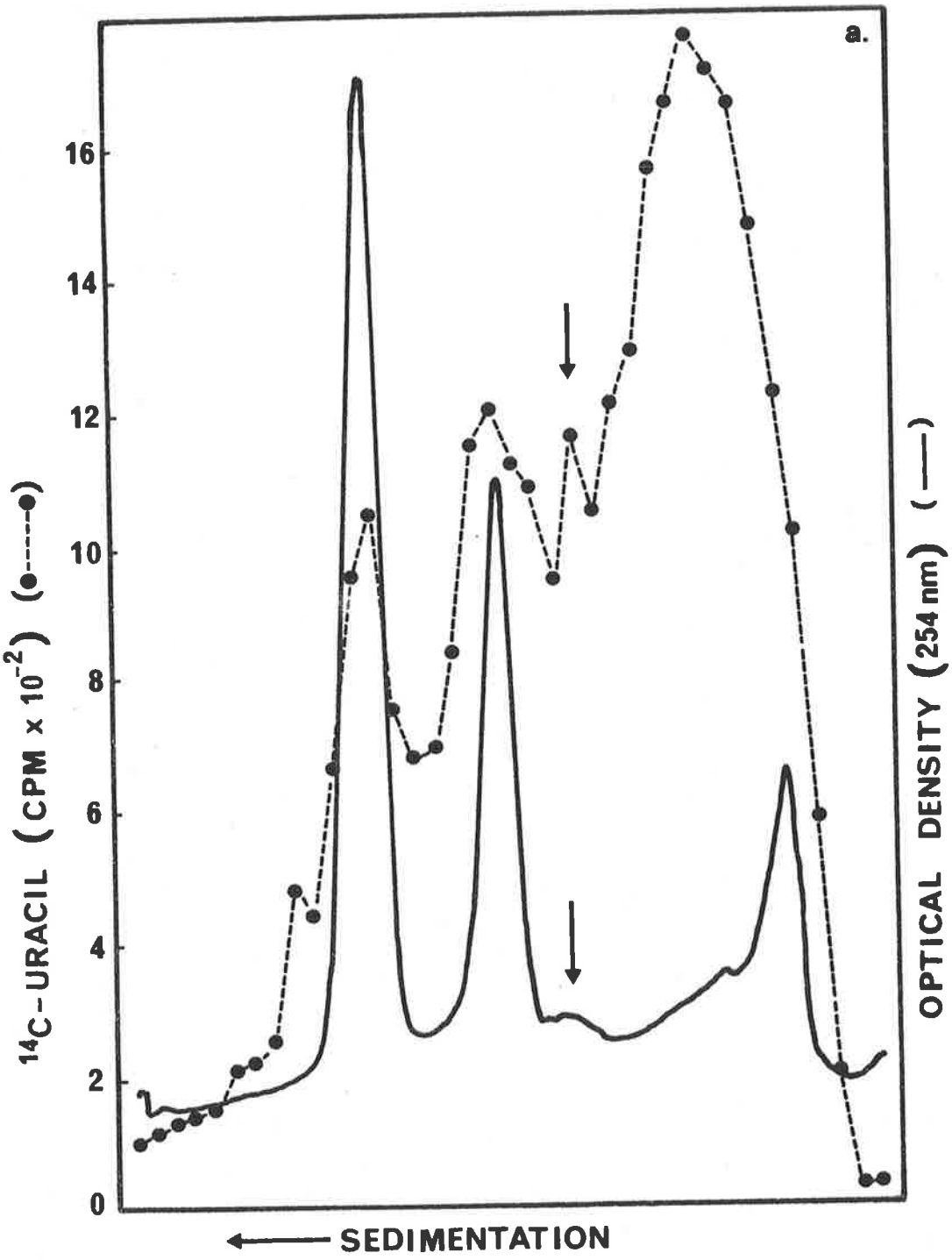

a.
16
14
12
10
8
6
4
2
0
^{14}C-URACIL (CPM x 10^{-2}) (●----●)
OPTICAL DENSITY (254 nm) (——)
SEDIMENTATION

FIG. 4,3b

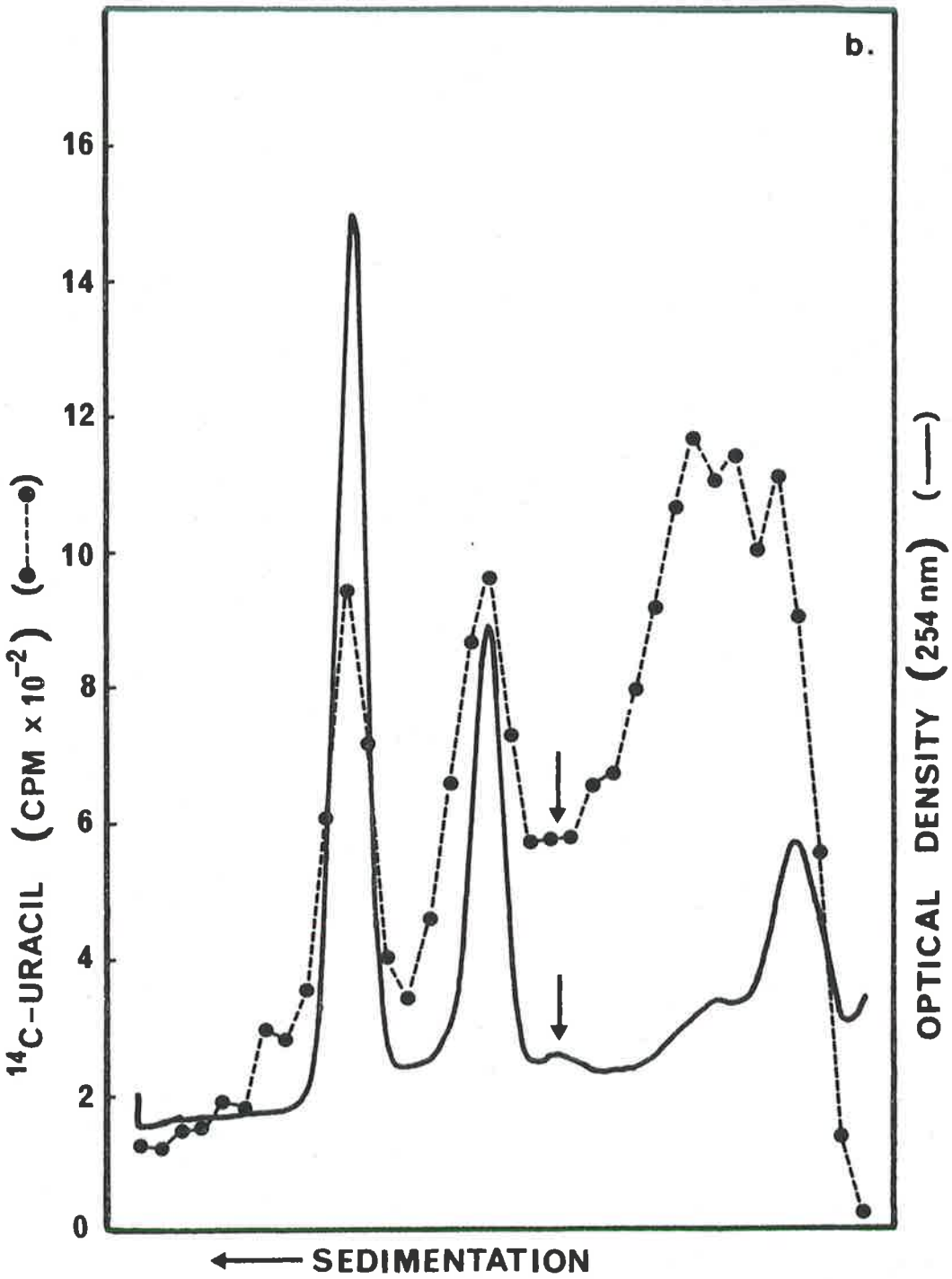

b.
16
14
12
10
8
6
4
2
0
^{14}C-URACIL (CPM x 10^{-2}) (●----●)
OPTICAL DENSITY (254 nm) (——)
SEDIMENTATION

FIG. 4,3c

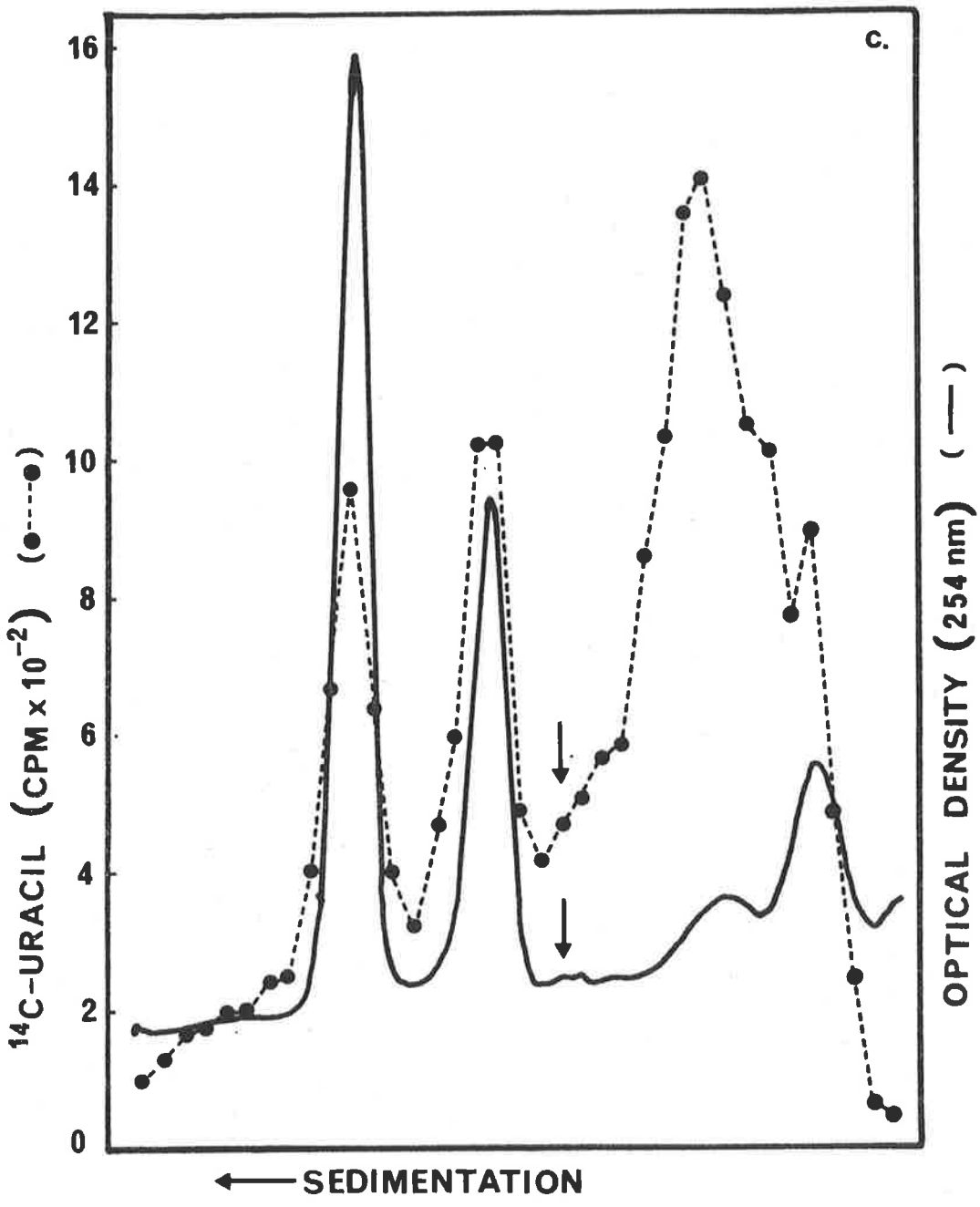

c.
16
14
12
10
8
6
4
2
0
^{14}C-URACIL (CPM x 10^{-2}) (●---●)
OPTICAL DENSITY (254 nm) (——)
SEDIMENTATION

FIGURE 4,4. THE OPTICAL DENSITY AND RADIOACTIVITY PROFILES OF TOTAL CELLULAR RNA PREPARED FROM CELLS INCUBATED FOR 75 MINUTES IN LOW AMINO ACIDS AND TREATED WITH RIFAMPICIN... A REPEAT EXPERIMENT.

This experiment was a repeat of that described in Fig. 4, 3.

Fig. 4,4 is also presented in three sections, showing the optical density and radioactivity profiles of RNA prepared from:

a) cells incubated for 75 minutes, i.e., zero minutes of rifampicin treatment;

b) 10 minutes of rifampicin treatment;

c) 20 minutes of rifampicin treatment.

The arrow indicates the position of 13S RNA.

FIG. 4,4a

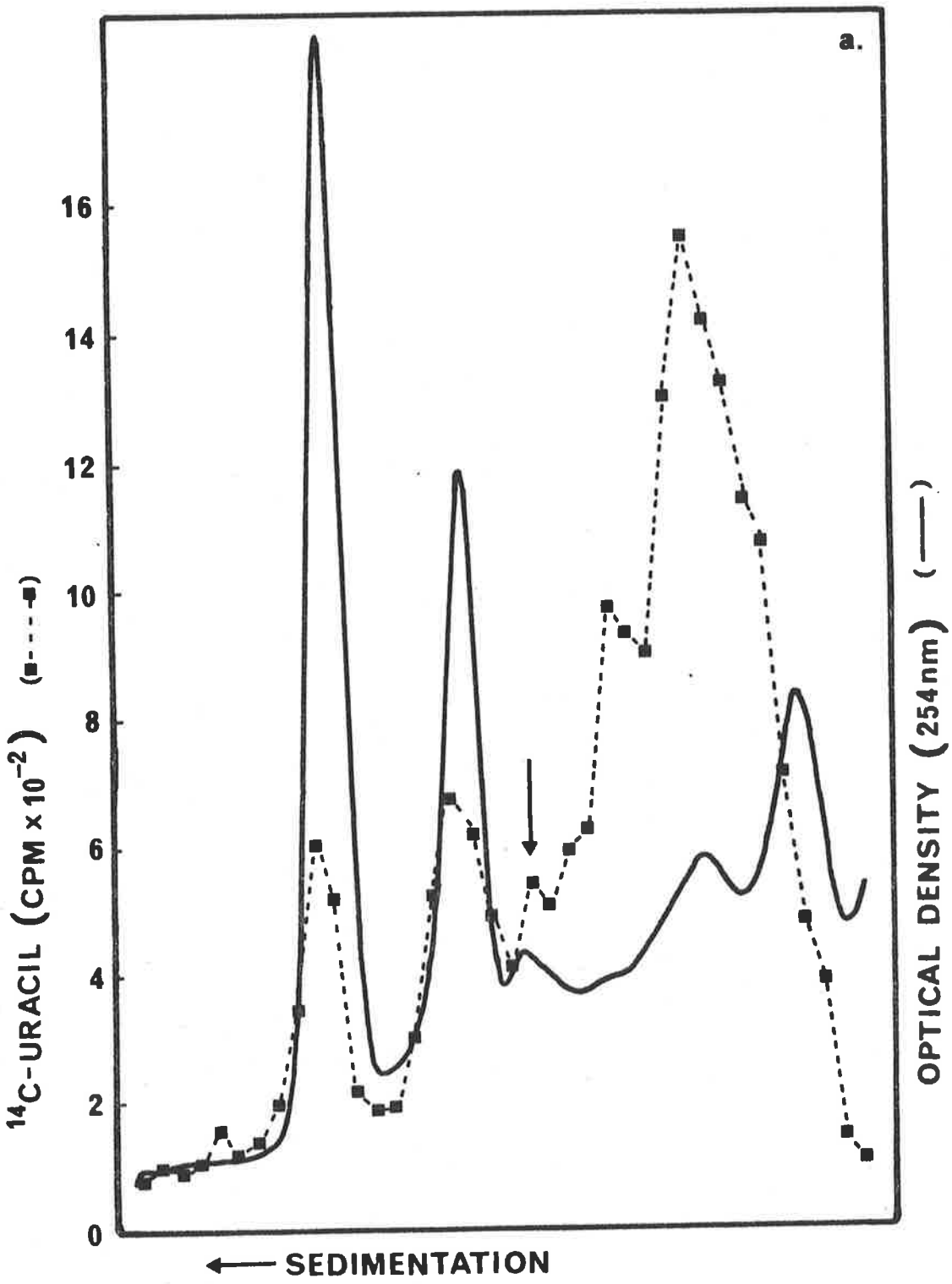

a.
16
14
12
10
8
6
4
2
0
^{14}C-URACIL (CPM x 10^{-2}) (■- - -■)
OPTICAL DENSITY (254nm) (——)
← SEDIMENTATION

FIG.4,4b

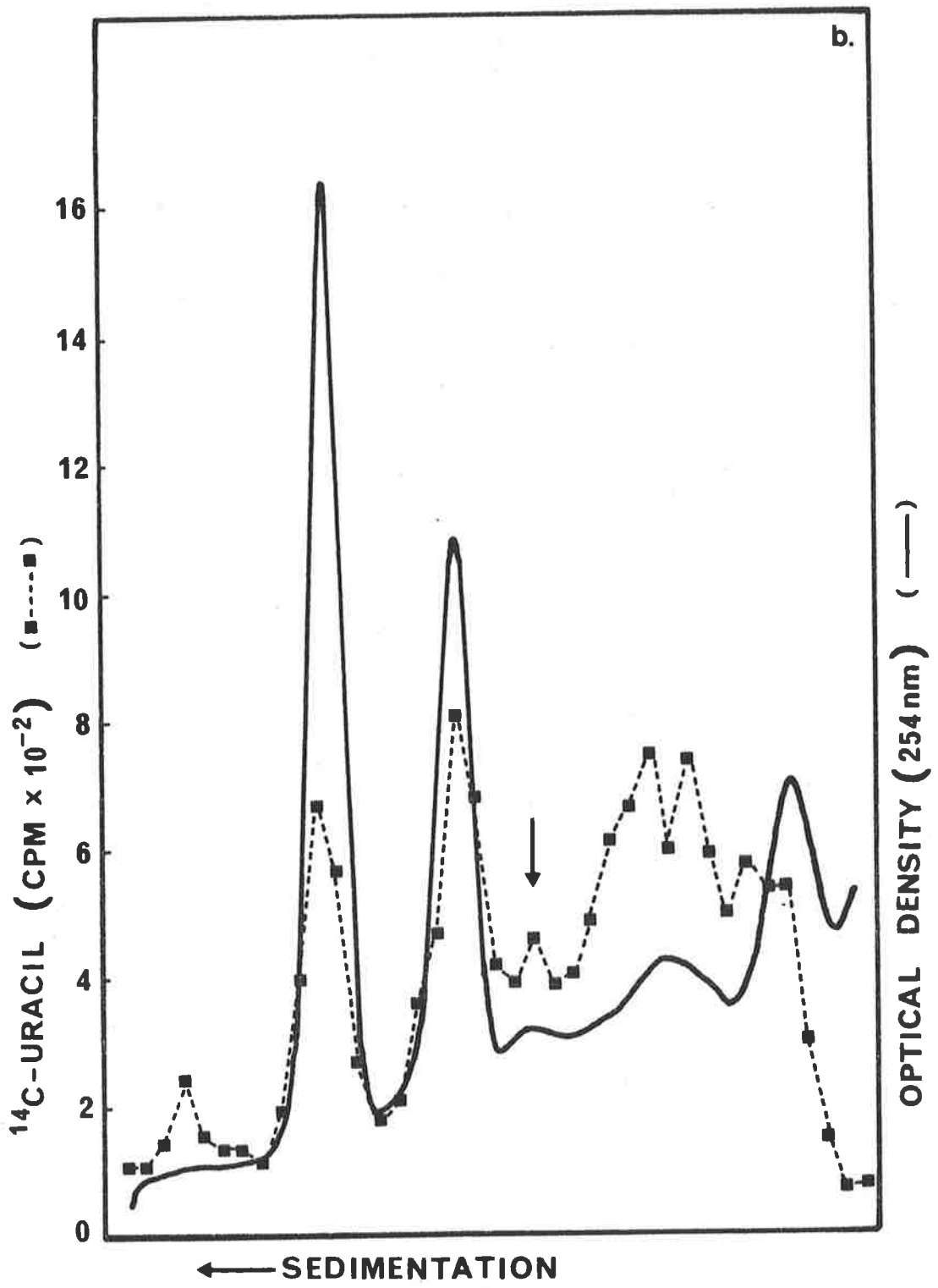

b.
16
14
12
10
8
6
4
2
0
^{14}C-URACIL (CPM x 10^{-2}) (■----■)
OPTICAL DENSITY (254 nm) (——)
SEDIMENTATION

FIG. 4,4c

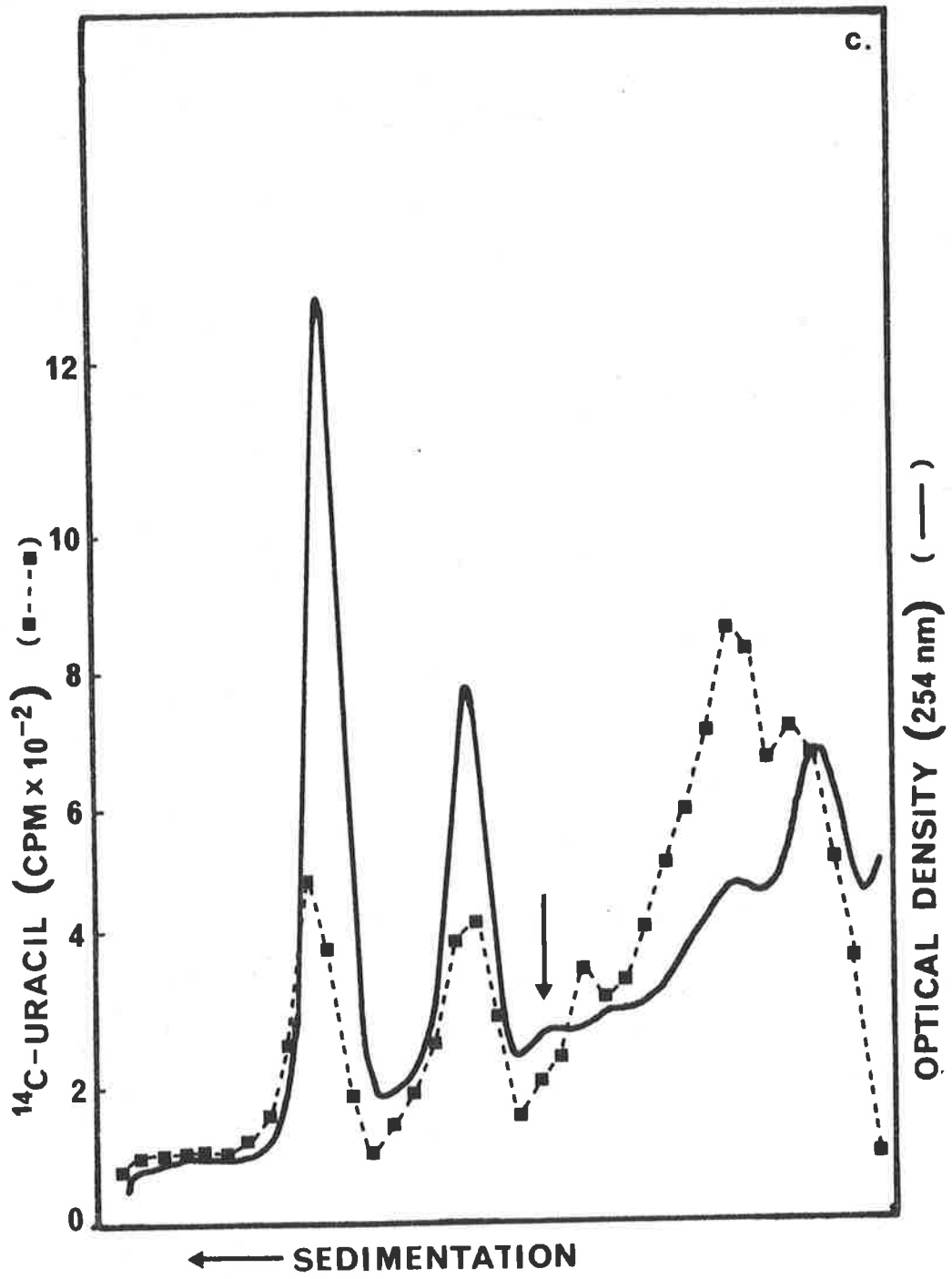

c.
12
10
8
6
4
2
0
^{14}C-URACIL (CPM x 10^{-2}) (■---■)
OPTICAL DENSITY (254 nm) (——)
SEDIMENTATION

FIGURE 4,5. OPTICAL DENSITY AND RADIOACTIVITY PROFILES OF TOTAL CELLULAR RNA PREPARED FROM CELLS INCUBATED FOR 100 and 150 MINUTES IN LOW AMINO ACIDS.

B.amyloliquefaciens cells were prepared as described previously (Fig. 3,10).

Total cellular RNA was prepared as described in the text.

4 µC of ^{14}C-uracil (spec. act. 55 mC/mmole) plus 50 µg unlabelled uracil per 5 ml of cells was added at the beginning of incubation in low amino acids. At 100 and 150 minutes, 5 ml cell samples were removed for RNA extraction.

100 µg of each RNA dissolved in TEN buffer (200 µl) was centrifuged on a 10-40% sucrose density gradient for 15¼ hours at 34,000 r.p.m. and 3°C in a 6 x 14 swing-out rotor in an MSE Super Speed 65 ultracentrifuge. The OD_{254} was recorded and 0.3 ml fractions assayed for radioactivity as described in Chapter 2.

The optical density and radioactivity profiles for the two RNA preparations are shown on separate pages as follows:

a) RNA from cells incubated for 100 minutes.

b) RNA from cells incubated for 150 minutes.

The arrow indicates the position of 13S RNA.

FIG. 4,5a

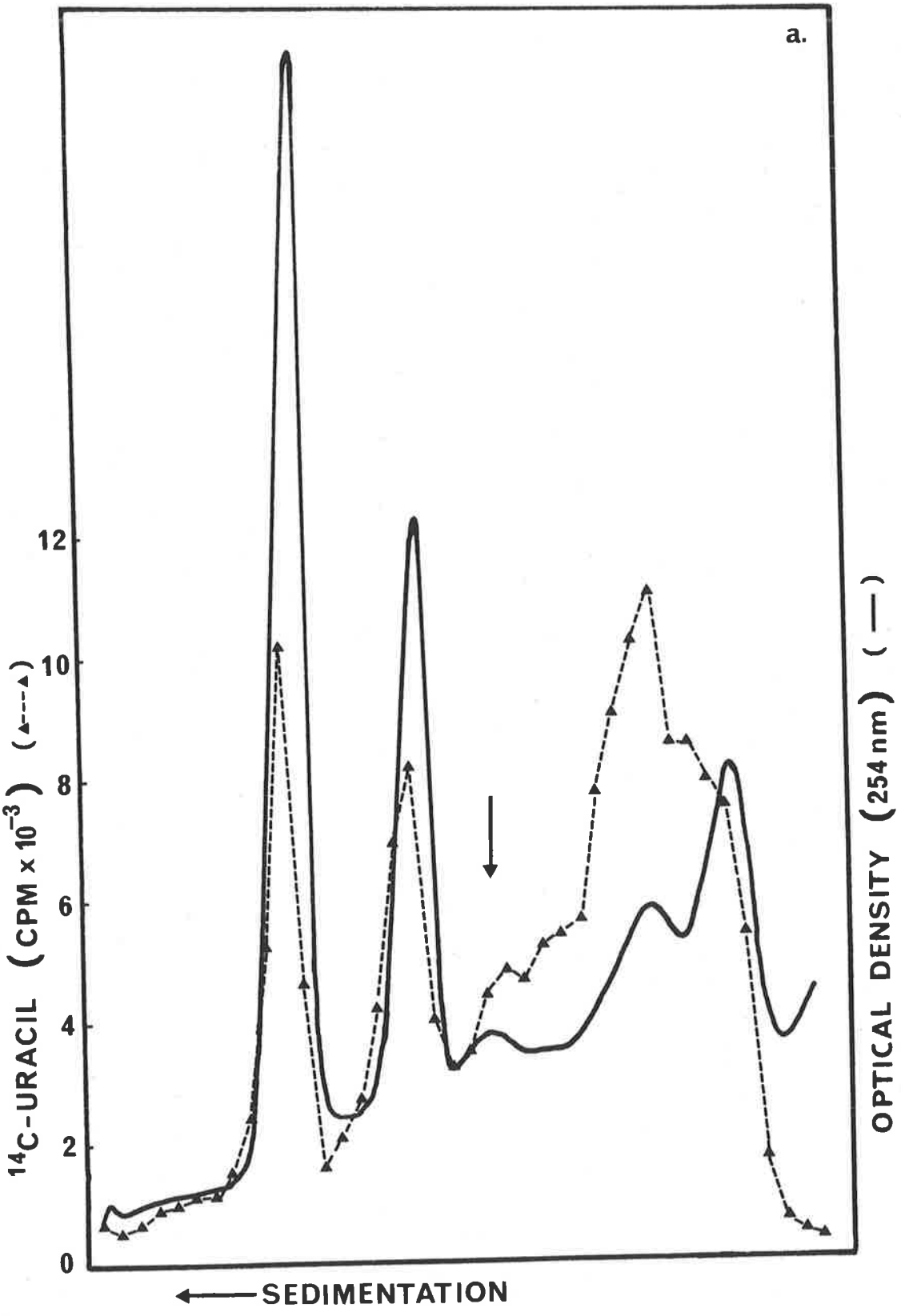

a.
12
10
8
6
4
2
0
^{14}C-URACIL (CPM x 10^{-3}) (▲---▲)
OPTICAL DENSITY (254 nm) (—)
SEDIMENTATION

FIG.4,5b

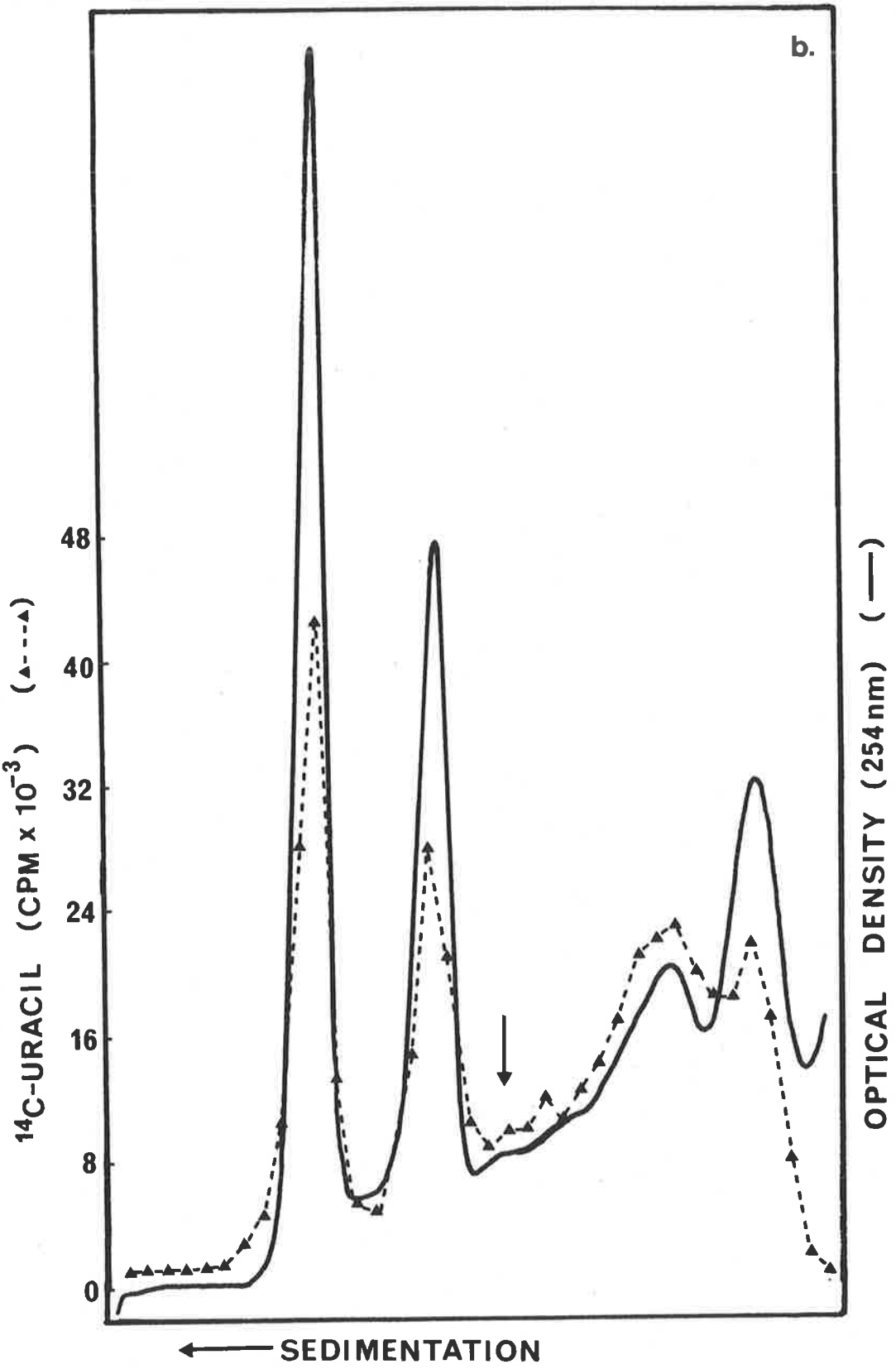

b.
48
40
32
24
16
8
0
^{14}C-URACIL (CPM x 10^{-3}) (▲---▲)
OPTICAL DENSITY (254nm) (——)
SEDIMENTATION

FIGURE 4,6. OPTICAL DENSITY PROFILES OF TOTAL CELLULAR RNA PREPARED FROM CELLS INCUBATED FOR 150 AND 300 MINUTES IN LOW AMINO ACIDS.

B.amyloliquefaciens cells were prepared as described previously (Fig. 3,10).

Total cellular RNA was prepared as described in the text.

Cells were incubated in low amino acids medium. At 90 minutes 4 µC of ^{14}C-uracil (spec. act. 55 mC/mmole) plus 50 µg unlabelled uracil per 5 ml of cells was added and incubation continued until 150 minutes when the RNA was extracted. To another sample of cells the same quantity of radioactive and unlabelled uracil was added at 180 minutes and the incubation continued until 300 minutes when the RNA was extracted.

100 µg Of each RNA dissolved in TEN buffer (200 µl) was centrifuged on a 10-40% sucrose density gradient for 15½ hours at 34,000 r.p.m. and 3°C in a 6 x 14 ml swing-out rotor in an MSE Super Speed 65 ultracentrifuge. The OD_{254} was recorded and 0.3 ml fractions assayed for radioactivity as described in Chapter 2.

The optical density and radioactivity profiles of the two RNA preparations are shown on separate pages as follows:

a) RNA from cells incubated for 150 minutes.

b) RNA from cells incubated for 300 minutes.

The arrow indicates the position of 13S RNA.

FIG. 4,6a

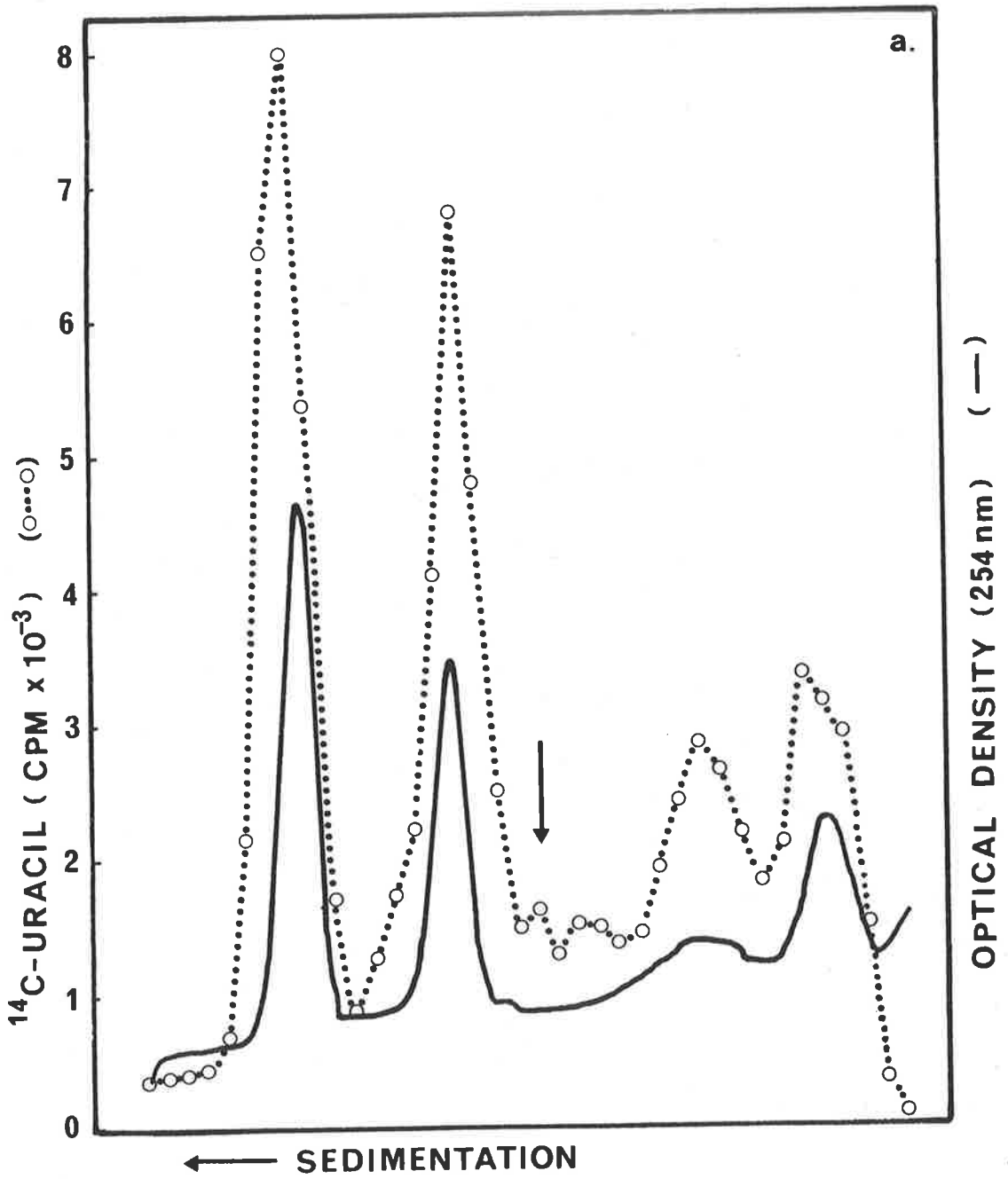

a.
8
7
6
5
4
3
2
1
0
^{14}C-URACIL (CPM x 10^{-3}) (o····o)
OPTICAL DENSITY (254 nm) (——)
SEDIMENTATION

FIG. 4,6 b

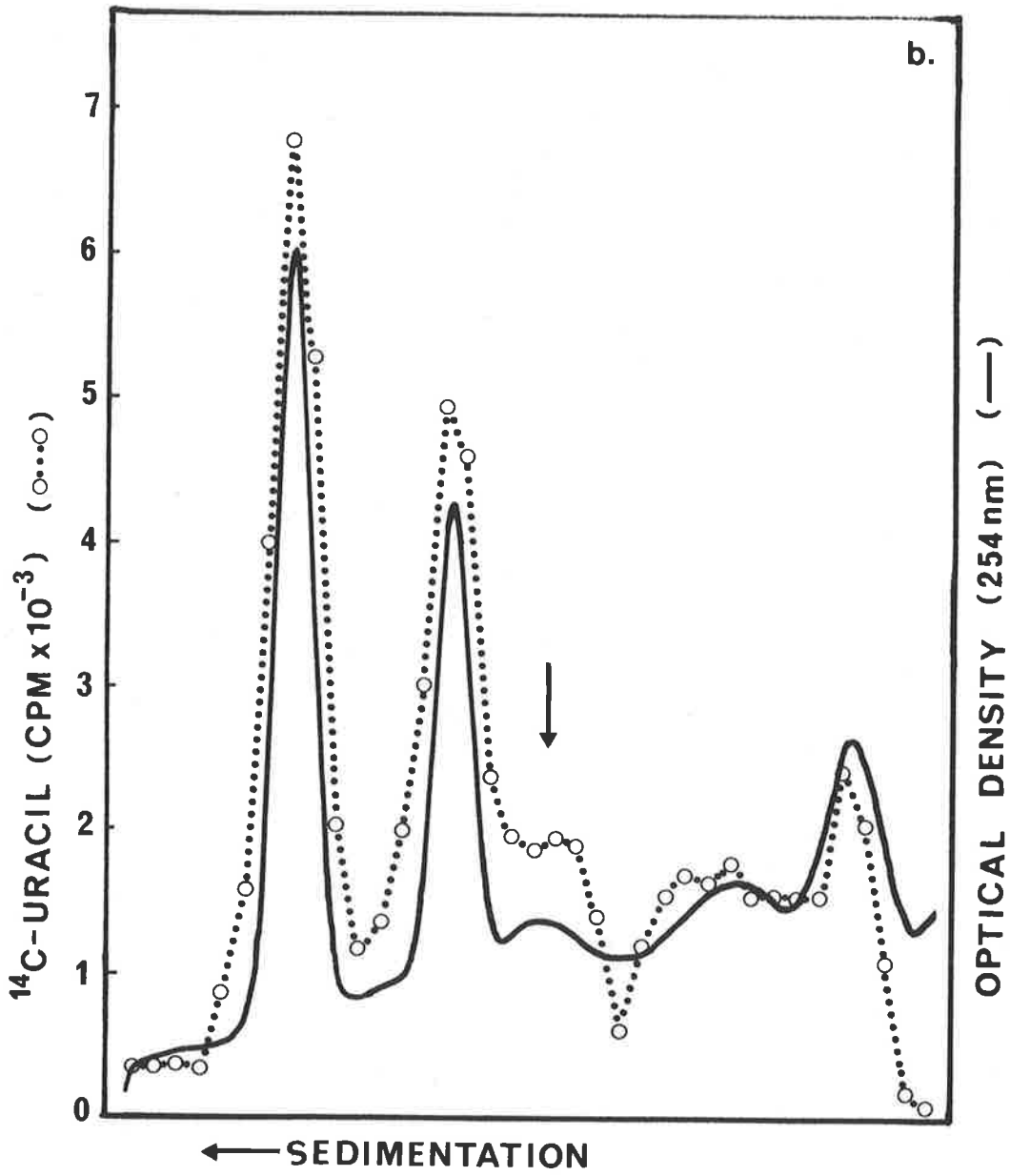

b.
7
6
5
4
3
2
1
0
^{14}C-URACIL (CPM x10^{-3}) (○···○)
OPTICAL DENSITY (254 nm) (—)
SEDIMENTATION

FIGURE 4,7. PROTEASE PRODUCTION BY CELLS OF B.AMYLOLIQUEFACIENS DURING A 300 MINUTE INCUBATION IN LOW AMINO ACIDS.

B.amyloliquefaciens cells incubated for 75 minutes in high amino acids medium were transferred to low amino acids medium (as previously described, Fig. 3,10) and further incubated. At 30-minute intervals 1 ml samples were taken, centrifuged, and the supernatants assayed for protease activity as described in Chapter 2.

[Samples were also taken for RNA extraction, see Fig. 4,8.]

FIG. 4,7

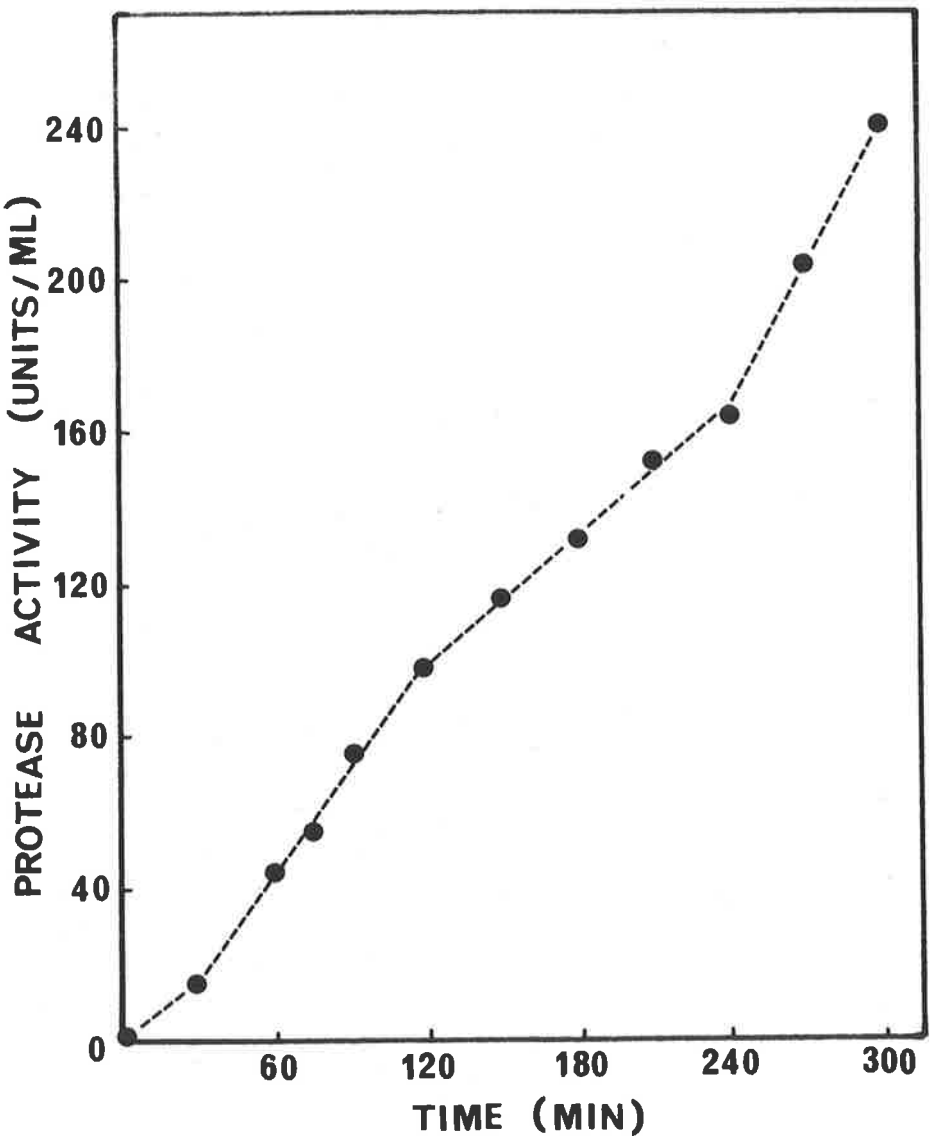

PROTEASE ACTIVITY (UNITS/ML)
240
200
160
120
80
40
0
60
120
180
240
300
TIME (MIN)

FIGURE 4,8. OPTICAL DENSITY PROFILES OF TOTAL CELLULAR RNA PREPARED FROM B.AMYLOLIQUEFACIENS CELLS AT VARIOUS TIMES DURING A 300 MINUTE INCUBATION IN LOW AMINO ACIDS.

B.amyloliquefaciens cells were prepared as described in Fig. 4,7.

Total cellular RNA was prepared as described in the text.

During the incubation of cells in low amino acids, 20 ml samples were taken, centrifuged and the pellet resuspended in 5 ml of 25 mM-Tris-HCl, pH 8.5 and 5 mM-Mg-acetate before extracting the RNA.

100 µg Of each RNA, dissolved in 200 µl of TEN buffer was centrifuged on a 10-40% sucrose density gradient for 15 hours at 34,000 r.p.m. and 3°C in a 6 x 14 ml swing-out rotor in an MSE Super Speed 65 ultracentrifuge. The OD_{254} was recorded as described in Chapter 2.

The profiles of the 12 RNA preparations are shown on three pages and are of RNA prepared from cells incubated for the following times:

a-d:	a,	zero minutes.
	b,	30 minutes.
	c,	60 minutes.
	d,	75 minutes.
e-h:	e,	90 minutes.
	f,	120 minutes.
	g,	150 minutes.
	h,	180 minutes.
i-l:	i,	210 minutes.
	j,	240 minutes.
	k,	270 minutes.
	l,	300 minutes.

The position of 13S RNA is indicated by the arrows.

FIG. 4, 8
(a-d)

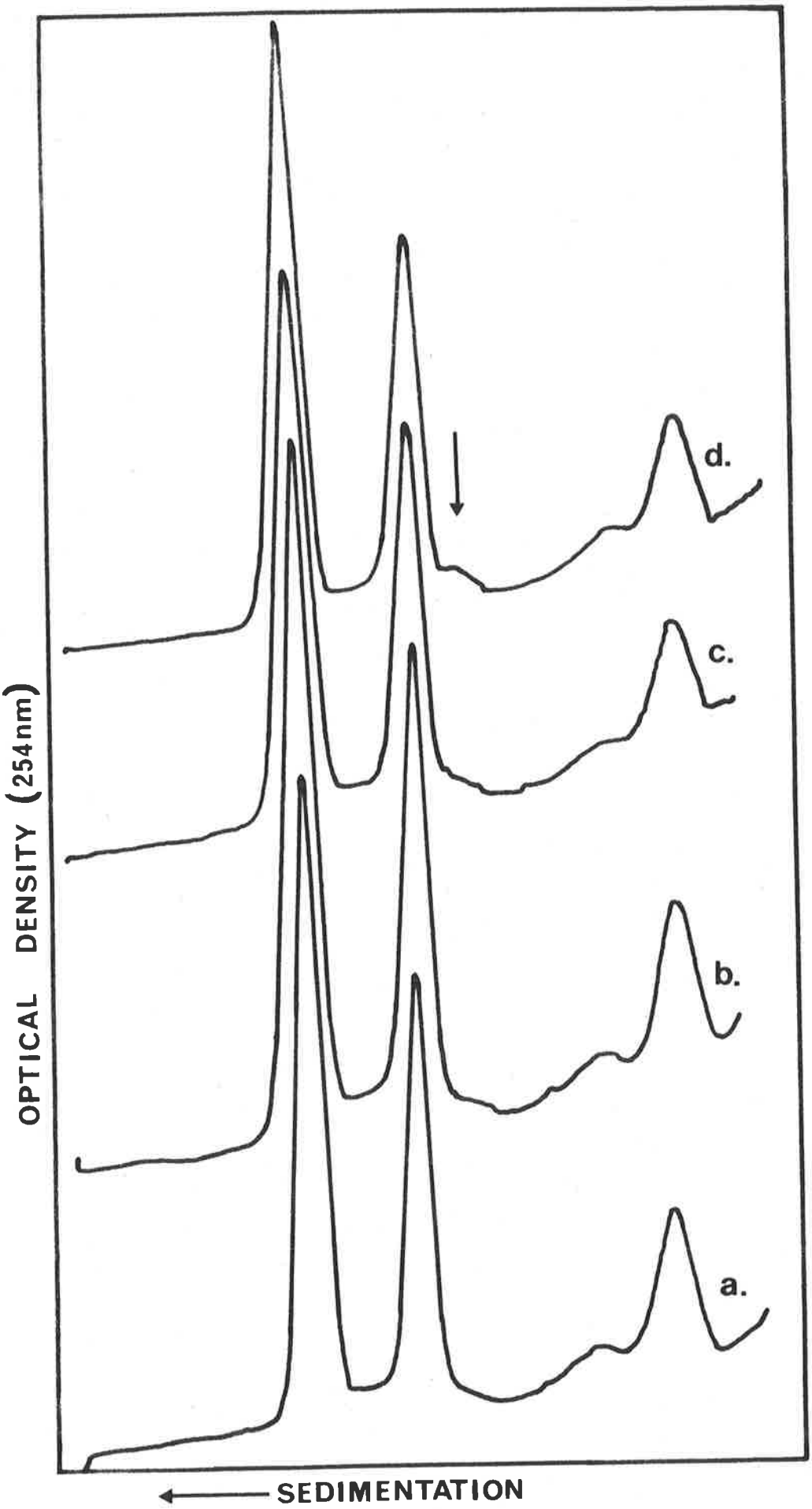


FIG. 4, 8
(e–h)

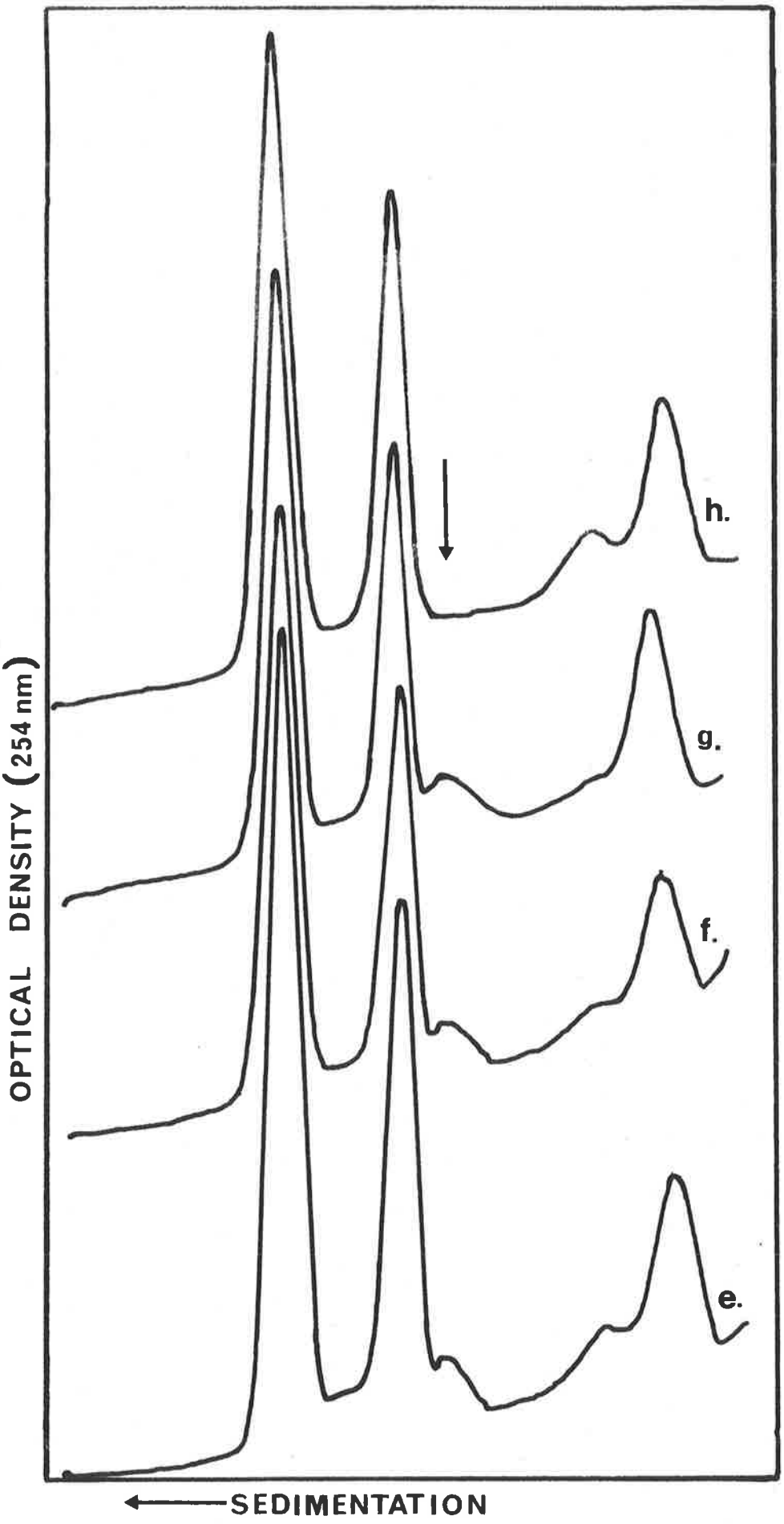


FIG. 4,8
(i–l)

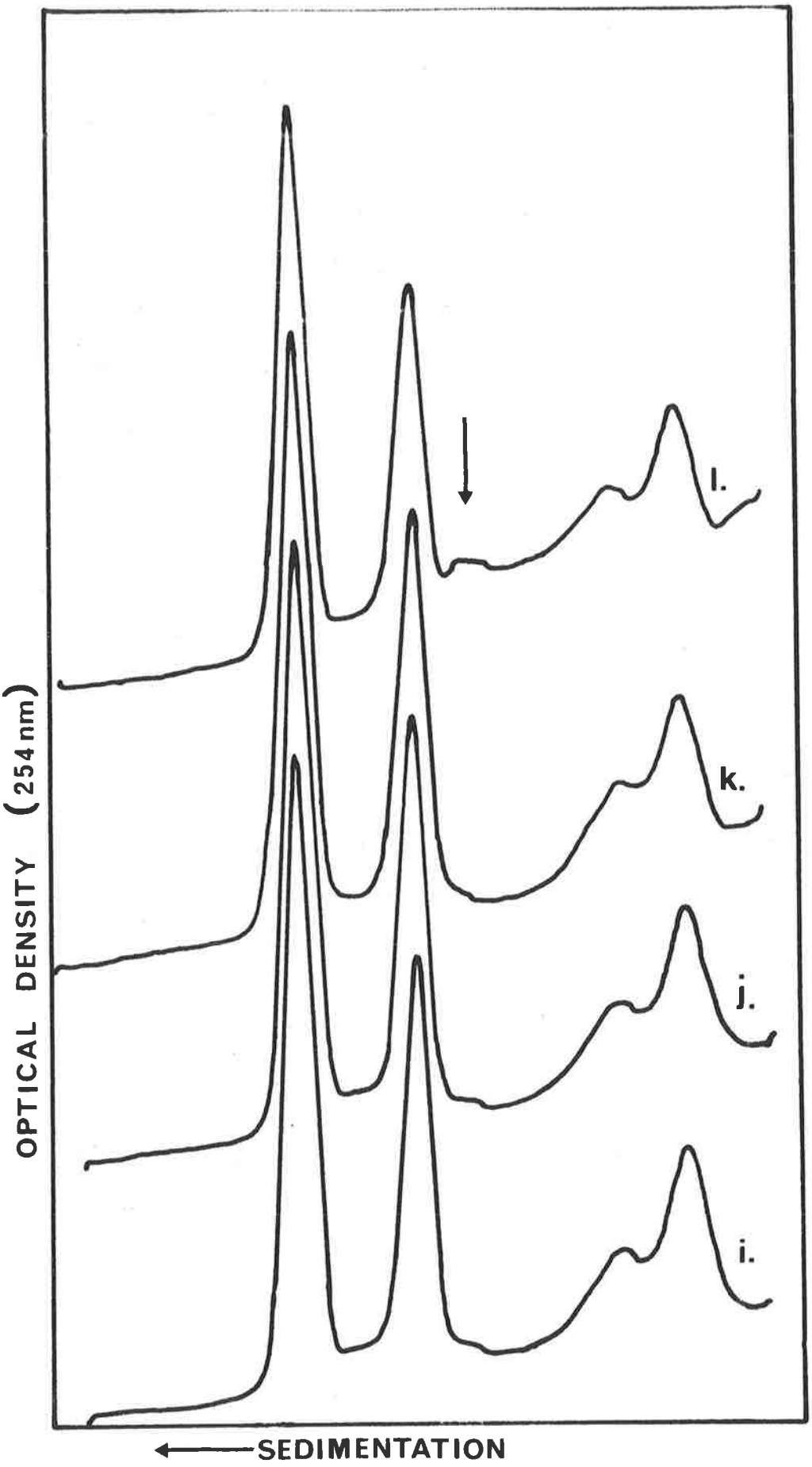


FIGURE 4,9. OPTICAL DENSITY PROFILES OF 13S RNA FROM B.AMYLOLIQUEFACIENS.

B.amyloliquefaciens cells were incubated for 75 minutes in high amino acids and then 75 minutes in low amino acids as has been previously described (Fig. 3,10).

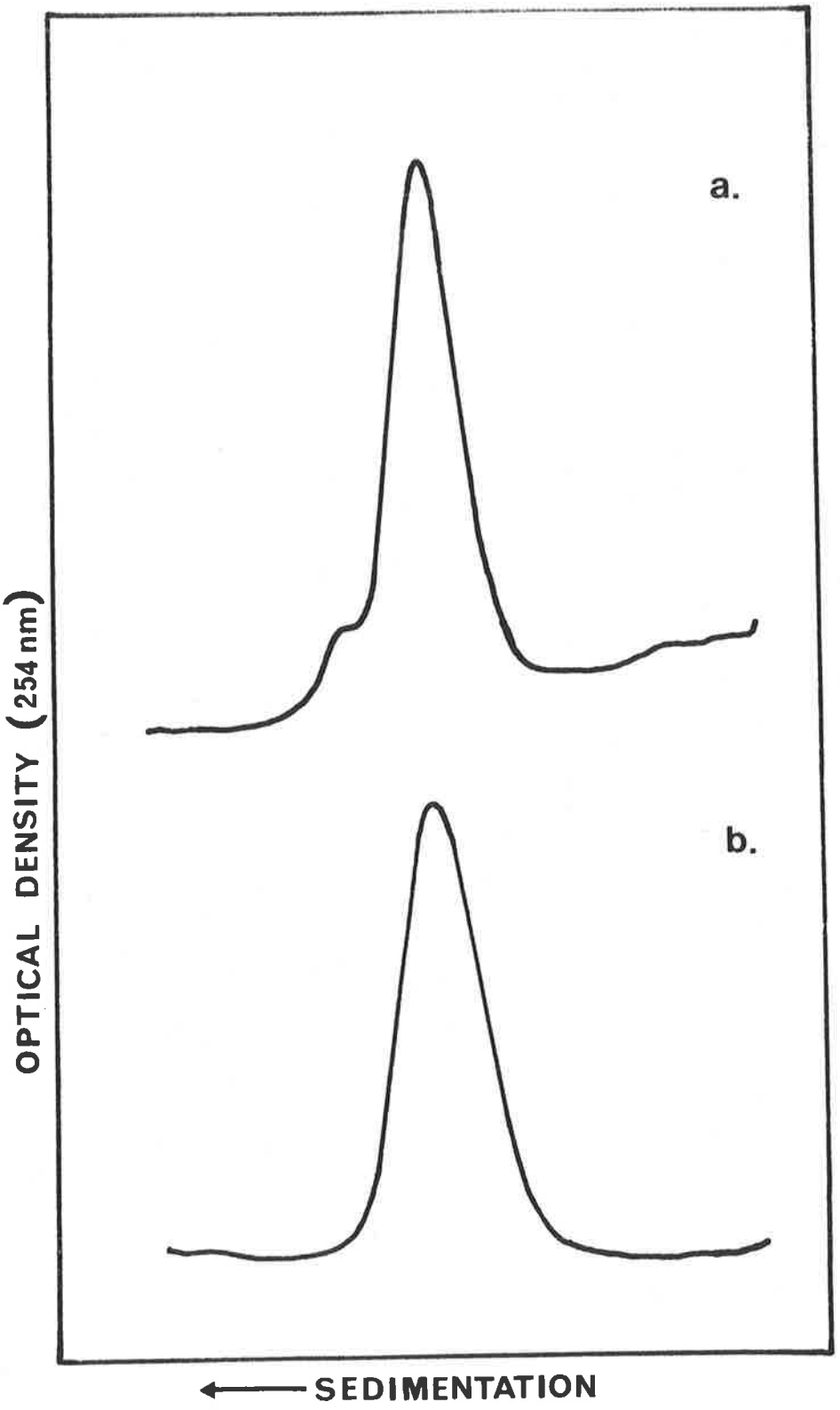
At 75 minutes total cellular RNA was prepared as described in the text. This RNA was centrifuged on a 10-40% sucrose density gradient as described in Fig. 4,8.

The 13S fraction of RNA was hand fractionated from this gradient, precipitated with 2.5 volumes of Ethanol - 100 mM Na acetate, pH 6.0. The precipitated RNA was dissolved in TEN buffer and centrifuged on a 5-20% sucrose density gradient for 15 hours at 34,000 r.p.m. and 3°C in an SW-41 rotor in a Beckman L265B ultracentrifuge.

a) The 13S peak which resulted is shown. It was further fractionated by removing the 16S peak and lower molecular weight material as indicated by the arrows.

b) The 13S peak after the second fractionation and recentrifuged on a similar 5-20% sucrose density gradient.

FIG. 4,9



CHAPTER 5

RESULTS

RESULTS

TRANSLATION OF THE PUTATIVE PROTEASE mRNA

5,1 INTRODUCTION

In Chapter 4 the tentative conclusion was made that the 13S RNA species, which appeared to oscillate in concert with the capacity for rifampicin-insensitive protease production, had properties consistent with it being the protease mRNA. Conclusive proof of this would be the demonstration that this RNA directs *de novo* synthesis of protease in an *in vitro* system.

In doing this work the author was concious of two major problems which could be encountered. Firstly, should it eventuate, the synthesis of an active protease in a cell-free system could prove detrimental to that system, and, because of its ability to autodigest, may also be difficult to identify. Secondly, it is realized that the 13S RNA as isolated may be the 'reserve' form of protease mRNA and as such may be in a non-translatable state (see Chapter 3). However, the reserve model is an hypothesis consistent with observations, but presumably could be incorrect; this aside, the reserve form could be contaminated with sufficient translatable mRNA or could be converted to this form in a cell-free system so that analysis of it would still be possible.

It was thought expedient to attempt translation of

the 13S RNA species in as many *in vitro* amino acid incorporating systems as could readily be made available. The most obvious system to use is perhaps an S-30 extract prepared from *Bacillus amyloliquefaciens*. Such a system was prepared using a method based on that of Modollel (1971). The *B.amyloliquefaciens* S-30 extract is more likely to contain specific factors which may be required for extracellular enzyme synthesis. On the other hand, synthesis of protease in this system would not be conclusive proof that the added RNA was the protease message since it could be argued that the *bone fide* mRNA was present in the extract; it is also known that the protein synthesizing system in bacilli extracts is relatively unstable (Doi, 1971; Takeda and Lipmann, 1966). An S-30 extract prepared from *E. coli* MRE 600 RNaseI$^-$ by the method of Modollel (1971) was used as an alternative bacterial system. It had recently been shown(Wang *et al*, 1976) that the mRNA for *E. coli* outer-membrane protein could direct the synthesis of this protein in a wheat-germ extract. Thus a wheat-germ amino acid incorporating system was prepared by the method of Roberts and Paterson (1973). The *Bufo marinus* oocyte amino acid incorporating system (May and Glenn, 1974) was used as the second eukaryotic system.

Thus preliminary studies were made to determine which systems would more readily allow identification of the 13S RNA. Varying quantities of the 13S RNA were added to each of the extracts, and the amino acid incorporating activity of these measured. As lack of stimulation of

incorporating activity does not necessarily imply that an mRNA has not been translated, the formation of protease antibody cross-reacting material was also measured. The wheat-germ system proved the most amenable to immediate investigation of the 13S RNA. Results from these studies will now be described.

5,2 STIMULATION OF AMINO ACID INCORPORATING ACTIVITY BY 13S RNA IN THE WHEAT-GERM SYSTEM

The wheat-germ system was prepared by the method of Roberts and Paterson (1973) (Methods, Chapter 2). The conditions were optimized for the translation of CMV (cucumber mosaic virus) RNA which a generous gift from M. Schwinghammer. These conditions are given in Methods, Chapter 2. 4 µg Of 13S RNA was found to stimulate the incorporation of ^{3}H-leucine into TCA-precipitable material from 7,875 c.p.m. to 57,500 c.p.m. per 50 µl reaction mixture, that is, a stimulation of approximately seven times above the background (endogenous) incorporation was achieved. This stimulation was considered reasonable and allowed for economic use of the RNA. At the end of a 60 minute incubation of 4 µg 13S RNA with the wheat-germ amino acid incorporating system, TCA (150 µl of 10% (w/v)-TCA containing 0.1% (w/v) unlabelled leucine) was added to the mixture. The resulting precipitate was washed, dissolved in SDS-gel electrophoresis loading buffer and electrophoresed as described in Methods, Chapter 2. The gels were sliced and assayed for radioactivity. It was seen from the

radioactivity profiles (Fig. 5,1) of the gels that the 13S RNA stimulated the synthesis of products of high molecular weight. The arrow indicates the expected position of protease (M.W. = 27,900) as determined from a duplicate gel on which pure protease was electrophoresed. A shoulder is evident in the position where protease is expected, but the main peak of radioactivity corresponds to a species of molecular weight, approximately 22,000. This was not seen as discouraging as a species of 22,000 molecular weight was found to be the major breakdown product when antibody precipitates of protease were not immediately bailed on solution in SDS-gel loading buffer prior to electrophoresis (E.V. Sêmets, personal communication). When protease antibodies were added to the cell-free system, above, no cross-reacting material was found and this is discussed later.

5,3 EFFECT OF OTHER CELLULAR RNA FRACTIONS ON AMINO ACID INCORPORATING ACTIVITY OF THE WHEAT-GERM SYSTEM

It seemed necessary to find out whether any other fraction of total cellular RNA obtained from B.amyloliquefaciens would also cause the stimulation of incorporation seen above. It was possible, for example, that the 13S RNA was in fact a ribosomal RNA breakdown product and was causing the stimulation of incorporation by protecting endogenous mRNAs from ribonuclease digestion. Thus, total cellular RNA on a sucrose density gradient was divided into five fractions (Fig. 5,2). 4 µg Of each were added to the

wheat-germ system and incubated for 60 minutes when the incorporation of ^{3}H-leucine into TCA-precipitable material was measured (Fig. 5,2). Addition of the 13S RNA gave maximum stimulation. Incorporation directed by the other fractions could be accounted for by contamination of these fractions by 13S RNA in the process of sedimentation through the sucrose density gradient.

5,4 TIME COURSE OF ^{3}H-LEUCINE INCORPORATION IN THE WHEAT-GERM SYSTEM BY 13S RNA

Protease is subject to autodigestion. It is possible that once active enzyme has been formed in an *in vitro* system, this will digest any incomplete enzyme forms. Thus it was necessary to find out whether incorporation was linear during the 60 minute incubation or whether the incorporation was rapid at first and then followed by a decline in TCA-precipitable counts. As it can be seen from Fig. 5,3, incorporation of ^{3}H-leucine into TCA-precipitable material in the presence of 13S RNA continued linearly for 60 to 90 minutes and then reached a plateau level.

5,5 SYNTHESIS OF PROTEASE ANTIBODY PRECIPITABLE MATERIAL

In the earlier experiment (Section 5,2) no protease antibody cross-reacting material was found to be made during the cell-free incubation in the presence of 13S RNA. The double-antibody procedure was used on this occasion;

that is, both rabbit-antiprotease and goat anti-rabbit gamma globulin were added to form the antibody precipitate. This procedure works well for the antibody to extracellular α-amylase prepared in this laboratory. However, when this technique was used to precipitate pure protease, several species of protein, one the size of native protease, the others smaller, were found in the precipitate. This occurred on occasions even though precautions were taken to minimize protease activity, e.g., incubation at 0°C during antibody precipitation, addition of protease inhibitors and boiling of the precipitate immediately on dissolution in SDS-gel loading buffer. The procedure was unreliable as it appeared that with obvious precautions being taken, the protease could, unpredictably, be degraded. The alternative antibody precipitation procedure involves the use of only the rabbit-antiprotease antibody. Because of the relatively small amount of protease that could be made in a cell-free system it is necessary to add extra protease as carrier to obtain a precipitate. It was necessary to add pure active protease as inactivated protease (EDTA-treated) is more susceptable to autodigestion and the partially digested protease molecules show reduced antibody cross-reacting properties.

To minimize the serious effects addition of pure protease to the cell-free system might have, the following procedure was used: after 60 minutes of incubation the cell-free reaction mix was placed on ice and protease antibody (420 μg) added. After 20 minutes, when it was

expected that the antibody would have reacted with all the protease present, the protease carrier (80 units) was added and the incubation continued on ice (Methods, Chapter 2). Using this procedure it was shown that the TCA-precipitable products formed in the cell-free reaction directed by 13S RNA were also precipitable by protease antibody, whereas little was precipitated from the reaction without added 13S RNA (Fig. 5,4). It could be argued that this difference was primarily due to the different levels of incorporation of ^{3}H-leucine in the two situations, and that the antibody was in fact acting in a non-specific manner in these experiments. However, two observations tend to rule this out. Firstly, if the similarly prepared amylase antibody is used instead, no counts are precipitated in either situation (Fig. 5,4). Secondly, if protease antibody and carrier are added to a reaction mixture where CMV RNA had been used to direct the incorporation of a large amount of ^{3}H-leucine (4 to 5 times more than with 13S RNA), then no precipitable counts are found.

Thus it does appear likely that protease is being synthesized in the <u>in vitro</u> system directed by the 13S RNA, but that it is either being degraded or the synthesis of the protease polypeptide is not being completed. It is of interest that 75% of the ^{3}H-leucine incorporated in the wheat-germ system directed by 13S RNA remained associated with the ribosome fraction, perhaps indicating that synthesis of the polypeptide chain was not being completed. No protease antibody cross-reacting material was found in

the ribosomal supernatant prepared from the 13S RNA-directed cell-free reaction mixture, indicating that either the protease was not released from the ribosomes or that during the preparation of the supernatant the protease had degraded.

5,6 TESTS ON AN ALTERNATIVE WHEAT-EMBRYO SYSTEM

The possibility was entertained that the wheat-germ system, as prepared here, was incapable of synthesizing high molecular weight proteins faithfully. The wheat-embryo system (Shih and Kaesberg, 1973) in which the wheat-germ is prepared in the laboratory rather than purchased commercially, had been reported to translate high molecular weight proteins (Shih and Kaesberg 1976). When this system was used it was found that the profile of TCA-precipitable products synthesized in the presence of 13S RNA (Fig. 5,5) was almost identical to that produced in the wheat-germ system (Fig. 5,4).

5,7 DISCUSSION

Although the results in this chapter are not yet conclusive, they show that it is very likely that the 13S RNA species which oscillates in concert with the capacity for rifampicin-insensitive protease production (i.e., the reserve mRNA pool) is the mRNA for extracellular protease. In the wheat-germ cell-free system this RNA stimulates the incorporation of ^{3}H-leucine into TCA-precipitable material. The stimulation caused by the addition of 4 µg of 13S RNA is about seven times above the background (endogenous) incorporation. This is comparable to the result of Wang *et al.* (1976) who found that 5 µg of *E. coli* outer-membrane protein stimulated a similarly prepared wheat-germ system 8-fold above background. No other reports of translation of a bacterial mRNA in a wheat-germ cell-free system have appeared in the literature to date. The products of the cell-free synthesis directed by 13S RNA cross-react with specific protease antibodies. However, the majority of the protease antibody cross-reacting material synthesized was a molecular weight lower than that of native protease. There could be two reasons for this:

(1) it is possible that much of the protease is being degraded, as protease not in the native form is susceptible to autodigestion,

(2) alternatively, the protease chains may not be completed during cell-free translation.

Preliminary steps were taken to minimize protease

digestion. Protease requires Ca^{++} for stability and Zn^{++} for activity. However, the addition of small quantities (0.001 mM) of Ca^{++} inhibited the amino acid incorporating ability of the wheat-germ system by 50%. PMSF (phenyl methyl sulfonyl fluoride) an inhibitor of the serine protease also was detrimental to the *in vitro* system. (A serine protease of similar molecular weight to the metallo-protease is also secreted by *Bacillus amyloliquefaciens* though the major one produced is the neutral metallo-protease, the one assayed for by the casein assay used for this work.) As another approach, small quantities of protease antibody were added to the *in vitro* system so that directly on synthesis of immunological regions of the protease molecule the antibody could bind and thus protect protease from degradation. However, this measure was also inhibitory to the *in vitro* system possibly due to non-specific binding of the antibody to the ribosomes.

The lower than native molecular weight products synthesized in the cell-free system may be due to incomplete or unfaithful translation of protease messenger. This occurred in both the 'wheat-germ' and the 'wheat-embryo' systems. If the theory that the protease mRNA forms a reserve of untranslatable RNA is correct, then it is conceivable that the RNA as isolated remains in an essentially non-translatable form, giving rise to the smaller molecular weight products observed. It is possible that the addition of soluble factors or membrane fractions from *Bacillus amyloliquefaciens* or even *E. coli*

is necessary for complete synthesis. Preliminary experiments have shown that addition of small quantities (4% of total incubation mixture) of B.amyloliquefaciens S-30 or S-100 (post-ribosomal supernatant) is very inhibitory (95% inhibition) to amino acid incorporation of the wheat-germ or wheat-embryo in vitro systems.

Another factor which could be affecting the translation of 13S RNA is the conditions used for cell-free synthesis. The wheat-germ system was optimized for the translation of CMV RNA but was also suitable for the translation of chick globin mRNA. mRNAs can have slightly different requirements with respect to Mg^{++}, K^{+}, spermine, and length of incubation. The requirements for protease synthesis may be different from those used in the preliminary experiments described in this chapter.

Thus it seems that the best approach to continuing this work of protease mRNA translation would be to firstly prepare large quantities of intact 13S RNA (as checked by formamide gel analysis). The next approach would be to optimize conditions, e.g., Mg^{++}, K^{+}, for 13S RNA-directed amino acid incorporation. Then the formation of protease antibody cross-reacting material with time of cell-free incubation should be followed. It may still prove necessary to use a protease inhibitor, e.g., p-aminobenzamidine, a substrate analogue (Mares-Guia and Shaw, 1965) which may prove a very successful inhibitor in this work, and to also add a bacterial extract.

Another approach to identifying a product of cell-free translation is to analyse the cyanogenbromide or tryptic-peptides of the products. To do this effectively two conditions must apply. Firstly, the product must be a completed protein and secondly, the background synthesis must be low. This is apart from the necessity of being able to inactivate protease sufficiently such that only tryptic-peptides result and not protease autodigested products. Once completed synthesis of protease is established it may be necessary to use, for example, the E. coli pre-incubated S-30 system to achieve low background synthesis. Translation with this system was not pursued beyond the preliminary trial which was unsuccessful, but may simply require further studies to optimize the conditions for protease translation.

If in all these attempts protease translation fails to be completed, it may become necessary to isolate the mRNA by immuno-precipitation of polysomes actively synthesizing protease. This, of course, requires that the reserve pool of protease mRNA is not in an untranslatable state in polysome form.

FIGURE 5,1. TCA-PRECIPITABLE PRODUCTS FROM A WHEAT-GERM AMINO ACID INCORPORATING SYSTEM DIRECTED BY 13S RNA.

B.amyloliquefaciens 13S RNA was prepared as described in Chapter 4. 4 µg of this RNA was added to the wheat-germ amino acid incorporating system described in Methods, Chapter 2. After incubation at 25°C for 60 minutes, the reaction tubes were placed on ice, and 150 µl of 10% (w/v) TCA containing 0.1% (w/v) unlabelled leucine was added to 40 µl of the reaction mixture. The precipitates were treated as described in Chapter 2 and electrophoresed on SDS-polyacrylamide gels (Weber and Osborn, 1967) at 8 mA/gel for 5 hours. Gels were then sliced (2 mm slices), treated with NCS and counted by liquid scintillation.

Shown in Fig. 5,1 are the radioactivity profiles of the gels:

—■—■— , 4 µg 13S RNA added to cell-free system.

—□—□— , no addition of RNA.

The arrow indicates the position of the marker protease electrophoresed on a separate gel.

Total incorporation (50 µl) was : no addition - 7,875 c.p.m.
plus 13S RNA - 57,500 c.p.m.
plus 2 µg CMV RNA - 215,000 c.p.m.

The tracker dye, bromophenol blue, migrated 76 mm.

FIG. 5,1

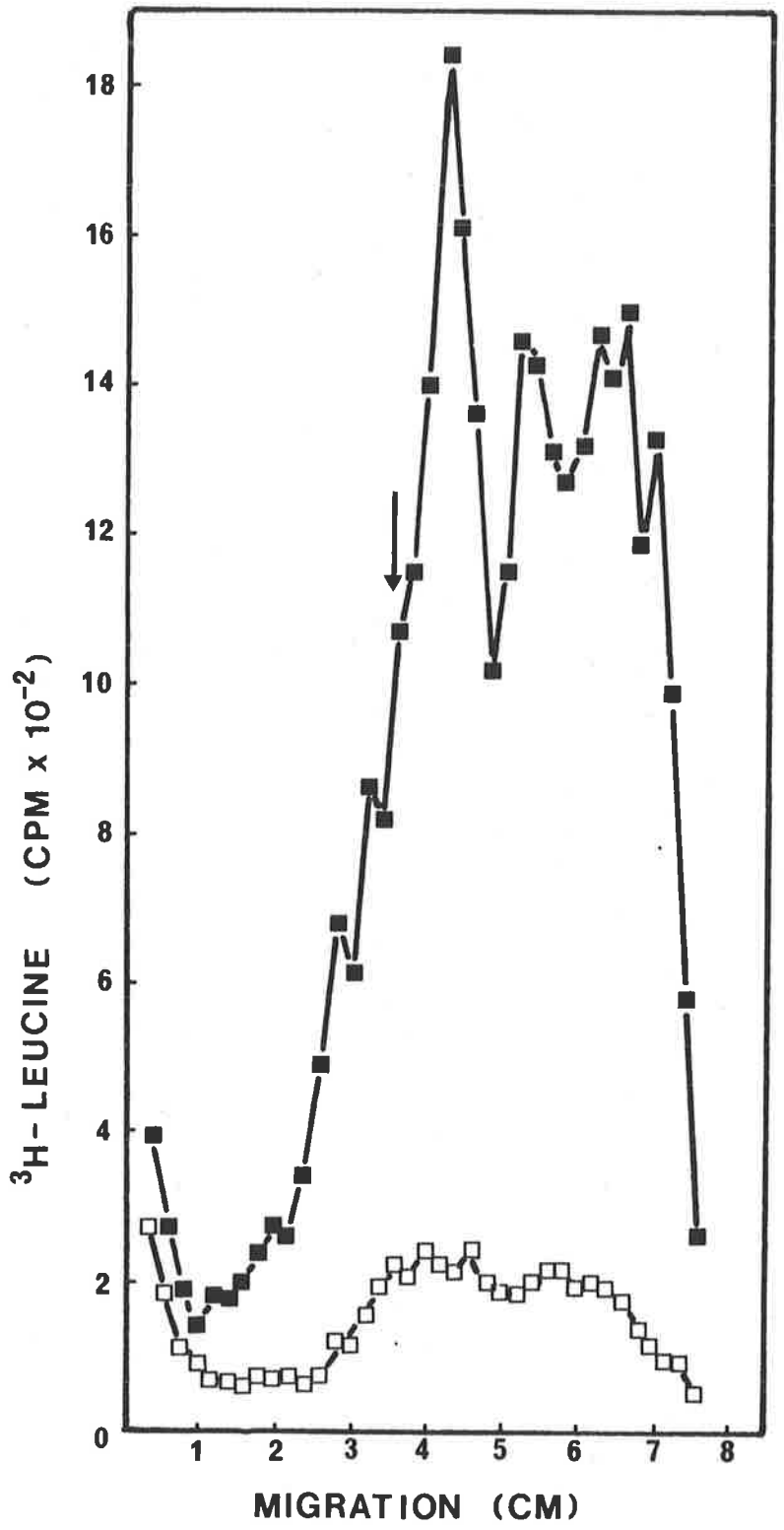

18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
^{3}H- LEUCINE (CPM x 10^{-2})
MIGRATION (CM)

FIGURE 5,2. STIMULATION OF THE WHEAT-GERM AMINO ACID INCORPORATING SYSTEM BY FRACTIONS OF TOTAL CELLULAR RNA FROM B.AMYLOLIQUEFACIENS.

Total cellular RNA was prepared as described in Chapter 4. Approximately 300 µg RNA was centrifuged on a 10-40% sucrose density gradient for 15 hours at 38,000 r.p.m. (SW41 rotor, Beckman L265B ultracentrifuge). The gradient was hand fractionated into 5 fractions, a-e. The arrows indicate the extent of the fractions. 4 µg Of RNA from each fraction was added to the wheat-germ amino acid incorporating system (Chapter 2) and after 60 minutes incorporation of ^{3}H-leucine into TCA-precipitable material was measured as described in Chapter 2.

Fig. 5,2 shows the optical density profile of the total RNA, the arrows indicating where fractions were taken.

The points (●) indicate the incorporation activity of 4 µg of the corresponding RNA fraction. 20 µl Samples from a 50 µl reaction mixture were taken. The dashed line (— —) was drawn to illustrate the trend in incorporation activity.

Background incorporation (no addition) was 4,150 c.p.m. per 20 µl.

Addition of 2 µg CMV RNA gave 120,000 c.p.m. per 20 µl.

FIG. 5,2

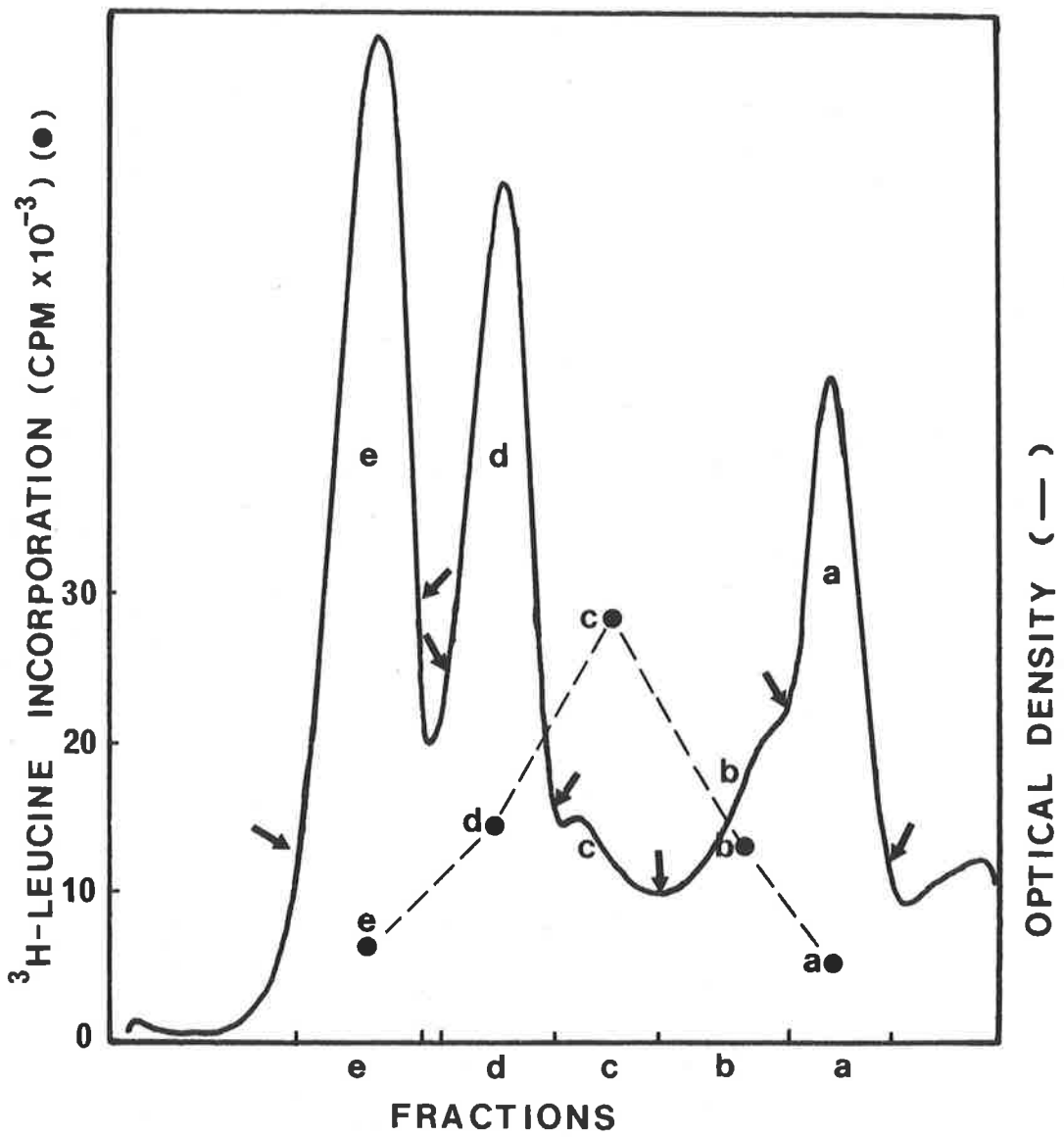

^{3}H-LEUCINE INCORPORATION (CPM x10^{-3}) (●)
OPTICAL DENSITY (—)
0
10
20
30
e
d
c
b
a
FRACTIONS

FIGURE 5,3. TIME COURSE OF ^{3}H-LEUCINE INCORPORATION BY THE WHEAT-GERM CELL-FREE SYSTEM DIRECTED BY 13S RNA.

13S RNA from B.amyloliquefaciens was prepared as described in Chapter 4. 4 µg of this RNA was added to the wheat-germ amino acid incorporating system which was as described in Chapter 2 except that 8 µCi/50 µl reaction mix of ^{3}H-leucine was added and the reaction mixture was adjusted to an 80 µl volume. 5 µl Samples were taken at the times indicated and pipetted into 150 µl of 10% (w/v)-TCA containing 0.1% unlabelled leucine and further treated as described in Chapter 2.

The graphs represent the average of duplicate trials.

-●—●- , 4 µg of 13S RNA added.

-○—○- , no addition of RNA.

FIG. 5,3

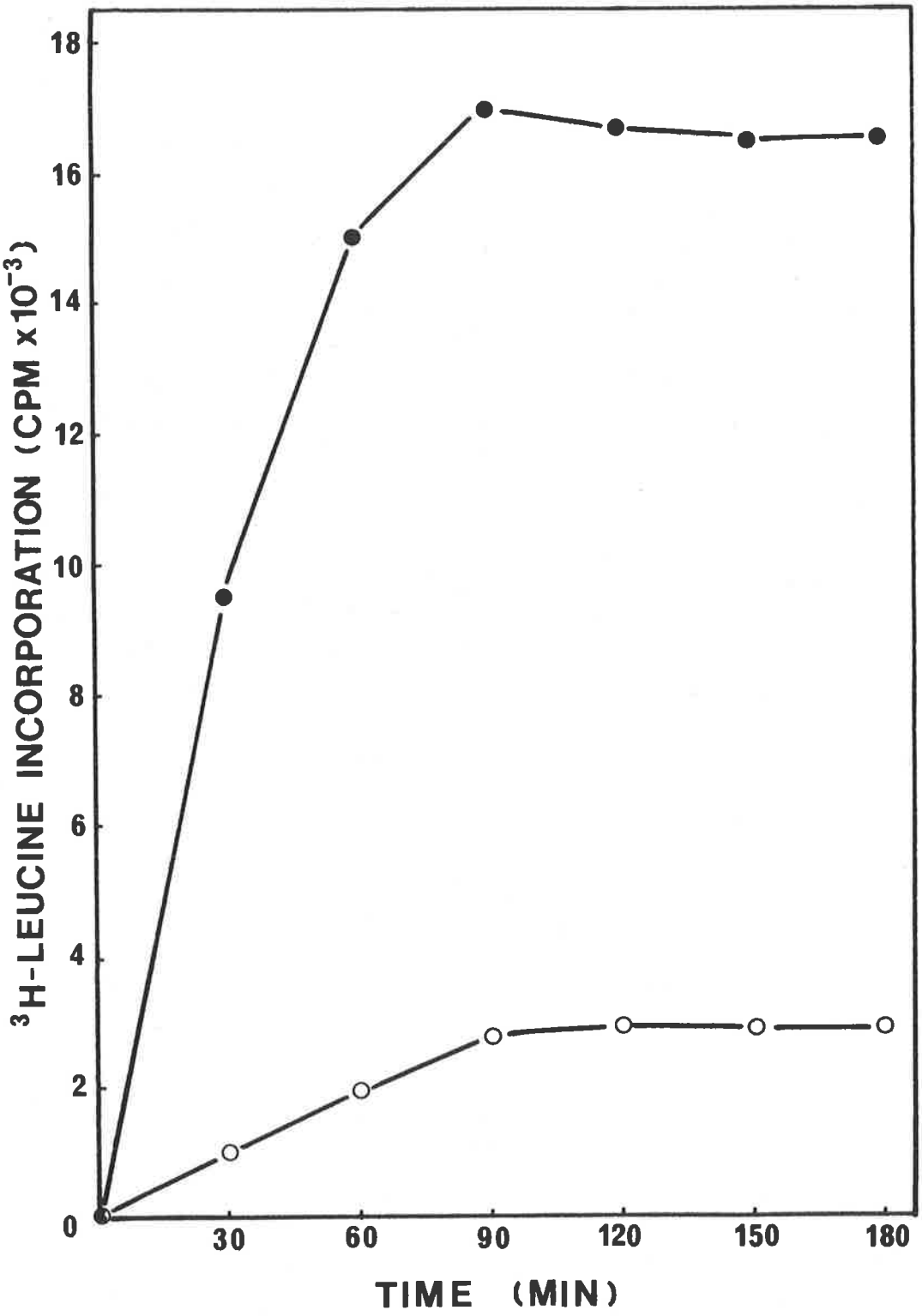

18
16
14
12
10
8
6
4
2
0
30
60
90
120
150
180
^{3}H-LEUCINE INCORPORATION (CPM x10^{-3})
TIME (MIN)

FIGURE 5,4. TCA AND SPECIFIC PROTEASE ANTIBODY PRECIPITATED PRODUCTS OF 13S RNA-DIRECTED SYNTHESIS IN THE WHEAT-GERM SYSTEM.

B.amyloliquefaciens 13S RNA was prepared as described in Chapter 4. 4 µg of this RNA was added to the wheat-germ amino acid incorporating system described in Chapter 2. After 60 minutes incubation, 20 µl of the reaction mixture was precipitated with 150 µl 10% (w/v)-TCA plus 0.1% (w/v)-unlabelled leucine and treated as described in Chapter 2. A further 20 µl sample was cooled on ice, protease antibody (420 µg rabbit antiprotease gamma globulin) added and after 20 minutes 80 units of protease added and the incubation continued at 4°C overnight. TCA and antibody precipitates were treated as described (Chapter 2) and electrophoresed on SDS-polyacrylamide gels (Weber and Osborn, 1969) at 8 mA/gel for 4½ hours. Gels were sliced into 2 mm slices, treated with NCS and counted by liquid scintillation.

a) Radioactivity profile of TCA-precipitable products

-■——■- , 13S RNA added (4 µg).

-□——□- , no addition of RNA.

b) Radioactivity profile of antibody precipitable products

-■——■- , 13S RNA added (4 µg); protease antibody.

-□——□- , no addition of RNA; protease antibody. A profile virtually indistinguishable from this one was obtained when products from a CMV-directed incorporation were precipitated with protease antibody.

-△——△- , 13S RNA added (4 µg); amylase antibody.

The arrow indicates the position of marker protease electrophoresed on a duplicate gel.

The marker dye, bromophenol blue, migrated to 6.4 cm.

FIG. 5,4

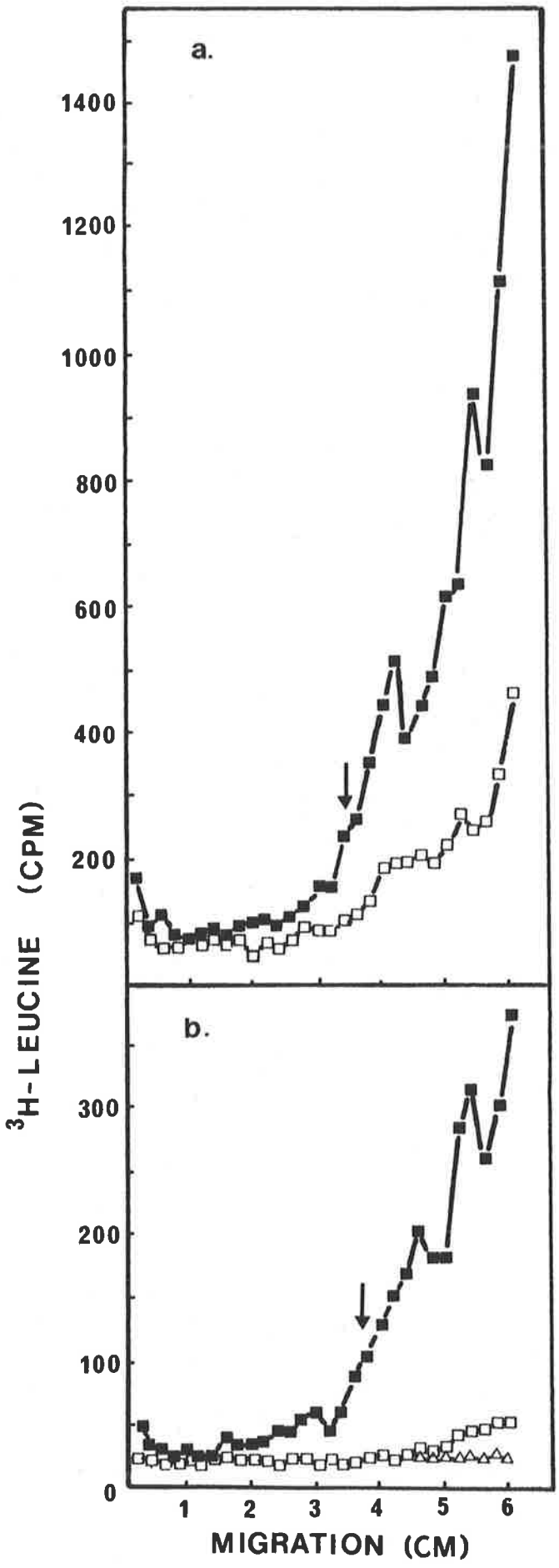

a.
b.
1400
1200
1000
800
600
400
200
300
200
100
0
1
2
3
4
5
6
^{3}H-LEUCINE (CPM)
MIGRATION (CM)

FIGURE 5,5. 13S RNA-DIRECTED AMINO ACID INCORPORATION IN THE WHEAT-EMBRYO SYSTEM.

13S RNA from B.amyloliquefaciens was prepared as described in Chapter 4. The wheat-embryo amino acid incorporating system was prepared as described by Shih and Kaesberg (1976). 4 µg Of 13S RNA was added to this system, incubated for 60 minutes and then 150 µl of 10% (w/v)-TCA containing 0.1% (w/v)-unlabelled leucine was added to a 20 µl sample. The precipitates were processed as described in Chapter 2, electrophoresed on SDS-polyacrylamide gels (Weber and Osborn, 1969) at 8 mA/gel for 4½ hours. The gels were sliced (2 mm slices), treated with NCS and counted by liquid scintillation.

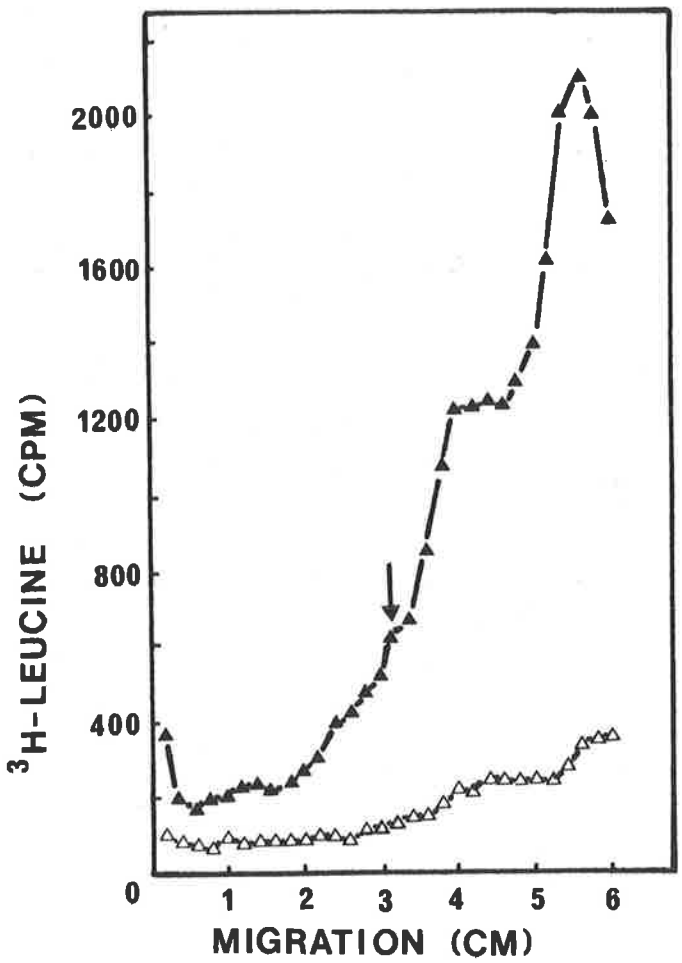
–▲——▲– , 13S RNA added (4 µg).

–△——△– , no addition of RNA.

The arrow indicates the position of marker protease electrophoresed on a duplicate gel.

Tracker dye, bromophenol blue, migrated to 6.5 cm.

FIG. 5,5



CHAPTER 6

FINAL SUMMARY AND DISCUSSION

FINAL SUMMARY AND DISCUSSION

It is not possible at this stage to arrive at final conclusions about the molecular biology of extracellular enzyme synthesis in Bacillus amyloliquefaciens, but the work in this thesis appears to leave no doubt that the system has remarkably different properties from any previously described. It is impossible to explain the findings on a conventional basis.

As described already, the best working hypothesis is that there are two forms of mRNA for protease, one a reserve untranslatable form, the other a translatable form; the first is converted to the second when transcription ceases. Transcription is able to supply mRNA for translation and accumulation of the reserve. The control mechanism also appears to be of a new type in that reserve mRNA builds up and declines in a cyclical fashion.

Before this hypothesis can be proved or rejected, the mRNA and its reserve form must be identified by translation in a cell-free system. Once this is done the characteristics (structural and functional) of the two forms can be examined. Moreover, a cDNA derived from protease mRNA will permit a more direct study of the control of its formation and accumulation by using hybridization assays. Hopefully, as described in Chapter 5, the mRNA may have been isolated and definitive proof of this is currently being sought.

There remain a large number of questions: where is the protease synthesized, how does it traverse the membrane,

and those already referred to above. Again, these are the subject of investigation in this laboratory.

A major question is, of course, how general are the phenomena discovered in the B.amyloliquefaciens protease system? The phenomenon of a pool of preformed mRNA for enzymes destined for secretion has been shown to be fairly widespread (Boethling, 1975; Gould et al., 1973; Priest, 1975; Sêmets et al., 1973; Stinson and Merrick, 1974). On the other hand, it cannot be asserted that synthesis of all proteins destined for secretion through the cell membrane show the same characteristics. In this laboratory (Saint, 1974) it was shown that an mRNA pool for B.amyloliquefaciens alkaline phosphatase does not exist. A similar report was made for alkaline phosphatase of E. coli (Wainwright and Beacham, 1977). There is no evidence for an mRNA pool for E. coli outermembrane protein, however, this mRNA is reported to be extremely stable (Hirashima et al., 1973 and 1974). Neither is there any evidence for an mRNA pool for Bacillus licheniformis penicillinase (Sargent et al., 1969; Yamamoto and Lampen, 1976). At the present time, therefore, generalized conclusions cannot be made. It may be speculated that synthesis of enzymes wholly secreted from the cell has characteristics similar to those for protease, while those which wholly (alkaline phosphatase, outermembrane protein) or partially (penicillinase) retained by the cell show different characteristics, but this is only conjecture.

The question of the mechanism of release of these

proteins likewise cannot be settled. The discovery of the penicillinase phospholipo-precursor with a hydrophobic 'leader' sequence (Dancer and Lampen, 1975; Yamamoto and Lampen, 1976) suggests that Blobel's 'signal' hypothesis (Blobel and Sabatini, 1971; Campbell and Blobel, 1976) may also apply in prokaryotes. Recent work by Smith et al. (1977) provided direct evidence for the direct synthesis and extrusion of nascent alkaline phosphatase of E. coli being synthesized on membrane-bound ribosomes. Sanders and May (1975) showed that B.amyloliquefaciens extracellular enzymes emerge from the protoplast in a trypsin-sensitive configuration implying that either a nascent form is extruded or that the enzyme is secreted in a form different from the final form which is insensitive to trypsin. It would seem that isolation of protease messenger would form a good basis for further studies on this basis.

In this regard, it may also be possible to identify and isolate the mRNA for B.amyloliquefaciens extracellular α-amylase. It was found that when cells were incubated in a low amino acids medium containing only half the normal amount of phosphate, that the α-amylase apparent mRNA pool increased markedly during the time that the protease mRNA pool was seen to decrease (Fig. 6,1). It is not yet known whether the α-amylase mRNA pool oscillates. Further investigation of the phenomenon of mRNA pool oscillation is currently in progress. In regard to the protease situation, it was of interest to determine whether the mRNA pool also oscillated during incubation in high amino

acids medium. Preliminary work (D. Love, personal communication) indicates that it does.

FIGURE 6,1. INCREASE IN RIFAMPICIN-INSENSITIVE α-AMYLASE PRODUCTION DURING INCUBATION IN A LOW AMINO ACIDS MEDIUM.

B.amyloliquefaciens cells were harvested at an OD_{600} of 3.6, washed twice and resuspended to the same cell density in high amino acids medium and incubated with shaking at 30°C for 75 minutes. Cells were then centrifuged, washed twice and resuspended in a low amino acids medium containing 17 mM-PO_4 instead of the normal 34 mM (Materials, Chapter 2). Rifampicin (0.075 µg/ml) was added to samples of cells (40 ml) at zero, 60 and 120 minutes and 2 ml samples were taken, centrifuged and assayed for both protease and α-amylase activity as described in Chapter 2.

a) This figure shows protease and α-amylase production by control cells (no rifampicin) during the incubation period.

b) This figure shows protease production after rifampicin was added at the following times:

–●—●– , zero minutes.

–■—■– , 60 minutes.

–▲—▲– , 120 minutes.

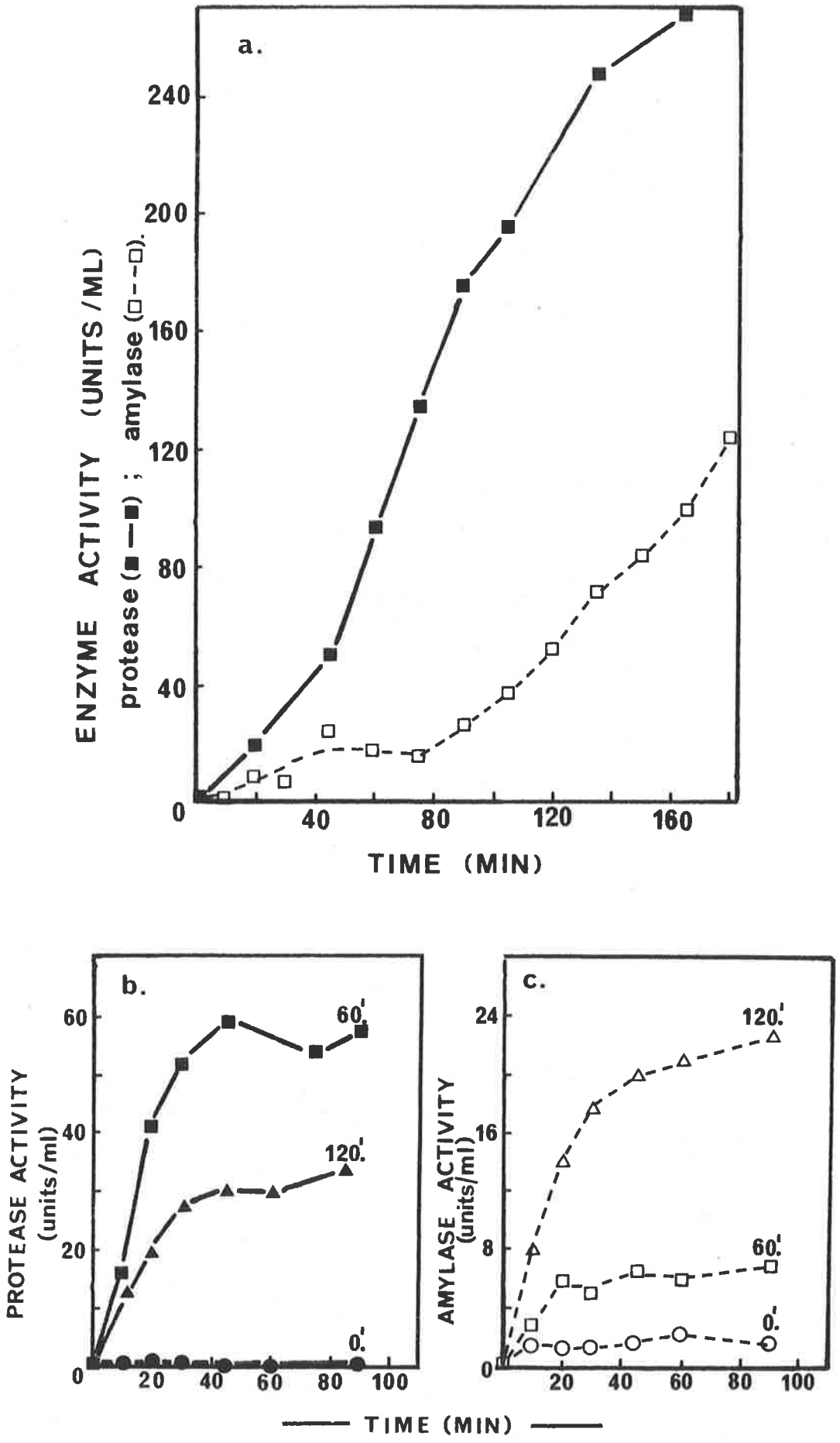
c) This figure shows α-amylase production after rifampicin was added at the following times:

–○--○– , zero minutes.

–□--□– , 60 minutes.

–△--△– , 120 minutes.

FIG. 6,1


a.
ENZYME ACTIVITY (UNITS/ML)
protease (■—■) ; amylase (□- -□).
0
40
80
120
160
200
240
40
80
120
160
TIME (MIN)
b.
PROTEASE ACTIVITY
(units/ml)
0
20
40
60
60'
120'
0'
20
40
60
80
100
c.
AMYLASE ACTIVITY
(units/ml)
0
8
16
24
120'
60'
0'
20
40
60
80
100
TIME (MIN)

CHAPTER 7

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APPENDICES

APPENDIX A

PUBLICATIONS

ABSTRACTS:

R. Douglas-Broers, B.K. May and W.H. Elliott (,976). 10th INTERNATIONAL CONGRESS OF BIOCHEMISTRY (Hamburg) p.134. "Modulation of capacity for rifampicin-insensitive synthesis of extracellular protease by Bacillus amyloliquefaciens."

R. Douglas-Broers, B.K. May and W.H. Elliott (1976). PROC. AUST. BIOCHEM. SOC. 8, 43. "Modulation of size of apparent pool of mRNA for extracellular protease in Bacillus amyloliquefaciens."