STUDIES ON EXTRACELLULAR ENZYME FORMATION BY BACILLUS AMYLOLIQUEFACIENS

A Thesis

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by

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SUMMARY

1. The synthesis of a peptide antibiotic by Bacillus amyloliquefaciens has been studied and shown to occur by a mechanism distinct from protein synthesis. Structural studies have led to an explanation for the surface active properties of the molecule as well as for the presence of D-leucine in the antibiotic. A mechanism for the synthesis of the molecule without disruption of the cell's own membrane has been proposed based on the necessity for the lactone ring in the molecule for activity. The possibility that the molecule is secreted as an obligatory part of the mechanism for extracellular enzyme secretion has been shown to be unlikely.

2. Previously it has been shown in this laboratory that protease secretion occurs for long periods in the absence of mRNA synthesis. It has now been demonstrated that the same is true for α-amylase and ribonuclease. The life time and accumulation of the apparent mRNA pools for these enzymes have been studied in detail.

3. Small amounts of the three extracellular enzymes were found to be associated with washed-cell suspensions. The enzyme pools were not due to adsorbtion phenomena. Release of these enzyme pools into the external medium occurred independent of protein synthesis and energy; however, release was markedly temperature dependent. These enzyme pools apparently represent

preformed enzyme *en route* to secretion.

4. Pulse labelling experiments showed that newly synthesised enzyme molecules are capable of immediate release into the external medium, but equilibrate with the preformed enzyme pool prior to eventual secretion.

5. Attempts were made to localise the position of this enzyme pool within the cell. Cell fractionation studies showed that the enzyme pool had no specific association with any cell structure(s). Evidence is presented which is compatible with the localisation of the enzyme pool between the permeability barrier of the cytoplasmic membrane and the cell wall.

6. Attempts were made to visualise this enzyme pool *in situ* and possibly locate the existence of any cytoplasmic zymogens for the extracellular enzymes by cytochemical techniques. These techniques involved the use of the electron microscope in conjunction with chemically conjugated ferritin and rabbit IgG antisera (specific for the extracellular enzymes). These conjugates were used to stain permeable thin sections of fixed cells embedded in bovine serum albumin. A similar technique, involving the use of fixed fragmented cells stained with 'hybrid' antibody molecules, was attempted. The antibody utilised above was prepared by linking together Fab fragments, of differing specificities (anti-ferritin and anti-exoenzyme), to form 'hybrid' $F(ab')_2$ molecules with dual specificity. These

attempts were unsuccessful.

7. In response to the addition of antibiotics to washed-cell suspensions it was found that phospholipid levels, rather than acetone-soluble lipid, were primarily affec t ed. It was noted that while five phospholipid species were present in whole cells, one of these actually accumulated in the external medium. A possible involvement of this phospholipid in the secretion of extracellular enzymes was implied; however, while of possible significance, conclusive proof of its involvement was not obtained.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. The work contained in this thesis contains, to the best of my knowledge, no material previously published or written by another person, except when due reference is made in the text.

Signed:

(ALLAN ROBERT GOULD)

ABBREVIATIONS

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

PUBLICATIONS

1. Gould, A.R., May, B.K. and Elliott, W.H. (1971).
"Studies on the protoplast-bursting factor from *Bacillus amyloliquefaciens*".
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J. Mol. Biol. 73, 213.

3. Glenn, A.R. and Gould, A.R. (1973).
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4. Gould, A.R., May, B.K. and Elliott, W.H. (1974).
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5. Glenn, A.R., Both, G.W., Gould, A.R. and Elliott, W.H. (1973).
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CHAPTER 1

INTRODUCTION

GENERAL INTRODUCTION

The work described in this thesis has been attempted to gain a better understanding of the synthesis and secretion of extracellular enzymes by bacteria. The organism we have chosen as a model system is Bacillus amyloliquefaciens* which secretes copious amounts of protease, α-amylase and ribonuclease into the external medium.

Extracellular enzymes occur widely in nature and many different types of cell secrete large amounts of enzymes into the external medium. For example, the pancreas secretes amylase, protease and ribonuclease (Redman, Siekevitz and Palade, 1966); the aleurone layer of germinating barley seeds secretes the same enzymes (Varner and Ram Chandra, 1964) while the liver secretes serum proteins as well as these enzymes. In fact, over 90% of the total protein synthesised by the liver is destined for export. Enzymes have been found in the external media of cultures of bacteria, particularly during the late stationary phase of their growth (Hagihara et al., 1958; Pollock, 1962; Lampen, 1965). Since these enzymes may have been liberated into the external medium by cell lysis rather than by a true process of secretion, Pollock (1962) defined an extracellular enzyme as one 'which exists in the medium around the cell, having originated from the cell without any alteration to cell structure greater than the maximum compatible with the cell's normal process of growth and reproduction'.

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

Bacterial extracellular enzymes are mainly degradative enzymes having as their substrates large molecular weight compounds such as starch, protein and nucleic acids. Apart from their physiological interest, the mechanism by which a cell can secrete large amounts of potentially lethal enzymes without either the cell being affected or the cell itself undergoing lysis or loss of membrane integrity poses an interesting biochemical problem. Some cells, such as the pancreatic cell, overcome this problem by utilising secretory granules. Investigations on secretion from the pancreas (Palade, Siekevitz and Caro, 1962), from leucocytes (Woodin, 1963) and the parotid gland (Schram and Bdolah, 1964) have all demonstrated that the proteins released from these cells are already packaged within a secretory granule whose membrane fuses with the cell membrane to discharge its contents outside the cell by reverse pinocytosis. However, the packaging of enzymes in zymogen granules and the process of reverse pinocytosis can be considered a secondary process. The primary mechanism of secretion of these proteins through the endoplasmic reticular membrane, prior to their enclosure within the secretory granules, is more important here due to its relevance to the bacterial system and will be discussed in some detail.

A. MECHANISM OF SECRETION OF MAMMALIAN SECRETORY PROTEINS

(1) Site of synthesis

Ribosomes in mammalian cells are known to occur in two locations, either free in the cytoplasm or attached to the endoplasmic reticulum to give the rough endoplasmic reticulum (Palade, 1955). It was suggested that proteins destined for secretion were synthesised on ribosomes attached to the endoplasmic reticulum and not by those free in the cytoplasm. Siekevitz and Palade (1960) showed that one minute after an intravenous injection of radioactive amino acids into a guinea pig, radioactive chymotrypsinogen (a secretory protein) was found attached to ribosomes bound to the endoplasmic reticulum but not to free cytoplasmic ribosomes. Caro and Palade (1964), using an autoradiographic technique, demonstrated that proteins synthesised by membrane-bound ribosomes in pancreatic acinar cells was first found within the lumen of the rough endoplasmic reticulum, then in the smooth endoplasmic reticulum and finally condensed into membrane-bounded packets of protein in the Golgi apparatus before being discharged from the cell. The rough endoplasmic reticulum has been shown to be the site of synthesis of α-amylase in guinea pig pancreas (Siekevitz and Palade, 1966; Redman, Siekevitz and Palade, 1966), serum albumin in rat liver (Campbell, Greengard and Kernot, 1960; Redman, 1968; Takagi, Tanaka and Ogata, 1969) and immunoglobulin by mouse myeloma cells (Sherr and Uhr, 1970; Pryme *et al.*, 1973). Free polysomes, it appears, synthesise those proteins not destined for secretion (Goldberg and Green, 1964; Redman, 1969; Ganoza and Williams, 1969; Hicks, Drysdale and Muro, 1969;

Andrews and Tata, 1971; Uenoyama and Ono, 1972a,b). However, non-secretory cells also contain membrane-bound ribosomes (i.e., brain and muscle) but it has been suggested that these may be synthesising membrane proteins (Dallner, Siekevitz and Palade, 1966).

(2) Evidence for the simultaneous synthesis and secretion of proteins

Redman, Siekevitz and Palade (1966) showed that after fractionation of pigeon pancreas cells, the rough endoplasmic reticulum broke down to give closed membranous vesicles (microscomes) in which the ribosomes were attached to the exterior surface. These microsomes were capable of incorporating radioactively labelled amino acids into α-amylase (a secretory protein from these cells). Tryptic digestion of isolated, labelled enzyme from this system showed that the label was uniformly distributed throughout the peptides thus confirming in vitro synthesis. After different periods of labelling, the microsomal system was fractionated. Initially, most of the total α-amylase radioactivity was associated with membrane-bound ribosomes indicating this as the site of synthesis. However, in addition to synthesising the enzyme, the microsomal system was capable of transporting a large fraction of enzyme produced on the ribosomes to a deoxycholate-soluble fraction, believed to represent the internal contents of the microsome. It was concluded that newly synthesised α-amylase had been transferred from its site of synthesis at the ribosome into the microsomal cavity (the microsomal equivalent of the cisternal space in vivo).

Similarly, Redman and Sabatini (1966) presented evidence that incomplete proteins were vectorially discharged into the interior of guinea pig liver microsomes on release from the ribosome by the addition of puromycin. This work interpreted the deoxycholate-soluble fraction as representing material from the microsomal vesicle. Streptovaricin A, which inhibits protein synthesis without causing release of incomplete protein chains, did not give this effect. The same situation was also shown to apparently be the case in a rat liver microsomal system by Redman (1967). The transfer of labelled incomplete proteins released by puromycin in this system occurred in the absence of adenosine triphosphate, at 0° and in the absence of changes in the membrane phospholipid metabolism suggesting that the only requirement for the transport of the nascent protein chain across the microsomal membrane was release from the membrane-bound ribosome. These results suggested that in vivo the nascent polypeptide chains are uni-directionally transferred through the membrane of the rough endoplasmic reticulum as they are synthesised and released into the cisternal space. Results obtained by Bevan (1971) using a microsomal system prepared from mouse plasmacytoma indicated that puromycin released peptides of nascent immunoglobulin remained associated with the microsomes after release from the ribosome, again implying uni-directional transfer of secretory proteins.

A comparison between microsomal systems prepared from secretory (rat liver) and non-secretory (rat cerebral cortex and skeletal muscle) tissues was performed by Andrews and Tata

(1971). They found that whereas most of the newly-synthesised polypeptides discharged by puromycin from membrane-bound ribosomes of liver were released into the deoxycholate-soluble fraction (corresponding to the microsomal cavity), those released from brain and muscle were released directly into the supernatant. This result was compatible with the relative lack of secretory function of the latter tissues and it has been suggested that this was due to a major difference in the attachment of ribosomes to the membrane of the endoplasmic reticulum of secretory and non-secretory tissues.

However, results which have relied upon deoxycholate fractionation of microsomes to determine the location of the puromycin mediated release of radioactive proteins or peptides synthesised in vitro have lately been subject to critism. Sauer and Burrow (1972) examined the puromycin-mediated release of labelled nascent proteins from rat liver microsomes labelled with ^{14}C-leucine. When the microsomes were fractionated with sodium deoxycholate the distribution of the radioactivity among the microsomal subfractions indicated that the released peptides could be either on the microsomal membrane or inside the microsomal vesicle. An unequivocal interpretation was not possible because of sub-fraction contamination and binding of the puromycin peptides to the membrane. However, the distribution of radioactivity following gel filtration of deoxycholate solubilised microsomal membrane, or dispersion of the labelled microsomes by sonication, suggested that the direction of the puromycin-mediated release was toward the microsomal membrane rather than toward the intravesicular space.

Similarly, Burke and Redman (1973) have shown that ^{14}C-polyphenylalanine released from the ribosomes by puromycin could adsorb non-specifically to membranes and be extracted into the deoxycholate soluble fraction. This suggests that the appearance of puromycin released peptides in the detergent soluble fraction of microsomes is not sufficient evidence for vectorial transport of nascent peptides. Further experiments are necessary to eliminate artefactual binding of the released peptides to the membrane.

(3) Model for secretion of proteins through the endoplasmic reticular membrane

The attachment of ribosomes to guinea pig liver microsomal membranes was investigated by Sabatini, Tashiro and Palade (1966) who showed that low concentrations of EDTA caused the release of the smaller (32s) subunit of the ribosome while at higher concentrations the larger subunit (approx. 47s) was eventually released from the membrane. In vivo labelling with ^{3}H-leucine showed that the large subunits contained 70% of the newly synthesised protein. It was concluded from this work and subsequently substantiated by electron microscopy, that the ribosomes were attached to the membrane by the large subunit.

On the basis of these observations Redman and Sabatini (1966) proposed a model for the synthesis and secretion of proteins. They envisaged that the nascent polypeptide chain was synthesised on membrane-bound ribosomes which accommodated the growing polypeptide chain within a central channel (the existence of which was suggested by electron microscopy)

present in the large subunit. This channel is assumed to be continuous with the cisternal space via a discontinuity in the membrane. The synthesis within the central channel is envisaged to be such that it confers uni-directionality to the growing polypeptide chain so that upon completion of translation it is released into the cisternal space.

Data obtained by Blobel and Sabatini (1970) and Sabatini and Blobel (1970) lends further weight to this concept. Studying the controlled proteolytic digestion of nascent polypeptide chains labelled in vitro in rat liver ribosomes they found that only limited digestion of the nascent chain occurred and that the resistant segment was located near the carboxy-terminal end of the nascent chain. From this they concluded that the resistant region was protected from proteolysis due to its location within the large subunit of the ribosome. Similarly, controlled proteolysis of rat liver microsomes at 0° suggested that when the nascent polypeptides growing within the large subunits of membrane-bound ribosomes emerge they enter directly into a close association with the microsomal membrane, since now virtually the entire polypeptide chain was resistant to proteolytic digestion.

The conclusion that the membrane-bound ribosomes of the rough endoplasmic reticulum are attached by the large (60s) subunit was originally based on electron microscopy studies (Redman and Sabatini, 1966). More recently, Baglioni, Bleiberg and Zauderer (1971) have shown that in intact plasmacytoma cells the 60s subunit can attach to the membrane independently of the 40s subunit even in the absence of protein synthesis.

The authors inferred that ribosomes do not attach to the membrane as polysomes. Attardi, Cravioto and Attardi (1969) and Rosbash and Penmann (1971) studying membrane-bound ribosomes from Hela cells, found that EDTA treatment released essentially all of the 40s subunits leaving significant amounts of the large subunits still bound to the membrane. This selective removal of small subunits from the membrane-bound ribosomes by EDTA is in agreement with the observations on rat liver microsomes by Sabatini, Tashiro and Palade (1966) and thus seems to be a general phenomenon. There seems to be conflicting evidence on the role of the nascent polypeptide chain in the attachment of ribosomes to the membrane. Treatment of rough microsomes with EDTA or trypsin tended to suggest that the nascent polypeptide chain was involved with the binding (Sabatini, Tashiro and Palade, 1966; Chefurka and Hayashi, 1966). However, since pretreatment of tissues with puromycin did not reduce the number of bound ribosomes (Blobel and Potter, 1967; Andrews and Tata, 1971; Rosbash and Penman, 1971) and 60s subunits could attach to membranes in the absence of protein synthesis (Baglioni, Bleiberg and Zauderer, 1971) the nascent polypeptide chain was suggested not to be involved.

A careful study by Adelman, Sabatini and Blobel (1973) however, seems to have resolved this issue. They found that the puromycin-dependent ribosome release from rat liver rough microsomes occurs only in a medium of high ionic strength. They concluded that ribosomes were attached to the membrane by two types of interaction: a direct one between the large subunit and the membrane (labile at high KCl concentrations)

and an indirect one in which the nascent polypeptide chain is involved with the membrane (puromycin labile).

Other factors such as steroid hormones (James, Rabin and Rabin, 1969; Sunshine, Williams and Rabin, 1971; Williams and Rabin, 1971) and specific binding proteins (Freidlender and Wettstein, 1970; Burka and Bulova, 1971) have also been implicated in the binding of ribosomes to membranes.

At the present time another mammalian system, the synthesis and secretion of immunoglobulins, is being studied. Sherr and Uhr (1970) and Pryme et al. (1973) studying immunoglobulin synthesis in mouse myeloma and plasmacytoma cells respectively, have shown in both systems that nascent immunoglobulins are synthesised on membrane-bound polysomes prior to their transport to the cisternae of the rough endoplasmic reticulum (Zagury et al., 1970). Bevan (1971) studying a mouse plasmacytoma that secretes immunoglobulin A claimed to have demonstrated the vectorial release of nascent peptides released from microsomes by puromycin. However, as has been previously mentioned, reservations must be held as to the interpretation of results obtained by detergent lysis of microsomes. Stevens and Williamson (1973a,b) have postulated that in vivo the regulation of H2L2 accumulation in the cisternal space (de Petris et al., 1963) is controlled by a feed-back repression of H2L2 on translation of H-chain mRNA. In vitro experiments have in fact demonstrated such a repression of H2L2 on H-chain mRNA translation, however, in vivo it is hard to visualise how H2L2 molecules in the cisternal space have access to the H-chain mRNA while separated by the endoplasmic reticular membrane. To over-

come this objection they have postulated that the mRNA actually protrudes through the membrane. However, it is possible that either the extrusion theory does not apply in the case of immunoglobulin secretion or that the binding of H2L2 to the H-chain mRNA and its subsequent repression is a remarkable fortuitous *in vitro* effect only.

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

It has been mentioned that secretory cells synthesise proteins for both intracellular and extracellular functions and that those proteins destined for export are synthesised on membrane-bound ribosomes. The occurrence of membrane-bound ribosomes in those cells, which lack in general, an obvious secretory activity suggests that the attachment of ribosomes to membranes may not be exclusively related to the synthesis of proteins to be exported. Indeed some exceptions to the general concept have been reported (Rolleston, 1972). Tata (1971), in particular, has suggested that the attachment of ribosomes to the membrane in non-secretory cells may be for the purpose of topographically separating different populations of ribosomes, presumably synthesising different proteins, a function which may be critical during rapid developmental changes. A similar conclusion was reached by Woodward *et al*. (1973) studying globulin synthesis by free and membrane-bound ribosomes in reticulocytes.

Thus, it seems that proteins destined for export are synthesised exclusively by membrane-bound ribosomes and that

unlike other proteins synthesised on membrane-bound ribosomes they are transported through the membrane. Several mechanisms have been proposed whereby membrane localised polysomes are segregated from free cytoplasmic polysomes. Blobel and Sabatini (1972) have suggested that polysome formation in the cytoplasm might precede the membrane attachment of the ribosomes. They proposed that the specificity lay in the mRNA sequence which coded for a unique N-terminal sequence not found in normal internal proteins and that this facilitated membrane binding. Tentative evidence from Milstein et al. (1972) on the in vitro translation of L-chain mRNA derived from mouse myeloma cells, indicates that the L chains synthesised have a higher molecular weight and an altered N-terminal tryptic peptide as compared to L chains derived from the secreted H2L2 molecule. They have postulated that these observed differences may play a role in the segregation and attachment of ribosomes devoted to secretory proteins. This is consistent with results which suggest that the binding of polysomes to the membrane occurs after protein synthesis has begun (Lisowska-Bernstein et al., 1970; Rosbash, 1972).

Baglioni, Bleiberg and Zauderer (1971) have postulated that the specificity resides in the 40s-mRNA complex which recognises the 60s subunit located at the membrane. Evidence compatible with this model was presented by Borgese, Blobel and Sabatini (1973) in that only the 40s subunit of membrane-bound ribosomes in rat liver can exchange with the free subunit pool after puromycin release of the nascent polypeptide chain in vitro. It appears that the 60s subunit remains membrane-bound; however,

this may result from the in vitro system lacking the necessary exchange factors for the large subunit.

There are several ways in which a specific mechanism for this process could be provided. It has been suggested that this difference resides in the ribosomes and is reflected in differences in Mg^{2+} dependence (Vernie, Bont and Emelot, 1972), in different sensitivities to growth hormone (McDonald and Korner, 1971) or in different sensitivities to antibiotics (Glazer and Sartorelli, 1972). Similarly, attachment of ribosomes to the membrane has been suggested to be dependent upon specificities residing in the mRNA (Murty and Sidransky, 1972) or in initiation factors (Uenoyama and Ono, 1972a,b).

However, little is known about what determines whether a protein synthesised on membrane-bound ribosomes is secreted or not. Perhaps this is regulated by some of the factors mentioned above or by interactions between the protein and the membrane. Since so little is known about the specificities involved at this stage, the actual mechanisms involved in secretion must remain speculative.

B. MECHANISM OF PROTEIN SECRETION BY BACTERIA

The mechanism of protein secretion by bacteria is not as well understood as that for mammalian systems. Many proteins secreted by bacteria are potentially lethal enzymes, for example, protease and ribonuclease and these cells must overcome the problem o secreting such molecules without damage to themselves. A simple solution to the problem can be found if it is postulated that the cytoplasmic membrane is the bacterial equivalent of the

mammalian endoplasmic reticulum and that a basic mechanism of vectorial discharge of secretory proteins is similar in the two cell types. There is indeed evidence claimed to indicate that protein synthesis occurs at the cytoplasmic membrane, although this has not been shown to be that for extracellular enzymes.

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

The evidence that the cytoplasmic membrane is a site of protein synthesis in prokaryotes comes primarily from the detection of membrane-bound polysomes which would incorporate radioactively labelled amino acids into protein in cell-free systems (Schlessinger, 1963; Schlessinger, Marchesi and Kwan, 1965; Moore and Umbreit, 1965). Moore and Umbreit (1965) and Cundliffe (1970) have suggested that the isolation of 50s membrane-bound ribosomal subunits from cell lysates may indicate that the ribosome is attached to the membrane via the large subunit, as found in rat liver microsomes (Sabatini et al., 1966). Aronson (1966), in a detailed in vitro study of polysome attachment to Bacillus megaterium membranes, presented evidence that the presence of the nascent polypeptides is a critical factor in the establishment of association with the membrane. This conclusion was based primarily on the finding that treatment of the polysomes with pronase reduced their binding capacity to the membrane.

A possible role for specific binding proteins in the

bacterial ribosome membrane attachment process has been suggested by Brown and Abrams (1970) and Scheinbuks et al., (1972). These workers found that in both Streptococcus faecalis and Azotobacter vinelandii that the cytoplasmic ribosomes differed from the membrane-bound ribosomes respectively by the presence and in the latter case, by the absence of an additional protein. However, the apparent proportion of membrane-bound as to cytoplasmic ribosomes appears to vary greatly. These differences may be influenced by the method of cell lysis (Hendler and Tani, 1964), the use of lysozyme to disrupt cells (Patterson et al., 1970), the Mg^{2+} concentration (Schlessinger et al., 1965), the age of the cell culture (Moore et al., 1966) and the K^{+} concentration (Coleman, 1969b). Thus it is evident that the variability of influences on the proportion of membrane-bound ribosomes may not be reflecting the true in vivo conditions and thus it is difficult to obtain unequivocal answers.

(2) Possible models for protein secretion in bacteria

Several bacterial secretion systems have been studied for some time. They are penicillinase production by Bacillus licheniformis, alkaline phosphatase production by Escherichia coli and Bacillus subtilis and the production of α-amylase, protease and ribonuclease by Bacillus amyloliquefaciens. The latter being the system used for present studies.

(3) Models for penicillinase secretion

Lampen (1965) proposed a mechanism of extracellular enzyme secretion in which enzyme synthesis is coupled with

membrane synthesis, occurring possibly at the mesosome, which is a complex internal invagination of the cytoplasmic membrane. It was suggested that the enzyme synthesised at the mesosome was inserted into and bound to the membrane at its point of growth in the mesosome. Release of the enzyme from the invagination was dependent on the membrane growing out of the mesosome. Beaton (1968) implicated the mesosome of Staphylococcus aureus in penicillinase secretion when it was found that the mesosome was structurally altered upon release of the enzyme from the cell.

Ghosh, Sargent and Lampen (1968) also found that in response to a penicillinase inducer, cells of B. licheniformis synthesised structures composed of tubules and vesicles which were enclosed by an internal invagination of the membrane. These structues were morphologically similar to mesosomes and appeared to be the cellular location of the cell bound penicillinases (Sargent, Ghosh and Lampen, 1968). Subsequent work, however, showed that these structures (previously termed 'secretory apparatus') were not essential for the actual secretion of penicillinase, since protoplasts which lacked these structures, were able to synthesise and secrete the enzyme. Therefore, it was postulated that although not obligatory, the insertion of newly synthesised enzyme into the mesosome was a normal process prior to the final secretion of the enzyme.

More recent work (Sargent and Lampen, 1970a,b) has shown that there are at least three forms of the active penicillinase produced by B. licheniformis. They are an hydrophylic extracellular form and two hydrophobic membrane associated forms. Of the latter type, one

exists tightly bound to the plasma membrane of the cell, while the other form is associated with the periplasmic vesicles and can be released even in the presence of protein synthesis or energy inhibitors. The cell bound penicillinases can be readily extracted in a state that is clearly different than that of the extracellular enzyme and can be converted, under certain conditions, to forms resembling the exo-enzyme. These variations in conformation and hydrophobicity are believed to account for the differences in membrane binding properties of the three forms of penicillinase. The above observations were unified in a proposed model of penicillinase secretion. It is proposed that as the polypeptide chain is synthesised by membrane-bound ribosomes and inserted into the membrane at special growing points, the enzyme either undergoes a conformational change to the hydrophylic exo-form, which is secreted, or it polymerises and assumes one of the two membrane-bound forms. This change in hydrophobicity to accommodate the enzyme to its final environment, is put forward as one of the most crucial steps in the secretion of the extracellular enzyme from this organism.

Bettinger and Lampen (1971) provided evidence that penicillinase from B. licheniformis was secreted through the membrane in an incompletely folded form. This came from the observation that although both the membrane-bound forms and the exo-penicillinase are resistant to trypsin or chymotrypsin digestion, protoplasts actively secreting penicillinase showed a sensitivity to these proteolytic enzymes. They suggested that as the incompletely folded chains emerged from the protective environment of the membrane, proteolytic digestion occurred

before the enzyme could assume its native, resistant conformation.

(4) Model for alkaline phosphatase secretion

Alkaline phosphatase has been extensively studied in E. coli (Heppel, Harkness and Hilmoe, 1962; Schlessinger, 1965) and in B. subtilis (Demain and Hendlin, 1967; Takeda and Tsugita, 1967). Although not a true extracellular enzyme (i.e, appearing in the external medium) it will be discussed here since the enzyme is periplasmic (located between the cell wall and the permeability barrier of the membrane) and therefore must involve passage of the enzyme across the membrane prior to its localisation in the periplasm.

It has been proposed by Schlessinger (1968) that this hydrolytic enzyme is synthesised as the inactive monomer on poly-ribosomes in the cell cytoplasm. After release from the ribosome these monomers diffuse through the bacterial cell membrane to the periplasmic space where subsequent dimersiation, in the presence of Zn^{2+}, to the active enzyme occurs. The presence of inactive monomers in the cytoplasm of E. coli was inferred by Schlessinger from the work of Torriani (1968). Since this was the first presented case for the presence of a possible cytoplasmic zymogen and thus not involving unidirectional synthesis and secretion by membrane-bound ribosomes, it will be dealt with in some detail.

Initially Torriani derepressed E. coli cells to promote synthesis of alkaline phosphatase, then chilled, washed and disrupted the whole cells. The lysate was fractionated and

inactive monomers found to be associated with a particulate fraction, presumably the membrane-cell wall particles. In an attempt to define the location of these monomers in relation to the cell membrane, derepressed cells were pulsed with ^{14}C-arginine and then quickly chilled, washed and converted to spheroplasts. This latter step released active enzyme from the periplasm. The spheroplasts were then pelleted by centrifugation and lysed. The whole cell lysate (membrane and cytoplasm) and the material released from the periplasm were assayed for monomers by specific antibody precipitation. The spheroplast lysate was found to contain the bulk (98%) of inactive monomers and sedimented with large particles, presumably membrane. This was presented as evidence that the monomers resided in the 'endoplasm' attached to the inside of the membrane. However, the possibility had not been excluded that the monomers resided on the exterior surface of the membrane (or even in the membrane itself) and not released with the periplasmic material. Therefore, the possibility exists that indeed alkaline phosphatase monomers are synthesised by ribosomes attached to the plasma membrane and extruded through it in the nascent form and only subsequent to its dimerisation to the active enzyme does it leave the membrane for the periplasmic space. However, until further experiments are performed on this aspect the possibility, in this case, of an inactive precursor in the cytoplasm cannot be eliminated.

It has been shown that E. coli alkaline phosphatase has no requirement for a specific membrane transport system to carry monomers across the plasma membrane and assemble

active dimers in the periplasm (Schlessinger and Olsen, 1968) nor that localisation in the periplasm requires the functional native structure of the polypeptide chain (Morris and Schlessinger, 1972). However, it has been claimed by Izui (1971) that there is a lipid requirement during the course of alkaline phosphatase induction in E. coli to permit formation of active dimers in the periplasm. This is not a prerequisite for the induction of β-galactosidase, a cytoplasmic enzyme in the same organism.

(5) Model for extracellular enzyme secretion in B. amyloliquefaciens

A question fundamental to the model of secretion in bacteria is whether the enzymes are synthesised and secreted as two separate events. It has been established that for all three extracellular enzymes of B. amyloliquefaciens that inhibition of protein synthesis stops secretion rapidly. The amount of preformed enzyme detectable in cell lysates is small (and has been the subject of investigations in Chapter 4). At no time does there seem to be a substantial accumulation of enzyme within the cell (Coleman and Elliott, 1962; Coleman and Elliott, 1965; May and Elliott, 1968a). Indeed, Smeaton and Elliott (1967) presented evidence that at least in the case of extracellular ribonuclease the enzyme cannot have existed inside the cell as the active enzyme. There exists in the cytoplasm of the cell a protein which specifically inhibits the extracellular ribonuclease. Formation of the enzyme-inhibitor complex is rapid and essentially irreversible (Hartley, 1970) and only drastic conditions in denaturing solvents will recover

active enzyme from this complex. It is therefore unlikely, although not excluded, that the enzyme could have been in contact with the cytoplasm. It is conceivable that formation of this complex in the cytoplasm does occur and that a biological mechanism operates to separate them immediately prior to secretion; no such cytoplasmic inhibitors for the protease or α-amylase have been found, and thus the idea would not constitute a general mechanism of protein secretion. The enzyme and inhibitor are the products of two distinct genes and hence could not represent secreted and non-secreted portions of a precursor zymogen.

The hypothesis has therefore been proposed (May and Elliott, 1968a) that at no time have the extracellular enzymes existed in the cell cytoplasm but that the nascent polypeptide chain is extruded through the plasma membrane as it is synthesised by membrane-bound ribosomes to take up its active configuration outside the permeability barrier of the membrane. This is analogous to the secretion of proteins through the pancreatic endothelial reticular membrane as proposed by Redman (1967). This idea is central to the mechanism of secretion of proteins by bacteria and its validity was the subject of investigations reported in this thesis.

(6) Properties of bacterial extracellular enzymes

Proteins secreted by bacterial cells into the culture medium are usually degradative enzymes (proteases, amylases and ribonucleases) which have as their substrates large molecular weight molecules, which are broken down into products

suitable for use as nutrients (Pollock, 1962). Bacterial penicillinases seem to be the exception to the rule as the role of this enzyme is probably defensive.

Most of the organisms known to produce extracellular enzymes are Gram-positive bacteria and fungi and it is noteworthy that exo-protein production by Gram-negative organisms is fairly rare, being restricted usually to enzymes located in the periplasmic space; for example, E. coli phosphatases, penicillinase and phosphodiesterases.

In general, bacterial extracellular enzymes have molecular weights no usually in excess of 80,000 (Pollock, 1962). In Bacilli most of the alkaline proteases are single polypeptide chains (subtilisin BPN', Novo and Carlsberg) with molecular weights in the range 27-30,000, whilst neutral proteases have molecular weights in the range 40-45,000. Most of the bacterial amylases have molecular weights of approximately 50,000 and ribonucleases a small range around 13,000. Surveys of the amino acid compositions of bacterial exo-enzymes reveal the striking fact that they all apparently lack cyst(e)ine, while mammalian exo-enzymes are known to contain disulphide bonds (Pollock and Richmond, 1962). Pancreati exo-ribonuclease contains two disulphide bonds even though its molecular weight is very nearly the same as that for the exo-ribonuclease from B. amyloliquefaciens which is completely devoid of cyst(e)ine (Hartley and Barker, 1972). Exceptions to this are known. Hofsten and Tjeder (1965) found two disulphide bonds in an extracellular protease from Arthrobacter, while an exo-streptococcal protease was shown to contain a thiol group (Ferdinand, Stein and Moore, 1965). The latter enzyme is unique in another

aspect in that it is released as an inactive extracellular zymogen, which on reduction by cyst(e)ine residues in the cell wall, is converted to the active enzyme (Liu and Elliott, 1965).

Although lacking disulphide bonds, bacterial extracellular enzymes do not necessarily lack a strong three-dimensional structure. It has been noted that a large number of the enzymes require divalent cations (usually Ca^{2+} or Zn^{2+}) for activation and stabilisation. Pollock (1962) has suggested that this cation requirement may replace cystine bonds in conferring the correct conformation for activity of the proteins.

AIMS OF THE WORK IN THIS THESIS

The research described here was designed to contribute to our knowledge on how extracellular enzymes are synthesised and secreted. The work consisted of several phases.

Firstly, at the start of this project, it was believed possible that a recently discovered 'lytic factor' produced by B. amyloliquefaciens might be associated with the mechanism of secretion. The idea was that the 'lytic factor' molecule (which contains a fatty acid) might be attached to the N-terminus of peptide chains to be secreted. This would dissolve in the membrane and act as a 'lead-out' head for the secreted protein. An association between production of peptide antibiotics, extracellular enzymes and sporulation in B. subtilis had been genetically established, though not understood (Schaeffer, 1969). Chapter 3 of this thesis described studies on the production of the lytic factor and some of its properties.

Following this, Chapter 4 covers investigations into the apparent accumulation of messenger RNA specific for the extracellular enzymes. Studies by Both et al. (1972) had shown that a pool of messenger RNA specific for the extracellular protease was present in washed-cells. It was of importance to determine whether this situation was unique for the extracellular protease or was a general phenomenon applicable to the other extracellular enzymes, α-amylase and ribonuclease.

During the course of the studies reported in Chapter 4 it was noted that there seemed to be a small pool of preformed extracellular enzyme associated with washed-cells. This enzyme pool continued to accumulate in the external medium for a defined period in the presence of protein synthesis inhibitors. Investigations into this phenomenon are detailed in Chapter 5 and attempts to localise its position within the bacterial cell concern Chapter 6.

Chapter 7 describes the finding of an accumulation of one species of phospholipid in the external medium of washed-cell suspensions. The accumulation of this phospholipid qualitatively might be correlated with the accumulation of extracellular enzymes in the presence of antibiotics. Its possible relationship in a mechanism of secretion is discussed.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

A. MATERIALS

1. Bacterial strains

The organism used was originally classified as B. subtilis; however, it has since been designated Bacillus amyloliquefaciens by Welker and Campbell (1967) on the basis of DNA base composition. This strain was obtained from the Takamine Laboratories Inc., Clifton, N.J., U.S.A.

2. Radioisotopes

All radioactive materials were obtained from Schwartz/Mann, Orangeburg, New York.

3. Antibiotics

Rifampicin was obtained from Schwartz/Mann, Orangeburg, New York. Actinomycin D was a generous gift from Merck, Sharp and Dohme. Chloramphenicol was obtained from Parke Davis and Co., Sydney, Australia. Puromycin dihydrochloride from Nutritional Biochemicals Corp., sodium Fusidate from Leo Pharmaceutical Products, 2,4-dinitrophenol and sodium azide from British Drug Houses Chemicals, Poole, England.

4. Liquid growth media

Liquid growth medium was essentially that described by Coleman and Elliott (1965). The medium contained $(NH_4)_2HPO_4$ (34 mM), $MgSO_4$ (1 mM), KCl (5 mM), 0.5% (w/v) casein hydrolysate (British Drug Houses Ltd.), sodium citrate (4.25 mM), $CaCl_2$ (0.125 mM), $ZnSO_4$ (0.0125 mM), $FeCl_3$ (0.5 mM) which was added as

a solution in 0.01 N-HCl, 0.05% (w/v) Bacto Difco yeast extract, trace metal solution (0.25 ml/L.) and 1% (w/v) maltose. The final pH was adjusted to 7.3 with H_3PO_4. The trace metal solution consisted of $CoCl_2.6H_2O$ (0.5 mg), ammonium molybdate (0.5 mg), $MnCl_2,4H_2O$ (5.0 mg) and $CuSO_4.5H_2O$ (0.01 mg) dissolved in 1.0 litre of water.

5. Washed-cell suspending media

This was the same as the liquid growth medium except that $FeCl_3$ and yeast extract were omitted.

6. Buffers

(a) Phosphate buffers were prepared from K_2HPO_4 and KH_2PO_4.

(b) Crystalline tris was obt ained from Sigma Chem. Corp. as 'Sigma 7-9'. When maximum purity was essential 'Trisma base, reagent grade' was used. Solutions of tris were adjusted to the required pH value by the addition of HCl.

(c) Other buffers were as detailed in Data for Biochemical Research (Dawson, Elliott, Elliott and Jones, 1969).

7. Lysozyme was three-times crystallised from egg white and was supplied by Sigma Chemical Co.

8. Agar was Difco Bacto-Agar and used as supplied by the Difco Labs., U.S.A.

9. Sucrose. Special enzyme grade sucrose (M.A.) was purchased from the Mann Research Labs., New York, U.S.A.

10. Ethyl methane sulphonate was obtained from the Eastman Organic Chemicals, Rochester 3, New York, U.S.A.

11. Millipore filters were obtained as Hawg; 0.45 μ; 25 or 47 mm white grid millipore filters from Millipore Filter Corp., Bedford, Mass., U.S.A.

12. Enzyme substrates

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

(b) Soluble starch for α-amylase assays was A.R. grade (Batch number 0550) supplied by British Drug Houses Ltd. Starch from this batch number was found to dissolve readily and give consistently high blank readings in the assay.

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

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

13. Chloroform, Methanol and Ether were all A.R. reagent grade and obtained from British Drug Houses Ltd.

14. Silica gel plates were Eastman 6061 Silica gel chromatogram sheets without fluorescent indicator and obtained from the Eastman Chem. Co., U.S.A.

15. Amino acids used to supplement synthetic media were either Mann assayed amino acids or B.D.H. amino acids depending on the purity needed for the experiment.

16. Rabbits (female, long-eared) were obtained from the Central Animal House, University of Adelaide.

17. Immunological reagents.

(a) Goat anti-rabbit IgG antiserum was a generous gift from Mr. A. Osmond, Department of Microbiology, University of Adelaide.

(b) Freunds Adjuvant (complete and incomplete) were obtained from the Commonwealth Serum Labs., Melbourne, Australia.

(c) Toluene 2,4-diisocyanate was a generous gift from Professor A.J. Wicken, School of Microbiology, University of New South Wales, and was EX lot 87754, K and K Labs., Plainview, New York.

18. Diethylamino ethyl cellulose 2 x8 (100 - 200 mesh) was obtained from Whatman W and B, Balson, London.

19. Aquacide 11 (lot 001036) was obtained from Calbiochem, Los Angeles.

20. Collodion bags were used as supplied by Sartorious Membranfilters.

21. Ferritin (horse) amorphous, cadmium free and 5x crystallise

was obtained from Schwarz/Mann, Orangeburg, New York (Lot no. X1859).

B. METHODS

1. Preparation of spore suspensions

B. amyloliquefaciens cultures were grown overnight in the liquid growth medium described earlier. The cells were then centrifuged and resuspended to the same cell density in sterile growth medium which had previously been diluted 25-fold with sterile water. The cultures were again shaken for 24 hour when spores and intact cells were centrifuged down and resuspended in sterile water. To destroy the remaining vegetative cells the suspension was incubated with lysozyme (3 µg/ml of cell culture) for 20 minutes at 37°. The spores were further purified by incubation in 1% sodium dodecyl sulphate at 37° for 1 hour, followed by successive washings, once with 1 N-NaCl, twice with 0.14 N-NaCl and twice with water. The spores were finally resuspended in sterile water.

2. Culture of B. amyloliquefaciens

(a) Incubation conditions for the growth of the organism

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

(b) Incubation conditions for washed-cell suspension experiments

Sterile conditions are not necessary for washed-cell experiments. After 25 hour growth (A_{600nm} = 3.6), unless otherwise stated, the culture cells were harvested, washed twice by resuspension and centrifugation at 30° with the appropriate suspending medium. The suspending medium was the same as the culture medium except that $FeCl_3$ and yeast extract were omitted, thereby limiting cell growth. A sample of the cell suspension 20 to 40 ml was shaken in a 250 ml conical flask at 30° and samples (1.0 ml) were withdrawn at the appropriate times, centrifuged and the supernatants assayed for extracellular enzyme activity.

3. (a) Measurement of total protein synthesis

To measure total cellular protein synthesis, 2.0 ml of a washed-cell suspension were shaken with 0.5 μC of uniformly labelled L-[^{14}C]leucine (spec. act. 316 mCi/mmole). At appropriate times, 0.1 ml samples were withdrawn and pipetted onto tubes at 0° containing 1% (w/v) Bacto casamino acids in 3.0 ml of 10% (w/v) trichloroacetic acid and left for 30 minutes. The precipitates were centrifuged and suspended in 1 N-NaOH (1.5 ml) for 20 minutes at room temperature. After adding 6.0 ml of 1% casamino acids in 10% TCA, the tubes were heated at 95° for 30 minutes, cooled and the contents of each filtered through a 2.0 cm diameter Oxoid membrane filter. Each tube was washed repeatedly with 1% casamino acids in 10% TCA (5.0 ml) and the washings transferred to the filter. The filters were

then washed with 10.0 ml of 1% (v/v) acetic acid and dried at 100° for 90 minutes. The filters were counted by liquid scintillation in a Packard Tri-Carb spectrometer (90% efficiency) using scintillation fluid containing 3 g. of 2,5 diphenyloxazole (PPO) and 0.3 g of 1,4-bis-(2-(4-methyl-phenyloxazolyl)benzene (POPOP) per litre of toluene.

(b) Measurement of 2-[^{14}C]uracil incorporation into total cellular RNA

2-[^{14}C]Uracil incorporation into total cellular RNA was measured by shaking 3.0 ml of a washed-cell suspension with 0.75 µCi of [^{14}C]uracil (spec. act. 52 mCi/mmole) in a suspending medium supplemented with 4.0 µg of unlabelled uracil per ml. Samples were withdrawn at various times and pipetted into tubes containing 1% (w/v) Bacto casamino acids in 3.0 ml of 5% (w/v) trichloroacetic acid, with an excess of unlabelled uracil (1.0 mg/ml) at 0°. The resulting preparations were kept at 0° for 10 minutes and then filtered through Oxoid membrane filters. The filters were washed repeatedly with a total volume of 15 ml of cold 5% TCA-uracil and finally with 5.0 ml of cold 1% (w/v) acetic acid. They were prepared for counting as described above.

4. Enzyme assay methods

(a) α-Amylase estimation

α-Amylase was measured by a modification to the method of Coleman and Elliott (1962).

A stock solution contained 2.3 ml of 3% (w/v) soluble

starch, 250 ml of 0.1 M potassium phosphate buffer (pH 6.2) with 0.025 M NaCl, 200 ml of water and 1.0 ml of 0.1 M $CaCl_2$. The assay mixture contained 4.5 ml of the stock solution and 0.5 ml of suitably diluted enzyme sample. The reaction was stopped with 0.9 ml of 1 M-HCl after 30 minutes incubation at 37°. To each tube, 0.1 ml of I_2 reagent (0.3% I_2, 3% (w/v) KI) and 4 ml of water were added and the absorbance measured at 620 nm. Control incubations without enzyme were carried out.

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

(b) Protease estimation

This enzyme was assayed in two ways:

1. Protease estimation using casein as substrate (May and Elliott (1968a).

A stock solution of casein (British Drug Houses Ltd.) was prepared by boiling for5 minutes a solution containing 1.0 g of casein and 100 ml of Sorenson's buffer, pH 7.6 (12.2 g Na_2HPO_4 and 1.82 g KH_2PO_4 per litre of water). The assay mixture contained 1.0 ml of enzyme suitably diluted with Sorenson's buffer. After the appropriate time of incubation at 35°, the reaction was stopped with 3.0 ml of a solution containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid. The precipitated protein was chilled at 4° for

60 minutes, centrifuged and the absorbance of the supernatant measured at 280 nm. Blanks were run for appropriate samples by adding the trichloroacetic acid reagent before the enzyme. A unit of protease activity is defined as that amount of enzyme which produces an increase in A_{280nm} of 0.05 in 40 minutes at 35°. The relationship between A^{1cm}_{280nm} and enzyme concentration was linear up to a value of 1.0.

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

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

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

(c) Ribonuclease estimation

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

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

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

5. Electron microscopy

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

(a) Preparation of negatively stained specimens

A drop of suspension was placed on a carbon-coated grid for about 20 seconds. Excess fluid was then removed with a piece of filter paper and several drops of stain (2% aqueous uranyl acetate) were immediately placed on the grid. The

fluid from the grid was removed after about 10 seconds with filter paper and the dried grid examined immediately.

(b) Preparation of sectioned specimens

Cells were pelleted by centrifugation and treated for 1 hour in gluteraldehyde (sodium cacodylate buffer, 0.1 M, pH 7.2) After washing for 30 minutes in this buffer, the pellet was fixed in 1% osmium tetroxide for 1 hour and taken to 1% uranyl acetate for 30 minutes. The fixed material was progressively dehydrated in acetone and finally embedded in 'Araldite'. Thin sections were cut on a LKB microtome and post-stained for 3 minutes in lead citrate.

(c) Preparation of thin sections embedded in albumin

The cells to be embedded were placed in 0.5 ml of a 30% (w/v solution of bovine serum albumin (Calbiochem) with 0.15 M NaCl. This paste was allowed to gel in short tubes and sliced into approximately 3 mm blocks which were then placed in 2% formaldehyde-phosphate-buffered saline at 0° for 180 minutes. The blocks were washed with water, drained and dried in a dessicator over silica gel. The cell blocks were sectioned with the aid of a microtome to a silver-gold interface. The sections, placed on grids, were floated face down successively in 4% Bovine Serum Albumin-phosphate-buffered saline (0° for 5 min), Ferritin-conjugate (15 min. at 0°), phosphate-buffered saline (0° for 1 min) three times, 2% formaldehyde-phosphate-buffered saline (20 minutes at 0°) followed by a water wash at 0°. The

stained sections were then examined using a Siemens Elmiskop I microscope operated at 80 Kv with a 50 μ objective aperture.

6. Preparation of antisera

(a) Preparation of proteins for injection

Protease purified by the method of Both et al. (1972) or α-amylase, purified by the method of Grant (1967), was dissolved in sterile saline (1.0 ml) to a concentration of 3 - 5 mg/ml. This was emulsified with an equal volume of Freund's complete adjuvant.

(b) Injection of rabbits

Initial injections were administered at multiple subcutaneous sites at the back of the neck. Subsequent injections were performed similarly after the primary antibody response reached a level which was undetectable by immunodiffusion.

(c) Collection of blood and purification of serum

Blood was collected from a small incision in the marginal ear vein into a suitably sized bottle and allowed to clot overnight in the presence of 0.05% sodium azide. The serum was decanted and centrifuged at 2,000 g in an MSE Super Minor centrifuge for 5 minutes. The clear serum was then mixed with an equal volume of saturated ammonium sulphate and allowed to stand for 1 hour at 0°. The precipitated protein was centrifuged and re-dissolved in distilled water. The protein solution was exhaustively dialysed against 0.0175 M phosphate buffer

(pH 7.2) to remove ammonium sulphate. The partially purified serum sample was then applied to a DEAE-cellulose column (2.5 times the original serum volume) and eluted with 0.0175 M phosphate buffer (pH 7.2). The column effluent was monitored at $A_{280\ nm}$ and the fractions containing A_{280nm} adsorbing material were pooled and concentrated by vacuum dialysis in collodion bags.

7. Coupling procedure for linking ferritin to γ-globulin

Basically this method is a modified form of the procedure of Schick and Singer (1961).

Toluene diisocyanate (0.3 ml) was emulsified with brief sonication in 7.5 ml of water, cooled to 0° with vigorous stirring and a cold solution of 25 mg ferritin in 7.5 ml 0.05 M sodium phosphate buffer (pH 7.5) added. Stirring was continued for a further 25 minutes. This procedure gives the desired solution of ferritin (1.5%) in an ionic strength of 0.1 as well as ensuring that the toluene diisocyanate is finely divided to give a large surface area for reaction. Unreacted toluene diisocyanate was removed by centrifugation, the supernatant filtered through a No.3 glass sinter and stored for 1 hour on ice to complete reaction with dissolved toluene diisocyanate.

Purified γ-globulin (210 mg) in 14 ml 0.05 M borate buffer, pH 9.3 - 9.5 was warmed to 37° and the ferritin-toluene diisocyanate solution added. The pH was adjusted to 9.3 - 9.5 and the mixture incubated at 37° for 1 hour. After dialysis in the cold against 0.1 M $(NH_4)_2CO_3$ (overnight) and two changes of phosphate-buffered saline, the conjugate was purified by

ultracentrifugation (three times, each at 150,000g for 180 min.) and passage down DEAE-cellulose.

8. Estimation of lytic factor

The protoplast bursting ability of the lytic factor was used as a basis for its quantitative estimation. Protoplasts used in the assay system were prepared from culture cells (A_{600nm} = 1.2): cells from 35 ml of culture were centrifuged, washed twice with 0.01 M magnesium acetate buffer, pH 7.0 (30 ml) equilibrated at 30° and resuspended in 30 ml of the same buffer containing 0.6 M sucrose. A glass rod was used to evenly suspend the cells in buffer during the washing procedure. Lysozyme (4 mg) was added and the cells were incubated at 30° as a thin layer with gentle agitation until phase contrast microscopy showed that the conversion of rod-shaped cells to spherical protoplasts was complete. This usually took 30 - 50 minutes. To these protoplasts, 2,4-dinitrophenol (1 mM) was added. The final $A^{1cm}_{600\ nm}$ of the 2,4-dinitrophenol treated protoplast suspension was approximately 0.75. To a cuvette containing 1.8 ml of suitably diluted test solution (made 0.6 M with respect to sucrose), 1.2 ml of protoplast suspension was added and the cuvette solution mixed by inversion and incubated at 30°. The decrease in A^{1cm}_{600nm} associated with the lysis of protoplasts by lytic factor was followed. After 15 minutes incubation, when protoplast lysis had ceased, the extent of lysis produced by a particular volume of the supernatant was measured as the difference in A^{1cm}_{600nm} between a control cuvette containing 0.6M sucrose-phosphate buffer instead of supernatant

and a cuvette containing the supernatant. The respective A^{1cm}_{600nm} values were plotted against the different volumes of the supernatant added to the protoplasts. A linear inverse relationship exists between the amounts of lytic factor in different supernatant samples and the volumes of the respective supernatants needed to produce a 50% lysis of protoplasts. An undiluted 25 hour culture supernatant sample was arbitrarily defined as containing 100 units/ml of lytic factor and was used as a standard to compare the lysis produced by an unknown supernatant sample.

9. Extraction of the lytic factor

B. amyloliquefaciens cultures were grown for 27 hours, at which time cells were removed by centrifugation and the supernatant (10 litres) cooled to 4° and adjusted to pH 2 with HCl. The resulting precipitate was collected by centrifugation at 4°, suspended in 1 litre of alkaline water (pH 8.5) and stirred at 4° for several hours until the precipitate had completely dissolved. During this procedure the pH was maintained at 8 - 9 using NaOH. To the resulting brown solution, $CaCl_2$ (2×10^{-2} M) was added and the mixture left overnight at 4°. The calcium precipitate was collected by centrifugation, washed once with 0.01 N-HCl and finally ground up in 50 ml of 0.01 N HCl using a mortar and pestle. This suspension was extracted six times with equal volumes of diethyl ether. The upper ethereal layers were combined, concentrated to about 40 ml and shaken with an equal volume of 0.01 N-HCl. The ethereal layer was again collected and filtered

through Whatman No.1 filter paper to remove any remaining traces of precipitate which had not passed into the aqueous layer. The ether was then removed by evaporation and the resulting residue dissolved in about 50 ml of acetone to give a clear yellow solution. This solution was de-colourised by active charcoal which was removed by filtration through Whatman No.542 filter paper. A clear colourless acetone solution resulted which contained the pure lytic factor. Further purification by passage through a 1.5 x 70 cm Sephadex LH-20 column was found to be unnecessary.

10. Extraction of acetone soluble and phospho-lipids

The procedure used was a modified version of that by Houtsmeuller and van Deenan (1965).

Samples from 40 ml of freeze-dried cells or supernatant were added to 30 ml of Sorrenson's buffer (pH 7.6). To this was added 30 ml chloroform and 65 ml of methanol and the resulting mixture stored at 2° for 120 minutes. The undissolved material was pelleted by centrifugation and re-extracted two more times with the above solutions. The extracts were pooled and a 0.25 volume of water and a 0.25 volume of chloroform added and left overnight at 4°. The aqueous phase was gently removed and discarded. The chloroform was removed from the lower phase by rotary evaporation under reduced pressure. The residue was dissolved in chloroform:methanol (2:1 (v/v)) and 6 volumes of acetone added. The temperature was dropped to -20° and the precipitated phospholipids removed by centrifugation at -20°. The phospholipids were washed once and the supernatant

pooled with the acetone-soluble lipids. The solvents were removed by a stream of nitrogen gas.

11. pH Measurements

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

12. Spectrophotometric measurements

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

CHAPTER 3

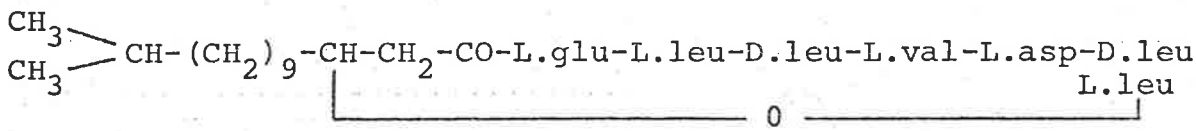
THE SYNTHESIS AND PROPERTIES OF THE PROTOPLAST-BURSTING FACTOR OF BACILLUS AMYLOLIQUEFACIENS

THE SYNTHESIS AND PROPERTIES OF THE PROTOPLAST-BURSTING FACTOR OF B. AMYLOLIQUEFACIENS

A. INTRODUCTION

The cells of 18 hour cultures of B. amyloliquefaciens, which are not producing significant amounts of extracellular enzymes, are converted to stable protoplasts by the action of lysozyme in 22% (w/v) sucrose; by contrast, secreting cells a few hours older fail to give protoplasts but are lysed. It was found that this failure was not due to a change in membrane stability of secreting cells, but due to the presence of a heat-stable protoplast-bursting factor in the medium which was secreted simultaneously with the extracellular enzymes (May and Elliott, 1970). It has since been shown that this protoplast-bursting factor (hereafter referred to as the 'lytic factor') is a peptide-lipid antibiotic which appears to be identical to the compound Surfactin isolated and characterised by Arima et al. (1968). This was suggested by amino acid analyses and the infra-red spectra of the free fatty acid and the antibiotic itself (May, Ph.D. thesis, 1970).

The structure shown in Scheme 1 was proposed by Kakinuma et al. (1969) after nuclear magnetic resonance, amino acid, infra-red and gas chromatographic studies.



Scheme 1.

It had been thought (May and Elliott, personal communication) that this lytic factor might act as a lead-out molecule for nascent polypeptide chains of the extracellular enzymes. This idea had some merit as the fatty acid moiety of the lytic factor is one of the major constituents of Bacillus subtilis membranes (Bishop, Rutberg and Samuelson, 1967). Thus, due to it similarity to a basic structural component, it may easily assimilate into the membrane and facilitate passage of the extracellular enzymes through the membrane and into the external medium. It was envisaged that to do this the molecule may be attached to the N-terminal end of the extracellular enzyme polypeptide chain by a transpeptidation mechanism which possibly splits off N-formyl methionine. At the time it was also thought that when protoplasts of B. amyloliquefaciens were prepared from intact secreting cells, extracellular enzyme synthesis and production of the lytic factor both ceased.

Thus, an investigation into the relationship between the lytic factor and extracellular enzyme secretion was initiated. However, a peculiarity was noticed during a study on the effect of antibiotics on the synthesis of the lytic factor by washed-cell suspensions (May, Ph.D. thesis, 1970).

When chloramphenicol (10 µg/ml) was added to a washed-cell suspension, general protein synthesis was immediately inhibited by greater than 90% but the accumulation of the lytic factor in the external medium continued for one hour at a rate comparable to that of control cells not exposed to chloramphenicol. This was true whether chloramphenicol was added at time zero or 45 minutes after the start of a washed-cell suspension experiment.

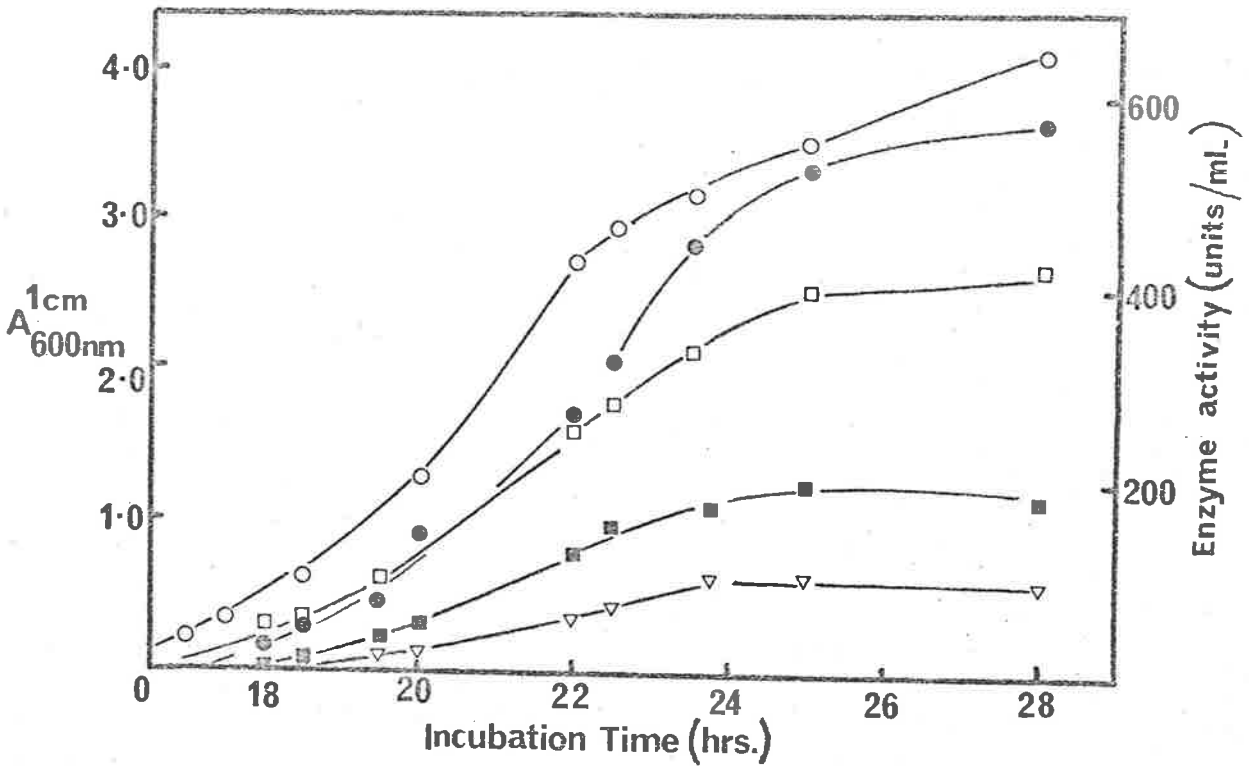
When actinomycin D (10 µg/ml) or puromycin (20 µg/ml) was added at time zero to a washed-cell suspension, production of the lytic factor ceased immediately. However, when these drugs (at the same concentration) were added 45 minutes after the commencment of a washed-cell experiment, production of the lytic factor continued at the same rate as control cells for one hour. Hence there was an anomaly between the actions of the drugs when added to washed-cells at different times and their effect on the production of the lytic factor, which needed further investigation.

B. GROWTH OF B. AMYLOLIQUEFACIENS AND PRODUCTION OF THE LYTIC FACTOR IN CULTURE

1. B. amyloliquefaciens culture was grown as described in Chapter 2. At various times samples were t aken and the bacterial cells removed by centrifugation. The supernatant samples were assayed for extracellular enzymes and the lytic factor. The results are shown in Figure 1. At all stages of the growth cycle essentially all of the lytic activity was found in the culture medium rather than associated with the bacterial cells, since washed and freeze-dried bacterial culture cells showed little or no lytic activity. The accumulation of the lytic factor in the supernatant parallels cell growth and extracellular enzyme production, being easily detected in the culture medium during the early log phase of the culture. This contrasts with many antibiotics whose production appears to commence at the beginning of the stationary phase (Nomura and Hosoda, 1956; Aida and Ito, 1962; Mach, Reich and Tatus, 1963).

FIGURE 3.1. THE ACCUMULATION OF THE EXTRACELLULAR ENZYMES AND LYTIC FACTOR IN THE CULTURE MEDIUM OF B. AMYLOLIQUEFACIENS.

Growth of B. amyloliquefaciens was followed by measuring the A_{600nm} (o). α-Amylase (●), protease (□), ribonuclease (■) and the activity of the lytic factor (▽) were determined from supernatant samples taken at the various times indicated. α-Amylase and protease are expressed as units/ml of supernatant, ribonuclease expressed as units/ml (x10) and lytic factor as units/ml (x2).


4·0
3·0
2·0
1·0
A^{1cm}_{600nm}
0
18
20
22
24
26
28
Incubation Time (hrs.)
600
400
200
Enzyme activity (units/ml.)

2. Production of the lytic factor by washed-cell suspensions

Since it was more convenient to study the production of lytic factor by washed-cell suspensions than in cell culture, conditions for the optimal rate of production and its subsequent assay were studied.

It was found that the lytic factor not only lysed protoplasts prepared from B. amyloliquefaciens, B. stearothermophilus and B. subtilis W168 but also lysed human, avian and rabbit red blood cells. The conditions of May and Elliott (1970), in which the decrease in absorbance (measured at 600 nm) of a protoplast suspension of B. amyloliquefaciens incubated at 30°C for 15 minutes, was found to be the most sensitive. When the decrease in absorbance was plotted against the amount of culture supernatant added, it was found that a sigmoidal curve resulted (Fig. 2, curve 1). Similar plots were made for serial dilutions of the supernatant (Fig. 2, curves 2, 3 and 4). It can be seen that a linear inverse relationship exists between the amount of lytic factor added (in different dilutions of supernatant) and the volumes of the respective supernatants needed to produce 50% lysis of the protoplasts (Arrows Fig. 2).

To ensure that the cells used in subsequent washed-cell suspensions were those which were producing the maximum level of lytic factor, washed-cell suspensions were prepared from cells taken from 21, 23 and 24 hour cultures ($A600_{nm}$ = 1.4, 2.52 and 3.36 respectively).

These washed-cell suspensions were then shaken at 30°C and at the times indicated (Fig. 3) supernatant samples were

FIGURE 3.2. RELATIONSHIP BETWEEN THE EXTENT OF PROTOPLAST LYSIS (A^{1cm}_{600nm}) AND AMOUNT OF LYTIC FACTOR ADDED (VOLUME OF SUPERNATANT).

Different volumes of a 25 hour culture supernatant were added to cuvettes containing 1.2 ml of 2,4-dinitrophenol-treated protoplasts. The final volume of the mixture was 3.0 ml. The cuvettes were incubated at 30° and the A^{1cm}_{600nm} measured over 15 minutes. The difference in A_{600nm} between a control cuvette without supernatant and a cuvette containing supernatant was measured (the arrows and straight lines are referred to in the text).

CURVE 1, undiluted supernatant
CURVE 2, supernatant diluted two-fold
CURVE 3, supernatant diluted four-fold
CURVE 4, supernatant diluted eight-fold.

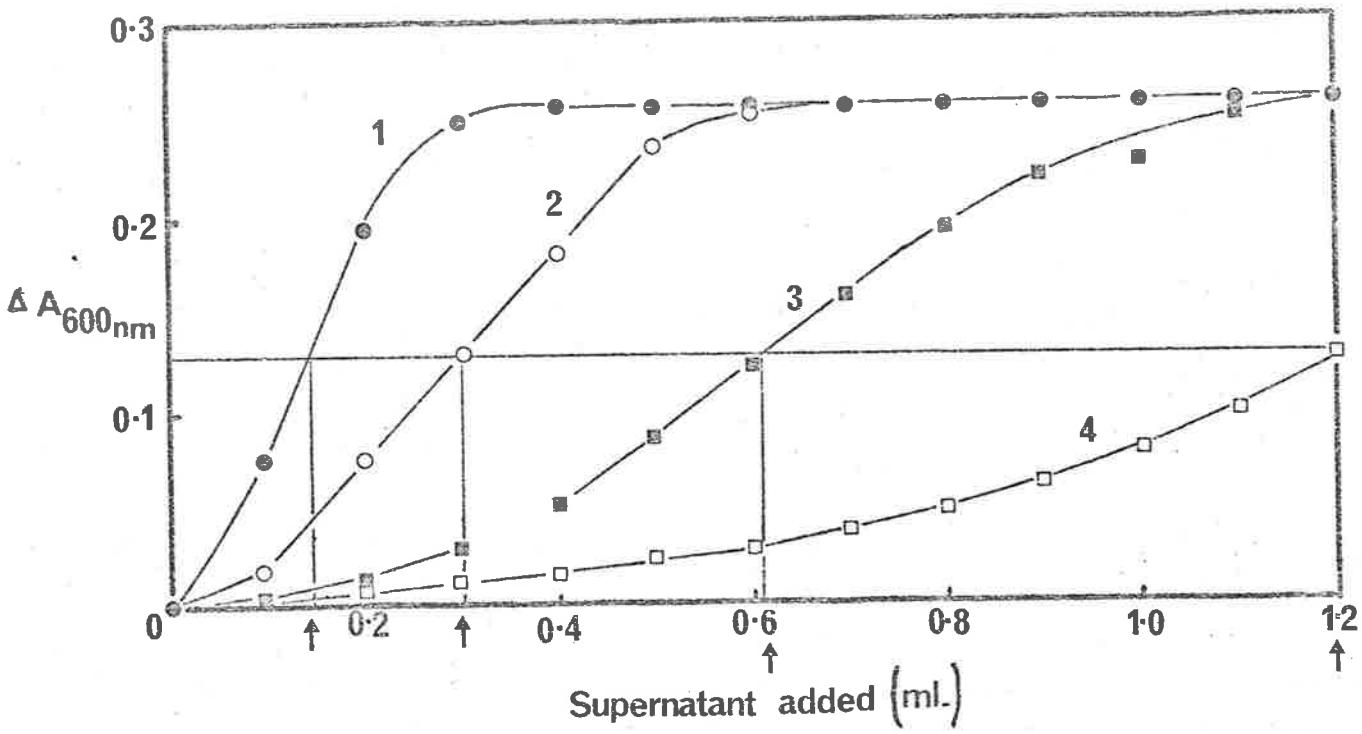

0·3
0·2
0·1
0
Δ A600nm
1
2
3
4
0·2
0·4
0·6
0·8
1·0
1·2
Supernatant added (ml.)

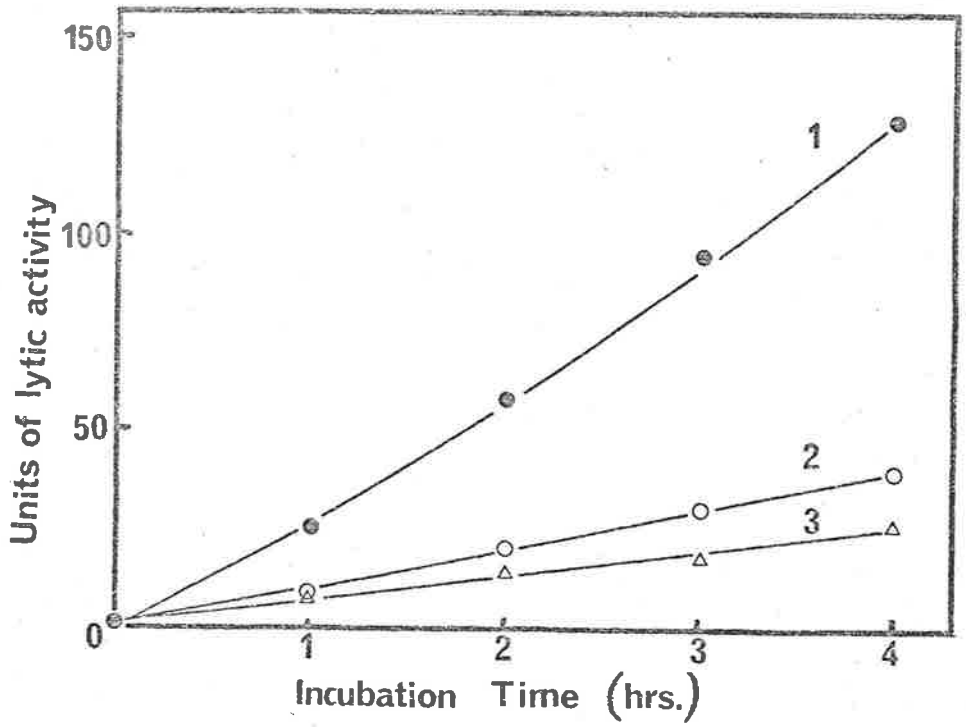
FIGURE 3.3. THE ACCUMULATION OF LYTIC FACTOR IN THE EXTERNAL MEDIUM OF WASHED-CELL SUSPENSIONS PREPARED FROM CULTURE CELLS OF DIFFERENT AGES.

Washed-cell suspensions were prepared as described in the text. Samples were withdrawn, centrifuged and the supernatants assayed for lytic factor activity. Washed cell suspensions were prepared as follows:

CURVE 1, from 21 hr cultures (A_{600nm} = 1.4)

CURVE 2, from 23 hr cultures (A_{600nm} = 2.52)

CURVE 3, from 24 hr cultures (A_{600nm} = 3.36).


150
100
50
0
Units of lytic activity
1
2
3
1
2
3
4
Incubation Time (hrs.)

prepared and assayed for lytic factor activity. It was apparent that washed-cell suspensions prepared from younger cultures had a greater rate of lytic factor production than those prepared from older cultures. Washed-cell suspensions prepared from 21 hour cell cultures ($A600_{nm}$ = 1.4) were 12 times more active (per unit cell density) than those prepared from 24 hour cell cultures ($A600_{nm}$ = 3.36). These results were fully repeatable if the cells were harvested at the same cell density as the time to reach a particular stage of growth varied slightly with each inoculum.

In the following experiments culture cells which had reached a density giving an $A600_{nm}$ = 1.4 (approximately 21 hour) were used since these cells produced maximal amounts of the lytic factor during the course of a washed-cell experiment.

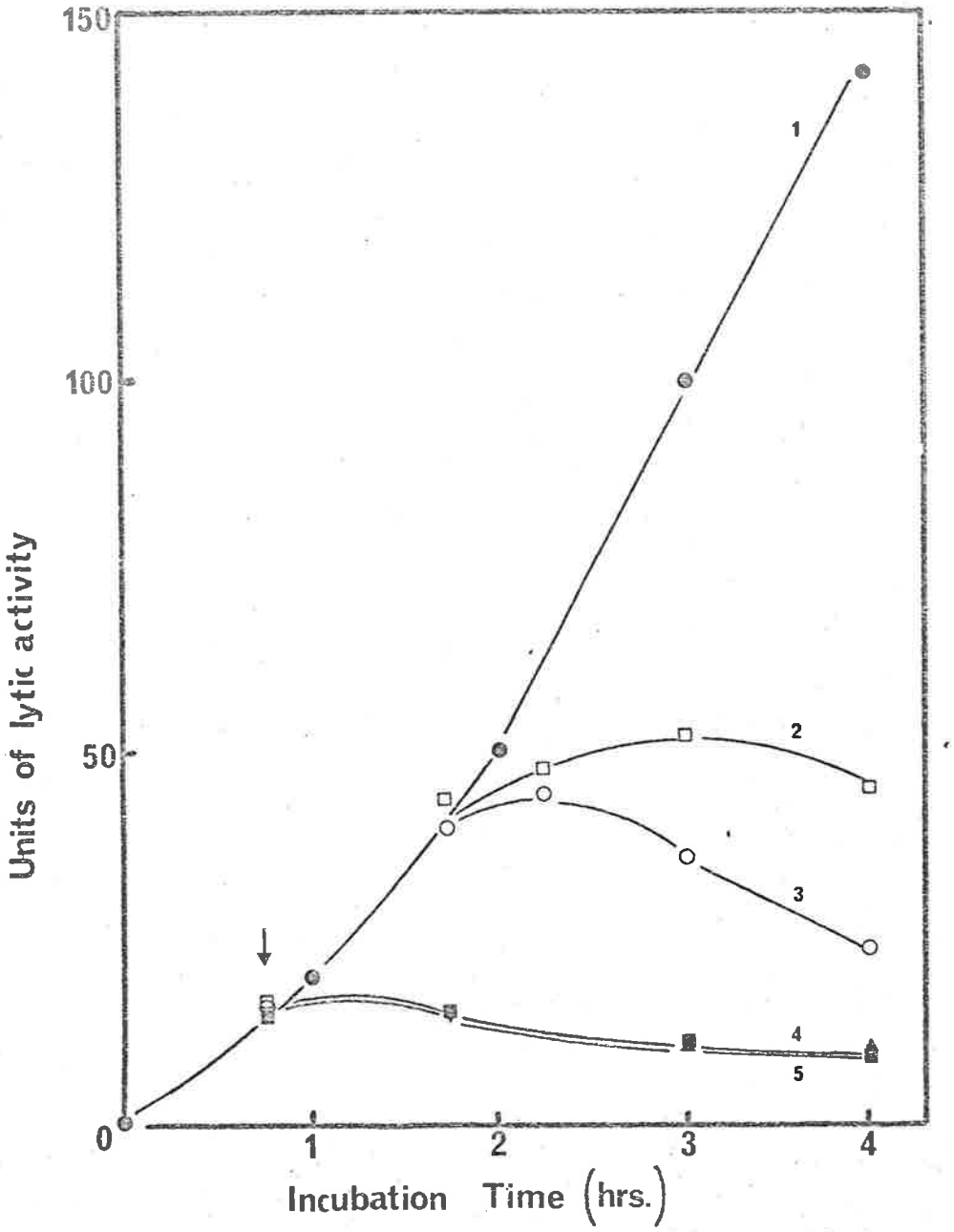
3. The effect of antibiotics on the production of the lytic factor by washed-cell suspensions

The effects of actinomycin D (2 µg/ml), chloramphenicol (10 µg/ml), puromycin (20 µg/ml) and rifampicin (0.5 µg/ml) added 45 minutes after the start of a washed-cell suspension experiment on lytic factor production are shown in Figure 4. In the presence of chloramphenicol, production of the lytic factor continued for approximately one hour, at the same rate as in control cells and then ceased. Actinomycin D gave a similar effect. However, puromycin or rifampicin caused an almost immediate cessation of lytic factor synthesis. The results with chloramphenicol were similar to those obtained previously (May, Ph.D. thesis, 1970) but those with puromycin at 60 minutes

FIGURE 3.4. EFFECT OF INHIBITORS OF PROTEIN SYNTHESIS ON THE APPEARANCE OF LYTIC FACTOR IN THE EXTERNAL MEDIUM OF WASHED-CELL SUSPENSIONS.

Washed-cells were prepared and incubated as described in the text. Samples were withdrawn, centrifuged and the supernatants assayed for lytic factor activity. Inhibitors were added (arrow) to cells after 45 minutes incubation.

CURVE 1, no addition
CURVE 2, chloramphenicol (10 µg/ml)
CURVE 3, actinomycin D (2 µg/ml)
CURVE 4, rifampicin (0.5 µg/ml)
CURVE 5, puromycin (20 µg/ml).


150
100
50
0
Units of lytic activity
1
2
3
4
5
1
2
3
4
Incubation Time (hrs.)

were at variance with these previous results.

A possible explanation for the unusual results was that the antibiotic concentrations used had a general toxic effect on the cells, leading to cell lysis. The latter would expose the lytic factor, which had accumulated during the course of the experiment, to membrane fragments which would neutralise its activity. This would account for the apparent decrease in total lytic factor activity after one hour's exposure to actinomycin D.

Experiments to determine the minimal concentration of each antibiotic to be added to the washed-cell suspensions at 60 minutes were therefore performed. Figures 5b, 6b and 7b show that the minimal levels of chlormaphenicol, actinomycin D and puromycin which immediately inhibit protein synthesis by greater than 90% were 5, 1 and 10 µg/ml respectively. At these concentrations there was no cell lysis as determined by phase contrast microscopy and the release of $A260_{nm}$ absorbing material. When the production of the lytic factor, in the presence of these drugs at their respective concentrations, was followed (Figs. 5a, 6a and 7a) it was seen that the factor continued to accumulate in the external medium for one hour after the addition of the drugs and at a rate comparable to that of control cells. Similar results were obtained when the same concentration of the drugs were added at time zero instead of 60 minutes after the commencement of the washed cell experiment.

These results show that although general protein synthesis was immediately inhibited by greater than 90%, the production of the lytic factor continued unimpaired for

FIGURE 3.5a. EFFECT OF CHLORAMPHENICOL CONCENTRATION ON THE APPEARANCE OF LYTIC FACTOR IN THE EXTERNAL MEDIUM OF WASHED-CELL SUSPENSIONS.

● , no addition of drug
▼ , 1 µg/ml
□ , 2 µg/ml
■ , 5 µg/ml.

FIGURE 3.5b. EFFECT OF CHLORAMPHENICOL CONCENTRATION ON ^{14}C-PROTEIN HYDROLYSATE INCORPORATION BY A WASHED-CELL SUSPENSION

● , no addition of drug
□ , 2 µg/ml
■ , 5 µg/ml.

The chloramphenicol was added at 60 min in all cases (arrow).

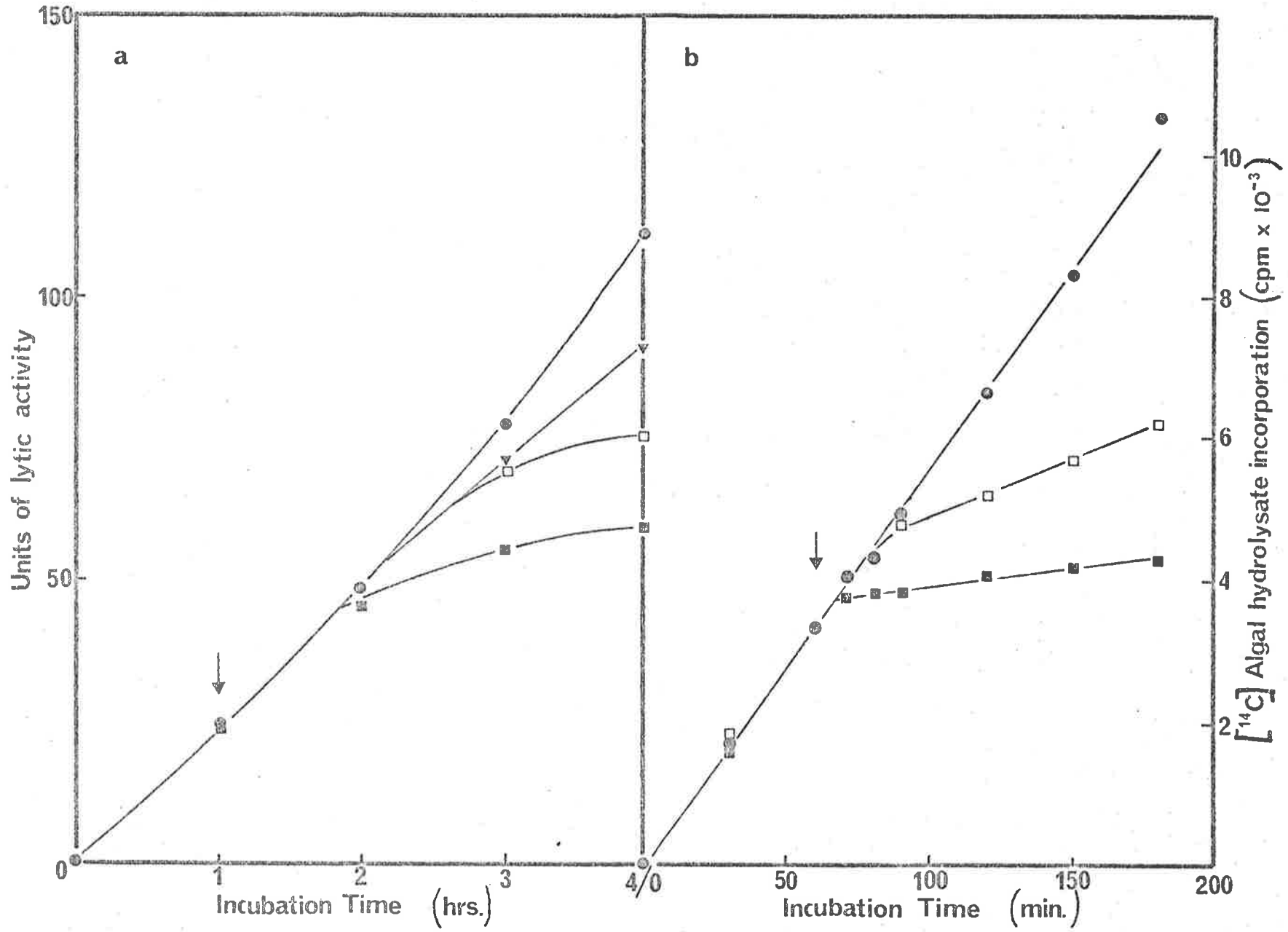

a
b
150
100
50
0
Units of lytic activity
1
2
3
4/0
Incubation Time (hrs.)
50
100
150
200
Incubation Time (min.)
10
8
6
4
2
[14C] Algal hydrolysate incorporation (cpm x 10-3)

FIGURE 3.6a. EFFECT OF ACTINOMYCIN D CONCENTRATION ON THE APPEARANCE OF LYTIC FACTOR IN THE EXTERNAL MEDIUM OF WASHED-CELL SUSPENSIONS.

● , no addition
□ , 0.1 µg/ml
■ , 0.5 µg/ml
▽ , 1.0 µg/ml.

FIGURE 3.6b. EFFECT OF ACTINOMYCIN D CONCENTRATION ON ^{14}C-PROTEIN HYDROLYSATE INCORPORATION BY A WASHED-CELL SUSPENSION

● , no addition
□ 0.1 µg/ml
■ 0.5 µg/ml
▽ 1.0 µg/ml

Actinomycin D was added in all cases at 60 min (arrow).

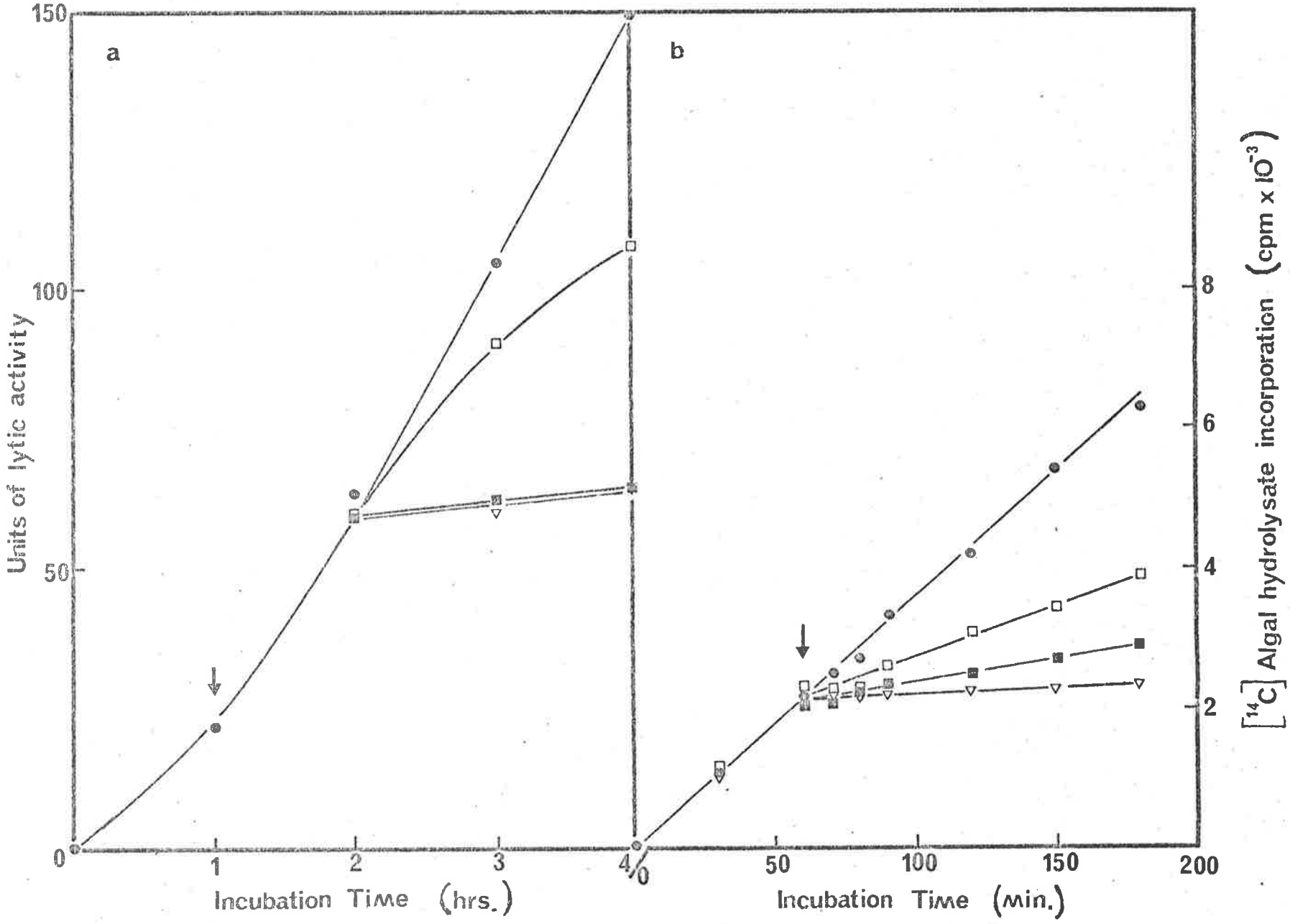

a
b
150
100
50
0
Units of lytic activity
1
2
3
4
0
50
100
150
200
Incubation Time (hrs.)
Incubation Time (min.)
8
6
4
2
[14C] Algal hydrolysate incorporation (cpm x 10-3)

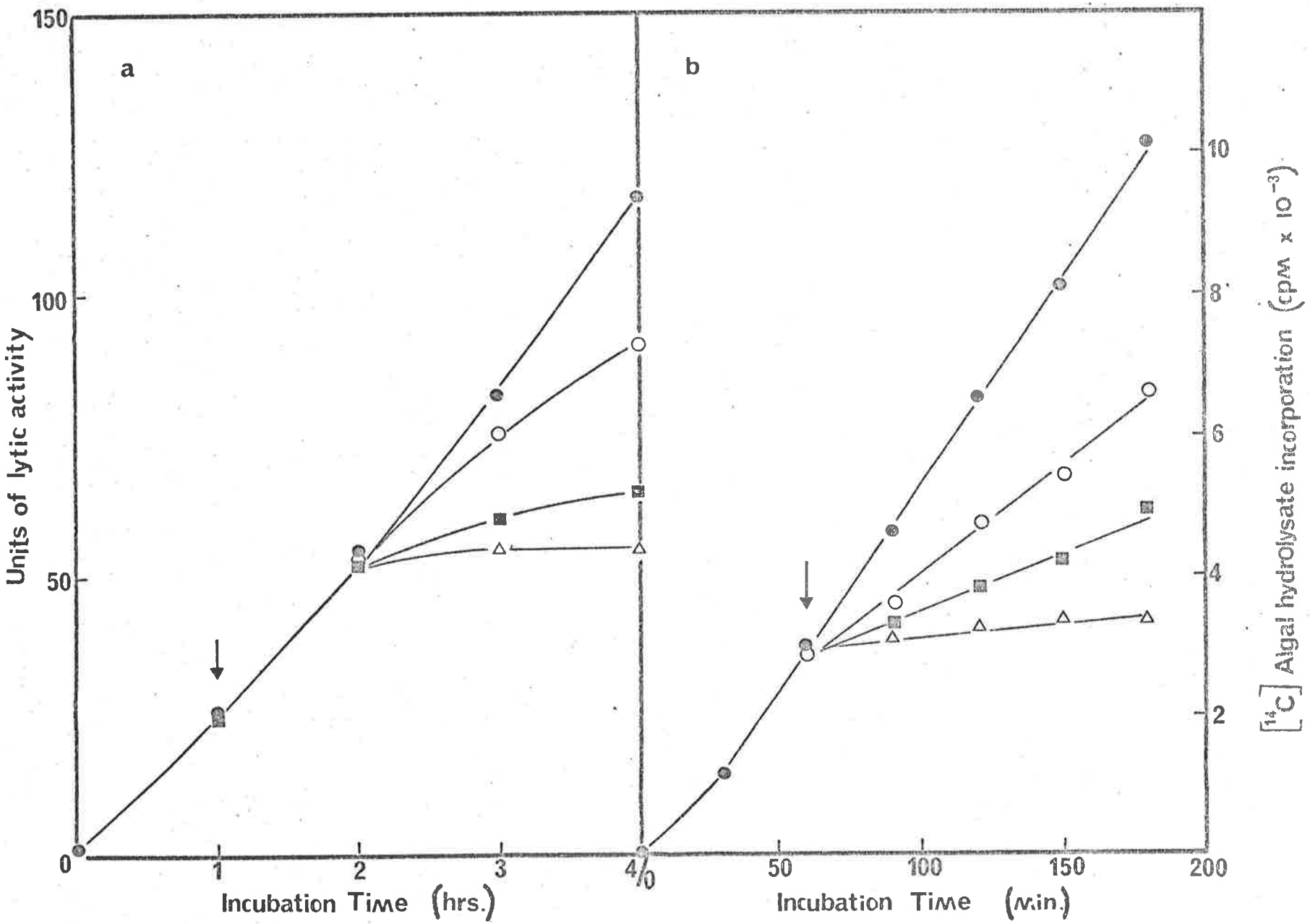
FIGURE 3.7a. EFFECT OF PUROMYCIN CONCENTRATION ON THE APPEARANCE OF LYTIC FACTOR IN THE EXTERNAL MEDIUM OF WASHED-CELL SUSPENSIONS.

● , no addition
o , 2 µg/ml
■ , 5 µg/ml
△ , 10 µg/ml.

FIGURE 3.7b. EFFECT OF PUROMYCIN CONCENTRATION ON ^{14}C-PROTEIN HYDROLYSATE INCORPORATION BY WASHED-CELL SUSPENSIONS

● , no addition
o , 2 µg/ml
■ , 5 µg/ml
△ , 10 µg/ml

Puromycin was added in all cases at 60 min (arrow).


a
b
150
100
50
0
Units of lytic activity
1
2
3
4/0
Incubation Time (hrs.)
50
100
150
200
Incubation Time (min.)
10
8
6
4
2
[14C] Algal hydrolysate incorporation (cpm x 10-3)

approximately one hour in the presence of these drugs. It has previously been shown (May and Elliott, 1970) that the lytic factor is synthesised de novo and thus, it appears that the synthesis of this factor proceeds by a mechanism differing from protein synthesis, as would indeed be expected from the presence of D-leucine and the fatty acid moiety (Daniels, 1968; Kleinkauf, Gievers and Lipmann, 1969; Coleman, 1969).

These results highlight the importance of carefully examining drug concentrations on cell integrity. This was done in all subsequent experiments.

4. The possible relationship between the secretion of extracellular enzymes and the production of the lytic factor

It has been mentioned in the introduction to this Chapter that it was considered possible that the secretion of extracellular enzymes and the lytic factor may be linked and that the lytic factor is utilised as a 'lead-out' molecule for these enzymes. This possibility was examined and found to be unlikely by the isolation and characterisation of a mutant lacking the lytic factor.

Bacterial spores were mutagenised using ethylmethyl-sulphonate (as described in Chapter 2) and outgrowth of the spores in culture medium permitted until an A_{600nm} = 0.3 was reached. The cells were washed three times by centrifugation and then plated onto a soft-agar overlay containing rabbit red blood cells (Materials and Methods, p.). As the bacterial colonies grew at 37°C the lytic factor diffused through the

agar and produced a zone of clearing around the colony, due to lysis of the red blood cells. Several colonies that failed to produce a zone of clearing were isolated by this method. The most promising of these (Lyt^-_9) was characterised further.

Growth and extracellular enzyme formation in liquid culture were comparable to the normal levels found in the wild type organism; however, no lytic activity could be detected in the medium even after 31 hours growth at 30°C (Fig. 8). Even when the culture supernatant was concentrated 20-fold no activity could be detected (i.e., less than 0.4 units/ml). This does not rigorously exclude the possibility that the lytic factor is utilised in the process of enzyme secretion as an inactive molecule may have sufficed. However, no material corresponding to lytic factor could be extracted, when examined by silica gel thin layer chromatography, from 30 hour Lyt^-_9 supernatant using the extraction procedure of Arima et al. (1968).

Since the Lyt^-_9 mutant sporulates with a frequency of 100 times less than the wild type organism, a possible connection may exist between sporulation and the production of this antibiotic as discussed by Schaeffer (1969), but this possibility was not investigated further.

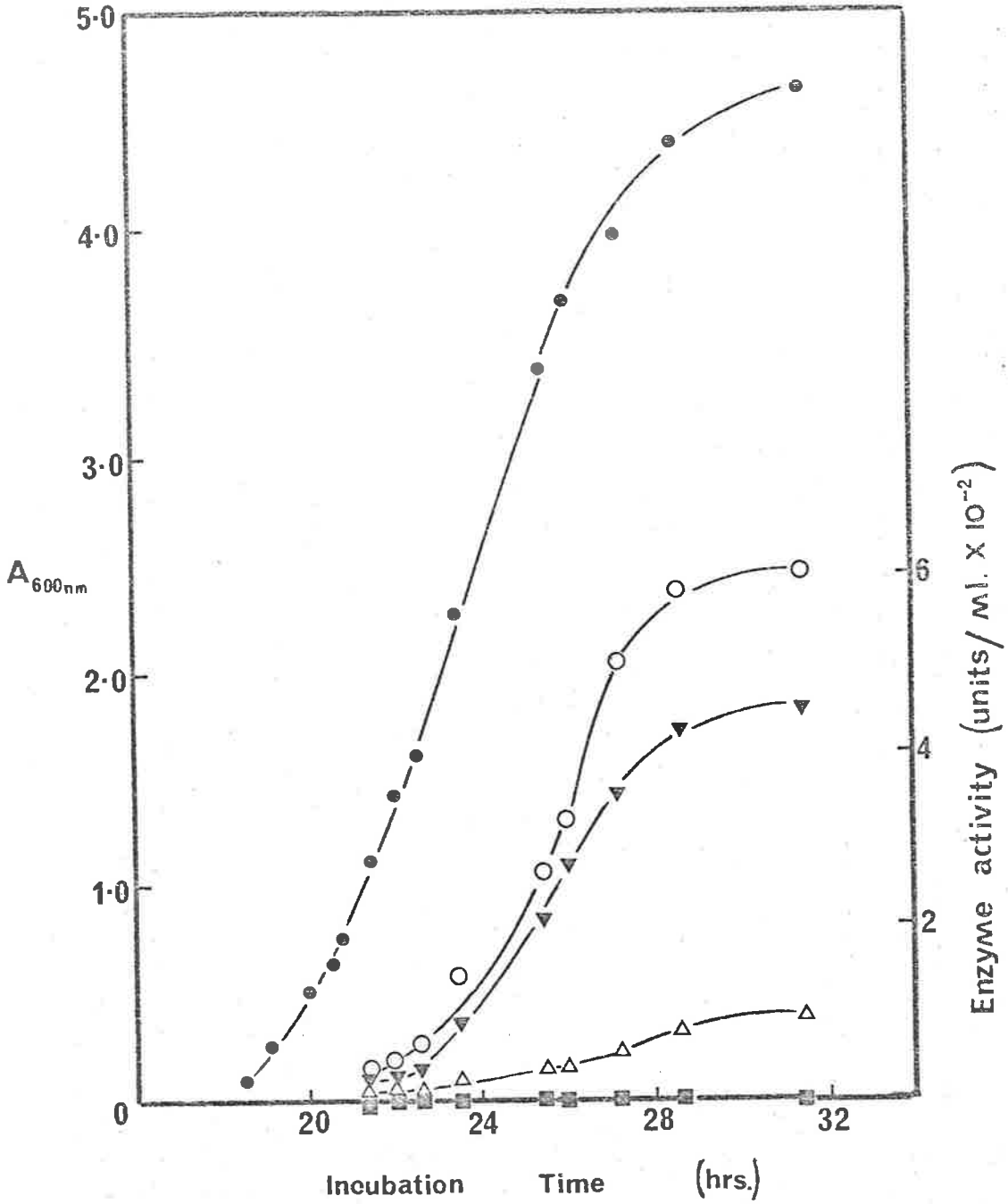
5. Structure and properties of the lytic factor

Although the secretion of the lytic factor may not be directly related to that of extracellular enzyme secretion, it still poses an interesting question as to the mechanism of its own secretion. The lytic factor has been shown to have antibiotic activity against many Gram-positive organisms (May and Elliott,

FIGURE 3.8. THE ACCUMULATION OF EXTRACELLULAR ENZYMES AND LYTIC FACTOR IN THE EXTERNAL CULTURE MEDIUM OF BACILLUS AMYLOLIQUEFACIENS Lyt_9^-.

● , Cell growth (A_{600nm})
○ , α-Amylase (units/ml)
▼ , protease (units/ml)
△ , ribonuclease (units/ml x 10)
■ , lytic factor (units/ml x 10).

The final levels of enzyme accumulated in the external medium were 568 units/ml of α-amylase, 420 units/ml of protease and 9.5 units/ml of ribonuclease. The levels of lytic factor were undetectable as explained in the text.


5·0
4·0
3·0
2·0
1·0
0
A_{600nm}
20
24
28
32
Incubation Time (hrs.)
6
4
2
Enzyme activity (units/ml. x 10^{-2})

1970) and it is capable of rapidly lysing protoplasts of B. amyloliquefaciens and red blood cells. The problem of how a cell can secrete a substance which is capable of rapidly disrupting its own cytoplasmic membrane is an intriguing one.

The structure proposed by Kakinuma et al. (1968) and shown in scheme 1 of the introduction to this Chapter was used as the basis for building a space filling model (Fig. 9a-d). From a study of the model it can be seen that the molecule possesses a hydrophylic side (Fig. 9a), a hydrophobic reverse side (Fig. 9b) and a hole bounded by hydrophylic groups. The ability of the lytic factor to disrupt membranes is probably due to a detergent-like hydrophylic and hydrophobic orientation of the molecule, permitting it to assimilate into the membrane and cause disorganisation of the latter.

When the two D-leucine residues in the space filling model were changed to the laevo configuration (Fig. 9c) it was not possible to obtain a conformation of the molecule which did not have at least one peptide bond grossly distorted also together with stearic interference between side chains. Moreover, it was observed that under such conditions the molecule no longer possessed its detergent-like orientation of hydrophobic and hydrophylic groups. Thus, the leucine residues are presumably needed in the dextro configuration to allow the formation of an unstrained cyclic peptide with surface active properties.

Disruption of the lactone bond (k), formed between the β-OH group of the fatty acid (l) and the C-terminal leucine residue, enabled the model to assume many random conformations (Fig. 9d). It was thought that this loss of molecular organisa-

FIGURE 3.9. SPACE FILLING MODEL OF THE LYTIC FACTOR

Construction of the model was based on the structure proposed by Kakinuma et al. (1969).

FIGURE 3.9a - hydrophylic side of the molecule;

FIGURE 3.9b - hydrophobic side of the molecule;

FIGURE 3.9c - conformation of the lytic factor molecule when all amino acids are in the laevo configuration

FIGURE 3.9d - molecule after alkaline hydrolysis of the lactone bond.

The labelling on the photographs is as below:

(a) C_{15} - B OH-isoacid; (b) L-Glu; (c) L-Leu;

(d) D-leu; (e) L-Val; (f) L-Asp; (g) D-Leu;

(h) L-Leu; (i) water molecule; (j) hydrophylic hole;

(k) lactone bond; (l) fatty acid hydroxyl group;

(m) C-terminal leucine carboxyl group; (n) L-Leu replacing D-Leu (d); (o) L-Leu replacing D-Leu (g).

Fig.3.9a

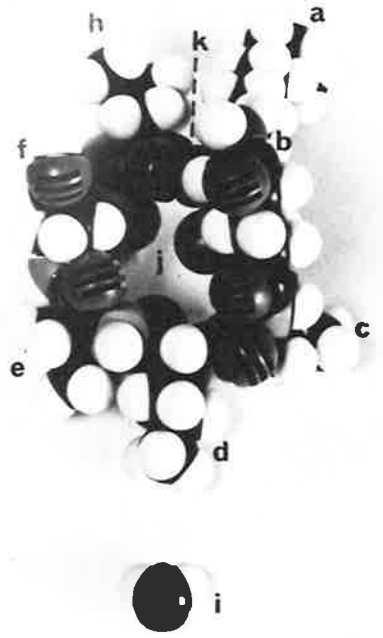


Fig.3.9b

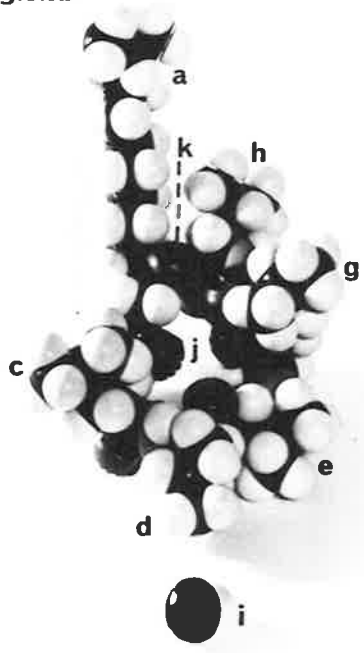


Fig.3.9c

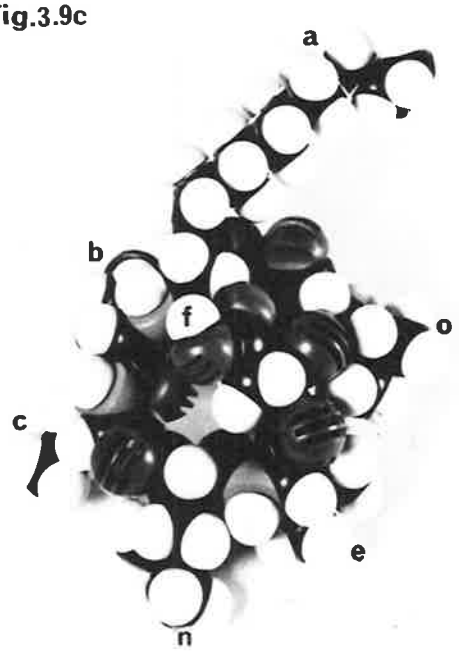
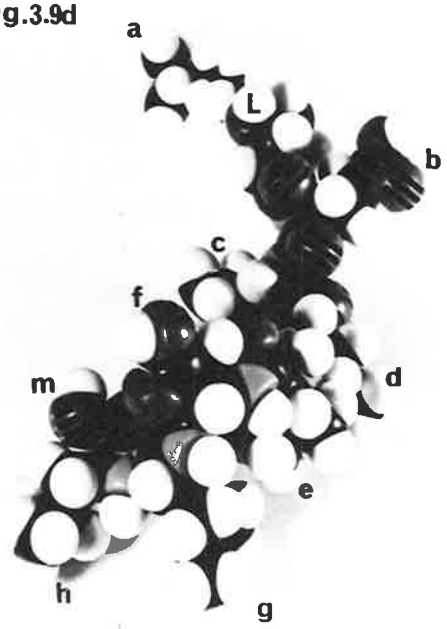


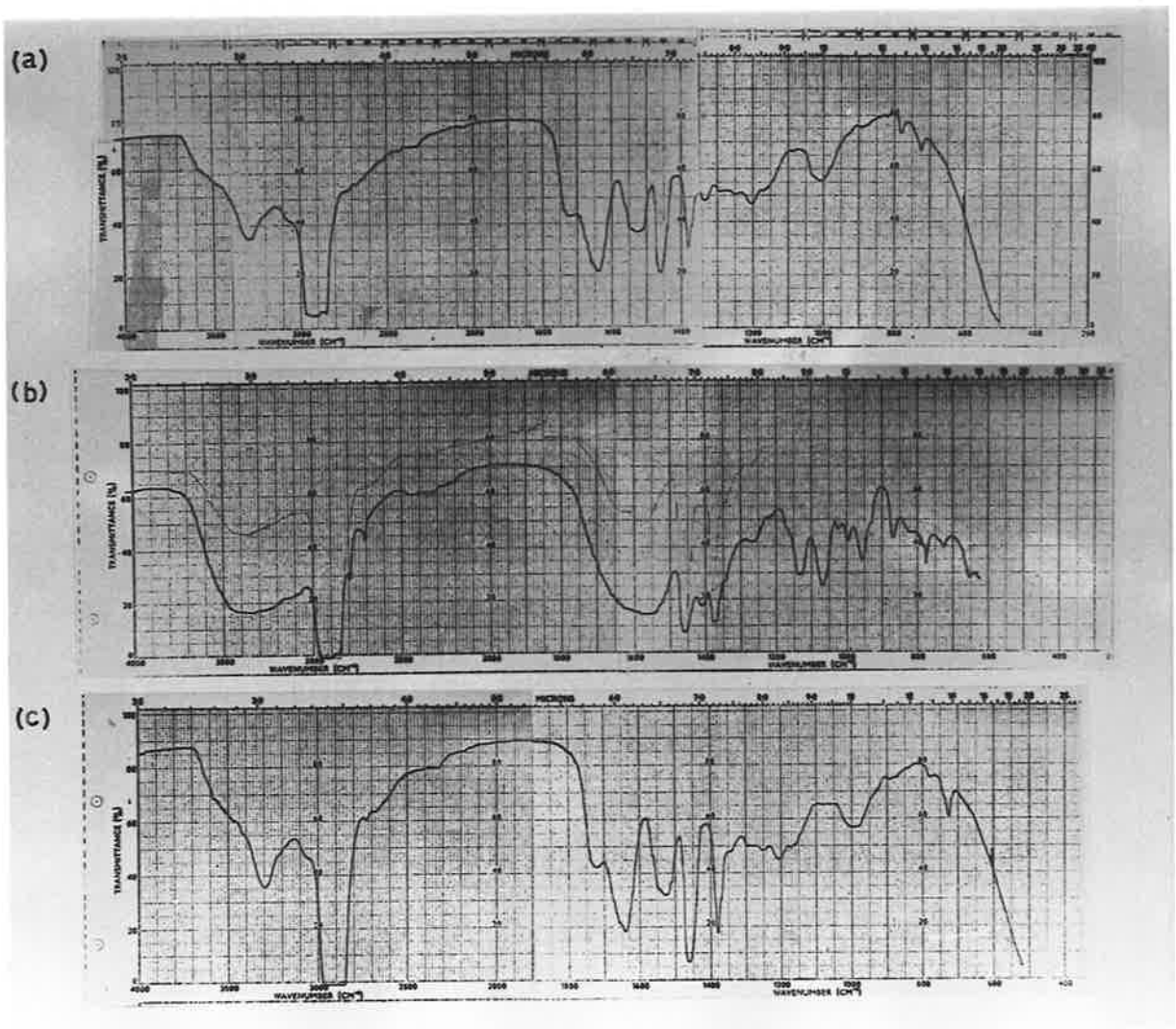
Fig.3.9d



tion could result in a loss of biological activity. This possibility was tested by alkaline hydrolysis of the lactone bond, followed by ether extraction to remove any unchanged starting material. The infra-red spectrum of the antibiotic shows a prominent absorbance in the region 1740 - 1710 cm^{-1}, which is characteristic for large ring lactones (Fig. 10a). On alkaline hydrolysis (0.1 N - NaOH, 60 min. at room temperature) this peak diminished and was accompanied by an increase in absorbance in the regions 3650 - 3390 cm^{-1} and 1650 - 1500 cm^{-1}, due to the formation of a hydrogen bonded hydroxyl group and the C-terminal leucine carboxylate anion respectively (Fig. 10b). This opening of the lactone ring was accompanied by a loss of 97% of the lytic activity. When this alkali-hydrolysed preparation was exposed to acid conditions (0.1 N-HCl, 60 min. at room temperature), followed by ether extraction 92% of the original lytic activity was regained in the ether-soluble fraction. The infra-red spectrum of the restored antibiotic was identical to that of the starting material (cf. Figs. 10c and10a) indicating that the lactone ring had been reformed. The presence or absence of the lactone ring in either the starting product (or the reformed product) or the alkali-hydrolysed antibiotic respectively, was confirmed by testing for the presence of ester linkages by the method of Whittaker and Wijesundera (1952). The former compounds gave a positive reaction while the latter did not. A sample of the antibiotic stored in phosphatebuffer (0.5 M, pH 8.0) gradually lost its lytic ability, the infra-red spectrum becoming identical to that of the alkali-treated preparation. Similarly,

FIGURE 3.10. INFRA-RED SPECTRA OF (a) LYTIC FACTOR, (b) ALKALINE HYDROLYSED LYTIC FACTOR AND (c) A SAMPLE OF ALKALINE HYDROLYSED LYTIC FACTOR EXPOSED TO ACID CONDITIONS

The samples were prepared and dissolved in Nujol before obtaining the infra-red spectrum.


(a)
(b)
(c)

an alkali-treated sample of the lytic factor was seen to gradually regain its ability to lyse protoplasts on standing in phosphate buffer (0.5 M, pH 7.0).

Thus, it seems that the biological activity of the antibiotic is dependent on the integrity of the lactone bond, which can easily open and close depending on the pH of the environment. The dependence of a molecule's biological activity on a lactone bond is not unique. Actinomycin D is inactive when its lactone ring is opened to form actinomycinic acid (May, Walsh, Elliott and Smeaton, 1968)

C. DISCUSSION

This work in this Chapter does not give support to the idea that the 'lytic factor' acts as a "lead out" molecule for extracellular enzyme secretion. It has been established that the appearance of the lytic factor in the external medium occurs in the absence of protein synthesis. However, despite this lack of obligatory coupling of its production to secretion of enzymes, its involvement was not rigorously excluded, since in the presence of inhibitors of protein and RNA synthesis it was possible that synthesised lytic factor diffused out independently. The observation that the Lyt^-_9 mutant still grew and secreted extracellular enzymes in a manner comparable to the wild type organism, tends to eliminate a possible correlation between the two.

The finding that the synthesis of the lytic factor continues in the presence of protein and RNA synthesis inhibitors is analogous to the findings with other antibiotics,

i.e., polymixin (Daniels, 1968) and Gramicidin S (Berg et al., 1965; Kurylo et al., 1966). These have been shown to be synthesised by a non-ribosomal mechanism. Kleinkauf, Gevers and Lipmann (1969) demonstrated that gramicidin S was synthesised as a result of the interaction of a polyenzyme system, charged with four component amino acids and another enzyme bearing the other remaining amino acid, located in the plasma membrane of B. brevis (ATCC 9999). Tyrocidine biosynthesis in cell-free extracts from B. brevis (ATCC 8185) have also been studied by Kleinkauf et al. (1970) and shown to be synthesised by a multi-enzyme complex utilising phosphopantetheine as a cofactor.

The presence of D-leucine and a fatty acid moiety in the lytic factor indicates that thismolecule may also be synthesised by such an enzymically controlled mechanism. It is now well-established that biosynthesis of polypeptide antibiotics is independent of an mRNA requiring process (Daniels, 1968).

With regard to the production of the lytic factor there is an interesting problem of how such a potent membrane lysing agent can be synthesised and secreted without damage to the cell. The ability of B. amyloliquefaciens to do this may lie in the dependence of the factor on an intact lactone ring for its biological activity. Possibly the active ring is assembled only after the molecule has passed through the cell wall, the latter presumably protecting the underlying membrane from its action. This could be accomplished by the cell wall or periplasmic space having an ionic environment incompatible with the closed-ring structure and even perhaps an enzyme to cleave the lactone bond, thus ensuring only an inactive molecule can come

into contact with the membrane.

In comparison with other peptide antibiotics the appearance of the lytic factor in the external culture medium occurs earlier than usual. The lytic factor can be easily detected in the early log phase with synthesis ceasing in the stationary phase. Synthesis of most antibiotics only commences when the cell culture has entered the stationary phase (Daniels, 1968).

The function of peptide antibiotic production is open to debate. Many sporulating bacteria produce peptide antibiotics during the first stage of sporulation (Schaeffer, 1969) and evidence has been presented that they are needed for the actual process of sporulation, possibly initiated by interaction with the cytoplasmic membrane (Bodansky and Perlman, 1969; Urry, 1971). It has also been suggested that antibiotics are either 'dust bins' for surplus metabolism (Woodroff, 1966) or that their function may be to eliminate competition from other organisms. This latter idea may have some merit, but the former seems improbable. An alternative idea (Elliott and Mandelstam, personal communication) is that some of the antibiotics may have a nutritive role by lysing bacteria so that extracellular enzymes in the external medium may act on their contents. Since amino acids and other nutrients will revert cells back to the vegetative state during the first part of their commitment to sporulation (Sterlini and Mandelstam, 1969), a nutritive role for extracellular enzymes and antibiotics is at least plausible.

CHAPTER 4

STUDIES ON THE APPARENT ACCUMULATION OF MESSENGER RNA FOR THE EXTRACELLULAR ENZYMES OF BACILLUS AMYLOLIQUEFACIENS

STUDIES ON THE APPARENT ACCUMULATION OF MESSENGER RNA FOR THE EXTRACELLULAR ENZYMES OF BACILLUS AMYLOLIQUEFACIENS

A. INTRODUCTION

The production of extracellular enzymes by washed-cell suspensions of Bacillus amyloliquefaciens has been investigated with the aim of elucidating the mechanism of extracellular enzyme synthesis and secretion. It has been shown previously that such production of each of these enzymes is subject to separate controls (though whether they have some primary control mechanism, such as activation of a sporulating genome, is unknown). None of these enzymes are substrate induced; ribonuclease synthesis is partially repressed by inorganic phosphate (May, Walsh, Elliott and Smeaton, 1968), protease synthesis is almost completely repressed by amino acids (May and Elliott, 1968a) while no definite control has been established for α-amylase (Coleman, 1967).

It has now been shown (Both et al., 1972) and reported in a preliminary paper (Both et al., 1971) that the initial phase of protease production in washed-cell suspension experiments is independent of messenger RNA synthesis. In a medium containing low concentrations of amino acids the production of the neutral protease of B. amyloliquefaciens occurs almost linearly, but in a high concentration of amino acids there is a rapid phase (phase I) of production lasting for 30 minutes, followed by a levelling for 50 minutes after which synthesis resumes linearly (phase II) (May and Elliott, 1968a; Both et al., 1972; Fig. 1). The inhibitors of RNA synthesis, rifampicin and actinomycin D,

inhibit phase II promptly but do not inhibit phase I production. The latter is nevertheless sensitive to inhibitors of protein synthesis and labelling studies have confirmed that the protease production involves de novo synthesis of the enzyme (Both et al., 1972).

This evidence suggests that there is present in harvested cells of B. amyloliquefaciens an accumulated pool of messenger RNA for protease, capable of supporting synthesis of the enzyme for 80 minutes. The biphasic time-course in the presence of high concentrations of amino acids was attributed to amino acid repression of protease mRNA formation, phase I representing translation and exhaustion of the accumulated pool. During the plateau phase of protease production, cell metabolism may reduce the internal amino acid level to the point where de-repression occurs at about 80 minutes and protease gene transcription and mRNA translation results in phase II synthesis. This interpretation assumes, (a) that the proteases in phases I and II are identical and (b) that any mRNA has the same stability in phases I and II. There is evidence supporting the first assumption (Both et al., 1972); while the second cannot be proven it seems a reasonable one.

It was of importance to see whether this was an isolated case of messenger accumulation or whether the same situation existed for the other two extracellular enzymes of B. amyloliquefaciens, α-amylase and ribonuclease.

B. RESULTS

1. Time-course of protease, α-amylase and ribonuclease formation by washed-cell suspensions

Figure 1 shows the time-course of production of the three extracellular enzymes by washed-cell suspensions. The curves for protease and ribonuclease are as described previously (May and Elliott, 1968a; Coleman and Elliott, 1965) but that for α-amylase differs from earlier findings in which a prolonged lag phase was observed (Coleman and Elliott, 1962). The latter has now been shown to occur with cells harvested at 22 hours (Fig. 2), as used for earlier studies, while in the present study cells were grown for 25 hours (A_{600nm} = 3.6) before they were harvested for washed-cell experiments.

2. Effect of rifampicin and chloramphenicol on extracellular enzyme formation

(a) Preliminary studies on the effects of rifampicin and chloramphenicol on ^{14}C-uracil and ^{14}C-leucine incorporation into washed-cell suspensions

When rifampicin, at a concentration of 0.5 µg/ml, was added to washed-cells L-[^{14}C]leucine incorporation into total protein synthesis was inhibited almost immediately by 96% (Fig. 3). Total cellular RNA synthesis, as measured by [^{14}C] uracil incorporation, was likewise inhibited by 97% (Fig. 4). When chloramphenicol, at a concentration of 10 µg/ml, was added to washed-cell suspensions general protein synthesis as measured by the incorporation of L-[^{14}C]leucine into total protein was inhibited almost immediately by 95% (Fig. 5).

FIGURE 4.1. TIME COURSE OF PROTEASE (—●—●—), α-AMYLASE (—□—□—) AND RIBONUCLEASE (—△—△—) FORMATION BY A WASHED-CELL SUSPENSION.

Enzyme activity is expressed as a percentage of that at 120 minutes. The 100% values were 17.9 units/ml for protease, 30.2 units/ml for α-amylase and 8.1 units/ml for ribonuclease.

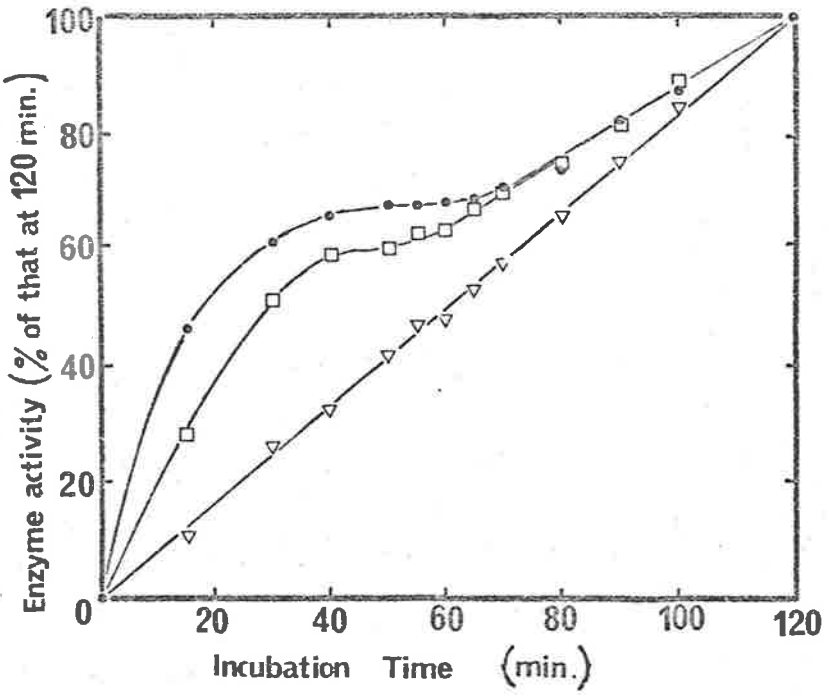

Enzyme activity (% of that at 120 min.)
100
80
60
40
20
0
20
40
60
80
100
120
Incubation Time (min.)

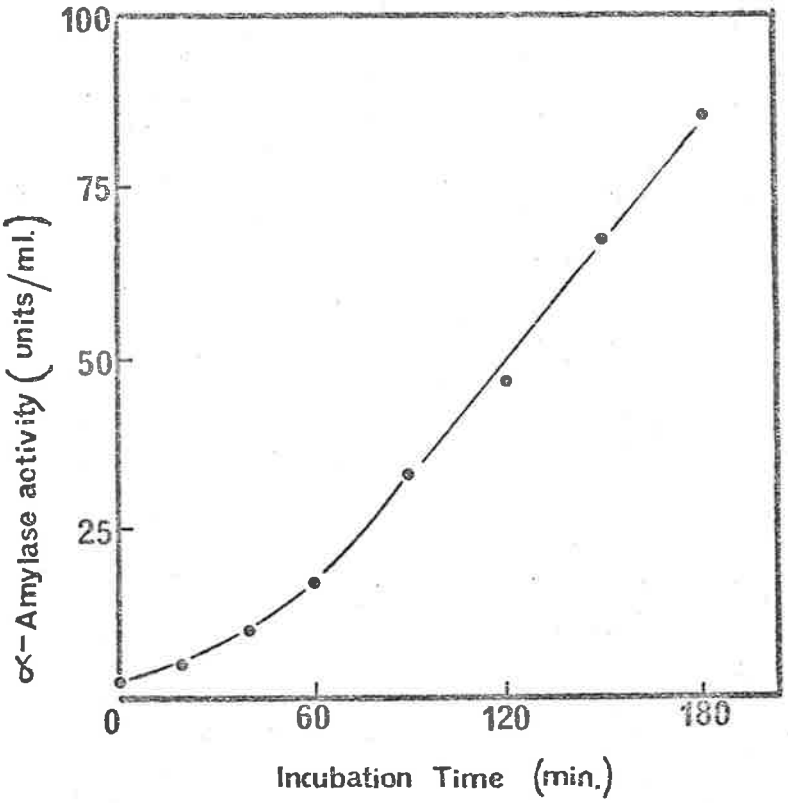
FIGURE 4.2. TIME COURSE OF α-AMYLASE ACCUMULATION IN THE EXTERNAL MEDIUM OF A WASHED-CELL SUSPENSION PREPARED FROM YOUNG CULTURE CELLS.

Culture cells, grown to an A_{600nm} = 1.8 (approx. 22 hr.) were used to prepare a washed-cell suspension. At the various times indicated samples were taken, centrifuged and the supernatants assayed for α-amylase.

FIGURE 4.3. EFFECT OF RIFAMPICIN (0.5 µg/ml) ADDED AT ZERO TIME ON [^{14}C]LEUCINE INCORPORATION INTO TOTAL PROTEIN BY A WASHED-CELL SUSPENSION.

● , no addition of drug
△ , rifampicin (0.5 µg/ml) added at time zero.

The 100% value for L-[^{14}C]leucine incorporation was 8020 c.p.m.


100
75
50
25
0
60
120
180
α- Amylase activity (units/ml.)
Incubation Time (min.)

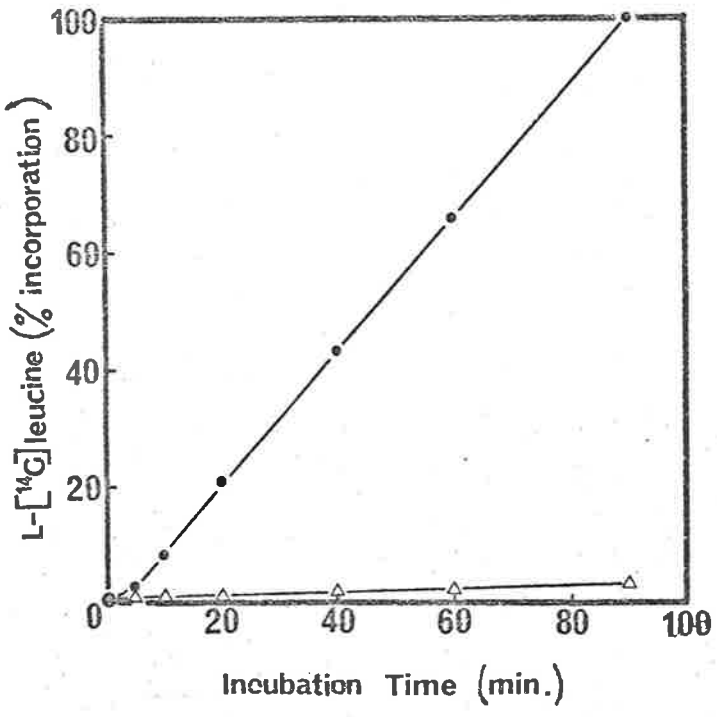

100
80
60
40
20
0
20
40
60
80
100
L-[14C]leucine (% incorporation)
Incubation Time (min.)

FIGURE 4.4. EFFECT OF RIFAMPICIN ON [^{14}C]URACIL INCORPORATION BY A WASHED-CELL SUSPENSION. RIFAMPICIN AND [^{14}C]URACIL WERE BOTH ADDED AT ZERO TIME.

■, no addition
□, rifampicin (0.5 µg/ml).

The 100% value for [^{14}C]uracil incorporation was 5,800 c.p.m.

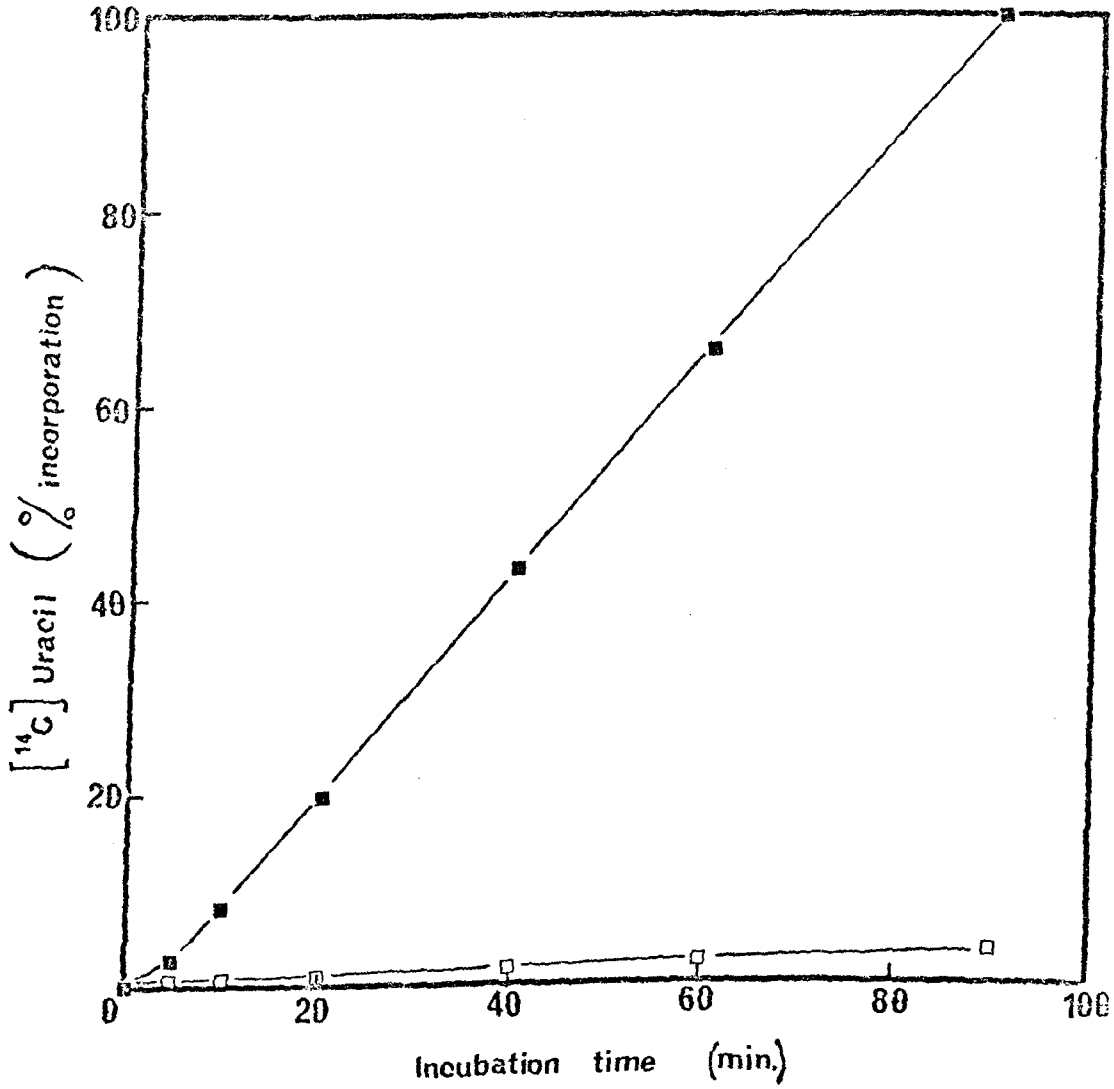
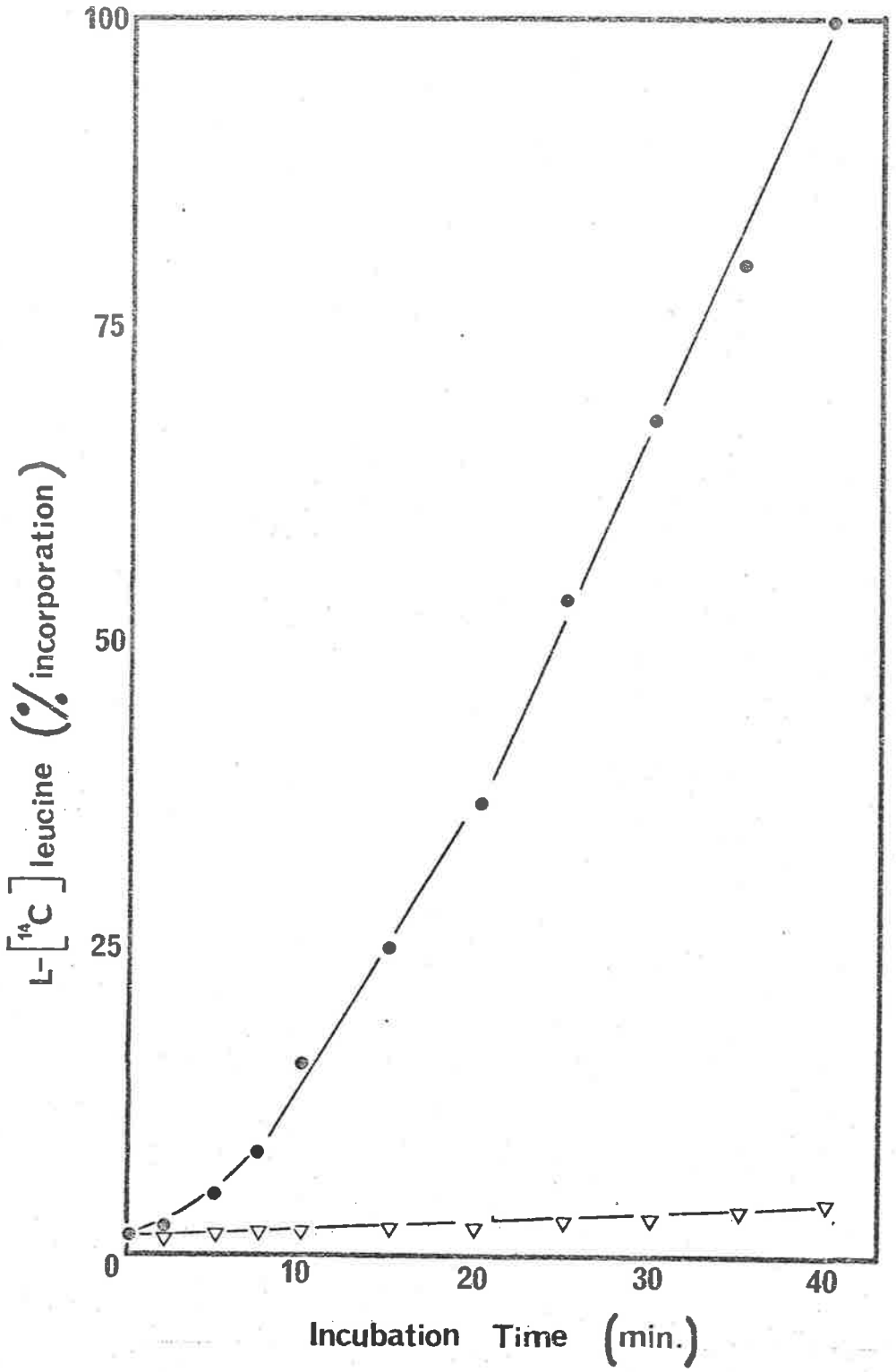

100
80
60
40
20
0
20
40
60
80
100
[14C] Uracil (% incorporation)
Incubation time (min.)

FIGURE 4.5. EFFECT OF CHLORAMPHENICOL ON L-[^{14}C]-LEUCINE INCORPORATION INTO TOTAL PROTEIN BY A WASHED-CELL SUSPENSION.

Chloramphenicol and L-[^{14}C]leucine were both added at zero time.

● , no addition
▽ , rifampicin (0.5 µg/ml)

The 100% value for L-[^{14}C]leucine incorporation was 10,500 c.p.m.


100
75
50
25
0
10
20
30
40
L-[14C]leucine (% incorporation)
Incubation Time (min.)

(b) Effect of drugs on protease formation by washed-cell suspensions

Figure 6 shows the effect of the addition of both rifampicin (0.5 µg/ml) and chloramphenicol (10 µg/ml) on the accumulation of protease by washed-cell suspensions. The drugs were added either at zero time or 70 minutes after the start of a washed-cell experiment.

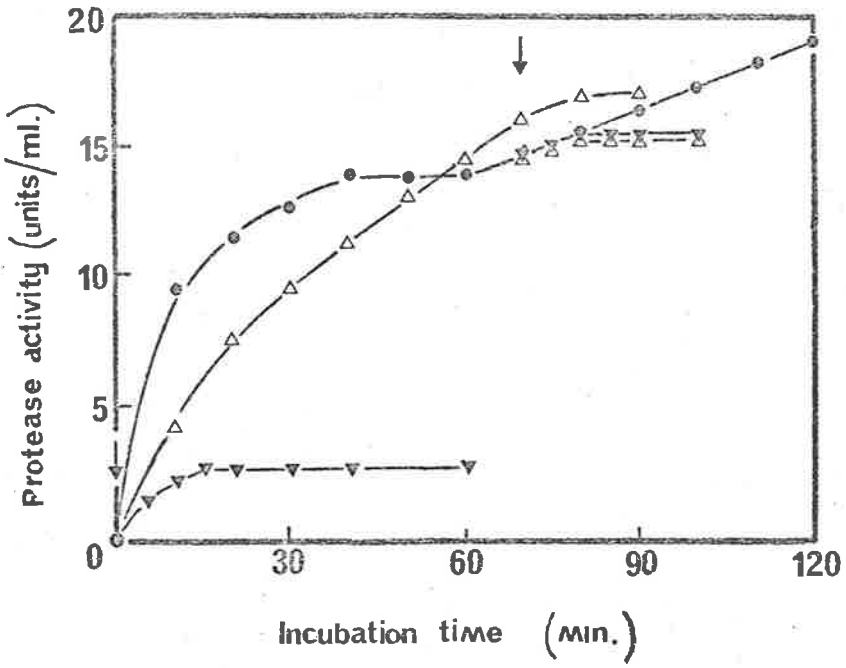
It was found that as reported earlier (Both et al., 1972) that rifampicin added at zero time to the washed-cell suspension had little effect on phase I protease accumulation. In marked contrast to this, when rifampicin is added to a washed-cell suspension after 70 minutes incubation (that is, during phase II synthesis), protease production is sensitive to the drug; inhibition being complete within a few minutes. The rifampicin-insensitive production of protease is signficantly inhibited by chloramphenicol (10 µg/ml) as is phase II of protease accumulation, however, in each case a small amount of enzyme accumulates in its presence. Separate experiments showed that the same amount of protease accumulates in the presence of chloramphenicol alone as does in the presence of rifampicin and chloramphenicol. Studies to be described later have shown that this chloramphenicol-insensitive appearance of enzyme represents pre-formed enzyme probably en route to the external medium and not enzyme adsorbed to the cells (Chapter 5). Enzyme appearance in the presence of chloramphenicol is not due to an initial insensitivity of the cells to the drug since total protein synthesis is immediately inhibited by 95% (Fig. 5).

FIGURE 4.6. EFFECT OF RIFAMPICIN AND CHLORAMPHENICOL ADDED AT ZERO TIME OR AFTER 70 MIN. INCUBATION ON PROTEASE ACCUMULATION IN THE EXTERNAL MEDIUM OF A WASHED-CELL SUSPENSION.

● , no addition of drugs

△ , 0.5 µg rifampicin/ml added at zero time (arrow) or after 70 min. incubation (arrow)

▼ , 10 µg chloramphenicol/ml and 0.5 µg rifampicin/ml both added at zero time or after 70 min. incubation.


20
15
10
5
0
30
60
90
120
Protease activity (units/ml.)
Incubation time (min.)

(c) Effect of drugs on α-amylase formation by washed-cell suspensions

As already mentioned (Fig. 1), the progress curve of α-amylase formation by washed-cells in the absence of added drugs resembles that for protease. A more detailed study is now described (Fig. 7a); the protease system is shown in Fig. 6 for comparison and confirms the results obtained by Both et al., (1972). There is an initial phase lasting for 40 minutes (phase I) followed by a second linear phase (phase II). When rifampicin was added to washed-cells at time zero, α-amylase production continues for 40 minutes, but the amount made is less than that in the absence of rifampicin (Fig. 7a). Again, in contrast to the observed effect of rifampicin on phase I accumulation, phase II is markedly inhibited by the drug when added at 70 minutes to a washed-cell suspension. The rifampicin-insensitive accumulation of α-amylase in phase I is sensitive to chloramphenicol while that appearing in the presence of rifampicin during phase II, was insensitive to chloramphenicol and must represent release of preformed enzyme. This implies that rifampicin inhibits α-amylase accumulation during phase II of synthesis almost immediately.

If rifampicin is added to washed-cells after 10 minutes incubation, α-amylase accumulation continues for 40 minutes at the same rate as without rifampicin, but then ceases completely (Fig. 7b).

FIGURE 4.7a. EFFECT OF RIFAMPICIN AND CHLORAMPHENICOL ADDED AT ZERO TIME OR 70 MIN. ON α-AMYLASE PRODUCTION BY A WASHED-CELL SUSPENSION.

● , no addition of drug

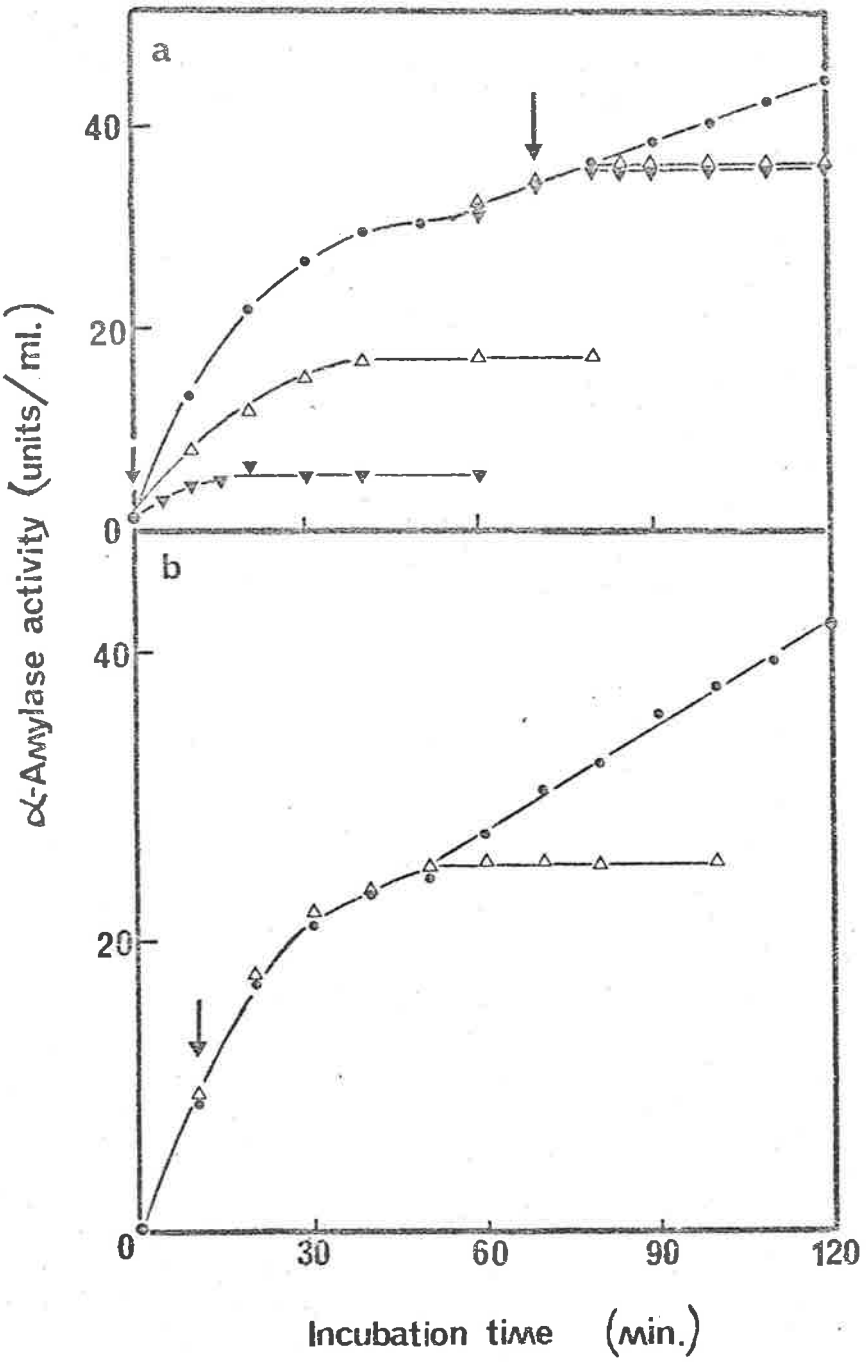
△ , 0.5 µg rifampicin/ml added at zero time (arrow) or after 70 minutes incubation (arrow).

▼ , 10 µg chloramphenicol/ml and 0.5 µg rifampicin/ml both added at zero time or after 70 min. incubation.

FIGURE 4.7b. EFFECT OF RIFAMPICIN ADDED AFTER 10 MIN. INCUBATION ON α-AMYLASE PRODUCTION BY A WASHED-CELL SUSPENSION.

● , no addition of drug

△ , 0.5 µg rifampicin/ml added after 10 min. incubation (arrow).


a
b
40
20
0
40
20
0
30
60
90
120
α-Amylase activity (units/ml.)
Incubation time (min.)

(d) Effect of drugs on ribonuclease accumulation by washed-cell suspensions

Ribonuclease continues to accumulate in the external medium of washed-cell suspensions for 40 minutes after the addition of rifampicin (0.5 µg/ml). This occurs irrespective of whether the drug was added at time zero or 70 minutes after the commencement of the washed-cell experiment (Fig. 8). Approximately half of the enzyme appearing in the presence of rifampicin may be accounted for by secretion of preformed enzyme, the appearance of which is chloramphenicol insensitive.

It may be argued that the appearance of extracellular enzymes in the presence of rifampicin added at the commencement of a washed-cell experiment, may be an effect peculiar to rifampicin. Therefore, another inhibitor of transcription was tested. Actinomycin D (2 µg/ml) was added at time zero or after 70 minutes incubation, either in the presence or absence of chloramphenicol (10 µg/ml), to a washed-cell suspension. It was found that actinomcyin D gave identical results to those obtained with rifampicin, for each of the three extracellular enzymes (Fig. 9a-c). (The studies on protease production confirmed earlier results of Both et al., 1972, and were included for comparison.)

3. The apparent accumulation of messenger RNA for extracellular enzymes as related to the age of the washed-cells

Preliminary evidence (Both et al., 1972) had indicated that an apparent mRNA pool for protease could be detected in

FIGURE 4.8. EFFECT OF RIFAMPICIN AND CHLORAMPHENICOL ADDED AT TIME ZERO OR 70 MIN. ON RIBONUCLEASE PRODUCTION BY A WASHED-CELL SUSPENSION.

● , no addition of drugs

▽ , 0.5 µg rifampicin/ml added at zero time (arrow) or after 70 min (arrow)

□ , 10 µg chloramphenicol/ml and 0.5 µg rifampicin/ml both added at zero time or after 70 min. incubation.

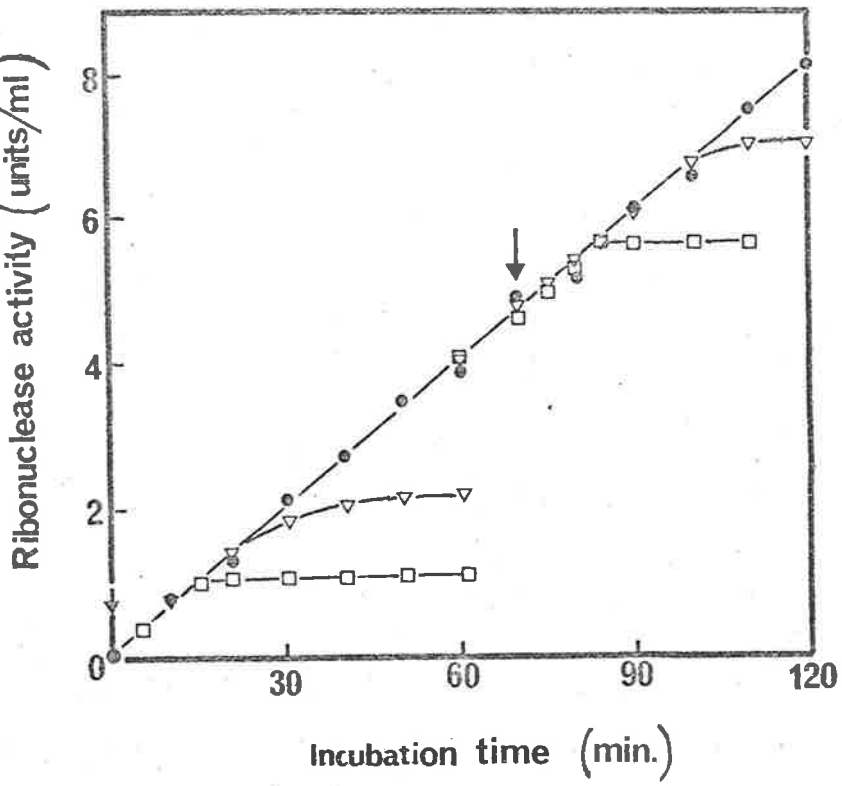

Ribonuclease activity (units/ml)
8
6
4
2
0
30
60
90
120
Incubation time (min.)

FIGURE 4.9a. EFFECT OF ACTINOMYCIN D AND CHLORAMPHENICOL ADDED AT ZERO TIME OR AFTER 70 MIN. INCUBATION ON PROTEASE ACCUMULATION IN THE EXTERNAL MEDIUM OF A WASHED-CELL SUSPENSION.

● , no addition of drugs

▲ , 2 µg actinomycin/ml added at zero time (arrow) or after 70 min. incubation (arrow).

□ , 10 µg chloramphenicol/ml and 2 µg actinomycin/ml both added at zero time or after 70 min. incubation

FIGURE 4.9b. EFFECT OF ACTINOMYCIN D AND CHLORAMPHENICOL ADDED AT ZERO TIME OR AFTER 70 MIN. INCUBATION ON α-AMYLASE PRODUCTION BY A WASHED-CELL SUSPENSION

● , no addition of drugs

▲ , 2 µg actinomycin/ml added at zero time (arrow) or after 70 min incubation (arrow)

□ , 10 µg chloramphenicol/ml and 2 µg actinomycin/ml both added at zero time or after 70 min. incubation.

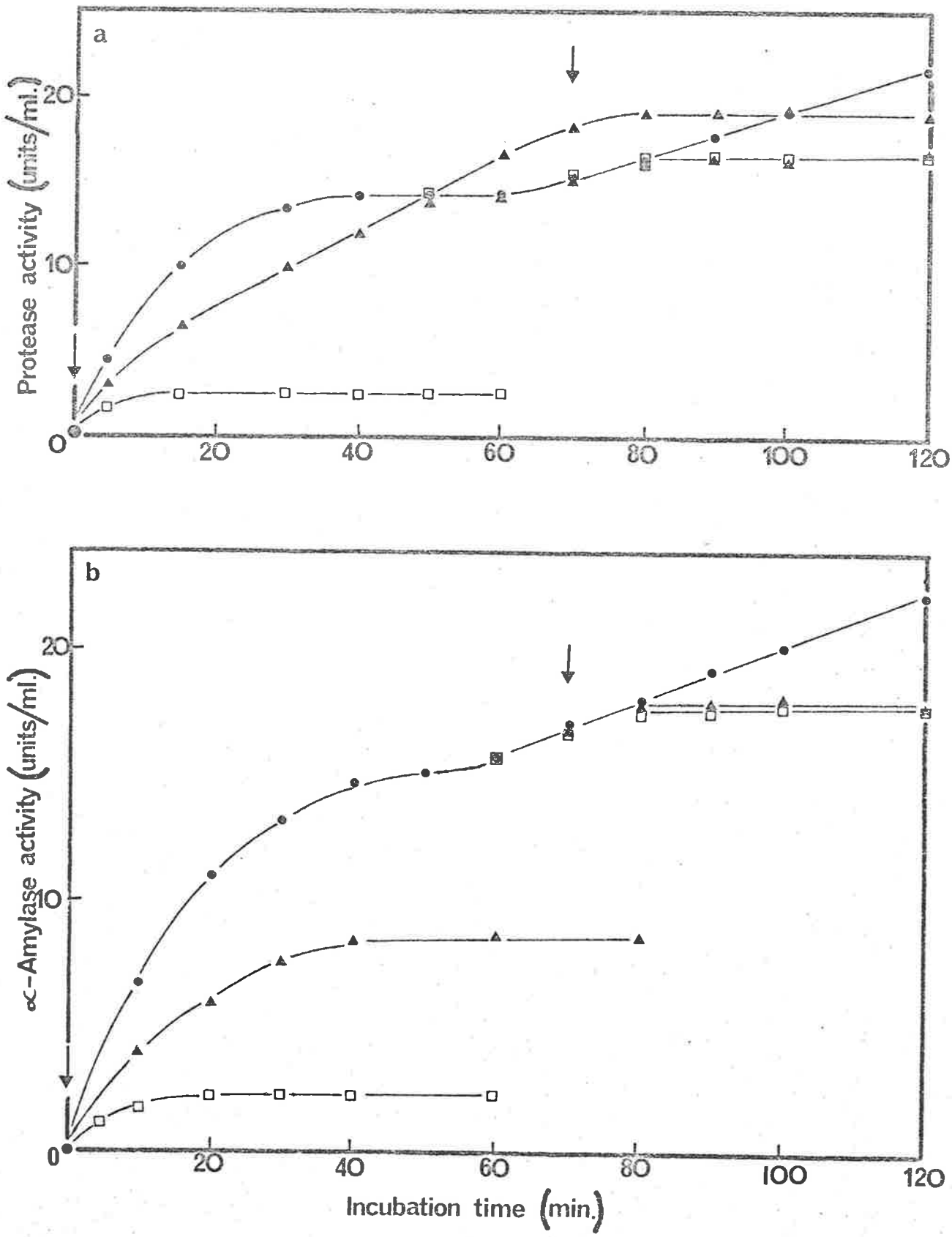
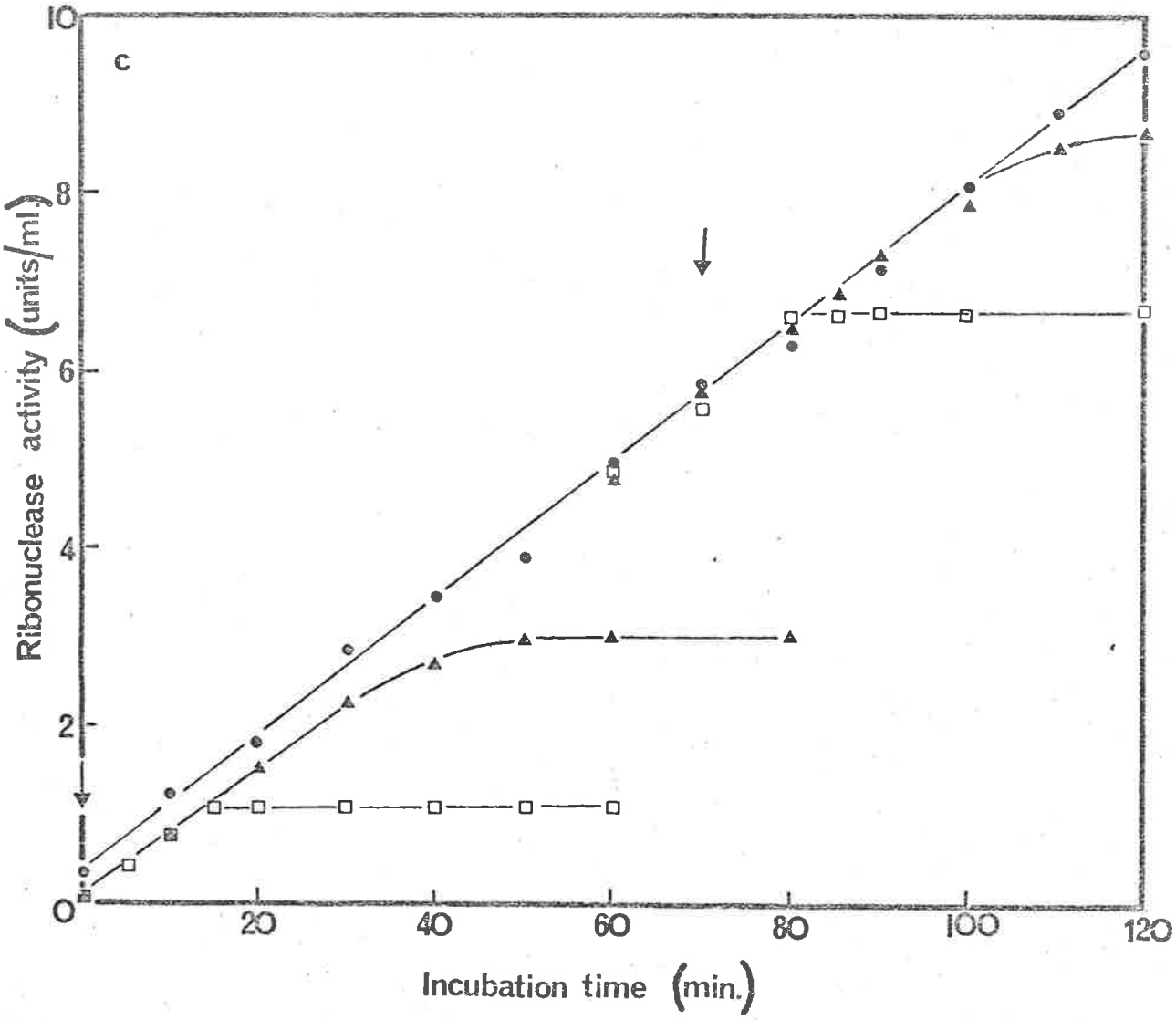

a
Protease activity (units/ml.)
20
10
0
20
40
60
80
100
120
b
∝-Amylase activity (units/ml.)
20
10
0
20
40
60
80
100
120
Incubation time (min.)

FIGURE 4.9c. EFFECT OF ACTINOMYCIN D AND CHLORAMPHENICOL ADDED AT ZERO TIME OR AFTER 70 MIN. INCUBATION ON RIBONUCLEASE PRODUCTION BY A WASHED-CELL SUSPENSION.

● , no addition of drugs

▲ , 2 µg actinomycin/ml added at zero time (arrow) or after 70 min. incubation (arrow)

□ , 10 µg chloramphenicol/ml and 2 µg actinomycin/ml both added at zero time or after 70 min. incubation.


c
Ribonuclease activity (units/ml)
Incubation time (min.)
0
2
4
6
8
10
20
40
60
80
100
120

young culture cells. The question then arises as to whether an accumulation of extracellular enzyme-specific mRNA occurs in cells of cultures growing from early log phase to stationary phase.

Cells were prepared for washed-cell experiments when the cultures showed an A_{600nm} = 1.8 (approximately 21.5 hours). These washed-cells were resuspended to the original cell density of the culture from which they were harvested and exposed to chloramphenicol (5 µg/ml) and rifampicin (0.25 µg/ml) and the amounts of extracellular enzymes produced over a 70 minute period determined. The results are shown in Figures 10a-c. Both protease and ribonuclease show an apparent accumulation of mRNA since the levels of enzyme produced in the presence of rifampicin are in excess of that accounted for by the preformed enzyme pool (that is, that level of enzyme which accumulates in the presence of chloramphenicol). However, α-amylase does not show an apparent accumulation of the messenger since the levels of enzyme accumulated in the presence of rifampicin and chloramphenicol are the same (Fig. 10c). It should be noted that in the above experiments, protease accumulates in the external medium for approximately 30 minutes whereas in 25 hour cells accumulation continues for 80 minutes in the presence of rifampicin. In the case of ribonuclease the appearance of this enzyme continues to appear in the external medium, in the presence of rifampicin, for the same time as in 25 hour cells.

The above experiments were repeated using cells harvested from cultures at an earlier stage of growth (A_{600nm} = 0.8; approx. 18.5 hour cultures). Since the levels of extracellular

FIGURE 4.10a. EFFECT OF CHLORAMPHENICOL AND RIFAMPICIN ON PROTEASE PRODUCTION BY A WASHED-CELL SUSPENSION PREPARED FROM YOUNG CULTURE CELLS (A_{600nm} = 1.8; approx. 21.5 hr. cells)

● , no addition of drug

□ , 0.25 µg rifampicin/ml added at zero time

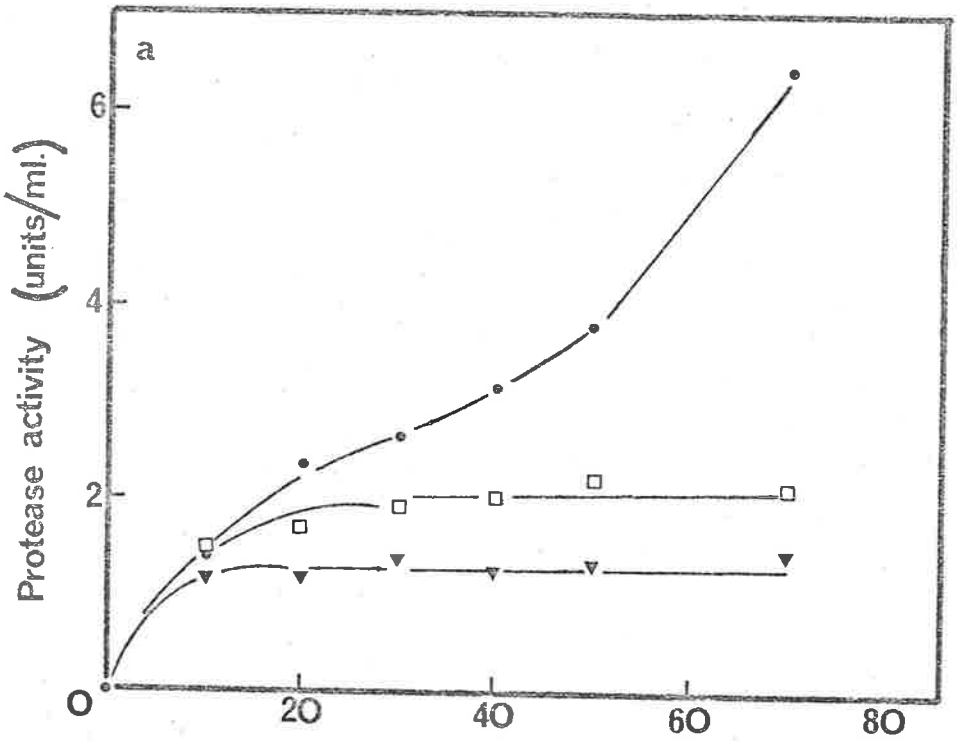
▼ , 0.25 µg rifampicin/ml and 5 µg chloramphenicol/ml both added at zero time.

FIGURE 4.10b. EFFECT OF CHLORAMPHENICOL AND RIFAMPICIN ON RIBONUCLEASE PRODUCTION BY A WASHED-CELL SUSPENSION PREPARED FROM YOUNG CULTURE CELLS (A_{600nm} = 1.8; approx. 21.5 hr. cells).

● , no addition of drug

□ , 0.25 µg rifampicin/ml added at zero time

▼ , 0.25 µg rifampicin/ml and 5 µg chloramphenicol/ml both added at zero time


a
Protease activity (units/ml.)
6
4
2
0
20
40
60
80

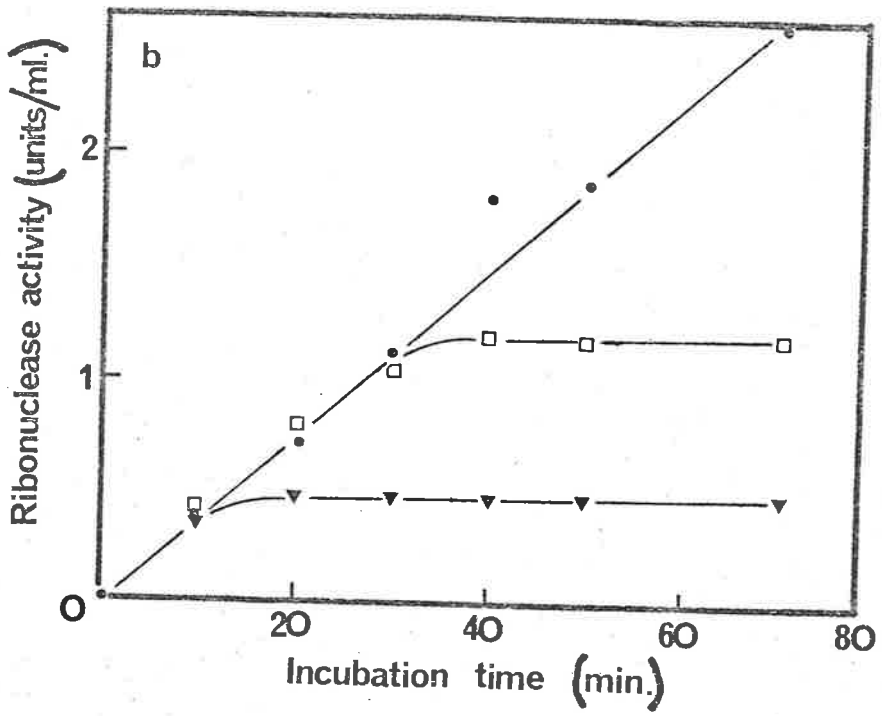
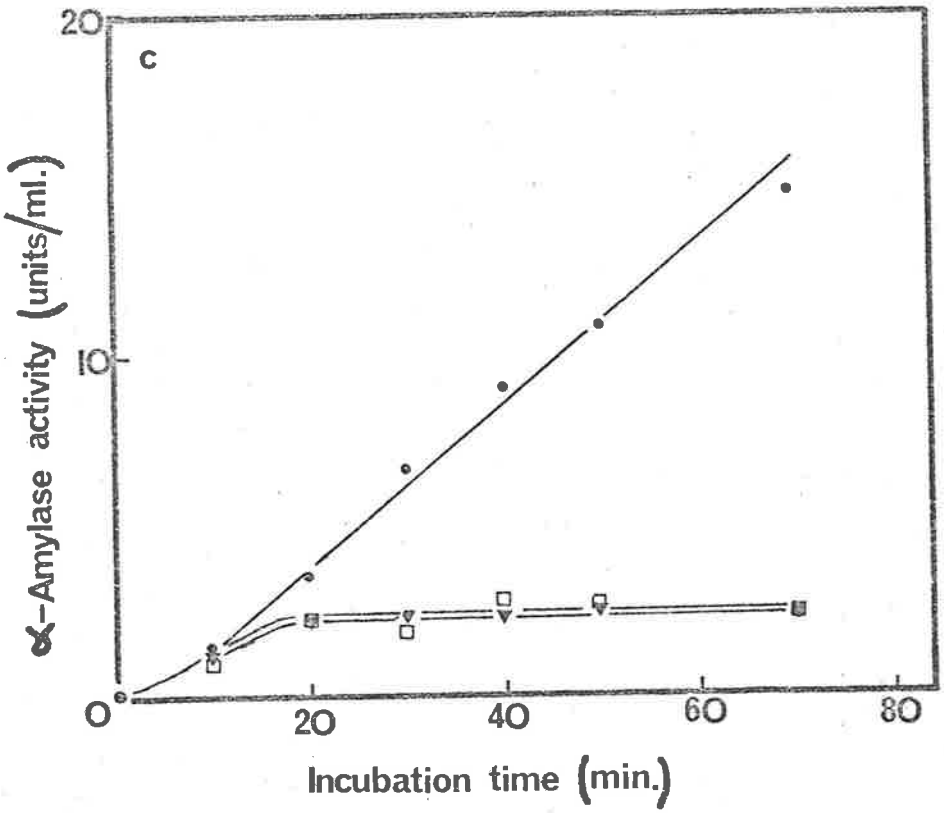

b
Ribonuclease activity (units/ml.)
2
1
0
20
40
60
80
Incubation time (min.)

FIGURE 4.10c. EFFECT OF CHLORAMPHENICOL AND RIFAMPICIN ON α-AMYLASE PRODUCTION BY A WASHED-CELL SUSPENSION PREPARED FROM YOUNG CULTURE CELLS (A_{600nm} = 1.8; approx. 21.5 hr. cells)

● , no addition of drug

□ , 0.25 µg rifampicin/ml added at time zero

▼ , 0.25 µg rifampicin/ml and 5 µg chloramphenicol/ml both added at time zero.


c
20
10
0
20
40
60
80
α-Amylase activity (units/ml.)
Incubation time (min.)

enzymes produced by young cells is low the washed-cell suspensions were made up to the same cell density as used in the first set of experiments (A_{600nm} = 3.6). It was found that with these cells the levels of the three extracellular enzymes accumulating in the presence of rifampicin or chloramphenicol were the same, indicating only the release of the preformed enzyme pools from these cells was occurring.

C. DISCUSSION

α-Amylase synthesis shows characteristics which closely parallel those described for protease (May and Elliott, 1968a; Both et al., 1972). Using the same reasoning as the latter, it appears that there is a pool of mRNA specific for the α-amylase in 25 hour cells which supports synthesis of the enzyme for about 40 minutes in the absence of continuing transcription. The existence of phases I and II in protease production has been ascribed to amino acid repression (Both et al., 1972) of transcription during phase I. This causes the mRNA pool to be exhausted while phase II is dependent upon derepression of the cells and continued transcription. It is not understood what causes the protease de-repression; a likely possibility is that the metabolism of the cells becomes such that the intracellular levels of amino acids is kept sufficiently low to permit de-repression of protease gene transcription. It is perhaps important to note that separate experiments (May, unpublished observations) have shown that exhaustion of amino acids in the medium is not responsible since added amino acids are incapable of repressing phase II synthesis - that is, the

derepression is a consequence of a cellular characteristic and not of a change in the medium. Cells from phase II, harvested and resuspended in new medium behave the same as control cells not resuspended in the new medium.

It would seem reasonable from the results described here to postulate that when cells are placed in the suspending medium, transcription of α-amylase mRNA is also repressed; the difference between adding rifampicin at 0 and 10 minutes indicates that the repression takes some minutes to establish. Attempts to find out what causes repression have not defined any specific component(s) of the medium as being responsible. It cannot be attributed to obvious candidates such as carbohydrate. Studies by Coleman (1967) on α-amylase control also showed that the situation is a complex one and he concluded that any medium which stimulates cellular growth represses α-amylase. For the moment the simplest explanation is that in cells in culture the transcription of α-amylase mRNA becomes maximal between 22 and 25 hours and results in a pool of mRNA being present in cells harvested at 25 hours. On resuspension in a new medium, repression is established which leads to exhaustion of the mRNA pool and phase II of synthesis is due to de-repression and continued transcription. As already stated, however, it is not possible to offer any suggestions as to what it is in the new medium which effects the repression of α-amylase, unlike the protease situation where high levels of amino acids are essential for repression in new medium (for clarity it should perhaps be emphasised that this does not contradict the earlier statement that amino acids do not repress cells which have

spontaneously become de-repressed in phase II. The important point is that no repression occurs in culture cells re-suspended in a low amino acids medium.)

In the case of ribonuclease, synthesis continues for 40 minutes in the presence of rifampicin while general protein and RNA synthesis is inhibited after 5 minutes. This again suggests the existence of a pool of accumulated mRNA specific for the ribonuclease, though in this case the evidence does not exclude the possibility that the mRNA is intrinsically long-lived. Since transcription of mRNA specific for the ribonuclease was apparently not repressed in the suspending medium, as appears to be the case for α-amylase and protease, the postulated pool of mRNA for ribonuclease could not be exhausted. Thus the sensitivity of transcription in the absence of the pool could not be tested.

Studies on the apparent accumulation of mRNA pools by washed-cell suspensions prepared from cultures of different ages, indicate that maximal mRNA accumulation for α-amylase and protease occurs between 22 and 25 hours. The apparent mRNA pool for ribonuclease seems to accumulate earlier; no pool is detected in 18.5 hour cells but it is present in 22 hour cells. It seems unlikely that the mRNA for ribonuclease becomes more stable as the culture ages and a more reasonable assumption is that a pool of mRNA accumulates with time as demonstrated for α-amylase and protease.

Several cases have been reported of the accumulation of specific mRNA's in bacteria. The most striking and recent

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

It has been proposed that the accumulation of the pool of protease mRNA was a repercussion of the mechanism of synthesis of extracellular enzymes. It has previously been postulated (May and Elliott, 1968a) that extracellular enzyme synthesis by this organism occurs on ribosomes bound at special translation-extrusion sites located at the membrane. It has been further proposed (Glenn et al., 1973) that the messenger for the extracellular protease migrates from the gene to a limited number of translation-extrusion sites and also shown that the accumulation of mRNA results from a positive imbalance of transcription over translation. Thus, the prolonged protease synthesis in the presence of rifampicin is a reflection of the fact that after many half life decay periods the amount of mRNA remaining is still adequate to saturate the translational sites. There is no direct evidence as to the form in which the pools of mRNA exist. The first possibility is that there is an accumulation of free polysomes. However, Both et al. (1972) reported that pactamycin (0.05 µg/ml) almost immediately inhibited the production of the extracellular protease during phase 1. This would tend to rule out a pool of polysomes, assuming that the pactamycin concentration used was specific for inhibiting the

initiation of protein synthesis as is claimed by Cohen, Herner and Goldberg (1969) since the pre-initiated ribosomes of polysomes would be expected to continue producing protease for some time if a large polysome pool existed. Alternatives are that the messenger is free, or bound only to a 30S subunit. In either situation translation would only begin after the 70S ribosome is fully assembled at the membrane.

The model of secretion in B. amyloliquefaciens raises several problems such as, (1) the nature of the translational-extrusion sites, (2) whether specific ribosomes exist, and (3) the basis of the specific interaction between the messenger and the ribosomes. Nevertheless, the extrusion model appears to fit the facts better than any other. It explains the immunity of the cells to protease and ribonuclease and also explains how the cell is able to secrete ribonuclease despite the presence in the cytoplasm of a vast excess of an almost irreversible inhibitor of the enzyme (Smeaton and Elliott, 1967; Hartley, 1970).

The main value of the results in this Chapter has been to indicate that the unique properties of the protease synthesis system, as established by Both et al. (1972), are not a peculiar aberration of the one system but also apply to α-amylase and almost certainly to ribonuclease. In view of the fact that synthesis of the three enzymes are separately controlled (as evidenced by their different repression characteristics) it would seem most likely that the enzymes are coded for by its own mRNA rather than by a polycistronic messenger. Given this, the results in this Chapter suggest that the apparent pools of

mRNA's are a reflection of the mechanism of extracellular enzyme synthesis by B. amyloliquefaciens rather than a fortuitous occurrence for some unknown reason.

CHAPTER 5

STUDIES ON THE RELEASE OF EXTRACELLULAR ENZYMES FROM WASHED-CELL SUSPENSIONS

STUDIES ON THE RELEASE OF EXTRACELLULAR ENZYMES FROM WASHED-CELL SUSPENSIONS

A. INTRODUCTION

As mentioned in the previous Chapter, it was consistently observed that small amounts of the three extracellular enzymes were released into the external medium, when washed-cell suspensions were exposed to inhibitors of protein synthesis such as chloramphenicol.

It was of importance to define what this release of enzyme represented. The most likely possibilities were that it was a trivial phenomenon representing elution of enzyme adsorbed to the cells, which had not been removed by previous washing, or that it represented preformed enzyme *en route* to secretion. The results presented in this Chapter are compatible with the latter and with a model of secretion whereby newly synthesised extracellular enzymes can be secreted directly from the cell or become cell-associated prior to release.

B. RESULTS

1. Effect of chloramphenicol on extracellular enzyme formation by washed-cell suspensions

Chloramphenicol (10 µg/ml) stops the accumulation of extracellular enzymes in the external medium of washed-cell suspensions of *B. amyloliquefaciens*, but this takes approximately 15 minutes to be complete. Over the first 10-15 minutes after the addition of the antibiotic - α-amylase, protease and ribonuclease continue to appear in the medium (Figs. 1a-c). In the

FIGURE 5.1a. EFFECT OF CHLORAMPHENICOL ON α-AMYLASE PRODUCTION BY A WASHED-CELL SUSPENSION.

● , no addition of drug

o , 10 µg chloramphenicol/ml added at time zero.

FIGURE 5.1b. EFFECT OF CHLORAMPHENICOL ON RIBONUCLEASE PRODUCTION BY A WASHED-CELL SUSPENSION

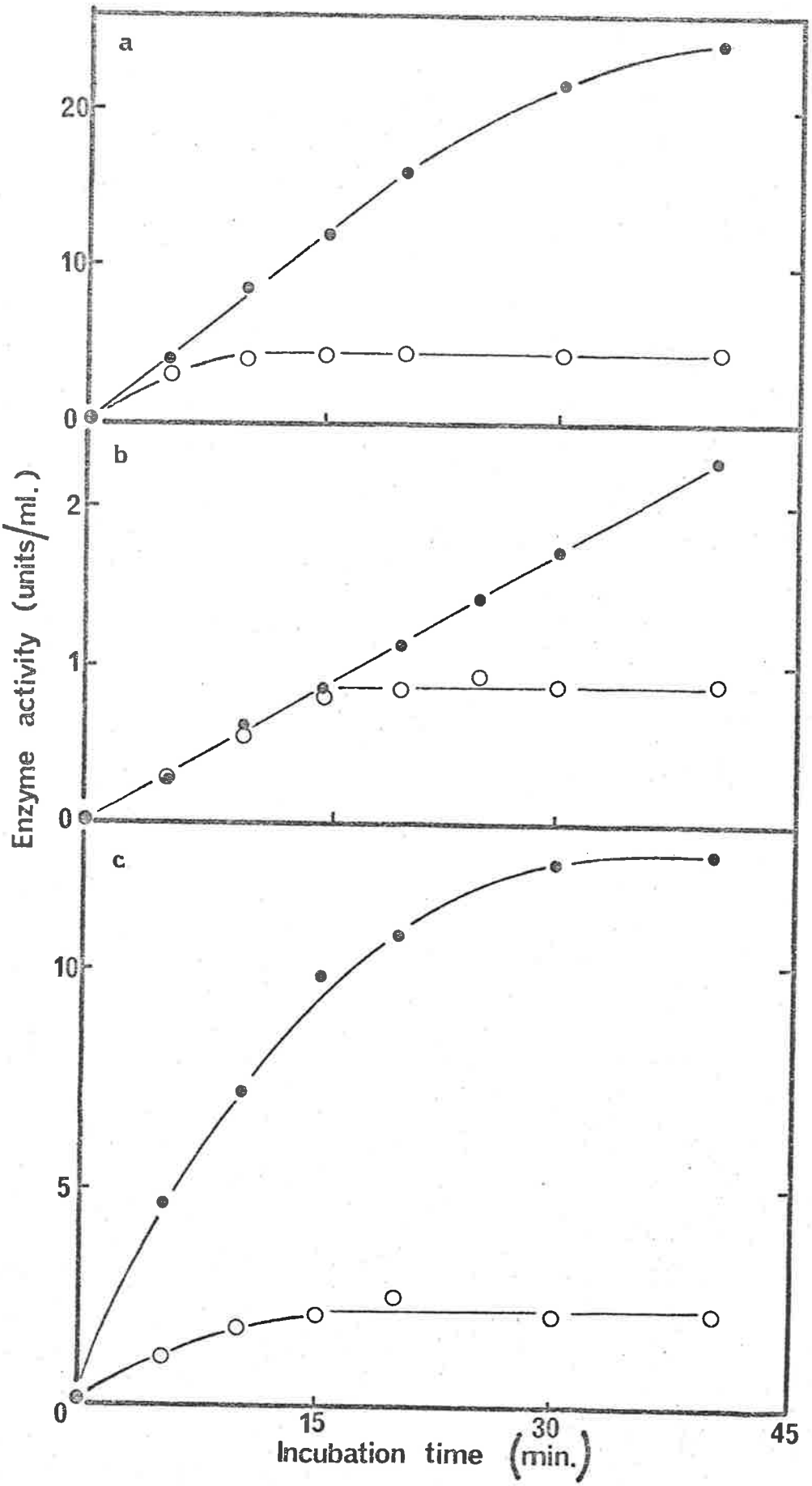
● , no addition of drug

o , 10 µg chloramphenicol/ml added at time zero

FIG. 5.1c. EFFECT OF CHLORAMPHENICOL ON PROTEASE PRODUCTION BY A WASHED-CELL SUSPENSION

● , no addition of drug

o , 10 µg chloramphenicol/ml added at time zero.


a
b
c
20
10
0
2
1
0
10
5
0
Enzyme activity (units/ml.)
15
30
45
Incubation time (min.)

case of ribonuclease the rate of accumulation parallels the control rate for 15 minutes, whereas this is not so for protease and α-amylase. The reason for this apparent difference in the behaviour of ribonuclease is unknown. This accumulation of enzymes is not due to an initial period of chloramphenicol insensitivity as general protein synthesis is immediately and almost completely inhibited under the same conditions (See Fig. 4.5). An identical result was obtained with 100 µg/ml chloramphenicol, with puromycin (20 µg/ml) and sodium fusidate (22.5 µg/ml); at these concentrations the two latter drugs immediately and completely inhibited general protein synthesis.

2. Nature of the chloramphenicol insensitive release of enzyme

The following experiment was performed to test whether the chloramphenicol insensitive appearance of extracellular enzymes was due to elution of enzyme non-specifically adsorbed to the cells and not removed by previous washings. Twenty-five hour cell cultures, containing large amounts of the three extracellular enzymes in the medium (approximately 700 units/ml of protease, 600 units/ml of α-amylase and 12 units/ml of ribonuclease), were treated with chloramphenicol (10 µg/ml) and incubated for 15 minutes. The cells were collected by centrifugation, washed twice in suspending medium and finally resuspended in this medium to the original cell density. Chloramphenicol (10 µg/ml) was then added and the production of extracellular enzymes was followed for 40 minutes. This was compared with enzyme production by cells which had not been

pre-exposed to chloramphenicol in the culture medium but which were treated with the drug at time zero in the washed-cell suspension experiment. Cells not pre-treated with chloramphenicol immediately released the expected amount of enzymes in the presence of the drug (curve 2, Figs. 2a-c), while cells which had been pre-exposed to chloramphenicol in the culture medium did not release any enzyme (curve 3, Figs. 2a-c). These results show that the chloramphenicol insensitive release is not a trivial phenomenon due to release of enzyme non-specifically adsorbed to the cells and most probably represents enzyme en route to secretion.

3. Effect of sodium azide and 2,4-dinitrophenol on the chloramphenicol insensitive release of enzyme

The energy requirements of the chloramphenicol insensitive release are shown in Figure 3. Neither 2,4,-dinitrophenol (2 mM) nor sodium azide (20 mM) affec t ed the chloramphenicol insensitive release of the extracellular protease by washed-cell suspensions, although it has been shown that these energy inhibitors separately inhibit general protein synthesis by greater than 95%. Similar results were obtained for the extracellular α-amylase and ribonuclease, thus the release of these pools of enzyme does not require energy.

4. Effect of temperature on the chloramphenicol insensitive release of extracellular enzymes

Washed-cell suspensions (both in the presence and absence of chloramphenicol (10 µg/ml) were cooled slowly to

FIGURE 5.2a. EFFECT OF CHLORAMPHENICOL ON THE PRODUCTION OF RIBONUCLEASE BY NORMAL WASHED-CELL SUSPENSIONS AND WASHED-CELLS PREPARED FROM CULTURE CELLS PRE-EXPOSED TO CHLORAMPHENICOL.

● , no addition of drug

△ , 10 µg chloramphenicol/ml added at time zero to a normal washed-cell suspension

□ , 10 µg chloramphenicol/ml added at time zero to a washed-cell suspension prepared from culture cells pre-exposed to chloramphenicol

FIGURE 5.2b. EFFECT OF CHLORAMPHENICOL ON THE PRODUCTION OF α-AMYLASE BY NORMAL WASHED-CELL SUSPENSIONS AND WASHED-CELL PREPARED FROM CULTURE CELLS PRE-EXPOSED TO CHLORAMPHENICOL.

● , no addition of drug

△ , 10 µg chloramphenicol/ml added at time zero to a normal washed-cell suspension

□ , 10 µg chloramphenicol/ml added at time zero to a washed-cell suspension prepared from culture cells pre-exposed to chloramphenicol.

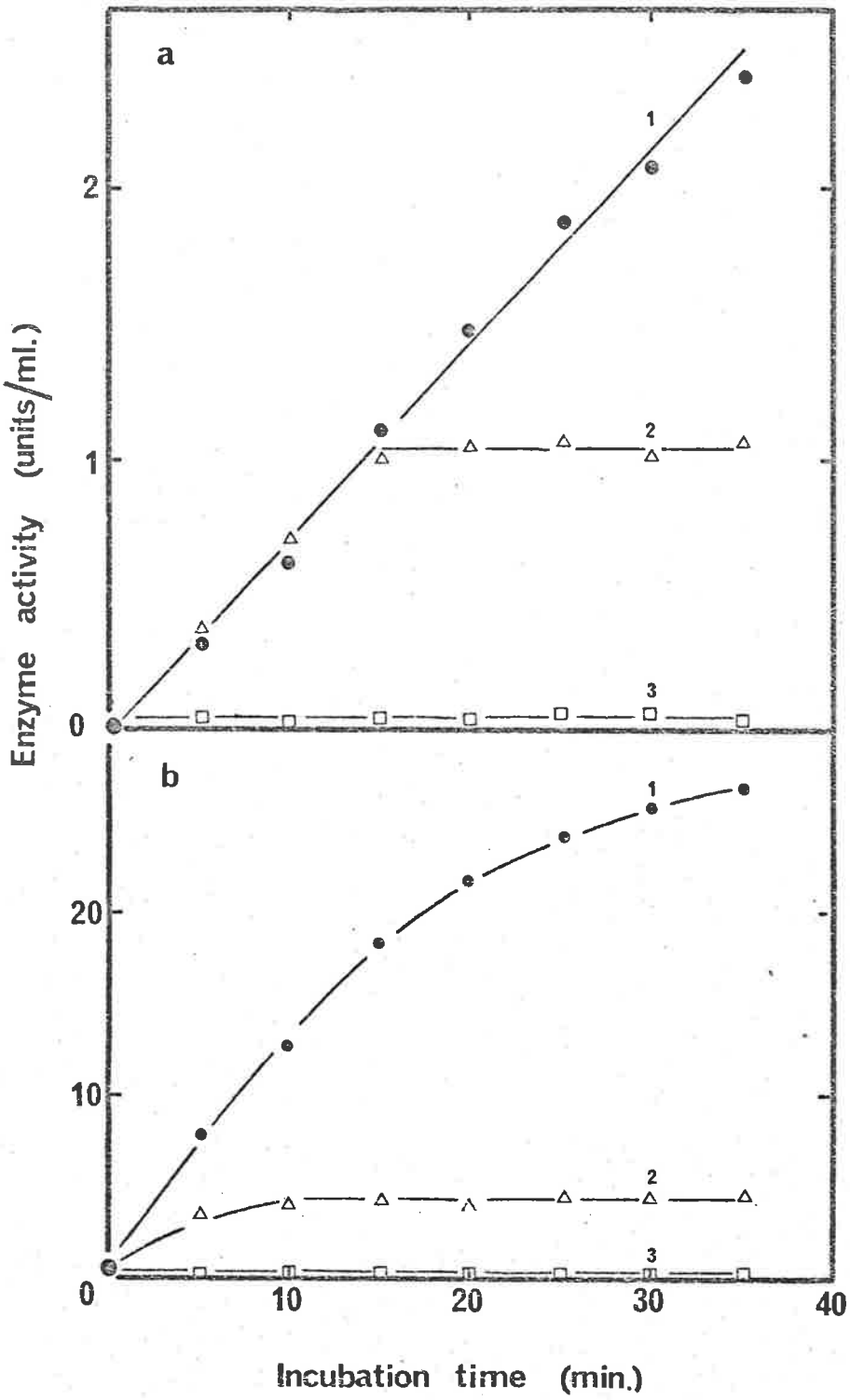

a
b
1
2
3
Enzyme activity (units/ml.)
Incubation time (min.)
0
1
2
10
20
30
40

FIGURE 5.2c. EFFECT OF CHLORAMPHENICOL ON THE PRODUCTION OF PROTEASE BY NORMAL WASHED-CELL SUSPENSIONS AND WASHED-CELLS PREPARED FROM CULTURE CELLS PRE-EXPOSED TO CHLORAMPHENICOL.

● , No addition of drug

△ , 10 µg chloramphenicol/ml added at time zero to a normal washed-cell suspension

□ , 10 µg chloramphenicol/ml added at time zero to a washed-cell suspension prepared from culture cells pre-exposed to chloramphenicol.

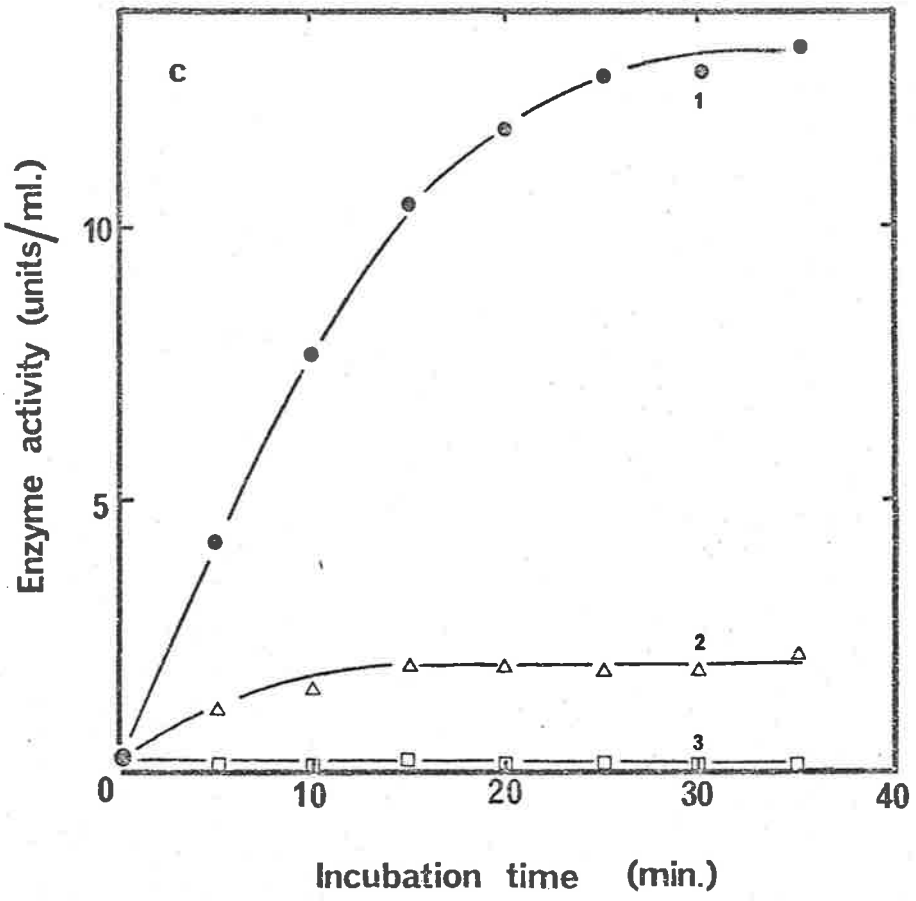

c
1
2
3
10
5
0
10
20
30
40
Enzyme activity (units/ml.)
Incubation time (min.)

FIGURE 5.3. EFFECT OF SODIUM AZIDE AND 2,4-DINITROPHENOL ON PROTEASE ACCUMULATION, IN THE PRESENCE OF CHLORAMPHENICOL, BY WASHED-CELLS.

All drugs were added to washed-cells at time zero.

● , no addition of drugs

□ , 10 µg chloramphenicol/ml

▼ , 10 µg chloramphenicol and sodium azide (20 mM) both added at time zero

△ , 10 µg chloramphenicol/ml and 2,4-dinitrophenol (2 mM) both added at time zero.

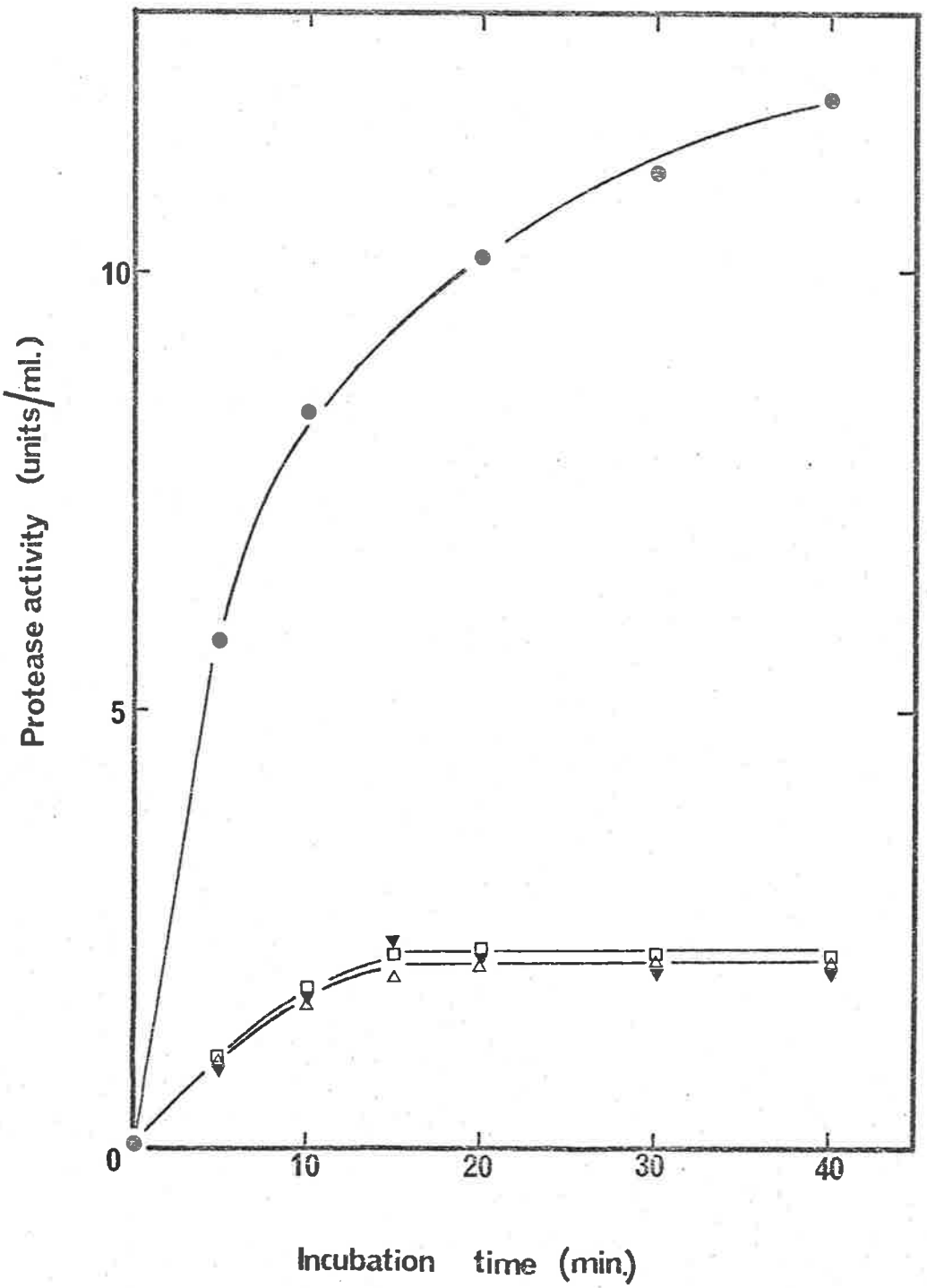

Protease activity (units/ml.)
10
5
0
10
20
30
40
Incubation time (min.)

FIGURE 5.4. EFFECT OF TEMPERATURE ON PROTEASE PRODUCTION BY WASHED-CELLS EITHER IN THE PRESENCE OR ABSENCE OF CHLORAMPHENICOL

Washed-cell suspensions were prepared as described in the text. The temperature was slowly dropped to 0° and to a sample of the washed-cell suspension, chloramphenicol was added to a final concentration of 10 µg/ml. After 30 min. incubation at 0° the temperature was raised to 37° and the incubation continued (arrow). At the various times indicated samples were taken, centrifuged and the supernatants assayed for protease activity.

● , no addition

□ , 10 µg chloramphenicol/ml added at time zero.

FIGURE 5.5. EFFECT OF TEMPERATURE ON PROTEASE PRODUCTION BY WASHED-CELLS TREATED WITH CHLORAMPHENICOL.

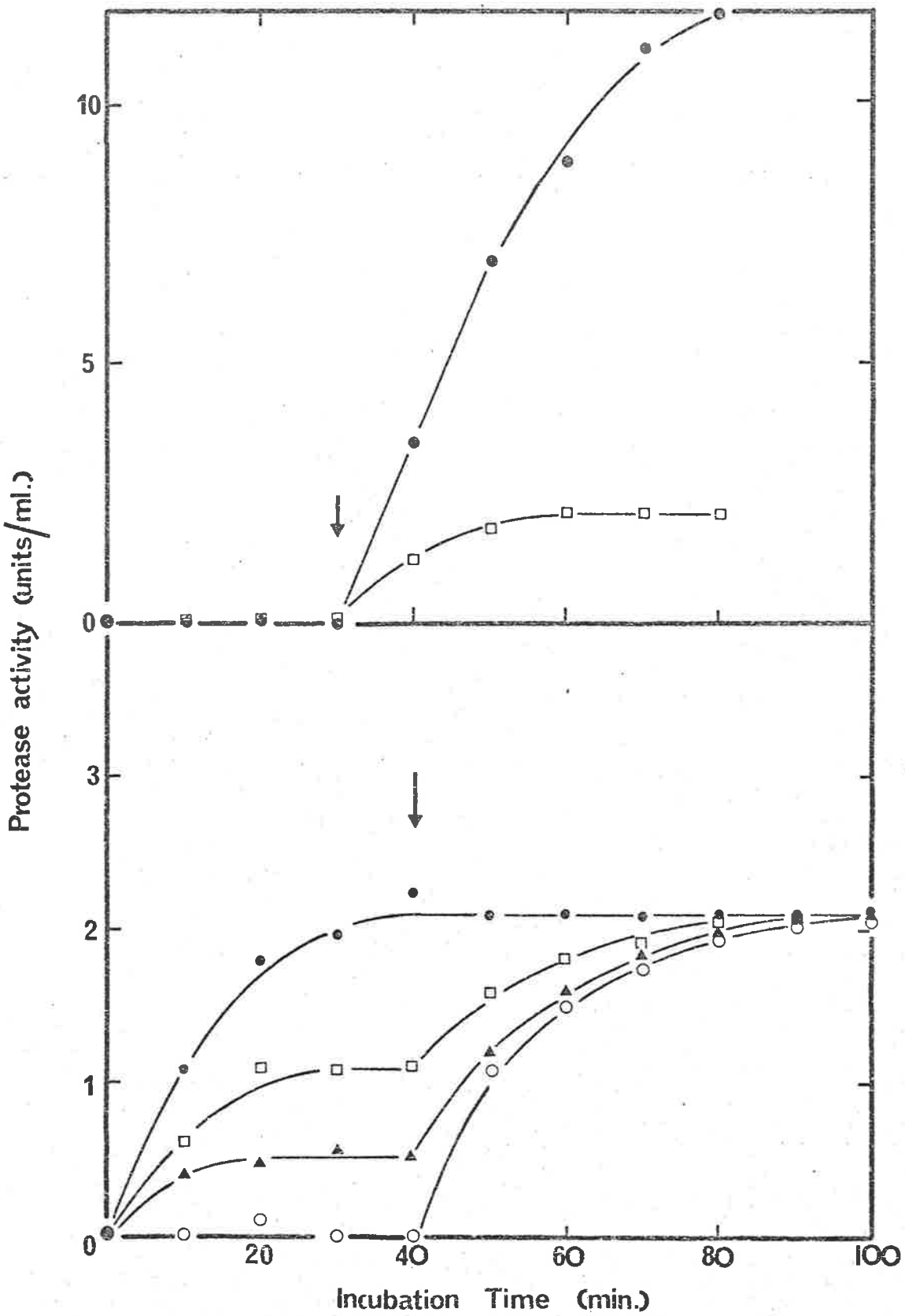
Washed-cell suspensions were prepared and cooled to 0° in the presence of chloramphenicol (10 µg/ml), as described in the text. Samples of this suspension were then incubated at either 0°, 10°, 20° or 30° and supernatants samples prepared at the times indicated. After 40 min. incubation at these various temperatures the temperature was raised to 30° in each case (arrow) and supernatant samples prepared.

○ , 0°

▲ , 10°

□ , 20°.

● ,30°.


Protease activity (units/ml.)
10
5
0
3
2
1
0
20
40
60
80
100
Incubation Time (min.)

0°C over a five minute time period by swirling in ice-water (it is necessary to slowly cool the cells since upon instantaneous cooling, cells become 'cold-shocked', a phenomenon described by Smeaton and Elliott (1967) in which the cell's normal permeability characteristics are lost). The washed-cell suspensions were then incubated at 0° with shaking, samples removed and the supernatants assayed for extracellular enzymes. After 30 minutes at 0° the temperature was raised to 37° and the incubation continued with shaking. It can be seen from Fig. 4 that at 0° no protease from control or chloramphenicol treated cells was released, but on raising the temperature to 37°C, release of protease occurred both in the presence and absence of chloramphenicol. Identical results were seen with α-amylase and ribonuclease. The total amount of enzyme released in the presence of chloramphenicol corresponded with the normal chloramphenicol insensitive release, although the pre-cooling resulted in a somewhat slower rate of release. This effect can be seen more clearly in the experiments described below and illustrated in Fig. 5.

The effect of intermediary temperatures on enzyme release was studied by cooling washed-cell suspensions to 0° in the presence of chloramphenicol (10 μg/ml) and then incubating the cells at the selected temperature for 50 minutes. At 0°, 10°, 20° and 30° differing amounts of protease were released and then remained at a constant plateau value for each temperature (Fig. 5). After 40 minutes preincubation at the appropriate temperature, the cells were transferred to a 30° shaking water bath (the incubations equilibrated to 30° in

less than one minute). The levels of protease now increased from the plateau amounts to that of a control suspension incubated from time zero at 30° in the presence of chloramphenicol.

Again it was noticeable that the chloramphenicol insensitiv release of protease from cells pre-exposed to 0° took considerably longer than 15 minutes; in the same experiment, α-amylase and ribonuclease release were examined and the results were identical to those described for protease.

5. Appearance of pulse labelled protease and α-amylase in the external medium of washed-cell suspensions

If the chloramphenicol insensitive release represents preformed enzyme en route to the external medium, the question arises as to why 15 minutes is needed before all of the enzyme is externalised. Two obvious possibilities exist. Either an enzyme molecule may obligatorily require 15 minutes to complete its passage to the exterior, the last enzyme molecule to be released into the medium representing the last to be synthesised. Alternatively, newly-synthsised molecules may feed into and equilibrate with a pool of enzyme which is released at a rate requiring 15 minutes for release of the entire pool. In the latter case an individual molecule has a statistical chance of being released from the cell virtually instantaneously after its synthesis. The following experiment was designed to see if there was a significant delay in the time of appearance of newly-synthesised extracellular enzymes into the external medium. Cells from 40 ml of culture were harvested at 25 hours, washed twice in suspending medium which lacked amino acids.

Finally, the cells were resuspended in this same medium and labelled for 90 seconds using ^{14}C-reconstituted protein hydrolysate (0.5 µC/ml). The labelled amino acids were diluted by the addition of 2.0 ml of casamino acids (40% (w/v)) and the incubation continued. To measure total cellular protein synthesis, samples (0.1 ml) were taken at appropriate times, and directly added to 10% TCA and 1% casamino acids (3.0 ml) and processed as in Materials and Methods. To measure the radioactive material accumulating in the external medium 3.5ml samples were centrifuged and any remaining bacterial cells removed by millipore filtration. Samples (1.0 ml) of the supernatant were then added to 10% TCA - 1% casamino acids (6.0 ml) and the precipitated material washed and counted by liquid scintillation. The omission of casamino acids from the suspending medium was separately shown not to affect total protein synthesis during the 90 second labelling period (Fig. 6a).

The addition of the casamino acids after the 90 second labelling period inhibited incorporation of ^{14}C-amino acids into total cellular proteins after about 4 minutes (Fig. 6a), although radioactive TCA precipitable material continued to accumulate in the external medium for about 15 minutes (Fig. 6b). Similar results were obtained when either streptomycin (20 µg/ml) or puromycin (20 µg/ml) were used to instantly stop ^{14}C-amino acid incorporation.

Using an immunological assay it was possible to determine how much of this radioactive TCA-precipitable material inthe external medium was due to the two extracellular enzymes, protease and α-amylase (ribonuclease was not examined since its

FIGURE 5.6a. [^{14}C]-PROTEIN HYDROLYSATES INCORPORATION BY A WASHED-CELL SUSPENSION.

Washed-cells were pulsed for 90 seconds by the addition of [^{14}C]-protein hydrolysate (0.5 µc/ml). After 90 sec. the incorporation was quenched by the addition of 2.0 ml casamino acids (40% w/v)) (arrow) and the incubation continued.

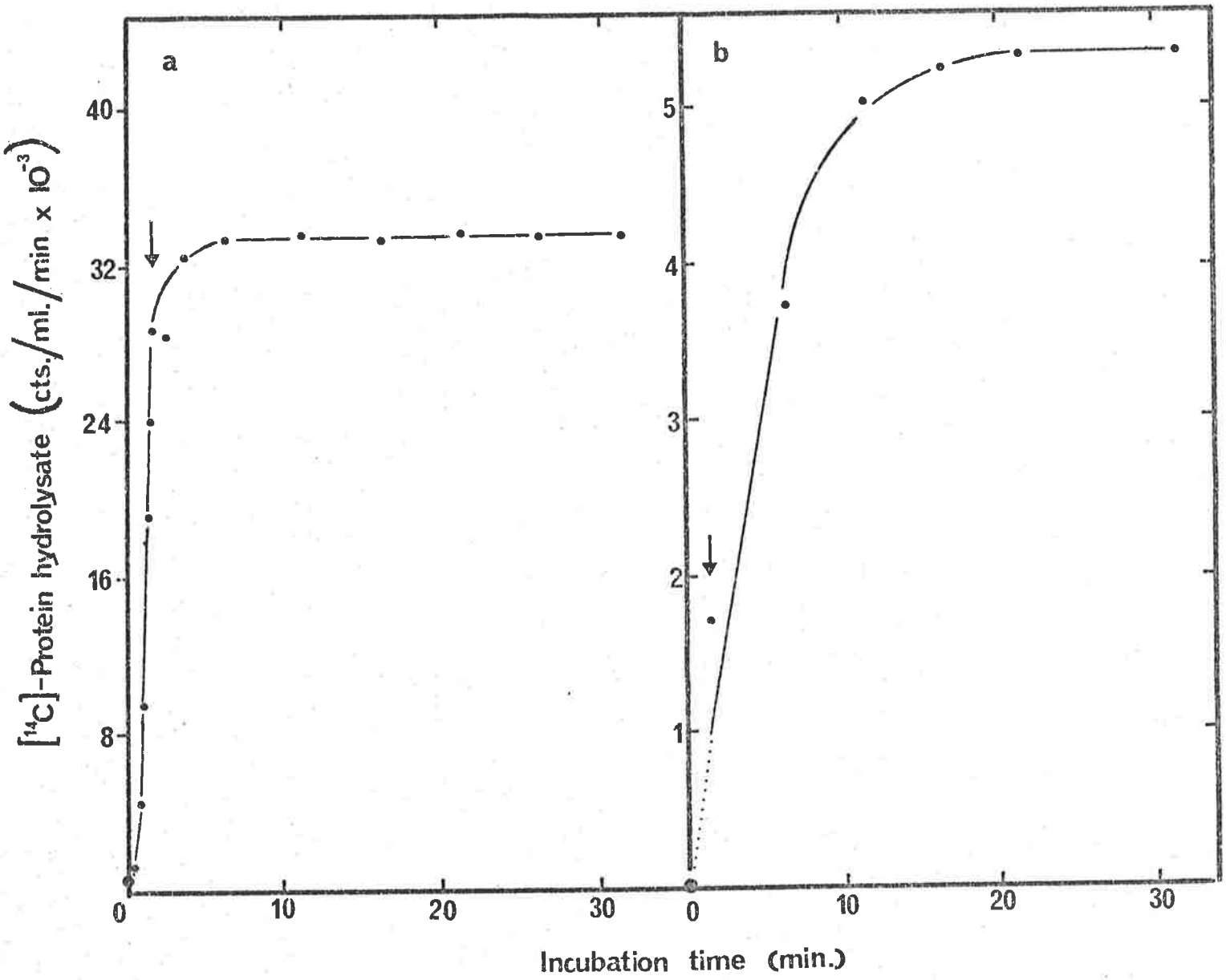
At the various times indicated, 0.1 ml samples were taken and directly added to 10% TCA - 1% casamino acid and the total TCA precipitable counts determined as explained in the text.

FIGURE 5.6b. THE LEVEL OF RADIOACTIVE TCA PRECIPITABLE MATERIAL ACCUMULATING IN EXTERNAL MEDIUM AFTER A 90 SEC. PULSE WITH [^{14}C]-PROTEIN HYDROLYSATE.

The labelling conditions were identical to those described for Fig. 5.6a. At the various times indicated, samples (3.5 ml) were taken and the bacterial cells removed by centrifugation and millipore filtration. The supernatant samples (1.0 ml) were then added directly to 10% TCA-1% casamino acids (6.0 ml) and the TCA precipitated radioactivity determined as in the text.

..... , 90 second pulse period

●—● , total supernatant TCA precipitable counts .


a
b
40
32
24
16
8
5
4
3
2
1
0
10
20
30
0
10
20
30
[14C]-Protein hydrolysate (cts./ml./min x 10-3)
Incubation time (min.)

antibody was not available). To supernatant samples, previously filtered through millipore filters, a fixed amount of either protease or α-amylase rabbit antiserum (purified as described in Materials and Methods) was added at a two-fold excess to neutralise the extracellular enzyme. This was then incubated at 37° for 60 minutes and then a predetermined amount of goat anti-rabbit antiserum was added to maximally precipitate the previously added rabbit anti-extracellular enzyme antibody. Incubation was continued for a further 60 minutes at 37° and then for 16 hours at 4°. The immune precipitate was washed three times with 5.0 ml lots of cold 0.9% (w/v) saline by centrifugation (4,500 g/10 min.) and resuspension, collected onto 47 mm millipore filters and the filters washed 5 times with 10.0 ml lots of cold saline. The filters were then dried and counted by liquid scintillation. Controls which contained goat anti-rabbit IgG antiserum alone or the specific antibody replaced by non-immune rabbit IgG were treated identically. After correction for the low non-specific precipitation of radioactive material in the controls, it can be seen (Fig. 7) that both protease and α-amylase appear in the external medium of the washed-cell suspension andthat there is no apparent delay before this pulse labelled enzyme emerges.

It is significant that after the pulse period it takes 15 minutes before all of the α-amylase or protease synthesised in 90 seconds is externalised. This is similar to the result where chloramphenicol addition to washed-cell suspensions resulted in the accumulation of enzyme in the external medium over the next 15 minutes.

FIGURE 5.7. THE ACCUMULATION OF RADIOACTIVE PROTEASE AND α-AMYLASE IN THE EXTERNAL MEDIUM OF A WASHED CELL SUSPENSION AFTER A 90 SECOND PULSE WITH [^{14}C]-PROTEIN HYDROLYSATE (AS DETERMINED BY IMMUNE PRECIPITATION).

The pulse labelling and immune precipitation conditions were as described in the text.

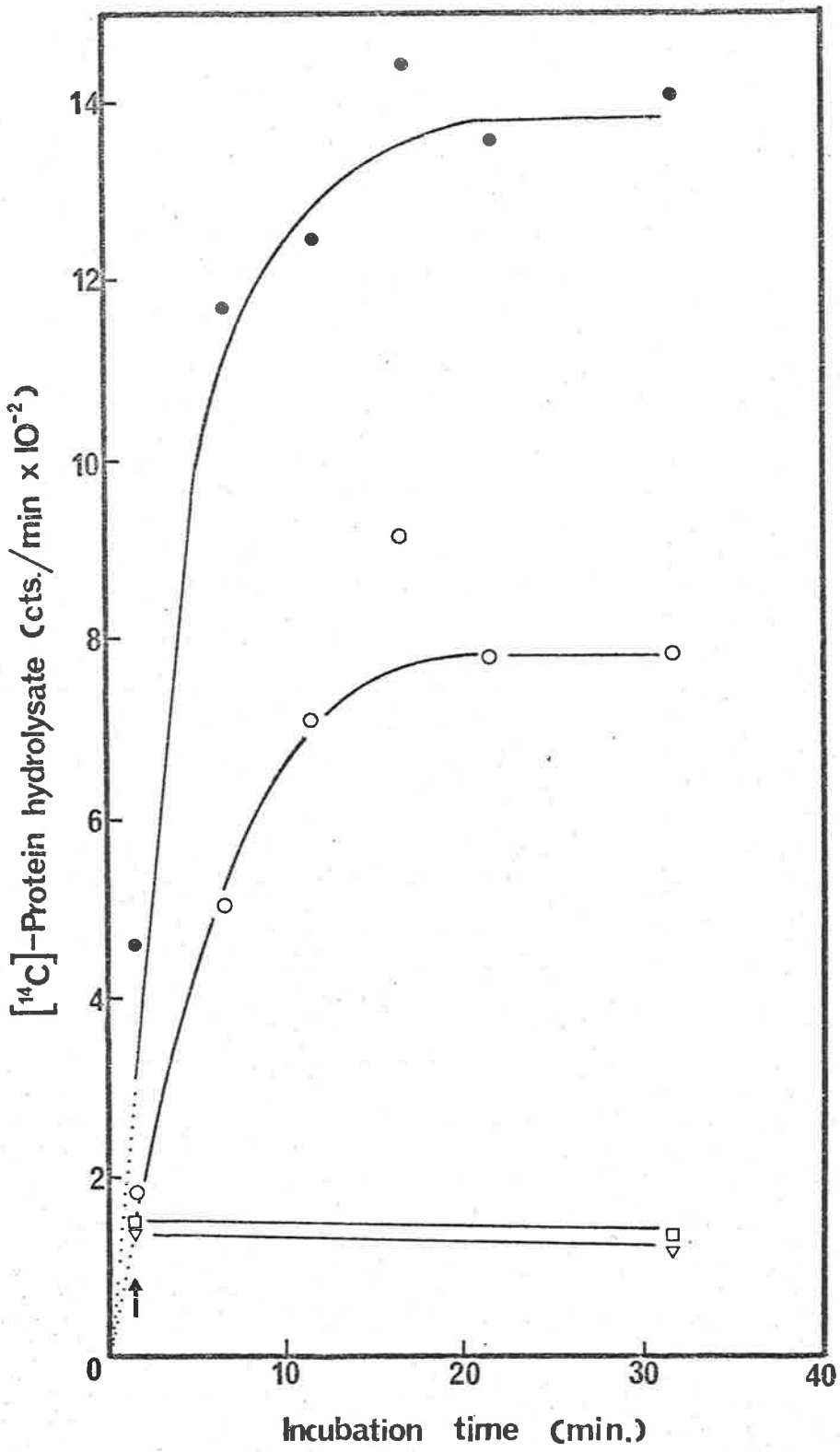
● , protease

○ , α-amylase

□ , level of radioactivity associated with non-specific precipitation when non-immune γG was substituted for immune rabbit γG

▽ , level of radioactivity associated with non-specific precipitation due to the goat anti-rabbit γG antiserum

....., 90 second pulse period (arrow).


[14C]-Protein hydrolysate (cts./min x 10-2)
0
2
4
6
8
10
12
14
10
20
30
40
Incubation time (min.)

If the newly-synthesised enzyme equilibrates with a pool of pre-formed enzyme awaiting release, then the prediction may be made that pulse labelled cells will secrete enzyme of a higher specific activity in the absence of the pool than will normal cells containing the pool (since the labelled enzyme will not be subject to dilution by equilibration with a pool of unlabelled enzyme). Such a finding would also eliminate the unlikely possibility that chloramphenicol causes release of a fraction which is not normally secreted but remains associated with the cell. To test this prediction the following experiment was carried out. A washed-cell suspension was exposed to chloramphenicol (10 µg/ml) for 15 minutes at 30° to release the pool of preformed enzyme into the external medium. A control sample of washed-cell suspension, not exposed to chloramphenicol, was also incubated for 15 minutes at 30°; these cells retained their preformed pool of enzyme. After 15 minutes, a 1.0 ml sample from each of the two washed-cell suspensions were taken and diluted 15 times in suspending medium lacking casamino acids at 30°. A 10.0 ml sample of these diluted cells was then filtered on a 47 mm millipore filter and washed five times with 10.0 ml washed-cell suspending medium lacking casamino acids at 30° (this procedure removes chloramphenicol from cells so that general protein synthesis and extracellular enzyme secretion resume immediately, although at a slightly reduced rate (Glenn et al., 1973)). The cells on the filter were then placed into a flask containing 10.0 ml of suspending medium lacking casamino acids but containing 0.5 µC/ml of ^{14}C-reconstituted protein hydrolysate. The flask was then

shaken at 30° for 90 seconds and 1.0 ml of a 40% (w/v) casamino acids solution added. After the addition of the casamino acids a 5.0 ml sample was taken immediately and again after 5 minutes incubation at 30°. Each sample was centrifuged and the supernatant passed through a millipore filter to remove any remaining bacterial cells. The filtrates were then assayed for protease activity using the Remazo brilliant blue-hide powder assay of Rinderkneckt et al. (1968). The radioactivity in the protease was measured after precipitation by the immunological procedure described earlier. In three separate experiments the specific activity of the protease (counts per minute per unit of activity x 10^2) from cells depleted of their pool of preformed enzyme was approximately 2.5 times greater than that from control cells (Table 1).

There existed the possibility that these results could be due to the internal amino acid pools of the drug treated cells being reduced so that the specific activity of the amino acids during the labelling period was greater than in the control cells not exposed to chloramphenicol. This would lead to an increase in the specific activity of any protein synthesised. To test this possibility the specific activities of the internal amino acid pools of the control and chloramphenicol treated cells after the pulse labelling period were determined. The cells were treated and labelled for 90 seconds as in the previous experiment; the cells were then quickly transferred to a 47 mm millipore filter, rapidly washed four times with suspending medium lacking casamino acids and then the filter with the retained cells plunged into 10.0 ml

TABLE 5.1.

Incubation time	A			B		
	0	5'	Increase	0'	5'	Increase
1. Radioactivity in protease immune precipitate (cpm)*	38	122	84	9	134	126
2. Protease activity (units/ml x 10^2)	0.80	3.21	2.4	0.32	1.72	1.4
3. Specific activity of protease (cpm/unit of protease x 10^2)	-	3.5	-	-	9.0	-
4. Ratio of specific activities of protease	-	[1.0+]	-	-	[2.57+]	-

THE SPECIFIC ACTIVITIES OF PROTEASE SECRETED INTO THE EXTERNAL MEDIUM BY CHLORAMPHENICOL TREATED AND UNTREATED DILUTE WASHED-CELL SUSPENSIONS, AFTER REMOVAL OF THE DRUG FROM THE CELLS.

Washed-cell suspensions were prepared and treated with chloramphenicol as detailed in the text. After the washing procedure the dilute cells were pulsed for 90 seconds with [^{14}C]-protein hydrolysate. The specific activity of protease secreted during the initial 5 min. incubation after the pulse period was determined as detailed in the text.

A. Cells preincubated for 15 min. in the absence of chloramphenicol (10 g/ml)
B. Cells preincubated for 15 min. in the presence of chloramphenicol (10 g/ml).

* Corrected for control value of 183 cpm obtained using non-immune rabbit IgG on the 5 min. labelled samples instead of anti-protease IgG. This value was the average of 189 cpm for the preincubation without chloramphenicol and 178 cpm for that with chloramphenicol.

\+ Ratios of 2.30 and 3.01 were obtained in duplicate experiments, the average of the 3 experiments being 2.63.

distilled water at 100° and kept at this temperature for 10 minutes. The cell debris was removed by centrifuation (25,000g/ 30 min.) and the supernatant added to five volumes of saturated (1%) picric acid and left for 10 minutes at room temperature. After centrifugation (4,500g/15 min.) the supernatant was passed down a 1.0 x 4.0 cm Dowex 2 x 8 (100 - 200 mesh) column to remove the picric acid. The column was washed with five bed volumes of 0.02 N-HCl and the eluate collected and freeze-dried. The samples thus collected were divided into equal portions; one half of the sample was analysed on the Technicon amino acid analyser, the other half was similarly loaded onto the Technicon analyser and 5.5 ml fractions of the column effluent directly collected. Samples (2.0 ml) of each fraction were then put into Bray's scintillation fluid (18.0 ml) and radioactivity determined by liquid scintillation. Due to the low levels of amino acids present and the problems of incomplete separation in some instances, the specific activity (expressed as total counts per minute per μmole of amino acid) of only seven amino acids could be determined (Table 2). However, the results do not support the idea that the chloramphenicol treated cells contain internal amino acid pools whose specific activities were 2.5 times greater than those of the control cells. It is therefore concluded that the pool of enzyme released in the presence of chloramphenicol and newly synthesised enzyme are secreted simultaneously.

TABLE 5.II

Amino Acid	Chloramphenicol treated			Untreated		
	μ mole	total cpm	Specific Activity	µmole	total cpm	Specific Activity
Glutamic	0.092	21816	237	0.122	26329	216
Glycine	0.005	259	52	0.011	336	31
Alanine	0.022	1262	57	0.025	1419	57
Valine	0.007	237	34	0.004	270	68
Iso-leucine	0.003	558	186	0.002	580	290
Leucine	0.002	553	277	0.004	732	183
Ornithine	0.002	261	131	0.005	663	133

THE SPECIFIC ACTIVITY OF INTERNAL AMINO ACIDS PRESENT IN CHLORAMPHENICOL TREATED AND UNTREATED WASHED-CELL SUSPENSIONS AFTER A 90 SECOND PULSE WITH [^{14}C]-PROTEIN HYDROLYSATE.

Washed-cell suspensions were prepared and pulsed for 90 seconds as detailed in the text. The internal amino acids were separated with the aid of a Technicon amino acid analyser and counted by liquid scintillation, as detailed in the text.

6. Time-course of accumulation of the cell associated pool of enzyme

As shown above chloramphenicol treated cells, after removal of the drug, immediately synthesise and secrete extracellular enzymes into the external medium. It was of interest to see whether such cells re-accumulate the cell associated pool of enzyme which was released in the presence of chloramphenicol and at what rate; protease was chosen for this study since the α-amylase and ribonuclease assay procedures are not sensitive enough.

A washed-cell suspension was treated with chloramphenicol (10 μg/ml) for 15 minutes to deplete the cells of enzyme and then the drug washed away as described previously. The dilute washed-cell suspensions were shaken in suspending medium in a number of separate flasks and then after various times of incubation, the amount of protease enzyme pool accumulated in the cells was determined. To do this chloramphenicol was added at different times and the extracellular protease determined in supernatant samples taken immediately after drug addition and again after 20 minutes further incubations at 30°. The difference between these protease levels represented the amount of preformed enzyme pool associated with the cells. It can be seen (Fig. 8) that the chloramphenicol treated cells began to accumulate a protease pool almost immediately after the removal of the drug and which had increased to a maximum in about 20 minutes.

FIGURE 5.8. THE TIME COURSE OF PRE-FORMED PROTEASE ACCUMULATION BY DILUTE WASHED-CELL SUSPENSIONS.

The experimental conditions were as given in the text.

— ● — ●— preformed protease pool (units/ml) present in a dilute suspension of chloramphenicol treated washed-cells after removal of the drug.

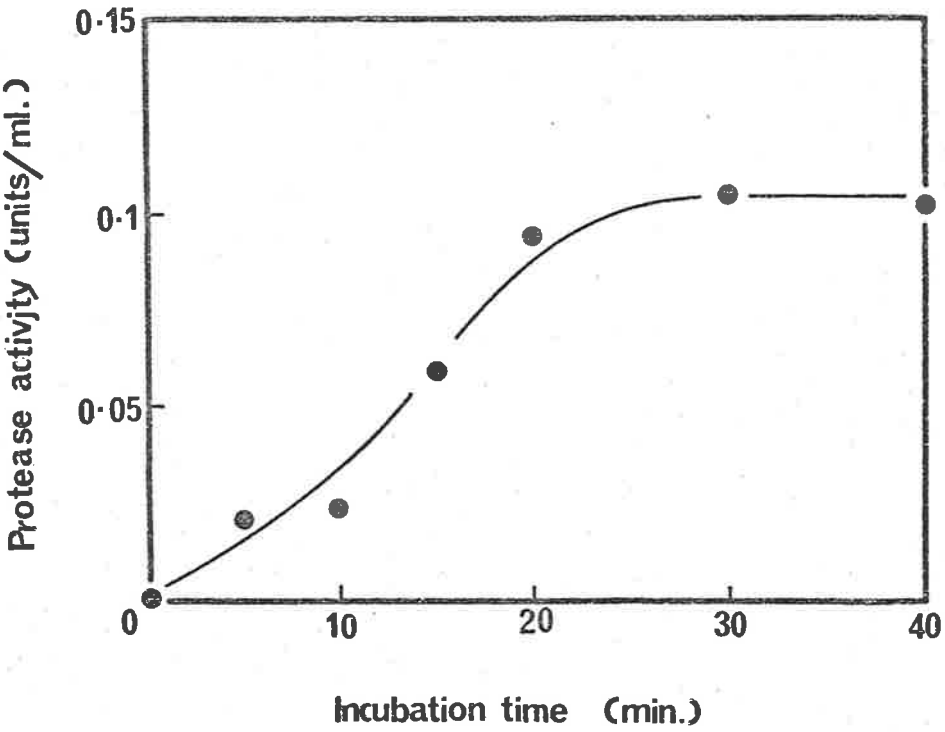

0·15
0·1
0·05
0
10
20
30
40
Protease activity (units/ml.)
Incubation time (min.)

FIGURE 5.9. EFFECT OF CHLORAMPHENICOL ON RIBONUCLEASE PRODUCTION BY NORMAL AND ACTINOMYCIN D STIMULATED WASHED-CELL SUSPENSIONS.

■ , no addition of drug

● , 0.667 µg actinomycin D/ml added at time zero

▽ , 10 µg chloramphenicol/ml added after 50 minutes incubation (arrow) to a normal washed-cell suspension

▲ , 10 µg chloramphenicol/ml added after 50 minutes incubation (arrow) to an actinomycin D stimulated washed-cell suspension (actinomycin D (0.667 µg/ml) added at time zero).

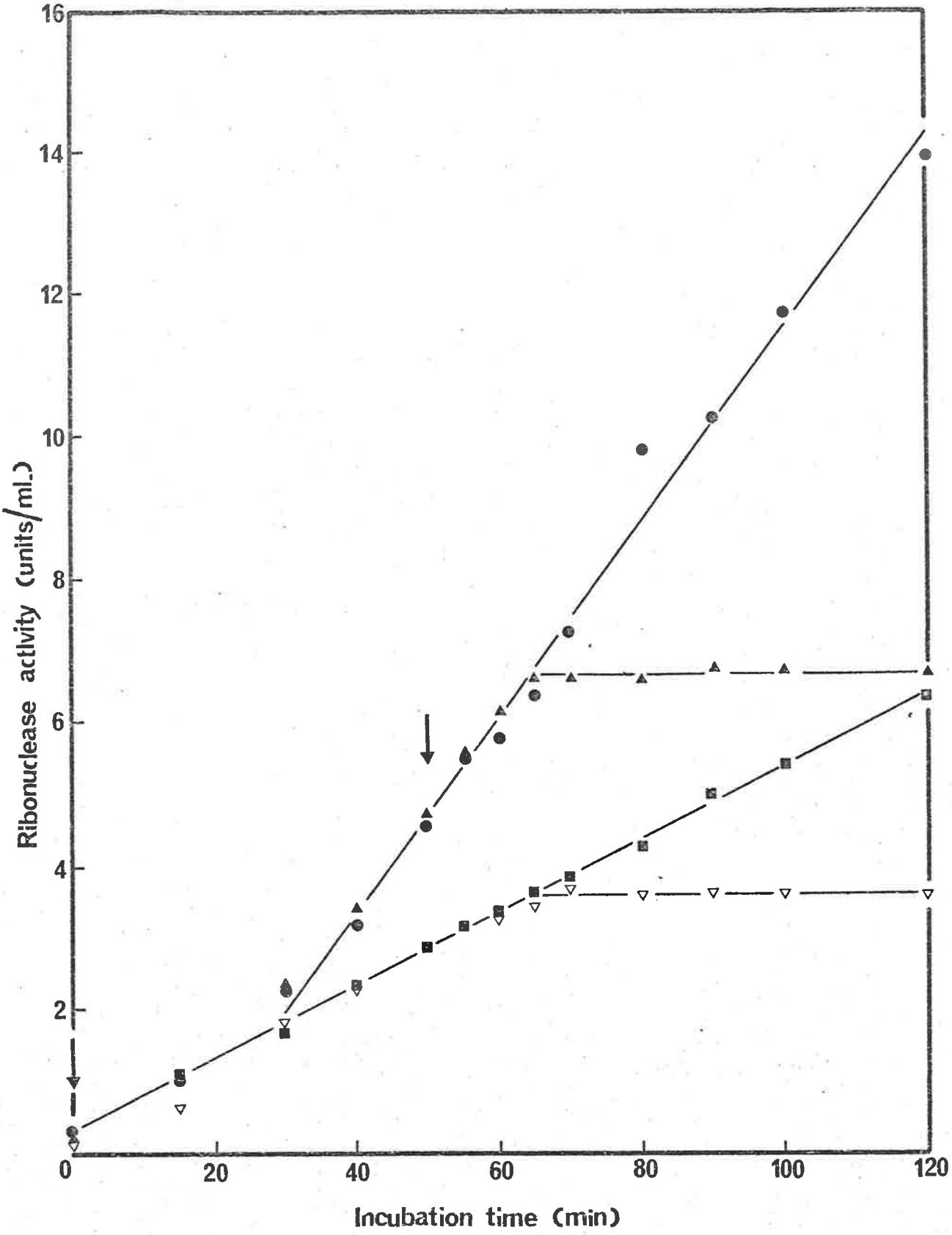

Ribonuclease activity (units/ml.)
16
14
12
10
8
6
4
2
0
20
40
60
80
100
120
Incubation time (min)

FIGURE 5.10. EFFECT OF RIFAMPICIN AND CHLORAMPHENICOL ON RIBONUCLEASE PRODUCTION BY NORMAL AND ACTINOMCYIN D STIMULATED WASHED-CELL SUSPENSIONS.

■, no addition of drug

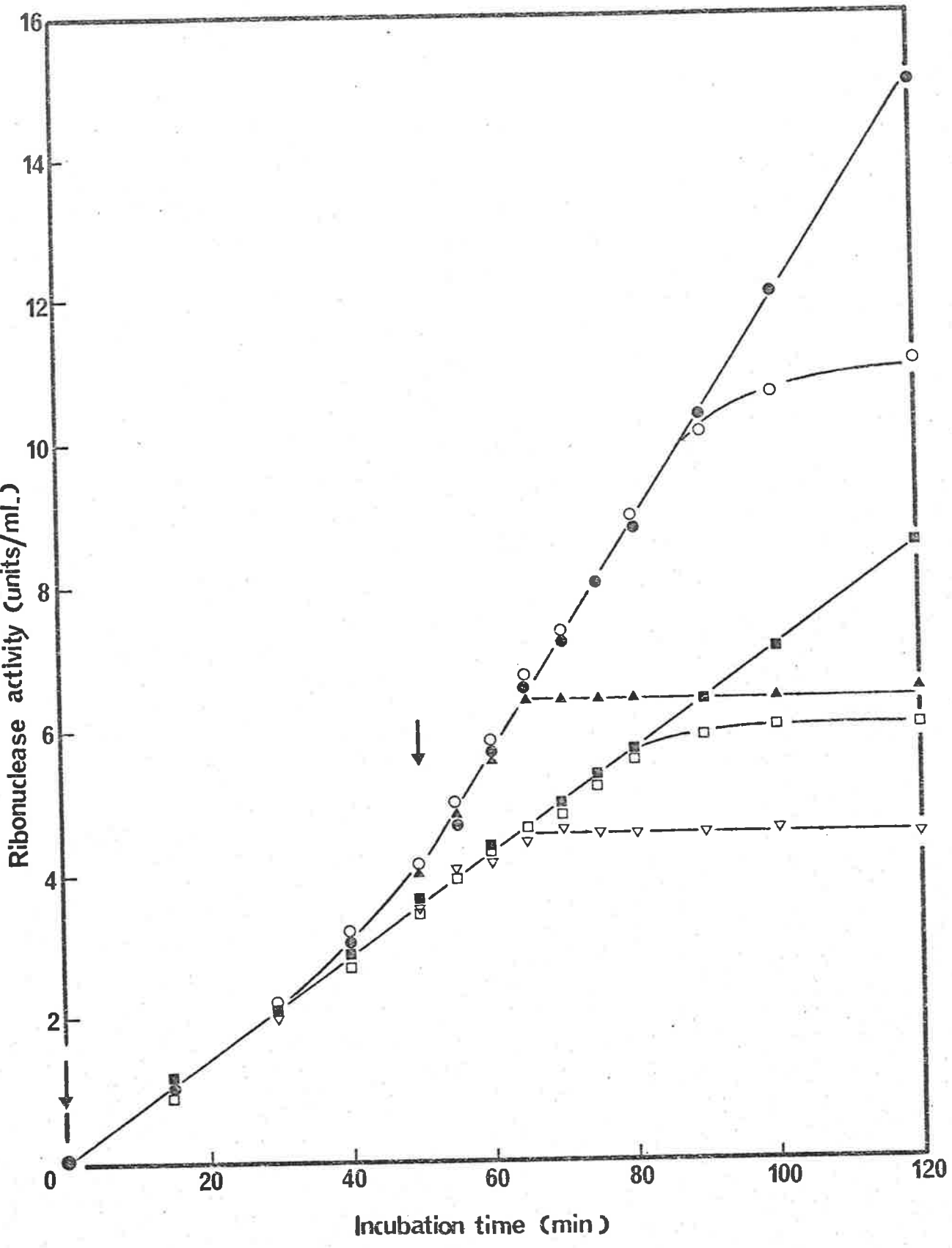
●, 0.667 µg actinomycin D/ml added at zero time

▽, 10 µg chloramphenicol/ml added at 50 minutes incubation (arrow) to normal washed-cells

□, 0.5 µg rifampicin/ml added at 50 minutes incubation (arrow) to normal washed-cells

▲, 10 µg chloramphenicol/ml added at 50 minutes incubation (arrow) to an actinomycin D (0.667 µg/ml; added at time zero) stimulated washed-cell suspension

○, 0.5 µg rifampicin/ml added at 50 minutes incubation (arrow) to an actinomycin D (0.667 µg/ml; added at time zero) stimulated washed-cell suspension.


16
14
12
10
8
6
4
2
0
20
40
60
80
100
120
Ribonuclease activity (units/ml.)
Incubation time (min)

7. The size of the preformed ribonuclease pool in actinomycin D stimulated washed-cell suspensions

It has been shown that ribonuclease formation by washed-cell suspensions can be stimulated by critically low concentrations of actinomycin D (Coleman and Elliott, 1965; May et al., 1968). When chloramphenicol (10 µg/ml) was added to either an actinomycin D (0.67 µg/ml added at time zero) stimulated washed-cell suspension, or an unstimulated control, a pool of preformed ribonuclease was present which continued to accumulate in the external medium for 15 minutes. However, in the actinomycin D stimulated suspension, the pool size was 2.5 - 3 times greater when compared to that present in control cells (Fig. 9). When rifampicin (0.5 µg/ml) was added to either an actinomycin D stimulated washed-cell suspension or an unstimulated control suspension, it was found that not only was the preformed enzyme pool increased in the stimulated suspensions but also the level of the apparent mRNA pool specific for the ribonuclease (Fig. 10 and Chapter 4). Actinomycin D stimulation has been shown to be specific for the ribonuclease as α-amylase and protease in the same experiments decreased by approximately 40% over a three-hour time period.

C. DISCUSSION

It has been shown that B. amyloliquefaciens cells contain a preformed pool of α-amylase, protease and ribonuclease enzymes. This has been shown not to be a trivial phenomenon of non-specific adsorption of enzymes to the cell surface. The pool is released in approximately 15 minutes (in the presence

of chloramphenicol) and this energy independent release provides a means of studying the final process of extracellular enzyme secretion. That the pool represents enzyme molecules *en route* to the exterior is indicated by several observations; it is constantly present in normally secreting cells, it is exhausted when cells are allowed to release it in the absence of further protein synthesis and it is restored again when such depleted cells are permitted to resume protein synthesis.

The pulse labelling experiments provide some information about the nature of the pool and its position in the secretory process. Since newly synthesised enzyme molecules begin to emerge immediately from the cell (as shown in the labelling studies) there is no obligatory delay of 15 minutes before a completed molecule is secreted, that is, the pool is not a 'pipeline' from which enzyme molecules emerge in the order in which they were synthesised. The fact that the enzyme molecules labelled during a 90 second pulse period continue to emerge (in the presence of chloramphenicol) at a constant rate for 15 minutes suggest that newly synthesised enzyme molecules immediately equilibrate with the entire enzyme pool. Compatible with this is the finding that pulse labelled enzyme emerges with a higher specific activity from enzyme depleted cells, than from control cells containing the pool of preformed enzyme.

It is impossible at this stage to decide on the location of the pool of enzyme molecules associated with the cell. It has previously been proposed that enzyme synthesis occurs on ribosomes at the membrane such that polypeptides are secreted into or through the membrane (Both *et al.*, 1972; Glenn *et al.*,

1973) and it is therefore tempting to speculate that the pool of enzyme molecules is located in the region of the membrane. Indeed, in the case of the extracellular ribonuclease there is indirect evidence which suggests that it does not exist as such in the cytoplasm of the cell. As referred to earlier, Smeaton and Elliott (1967), have shown that there is a protein in the cell cytoplasm which specifically binds to and inhibits the enzyme such that only drastic conditions in denaturing solvents will recover active enzyme when the inhibitor and ribonuclease are mixed together (Hartley, 1970). It is therefore unlikely that the active enzyme could have existed in the cytoplasm (though not excluded since one cannot eliminate the possibility that the membrane pulls apart such a complex). No such cytoplasmic inhibitors for the extracellular protease or α-amylase have been detected.

The effects of temperature on the release of the cell associated pool of enzyme cannot be interpreted in terms of a simple effect on an enzymic process or simple diffusion. In either case temperature would be expected to affect the rate of release of the enzyme pool but not the final amount secreted, as is in fact observed. No secretion occurs at 0° while complete pool release occurs at 30°. Surprisingly, at 20° enzyme secretion ceases after approximately 50% is released and approximately 20% at 10°, and so on (Fig. 4). The temperature effects might be related to temperature changes induced in the structure or fluidity of the membrane (Frye and Edidin, 1970; Taylor et al., 1971; Singer and Nicolson, 1972; Sundquvist, 1972) such that it exists in a form incapable of enzyme release at 0° but

permitting complete release at 30°. However, it is further necessary to postulate that at 20° half of the enzyme is unavailable for release and proportionately so for the other intermediary temperatures.

It is not understood why it should take 15 minutes for the total pool to be released in the presence of chloramphenicol. Perhaps a lipid (or protein) carrier, needed for transport through the membrane may be present in limiting amounts or even a physical restriction by the cell wall to the rate of enzyme release.

The level of the preformed enzyme pool seems to be variable under certain conditions. In the case of actinomycin D stimulated washed-cell suspensions the pool of preformed ribonuclease was found to increase although it still required 15 minutes before it was fully externalised. Similarly, the apparent mRNA pool specific for the ribonuclease was increased to the same degree. It has been proposed that the method of actinomycin D stimulation operates by the inhibition of a phosphate requiring repressor molecule for the ribonuclease gene (May et al., 1968). In critically low doses, actinomycin selectively inhibits the production of the apo-repressor (which has a short life time of approximately 30 minutes) and thus the ribonuclease gene is maximally de-repressed. It has been shown that the mRNA for protease has a rapid turn-over rate (Glenn et al., 1973) and it was proposed that the excessive production of mRNA may be a mechanism to ensure that sufficient intact mRNA molecules reach membrane localised translational sites to support extracellular enzymes synthesis. In the case

of actinomycin stimulated cells it is not necessary to postulate that new translation-extrusion sites are created to handle the increased amount of ribonuclease mRNA; it may be in fact that the messengers compete for a limited number of existing sites. If one messenger is specifically increased then this will increase its percentage probability of being translated. In fact, actinomycin stimulation of ribonuclease results in a decrease in the levels of α-amylase and protease accumulation (May et al., 1968). This pressure of mRNA competition may be reflected in the level of the preformed enzyme pools. The more mRNA present for a particular extracellular enzyme then the more translational-extrusion sites occupied by that messenger and thus the pool size of preformed enzyme might be increased. This situation is reflected with the protease and α-amylase biphasic accumulation in the supernatant of washed-cell suspensions. In phase I of the accumulation of these enzymes, when mRNA pools specific for the extracellular enzymes exist, the preformed enzyme pools are greater than those found in the phase II of synthesis when no such mRNA pools exist (Both et al., 1972; Chapter 4, Figures 6 and 7a). Therefore, the variability in the size of the preformed enzyme pools may reflect the level of translation of that specific mRNA which is available for the translation-extrusion sites.

Pollock (1961) and Lampen (1967) have previously reported a temperature dependent, energy independent release of penicillinase from chloramphenicol treated B. subtilis and B. licheniformis cells, respectively. Sargent and Lampen

(1970b) have established that there are three forms of the penicillinase and described the cellular location of each of these different forms. The cellular location of the preformed enzyme pools is the subject of the next Chapter and was initiated in the hopes of obtaining a more detailed knowledge of the actual secretion process.

CHAPTER 6

ATTEMPTS TO LOCALISE THE CELL ASSOCIATED ENZYME FRACTION IN WASHED-CELL SUSPENSIONS OF BACILLUS AMYLOLIQUEFACIENS.

ATTEMPTS TO LOCALISE THE CELL ASSOCIATED ENZYME FRACTION

A. INTRODUCTION

As mentioned in the previous Chapter the cellular location of the preformed pool of extracellular enzymes released in the presence of chloramphenicol was a matter of speculation.

It has been shown by Sargent, Ghosh and Lampen (1968) that on the formation of protoplasts from Bacillus licheniformis, penicillinase was found attached not only to the plasma membrane but also associated with a periplasmic vesicle fraction released upon protoplast formation. Kushner and Pollock (1961) and Lampen (1967) indicated that penicillinase may be covalently bound to the membrane and that enzyme secretion involves cleavage of this bond. Similarly, it has been reported that the alkaline phosphatase from Bacillus subtilis A was located in the cytoplasmic membrane (Wood and Tristram, 1970). The enzyme was not liberated by the formation of protoplasts nor by osmotically shocking whole cells. A different situation occurs for the same enzyme in E. coli. Here the enzyme is liberated into the surrounding medium on the formation of spheroplasts (Malamy and Horecker, 1961; Neale and Tristram, 1963) and by osmotic shock (Neu and Hepple, 1965). It is suggested that in E. coli the alkaline phosphatase is a periplasmic enzyme and has been confirmed by Done et al. (1965) with the aid of cytochemical techniques.

In this Chapter work is described which attempted to find the position and nature of the association between the cell and the enzyme pool which is released in the presence of chloramphenicol. For convenience this fraction will be

referred to as the 'enzyme pool' without further qualification.

B. RESULTS

1. Attempts to see whether the enzyme pool is associated with any cellular component

It has been shown that washed-cell suspensions may undergo what is termed, a 'cold shock'. This phenomenon occurs when cells at 30° are squirted into a stirred medium at 0° and are thus subjected to a very rapid (essentially instantaneous) temperature transition from 30° - 0° (Smeaton and Elliott, 1968). Cells cooled over a period of a minute or so are unaffected. When washed-cells are subjected to a cold shock the normal permeability characteristics of the membrane are lost. Leakage of nucleotides and small molecular weight proteins occurs and stable protoplasts cannot be prepared from such cells (May and Elliott, personal communication). The effect of this procedure on the enzyme pool was studied.

Cold shocked and unshocked cells were incubated at 0° for 120 minutes, either in the presence or absence of chloramphenicol (10 µg/ml) and enzyme release measured. The cells were then warmed to 30° and the release of enzyme into the supernatant again followed. It was observed that the enzyme pool was not released from cold shocked cells at either temperature, whereas control cells did release this fraction, in the presence of chloramphenicol, at 30° (Fig. 1). α-Amylase showed similar characteristics; ribonuclease was not assayed due to the release of a cytoplasmic inhibitor specific for the extracellular ribonuclease when cells are cold shocked (Smeaton

FIGURE 6.1. EFFECT OF COLD-SHOCK ON THE RELEASE OF THE PREFORMED PROTEASE POOL BY WASHED-CELL SUSPENSIONS.

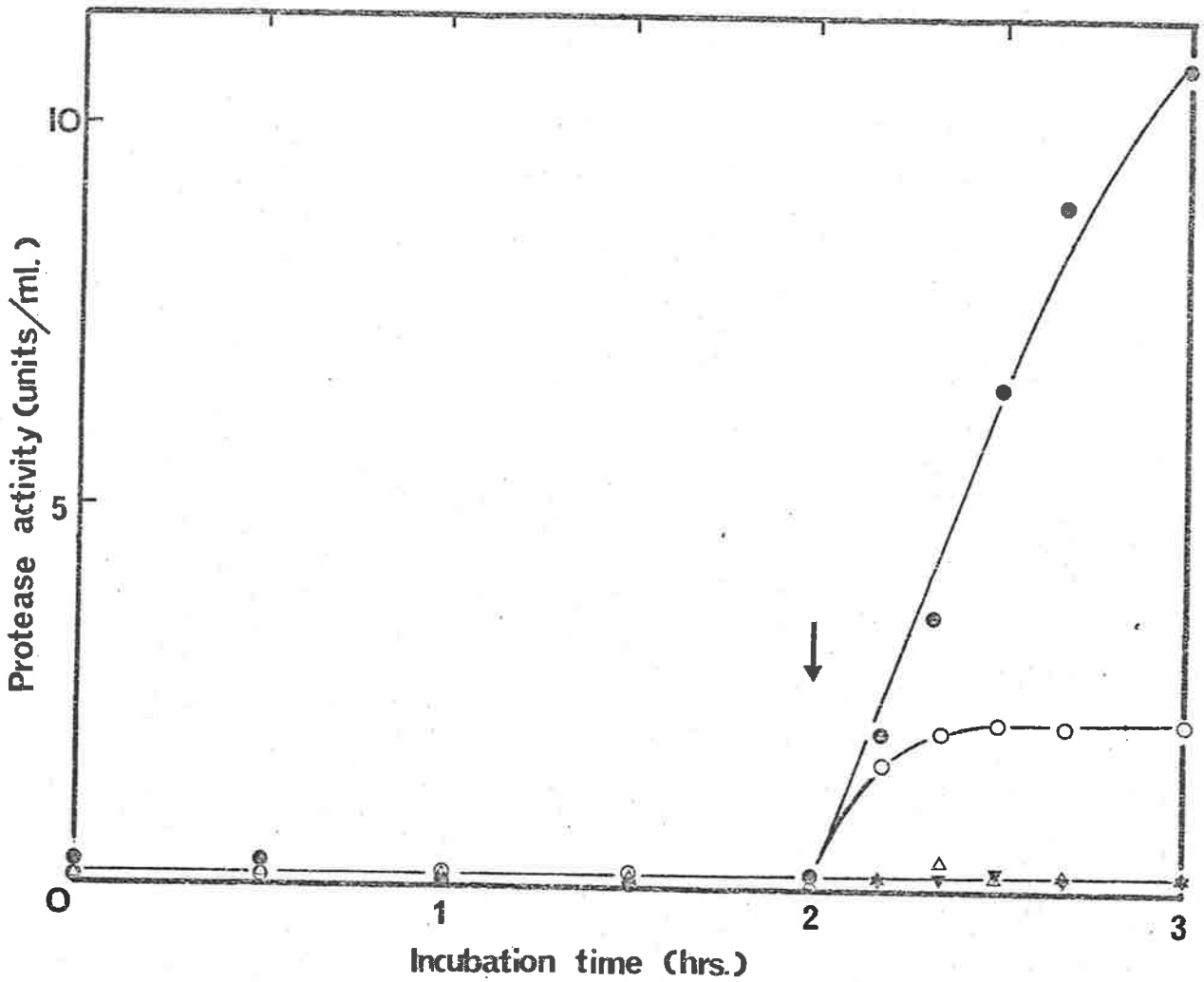
● , no addition of drug

o , 10 µg chloramphenicol/ml added at time zero

△ , no addition of drug (cold shocked) washed-cells

▼ , 10 µg/ml chloramphenicol added to cold shocked washed-cells at time zero.

All washed-cell suspensions were held at 0° for 120 minutes, whereupon they were transferred to a 30° shaking water bath (arrow) and the incubation continued.


Protease activity (units/ml.)
10
5
0
1
2
3
Incubation time (hrs.)

and Elliott, 1968). Attempts were now made to disrupt cells and see whether the enzyme pool was associated with any fraction.

When cold shocked or normal washed-cells, at 0° and in the presence or absence of chloramphenicol, were disrupted by passage through the French pressure cell (operated at 9 ton per square inch and 0°), the enzyme pools were found to be present in the supernatant after centrifugation of the cell debris (150,000g/120 min./0°).

Gentler methods of cell disruption were then attempted. Since the enzyme pool was not released by cold shocked cells at 30°, enzymic digestion of the cell wall in the presence of lysozyme and sucrose was attempted. Cold shocked washed-cell suspensions were resuspended (in the presence and absence of chloramphenicol (10 µg/ml)) in TMK-sucrose buffer (50 mM tris-HCl buffer, pH 7.6; 10mM magnesium acetate; 85 mM KCl; 22% (w/v) sucrose) containing lysozyme (150 µg/ml) and incubated at 30° for 40 minutes. Such treatment of cold shocked cells does not result in the formation of protoplasts (due to induced membrane instability) but in small membranous vesicles (May, personal communication). The incubation mixture was then centrifuged (150,000g/120 min./0°) to pellet membranous vesicles and ribosomes and then the pellet and the supernatant assayed for protease and α-amylase. All of the enzyme present was found to remain free in the supernatant after centrifugation.

Similarly, normal washed-cell suspensions were incubated with TMK-sucrose buffer and lysozyme (150 µg/ml) at

30° for 40 minutes in the presence of chloramphenicol (10 µg/ml). At the end of this period protoplast formation was complete. The protoplasts were removed by centrifugation and the supernatant assayed for enzyme. Again, it was found that the enzyme pool was liberated into the supernatant and no enzymic activity could be found associated with either whole intact protoplasts or osmotically lysed protoplasts (membrane and cytoplasm). However, during protoplast formation, the bacterial mesosomes (which have the appearance of vesicles or layers of membrane surrounded by an invagination of the cytoplasmic membrane (Plate 1)), extrude to take a string of pearl-like form of extended tubules. These structures are seen attached to protoplasts and free in the supernatant when protoplasts are prepared in the manner described above and are shown in Plate 2. After pelleting the vesicles by centrifugation of the protoplast supernatant (150,000g/120 min./0°) again the enzyme pool remained free in the supernatant.

Thus, by all procedures attempted, no particulate association of the enzyme pool could be established. However, the action of lysozyme on washed-cell suspensions, in TMK-sucrose buffer and chloramphenicol (10 µg/ml), results in an increased rate of appearance of the enzyme pool in the external medium when compared to that occurring in the presence of chloramphenicol (10 µg/ml) alone (Fig. 2). Essentially all of the enzyme pool was released during the initial 5 minutes of incubation in the presence of lysozyme (150 µg/ml), whereas 15 minutes is needed to externalise it in the presence of chloramphenicol alone. A possible explanation for this

PLATE 6.1. SECTION OF INTACT B. AMYLOLIQUEFACIENS CELLS SHOWING THE PRESENCE OF THE MESOSOMAL STRUCTURE (M).

Sections were prepared as described in Chapter 2. Spherical membranous beads are contained within a bag which represents an internal invagination of the cytoplasm membrane.

Magnification: electron microscope, 30,000x
optical, 3.3x.

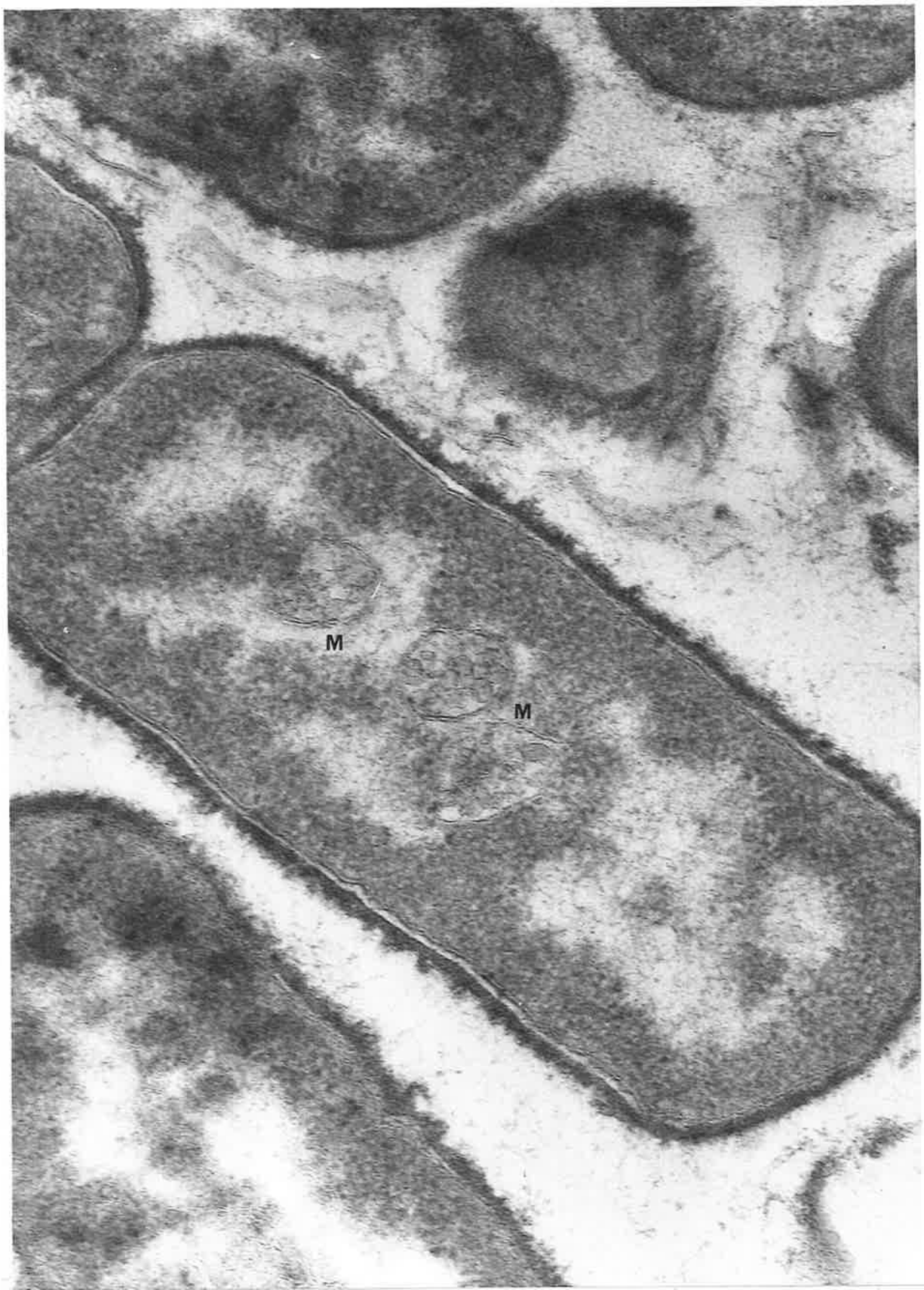

M
M

PLATE 6.2. NEGATIVELY STAINED PROTOPLAST (P) TO WHICH IS ATTACHED A LONG APPENDAGE CONSISTING OF A STRING OF VESICLES (V).

The preparation was negatively stained with uranyl acetate as described in Chapter 2.

Magnification: electron microscope, 40,000x
optical, 3.3x.

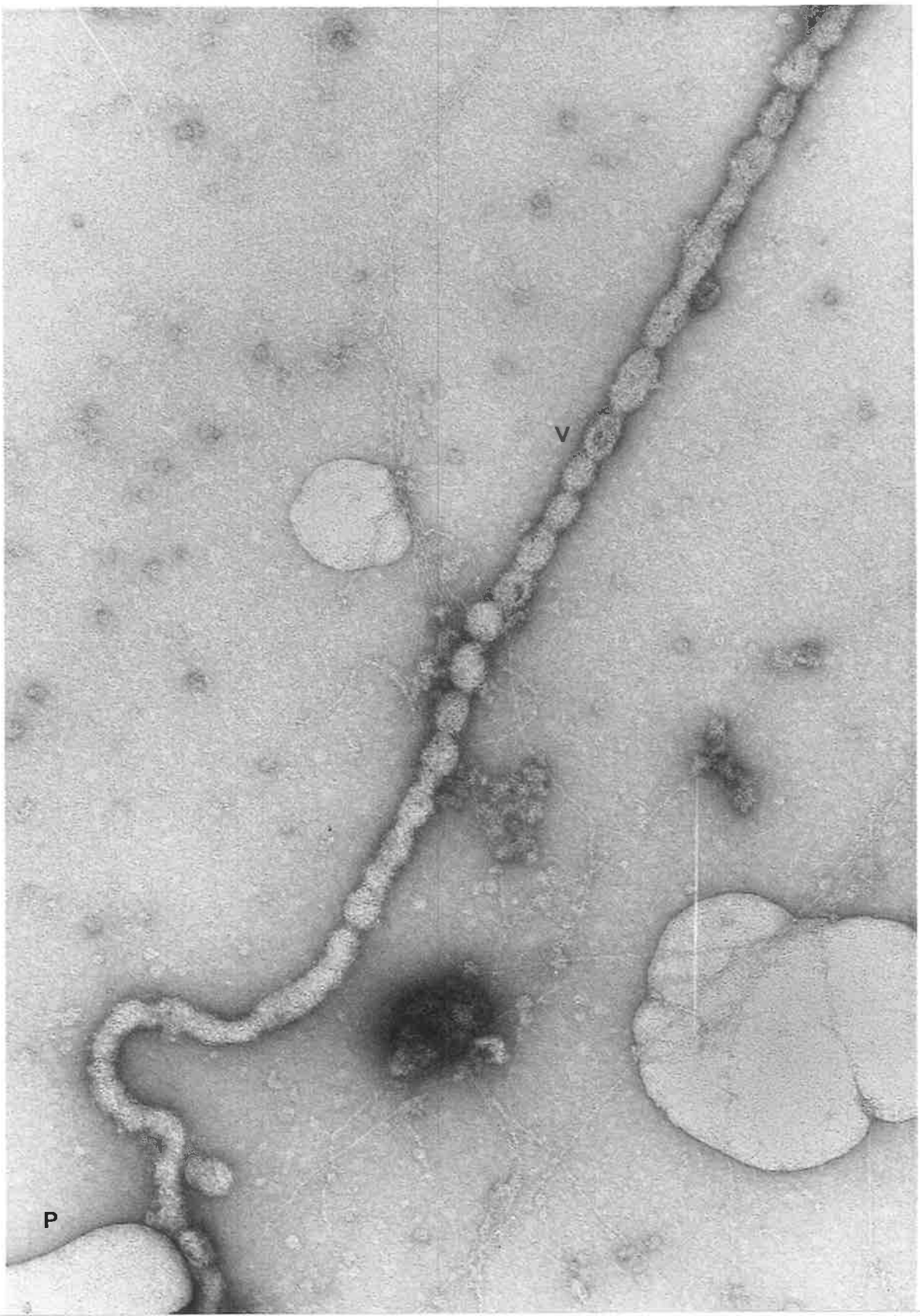
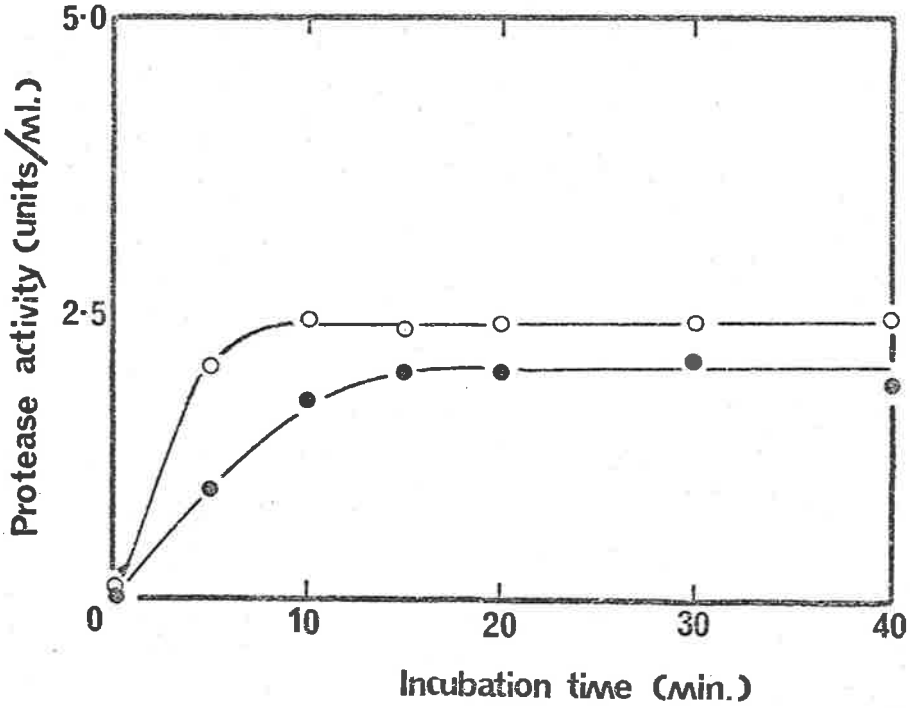

V
P

FIGURE 6.2. EFFECT OF LYSOZYME AND CHLORAMPHENICOL ON PROTEASE FORMATION BY WASHED-CELLS IN TMK-SUCROSE BUFFER

● , chloramphenicol (10 µg/ml)

o , lysozyme (150 µg/ml) and chloramphenicol (10 µg/ml)

Lysozyme and chloramphenicol were added at time zero, protease activity was determined by the remazobrilliant blue/Hide assay.


5·0
2·5
0
10
20
30
40
Protease activity (units/ml.)
Incubation time (min.)

increased rate of release may lie in electron microscopic observations made by J. McInnes (in this laboratory). It was found that on close examination of sectioned cells, during the initial 5 minutes of incubation in the presence of lysozyme, a band of dense material lying near the cytoplasmic membrane appeared to have been removed from the cell wall; leaving the outer side of the membrane with some cell wall material still attached (Plate 3). The possibility that the cell membrane had pulled away from the cell wall during this treatment was unlikely as measurements from the plasma membrane to the exterior surface of the cell wall were the same in both cases and in fact the dimensions of the cell wall were actually reduced after lysozyme treatment.

2. Attempts to fix the enzyme pool in situ with formaldehyde

It seemed possible that if cells were fixed with formaldehyde to cross link proteins (Steck, 1972), the enzyme pool might become attached covalently to neighbouring proteins. Provided the enzyme still retained activity, it might then be possible to fractionate the cells and determine the location of the enzyme. Preliminary experiments showed that protease and amylase retained some activity (about 20%) under the fixing conditions and attempts were made to carry out the above experiment. Preliminary results appeared promising in that formaldehyde fixed cells subjected to the French pressure cell at 0° retained the enzyme pool at 0° and released enzyme at 30°. However, electron microscopy of the preparation showed it to be a complex mixture of cells and particles which made fractionation

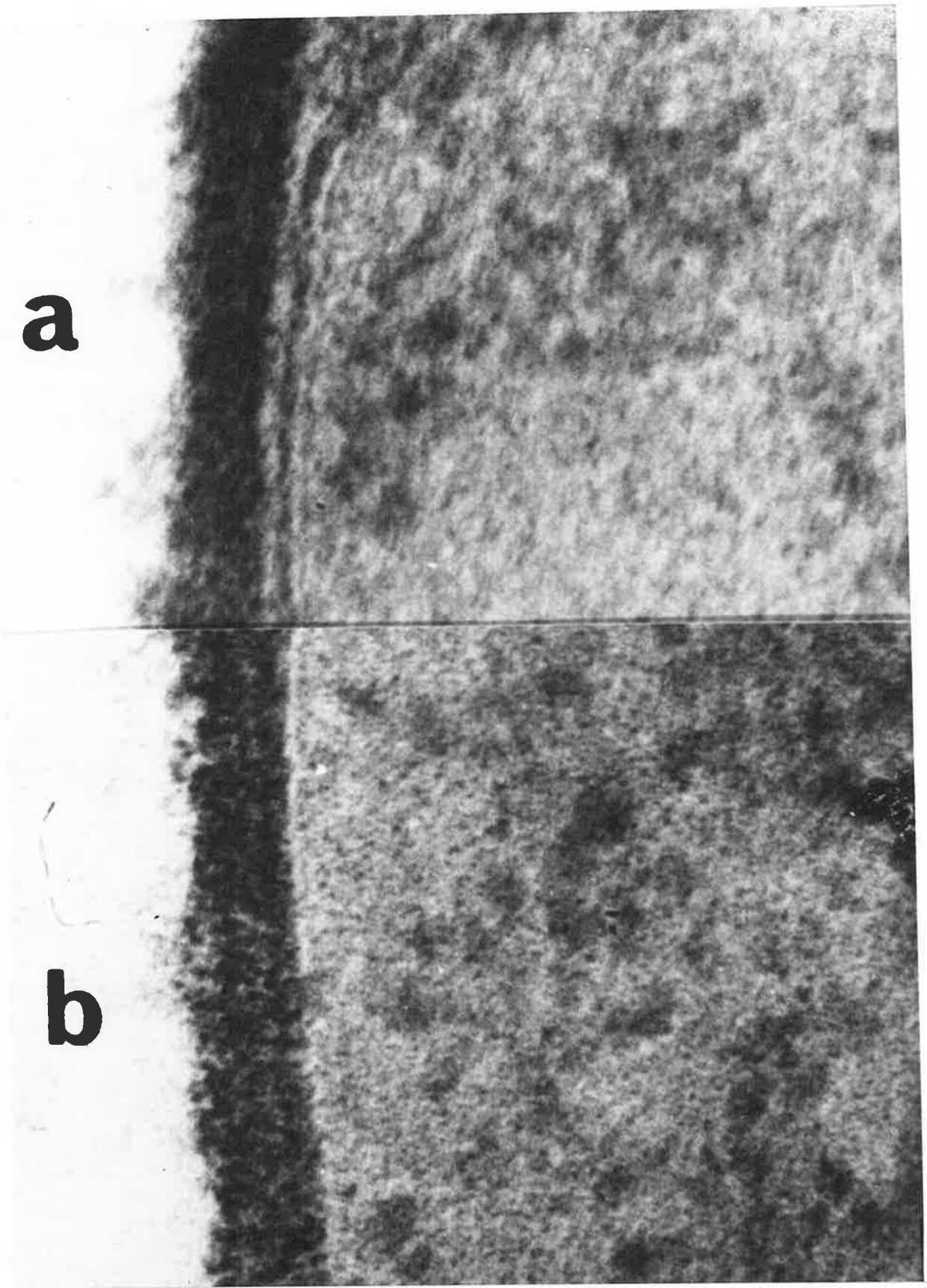
PLATE 6.3. COMPOSITE ELECTRON MICROGRAPH OF SECTIONED SPECIMENS OF B. AMYLOLIQUEFACIENS

(a) FIVE MINUTE LYSOZYME-TREATED CELLS, and
(b) CONTROL CELLS

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

Magnification, 408,000x.

This photograph was reproduced with the kind permission of J.L. McInnes (of this laboratory).


a
b

studies almost impossible. The approach was therefore abandoned.

3. The effect of osmotic shock on washed-cell suspensions at 0°

Washed-cell suspensions were cooled slowly to 0° as described previously and resuspended to the original cell density in 25 mM phosphate-22% (w/v) sucrose buffer (pH 7.5) at 0°, in the presence of chloramphenicol (10 µg/ml). The cells were then shaken at 0° for 15 minutes. After this period the cells were then resuspended in 25 mM phosphate buffer (pH 7.5) without sucrose at 0°. This procedure leads to osmotic shock (Heppel, 1965). After 15 minutes incubation at 0° the cells were then pelleted by centrifugation and the supernatants assayed. It was found that approximately 3% of the enzyme pools of both α-amylase and protease appeared in the 'shock fluid'. Thus, it appeared that the enzyme pools are not released by osmotic shock at 0°.

4. Attempted localisation of the enzyme pool by the use of electron microscopy and ferritin conjugated antibodies. Attempts to see whether inactive enzyme precursors exist within the cell.

In an attempt to directly localise the enzyme pool in the cell, the method of McLean and Singer (1970) was utilised. Intracellular antigens may be visualised with the aid of electron dense conjugates of ferritin and antibody (directed against the intracellular antigen) which are used to stain thin sections of

cells which have previously been embedded in albumin and cross-linked with formaldehyde. By the use of this technique it was thought to be possible to show not only the location of the preformed enzyme pool but also and equally important to test for the existence of cytoplasmic inactive zymogens of extracellular enzymes.

Ferritin was conjugated to purified anti-protease IgG by the method of Schick and Singer (1961) except that the toluene diisocyanate (TDC) was sonicated briefly to give a finely dispersed emulsion before the temperature was dropped to 0° for the reaction with ferritin. The resulting conjugate was purified from unreacted antibody by centrifugation (150,000g/180 min./4°) and washed three times with phosphate buffered saline (PBS) at 4°. The pellet (which contains ferritin conjugated antibody and unreacted ferritin) was further purified by DEAE chromatography to separate the unwanted ferritin (Fig. 3). It was observed that on immunodiffusion analysis of the purified conjugate that the anti-protease IgG was indeed linked to ferritin, but it seemed that the antibody combining activity had been severely impaired after conjugation. The majority of the IgG linked to the ferritin seemed to have been denatured (Fig. 4a-b) and little combining activity remained. Goat antiserum raised against purified rabbit IgG readily demonstrated the presence of rabbit IgG linked to the ferritin. Many attempts to modify conditions such that the denaturation of the specific antibody did not occur during the conjugation to ferritin were of little avail. Thus, when staining the embedded, thin sectioned cells with this conjugate, large

FIGURE 6.3. ELUTION OF γ^G, FERRITIN AND γ^G-FERRITIN CONJUGATE FROM DEAE-CELLULOSE.

After ultracentrifugation the reaction mixture from the toluene 2,4-diisocyanate conjugation procedure was applied to a DEAE-cellulose column. The $\gamma\alpha$ was eluted from the column with 0.0175 M sodium phosphate buffer (adjusted to pH 7.2 with KOH). The column was then eluted with the above buffer and a continuous sodium chloride gradiant from 0 to 3 M.

Material eluted from the column in essentially 3 peaks. Immunodiffusion studies indicated peak I contained a slight trace of γG and no ferritin, peak II contained ferritin and no γG whereas peak III contained both ferritin and γG and was presumed to be the desired conjugate.

o—o , A_{280nm}

....., Molarity (NaCl).

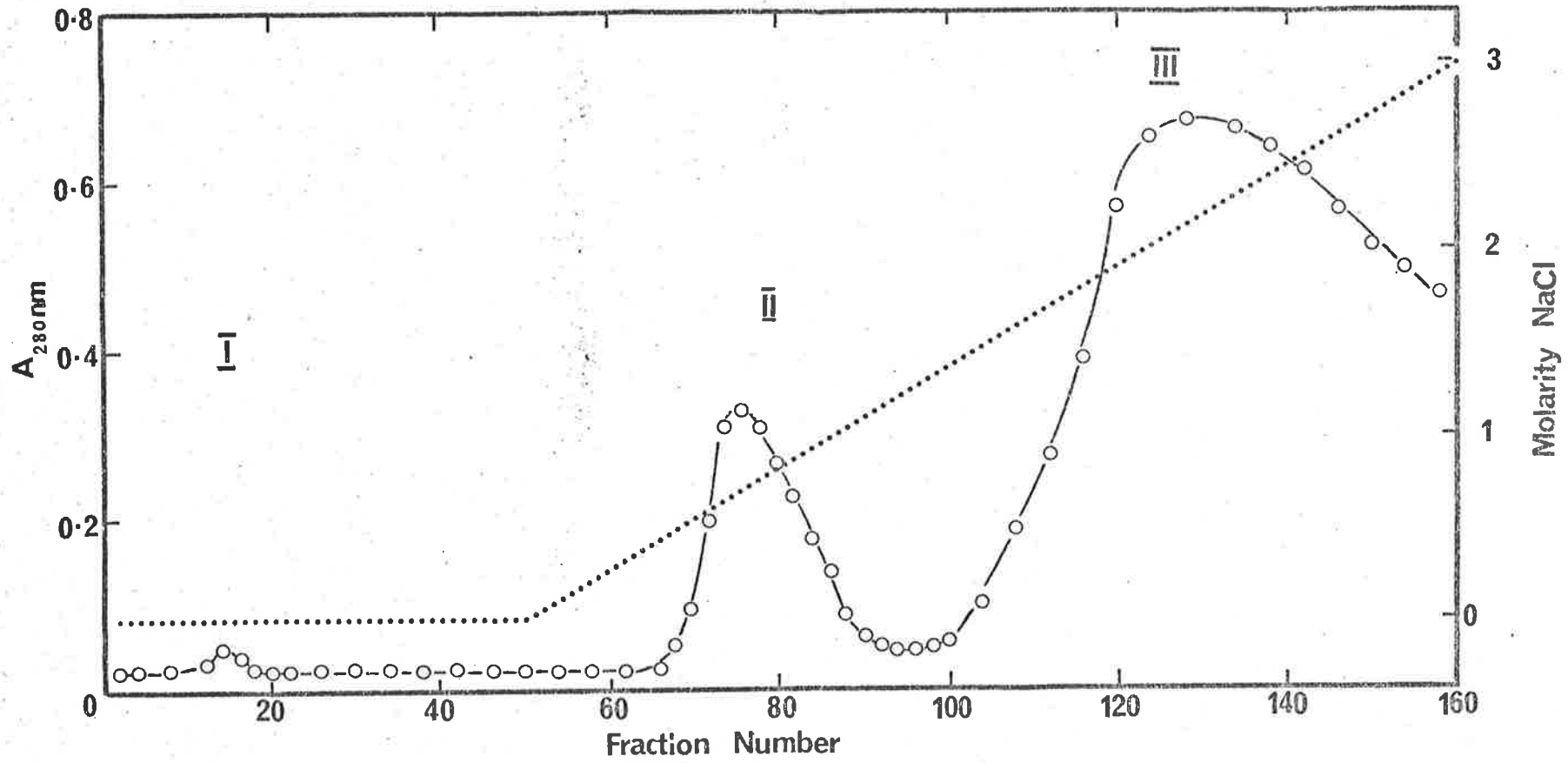

0·8
0·6
0·4
0·2
0
A_{280nm}
I
II
III
20
40
60
80
100
120
140
160
Fraction Number
3
2
1
0
Molarity NaCl

FIGURE 6.4a. IMMUNODIFFUSION ANALYSIS OF FERRITIN AND RABBIT ANTI-PROTEASE ANTISERUM PRIOR TO CHEMICAL CONJUGATION WITH TOLUENE DIISOCYANATE

(a) Goat anti-rabbit γG antiserum
(b) Ferritin + rabbit anti-protease antiserum
(c) purified protease (neutral species).

(The wells for the diffusion studies decrease serially in volume, such that each preceeding well contains twice the volume of the next. This enables a quick system for evaluating the relative concentrations of antibody or antigens against known standard antigens or antibodies (respectively).

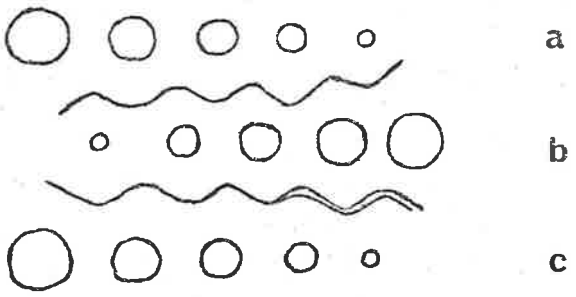
FIGURE 6.4b. IMMUNODIFFUSION ANALYSIS OF THE PURIFIED FERRITIN-ANTI-PROTEASE CONJUGATE COUPLED BY THE TOLUENE DIISOCYANATE PROCEDURE OF SCHICK AND SINGER (1961).

(a) Goat anti-rabbit γG antiserum
(b) Ferritin-anti-protease conjugate
(c) Purified protease (neutral species).

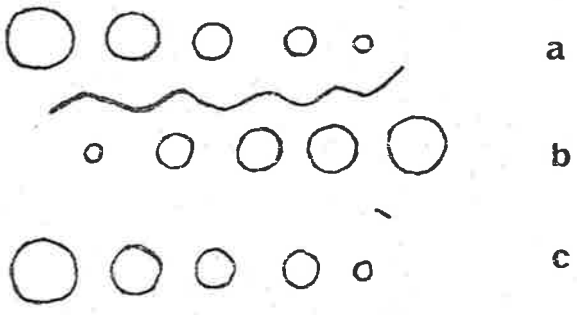
(NOTE ON INTERPRETATION OF FIGS. 6.4a, b, and 6.9.)

*It was originally thought (Both et al., 1972) that B. amyloliquefaciens secreted two proteases into the external medium during the course of a washed-cell experiment. They are the major (neutral) and minor (alkaline) proteases and were readily separated by electrophoresis on sepraphore strips. The protease purification of Both et al. (1972) isolated the major protease as what was thought to be a pure enzyme. However, on immune diffusion between the purified major protease and its antisera, two discrete precipitin lines could be seen (Fig. 6.4a). Therefore, the possibility existed that the major protease activity is actually due to two distinct proteolytic enzymes. This has very recently been shown to be case by polyacrylamide gel electrophoresis (J. Bielicki, personal communication). The minor (alkaline) protease will also cross react with the antiserum raised against the major protease. Thus, with a mixture of the isolated proteases or culture supernatant, 3 precipitin lines are seen (Fig. 6.9).

A



B



amounts of ferritin conjugated to what was in essence non-immune IgG were also added.

The final procedure adopted was as follows: washed-cell suspensions (40 ml) were prepared (as previously described) and incubated either in the absence or presence of chloramphenicol (10 μg/ml) for 20 minutes at 30°. This step results in cells which contain the enzyme pool and those which lack this fraction respectively. The cells were then washed in suspending medium and slowly cooled to 0°. They were then centrifuged (4,500g/ 5 min./0°) and finally resuspended in 1% formaldehyde-phosphate buffered saline (0.05 M phosphate-0.15 M, NaCl, pH 7.4) and fixed at 0° for 120 minutes. The embedding, sectioning and staining procedures were essentially those of McLean and Singer (1970) and are described in Materials and Methods.

The staining of chloramphenicol treated and untreated sectioned cells with ferritin conjugated to either anti-protease IgG or non-immune IgG are shown in plates 4-6. Plates 4 and 5 are representative of washed-cell suspensions treated with chloramphenicol for 20 minutes or untreated cells stained with ferritin conjugated to rabbit anti-protease IgG, respectively. Plate 6 is representative of cells treated with non-immune IgG conjugated to ferritin. As the latter photograph shows, there is a high non-specific background staining of the sections which made it extremely difficult to visualise any intracellular antigen present.

Thus, it was necessary to resort to statistical counting of ferritin granules located over the bacterial cells and the supporting matrix. Photographs of chloramphenicol treated and

PLATE 6.4. PERMEABLE THIN SECTIONS OF CHLORAMPHENICOL-TREATED WASHED-CELLS, EMBEDDED IN ALBUMIN AND STAINED WITH FERRITIN-ANTI-PROTEASE CONJUGATE.

The cells were treated with chloramphenicol, embedded, sectioned and stained with the immune-ferritin conjugate as described in the text. The dark spots lying over areas of the photograph are due to the electron-dense ferritin molecules of the immune conjugate.

Magnification: electron microscope, 20,000x.
optical, 5x.

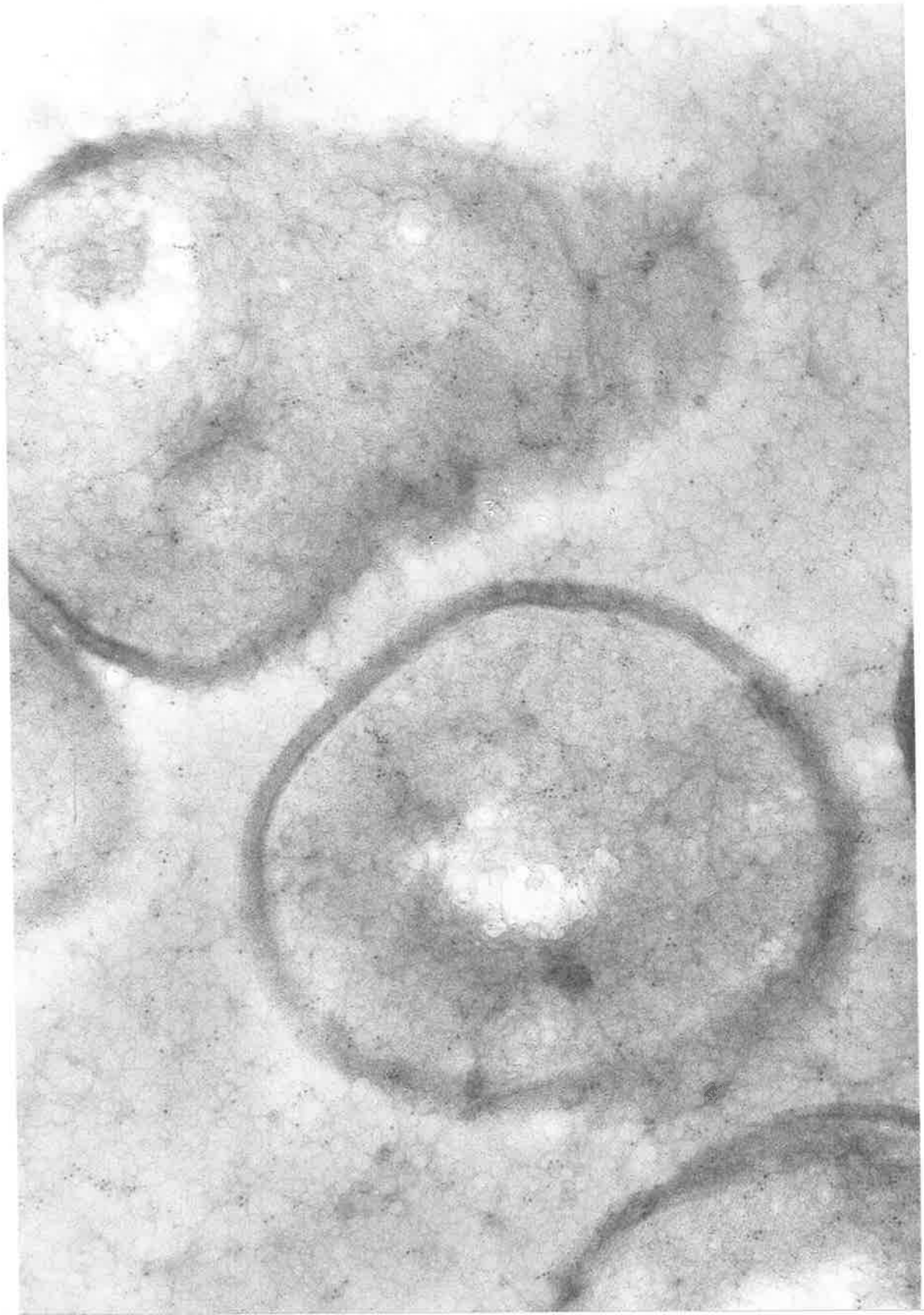


PLATE 6.5. ELECTRON MICROGRAPH OF PERMEABLE THIN SECTIONS OF UNTREATED WASHED-CELLS OF B. AMYLOLIQUEFACIENS EMBEDDED IN ALBUMIN AND STAINED WITH THE FERRITIN-ANTI-PROTEASE CONJUGATE.

The cells were embedded, sectioned and stained with the immune-ferritin conjugate as described in the text. The dark spots lying over areas of the photograph are due to the electron-dense ferritin molecules in the immune conjugate.

Magnification: Electron microscope, 20,000x
optical, 5x.

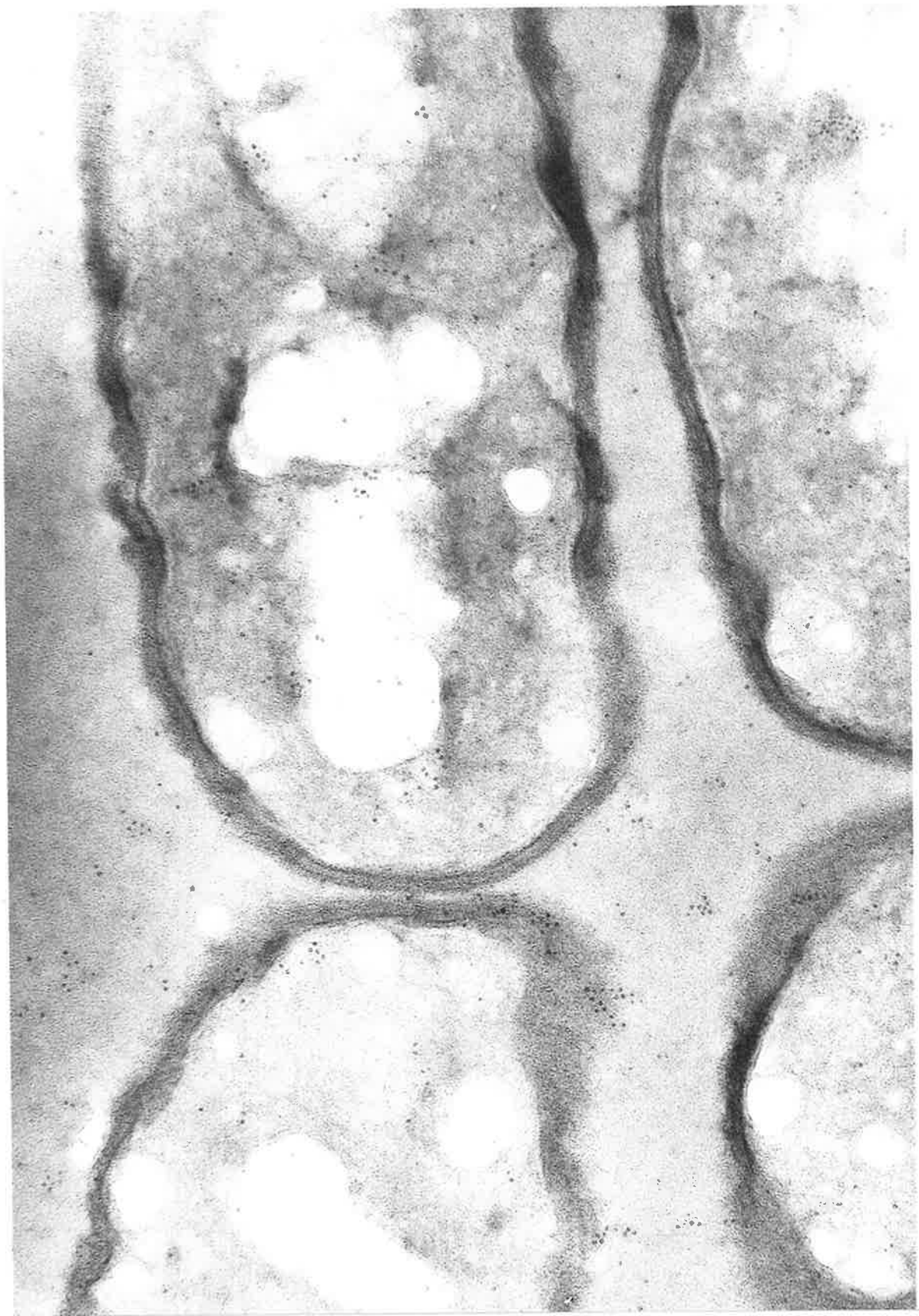
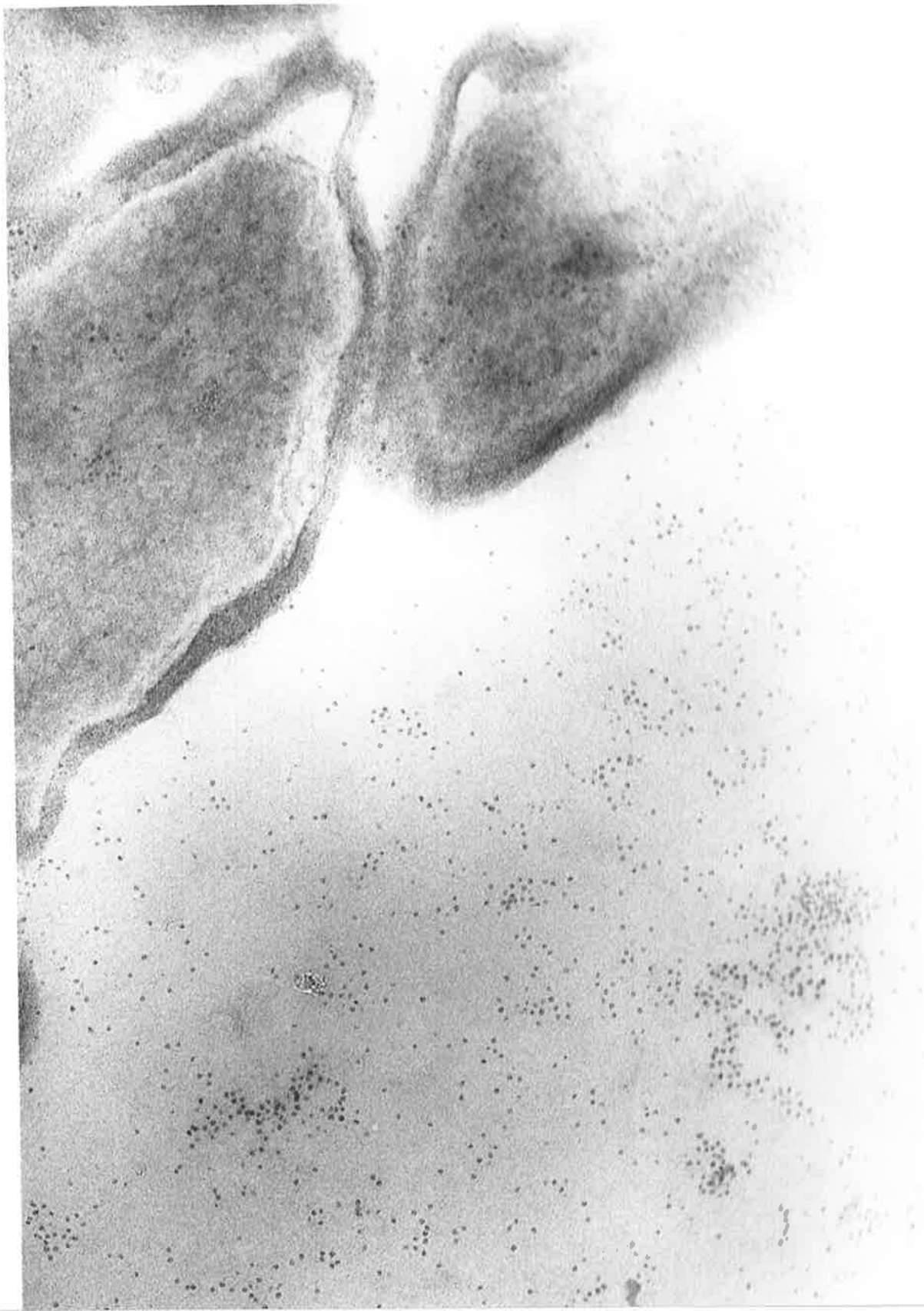


PLATE 6.6. ELECTRONMICROGRAPH OF PERMEABLE THIN SECTIONS OF UNTREATED WASHED-CELLS OF B. AMYLOLIQUEFACIENS EMBEDDED IN ALBUMIN AND STAINED WITH A NON-IMMUNE ANTISERA-FERRITIN CONJUGATE

The cells were embedded, sectioned and stained with the non-immune - ferritin conjugate as described in the text. The dark spots lying over areas of the photograph are due to the electron-dense ferritin molecules in the conjugate.

Magnification: Electron microscope: 20,000x
Optical, 6x.



untreated cells, stained with the immune conjugate, were carefully cut up to give areas of cytoplasm, membrane-cell wall and the matrix outside the cell. These portions of each photograph were then weighed and the weights taken as representing the relative areas for each portion. Duplicate photographs were then scored for ferritin granules lying in each of the three areas. The density of ferritin granules could therefore be expressed as grains per unit area. At least 10 different photographs of either chloramphenicol treated or untreated cells were counted in this fashion, plates 4 and 5 being representative photographs of each class.

It was found that in washed-cells treated with chloramphenicol (10 µg/ml) for 20 minutes at 30° that the intensity of staining of the three representative areas were essentially the same. However, cells of the type which had not been treated with chloramphenicol gave a different localisation. It was found that whereas the matrix and cytoplasm of the cells had approximately the same levels of staining (equal to the levels found in chloramphenicol treated cells), the membrane-cell wall of these cells showed a grain density that was approximately 2 times greater (Fig. 5). This was perhaps suggestive that the preformed enzyme pool was located in the region of the membrane-cell wall.

However, this technique may be subject to many criticisms, perhaps the major ones being the quite high backgrounds of staining with the non-immune conjugate which occurs in some preparations. This staining could not be eliminated by pre-treatment of the grids with non-immune serum nor by

FIGURE 6.5. STATISTICAL COUNTS OF FERRITIN CONJUGATED ANTI-PROTEASE ANTIBODY LOCATED ON PERMEABLE THIN SECTIONS OF CHLORAMPHENICOL TREATED AND UNTREATED WASHED-CELLS.

The cell specimens were prepared, stained and scored as described in the text.

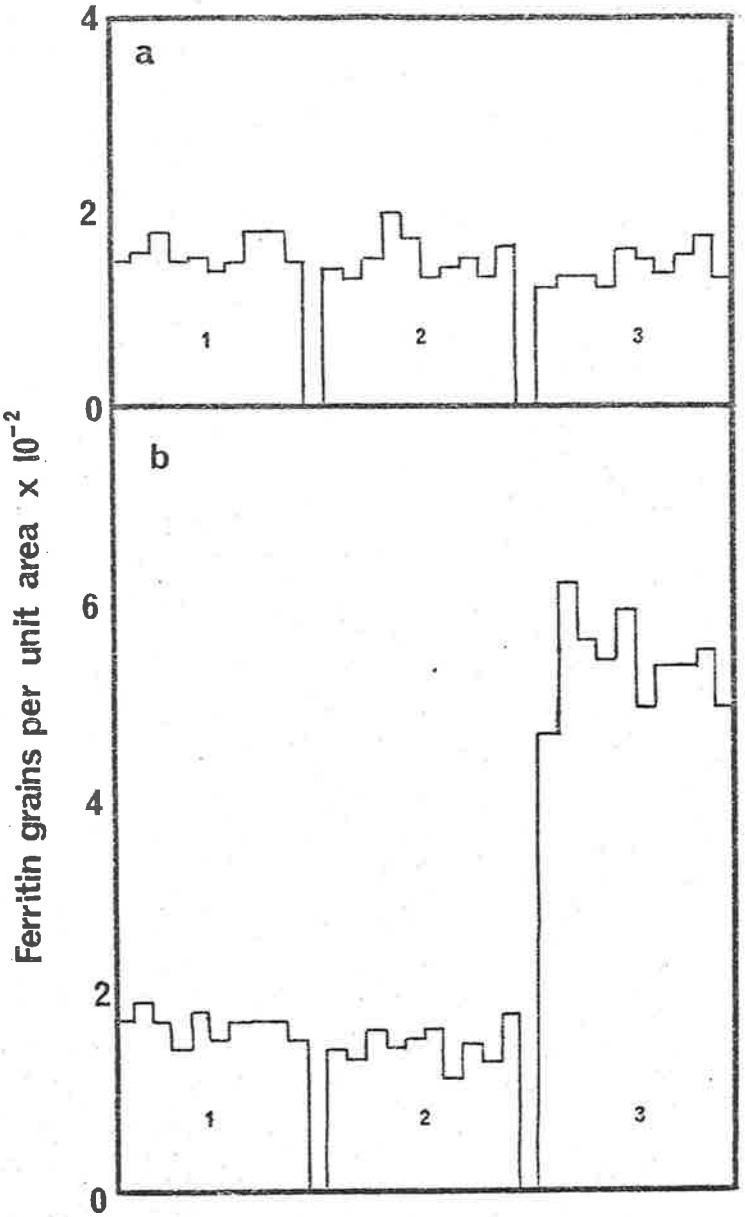
(a) chloramphenicol treated cells

(b) untreated cells

1. Cytoplasm

2. Matrix

3. Membrane/Cell Wall.


Ferritin grains per unit area x 10^{-2}
a
b
1
2
3
0
2
4
6

washing with buffers of high salt concentration (up to a maximum of 1.5 M). Also the large white areas found in the cytoplasm of these cells are indicative of extraction of nuclear material which may occur either during the embedding or staining procedures (Theoretical and Applied Histochemistry (1968)). Finally, this technique does not lend itself to visualisation of fine detail within cells, which may be a drawback in the adequate localisation of antigens within the cell. For these reasons, the results obtained by the above technique were regarded as inconclusive though perhaps encouraging. It was thought that by the use of either different localisation procedures involving ferritin as the visual marker, or cell preparations, that these problems above might be overcome.

Since even non-immune IgG conjugated to ferritin resulted in high non-specific backgrounds and chemical conjugation resulted in a considerable loss of antibody activity, the hybrid antibody procedure of Hämmerling et al. (1968) was resorted to.

5. The use of hybrid antibodies in an attempt to localise the enzyme pool and detect possible intracellular zymogens

Incubation of γ^Gantibody with pepsin causes cleavage and degradation of the Fc fragment, the combining activity of the antibody being preserved (Nisonoff et al., 1960a,b; Nisonoff and Dixon, 1964). Pepsin treated antibody is a dimer ($F(ab')_2$) of two identical units, the Fab' fragments, each with one combing site, linked by at least one disulphide bond.

Loss of the Fc fragment renders this disulphide bond highly susceptible to reduction by mercaptans and it can be selectively split because its oxidation potential is smaller than that of the disulphide bonds linking the light chain to the remainder of the heavy chain in each Fab' fragment. Thus, in low concentrations of mercaptoethylamine the bivalent $F(ab')_2$ fragment dissociates into two Fab' fragments (Nisonoff et al., 1960a). This is reversible by mild oxidation which reconstitutes the bivalent $F(ab')_2$ (Nisonoff et al., 1960b). When reconstitution takes place in a mixture of Fab' pieces from antibodies of different specificities, some of the $F(ab')_2$ molecules so formed are 'hybrids' with double specificity (Nisonoff and Rivers, 1961). Recombination of Fab' fragments is thought to occur at random, i.e., without preferential union of Fab' pairs with the same serological specificity (Hämmerling et al., 1968).

Thus, a hybrid antibody with one specificity for an extracellular enzyme and the other for ferritin should provide an ideal reagent for labelling and since chemical manipulation of the antibody is not involved, the combining activity of the antibody should not be greatly impaired. Another feature in favour of this type of visualising agent is that γG coupled chemically to ferritin in the conventional manner frequently shows ferritin granules clumped together, whereas hybrid antibodies do not exhibit this tendency (Hämmerling et al., 1968).

6. Preparation and hybridisation of $F(ab')_2$ fragments of dual specificity

Anti-amylase or anti-ferritin γG (purified as previously described) at a concentration of 10 mg/ml in 0.1 M sodium acetate buffer (pH 4.5), was incubated with 2% of its weight of pepsin (3 x cryst.; Sigma Chem. Co.) for 16 hours at 37°. The mixture was neutralised with 1 N-NaOH and chromatographed on Sephadex G 150 (10 x 120 cm) by upward flow; 3.0 ml fractions were collected (Fig. 6). The resultant $F(ab')_2$ preparation was concentrated by vacuum dialysis in collodion bags.

At this stage anti-ferritin $F(ab')_2$ molecules were separated from non-immune $F(ab')_2$ molecules also present by immune precipitation on the addition of a slight excess of ferritin. The immune precipitate was washed three times with phosphate-buffered saline at 4° and then homogenised in 0.3 M glycine buffer at pH 2.5, in the cold. The released ferritin was then removed by centrifugation (150,000g/180 min.) and the purified anti-ferritin $F(ab')_2$ molecules concentrated by vacuum dialysis in collodion bags.

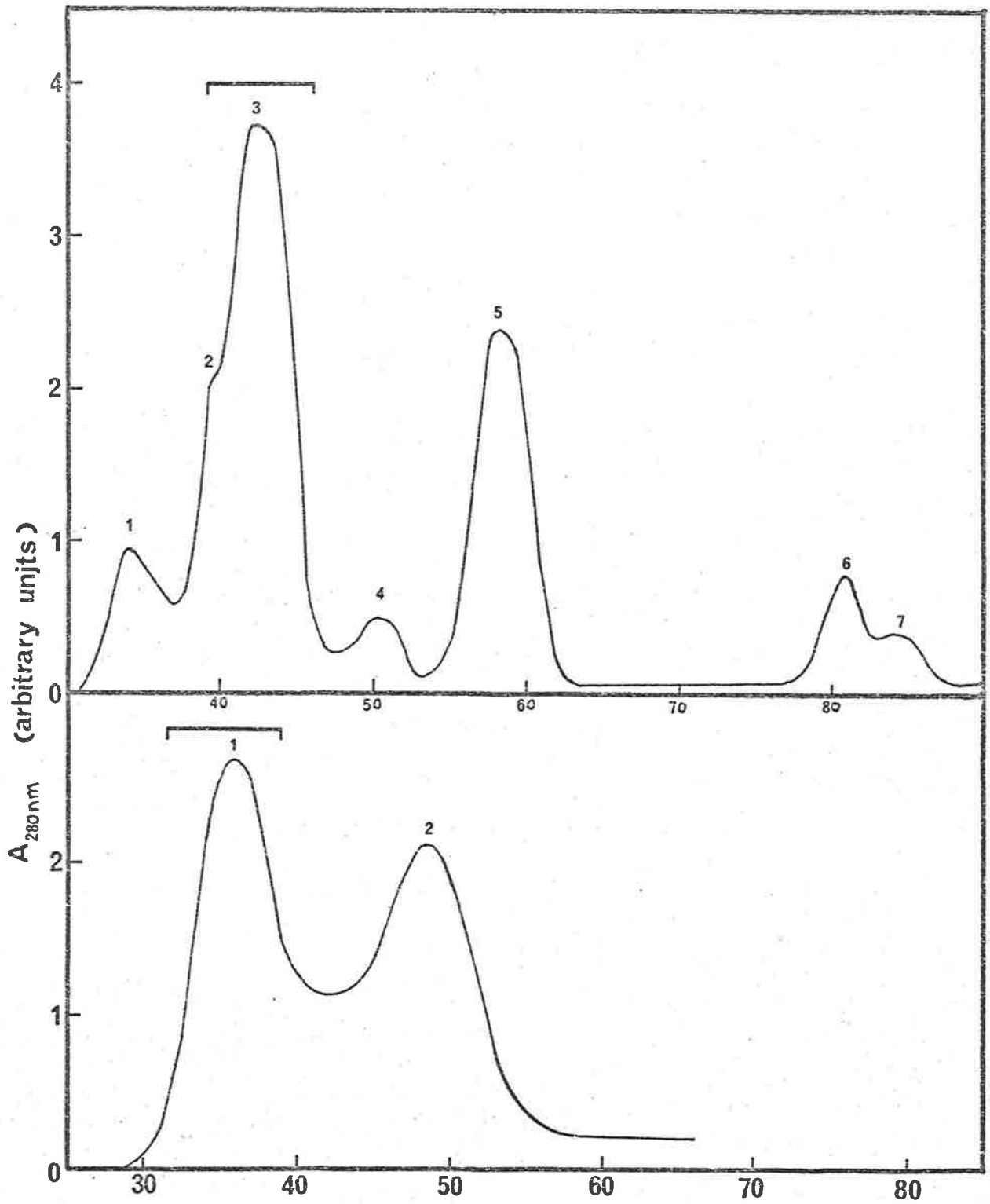
The purified anti-ferritin $F(ab')_2$ molecules (8 mg) were mixed with anti-amylase $F(ab')_2$ (2 mg) in 1.0 ml sodium acetate buffer (0.1 M, pH 5.0). 2-Mercaptoethylamine-HCl was added to a final concentration of 0.015 M and the mixture incubated under nitrogen at 37° for 60 minutes. The reducing agent was removed by exhaustive dialysis against sodium acetate buffer (0.1 M, pH 5.0) at 4°. The mixture was then neutralised with 1 N-NaOH and reoxidised gently by stirring in an atmosphere

FIGURE 6.6. SEPHADEX G-150 CHROMATOGRAPHY OF PEPSIN DIGESTED PURIFIED ANTIBODY.

The solid line represents the tracing obtained from an LKB Uvicord tracer monitoring A_{280nm} material. Peak 3 ($F(ab')_2$) fragments was collected and concentrated by vacuum dialysis in collodion bags prior to hybrid antibody preparation, as described in the text.

FIGURE 6.7. SEPHADEX G-100 CHROMATOGRAPHY OF RECONSTITUTED $(Fab')_2$ FRAGMENTS

The solid line represents the tracing obtained from an LKB uvicord tracer monitoring A_{280nm} material eluted from the column. Peak 1 represents reconstituted $F(ab')_2$ fragments while peak 2 is undimerised Fab' fragments.


A280nm (arbitrary units)
1
2
3
4
5
6
7
0
1
2
3
4
30
40
50
60
70
80

of oxygen for 120 minutes. Fab' fragments which had failed to dimerise were removed by chromatography on Sephadex G100 (10 x 120 cm) in 0.1 M sodium acetate buffer, pH 7.0 (Fig. 7). The reconstituted $F(ab')_2$ molecules arise from Fab' fragments of three different specificites (anti-amylase, anti-ferritin and non-immune. The latter was added with the anti-amylase F(ab') fragments, since this was not purified). The reformation of anti-amylase-anti-amylase, non-immune-non-immune and anti-amylase-non immune $F(ab')_2$ molecules was kept to a minimum by the addition of an excess of anti-ferritin $F(ab')_2$ molecules. Therefore, the majority of reconstituted $F(ab')_2$ molecules formed will be anti-ferritin-anti-ferritin, anti-ferritin-anti-amylase, anti-ferritin-non-immune with some anti-amylase-anti-amylase, anti-amylase-non-immune and non-immune-non-immune.

Further purification of the hybrid $F(ab')_2$ molecules was achieved by immune precipitation (using a slight excess of ferritin) and dissociation of the complex as described above. This final purification step results in a mixture of $F(ab')_2$ molecules of the following specificities: anti-ferritin-anti-ferritin, anti-ferritin-anti-amylase, and anti-ferritin-non-immune. The former and latter hybrids should not participate in the procedure for localisation of the extracellular enzyme since they lack the immune specificity for the enzyme.

To ensure that the hybridisation process had been successful, immunodiffusion analysis of the hybrid was performed (Fig. 8). It can be seen that the hybrid reacts with the ferritin and ferritin-amylase mixture of antigens, whereas no immune precipitate is formed between the hybrid and

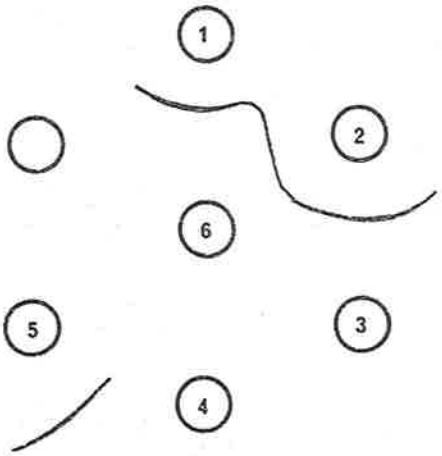
FIGURE 6.8. IMMUNODIFFUSION ANALYSIS OF THE HYBRID $F(ab')_2$ ANTIBODY

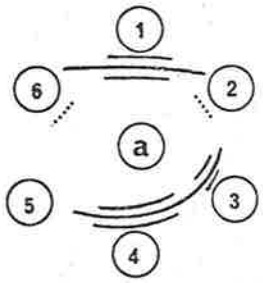
1. Ferritin
2. Mixture of α-amylase and ferritin
3. Anti-ferritin antiserum
4. Anti- α-amylase antiserum
5. α-Amylase
6. Hybrid antibody preparation containing $F(ab')_2$ molecules of the following specifications:
   (a) anti-ferritin - anti-ferritin
   (b) anti-ferritin - anti-amylase
   (c) anti-ferritin - non-immune.

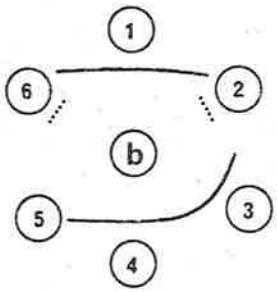
FIGURE 6.9. IMMUNODIFFUSION ANALYSIS OF THE MATERIAL RELEASED FROM CHLORAMPHENICOL TREATED AND UNTREATED WASHED-CELLS AFTER RUPTURE BY THE FRENCH PRESSURE CELL

1. 26 Hour culture supernatant
2. Pellet from cell lysate (no pretreatment with chloramphenicol)
3. Supernatant from cell lysate (no pretreatment with chloramphenicol)
4. 26 Hour culture supernatant
5. Pellet from cell lysate (cells pretreated with chloramphenicol)
6. Supernatant from cell lysate (cells pretreated with chloramphenicol)

A. Anti-protease antiserum
B. Anti-amylase antiserum.


1
2
6
5
3
4


1
6
2
a
5
3
4


1
6
2
b
5
3
4

the amylase antigen (the ferritin alone gives a precipitin reaction since the preparation contains anti-ferritin-anti-ferritin $F(ab')_2$). The hybrid actually inhibits the formation of an immune precipitate between amylase and anti-amylase antiserum (as seen from the assymetry of the precipitin band) since $F(ab')_2$ molecules of dual specificity although retaining antibody combining activity cannot form immune precipitates against a single antigen due to their monovalency.

7. Labelling of cell preparations with hybrid antibody

Washed-cell suspensions were slowly cooled to 0° and fixed with 4% (w/v) formaldehyde for 120 minutes at 0°. The cells were washed three times with phosphate-buffered saline at 0° and then fragmented by passage through the French pressure cell operated at 9 tons per square inch and 0° (in the presence of non-immune γG (2mg/ml). The cell fragments were then incubated in the presence of the hybrid $F(ab')_2$ at 0° for 60 minutes, ferritin (5 mg/ml) for 60 minutes at 0°, washed three times with phosphate-buffered saline at 0° and then fixed at 0° for 60 minutes with 4% (w/v) formaldehyde. These stained fragments were then sectioned and prepared for electron microscopy (Materials and Methods).

Washed-cell suspensions, either pretreated or untreated with chloramphenicol (10 µg/ml) for 20 minutes, were fragmented, stained and sectioned, as described above. It was found that these fragments were stained heavily over areas of cell cytoplasm. Only very occasionally could ferritin grains be seen either at the cell wall or membrane (plates 7 and 8). This pattern

PLATE 6.7. ELECTRON MICROGRAPH OF FIXED, FRAGMENTED WASHED-CELLS OF B. AMYLOLIQUEFACIENS AND STAINED WITH HYBRID ANTIBODY AND FERRITIN

The cells were fixed, fragmented and reacted with the hybrid antibody and ferritin as described in the text. The dark spots lying over areas of the fragmented cells depicted in the photograph are due to the electron-dense ferritin molecules.

Magnification: electron microscope: 20,000x
optical, 8x.

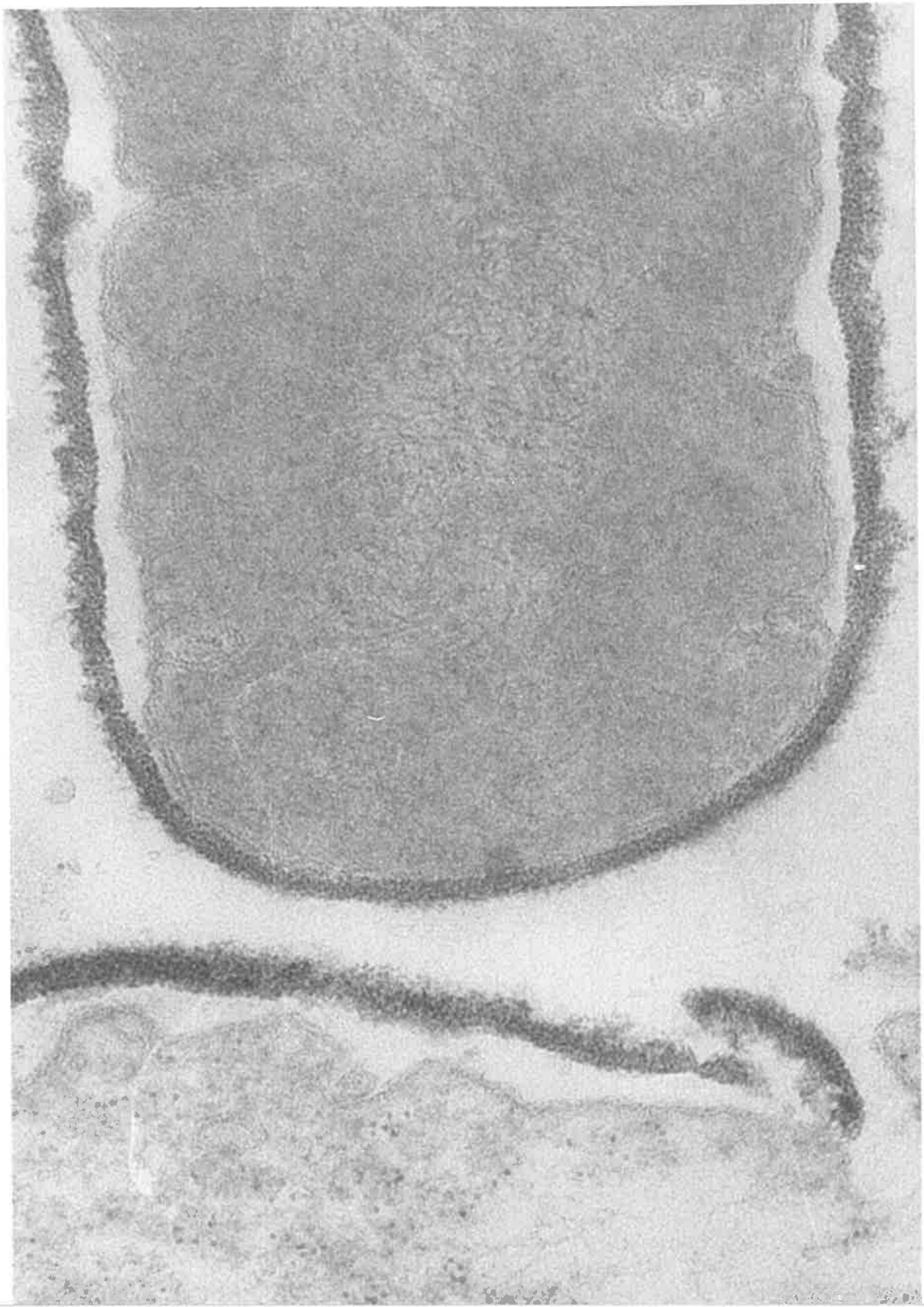
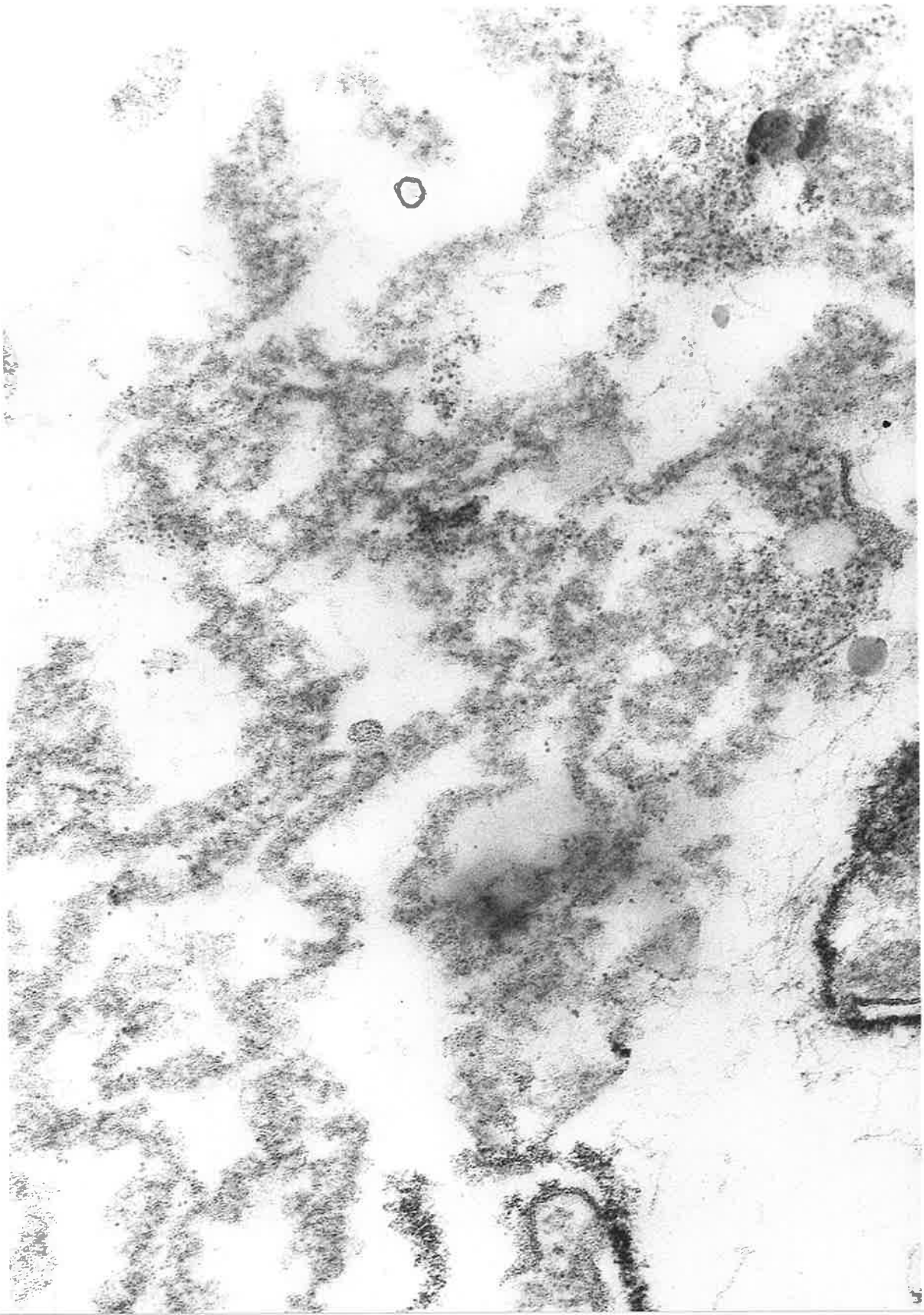


PLATE 6.8. ELECTRONMICROGRAPH OF THE CYTOPLASM FROM FIXED, FRAGMENTED WASHED-CELLS OF B. AMYLOLIQUEFACIENS STAINED WITH HYBRID ANTIBODY AND FERRITIN.

The cells were fixed, fragmented and reacted with the hybrid antibody and ferritin as described in the text. The dark spots lying over the cell cytoplasm are due to electron dense ferritin molecules.

Magnification: electron microscope, 20,000x
optical, 4.5x.



of staining was the same irrespective of whether the cells had been pre-exposed to chloramphenicol or not.

To check whether the intense cytoplasmic staining is an artefact, the hybrid antibody was omitted from the staining procedure. Thus, the cells were exposed only to ferritin and there should not have been any staining evident. However, as can be seen in plate 9 that although the hybrid antibody was omitted during the staining procedure the cell cytoplasm is quite heavily stained with ferritin. A possible explanation for the dense staining is that after fixing the cells the washing procedure does not remove fixative within the cell but only that on the cell surface. On fragmentation this fixative is still available to cross-link ferritin (even though non-immune γG was present during fragmentation).

8. Attempts to locate intracellular zymogens of the extracellular enzymes other than by ferritin labelling

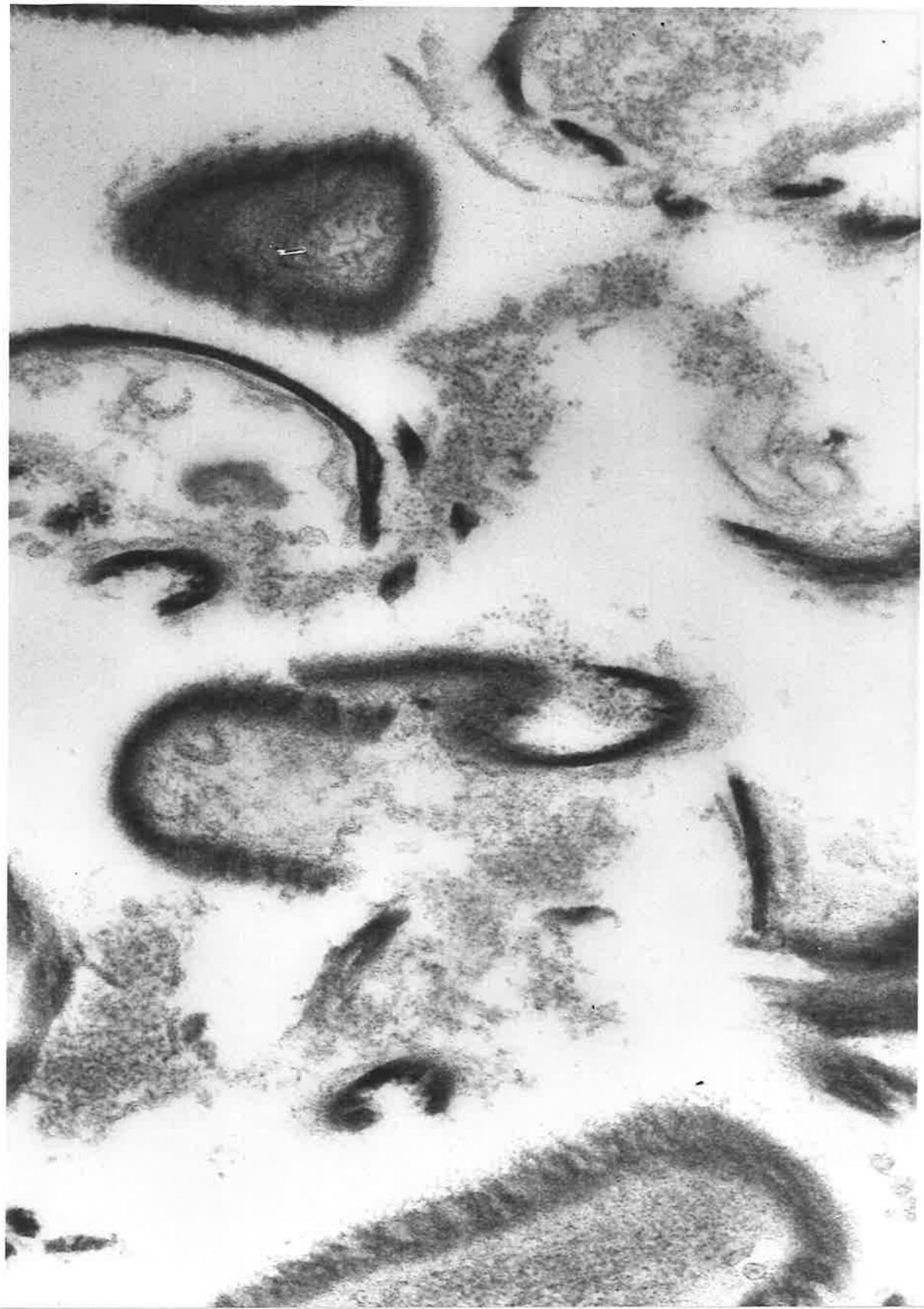
The possibility exists that inactive intracellular zymogens are present within the cytoplasm and cannot be detected by the use of cytochemical techniques using antibodies and ferritin due to the high levels of non-specific staining. Therefore, other approaches were used in an attempt to answer this question. Since no active enzyme (apart from the preformed enzyme pool) can be located within washed-cell suspensions, attempts were made to locate an intracellular zymogen by immuno-diffusion.

Washed-cell suspensions (100 ml) were incubated at 30° in the presence or absence of chloramphenicol (10 µg/ml) for

PLATE 6.9. ELECTRON MICROGRAPH OF FIXED, FRAGMENTED WASHED-CELLS OF B. AMYLOLIQUEFACIENS STAINED WITH FERRITIN ALONE.

The cells were fixed, fragmented and treated with ferritin as described in the text. The dark spots lying on the cell cytoplasm are due to electron-dense ferritin molecules.

Magnification: Electron microscope, 20,000x
Optical, 3.7x.



20 minutes. The cells were removed by centrifugation and washed. The cells were then resuspended in distilled water at 0° and passed through the French pressure cell (operated at 9 ton per square inch and 0°). The cell debris was removed by centrifugation (30,000g/30 min./0°) and the debris and lysate supernatant freeze-dried. These were then resuspended in a total of 1.0 ml phosphate-buffered saline and examined by immunodiffusion (Fig. 9). When the immunodiffusion patterns were examined it could be seen that in the case of both α-amylase and protease that the preformed enzyme pools were liberated into the supernatant on cell lysis, as expected, in those cells not exposed to chloramphenicol. However, in the case of cells exposed to chloramphenicol, no evidence of a cytoplasmic precursor (which would have been released into the lysate supernatant) could be detected. However, extremely faint precipitin lines with both antisera could be detected in the cell debris pellet, in cells either exposed or those not exposed to chloramphenicol, and which remained with the cell debris pellet even after washing three times by centrifugation. This may be due to either an inactive zymogen located at the membrane (as proposed by Torriani for alkaline phosphatase monomers) or due to the presence of nascent polypeptide chains on membrane bound ribosomes. This technique will not resolve between these possibilities; however, if a zymogen does exist it must be present in extremely low levels.

C. DISCUSSION

Unfortunately, no definite conclusions can be drawn from this ferritin labelling work described here. The first studies with the chemically conjugated antibody did give an indication of the enzyme pool being localised in the membrane-cell wall region but the non-specific labelling which occurred made the detection of intracellular zymogens impossible and made specific localisation doubtful. Similarly, ferritin clumping which is known to occur (Hämmerling et al., 1968) is a further worry when resorting to statistical counting methods. The hybrid antibody staining work was disappointing in that it gave artefactual staining of the cytoplasm but no evidence for pool localisation in the membrane-cell wall region. Even the negative result cannot be interpreted as meaningful since it is difficult to be certain that the concentration of enzyme pool would be sufficient to be detected if it was evenly spread throughout a particular fraction of a cell. If it was concentrated in specific areas there is no guarantee that a given section or sample of fragments would encounter one of these specific areas. A qualitative study of ferritin labelling for a given amount of enzyme would be necessary to give a guide on these questions. These would be time-consuming and there has been no opportunity to carry them out.

The dense staining of the cytoplasm in the hybrid antibody work is apparently artefactual. This does not eliminate the possibility of an inactive precursor of the extracellular enzymes existing in the cytoplasm in small amounts. The diffusion studies on cell extracts do not eliminate the above

possibility since any precursors may be ribosome bound and fail to diffuse. The existence of precursors in the cytoplasm is a crucial question and must ultimately be answered by the development of an in vitro cell-free system and isolation of the synthesised extracellular enzymes.

The most likely location of the enzyme pool is that it lies free between the membrane and cell wall. The reason for this conclusion comes from very recent studies performed by R.L. Sanders. It has been shown that protoplasts which are actively synthesising and secreting extracellular enzymes do not contain a pool of preformed enzymes. This observation, together with the inability to show any particulate association of the enzyme pool, tends to indicate that the cell wall is needed in an intact state to give rise to this faction. The rapid release of the preformed enzyme pool on treatment with lysozyme, which is apparently due to the removal of a dense band of material lying between the cell wall and membrane, is in agreement with this theory.

The possible relationship of the enzyme pool to the overall process of secretion may now be attempted. Since its existence is dependent on an intact cell wall, its location may be defined as outside the permeability barrier of the membrane but within the cell wall. It is assumed that after passage of the nascent protein through the membrane, a temporary restriction by the cell wall causes a small pool of active enzyme to accumulate in this region. This would explain the independence from energy and continuing protein synthesis, for its eventual release into the external medium. The peculiar

temperature effects may be due to a phenomenon which occurs due to the enzyme pool's close physical association with the membrane. At low temperatures the enzyme pool may be more tightly associated with the membrane than at higher temperatures. However, on disruption of the cell or removal of the cell wall this association may be lost or disrupted and the enzyme pool thus freely able to appear in the supernatant.

The finding that protoplasts do not accumulate a preformed enzyme pool argues that the accumulation of the enzyme pool is not a fundamental step in the process of secretion, but merely arises due to physical restrictions offered by the cell. In this event the enzyme fraction has no significance in the elucidating of a central concept of secretion involving either translation and secretion by membrane-bound ribosomes or in the passage of preformed enzyme zymogen precursors through the membrane.

ADDENDUM:

INHIBITION OF LIPID SYNTHESIS BY INHIBITORS OF PROTEIN AND RNA SYNTHESIS

INHIBITION OF LIPID SYNTHESIS BY INHIBITORS OF PROTEIN AND RNA SYNTHESIS

A. INTRODUCTION

During the course of investigation into the possible accumulation of mRNA specific for the extracellular enzymes (Chapter 4) an unusual accumulation of lipid in the external medium of washed-cell suspensions was also noticed. It was noted that this accumulation occurred when extracellular enzyme synthesis occurred and ceased in response to inhibition of the extracellular enzymes.

It was felt at this time that an investigation into this phenomenon would possibly throw more light on secretion in our model system, particularly as reports by Izui (1972) and Yoneda *et al.* (1973) had linked lipid metabolism and the accumulation of periplasmic and extracellular enzymes, respectively. This work is presented as an addendum since it is of some potential significance but does not fit into the main sequence of the work described earlier.

B. RESULTS

1. The occurrence of lipid and phospholipid material in washed-cell suspensions

A washed-cell suspension (40 ml) was incubated at 30° for 90 minutes and the cells then pelleted by centrifugation. The supernatant and the cell pellet were separately freeze-dried and then lipids extracted from this material three times by the method of Houtsmeuller and van Deenan (1965), except that the

buffer was citrate-phosphate (pH 6.5). From the pooled extracts phospholipids were precipitated from the total lipid by the addition of six volumes of redistilled acetone at -20°. The acetone-soluble lipids and phospholipids from each fraction were then dried by rotory evaporation and overnight in a dessicator *in vacuo* (over P_2O_5). When the samples were completely dry they were carefully weighed. These samples were then taken up in chloroform:methanol (2:1) to a concentration of 2.0 mg/ml. Samples (0.05 ml) were subjected to thin layer chromatography on silica gel.

Itwas found that in 40 ml of washed-cell suspensions the cells contained acetone-soluble lipids (5888 µg) and phospholipid (1068 µg) as did the supernatant (4042 and 473 µg respectively) after 90 minutes incubation. Separate control experiments in which the suspending medium itself (i.e., no cells added) was extracted showed a background of 518 µg of acetone-soluble lipid but no detectable phospholipid to be present.

When the components of the acetone-soluble lipid extracts from both cells and supernatant were examined by thin layer chromatography all those species present in the cells were also found in the supernatant (Fig. 1a-b). The appearance of large quantities of lipid in the supernatant was not unexpected as cell cultures of this organism, when centrifuged, often show a lipid pellicle. However, when the phospholipids from the washed-cells were compared to that of their supernatants obvious differences were observed. Whereas washed-cells contained five major phospholipids (Fig. 2a) the supernatants contained only one species (Fig. 2b). All spots stained with

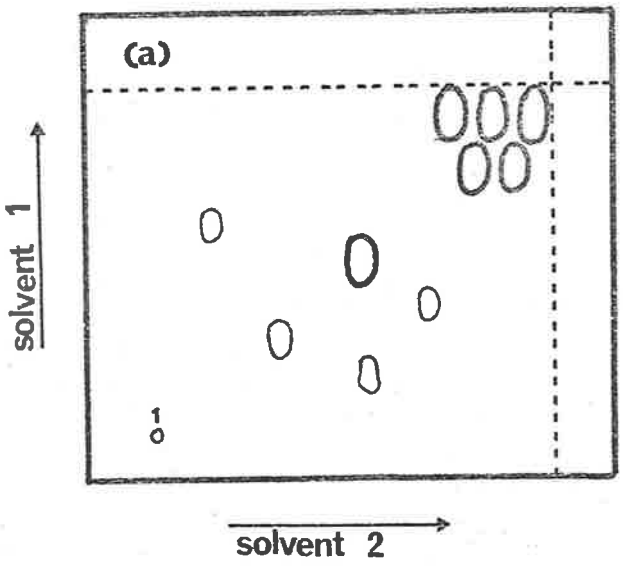
FIGURE 1a. ACETONE-SOLUBLE LIPIDS EXTRACTED FROM CELLS OF B. AMYLOLIQUEFACIENS.

FIGURE 1b. ACETONE SOLUBLE LIPIDS EXTRACTED FROM WASHED-CELL SUPERNATANT AFTER 90 MINUTES INCUBATION AT 30°C.

Species 1 marks unmigrated material from the origin.

Solvent 1. Chloroform: Methanol: 7N- NH_4OH (95: 5: 0.8 v/v)

Solvent 2. Chloroform: Acetone: Methanol: Acetic acid : water (8: 1.5; 0.25: 0.25: 0.2 v/v).


(a)
solvent 1
solvent 2

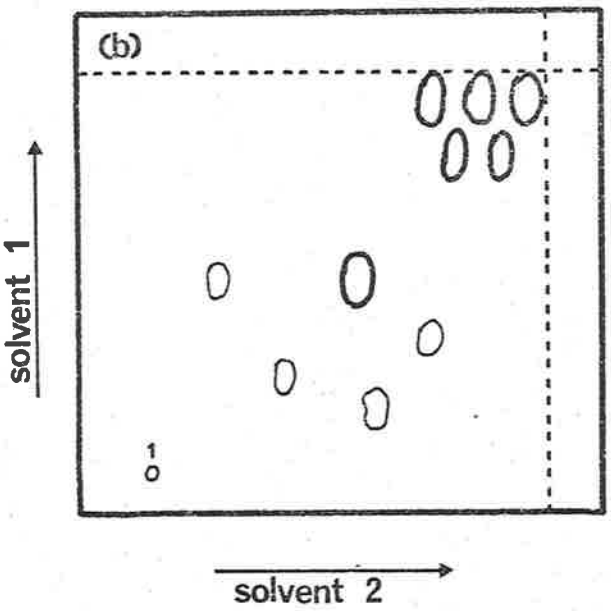

(b)
solvent 1
solvent 2

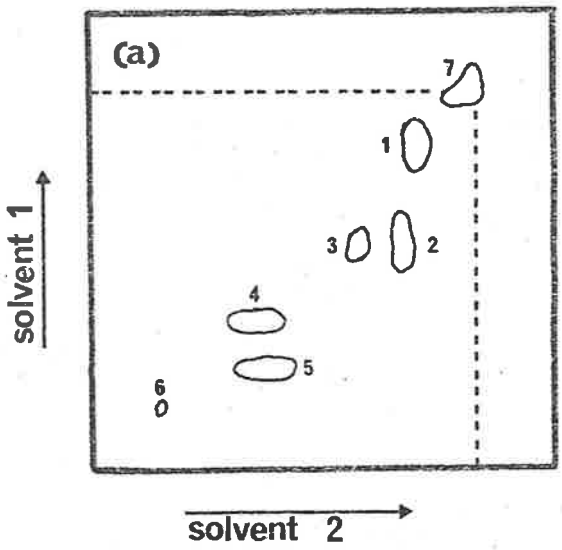
FIGURE 2a. PHOSPHOLIPIDS EXTRACTED FROM CELLS OF B. AMYLOLIQUEFACIENS.

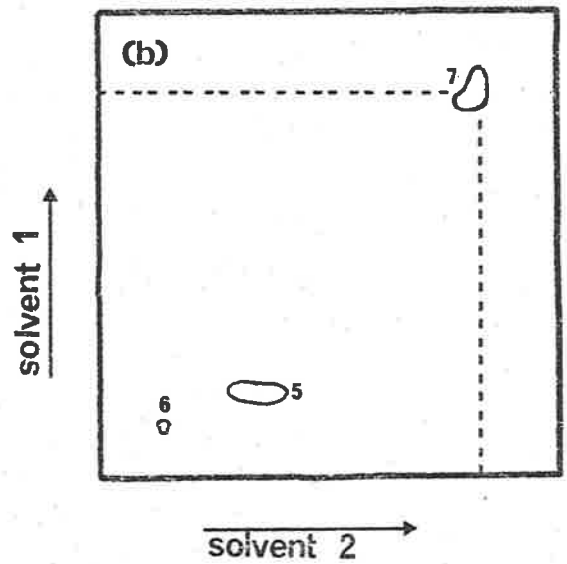
Species 1-7 stained with I vapour, 1-6 with molybdate reagent and 3 and 4 gave a positive reaction with ninhydrin (1% (w/v) in acetone). Species 6 is unmigrated phospholipid while species 7 are contaminating acetone-soluble lipids.

FIGURE 2b. PHOSPHOLIPID EXTRACTED FROM THE WASHED-CELL SUPERNATANT AFTER 90 MINUTES INCUBATION AT 30°C. SPECIES 5 CO-MIGRATES WITH THE SPECIES 5 EXTRACTED FROM WASHED-CELLS AND DOES NOT STAIN WITH NINHYDRIN.

Solvent 1. Chloroform: Methanol: Water (64: 25.4 v/v)

Solvent 2. Chloroform: Methanol: 7M-NH_4OH (60: 35:5 v/v)


(a)
7
1
3
2
4
5
6
solvent 1
solvent 2


(b)
7
5
6
solvent 1
solvent 2

the molybdate reagent described by Vaskovsky and Kostetsky (1972) and species 3 and 4 gave a positive reaction with ninhydrin (1% w/v in acetone). The pattern and staining of the phospholipids was almost identical to that reported by Oo and Lee (1972) from B. stearothermophilus. The supernatant phospholipid co-chromatographed with species 5 of the cellular phospholipids, which was tentatively identified as lysophosphatidyl glycerol. Thus, the appearance of a single phospholipid species in the supernatant contrasts with that of the acetone-soluble lipids.

2. The effect of antibiotics on the appearance of phospholipid in the supernatant of washed-cell suspensions

It has been shown in Chapter 4 that the accumulation of extracellular enzymes in the external medium of washed-cell suspensions was insensitive to rifampicin and sensitive to chloramphenicol when added at time zero to a washed-cell suspension. However, a differential effect on extracellular enzyme synthesis has since been reported (McInnes et al., 1972). Low concentrations of chloramphenicol (2 µg/ml) inhibit the accumulation of extracellular enzymes over a 60 minute time period by 90%, whereas general protein synthesis is not as severely inhibited (approximately 40%). If a connection between the appearance of extracellular enzymes and phospholipid in the supernatant exists, the appearance of the latter should be affected in a similar manner by the addition of these antibiotics.

Samples (40 ml) of a washed-cell suspension were

incubated with chloramphenicol (2 and 10 µg/ml) and rifampicin (0.5 µg/ml) for 90 minutes at 30°. A control suspension, with no drugs added, was incubated for the same period. At the end of incubation the suspensions were centrifuged, the supernatants and cells freeze-dried, extracted for acetone-soluble lipids and phospholipids and the extracts were weighed. The averaged results of two such experiments are shown in Figures 3 and 4. It can be seen that the amount of cellular acetone-soluble lipids was reduced but the addition of antibiotics by approximately 10% (Fig. 3a). On the other hand, there was a dramatic reduction in the level of phospholipids in the cells when compared with the untreated control cells (Fig. 3b). When the phospholipid samples extracted from the antibiotic treated cells were examined by thin layer chromatography, the results indicated a general reduction of all phospholipids, rather than a selective reduction.

When the effects of antibiotics on the levels of acetone-soluble lipids in the supernatant were compared to that of the control, it was seen that their accumulation was not severely affected (approximately 10% inhibition) (Fig. 4a). However, the phospholipid appearing in the supernatant was drastically reduced by both concentrations of chloramphenicol but was relatively unaffected by rifampicin (Fig. 4b). Thin layer chromatography of the phospholipid in the supernatant again revealed only one species (species 5). Thus, again the antibiotics altered the levels of phospholipid accumulation in the supernatant rather than that of the acetone-soluble lipids.

FIGURE 3. THE EFFECT OF CHLORAMPHENICOL AND RIFAMPICIN ON CELLULAR LIPIDS

(a) Effect on acetone-soluble lipids. The 100% value was 5188 µg

(b) Effect on phospholipids. The 100% value was 1405 µg.

1. Control
2. 2 µg chloramphenicol/ml
3. 10 µg chloramphenicol/ml
4. 0.5 µg rifampicin/ml.

The values are averaged from two separate experiments.

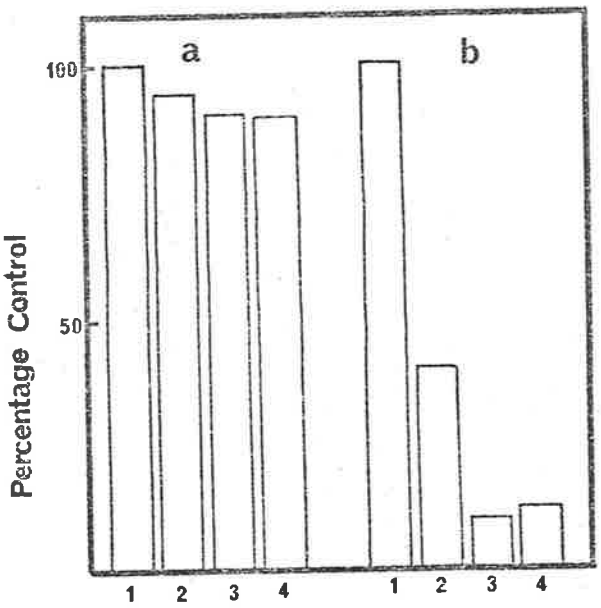
FIGURE 4. THE EFFECT OF CHLORAMPHENICOL AND RIFAMPICIN ON LIPIDS IN WASHED-CELL SUPERNATANTS.

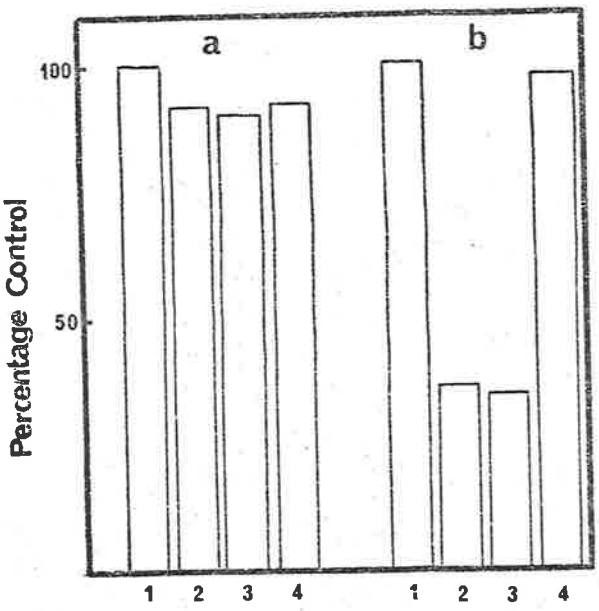
(a) Effect on acetone soluble lipids. The 100% value was 4693 µg.

(b) Effect on phospholipids. The 100% value was 445 µg.

1. Control
2. 2 µg chloramphenicol/ml
3. 10 µg chloramphenicol/ml
4. 0.5 µg rifampicin/ml.

The values are averaged from 2 separate experiments.


a
b
100
50
Percentage Control
1 2 3 4
1 2 3 4


a
b
100
50
Percentage Control
1 2 3 4
1 2 3 4

C. DISCUSSION

The appearance of a single phospholipid in the supernatant of cells secreting extracellular enzymes may be of significance. The reduction of secretion of the phospholipid in the presence of inhibitors of protein synthesis at least encourages interest in the observation. However, the work is clearly preliminary and there is no basis for presuming that a casual connection between enzyme secretion and phospholipid release exists. In order to examine this, attempts were made to isolate a glycerol requiring mutant of *B. amyloliquefaciens* to see whether glycerol deprivation inhibited enzyme secretion and phospholipid production. Initial attempts to obtain such a mutant were unsuccessful and there was no time to proceed further with this.

CHAPTER 7

FINAL SUMMARY AND DISCUSSION

FINAL SUMMARY AND DISCUSSION

The work in this thesis in showing that messenger RNA for all three extracellular enzymes apparently accumulates in the cell, strengthens the belief that the phenomenon is related to the mechanism of secretion rather than being an abberant situation. This conclusion has been further reinforced by other work in this laboratory (Semets *et al.*, 1973) showing that the same situation exists in a genetically distinct organism, *B. subtilis*.

There are many questions and problems remaining: prime among these is to define the cellular location of extracellular enzyme synthesis. The crucial question here is whether they are synthesised as are normal proteins in the cytoplasm and then secreted or whether synthesis and secretion are simultaneous events occurring at the membrane. My own work unfortunately has failed to conclusively answer this question. But it does show firstly that the level of any intracellular enzyme or precursor is extremely minute and not free in solution and secondly, that a finished protein molecule can emerge essentially instantaneously from the cell. All in all the work here is compatible with the nascent peptide extrusion theory, but it does not of course prove it.

It seems likely that resolution of the problems of secretion will depend on development of a cell-free system capable of synthesising extracellular enzymes or their precursors. Work to this end is in progress in this laboratory.

If the nascent peptide extrusion mechanism is correct it raises many interesting problems of molecular biology;

such as, why particular ribosomes at the membrane recognise particular messengers or alternatively, what causes ribosomes with extracellular enzyme messengers to attach to secretory sites. Irrespective of whether the nascent peptide theory or secretion of finished proteins proves to be correct, the problem of how the molecule vectorially traverses the membrane lipid barrier is an important and interesting one.

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